Cell Lineage Specification during Postembryonic Brain Development in *Drosophila:* *Expression and Function of the Cephalic Gap Gene empty spiracles*

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1 Summary

The cephalic gap gene *empty spiracles* (*ems*) encodes a homeodomain transcription factor that is essential for the regional specification of the early embryonic brain in *Drosophila*. This thesis presents the analysis of *ems* expression and function during larval and pupal development of the brain. In the late larval brain eight neuroblast lineages express *ems*. In seven lineages *ems* is only transiently expressed and expression disappears in the early pupa. In contrast, all adult-specific neurons of the medial-most lineage (EM lineage) continuously express *ems* throughout larval and pupal development as well as in the adult brain.

In a first study (Chapter II) we have investigated the function of *ems* in the EM lineage. The cell bodies of the EM lineage are located ventral to the antennal lobes from where they extend fine neurite arborizations into the suboesophageal ganglion and a prominent projection into the superior medial protocerebrum. Clonal mutant analysis of the adult-specific cells in the EM lineage has revealed three distinct functions of *ems* during larval development. First, the number of cells was reduced by half. This could be rescued by blocking apoptosis in *ems* mutant clones suggesting a function of *ems* in cell survival. Second, all mutant clones extended undirected misprojections into the surrounding neuropile. Third, the projection into the superior protocerebrum was missing in half of the clones. A closer examination of the projection patterns of *ems* mutant single-cell clones demonstrated that *ems* is required cell-autonomously in postmitotic neurons for the correct extension of the protocerebral projection.

In our second study (Chapter III) we have examined the role of *ems* in development of the olfactory projection neurons (PNs). Two of the transiently expressing *ems*-positive lineages in the larval brain correspond to the adult-specific anterodorsal and lateral PN lineages (adPN and lPN, respectively). Clonal mutant analysis of the GH146-positive PNs revealed different roles of *ems* in the two lineages. In the adPN lineage transient *ems* expression is required for precise dendritic targeting. In the lPN lineage *ems* function is necessary for the formation of the correct number of progeny during larval development. Furthermore, timely down-regulation of *ems* expression is necessary for the proper connectivity of PNs.

The finding that *ems* and its mammalian homologs *Emx1*/*Emx2* are both expressed in second order olfactory PNs suggests conserved genetic mechanisms for the specific relay of olfactory information to higher brain centres.

1 Introduction

1.1 DROSOPHILA NEUROGENESIS

In insects, the embryonic brain consists of a supraoesophageal ganglion that can be subdivided into the protocerebral, deutocerebral, and tritocerebral neuromeres and a suboesophageal ganglion that is subdivided into the mandibular, maxillary, and labial neuromeres. The developing ventral nerve cord extends posteriorly from the suboesophageal ganglion into the body trunk (Campos-Ortega and Hartenstein, 1997). In this work, we will use the term ‘brain’ equivalent to the supraoesophageal ganglion.

In *Drosophila*, most neuroblasts (Nbs) have two proliferative periods: an initial brief period during embryogenesis that generates the primary neurons of the functional larval central nervous system (CNS), and a second prolonged period during larval and early pupal stage that generates the secondary or adult-specific neurons (Fig. 1-1). The secondary neurons make up 90% of the adult CNS. These two neurogenic periods are separated by a time window lasting from late embryogenesis to approximately the second half of first instar stage where most brain Nbs persist in a cell-cycle arrested state (Prokop and Technau, 1991; Truman and Bate, 1988). Proliferating Nbs undergo sequential cycles of self-renewing divisions, dividing asymmetrically to produce ganglion mother cells (GMCs) that in turn divide once to produce two post-mitotic daughter cells (Truman and Bate, 1988). Thus during larval life the adult-specific progeny of each Nb accumulates in a growing cluster of immature neurons that extend fasciculated neurites (secondary lineage axon tracts = SATs) close to the neuropile but wait until metamorphosis to complete their extension to adult specific synaptic targets (Dumstrei et al., 2003; Truman et al., 2004; Zheng et al., 2006). Whereas the primary, larval-functional progeny of each Nb show a high degree of phenotypic diversity (Brody and Odenwald, 2000; Kambadur et al., 1998), the adult-specific cells in a given lineage are remarkably similar and typically project to only one or two initial targets in the larva (Pereanu and Hartenstein, 2006; Truman et al., 2004; Zheng et al., 2006). During metamorphosis the adult brain forms by neuronal remodelling of larval functional neurons and final morphogenesis of adult-specific neurons (Lee et al., 2000; Marin et al., 2005; Zheng et al., 2006).

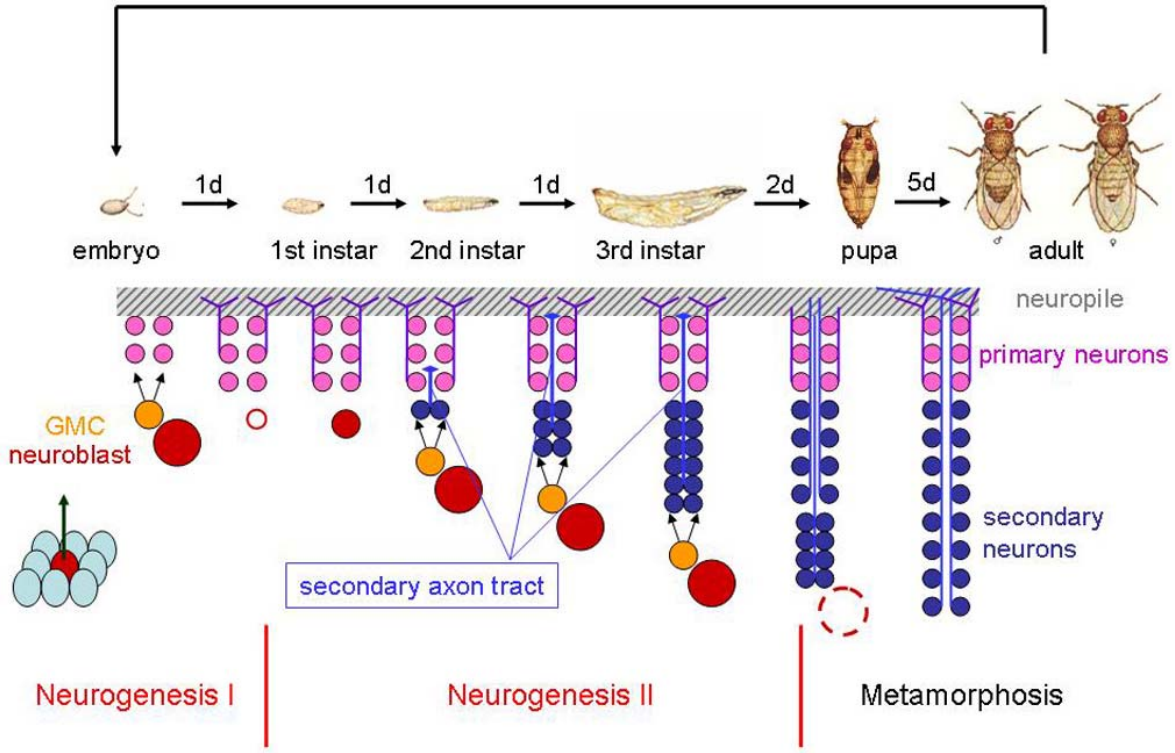

Figure 1-1 Lifecycle and neurogenesis in *Drosophila*. Two phases of neurogenesis, separated by a quiescent state of the neuroblast, produce primary and secondary neurons of the same lineage. Whereas the primary progeny of the neuroblast quickly differentiates into functional neurons of the larva, cells of the secondary lineage wait until metamorphosis to extend their projections (see text for more detail).

Classical neuroanatomical studies describe the architectural subunits of the adult *Drosophila* brain in terms of their spatial coordinates as they appear in the adult (Strausfeld, 1976). A number of recent publications have addressed the analysis of the developmental origin of adult brain units taking advantage of the MARCM (Mosaic Analysis with a Repressible Cell Marker) system (Jefferis et al., 2001; Jefferis et al., 2004; Kimura et al., 2005; Komiyama et al., 2003; Lee and Luo, 1999; Marin et al., 2005; Zheng et al., 2006). Upon heat-shock induced mitotic recombination in the Nb all clonally related cells are labelled with a membrane-bound marker, and therefore, projection patterns of neurons can be studied in the context of overall brain architecture. Furthermore, clonal mutant analysis enables us to study homozygous mutant clones in a heterozygous background. In addition, fine neuronal morphology or the timing of developmental processes can be studied at single-cell clone resolution with MARCM (reviewed in Lee and Luo, 2001).

A recently published Nb lineage atlas of the developing adult brain in the late larva subdivides each brain hemisphere into approximately 100 clonal lineages, each represented by a fasciculated neurite bundle that forms an invariant pattern in the neuropile (Pereanu and Hartenstein, 2006). To date it is largely unknown what developmental factors determine the anatomical and functional diversities and specificities of the clonal sub-units of the adult brain. Interestingly, a different molecular genetic analysis in early embryonic development has resulted in the identification of developmental control genes that are involved in generating the larval functional brain.

In the early embryo approximately 100 bilaterally symmetrical Nbs segregate from the neuroepithelium in a stereotyped array. A detailed analysis of the expression of over 30 developmental control genes has shown that specific combinations of gene expression uniquely identifies all embryonic brain Nbs (Urbach and Technau, 2003). For a number of these developmental control genes, loss of function analyses have revealed severe defects in neurogenesis, patterning, and circuit formation during embryonic brain development (Hirth et al., 1998; Hirth et al., 2003; Hirth et al., 1995; Kammermeier et al., 2001; Noveen et al., 2000; Urbach and Technau, 2003). However, it is still largely unclear how these genes, and the embryonic process that they control, relate to the clonal organization of the adult brain. Furthermore, to date, only a few early developmental control genes have been analysed in postembryonic brain development (Callaerts et al., 2001; Hassan et al., 2000; Hitier et al., 2001; Kurusu et al., 2000; Pereanu and Hartenstein, 2006). Therefore, the question arises whether classical developmental control genes implicated in early embryonic neurogenesis and neural patterning, are re-used at later developmental stages in the brain. Observations of that kind have been recently made in vertebrates (Zapala et al., 2005).

1.2 EARLY EMBRYONIC FUNCTIONS OF THE *EMPTY SPIRACLES* GENE IN *DROSOPHILA*

The *Drosophila ems* gene belongs to the cephalic gap genes together with *tailless* (*tll*), *orthodenticle* (*otd*), *buttonhead* (*btd*) and *sloppy paired* (*slp*). At the early blastoderm stage of embryogenesis the cephalic gap genes are broadly expressed in overlapping anterior stripes where their expression is initially regulated by maternal effect genes (Dalton et al., 1989; Walldorf and Gehring, 1992). The functional inactivation of any of these genes results in gap-like phenotypes where structures of several head segments are missing (Cohen and Jurgens, 1990; Grossniklaus et al., 1994). The cephalic gap genes *tll*, *otd*, *ems* and *btd* have been shown to be essential in early brain development. By the time of neuroblast delamination in the anterior brain their expression domains become restricted to specific subsets of Nbs (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). Mutational inactivation of a given cephalic gap gene results in the deletion of a specific brain area indicating the requirement of these genes in early specification of the anterior brain primordium (Hirth et al., 1995; Younossi-Hartenstein et al., 1997).

At the beginning of embryonic neurogenesis ems is expressed in 11 bilaterally symmetrical Nbs derived from the procephalic neuroectoderm (Urbach and Technau, 2003). Later in embryonic brain development *ems* expression is found exclusively in the deutocerebral and tritocerebral neuromeres of the anterior brain. A reiterated segmental expression pattern is also seen in the ventral nerve cord at later embryonic stages. Loss-of-function of the *ems* gene results in a gap-like phenotype in the brain due to the absence of cells in the deutocerebral and anterior tritocerebral neuromeres (Hartmann et al., 2000; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In the *ems* mutant domain the expression of the proneural gene *lethal of scute* (*l'sc*) is lost and neuroblasts fail to form (Younossi-Hartenstein et al., 1997). This phenotype can be rescued by ubiquitous over expression of *ems*, which results in proper brain development (Hartmann et al., 2000). Thus, ems function is required for the specification and formation of the anterior embryonic brain in *Drosophila*.

The two mouse orthologues, *Emx1* and *Emx2*, of *Drosophila* ems, show largely overlapping expression domains in the developing brain. Whereas *Emx1* mutant mice are postnatal viable and show rather subtle phenotypes restricted to the forebrain, *Emx2* mutant mice die immediately after birth (Pellegrini et al., 1996; Qiu et al., 1996; Yoshida et al., 1997). *Emx2* expression is seen in the germinative neuroepithelium of the presumptive cerebral cortex in

the developing forebrain (Gulisano et al., 1996; Mallamaci et al., 2000; Pellegrini et al., 1996). The anteriormost expression of *Emx2* in the brain is found in the olfactory epithelium, whereas posteriorly the expression domain extends into the roof plate of the diencephalon. *Emx2* is expressed throughout the developing neocortex in a graded manner. In the olfactory bulb *Emx1* and *Emx2* are expressed during later developmental stages in the subependymal layer and in the mitral layer. *Emx1* expression is restricted to mitral cells of the main olfactory bulb, whereas *Emx2* mostly to those of the accessory olfactory bulb. Mitral cells of the main olfactory bulb and accessory olfactory bulb receive chemical information coming from the main olfactory epithelium and the vomero-nasal organ, respectively, related to feeding and social/sexual behaviour, respectively. They both project to several specific targets in the basal telencephalon through independent ways (Mallamaci et al., 1998; Younossi-Hartenstein et al., 1997). Analysis of *Emx1/Emx2* double mutant mouse cortex has revealed important roles in neuroblast proliferation, migration and differentiation. The olfactory bulbs of double mutant mice are reduced in size and the mitral cell layer is disorganized (Bishop et al., 2003). In addition to its expression during development, *Emx2* has been found in the adult mouse brain expressed in neural stem cells (Cecchi, 2002).

1.3 THE OLFACTORY SYSTEM OF *DROSOPHILA*

In *Drosophila*, 1300 olfactory receptor neurons (ORNs) per side are concentrated in two peripheral appendages, the third antennal segments and the maxillary palps (Fig. 1-2). Chemical odorants bind to adequate olfactory receptors (ORs) expressed on the dendrites of ORNs and initiate signal transduction. The first olfactory relay in the fly brain are the antennal lobes, the insect equivalent of the vertebrate olfactory bulb. Discrete subdivisions, the glomeruli, correspond to both morphological and functional units. In *Drosophila*, 45–50 glomeruli can be uniquely identified by position, size, and shape and have been catalogued in atlases (Couto et al., 2005; Laissue et al., 1999). In each glomerulus, axons of ORNs of a single class that express the same olfactory receptor converge and synapse typically with a single class of second order projection neurons (PNs), the insect equivalent of vertebrate mitral cells. Thus, a total of approximately 50 classes of ORNs form one-to-one connections with 50 uniglomerular PNs classes (Axel, 1995; Jefferis et al., 2002).

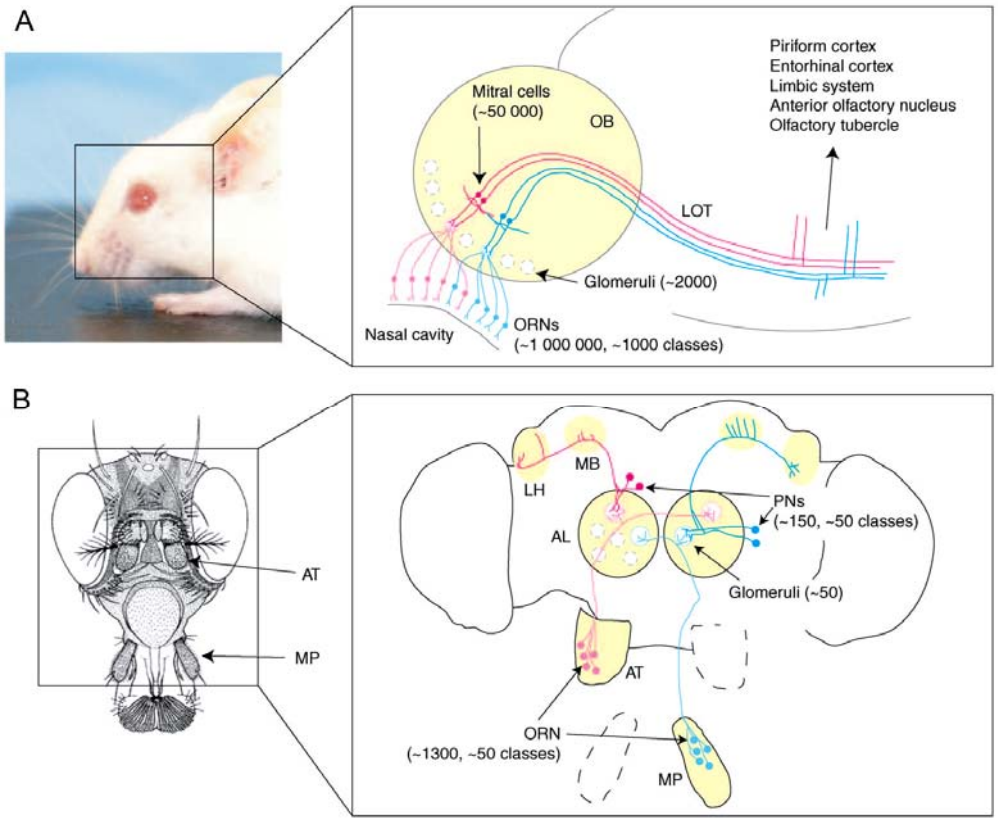

Figure 1-2 Similar organization of the olfactory system in mouse (A) and *Drosophila* (B). Olfactory receptor neurons (ORNs) expressing the same receptor (same color) target their axons to the same glomerulus in the olfactory bulb (A) and antennal lobe (B). The dendrites of fly second order projection neurons (PNs) and the apical dendrites of mouse mitral cells also target to single glomeruli, and their axons project to specific parts of

higher olfactory centers. Numbers in parentheses refer to numbers of neurons and glomeruli. Abbreviations: AL, antennal lobe; AT, 3rd antennal segment; LH, lateral horn; LOT, lateral olfactory tract; MB, calyx of the mushroom body; MP, maxillary palp; OB, olfactory bulb; ORN, olfactory receptor neuron; PN, projection neuron (modified after Komiyama and Luo, 2006).

Approximately 150 PNs originating from three distinct Nb lineages relay the olfactory information to higher brain centres at the mushroom body calyx and the lateral horn (Jefferis et al., 2001; Marin et al., 2002; Stocker et al., 1997; Wong et al., 2002). In the mushroom body calyx and the lateral horn different PN classes form highly stereotypical axon patterns. Axon pattern maps have been created of the axon terminal arborizations of most PN classes (Marin et al., 2002; Wong et al., 2002). Glomerular targeting of PN dendrites is prespecified by their lineage and birth order. Furthermore, the initial dendritic targeting of PNs in the antennal lobe occurs prior to invasion of ORN axons. The dendritic innervation pattern of PNs in the antennal lobe starts approximately at pupa formation and is largely established at 18 hours after pupa formation (APF), although it becomes further refined upon arrival of ORN axons (Jefferis et al., 2004). The development of the axon terminal arborizations lags behind the dendritic maturation. However, between 24 and 30 hours APF the stereotypic axon patterns starts to be recognizable (Jefferis et al., 2004).

Intrinsic action of the two POU-domain transcription factors, Acj6 and Drifter, regulates the dendritic targeting in a lineage specific way (Komiyama et al., 2003; Komiyama and Luo, 2006). The *acj6* gene is specifically expressed in the anterodorsal PNs (adPNs) and its function is required for the correct dendritic targeting to adPN-specific glomeruli. A similar role has been found for *Dfr* in lateral PNs (lPNs). In contrast, two cell surface molecules, N-cadherin and Dscam, are present on the dendritic projections of both PN lineages and affect all PN classes equally. N-cadherin is required to restrict dendritic targeting of PNs to the target glomerulus and it has been implicated in the formation of correct terminal arborizations of PN axons in the lateral horn (Zhu and Luo, 2004). Dscam is responsible for the elaboration of the dendritic field based on the repulsive interactions between dendrites of the same cell (Zhu et al., 2006). Another cell-intrinsic component involved in the spatial specificity of dendritic targeting is the transmembrane receptor Semaphorin-1a. It has been implicated in directing the dendritic targeting of PNs along the dorsolateral to ventromedial axis of the antennal lobe based on graded expression of the receptor in PNs. In addition, Semaphorin-1a also regulates axon targeting of PNs in higher brain centres (Komiyama et al., 2007). As

opposed to the contribution to spatial specificity of dendrite targeting, it has been recently shown, that the BTB-Zinc Finger protein Chinmo (Chronologically inappropriate morphogenesis) regulates neuronal temporal identity in mushroom body and PN lineages. Loss of Chinmo cell-autonomously caused early-born class of PNs to adopt the fates of late-born class of PNs within the same lineage (Zhu et al., 2006). Taken together, the olfactory system of *Drosophila* provides an excellent model system to study mechanism involved in specific targeting at the dendritic and axonal terminals.

1.4 THIS THESIS

The analysis of the cephalic gap gene *ems* in the *Drosophila* brain has been limited to the early functions in anteroposterior regionalization during early embryonic development. This is in contrast to the data available from vertebrate species, where *Emx1/Emx2* has been shown to be involved in later developmental processes and also in the adult brain. Here we have found that *ems* is expressed at larval, pupal and adult stages in the fly brain. From eight *ems*-positive secondary lineages, three have been characterized in more detail and the function of *ems* during their development was studied. Based on which criteria have we selected our candidate lineages? The first lineage we have analysed in this study was selected based on its unique *ems* expression pattern during larval and adult stages. The other two lineages attracted our interest because of their function in the olfactory pathway of Drosophila where the vertebrate *ems* homologues have been found to be expressed. The two resulting data sets will be presented in the chapters 2 and 3.

2 Cell lineage-specific expression and function of the *empty spiracles* gene in adult brain development of *Drosophila melanogaster*

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2.1 SUMMARY

The homeodomain transcription factor encoding *empty spiracles (ems)* gene is a member of the cephalic gap gene family that acts in early specification of the anterior neuroectoderm in the embryonic brain of *Drosophila*. Here we show that *ems* is also expressed in the mature adult brain in the lineage-restricted clonal progeny of a single neuroblast in each brain hemisphere. These *ems*-expressing neuronal cells are located ventral to the antennal lobes and project a fascicle to the superior medial protocerebrum. All adult-specific secondary neurons in this lineage persistently express *ems* during postembryonic larval development and continue to do so throughout metamorphosis and into the adult. Mosaic-based MARCM mutant analysis and genetic rescue experiments demonstrate that *ems* function is autonomously required for the correct number of cells in the persistently expressing adult-specific lineage. Moreover, they indicate that *ems* is also required cell autonomously for the formation of the correct projections in this specific lineage. This analysis of *ems* expression and function reveals novel and unexpected roles of a cephalic gap gene in translating lineage information into cell number control and projection specificity in an individual clonal unit of the adult brain.

2.2 INTRODUCTION

The insect brain is generated by stem cell-like neuroblasts that derive from the cephalic neuroectoderm. Neuroblasts divide repeatedly in an asymmetric mode which is self-renewing and generates smaller ganglion mother cells, which usually divide once to produce two postmitotic progeny (Pearson and Doe, 2004; Skeath and Thor, 2003). In insects such as *Drosophila*, which have complete metamorphosis, neuroblasts generate the primary neurons of the larval brain during embryonic development. Following a period of quiescence, most neuroblasts resume their asymmetric mode of proliferation during postembryonic larval development and generate the adult-specific secondary neurons which make up the bulk of the adult CNS (Prokop and Technau, 1991; Truman and Bate, 1988). The adult-specific neurons that are generated during larval life from each persistent neuroblast form a lineage-related cluster of immature neurons that extend fasciculated primary neurites into the neuropile but wait until metamorphosis to complete their extension to synaptic targets (Dumstrei et al., 2003; Pereanu and Hartenstein, 2006; Truman et al., 2004; Zheng et al., 2006). During metamorphosis development of the adult brain is completed through neuronal remodelling of larval functional neurons and final morphogenesis of adult-specific neurons (Lee et al., 2000; Marin et al., 2005; Zheng et al., 2006).

Recent analyses of *Drosophila* neurogenesis have identified developmental control genes that are involved in generating the larval brain. Expression analysis for over 30 of these genes has shown that specific combinations of gene expression characterize each of the approximately 100 embryonic brain neuroblasts (Urbach and Technau, 2003). For a number of these genes, loss of function analyses have revealed severe defects in neurogenesis, patterning, and circuit formation during embryonic brain development (Hirth et al., 1998; Hirth et al., 2003; Hirth et al., 1995; Kammermeier et al., 2001; Noveen et al., 2000; Urbach and Technau, 2003). In contrast, only a few of these developmental control genes have been studied in postembryonic development of the adult brain (Callaerts et al., 2001; Hassan et al., 2000; Hitier et al., 2001; Kurusu et al., 2000; Pereanu and Hartenstein, 2006).

The *empty spiracles* (*ems*) gene plays a central role in embryonic development of the brain (Lichtneckert and Reichert, 2005). The *ems* gene encodes a homeodomain transcription factor that acts as a cephalic gap gene during early embryogenesis (Cohen and Jurgens, 1990; Dalton

et al., 1989; Walldorf and Gehring, 1992). During embryonic neurogenesis, *ems* is expressed in 11 bilaterally symmetrical neuroblasts and later is found in the deutocerebral and tritocerebral embryonic brain neuromeres (Hirth et al., 1995; Urbach and Technau, 2003). Mutation of the *ems* gene results in the absence of cells in the deutocerebral and tritocerebral anlagen; this is due to the failure of neuroblasts to form in the mutant domain (Younossi-Hartenstein et al., 1997). In contrast to the insight into the role of *ems* in embryonic brain development, virtually nothing is known about expression or function of *ems* during postembryonic development of the adult brain.

This lack of information on *ems* action in postembryonic brain development in *Drosophila* contrasts with the wealth of information on the role of the *ems* orthologs, *Emx1* and *Emx2*, in mammalian brain development. Both of these mammalian genes are expressed in the early neuroectoderm and the embryonic progenitor cells that give rise to telencephalic brain regions (Simeone et al., 1992a; Simeone et al., 1992b). Mutant analysis indicates that these genes play important roles in early patterning and proliferation of anterior brain regions (Bishop et al., 2003; Cecchi and Boncinelli, 2000; Shinozaki et al., 2002). Mammalian *Emx* genes also act in later phases of brain development and are expressed in cells of the adult brain (Briata et al., 1996; Cecchi, 2002; Gulisano et al., 1996). Moreover, mutant analysis suggests a role of *Emx* genes in differentiation and maintenance of cortical neurons and in pathfinding of cortical efferents (Bishop et al., 2003; Shinozaki et al., 2002).

The finding that mammalian *Emx* genes play multiple roles in different stages of brain development underscores an emerging theme in vertebrate neuronal development. Thus, many developmental control genes implicated in early neurogenesis and patterning, are re-expressed and have different roles in later embryogenesis and postembryonic brain development (Salie et al., 2005; Zapala et al., 2005). Given the conservation of expression and function of fly *ems* and mammalian *Emx* genes in embryonic brain development, we set out to determine if the *Drosophila ems* gene might also play important roles in postembryonic development of the adult brain.

Here we show that *ems* is expressed in the adult brain in the clonal progeny of a single neuroblast in each brain hemisphere. All adult-specific secondary neurons in this lineage already express *ems* during larval development and continue to do so throughout

metamorphosis and into the adult. To investigate the role of the *ems* gene in the persistently expressing, adult-specific brain lineage, we use mosaic-based MARCM mutant analysis. Our findings demonstrate that *ems* function is cell autonomously required for the correct number of cells in this lineage. Moreover, they indicate that *ems* is also required cell autonomously for the formation of the correct neuritic projections in this specific lineage. This analysis of *ems* function reveals novel and unexpected roles of a cephalic gap gene in determining the anatomical features of an individual lineage-based unit in the adult brain of *Drosophila*.

2.3 RESULTS

2.3.1 The ems gene is expressed in the adult brain of Drosophila

To investigate if the *ems* gene is expressed in cells of the adult brain, we carried out an immunocytochemical analysis of whole mount brains 1-10 days after eclosion using an anti-Ems antibody. In all cases, *ems* expression was detected in two bilaterally symmetrical cell clusters of the central brain (Fig. 2-1A). To locate these *ems*-expressing cell clusters relative to the neuropile, double labelling experiments were carried out using the neuropile marker anti-nc82 in combination with the anti-Ems antibody. These studies showed that the two symmetrically arranged *ems*-expressing cell clusters are located ventral to the antennal lobes and dorsal to the suboesophageal ganglion near the anterior midline of the brain (Fig. 2-1B, C). No other cells in the central brain or optic lobes expressed *ems* in the adult brain.

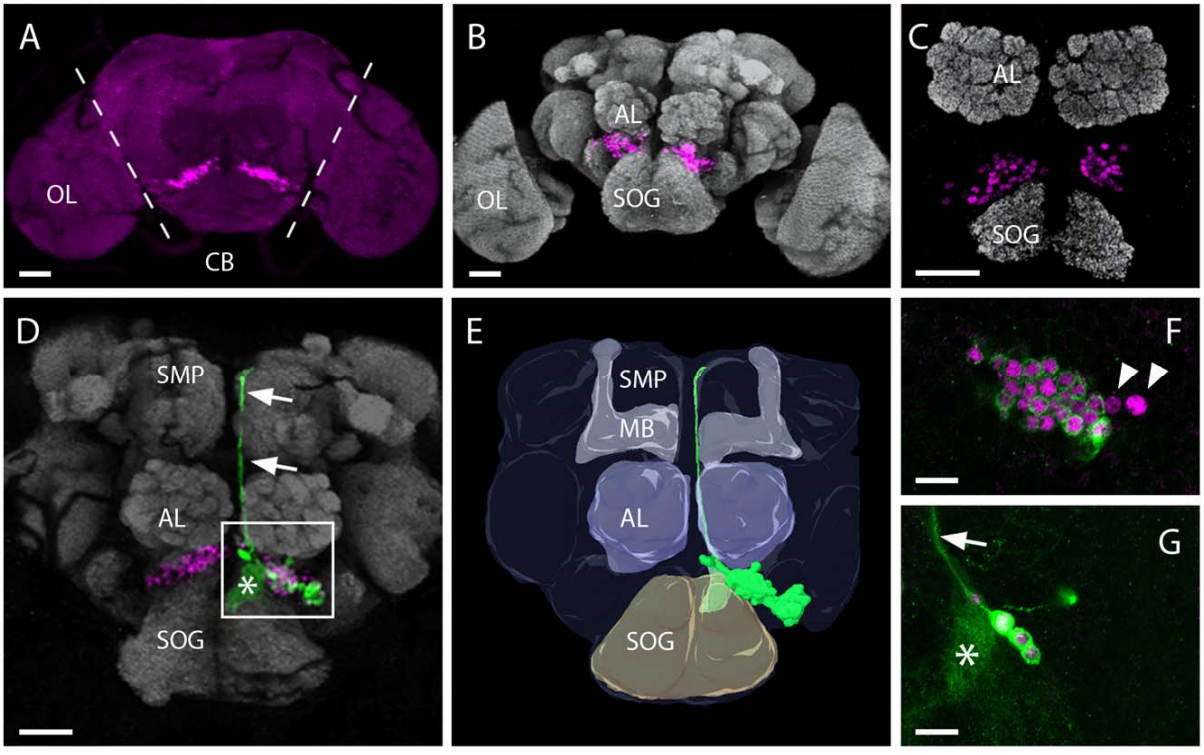

Figure 2-1 *ems* is expressed in one cluster of clonally related cells per hemisphere. Frontal views of adult brains. Anti-Ems labelling is magenta. (B-D) The neuropile marker Nc82 is white. (D-G) GFP labelled wild-type MARCM clones are green. (A,B) Z-projection of optical sections. Dashed line indicates border between central brain (CB) and optic lobes (OL). (C) Single optical section showing *ems* expression in cells between antennal lobes (AL) and suboesophageal ganglion (SOG). (D) Individual wild-type clone shows co-localization of GFP with Ems in adult-specific cells. Single optical section (higher magnification of selected area, box) reveals that

all GFP labelled cells express *ems* whereas a small subset of *ems*-expressing cells lack GFP (arrowheads in F). At deeper focal plane, the same clone as in F extends projection medially into superior medial protocerebrum (SMP; arrows in D,G) and arborizations into the adjacent SOG neuropile (asterisk in D,G). (E) 3D-model of (D) illustrating position of MARCM clone related to major neuropile compartments such as SOG, AL, mushroom bodies (MB) and SMP. Scale bars: 50 μm (A-D) and 5 μm (F,G).

The compact aspect of the *ems*-expressing cell clusters suggests that they might represent clonally restricted neuroblast lineages. To investigate this, we carried out a MARCM-based analysis (Lee and Luo, 1999; Lee and Luo, 2001). In these experiments, GFP-labelled wild-type clones were induced at random in early first instar larvae (21-25 hours after egg laying) to specifically label the secondary, adult-specific lineage of individual larval neuroblasts. The brains of adult flies that contained GFP-labelled MARCM clones were then co-labelled with anti-Ems and anti-Nc82. Brains, in which GFP-labelled clones were anti-Ems-immunoreactive, were analyzed further by confocal microscopy.

Co-labelling of cell bodies with GFP and anti-Ems was restricted to one clone per brain hemisphere (Fig. 2-1D). All of the GFP-labelled cells of this particular clone co-expressed *ems*. In addition, a few *ems*-expressing cells that were not GFP-labelled were closely associated with the cell cluster that co-expressed GFP and *ems* (Fig. 2-1F). These findings indicate that the majority of the cells in the *ems*-expressing clusters of the adult brain are secondary adult-specific neurons that derive in a clonal manner from a single larval neuroblast. (The non-GFP-labelled in the *ems*-expressing clusters may represent primary neurons generated by the same persistent neuroblast during embryogenesis.)

The *ems*-expressing cells in the GFP-labelled neuroblast clones were associated with a GFP-labelled fascicle that extended to more dorsal brain regions. To facilitate the analysis of this fascicle, a digital 3D-model of the projection and the major neuropile compartments along which the fascicle projected was generated (Fig. 2-1E). This showed that the fascicle projected from the GFP-labelled cell bodies medially along the antennal lobe to the ipsilateral superior medial protocerebrum. Hereafter this will be referred to as the “protocerebral fascicle” of the *ems*-labelled clonal cells. Close to the cell bodies, a dense arbor of labelled,

dendrite-like processes was observed; these short processes extended ventrally into the subesophageal neuropile (Fig. 2-1G, asterisk).

2.3.2 The ems gene is expressed in brain neuroblast clones during larval development

The restricted expression of *ems* in the adult brain suggested that it might be required for the development of the neuroblast lineage. This prompted us to examine the expression of *ems* at earlier stages. Anti-Ems immunolabeling was found in several distinct cell clusters in each brain hemisphere of late third instar larvae including a prominent cluster located near the medial edge of each hemisphere (Fig. 2-2A). (Additionally, scattered cells in the subesophageal ganglion also expressed *ems*; these cells were not considered further in this study.) No *ems* expression was seen in the developing optic lobes. The architecture of these *ems*-expressing clusters was further examined in double immunolabeling experiments using anti-Ems in combination with anti-Neurotactin (Fig. 2-2B). Neurotactin is highly expressed on fasciculated neurites of immature neurons, and anti-Neurotactin-labelling can therefore be used to reconstruct secondary lineages in the larval brain (de la Escalera et al., 1990; Pereanu and Hartenstein, 2006).

A total of eight cell clusters with *ems* expression were found in each brain hemisphere. These cell clusters could be unambiguously identified based on their relative positions and on the projection pattern of their primary neurite bundles (Pereanu and Hartenstein, 2006; Truman et al., 2004). Each cell cluster contained a large *ems*-expressing cell near the cortex surface associated with a columnar-like aggregate of smaller cells which co-expressed *ems* and *neurotactin* (Fig. 2-2C, D). In each cell cluster, a *neurotactin*-expressing fascicle emerged from the smaller *ems*-expressing cells (Fig. 2-2D). This fascicle extended towards the brain neuropile (Fig. 2-2E). A digital 3D-model of all eight *ems*-expressing cell clusters and their primary neurites is shown in Fig. 2-2F. The medial cluster (asterisk) with its dorsally projecting fascicle (arrow) is clearly identifiable in this model.

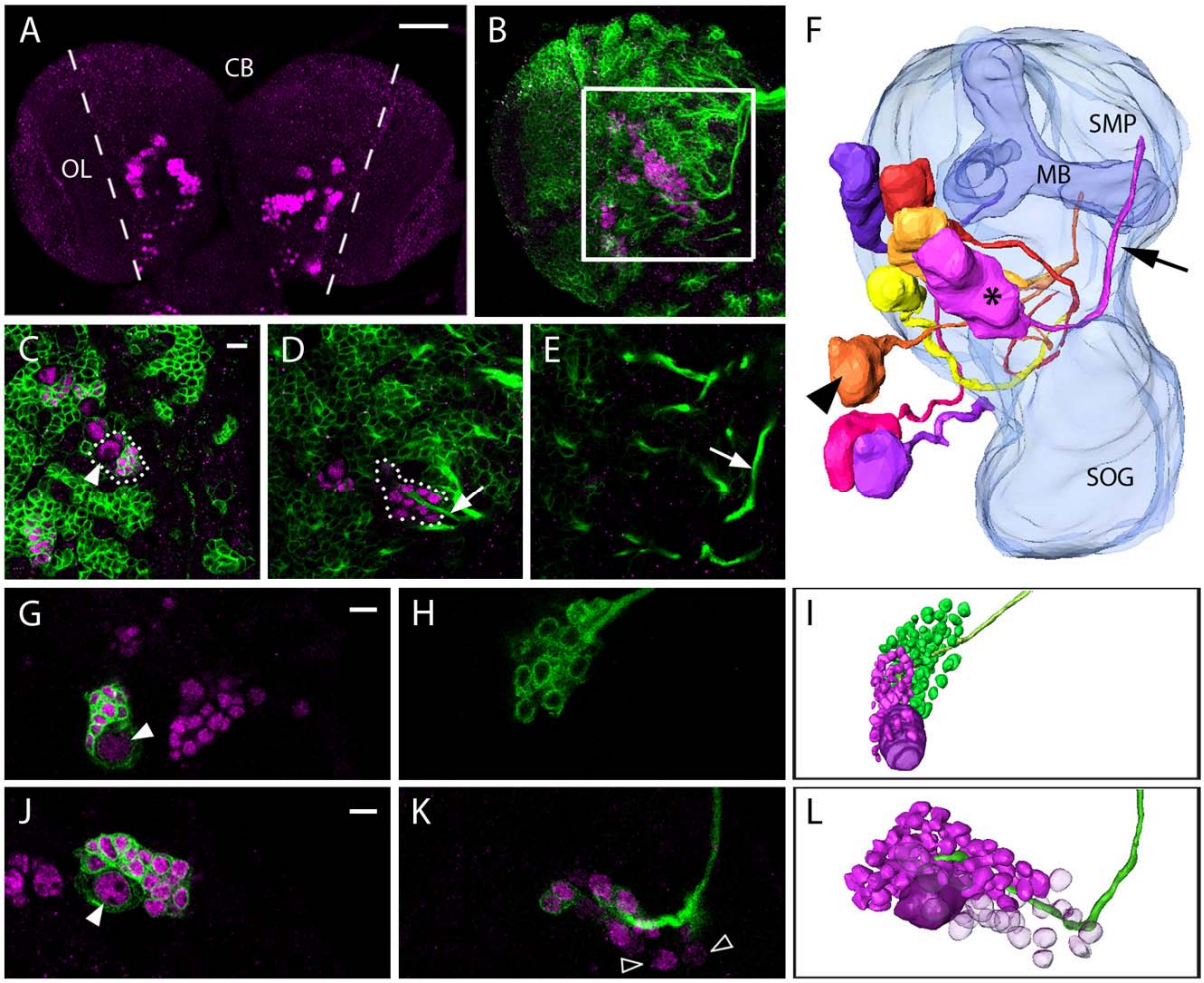

Figure 2-2 *ems* expression is restricted to eight neuroblast lineages in the larval brain. Ventral views wandering stage larva; anterior to top. Anti-Ems labelling is magenta. Green indicates anti-Neurotactin in (B-E) and membrane-bound GFP labelled wild-type MARCM clones in (G-L). (A) Z-projection of optical sections. Dashed line indicates border between CB and OL. Analysis of Ems-positive clusters was performed on stacks of optical sections (B-E) and a 3D-model (F) was generated. (B) Z-projection of one brain hemisphere showing selection (box) used for enlarged views of single optical sections (C-E). (C) Optical section close to surface of cortex showing neuroblast (arrowhead) in close contact with small cells of medial cluster (dotted). (D) At deeper focal plane, medial cluster cells (dotted) surround neurite bundle (arrow). (E) Optical section close to neuropile surface showing neurite bundle of medial cluster (arrow). (F) Digital 3D-model illustrates eight Ems-positive clusters and their neurite projections. Neuropile (blue) based on ChAT-promoter driven GFP expression. The eight *ems* expressing lineages could be tentatively assigned to the DAlv2, BAmas2, BAmv2, BAmv3, BAlp1, BAlp2, BAlp3, BAlc lineages of the late larval brain atlas (Pereanu and Hartenstein, 2006). Optical sections of wild-type MARCM clones recorded at different focal planes (G,J: superficial; H,K: intermediate) for *ems* expression analysis and 3D-modeling (I,L). In (F) an arrowhead marks the lineage corresponding to the clone shown in (G-I) whereas an asterisk indicates the EM lineage (J-L). Solid arrowheads indicate neuroblasts, open arrowheads indicate Ems-positive cells lacking GFP. Scale bars: 50 µm (A) and 5 µm (C, G, J). Abbreviations see Fig. 2-1.

These findings suggest that each *ems*-expressing cell cluster in the late third instar brain is composed of a persistent neuroblast and its progeny, which project fasciculated primary neurites into the neuropile. To determine if *ems* expression in the late third instar brain is restricted to adult-specific lineages, a MARCM based clonal analysis was carried out. GFP-labelled wild-type MARCM clones, induced in early first instar larvae, were recovered in the late third instar brain for all eight Ems-positive cell clusters. This indicates that each cell cluster represents a neuroblast clone. A clear difference in *ems* expression was observed between the medial *ems*-expressing lineage (hereafter referred to as the EM lineage) and the remaining seven *ems*-expressing lineages. In these seven lineages, *ems* expression was present in the neuroblast and in a small subset of the adult-specific neurons located adjacent to the neuroblast; these represent the late born cells in the neuroblast clone (Fig. 2-2G). In contrast, early born neurons located further away from the neuroblast did not express *ems* (Fig. 2-2H). This spatially restricted *ems*-expression pattern within neuroblast clones is illustrated in a digital 3D-model of one representative of the seven lineages (Fig. 2-2I; arrowhead in 2-2F).

A markedly different *ems*-expression pattern was observed in the EM lineage. In this lineage, *ems*-expression was present throughout the secondary lineage including the neuroblast and the adult-specific neurons, irrespective of their position within the clone (Fig. 2-2J, K). A digital 3D-model of the EM lineage (Fig. 2-2L) illustrates the fact that *ems* is expressed throughout the GFP-labelled clone. In addition, GFP-negative cells located close to the early born, adult-specific neurons of the GFP-labelled clone also expressed *ems*, albeit at a lower level (open arrowheads in Fig. 2-2K; lightly coloured cells in Fig. 2-2L). These could be progeny generated by the EM neuroblast during embryonic development before MARCM clone induction

2.3.3 ems-expression in the EM lineage persists through metamorphosis of the brain

Among the eight neuroblast lineages that express *ems* in the larval brain, only one, the EM lineage, expresses the gene in all of its secondary, adult-specific cells. This expression pattern also characterizes the single *ems*-expressing lineage in the adult brain and, together with their

similar location and neurite projection pattern, suggests that larval EM lineage might correspond to the adult lineage.

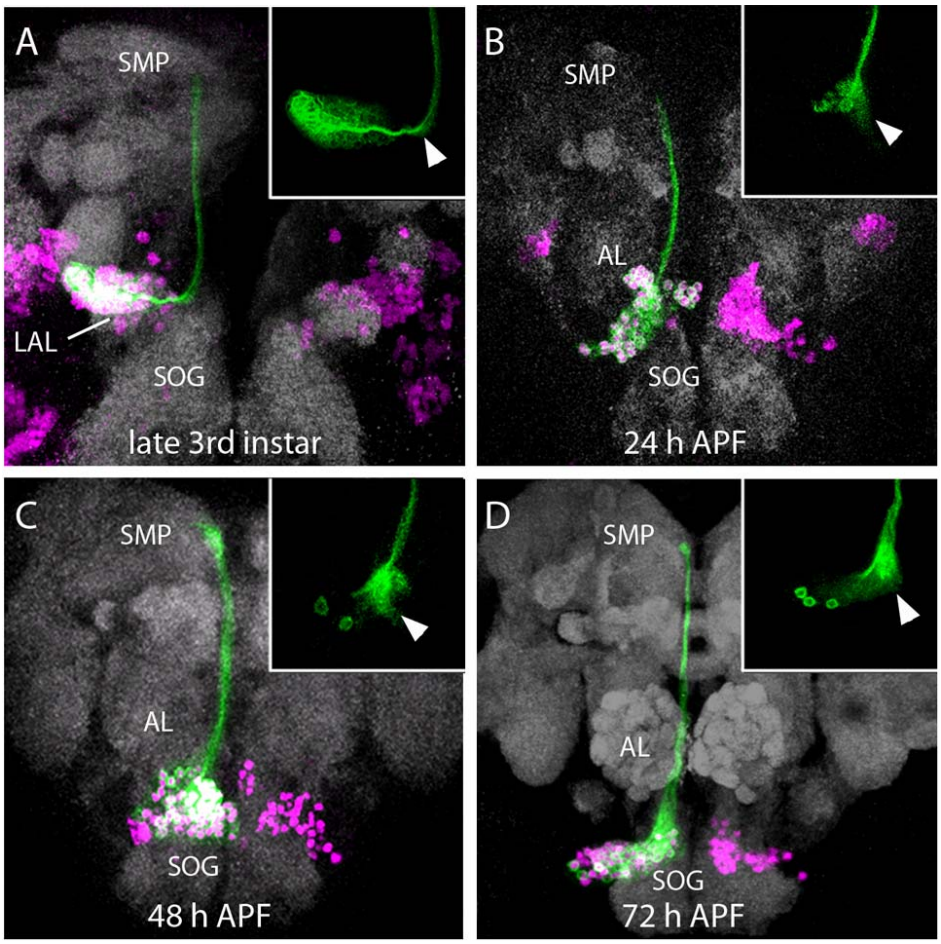

Figure 2-3 . *ems* expression in EM lineage persists through metamorphosis. (A-D) Brains double stained with anti-Ems (magenta) and nc82 (white). Specimens selected for GFP-labelled wild-type EM clones (green). Reconstructions of optical sections. Insets show optical sections at plane where clones extend neurites (GFP only). Short arborizations arise close to cell bodies during early pupal stages (arrowheads). Abbreviations: APF, after puparium formation; LAL, larval antennal lobe. Other abbreviations see Fig. 2-1.

To investigate this, GFP-labelled wild-type MARCM clones induced in early first instar larvae were examined at late third instar stage and at 24 hours, 48 hours, and 72 hours after puparium formation (APF). In all cases, only one GFP-labelled clone, which co-expressed *ems* in all labelled cells, was observed per brain hemisphere (Fig. 2-3). These clones were comparable in size and location in the brain cortex and had similar fascicle projections. Moreover, their overall morphology at 72h APF was very similar to that of the single *ems*-

expressing clone in the adult brain (compare Figs 2-3D and 2-1D). In contrast, *ems* expression in the other lineages was strongly reduced in the early pupa and completely disappeared at later pupal stages. This indicates that the larval EM lineage persists through metamorphosis maintaining both its *ems* expression features and its neurite fascicle projection pattern. One morphological change that did occur during metamorphosis in the cells of the EM lineage was the emergence of dense dendrite-like arborizations (Fig. 2-3 insets). In the early pupa, these short arborizations extended ventrally into the neuropile of the subesophageal ganglia. They were retained through metamorphosis and remained present in the adult brain (Fig. 2-1G).

Based on its overall morphology, we tentatively assign the EM lineage to the BAmas2 secondary lineage defined by Pereanu and Hartenstein (2006). Accordingly, this lineage is a member the basoanterior group of the ventral deuterocerebrum which surrounds the antennal compartment and projects its secondary lineage axon tract (SAT) upwards along the medial edge of the brain along the median bundle.

2.3.4 Neuronal precursors and postmitotic neurons are present in ems mutant EM lineages

To determine the role of the *ems* gene in the development of the EM lineage, *ems* mutant and wild-type MARCM clones were induced randomly in early first instar larvae and analyzed in late third instar brains. Mutant lineages were homozygous for ems^{9Q64}, an embryonic lethal loss-of-function allele of *ems*. This allele encodes a truncated non-functional protein that is detected by the anti-Ems antibody in the cytoplasm.

All labelled wild-type and *ems* mutant EM lineages contained one large cell, the neuroblast, which consistently expressed the transcription factor Grainyhead (Grh) (Fig. 2-4A, B). Smaller Grh-expressing ganglion mother cells (GMCs) were found directly adjacent to the neuroblast in both wild-type and mutant EM lineages. Moreover, expression of the mitotic markers Cyclin E (CycE) and anti-phosphorylated histone-H3 (H3p) was seen in neuroblasts and GMCs of both wild-type and mutant clones (Fig. 2-4C-F). These findings indicate that neuroblasts and GMCs are present and mitotically active in wild-type and *ems* mutant EM

lineages at late third instar stage (Almeida and Bray, 2005; Bello et al., 2003; Cenci and Gould, 2005).

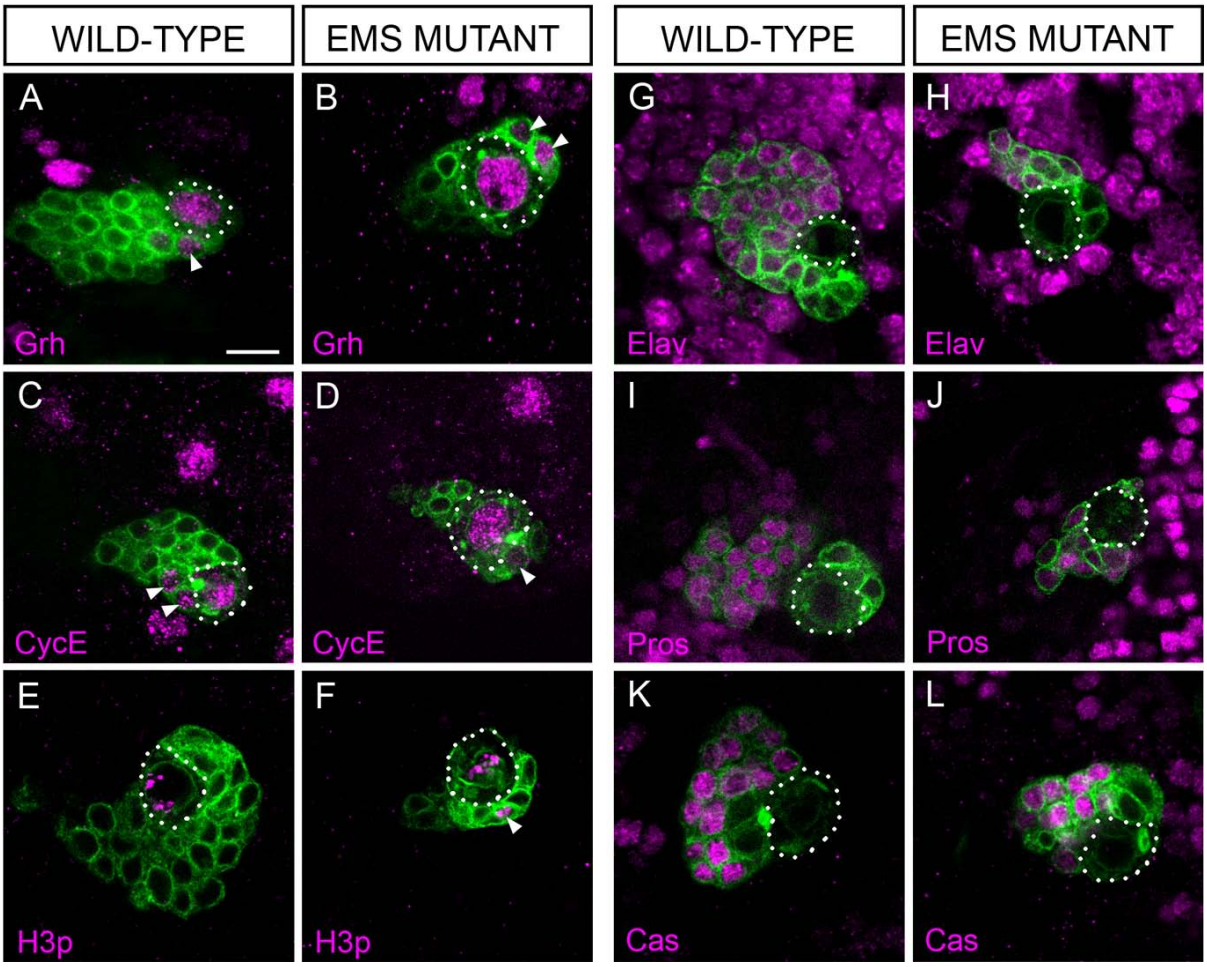

Figure 2-4 Cell types in the EM lineage are not altered in *ems* mutant clones. Single optical sections. Co-labelling of GFP-marked wild-type and *ems* mutant MARCM clones (green; for genotypes see Material and Methods) with antibodies against protein indicated on each panel (magenta). The anti-Ems immunoreactivity used for the identification of EM lineage is omitted for clarity. Neuroblasts encircled with dots; GMCs marked by arrowheads. Abbreviations see text. Scale bar: 5 μm.

In addition to precursors, the EM clones contained a number of smaller cells representing adult-specific neural progeny of the lineage which expressed the neuron-specific label Elav in wild-type and mutant clones (Fig 2-4G, H). In addition, two differentiation markers Prospero (Pros) and Castor (Cas) (Almeida and Bray, 2005) which were expressed in postmitotic neurons of the wild-type EM lineage, were also seen in the neural progeny of the *ems* mutant EM lineage (Fig. 2-4I-L). Taken together, this indicates that mitotically active progenitor cells

and differentiating neuronal progeny are present in the wild-type and *ems* mutant EM lineage in the late third instar brain.

2.3.5 The ems gene is required for correct neuronal cell number in the EM lineage

Although *ems* mutant EM lineages did contain postmitotic neurons, the number of neurons per clone seemed to be reduced (Fig 2-4). Reduction in clone size was clearly manifest in mutant clones of late third instar brains (Fig. 2-5B-G). Quantification of GFP-labelled cells revealed that the wild-type EM lineage contains on average 79 adult-specific cells (s.d. = 3.4; n = 7) while *ems* mutant clones had an average of only 36 labelled cells (s.d. = 12.8; n = 20) (Fig. 2-5A). To confirm that this reduction was due to *ems* loss-of-function, we carried out a clonal rescue experiment. For this, Ems expression was targeted in *ems* homozygous mutant clones using a UAS-*ems* transgene under the control of the MARCM *tub*-GAL4 driver. When examined in late third instar, the size of these rescued clones was restored to an average of 77 cells (s.d. = 4.5; n = 10) which was almost wild-type (Fig. 2-5A, also compare Fig. 2-5H-J and Fig. 2-5B-D). These findings indicate that the *ems* gene is required cell autonomously for the correct number of adult-specific neurons in the EM lineage.

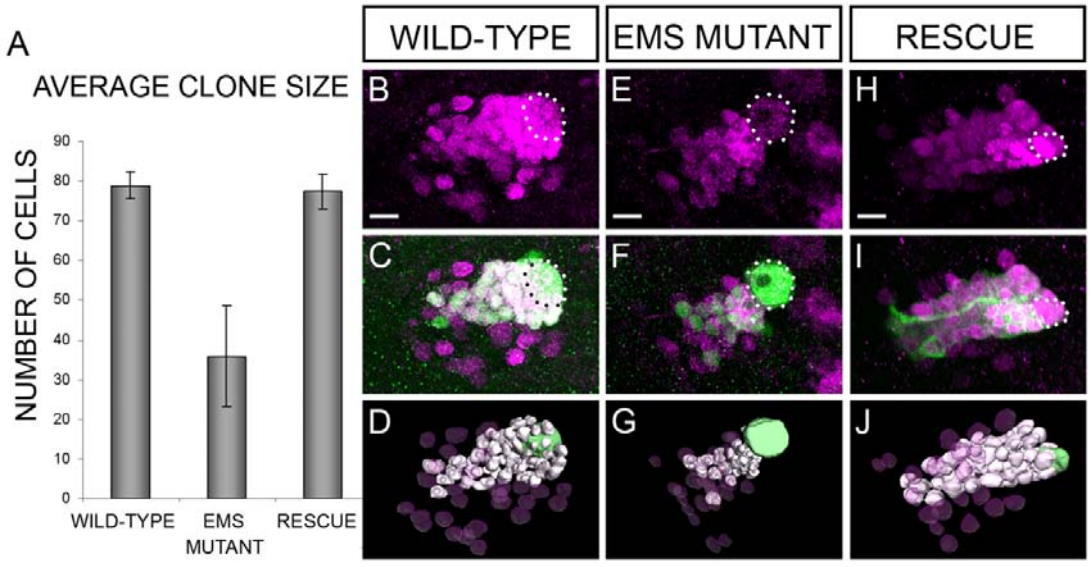

Figure 2-5 Reduction of cell numbers in *ems* mutant EM clones in the late larval brain. (A) Average cell numbers of wild-type, *ems* mutant, and rescued clones at late wandering larval stage (96 hours ALH) are indicated in bar graph (for genotypes see Material and Methods). Wild-type (B-D), mutant (E-G) and rescue (H-

J) clones co-labelled with anti-Ems (magenta), and anti-β-GAL (green in C,F) or GFP (green in I) and shown in Z-projections. Note that in (I) a membrane-bound GFP marker results in weaker overlap with the nuclear anti-Ems signal as compared to the nuclear anti-β-GAL in (C and F). Digital 3D-models were generated to visualize clone size (white in D,G,J). (Ems-positive cells not co-labelled with clonal marker are shown in light magenta.) Neuroblast outlined with dots in confocal images and in green in the 3D-models. Scale bars: 5 µm.

In order to obtain insight into the mechanisms of clone size reduction in *ems* mutants, a more detailed characterization of the EM lineage during postembryonic development was carried out for both wild-type and mutant clones. First, the number of cells in EM clones (induced at early first instar) was determined at different larval and pupal stages (Fig. 2-6). At 48h after larval hatching (ALH), mutant and wild-type clones contained a similar number of cells suggesting that initially postembryonic proliferative activity in the EM clones was not affected by *ems* loss-of-function. Marked differences between mutant and wild-type clones became apparent at 72h ALH in that the *ems* mutant clones contained fewer cells than the wild-type clones. This difference had increased at 96h ALH and remained large through pupal development and in the adult.

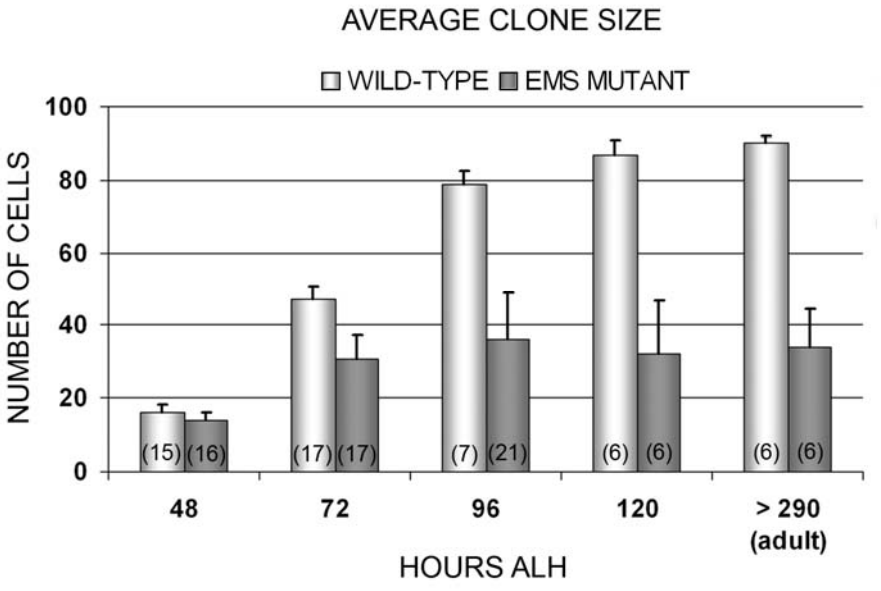

Figure 2-6 Wild-type and *ems* mutant EM clone size at different developmental stages. MARCM clone induction occurred at 0 h ALH (after larval hatching). Average number of cells is plotted against the time of analysis. Numbers of clones analysed indicated in brackets.

To determine if this difference in clonal cell number might be due to reduced proliferative activity in the *ems* mutants, we next studied the incorporation of BrdU into wild-type and mutant clones at the mid third instar stage (see Chapter 7 Experimental Procedures). In wild-type clones an average of 16.2 (s.d.=2.2., n=13) and in *ems* mutant clones and average of 15.9 (s.d.=1.9; n=13) labelled cells were observed indicating that mitotic activity was similar in the two cases. Furthermore, the percentage of EM neuroblasts expressing the mitotic marker H3p at mid third instar stages was comparable in both cases; 43% of wild-type neuroblasts (n=74) and 50% of *ems* mutant neuroblasts (n=14) expressed the marker. (Comparable findings were obtained for late third instar larva; data not shown). Taken together, these findings imply that the proliferative activity was not significantly reduced in *ems* mutant EM clones.

To investigate if the reduction in clonal cell number might involve the death of postmitotic cells, we initially stained *ems* mutant clones in late third instar brains with the apoptosis marker cleaved caspase 3. All of the *ems* mutant EM clones studied contained 1-4 cleaved caspase 3-positive cells (average=2.4, s.d.=1.0, n=10) indicating the presence of apoptosis in the mutant lineages. To determine if apoptosis can account for the reduction in clonal cell number observed in the *ems* mutant lineage, we next blocked cell death in *ems* mutant clones through misexpression of the pancaspase inhibitor P35. For this, clones were induced in early first instar larva and cell numbers determined at the late third larval stage. Blocking cell death resulted in mutant clones containing an average of 70 (s.d.=15; n=9) cells. This is comparable to an average of 79 cells in wild-type EM clones and an average of 77 cells in *ems* mutant clones misexpressing an *ems* transgene, and it is significantly higher than the average of 36 cells in *ems* mutant clones (see above). These findings imply that the reduction in clonal cell number in *ems* mutant EM lineages is due to apoptosis.

2.3.6 The ems gene is required for correct projections in the EM lineage

When examined in the adult brain, *ems* mutant MARCM clones in the EM lineage showed a second marked *ems* mutant phenotype. In many cases, mutant clones lacked the prominent protocerebral fascicle that projected to the superior medial protocerebrum in the wild-type

control (Fig. 2-7A-D). In other mutant clones a somewhat reduced protocerebral fascicle was formed (Fig. 2-7E, F). Moreover, in all *ems* mutant clones examined (n = 8), aberrant projections extended without obvious pattern towards adjacent neuropiles (Fig. 2-7C-F, arrowheads). Misdirected projections of this type were never observed in the wild-type control. These projection defects were fully restored in rescue experiments in which the *ems* transgene was misexpressed in the *ems* mutant EM clone (Fig. 2-7G, H). Rescued clones had a normal protocerebral fascicle and never showed short aberrant or misdirected process extensions. These findings indicate that the *ems* gene is required cell autonomously for the correct projection pattern of adult-specific neurons in the EM lineage.

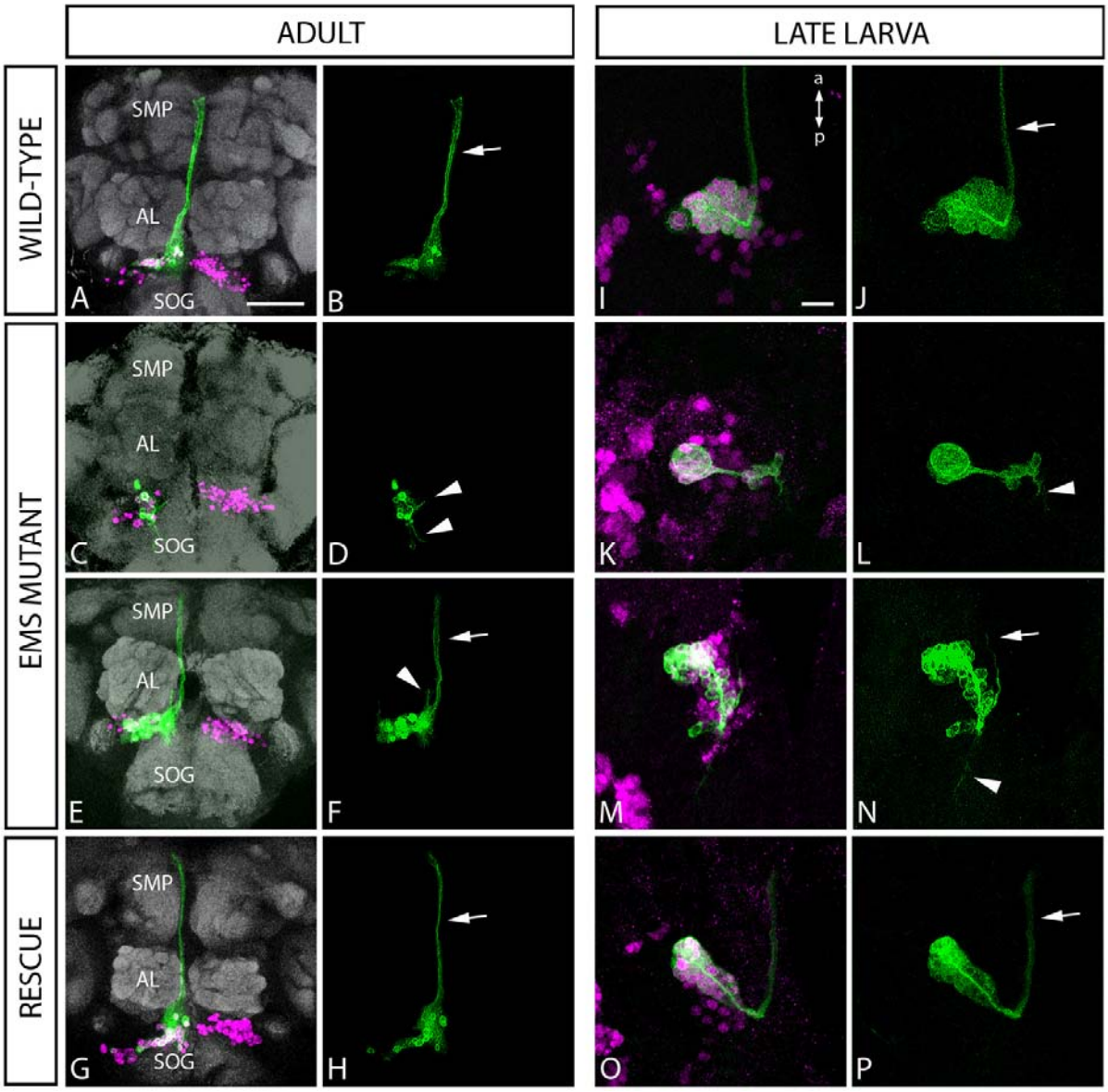

Figure 2-7 *ems* is required for the formation of correct projections of the EM lineage. GFP-labelled MARCM clones (green) analyzed in adult (A-H) or late wandering larval stage (I-P). Anti-Ems antibody

(magenta) labels both wild-type protein and truncated form. Neuropile labelled with Nc82 (white); relevant compartments labelled as in Fig. 2-1. Only one hemisphere is shown in larva; double-arrow indicates anterior (a) to posterior (p) axis. Merged images in left columns, clones (GFP channel only) in right columns. Protocerebral projection (arrow), ectopic neurites (arrowheads). Scale bars: 50 μm (A), 5 μm (I).

Since the primary fascicle of the EM lineage is formed in larval stages, it is possible that the projection phenotype observed in the adult *ems* mutant EM lineage first manifests itself in the larval brain. Alternatively, the larval fascicle may develop normally in the mutant lineage and then become disrupted during metamorphosis. To investigate this, we characterized the primary fascicle of the EM lineage in wild-type and *ems* mutant MARCM clones at the late third instar. In contrast to the wild-type clones, approximately half of the *ems* mutant EM lineages (11/20) showed a complete lack of the primary fascicle, and all of the mutant lineages (20/20) had ectopic misdirected process extensions (Fig. 2-7I-N). These larval projection defects were fully restored in rescue experiments in which the *ems* transgene was misexpressed in the *ems* mutant EM clone (Fig. 2-7O, P). This indicates that *ems* function is already required during larval stages for the formation of correct projections by adult-specific EM neurons.

To determine if the cell autonomous requirement of the *ems* gene for correct projection of the EM lineage occurs at the level of postmitotic cells, we analysed single cell MARCM clones in third instar larval brains. Single labelled wild-type cells had neuronal morphologies that were expected for the EM lineage (Fig. 2-8A, B). Thus, labelled cells had a cell body located in the appropriate region of the brain cortex as well as a projection pattern in the brain neuropile consisting of a single process that extended towards the midline, turned anteriorly, projected to the anterior protocerebrum, and there formed arborizations. In contrast, most of the single labelled *ems* mutant cells showed dramatic projection defects. In some cases, labelled cells extended processes posteriorly that arborized in the subesophageal ganglion (Fig. 2-8C, D). In other cases, labelled cells failed to extend any process (Fig. 2-8E, F). These observations indicate that individual postmitotic EM cells manifest a cell autonomous requirement for the *ems* gene in order to establish their appropriate neuronal morphology.

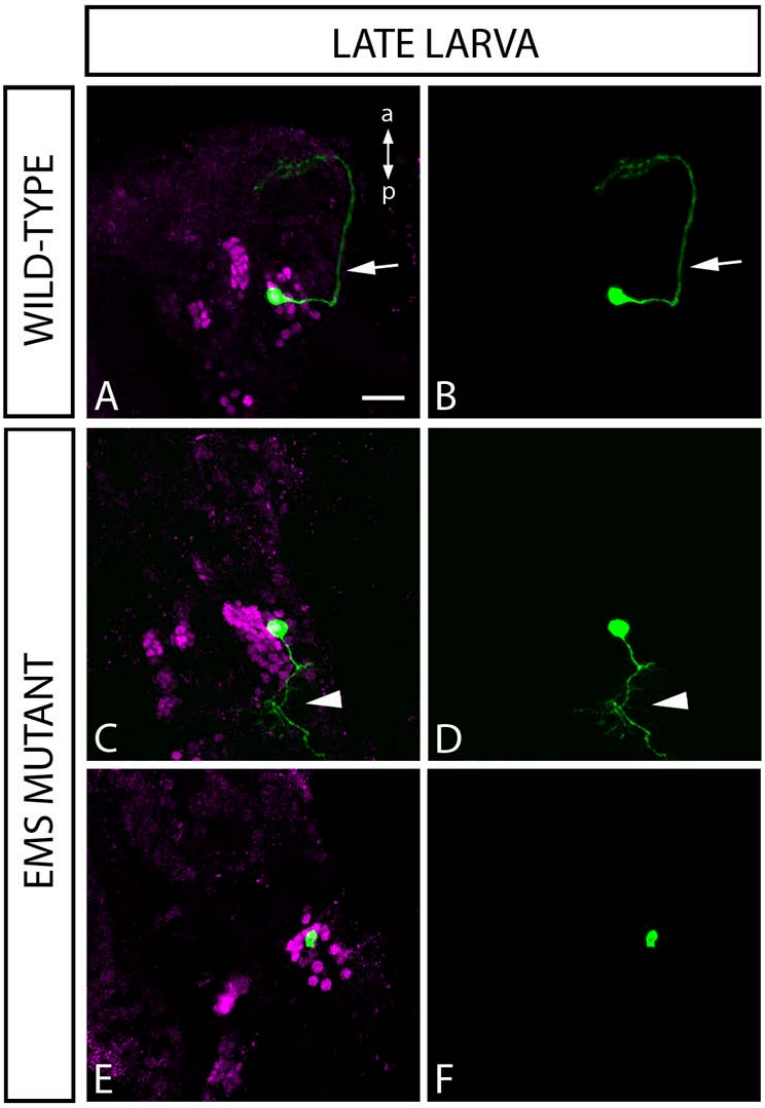

Figure 2-8 *ems* is required for the formation of correct projections in postmitotic neurons of the EM lineage. Single cell clone analysis. Viewed as in Fig. 7I-P. Single GFP-labelled cells (green) analyzed in late wandering larval stage. MARCM clone induction at 48 hours ALH. GFP-clones in right columns, merged images with anti-Ems signal (magenta) in left columns. Protocerebral projection (arrow), ectopic neurites (arrowheads). Scale bar: 5 μm.

Taken together, our MARCM-based mutant analysis indicates that *ems* loss-of-function leads to two cell autonomous phenotypes in the adult-specific EM lineage of the brain. First, the number of adult-specific cells in the mutant EM lineage is dramatically reduced. Second, marked projection defects occur in the protocerebral fascicle of the adult-specific neurons in the mutant EM lineage.

2.4 DISCUSSION

2.4.1 Expression of ems in postembryonic neuroblast lineages

During postembryonic development of the *Drosophila* brain, expression of the *ems* gene is observed in eight neuroblast lineages per hemisphere. In seven of these, *ems* expression is transient and disappears during pupal development. This cessation of expression during metamorphosis could be related to the dynamic pattern of *ems* expression within each lineage. Thus, during larval development of these lineages, *ems* expression appears limited to the neuroblast and its recently generated progeny, suggesting that expression in the progeny may be transient. This type of dynamic expression could explain the fading out of the Ems-signal in the seven lineages once their neuroblasts stop proliferation during pupation.

In contrast, in the eighth neuroblast lineage, *ems* expression is persistent. During larval development the neuroblast and all of its adult-specific progeny express *ems*; this expression continues throughout metamorphosis and into the adult in all postmitotic cells of the EM lineage. The mechanisms responsible for the maintenance of *ems* expression in the adult-specific cells of the EM lineage are currently unknown. However, there is some evidence that *ems* is also expressed and maintained in the primary neurons of the EM lineage generated during embryogenesis. In all postembryonic stages and in the adult, approximately 30 *ems*-expressing neurons are closely associated with the early born, adult-specific neurons of the EM clone. These neurons are not generated postembryonically, and their number does not change significantly during postembryonic development. This suggests that the mechanisms responsible for the persistence of *ems* expression in the EM lineage may operate in all cells of the lineage, embryonic and postembryonic.

During early embryogenesis, *ems* is expressed in a total of eleven neuroblasts per embryonic brain hemisphere (Urbach and Technau, 2003). An unambiguous link between these embryonic brain neuroblasts and the eight postembryonic *ems*-expressing neuroblasts has not yet been established. If the persistent expression of *ems* is a unique feature of the EM lineage, it should be possible to trace this lineage back into embryonic stages and identify its embryonic neuroblast of origin. For the remaining seven postembryonic *ems*-expressing neuroblasts this may be more difficult and require a combination of molecular markers and

neuroanatomical lineage mapping (Pereanu and Hartenstein, 2006; Younossi-Hartenstein et al., 2006).

The postembryonic expression of *ems* in the fly brain has interesting parallels to the expression of the *Emx1* and *Emx2* genes in the mammalian brain. In addition to early expression in the neural plate, the *Emx1* gene is expressed in a many differentiating and mature neurons of the murine cortex (Briata et al., 1996; Gulisano et al., 1996). Brain-specific expression of *Emx2* appears to be more transient in later stages and in the adult brain seems to be restricted to neural stem cells (Gangemi et al., 2001; Mallamaci et al., 1998; Mallamaci et al., 2000). Thus, spatially restricted persistent and transient expression patterns are observed for the *ems/Emx* genes in neural progenitors and in neurons during brain development and maturation in flies and mice.

2.4.2 Functional roles of ems in the EM lineage

For mutant analysis of *ems* function we focused on the EM lineage and used clonal techniques to ensure that the secondary adult-specific neurons are mutant from the time of their birth onwards. Two lineage-specific mutant phenotypes are apparent in these loss-of-function experiments. The number of adult-specific neurons is reduced and projection defects occur in mutant clones. Both phenotypes are cell-autonomous, and both can be fully restored in genetic rescue experiments. Moreover, both mutant phenotypes are seen in larval stages and persist in the adult brain. These findings implicate the *ems* transcription factor in translating lineage information into neuronal cell number control and neurite projection specificity.

There are several possible explanations for the 50% reduction in cell number observed in *ems* mutant EM clones. First, proliferation of the mutant neuroblast might cease due to cell cycle arrest or to premature neuroblast death. This seems unlikely since proliferating neuroblasts can be identified in larval *ems* mutant clones based on expression of specific markers. Second, cell division of ganglion mother cells might be suppressed in favour of a direct differentiation of each neuroblast progeny into a single neuron, resulting in a total clone size reduction of 50%. This also appears unlikely since GMCs expressing a cell proliferation marker can be identified repeatedly in mutant clones indicating that they divide normally to produce two

daughter cells. Third, the time window of proliferative activity or the proliferation rate of the persistent neuroblast is shortened in *ems* mutants. While we cannot rule out this possibility, it appears unlikely as well for the following three reasons. First, mutant and wild-type clones contain a similar number of cells at 48h ALH suggesting that the proliferation rate is not affected at this stage. Second, BrdU incorporation studies reveal no difference in mitotic activity at late larval stage brains of wild-type versus *ems* mutant clones. Third, the percentage of neuroblasts expressing the mitotic marker H3p at late larval stages was comparable for wild-type and *ems* mutant clones. The final explanation for the marked reduction in cell number seen in mutant clones is that postmitotic cells die due to apoptosis. This possibility is supported by two observations. Late larval *ems* mutant EM clones contain apoptotic cells as assayed by the apoptosis marker cleaved caspase 3. Blockage of cell death in the *ems* mutant lineage through a pancaspase inhibitor results in significant restoration of the clonal cell number to a value comparable to that observed in the wild-type. Based on these findings, we posit that *ems* is required in the adult-specific EM lineage for survival of clonal postmitotic progeny.

Two types of neurite projection defects are observed in *ems* mutant EM lineages. First, in the adult brain of all *ems* mutants, short aberrant projections extend from the cell bodies in a misdirected manner into adjacent neuropile. Misdirected projections of this type are also present in the larval *ems* mutant EM lineages. This suggests that *ems* is already required during larval stages to prevent the formation of these misprojections. Whether the aberrant projections formed in the larva persist into the adult or whether misprojections of this type are continuously formed (and retracted) during metamorphosis and in the adult is currently not known. However, the fact that neurite projections, albeit short and ectopic, are formed in all mutant EM clones implies that the *ems* gene is not required for process outgrowth per se. Rather, the *ems* gene appears to be required to prevent the formation of misdirected processes, suggesting a role of the gene in neuronal pathfinding.

A second projection defect is observed in the adult brain in approximately half of the *ems* mutant EM lineages. It consists in the complete absence of the fascicle projecting to the superior medial protocerebrum. This projection phenotype in the adult has a corresponding projection phenotype in the larva, in that the primary neurite bundle is missing in approximately half of the mutant lineages. These observations suggest that the formation of

the primary neurite bundle during larval development might be a prerequisite for the process extension to adult-specific targets during metamorphosis; this would indicate a larval requirement of *ems* for neurite fascicle formation.

Both projection phenotypes seen in mutant neuroblast clones, short ectopic neurite projections and the absence of the fascicle to the protocerebrum, are also apparent in *ems* mutant single cell clones of the larval brain. Given that all other cells in the lineage, including the EM neuroblast, are wild-type-like in these experiments, this finding indicates that individual postmitotic neurons of the EM lineage have a cell autonomous requirement for the *ems* gene in order to form correct projections in larval brain development.

2.4.3 Are features of ems function in brain development general?

Our analysis of *ems* function in the EM lineage demonstrates that homeobox transcription factors can influence adult brain architecture in a cell autonomous and lineage-specific way. A lineage-specific, cell autonomous requirement of other transcription factors in brain development has been shown for the olfactory projection neurons and for mushroom body neurons in *Drosophila* (Callaerts et al., 2001; Komiyama et al., 2003; Kurusu et al., 2000; Martini and Davis, 2005; Martini et al., 2000; Noveen et al., 2000). Thus, increasing evidence indicates that key developmental control genes, which operate early in embryogenesis, also act later in a lineage-specific manner in controlling anatomical features of the adult *Drosophila* brain. It may be a general feature of brain development, that developmental control genes implicated in early neurogenesis and patterning are re-expressed and have different roles in later embryogenesis and postembryonic brain development (Salie et al., 2005; Zapala et al., 2005).

A comparison of the role of *ems* in *Drosophila* brain development, with that of *Emx1* and *Emx2* in mammalian brain development is interesting especially when the cortical phenotypes of *Emx1/Emx2* double mutants are considered (Bishop et al., 2003; Shinozaki et al., 2002). The cortical surface area of *Emx1/Emx2* double mutants is about half that of wild-type and the thickness of the preplate and cortical plate is reduced, suggesting that *Emx* genes regulate the

numbers of cortical neurons. Moreover, *Emx1/Emx2* double mutants have major defects in the pathfinding of most cortical axons, implying an important role of *Emx* genes in axonal pathfinding in the brain. Thus, mutant analyses in *Drosophila* and mouse suggest that loss-of-function of *ems/Emx* genes may result in comparable brain phenotypes, namely in reduction of neuronal cell number and in neurite projection defects. This in turn suggests that the morphological differentiation of brain architecture in both flies and mammals may involve conserved functions of orthologous *ems/Emx* homeobox genes not only in the early embryo but also during later stages of brain development.

Acknowledgements:

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3 Precise connectivity of olfactory projection neurons requires transient *empty spiracles* expression during *Drosophila* larval development

Robert Lichtneckert, Lionel Nobs, and Heinrich Reichert

Manuscript in preparation

3.1 SUMMARY

In both insects and mammals distinct second order projection neurons make synaptic connections with specific olfactory receptor neurons (ORNs) and relay the olfactory sensory input coming from the periphery to higher brain centres. In *Drosophila* it has been previously shown that dendritic targeting of different projection neurons (PNs) is prespecified by their lineage identity and birth order. However, the genetic programs underlying the wiring specificity of the at least 34 distinct PN classes is still not understood. The cephalic gap gene *empty spiracles* (*ems*) encodes a homeodomain transcription factor that has been recently found to regulate correct cell number and neurite projection in an adult-specific neuronal lineage of the *Drosophila* brain. Here we show that transient *ems* function during early PN differentiation is required for precise dendritic targeting of the anterodorsal PN (adPN) lineage. In addition, transient *ems* function is essential for the formation of the correct number of lateral PNs (lPNs) suggesting that *ems* plays different roles in the two major PN lineages. Furthermore, we provide evidence that down-regulation of *ems* activity during later PN differentiation is crucial for correct dendritic and axonal targeting. Thus, tight temporal regulation of *ems* expression is required for proper connectivity of PNs. The finding that *ems* and its mammalian homologues *Emx1*/*Emx2* are both expressed in second order olfactory PNs (mitral cells in mammals) suggests that conserved genetic programs might be responsible for specific relay of olfactory information to higher brain centres.

3.2 INTRODUCTION

The formation of appropriate interconnections between specific neurons during development is a critical prerequisite for the proper functioning of adult central nervous systems (CNS). This requires the precise spatiotemporal generation of distinct sets of neurons and the extension of their dendrites and axons to the correct targets. Studies of CNS development in many model systems have demonstrated that transcription factors play key roles in these events at multiple levels: anteroposterior and dorsoventral patterning of the neuroepithelium, specification of neuronal fates, axon guidance to the target area, and selection of synaptic partners (Arber et al., 2000; Chen et al., 2003; Shirasaki and Pfaff, 2002). For example, ETS domain transcription factors coordinate synaptic connections between sensory and motor neurons in the reflex circuits of the vertebrate spinal cord (Arber et al., 2000). Furthermore, LIM-homeodomain as well as POU domain transcription factors specify distinct neuronal subpopulations and determine their axonal pathways in vertebrates and invertebrates (Certel and Thor, 2004; Thor et al., 1999; Tsuchida et al., 1994). In addition, the POU domain transcription factors, Acj6 and Drifter, regulate the connections between different subsets of ORNs and second order PNs of the olfactory system in Drosophila (Komiyama et al., 2004; Komiyama et al., 2003).

A typical neuroblast (Nb) in the *Drosophila* brain undergoes asymmetric division to generate again one Nb and a ganglion mother cell (GMC), which divides once to produce two postmitotic neurons. During a first, embryonic period of neuroblast divisions neurons are born that will form the functional larval CNS. Hereafter, these cells will be referred to as primary or larval-specific neurons. Following a period of quiescence, most Nbs resume proliferation during larval and early pupal stages to generate the vast majority of neurons of the adult brain (Prokop and Technau, 1991; Truman and Bate, 1988). These cells will be referred to as secondary or adult-specific neurons in this work. During the larval period, adult-specific neurons produced by one Nb (secondary Nb lineage) extend one collective neurite bundle (secondary axon tract or SAT) in a lineage-specific pattern towards the neuropile. Secondary neurons differentiate during metamorphosis, extending terminal axonal and dendritic branches into the target neuropile area (Pereanu and Hartenstein, 2006).

The olfactory pathway in *Drosophila* represents a suitable model system to investigate the genetic basis of neuronal wiring specificity down to single-cell resolution. From the periphery, ORNs bearing the same olfactory receptor converge their axons onto one of 45 – 50 individually identifiable glomeruli of the antennal lobe (Couto et al., 2005; Laissue et al., 1999). Distinct second order PNs connect to the ORNs in specific glomeruli and extend their axon to the mushroom body calyx and the lateral horn (Stocker et al., 1997). In the lateral horn, PNs form highly stereotyped axonal branching patterns, which are characteristic for each glomerular PN class (Marin et al., 2002; Wong et al., 2002). In the adult fly brain, 3 PN Nb lineages have been identified by clonal analysis (Jefferis et al., 2001). Two major lineages of PNs, the anterodorsal PNs (adPNs) and lateral PNs (lPNs), extend their dendrites to specific and mutually exclusive subsets of glomeruli in the antennal lobe. Birth order further specifies the identity of adPNs and thus their dendritic and axonal targets (Jefferis et al., 2001; Zhu et al., 2006). Several lines of evidence suggest that dendritic and axonal fine arborizations of PNs develop at the absence of olfactory input and is regulated by genetic programs (Jefferis et al., 2001; Jefferis et al., 2004; Komiyama et al., 2003; Marin et al., 2002; Wong et al., 2002; Zhu et al., 2006).

The cephalic gap gene *ems* was originally identified in a screen for zygotic patterning mutations (Dalton et al., 1989; Jurgens et al., 1984). It encodes a homeodomain transcription factor that is essential for proper brain development during embryogenesis. The *ems* mutant embryonic brain lacks the deutocerebral and tritocerebral neuromeres where the gene is normally expressed in the wild-type (Hirth et al., 1995). In a recent study, *ems* expression was described in 8 Nb lineages during larval brain development from which only one maintained *ems* expression into the adult stage. Clonal mutant analysis of *ems* function in the persistently expressing lineage revealed a role of *ems* in the establishment of correct neurite projections and in cell survival (Lichtneckert *et al*., 2007).

In this work, we show that two of the 8 Nb lineages, which express *ems* during larval development correspond to the adPN and lPN lineages. We provide evidence that *ems* is transiently expressed in all adult-specific adPNs and lPNs during early neuronal differentiation. Clonal loss of and gain of *ems* function revealed that transient *ems* expression is necessary for correct dendritic and axonal targeting of at least a subset of adPNs. In addition, we found that tight regulation of *ems* expression in lPNs is crucial for correct formation of the adult-specific lineage.

3.3 RESULTS

3.3.1 Two ems expressing neuroblast lineages show olfactory projection neuron-like morphology in the late larval brain

In an earlier study we have carried out a thorough clonal analysis of *ems* expressing neuroblast lineages and their secondary lineage axon tracts (SAT) in the late third instar larval brain. For this, we have induced wild-type MARCM clones in early first instar larvae and analysed the projection patterns of each of the secondary lineages based on labelling with a membrane-bound GFP (Lichtneckert *et al*., 2007). A comparison of the clonal morphologies revealed that two of totally eight *ems* expressing lineages followed a nearly identical SAT trajectory; they both first extended their axon bundle towards the ventral midline where they turned anteriorly to project towards the anterior lateral part of the central brain (Fig. 3-1 A, B). In spite of their similar projection pattern, the two lineages could be clearly distinguished based on the position of their cell bodies at the ventral side of the larval brain. The lineage, whose cell bodies were located more anteriorly was temporarily named E2 lineage (*ems*-positive 2), whereas the more posterior lineage was temporarily named E3 (*ems*-positive 3; Fig. 3-1 A', B').

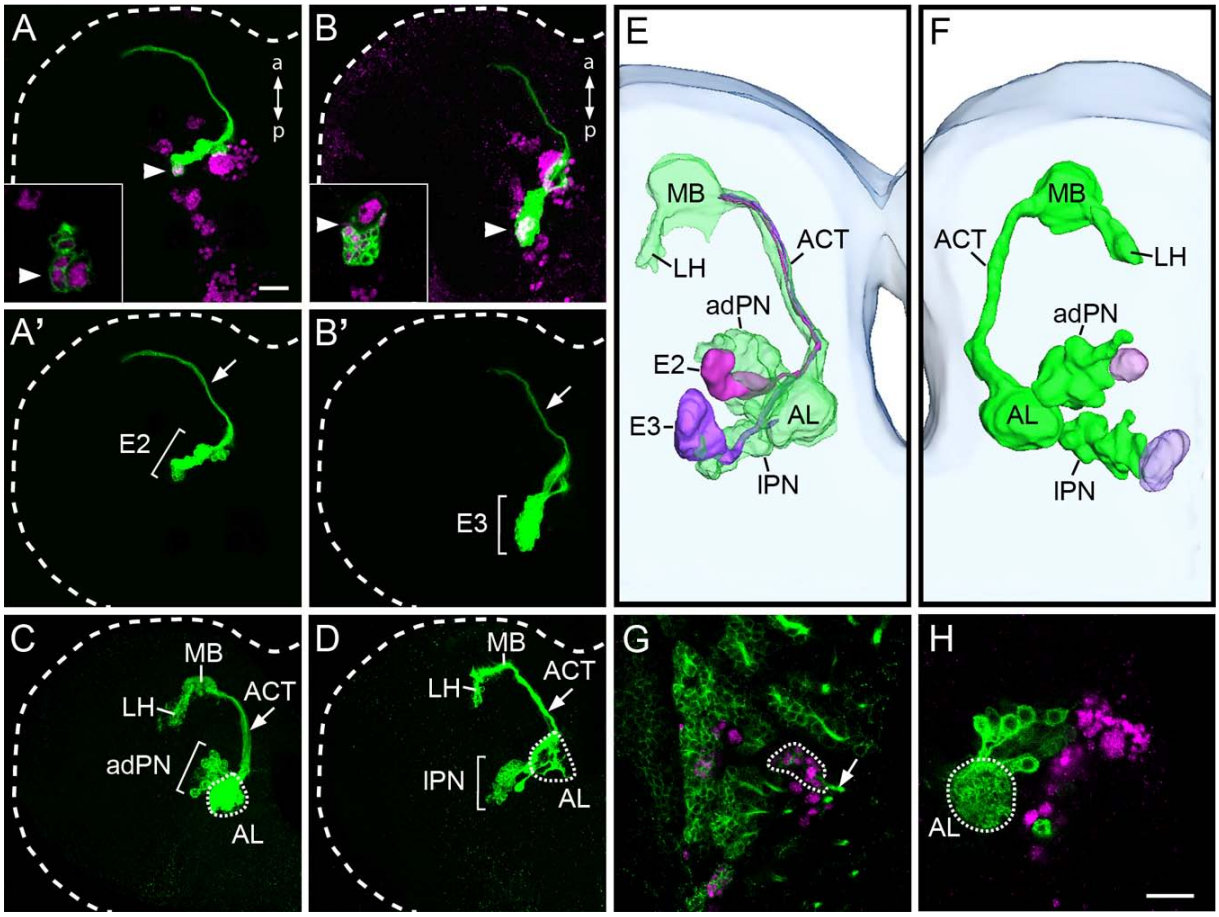

Figure 3-1 Two *ems* expressing neuroblast lineages show olfactory projection neuron-like morphology in the late larval brain. Ventral views of single hemispheres (outlined with dashed line in A – D, light blue in E, F). Double-headed arrow in (A) and (B) demarcates anterior (a) to posterior (p) axis on ventral midline. Anti-Ems is magenta. Green represents membrane-bound GFP, except in (G) Anti-Neurotactin. (A - D) z-projections of optical sections. Two identified wild-type MARCM clones express *ems*, E2 in (A and A') and E3 in (B and B'), and extend similar projections towards the anterior lateral brain (arrows in A' and B'). Insets in (A and B) show single optical sections through *ems* expressing cells at the position indicated by the arrowheads. Single brackets delimit the position of cell bodies in (A', B', C' and D'). (C and D) same orientation as (A and B). Two single projection neuron (PN) clones of the anterodorsal (adPN; C) and the lateral (lPN; D) lineages project to the mushroom body calyx (MB) and lateral horn (LH). Larval antennal lobe (AL) with dotted outline (C, D, H) and axonal projections along the antennal cerebral tract (ACT) with arrow. (E) digital 3-D model of left hemisphere illustrating E2 and E3 lineages (magenta and violet, respectively) projecting along the ACT labelled by GH146-positive adPN and lPN cells (transparent green). (F) digital 3-D model of right hemisphere illustrating position of *ems*-positive cells (transparent magenta and violet) relative to GH146-positive cells (green). (G) Co-labelling of Anti-Neurotactin (green) with Anti-Ems (magenta) and GAL4-GH146 UAS-mCD8::GFP (omitted for clarity) was used in the 3-D models (E and F) to reconstruct cell lineage surface (dotted) and projections (arrow). (H) single optical section showing absence of co-localization of Anti-Ems and GH146 signal in PNs. Scale bars: 10µm.

The analysis of the overall morphology of the E2 and E3 lineages revealed an unexpected similarity of the two *ems* expressing lineages with the projection pattern of the well characterized antennal projection neurons (PN) in the third instar larval brain. Therefore, we wanted to compare the E2 and E3 clones with single neuroblast MARCM clones in the PN lineages. For this purpose, we have used the enhancer trap line *GAL4-GH146* (hereafter referred to as GH146) as MARCM driver line that specifically labels a subset of all larval and adult olfactory PNs (Lai and Lee, 2006; Stocker et al., 1997). It has been shown before, that two major groups of PNs, which are located anterodorsal and lateral to the antennal lobe (adPNs and lPNs, respectively) are clonally derived from two separate neuroblasts (Jefferis et al., 2001). Both PN lineages connect the antennal lobe with the mushroom body calyx and the lateral horn of the protocerebrum.

A side-by-side comparison of single PN neuroblast clones that were induced in the early embryo with the *ems*-positive E2 and E3 clones was made in the late third instar brain (Fig. 3-1 A', B' versus C, D). Similarities as well as differences could be detected between the *ems*-positive clones and the PN clones. Striking resemblance was found for the relative positions of the cell bodies (brackets in Fig. 3-1 A-D) and the major neurite bundle (arrows in Fig. 3-1 A-D) with E2 being most similar to the adPN clone whereas E3 resembled more the lPN clone. The differences concerned mainly the neurite terminal projections where the PN clones showed more extensive dendritic and axonal innervations of the larval antennal lobe, mushroom body calyx and lateral horn (Fig. 3-1 C, D). However these differences can be explained by the different time of induction of the E2 and E3 clones with respect to the PN clones. The E2 and E3 clones were induced in the early first instar larva and thus only the postembryonic still undifferentiated portion of the lineages are labelled using a ubiquitous GAL4 reporter system (see Chapter 7 Experimental Procedures). In contrast, the PN clones were induced in the early embryo to label all GH146-positive cells in the respective lineages. Therefore, in PN lineages fully differentiated dendritic and axonal extensions could be observed in the respective neuropile compartments. Taken together, significant similarity between the E2 and the adPN clones and the E3 and lPN clones can be observed in the late larval brain. This indicates that *ems* might be expressed in adult-specific cells of the adPN and lPN lineages.

We next wanted to further test the hypothesis that the *ems* expressing lineages E2 and E3 correspond to the adPN and lPN lineages. For this, we have analyzed late third instar larval brains expressing a GFP reporter in GH146-positive cells that were co-stained with anti-Ems and anti-Neurotactin (Fig. 3-1 G-H). Anti-Neurotactin immunostaining labels fasciculated neurites of immature neurons and is therefore well suited to outline secondary lineages with their SATs in the larval brain (de la Escalera et al., 1990; Pereanu and Hartenstein, 2006). As described before, eight *ems* expressing lineages including their SATs were detected in each hemisphere and recorded as stacks of optical sections (Fig. 3-1 G; see also Lichtneckert *et al*., 2007). For better visualization, digital 3D-models were generated from the triple immunolabelled confocal stacks showing the two *ems* expressing lineages, E2 and E3, together with the GFP-labelled GH146-positive PNs in the central larval brain (Fig. 3-1 E, F). The cell bodies of the E2 and E3 secondary lineages which expressed *neurotactin* (brackets in Fig. 3-1 E) showed only very limited overlap with the 3-D rendered GH146 signal (transparent green in Fig. 3-1 E). However, this was expected since anti-Neurotactin labels immature neurons, whereas GH146 is restricted to differentiated PNs. In contrast, the corresponding SATs of the *ems*-positive cells, also labelled with anti-Neurotactin, showed a nearly perfect match with the axon bundle of the PNs along the antennal cerebral tract (ACT). No anti-Neurotactin signal could be recognized in the antennal lobe, mushroom body calyx and lateral horn neuropiles. Interestingly, no cellular co-localization of the anti-Ems and GH146 signals could be detected in any of the third instar larval brains examined (Fig. 3-1 H and Fig. 3-1 F).

Thus, in the third instar larval brain, identified secondary lineages that are characterized by *ems* expression show striking morphological similarities to the GH146-positive adPN and lPN lineages. This suggests, that the *ems* gene is expressed in a subset of cells of the adPN and lPN lineages in the third instar larva.

3.3.2 ems is expressed in the adPN and lPN lineages during larval development

The similar projection pattern of two identified *ems* expressing secondary lineages with the adPN and lPN lineages in the third instar larval brain suggested that *ems* might be expressed

in GH146 negative cells of the PN lineages during development. This prompted us to apply the dual-expression-control MARCM system (Lai and Lee, 2006) to the third instar larval brain when *ems* expression is observed in the E2 and E3 lineages. The dual-expression-control MARCM system allows the simultaneous labelling of homozygous GAL80-minus cells with two reporter systems that are differentially expressed within the Nb clone. Therefore, we have used the ubiquitous LexA::GAD driver labelling all the progeny of the GAL80-minus Nb, in combination with the GH146 driver to specifically label a subset of identified cells in the PN lineages (Lai and Lee, 2006). In case *ems* was expressed in the adPN and/or lPN lineage at third instar larval stage, we would expect to find single Nb clones labelled with the ubiquitous LexA::GAD driver that express *ems* in one subset and GH146 in a different subset of the clonally related cells.

In order to test this hypothesis, we have induced dual-expression-control MARCM clones in the early embryo for labelling of the complete lineage and analyzed the PN clones at 96 hours after larval hatching (ALH) in the late third instar brain (Fig. 3-2 A, B). Based on the morphology of the GH146 clones, we could identify the adPN and lPN lineages, respectively, and examine all GAL80-minus cells within the Nb clone using the ubiquitous lexA::GAD reporter system. As described in the adult, many additional cells that are negative for GH146 could be detected due to lexA::GAD expression (Lai and Lee, 2006). Furthermore, we have repeatedly found *ems* expression in one big cell, supposedly the Nb, and a number of adjacent smaller cells in both the adPN and lPN lineages. In both PN clones *ems* expression was mainly restricted to cells located close to the outer surface of the cortex (Fig. 3-2 A1, B1), whereas the GH146 signal was more frequent in cells deeper in the cortex and close to the antennal lobe (Fig. 3-2 A2, A3, B2, B3). Again, no overlap between the anti-*ems* and the GH146 signal in the same cell could be observed. Thus, applying the dual-expression-control MARCM system we have found molecular genetic evidence that *ems* is expressed in both the adPN and lPN lineages in the late third instar brain.

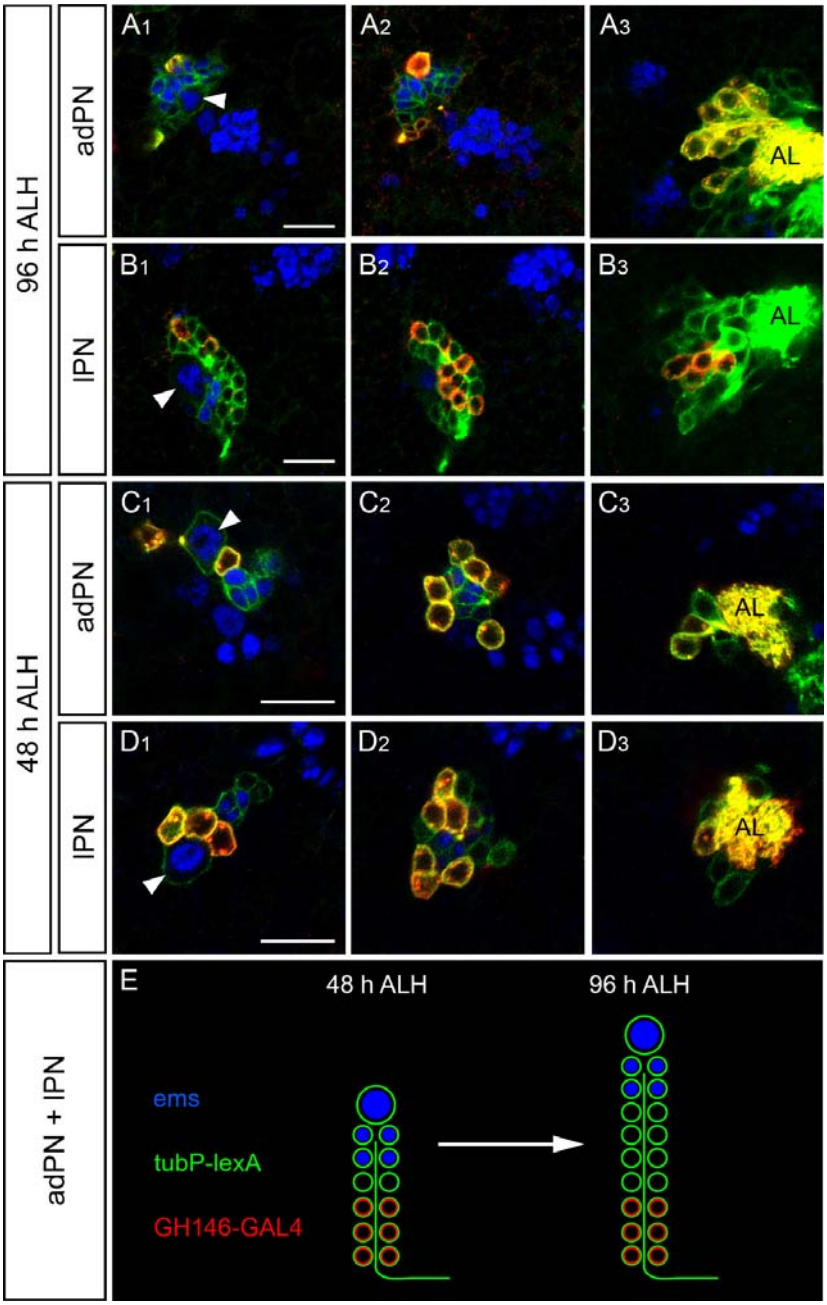

Figure 3-2 *ems* is expressed in adPN and lPN lineages during larval stages. Dual-expression control MARCM with ubiquitous *tubP-lexA::GAD* driving rCD2::GFP (green) and the subtype specific *GAL4-GH146* driving mCD8 in PN (red; see Materials and Methods for details). Co-localization of the two membrane-bound reporter constructs in yellow. Blue represents Anti-Ems. Clone induction by heat shock at 3-6 hours after egg laying. Clones were analyzed in late third instar larvae (A and B) at 96 hours after larval hatching (96 h ALH) and at second to third instar transition (C and D) at 48 hours ALH (48 h ALH). GAL80-minus adPN and lPN clones were identified via the GH146 signal and the whole lineage was labelled by the ubiquitous driver. (A – D) partial views from a ventral region including antennal lobes of single hemispheres. Anterior to the top, medial to the right. Image series (1 – 3) in (A – D) show single optical sections of same confocal stack with section (1) close to the outer cortical surface, (2) at deeper cortical level, (3) through antennal lobe at neuropile surface. *ems* expression is found in neuroblast (arrowhead) and adjacent cells but not in GH146-positive cells. (E) Model of transient *ems* expression during larval development (see text for details). Scale bars: 10µm.

Given the limited number of *ems*-positive cells and the lack of overlap with the GH146-positive cells within the PN lineages, we next wanted to test if *ems* expression in PN lineages is restricted to late third instar stage or if *ems* is also expressed earlier in larval development. Therefore, we have again induced dual-expression-control MARCM clones in the early embryo but now brains were analyzed at 48 h ALH. As expected, clones of both PN Nbs appeared smaller with respect to the late third instar stage. However, as in the late third instar brain, lexA::GAD expression revealed the presence of GH146-negative cells within the Nb clones. In both PN lineages, *ems* expression was again found in one Nb-like large cell and in adjacent smaller cells that were located close to the outer surface of the cortex (Fig. 3-2 C1, D1). No co-expression of *ems* and the GH146 signal in the same cells was found in any of the brains examined (Fig. 3-2 C2, C3, D2, D3). The same expression pattern of the *ems* gene within the adPN and lPN lineages was observed at 72 hours ALH and at 0 puparium formation (data not shown).

The restricted expression of *ems* to the putative Nb and its adjacent progeny is consistent with the observation that *ems* is transiently expressed in seven out of eight secondary lineages, during larval and early pupal development. Furthermore, *ems* expression has been shown to completely disappear from all transiently expressing brain lineages, including the E2 and E3 lineages, after 24 hours APF (Lichtneckert *et al*., 2007). Taken together, this suggests that *ems* is transiently expressed during the early differentiation steps of all larvally born PNs. As the PNs mature *ems* expression is down-regulated and stays off in the adult brain (Fig. 3-2 E).

3.3.3 Ems is required for correct neuronal cell number in the lPN lineage but not in the adPN lineage

We next wanted to further characterize the expression and function of *ems* in the adPN and lPN lineages at late third instar stage. For this, we have induced wild-type MARCM clones in the early first instar larvae and analyzed them in late third instar brains. A ubiquitous driver in combination with a membrane-bound GFP reporter was used in order to label all the adult-specific progeny of the PN Nbs. MARCM clones in the adPN and lPN lineages were recognized by the expression of *ems* and their projection pattern.

First, we have determined the average size of wild-type adPN and lPN clones as the total number of the GFP-labelled progeny and, additionally, the *ems*-positive cells within the clones were counted. Wild-type adPN clones had an average of 64 cells (s.d. = 2.3; n = 18) with 12 *ems* expressing cells (s.d. = 1.4; n = 18), whereas wild-type lPN clones had an average of 201 cells (s.d. = 4.9; n = 8) with 42 *ems*-positive cells (s.d. = 2.3; n = 8) (Fig. 3-3 A, B). For further characterization of *ems* expression within the PN lineages, we have analyzed wild-type clones co-labelled with *ems* and either the transcription factor Grainyhead (Grh), generally used as Nb and GMC marker in the larval brain, or the postmitotic neuron marker Elav (Almeida and Bray, 2005; Cenci and Gould, 2005; Robinow et al., 1988). All labelled wild-type PN clones contained one large cell, the Nb, that was characterized by the expression of Grh and the absence of Elav (dotted outline in Fig. 3-3 C, D, F, G). Similarly, one to three smaller cells, corresponding to GMCs, were located adjacent to the Nbs and also expressed Grh at the absence of Elav expression (arrow in Fig. 3-3 C, D, F, G). The *ems* gene was co-expressed with Grh in the Nbs and GMCs of both PN lineages. Clones co-stained with *ems* and Elav revealed that *ems* expression can be found in postmitotic neurons of both PN lineages (Fig. 3-3 D, G). Anti-*ems* staining, however, was always restricted to the Nb and its latest born progeny. This, further supports the notion that *ems* is transiently expressed in immature postmitotic neurons.

To determine the role of *ems* in the development of the secondary adPN and lPN lineages clones homozygous for the loss-of-function allele ems^{9Q64} were induced in the early first instar larva and analyzed in the late third instar larva, as done before for wild-type clones. The comparison of wild-type versus *ems* mutant clone size revealed a striking discrepancy in *ems* requirement of the two different PN lineages during larval development. *ems* mutant adPN clones had a virtually unchanged average number of cells (m = 62; s.d. = 7.3; n = 19) with respect to the wild-type (Fig. 3-3 A). In contrast, very few *ems* mutant lPN clones could be recovered as compared to wild-type lPN clone frequency and the average cell number in the mutant clones showed a 6-fold reduction (m = 33; s.d. = 21.9; n = 2) as compared to wild-type. This suggests, that in *ems* mutant lPN clones the cells are either not generated properly by the Nb or postmitotic cells die before late third instar stage. Thus, during larval development *ems* expression is required in the lPN lineage for the correct number of cells, whereas the average size of the adPN clones was not affected by *ems* loss-of-function.

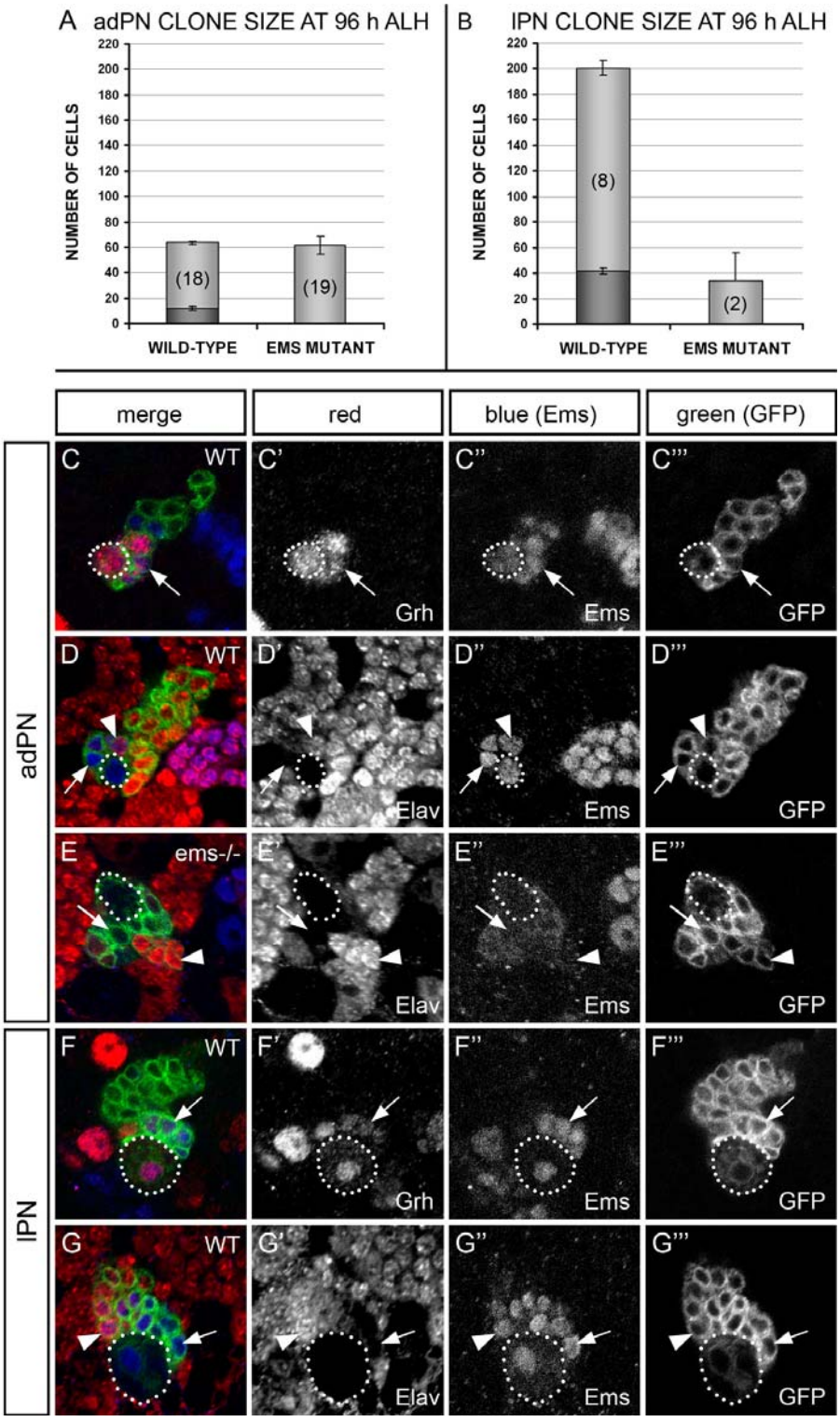

Figure 3-3 Reduction of cell numbers in *ems* mutant lPN but not adPN lineage in the late larval brain. (A and B) Average cell numbers of wild-type and *ems* mutant clones at late third instar larval stage (96 h ALH) are indicated in bar graph (for genotpyes see Materials and Methods). Clones were labelled with *tubP-GAL4* driven membrane-bound GFP and induced in early first instar larvae. (C – G) co-immunostaining of GFP-marked *tubP-GAL4* driven wild-type (WT in C, D, F, G) and *ems* mutant (ems-/- in E) clones (green) with Anti-Ems in (blue) and Grh or Elav (as inicated in second column; red). For each image (C – G) single channels are shown separately in a horizontal row. *ems* expression is found in neuroblasts (dotted outline), GMCs (arrow) and postmitotic neurons (arrowhead) in wild-type adPN and lPN lineages and in the *ems* mutant adPN lineage.

We next wanted to test whether neuronal differentiation is affected in *ems* mutant adPN clones by co-labelling with Elav and *ems* (the ems^{9Q64} allele encodes a truncated non-functional protein that is detected by the anti-Ems antibody in the cytoplasm and can therefore be used for the identification of the mutant lineage; see Fig. 3-3 E''). We have found that Anti-Elav staining was present in cells adjacent to the Nb in *ems* mutant adPN clones. Furthermore, wild-type and *ems* mutant adPN clones contained comparable percentages of Elav-positive cells, with an average of 96% (s.d. = 2.3; n = 7) in wild-type clones and 94% (s.d. = 7.4; n = 5) in mutant clones. This suggests, that the *ems* mutant adPN lineage generate normal numbers of progeny, which adopt a neuronal fate.

Thus, loss of *ems* function during larval development affects the number of cells in the lPN lineage whereas generation and differentiation of *ems* mutant adPN appears to be unchanged with respect to wild-type in the late third instar brain.

3.3.4 Ems is required for proper dendritic targeting of adPNs

Since *ems* is expressed in the anterodorsal and lateral PN Nbs and transiently in their larval progeny, we hypothesized that *ems* might play a role in proper innervation of PN targets in the adult brain. To test our hypothesis, wild-type and *ems* mutant MARCM clones were generated that specifically labelled single PN lineages with a membrane-bound GFP under the control of the GH146 driver (Fig. 3-4 H ; see Materials and Methods for details). Clones were induced in early first instar larvae in order to label all adult-specific GH146-positive cells. From 34 adult brains, 9 adNb and 11 lNb clones (neuroblast clones of adPN and lPN lineage, respectively) were recovered. All wild-type clones showed the typical overall PN morphology connecting the antennal lobe with the mushroom body calyx and lateral horn (Fig. 3-4 A, B). Moreover, all wild-type PN clones examined, densely and exclusively innervated the subset of glomeruli in the antennal lobe specific for either the adPNs or lPNs (Fig. 3-4 C, F). For the *ems* mutant analysis we have recovered 24 adNb clones from totally 126 adult brains examined, which corresponds to the frequency observed with wild-type control clones. In contrast, only 2 *ems* mutant lNb clones were detected in 126 brains, which is about 20-times less frequent than the occurrence of corresponding wild-type control clones. Furthermore, the two mutant lNb clones only contained 5 and 6 cells, respectively, which corresponds to an approximately 7-fold reduction in the number of GH146-positive cells as compared to wild-

type clones. This observation is in accordance with our data from the late third instar brain where the frequency of lPN clones is strongly reduced and identifiable lPN clones have strongly reduced cell numbers (see above).

We have therefore focused the analysis of *ems* mutant clones to the adPNs in the adult brain. No significant change in cell numbers could be found in *ems* mutant adNb clones (m = 30.0; s.d. = 5.3; n = 24) compared to the wild-type control clones (m = 33.3; s.d. = 3.6; n = 9). Thus, as observed before in the late third instar larva, *ems* is not required for the proliferation or survival of adult specific adPNs. Moreover, *ems* mutant clones retained their overall projection pattern from the antennal lobe to the mushroom body and lateral horn suggesting that the cells adopted general PN fate. We then set out to investigate the dendritic targeting specificity of *ems* mutant adNb clones in the antennal lobe. In our analysis we have found three types of *ems* mutant phenotypes: 1. absence of innervation of adPN specific glomeruli, 2. ectopic innervation of inappropriate glomeruli including lPN targets, 3. extension of ectopic projections into the suboesophageal ganglion.

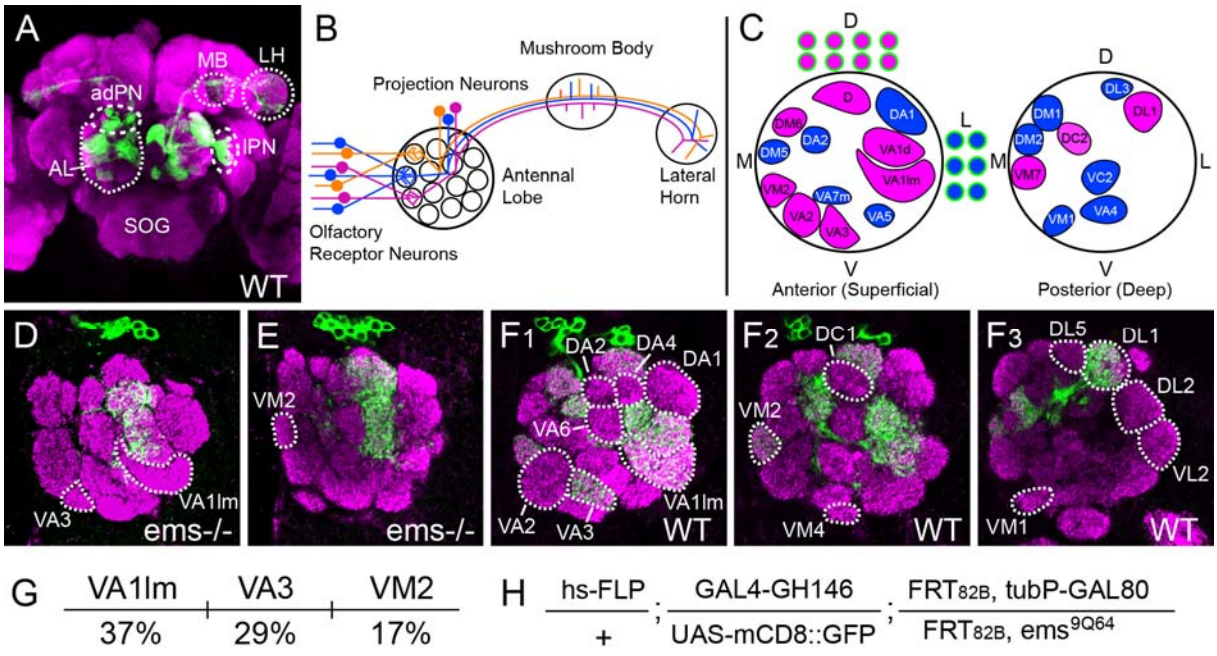

Figure 3-4 The dendrites of *ems* mutant adPNs fail to innervate adPN-specific glomeruli. In all confocal images green represents Anti-GFP labelled UAS-mCD8::GFP *GH146-GAL4* wild-type (WT) or *ems* mutant (*ems*-/-) MARCM clones. All clones were induced in the early first instar larva and analysed in the adult. Magenta represents mAb nc82, a synaptic marker demarcating neuropile compartments and glomeruli in the antennal lobe. (A) Frontal view of the central adult brain showing gross morphology of a adNb clone (adPN) in the left hemisphere and a lNb clone (lPN) in the right hemisphere. Both clones extend dendrites to specific glomeruli in the antennal lobe (AL) and project axons into the mushroom body calyx (MB) and the lateral horn (LH). No innervation of the suboesophageal ganglion (SOG) is observed. (B) Schematic representation of the PNs in the olfactory system of *Drosophila*. Olfactory receptor neurons expressing the same receptor (same colour) converge on the same glomeruli in the antennal lobe. Projection neurons send dendrites to glomeruli and axons to higher olfactory brain centres, the mushroom body and the lateral horn. (C) Schematic of the mutually exclusive subsets of glomeruli innervated either by adPNs (magenta) or lPNs (blue). The cell bodies are outlined with green. Glomeruli are shown at two different levels of the antennal lobe in the right side of the brain from a frontal view with dorsal to the top and medial to the left. Note that the VA2 glomerulus is targeted by adPNs born before clonal induction (Jefferis *et al*., 2001). In all subsequent images with single antennal lobes, the right side of the brain is shown with dorsal to the top and medial to the left. (D – F) Antennal lobe dendritic innervation pattern of *ems* mutant (D and E) and wild-type (F) adNb clones shown in anterior (C, F_1), intermediate (D, F_2) or posterior (F_3) single confocal sections. The *ems* mutant clone failed to innervate the VA3, VA1lm (D) and VM2 (E) glomeruli (outlined with dots). (G) Frequency of absence of innervation in totally 24 *ems* mutant adNb clones. (H) Genotype used to generate the *ems* mutant clones. For wild-type clones a simple FRT_{82B} chromosome was used instead of the FRT_{82B}, ems^{9Q64}.

Our analysis has shown that 6 of the wild-type adPN target glomeruli (D, DC2, DL1, DM6, VA1d, VM7) were not significantly affected *ems* mutant clones (data not shown). In contrast, *ems* mutant adNb clones failed to innervate 3 adPN-specific glomeruli with a frequency

ranging from 17-37% as compared to 100% innervation in wild-type clones (Fig. 3-4 D – G). These results indicate that *ems* is required for proper dendritic targeting to at least 3 adPN-specific glomeruli.

In addition to the failure of proper targeting of a subset of adPN-specific glomeruli, dendritic mistargeting to at least 9 inappropriate glomeruli was found in *ems* mutant adPN neuroblast clones (Fig. 3-5 A – F; Glomeruli were considered to be innervated ectopically only if dendritic arborizations penetrated the core of the glomerulus). 4 glomeruli (DA2, DA4, DL2, VL2) were ectopically innervated in more than 50% (54-71%) of the *ems* mutant clones, whereas mistargeting to 5 additional glomeruli (DA1, DC1, DL5, VA6, VM1) was less frequent (17-42%; Fig. 3-5 F). Two of the ectopically innervated glomeruli (DA1, VM1) are normally reserved for lPNs, whereas the other mistargeted glomeruli have no GH146-positive innervation in wild-type.

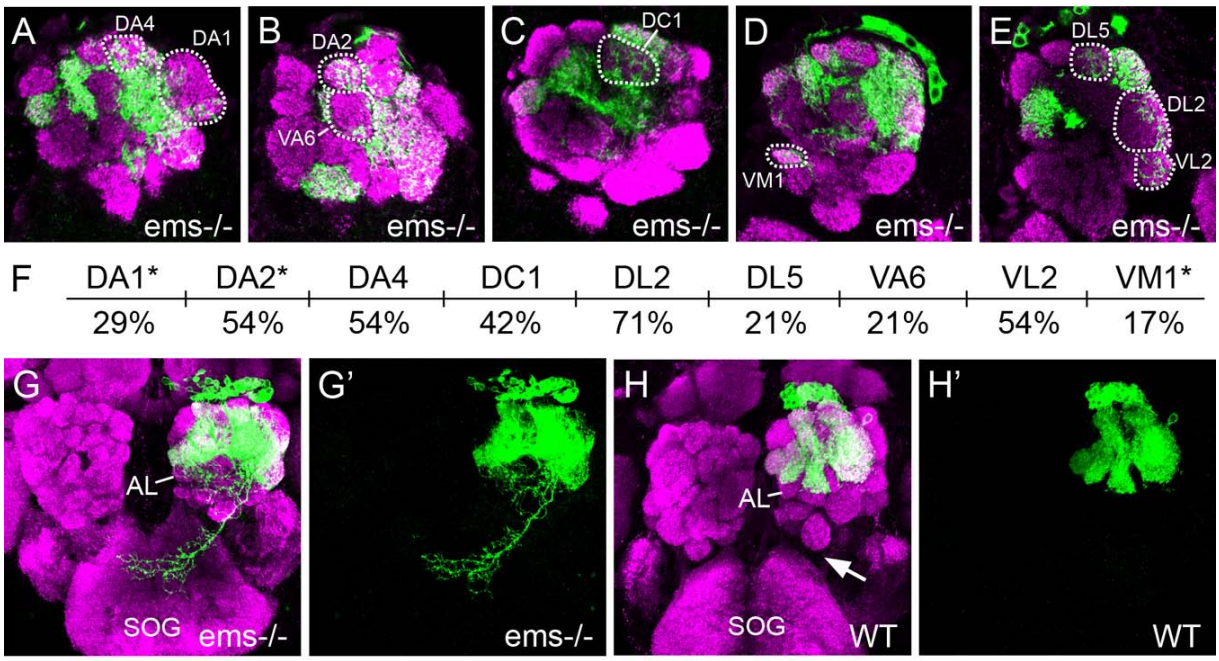

Figure 3-5 The dendrites of *ems* mutant adPNs innervate ectopic glomeruli and the suboesophageal ganglion. Green represents GFP labelled GH146 wild-type (WT) or *ems* mutant (*ems-/-*) MARCM clones. Magenta represents mAb nc82. (A – E) Antennal lobe dendritic innervation pattern of *ems* mutant adNb clones shown in anterior (A and B), intermediate (C) or posterior (D and E) single confocal sections (For wild-type adNb clone innervation pattern see Fig. 3-4 E). ems mutant clones ectopically innervated the DA1, DA4 (A), DA2, VA6 (B), DC1 (C), VM1 (D) , DL2, DL5, VL2 (E) glomeruli (outlined with dots). (F) Frequency of ectopic innervation in 24 *ems* mutant adNB clones. (G and H) Z-projections of frontal views of antennal lobes (AL) and the suboesophageal ganglion (SOG). (G and G') 8 out of 24 *ems* mutant adNb clones misproject to the SOG. (H and H') Wild-type adNb clones never innervate the SOG.

In one third of the cases (8 out of 24 clones), *ems* mutant adNb clones ectopically projected into the suboesophageal ganglion. The ectopic neurites typically extended from the ventral portion of the antennal lobe into the adjacent suboesophageal ganglion where it expanded fine arborizations into the ipsi- and contralateral neuropile compartments (Fig. 3-5 G, G'). No similar suboesophageal projection was observed in any of the wild-type control clones (Fig. 3-5 H, H'). Thus, transient *ems* expression during early differentiation of adPNs is required for proper targeting to adPN-specific glomeruli.

3.3.5 Axonal targeting is not to affected in ems-/- mutants

Since *ems* mutant adPN clones showed significant dendritic targeting defects in the adult brain, we next wanted to test if *ems* also regulates the highly stereotyped adPN axon terminal arborizations in the lateral horn. For a higher resolution of the axon terminals, we have performed a single-cell MARCM analysis of wild-type versus *ems* mutant DL1 class PNs. DL1 cells are the only class of PNs that can be unequivocally identified by the time of single-cell clone induction using the GH146 driver (Jefferis et al., 2001). Wild-type DL1 PNs have a uniglomerular dendritic innervation in the antennal lobe and a stereotyped axon branching pattern in the lateral horn (Fig. 3-6 A, B). DL1 axons bifurcate as they enter the lateral horn into a lateral and a dorsal branch (arrowhead in Fig. 3-6 B; Marin *et al*., 2002). All *ems* mutant DL1 single-cell clones exclusively innervated the DL1 glomerulus in the antennal lobe from where the axon projected to the lateral horn. Upon entry into the lateral horn neuropile compartment the axons normally bifurcated into the dorsal and lateral branch and no obvious alteration of the stereotyped wild-type pattern could be observed (Fig. 3-6 D, E). In order to gain insight into the general axon terminal arborization pattern of *ems* mutant adPNs, z-projections of confocal stacks encompassing the entire lateral horn were compared between wild-type and *ems* mutant adNb clones. No obvious axonal phenotype could be detected in the *ems* mutant (Fig. 3-6 C versus 3-6 F). Thus, we did not find any evidence of a requirement of *ems* in axonal outgrowth, guidance or terminal arborization in adPNs.

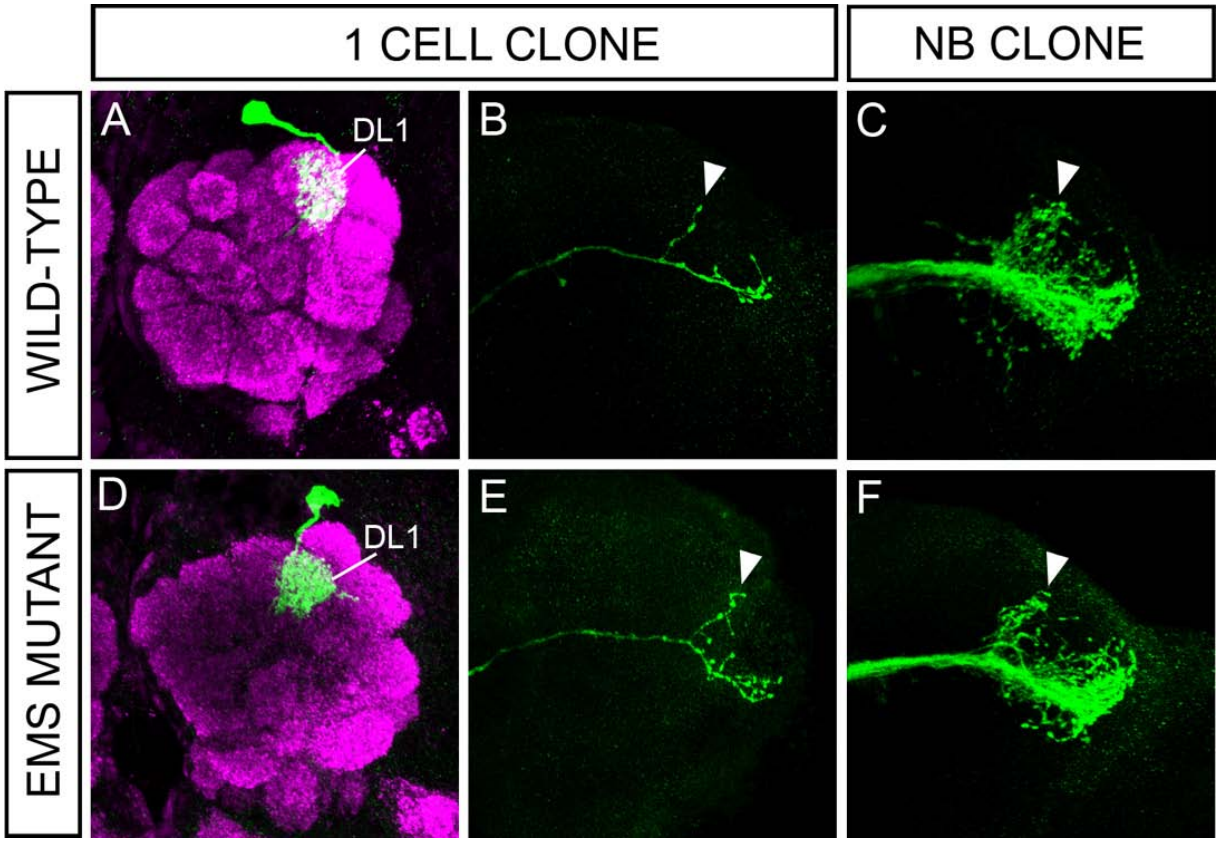

Figure 3-6 The dendrites and axon terminals of DL1 single-cells form correctly in the *ems* mutant. Green represents GFP labelled GH146 wild-type (WT) or *ems* mutant (*ems-/-*) MARCM clones. All clones were induced in the early first instar larva. Only adNb clones and DL1 class single-cell clones can be induced in the adPN lineage by that time. Magenta represents mAb nc82. (A and B) Wild-type DL1 single-cell clones innervate exclusively the DL1 glomerulus in the antennal lobe (A) and bifurcates into a characteristic dorsal (arrowhead) and lateral branch in the lateral horn (B). (C) The Axon arborization pattern of a adNb wild-type clone clearly shows a bundle of dorsal branches (arrowhead) as visualized in a z-projection encompassing the whole lateral horn. (D and E) *ems* mutant DL1 single-cell clones still innervate the correct glomerulus and show no obvious defects in the antennal lobe (D) or at the axon terminals in the lateral horn (E). (F) Z-projection of *ems* mutant adNb clone reveals no obvious axon targeting defect. The bundle of dorsal branches is well detectable (arrowhead).

3.3.6 Misexpression of ems in GH146-positive PNs causes dendritic and axonal targeting defects and the absence of lPNs

In wild-type adPN and lPN lineages, *ems* is only transiently expressed in postmitotic cells and expression disappears before the GH146-signal becomes detectable in the differentiating neurons. In both lineages, no *ems* expression is found later than 24 hours APF (Lichtneckert *et al.*, 2007). We therefore, wanted to test if down-regulation of *ems* expression in the

differentiating PNs is necessary for correct innervation of the antennal lobe glomeruli and the lateral horn. For this, we have misexpressed *ems* using a UAS-ems construct in otherwise wild-type GH146 MARCM clones that were induced in the early first instar larva.

To our surprise, no lPN clone could be detected in the 53 adult brains examined. This suggests that GH146-positive cells misexpressing *ems* in this lPN lineage did not survive to the adult stage. In contrast to the lPN lineage, 9 adPN neuroblast clones could be recovered from 53 adult brains and cell counts revealed that the average clonal cell number corresponded to wild-type levels (m = 33; s.d. = 0.4; see above). We next focused on the dendritic innervation of antennal lobe glomeruli by the adNb clones. We found that in adNb clones which misexpressed *ems* (expression confirmed by immunostaining; data not shown) 7 glomeruli were mistargeted which were never innervated by wild-type adNb clones induced in the early larva (Fig. 3-7 A – D). 2 glomeruli, DC1 and VM4, were mistargeted with a medium frequency (44%), whereas innervation of other 5 glomeruli was observed in more than 78 % (78-100%) of the cases. Interestingly, innervation of the VA2 glomerulus was found in all *ems* misexpressing adPN neuroblast clones induced in the early first instar larva. Although VA2 class PNs belong to the adPN specific set of GH146-positive glomeruli (see Fig. 3-4 B), they are only produced during embryonic development and no VA2 innervation is found in wild-type adNb clones induced during larval stages.

In addition to the 7 glomeruli which had ectopic innervation by *ems* misexpressing adNb clones we have found loss of innervation in one adPN specific glomerulus, the DL1 glomerulus. In all *ems* misexpressing adNb clones examined innervation of the DL1 glomerulus was completely absent (Fig. 3-7 C, D). In order to test if the lack of DL1 innervation is due to the absence of DL1 class neurons or the misprojection to other dendritic targets, we have analyzed DL1 class single-cell clones misexpressing *ems* that were induced in the early first instar larvae. Wild-type adPN single-cell clones induced by early larval heat shock (0 – 36 h) invariably innervate the DL1 glomerulus (see above; Jefferis *et al*., 2001). All 7 examples of *ems* misexpressing DL1 single-cell clones showed abnormal dendritic innervation. Whereas innervation of the DL1 glomerulus was never observed, two categories of misprojections were repeatedly found. In 5 of 7 single-cell clones dendritic innervation was mistargeted to the DA2 and DM6 glomeruli (Fig. 3-7 E, F). All of these clones showed additional dendritic branching in the dorsal part of the antennal lobe which could not be attributed to single glomeruli. In 2 of the 7 single-cell clones a diffused pattern of dendritic

branches was observed without dense innervation of single glomeruli (Fig. 3-7 G). Thus, misexpression of *ems* in GH146-positive adPNs causes dendritic mistargeting and the lack of DL1 innervation.

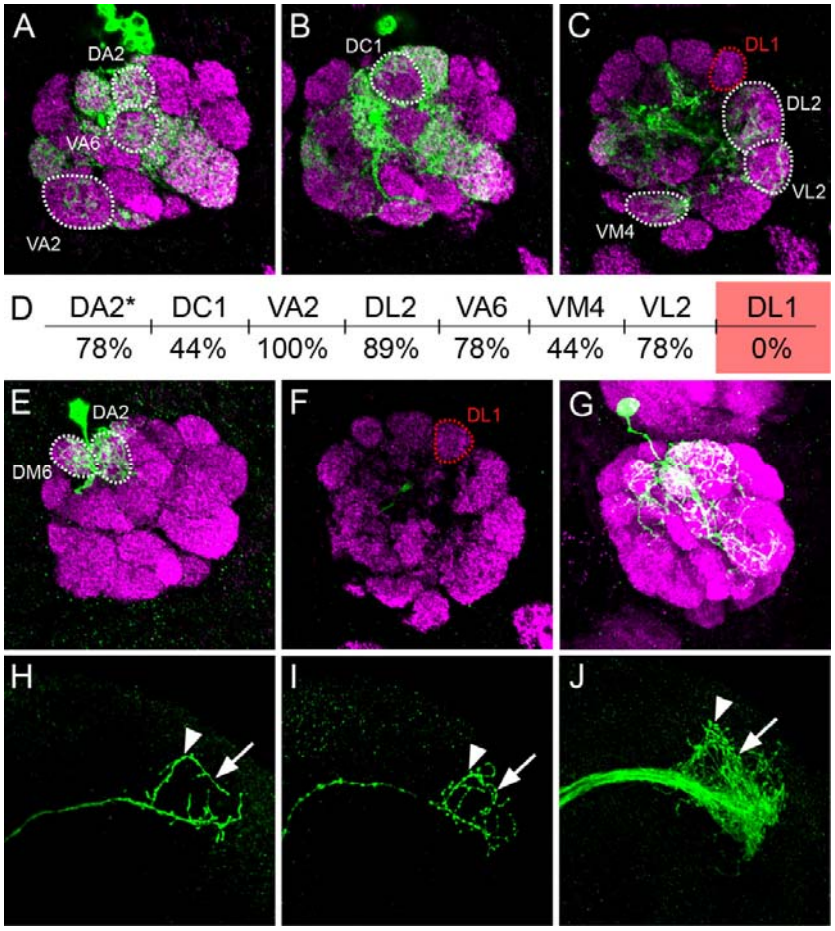

Figure 3-7 Misexpression of *ems* in mature adPN causes dendritic and axonal targeting defects. Green represents GFP labelled GH146-GAL4 UAS-Ems misexpression MARCM clones. All clones were induced in the early first instar larva. (A – C) Antennal lobe dendritic innervation pattern of *ems* misexpressing adNb clones shown in anterior (A), intermediate (B) or posterior (C) single confocal sections (For wild-type adNb clone innervation pattern see Fig. 3-4 E). The *ems* misexpression clone ectopically innervated the DA2, VA2, VA6 (A), DC1 (B), DL2, VL2 andVM4 (C) glomeruli (outlined with white dots) and lacked innervation in the DL1 glomerulus (outlined with red dots). (D) Frequency of innervation of 7 ectopic glomeruli (white background) and the adPN specific glomerulus DL1 (red background) in totally 9 *ems* misexpressing adNb clones. (E – G) Antennal lobe dendritic innervation pattern of *ems* misexpressing DL1 single-cell clones shown in anterior (E) or posterior (F) single confocal sections and as z-projection through the whole anennal lobe (G). The *ems* misexpressing DL1 class single-cell clone never innervated the DL1 glomerulus but either ectopically innervated the DA2 and DM6 glomeruli (E) or extended unspecific arborizations through a major part of the antennal lobe (G). (H and I) Two examples of lateral horn axon terminal arborization of *ems* misexpressing DL1 single-cell clones. A dorsal branch first extends dorsally (arrowheads) but then turns and expands towards the tip of the lateral branch (arrows). (J) Z-projection of *ems* misexpressing adNb clone shows a bundle of dorsal branches

(arrowhead) and dense innervation (arrowhead) on a hypothetical line connecting the tip of the dorsal branch with the lateral tip.

We next wanted to test if misexpression of *ems* could affect the axonal projection pattern of DL1 single-cell clones. All 7 single-cell clones extended an axonal projection from the antennal lobe to the mushroom body calyx and the lateral horn, suggesting that overall PN morphology was not affected. In order to examine the axonal morphology at higher resolution we have again focused on the terminal arborization in the lateral horn. In all 7 DL1 class single-cell clones misexpressing *ems* the two major branches characteristic for DL1 axon terminals could be observed. However, in all examples, the dorsal branch first projected dorsally, as in wild-type, but then made a 90° turn and continued to extend towards the tip of the lateral branch. This lateral turn and overgrowth of the dorsal branch was never observed in wild-type DL1 cells (compare Fig. 3-6 B with Fig 3-7 H, I).

Subsequently, we expanded the analysis from DL1 class single-cell clones to *ems* misexpressing adNb clones. For this, we examined their general axonal pattern as z-projections encompassing the entire lateral horn and compared it to the wild-type control. In both cases, the dorsally projecting branch is clearly distinguishable (arrowheads in Figs. 3-6 C and 3-7 J). However, as compared to the wild-type, the *ems* misepxressing adNb clones contained additional axonal branches in the dorsal part of the lateral horn (arrows in Figs. 3-6 C and 3-7 J). The position of these additional branches in the *ems* misexpressing adNb clones was coincident with the site where the overshooting dorsal branch in *ems* misexpressing DL1 single-cell clones was observed (arrows in Figs. 3-7 H – J). This suggests, that the axonal phenotype observed in the DL1 class neurons could, at least in part, account for the phenotype observed in the whole adNb clone.

Taken together, misexpression of *ems* in PNs beyond the time of endogenous *ems* expression leads to dendritic and axonal targeting defects of adPNs and the absence of GH146-positive lPN in the adult brain.

3.4 DISCUSSION

3.4.1 ems is expressed in the adPN and lPN secondary lineages

Here we show that two of the 8 *ems*-positive Nb lineages which had been described before during larval and early pupal stages correspond to the developing adPN and lPN lineages. In a previous work all 8 *ems*-positive lineages could be tentatively assigned to identified secondary lineages of a digital atlas of the third instar larval brain (Pereanu and Hartenstein, 2006; Lichtneckert *et al.*, 2007). Thus, based on anatomical and morphological criteria the two secondary lineages giving rise to the adPNs and lPNs in the adult most likely correspond to the BAmv3 and BAlc1/2 secondary lineages of the digital atlas (No clear distinction is made between the BAlc1 and BAlc2 lineages in the secondary lineage atlas). BAmv3 and BAlc1/2 are part of the basoanterior group of secondary lineages that forms the ventral deutocerebrum and surround the larval antennal neuropile compartment. In fact, both lineages have been described to extend their SATs along the larval inner antennal cerebral tract towards the anterior lateral protocerebrum as would be expected from prospective adult-specific PNs (Pereanu and Hartenstein, 2006). Interestingly, in the embryonic brain *ems* is also expressed in the deutocerebral neuromere. Moreover, at the absence of *ems* function cells of the deutocerebral neuromere are absent in the embryonic brain (Hirth et al., 1995). This raises the question if *ems* is already involved in the development of larval specific PNs during embryogenesis.

3.4.2 The ems/Emx genes are expressed in second order projection neurons in Drosophila and vertebrates

We have found *ems* expression during development of second order PNs known to make dendritic connections with ORNs in the antennal lobe and relay the sensory inputs to the higher olfactory centres in the *Drosophila* brain. The organization of the insect olfactory pathway is very similar to that of mammals (Hildebrand and Shepherd, 1997; Komiyama and Luo, 2006). ORNs of a single class that express the same olfactory receptor converge to discrete glomeruli of the olfactory bulb, the first olfactory relay in the mammalian brain, equivalent to the antennal lobe in *Drosophila*. In each glomerulus of the olfactory bulb, ORNs specifically synapse with a single class of second order projection neurons, called mitral cells

in mammals, which then send their axons to different target areas in higher brain centres. Two mammalian orthologs, Emx1 and Emx2, of the *Drosophila ems* gene are involved in patterning and proliferation of anterior brain regions during early development, which is comparable to the role of *ems* during development of the embryonic fly brain (Cecchi, 2002; Lichtneckert and Reichert, 2005). Moreover, both EMX proteins have been detected in mitral cells of the mouse olfactory bulb at later embryonic stages. Interestingly, the mitral cell layer of the olfactory bulbs of *Emx1/Emx2* double mutants are thin and disorganized although at least part of the mitral cells are produced (Bishop et al., 2003; Brox et al., 2004; Mallamaci et al., 1998; Yoshida et al., 1997). Together, these findings point to an evolutionary conservation of *ems*/*Emx* expression and function in secondary projection neurons of insect and mammalian olfactory systems.

3.4.3 Transient ems expression in adult-specific adPNs and lPNs

In several neurvous systems, transcription factors have been shown to determine synaptic specificity (Shirasaki and Pfaff, 2002; Skeath and Thor, 2003). Here we provide expression, loss- and gain-of-function data to demonstrate that transient *ems* expression is cell-autonomously required for precise dendritic and axonal connectivity of olfactory projection neurons. At all stages examined during larval and early pupal development of adPN and lPN lineages *ems* expression was detected in the Nbs and GMCs as well as in the latest-born postmitotic neurons. Still, *ems* was never co-expressed with the GH146 signal, which labelled preferentially deeper cells of the same lineage that were closely associated to the antennal lobe. The spatiotemporal expression patterns of *ems* within the adPN and lPN lineages is consistent with the notion that all adult-specific PNs transiently express *ems* during their early differentiation. No Ems protein is found in GH146-positive PNs by the time they extend their dendritic and axonal arborizations (Jefferis et al., 2004). Nevertheless, *ems* function in adPNs is required for the correct and exclusive innervation of adPN-specific glomeruli.

Thus, our results demonstrate the temporal separation of the physical presence of Ems from the process it finally regulates in differentiating adPNs. This is in contrast with two POU domain transcription factors, Acj6 and Dfr, which are expressed during larval and early pupal stages (and into the adult stage for Acj6) when they regulate distinct dendritic targeting in a lineage-specific way (Komiyama et al., 2003). To date, the factors that might mediate *ems* function during adPN differentiation are unknown.

3.4.4 Different roles of transient ems expression in adPN and lPN lineages

Analysis of *ems* mutant Nb clones in the secondary adPN and lPN lineages revealed a striking difference in *ems* requirement. Whereas, no significant change in cell numbers of *ems* mutant adNb clones were found compared to wild-type clones, *ems* mutant lNb clones were strongly reduced in size. In addition, *ems* mutant lNb clones were found 20-times less frequently than the corresponding wild-type control clones. Thus, at the absence of *ems* function lNb clones fail to provide the correct number of adult-specific progeny. Several possible explanations arise for this phenotype. First, proliferation of the Nb might be arrested or the Nb could have died during early larval development. Second, the Nb is present but proliferating at a lower rate. Third, postmitotic cells in the clone might die due to programmed cell death. We are currently investigating these 3 possibilities.

Functional inactivation of *ems* does not affect the correct cell number in adNb clones. Thus, *ems* is not required for proliferation or survival of adPNs. Furthermore, the *ems* mutant postmitotic progeny of adNb normally expressed the neuronal marker Elav and retained gross dendritic and axonal morphology of wild-type PNs in the olfactory pathway. This suggests, that *ems* function is not required for the specification of neuronal fate and neurite guidance to the target area of adPNs. However, three different dendritic targeting phenotypes could be observed. First, *ems* mutant adPNs failed to innervate a subset of adPN-specific glomeruli. Loss of proper dendritic targeting was reported from *acj6* mutant adNb clones (Komiyama et al., 2003). Second, *ems* mutant adPNs mistargeted other glomeruli which are not adPN-specific. Only one-third of the mistargeted glomeruli belonged to the lPN-specific group, whereas two-thirds of the glomeruli are not innervated by any adult-specific GH146 cells. Interestingly, dendritic mistargeting was limited to one-third of non-adPN-specific glomeruli, which argues against a random glomerular innervation. In addition, *ems* mutant DL1 class single-cell clones always retained their characteristic uniglomerular innervation, suggesting significant differences in *ems* requirement of different adPN classes.

The graded expression of Semaphorin-1a has been recently shown to direct the dendritic targeting of PNs along the dorsolateral to ventromedial axis of the antennal lobe (Komiyama et al., 2007). We could not observe a general trend of mistargeted glomeruli on the

corresponding axis of the antennal lobe suggesting that *ems* function does not interfere with Semaphorin-1a expression levels in adPNs.

Finally, in the third dendritic targeting phenotype extensive arborizations invaded the neighbouring suboesophageal neuropile compartment. Therefore, misprojections by *ems* mutant adPNs are not limited to closely related targets within the antennal lobe but can also be redirected to more distant target area. However, all misrouted dendrites extended from inside the antennal lobe into the suboesophageal ganglion suggesting that the antennal lobe was still correctly recognized as primary target area of the outgrowing dendrites.

Taken together, clonal analysis of *ems* function in adult-specific PNs revealed different roles of *ems* in the two major PN lineages. In the lPN lineage, *ems* function is required for the formation of the correct number of adult-specific cells, suggesting a role in proliferation or survival. In contrast, *ems* is not required for the correct number of progeny in adNb clones but instead for the correct targeting of dendrites. Axon terminal arborizations appear to be unaffected in *ems* mutant adNPs.

3.4.5 Down-regulation of ems expression during neuronal differentiation is critical for proper neurite targeting

Transient *ems* expression during larval development is required for the correct targeting of dendrites but not axons of adPNs. However, no Ems protein is found in PNs when they extend their dendritic and axonal arborizations during early pupal development. The absence of Ems at later PN differentiation steps could, on one hand, simply reflect the fact that *ems* function is no longer needed. On the other hand, the pulse of *ems* expression at the beginning of PN differentiation could be tightly regulated and *ems* function at later stages could have deteriorating effects. In fact, this turned out to be the case for both PN lineages. No *ems* misexpressing lPN cells could be detected, whereas *ems* misexpressing adNb clones were found at normal frequencies. One possible explanation for the absence of lPN clones would be that GH146-positive lPNs are eliminated by *ems* misexpression. It is important to note that *ems* misexpression using the GH146-GAL4 driver does not affect the Nb since GH146 is only expressed in the postmitotic progeny. Although the number of cells within the *ems* misexpressing adNb clones as well as their general projection pattern was unaffected, frequent

dendritic and axonal mistargeting was observed. Interestingly, axonal mistargeting was never observed in *ems* mutant adPNs. Furthermore, the axonal phenotype found in different *ems* misexpressing DL1 class single-cell clones did not correspond to any known axonal projection pattern of PNs (Marin et al., 2002; Wong et al., 2002). Taken together, precise regulation of transient *ems* expression is crucial for the establishment of correct connections by PN within the *Drosophila* olfactory system.

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4 Insights into the urbilaterian brain: conserved genetic patterning mechanisms in insect and vertebrate brain development

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4.1 SUMMARY

Recent molecular genetic analyses of *Drosophila melanogaster* and mouse central nervous system development revealed strikingly similar genetic patterning mechanisms in the formation of the insect and vertebrate brain. Thus, in both insects and vertebrates, the correct regionalization and neuronal identity of the anterior brain anlage is controlled by the cephalic gap genes *otd/Otx* and *ems/Emx*, whereas members of the *Hox* genes are involved in patterning of the posterior brain. A third intermediate domain on the anteroposterior axis of the vertebrate and insect brain is characterized by the expression of the *Pax2/5/8* orthologues suggesting that the tripartite ground-plan of the protostome and deuterostome brain share a common evolutionary origin. Furthermore, cross-phylum rescue experiments demonstrate that insect and mammalian members of the *otd/Otx* and *ems/Emx* gene families can functionally replace each other in embryonic brain patterning. Homologous genes involved in dorsoventral regionalization of the central nervous system in vertebrates and insects show remarkably similar patterning and orientation with respect to the neurogenic region (ventral in insects and dorsal in vertebrates). This supports the notion that a dorsoventral body axis inversion occurred after the separation of protostome and deuterostome lineages in evolution. Taken together, these findings demonstrate conserved genetic patterning mechanisms in insect and vertebrate brain development and suggest a monophyletic origin of the brain in protostome and deuterostome bilaterians.

4.2 INTRODUCTION

The question of whether the last common ancestor of bilaterians had an anatomically complex central nervous system (CNS) is controversial. The enigmatic *"Urbilateria"* is now long extinct and evidence from the new molecular based phylogeny implicates the absence of intermediate taxa at the basis of protostome-deuterostome lineage separation (Fig. 4-1) (Adoutte et al., 2000). Several attempts to reconstruct the last common bilaterian ancestor and determine the origin of the CNS of organisms as different as insects and vertebrates have been made in the past. Based on differences in embryonic topography and morphogenesis of the nervous system, bilaterian animals have been subdivided into different groups thought to be characterized by the evolutionary independent origin of their nervous systems (Brusca and Brusca, 1990).

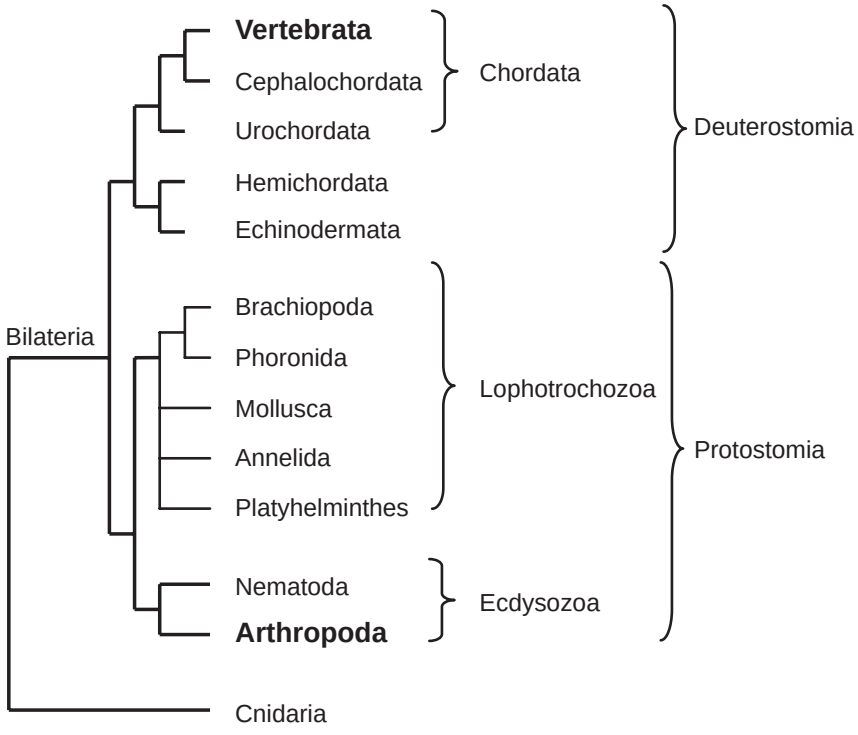

Figure 4-1 Phylogenetic relationship of mouse and *D. melanogaster*. Simplified version of the new molecular-based phylogeny showing a selection of bilaterian phyla with the Cnidaria as outgroup. Bilaterian phyla are grouped into major cladistic classifications indicated at the right side (modified after Adoutte *et al.*, 2000). Vertebrates and arthropods are evidenced in bold. The phylogenetic tree indicates that homologous features of mouse and *D. melanogaster* already existed in the common ancestor of all bilaterian animals.

Contrasting with this notion of independent origins, is a large amount of molecular genetic data generated in several vertebrate and invertebrate model systems which indicate that key developmental processes, such as proliferation, regionalization and specification of the embryonic nervous system are controlled by homologous genes in vertebrates and insects (Arendt and Nubler-Jung, 1999; Reichert and Simeone, 1999). Indeed, evidence from recent experiments in *Drosophila melanogaster* (*D. melanogaster*) and mouse indicate that basic genetic mechanisms involved in embryonic brain development are conserved and suggest a common evolutionary origin of the protostome and deuterostome brain. Here we review the basic genetic mechanisms of brain development in *D. melanogaster* and mouse from a comparative developmental genetic perspective. Recent expression data and functional experiments on key developmental control genes, such as the dorsoventral patterning genes, the cephalic gap genes *otd/Otx* and *ems/Emx*, or the *Hox* and *Pax2/5/8* genes are reconsidered in the light of a possible common origin of the bilaterian brain.

4.3 OVERVIEW OF EMBRYOGENESIS OF THE BRAIN IN INSECTS AND VERTEBRATES

The insect brain is composed of an anterior supraesophageal ganglion and a posterior subesophageal ganglion. The supraesophageal ganglion is subdivided into the protocerebrum (b1), the deutocerebrum (b2) and the tritocerebrum (b3), whereas the subesophageal ganglion is subdivided into the mandibular (s1), maxillary (s2) and labial (s3) neuromeres (Campos-Ortega and Hartenstein, 1997; Reichert and Boyan, 1997; Therianos et al., 1995; Younossi-Hartenstein et al., 1996). The anterior brain anlage of *D. melanogaster* derives from the procephalic neurogenic region, which is specified to become neuroectoderm through genetic interactions during gastrulation. The posterior embryonic brain derives from the rostral-most ventral neurogenic region and is specified in a manner similar to that of the ventral nerve cord (Doe and Skeath, 1996). Within the cephalic neuroectoderm, single progenitor cells called neuroblasts delaminate and start to proliferate giving rise to the developing brain of *D. melanogaster*.

In vertebrates, inductive interactions between germlayers during gastrulation cause an early regionalization of the developing neural tube. This leads to a rostrocaudal subdivision of the anterior neural tube into the rostral forebrain (prosencephalon or telencephalon/diencephalon) and midbrain (mesencephalon) regions and into the caudal hindbrain regions (rhombencephalon or metencephalon/myelencephalon). The developing hindbrain reveals a clear metameric organization based on seven or eight rhombomeres with pair-wise compartment-like organization (Lumsden and Krumlauf, 1996). The segmental organization of the embryonic prosencephalon is still debated, however a number of studies suggest that this region, like the hindbrain, is subdivided into six neuromeres known as prosomeres (Rubenstein et al., 1994; Rubenstein et al., 1998).

4.4 CONSERVED DORSOVENTRAL PATTERNING MECHANISMS INDICATE A CNS AXIS INVERSION IN PROTOSTOME AND DEUTEROSTOME EVOLUTION

One of the major arguments during the last two centuries against the common origin of the nervous systems of protostomes and deuterostomes has been the morphologically opposite position of the nerve cords in arthropods (ventral) and vertebrates (dorsal). This striking morphological discrepancy has lead to the concept of two taxonomic groups, whose CNS evolved independently from a primitive common ancestor. Invertebrates exhibiting a ventrally located nerve cord such as arthropods, annelids, and mollusks were grouped into the gastroneuralia whereas the notoneuralia include urochordates, cephalochordates and vertebrates that are characterized by a dorsal nerve cord (Brusca and Brusca, 1990; Hatschek, 1888). This general notion was first challenged by Geoffroy St. Hilaire in the early nineteenth century who argued, based on morphological considerations, that the ventral side of arthropods corresponds to the dorsal side of vertebrates. Molecular genetic evidence from recent developmental studies in *D. melanogaster* and different vertebrate model organisms have strengthened the view that the dorsoventral bauplan of protostomes, such as arthropods, represents an inversion of the bauplan of deuterostomes, such as vertebrates. From an evolutionary point of view this is thought to be the consequence of the inversion of dorsoventral body axis in one of the two animal groups (Arendt and Nubler-Jung, 1994; De Robertis and Sasai, 1996). The implications of the dorsoventral inversion theory are that the CNS of protostomes and deuterostomes derive from evolutionarily homologous body regions and that the last common ancestor might already have had a centralized nervous system that was inherited to both animal groups.

Recent developmental genetic evidence supports the dorsoventral inversion theory at two different levels of neuroectoderm specification (Fig. 4-2). At the level of regionalization of the dorsoventral axis with respect to the presumptive neurogenic region, the early embryos of vertebrates and insects are both patterned by two opposed gradients of homologous morphogens. In accordance with the dorsoventral hypothesis, the TGFβ (Transforming Growth Factor β) family member encoded by the *dpp* (*decapentaplegic*) gene is expressed dorsally in the insect *D. melanogaster* whereas its vertebrate orthologue *BMP4* (*Bone*

) is localized at the ventral side in vertebrates. These factors are antagonized by the secreted products of the homologous genes *sog* (*short gastrulation*) in *D. melanogaster* and *Chordin* in vertebrates (De Robertis and Sasai, 1996; Holley et al., 1995). The site of action where *sog/Chordin* expression inhibits *dpp/BMP4* signaling corresponds in fly and mouse to the region of the dorsoventral axis that gives rise to the neuroectoderm in the early embryos. Thus, in insects and vertebrates, the neurogenic potential of *sog/Chordin* function seems to be conserved, whereas their expression gradients are inverted with respect to the dorsoventral body axis.

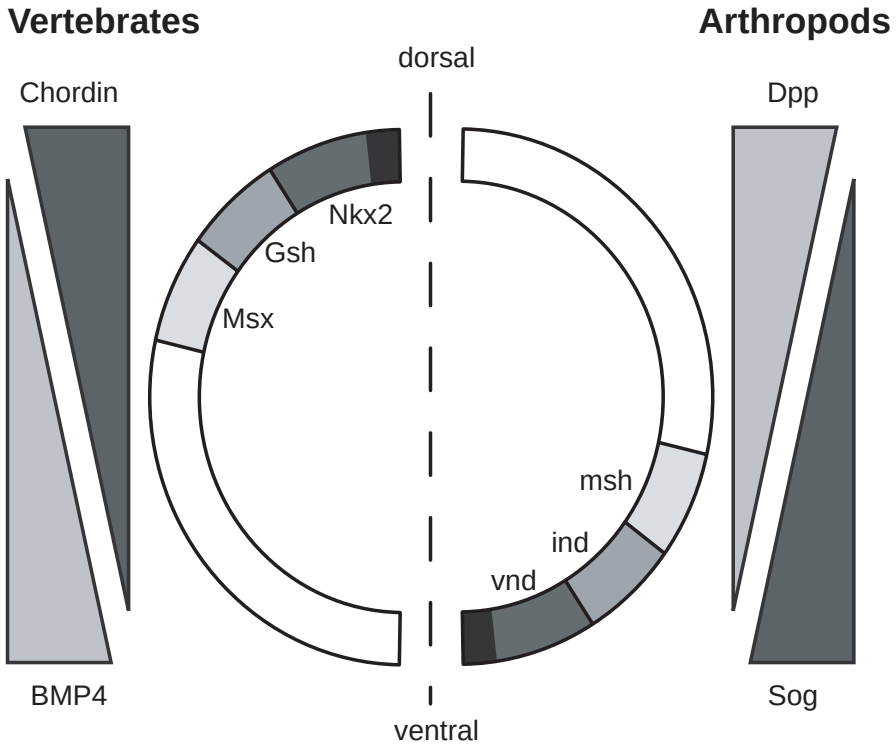

Figure 4-2 Schematic representation of the molecular genetic patterning of the dorsoventral axis in vertebrates and arthropods. Only half of the body wall is represented for vertebrates and arthropods in the schematic dorsoventral sections with dorsal to the top for both animal groups. The secreted products of the homologous genes *dpp/Bmp4* form a dorsoventrally inverted gradient in vertebrates with respect to *D. melanogaster*. They are antagonized by *sog/Chordin*, another homologous gene pair, from the region of the embryo that will adopt a neurogenic potential. This region is further patterned by a set of homeobox genes into medial (*vnd/Nkx2*), intermediate (*ind/Gsh*) and lateral (*msh/Msx*) neurogenic domains.

A second level of dorsoventral patterning of the neuroectoderm has been found to be conserved in evolution as well. A set of homologous genes are involved in the formation of dorsoventral regions of the developing CNS in insects and vertebrates. Again their relative

expression domains are inverted in the sense of a dorsoventral axis inversion between protostomes and deuterostomes (Chan and Jan, 1999; Cornell and Ohlen, 2000). In *D. melanogaster* proneural clusters and early delaminating neuroblasts in the ventral neurectoderm are arranged in three longitudinal columns (medial, intermediate and lateral) on either side of the midline cells (Skeath and Thor, 2003). Similarly, in vertebrates, such as frog (Chitnis et al., 1995) and zebrafish (Haddon et al., 1998), proneural clusters that give rise to primary neurons are arranged in three columns on each side of the neural plate (medial, intermediate and lateral). In *D. melanogaster* the homeobox genes *vnd* (*ventral nerve cord defective*), *ind* (*intermediate neuroblasts defective*) and *msh* (*muscle specific homeobox*) are essential for the formation and specification of neuroblasts in the ventral, intermediate and lateral longitudinal columns (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). In the neural plate of vertebrates the expression of the homologous genes of the *Nkx2* (*vnd*), *Gsh* (*ind*) and *Msx* (*msh*) families defines the medial, intermediate and lateral neurogenic columns and are involved in their specification (Arendt and Nubler-Jung, 1999).

The functional conservation and the similar relative expression patterns of these dorsoventral patterning genes in vertebrates and insects imply a common origin of the CNS of protostomes and deuterostomes. Accordingly, a reasonable explanation for the opposed positions of the CNS in these two animal groups is the dorsoventral axis inversion between protostomes and deuterostomes. Alternative scenarios for independent CNS evolution in protostomes and deuterostomes based on condensations of multiple nerve cords or nerve nets have been proposed (Gerhart, 2000; Holland, 2003; Lacalli, 2003). However, additional, independent evidence for a common origin of the CNS in protostomes and deuterostomes, is provided by key control genes involved in anteroposterior patterning of the CNS in insects and vertebrates.

4.5 THE HOMEOTIC GENES PATTERN THE POSTERIOR BRAIN IN INSECTS AND VERTEBRATES

The homeobox or *Hox* genes code for transcription factors with a characteristic helix-turn-helix DNA-binding motif called the homeodomain. Homeotic genes involved in specifying anteroposterior segment identity in the ectoderm were first discovered in *D. melanogaster*. Subsequently, similar clustered homeotic genes were found in a wide range of species where they have been shown to have an essential role in anteroposterior body axis patterning (Ferrier and Holland, 2001; Schilling and Knight, 2001; Carpenter, 2002; Hughes and Kaufman, 2002; Vervoort, 2002). In *D. melanogaster* the *Hox* genes are arranged in two gene clusters known as the *Antennapedia* (ANT-C) and *Bithorax* (BX-C) complex. The ANT-C contains the five more anteriorly expressed *Hox* genes: *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr)* and *Antennapedia (Antp)*. The BX-C contains the three posteriorly expressed genes: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*). Interestingly, the relative position of the genes within the clusters show a correlation with their spatial and temporal expression pattern in the body; genes located towards the 3' end of the cluster are expressed more anteriorly and earlier in development than genes closer to the 5' end. This correlation has been termed spatial and temporal colinearity (Mann, 1997). Furthermore, there appears to be a conserved functional hierarchy among the members of the homeotic gene clusters in that more posterior acting genes are functionally dominant over more anterior expressed genes, a fact that has been called 'phenotypic suppression' (Duboule and Morata, 1994). Mammalian *Hox* genes are aligned into 13 paralogous groups which are organized in four chromosomal clusters called *Hox A – Hox D*. The four clusters contain 9 – 11 *Hox* genes and only the *Hox-B* cluster comprises orthologues of all *D. melanogaster* homeotic complex genes. Similarly, as in *D. melanogaster*, the principle of spatial and temporal colinerarity among the paralogous groups is also observed for vertebrate *Hox* genes, and more posterior acting genes impose their developmental specificities upon anterior acting genes what has been termed 'posterior prevalence' (Duboule and Morata, 1994; Mann, 1997).

Hox genes are expressed in the developing CNS of insects and vertebrates in a remarkably similar anteroposterior order (Fig. 4-3 A). In *D. melanogaster* genes of the *Hox* clusters are expressed in discrete domains in the developing brain and the ventral nerve cord and their anterior expression boundaries often coincide with neuromere compartment boundaries. In

contrast to the embryonic epidermal structures of *D. melanogaster*, the anteroposterior arrangement of the homeotic genes in the fly CNS does not strictly fulfill the criterium of spatial colinearity (Kaufman *et al.*, 1990; Hirth *et al.*, 1998). The expression domains of the two 3' most *Hox* genes of the ANT-C are inverted in that the anterior expression boundary of *lab* is posterior to that of *pb*. Interestingly, with respect of the relative spatial order of homeotic gene expression, the CNS of *D. melanogaster* is more similar to the CNS of the mouse than to the epidermis of the fly itself. In vertebrates, *Hox* genes are expressed in the hindbrain and spinal cord of the developing CNS. Expression precedes rhombomere formation and becomes progressively restricted to specific domains during embryogenesis. The most anterior *Hox* gene expression in the mouse brain is at the boundary between rhombomeres 2 and 3. This is followed posteriorly by a set of *Hox* gene expression domains, which generally coincide at their anteriormost domains with rhombomere boundaries. As in the *D. melanogaster* CNS, the mouse orthologues of the *lab* and *pb* genes, *Hoxb-1* and *Hoxb-2*, show an same inversion concerning the spatial colinearity rule of *Hox* cluster genes (Fig. 4-3 B).

In *D. melanogaster*, mutational inactivation of either of the *Hox* genes *lab* or *Dfd* results in severe axonal patterning defects in the embryonic brain (Hirth *et al.*, 1998). In *lab* null mutants axonal projection defects occur in the posterior tritocerebrum where *lab* is expressed in the wild type brain. In the mutant, longitudinal pathways connecting supraesophageal and subesophageal ganglia as well as the projections in the tritocerebral commissure are absent or reduced. Interestingly, the brain defects are not due to a deletion in the tritocerebral neuromere; neuronal progenitors are present and give rise to progeny in the mutant domain. Theses postmitotic cells, however, do not form axonal and dendritic extensions and are not contacted by axons from other parts of the brain. The *lab* mutant cells do not acquire a neuronal fate, as revealed by the absence of neuronal markers, but rather remain in an undifferentiated state (Fig. 4-3 A). Comparable defects are seen in the *D. melanogaster Dfd* mutant in the corresponding mandibular domain, where the wild type expression of the gene is located. Thus, the appropriate expression of the homeotic genes *lab* and *Dfd* is essential for the establishment of regionalized neuronal identity in the brain of *D. melanogaster*.

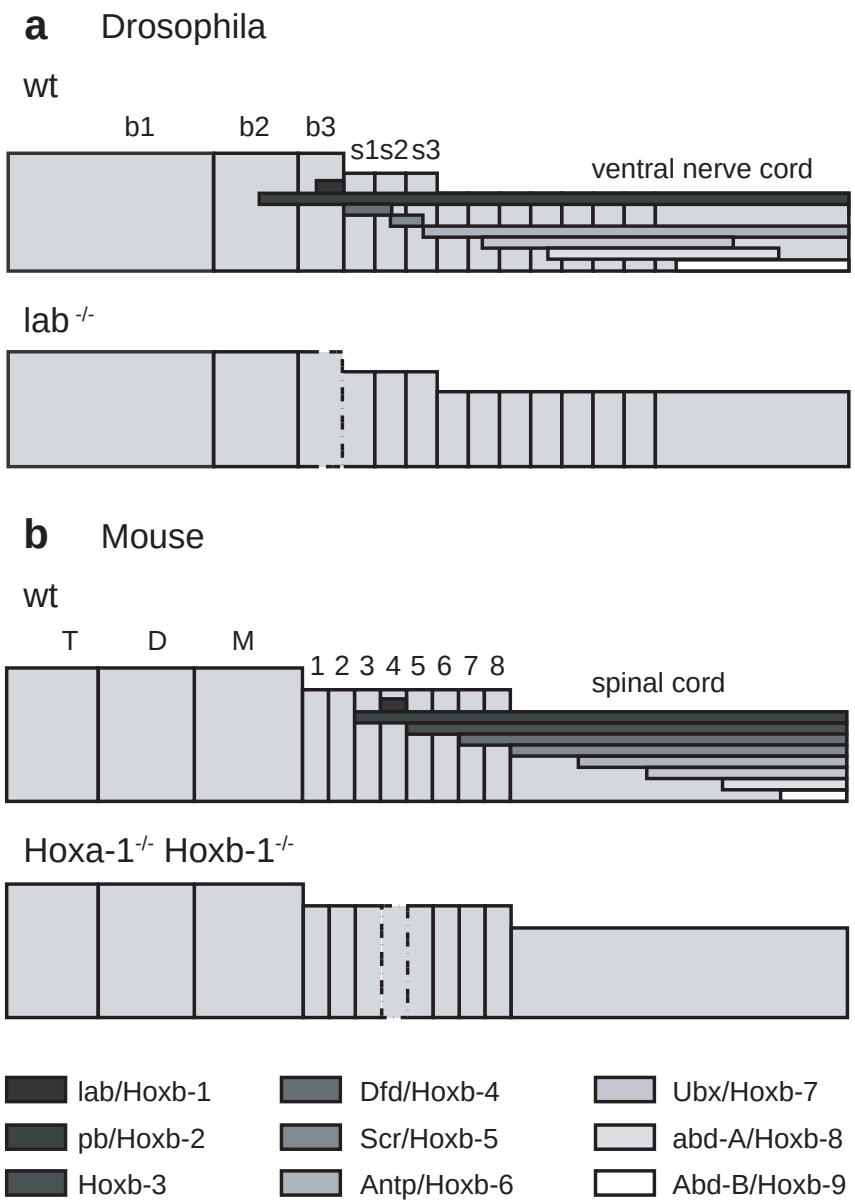

Figure 4-3 Simplified schematic comparison of *Hox* gene expression domains and mutant phenotypes in the CNS of *D. melanogaster* and mouse. (A) Expression domains of the homeotic genes of the Antennapedia and Bithorax complexes in the CNS of *D. melanogaster*: *lab* (*labial*), *pb* (*proboscipedia*), *Dfd* (*Deformed*), *Scr* (*Sex combs reduced*), *Antp* (*Antennapedia*), *Ubx* (*Ultrabthorax*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*). In *lab* null mutant embryos (*lab-/-*) cells of the posterior part of the tritocerebrum (b3) are correctly located in the mutant domain, but fail to assume their correct neuronal cell fate (indicated by dashed lines). (B) Expression of the homeotic genes *Hoxb-1*, *Hoxb-2*, *Hoxb-3*, *Hoxb-4*, *Hoxb-5*, *Hoxb-6*, *Hoxb-7*, *Hoxb-8* and *Hoxb-9* in the embryonic CNS of mouse. Double mutant embryos of *Hoxa-1* and *Hoxb-1* (*Hoxa-1-/-; Hoxb-1-/-*) result in a reduzed size of rhombomere 4 (4) and additionally a loss of expression of rhombomere 4 specific markers (indicated by dashed lines). The synergistic action of *Hoxa-1* and *Hoxb-1* in the specification of rhombomere 4 is comparable to the action of their single orthologue *lab* in the posterior tritocerebrum of *D. melanogaster*. Abbreviations: b1, protocerebrum; b2, deutocerebrum; b3, tritocerebrum; s1, mandibular neuromere; s2, maxillary neuromere; s3, labial neuromere; T, telencephalon; D, diencephalon; M, mesencephalon; 1 – 8, rhombomeres 1 – 8; wt, wild type (modified after Hirth and Reichert, 1999).

In mouse, the *lab* orthologues *Hoxa-1* and *Hoxb-1* are expressed in overlapping domains with a sharp anterior boundary coinciding with the presumptive rhombomere 3/4 border. Single loss-of-function mutations of particular *Hox* paralogues show marked phenotypic differences suggesting synergy or functional compensation mechanisms (Carpenter, 2002; Maconochie et al., 1996; Rijli et al., 1998). Functional inactivation of *Hoxa-1* causes segmentation aberrations leading to a reduced size of rhombomeres 4 and 5, defects of motor neuron axonal projections and malformations of the trigeminal and facial/vestibuloacoustic nerve, but the normal identity of rhombomere 4 is not altered (Gavalas et al., 1998; Rijli et al., 1998; Studer et al., 1998). In contrast, loss of *Hoxb-1* function has no effect on the size of rhombomere 4 but result in a loss of identity of the segment and a partial transformation into an rhombomere 2 identity (Goddard et al., 1996; Studer et al., 1996). The *Hoxa-1*, *Hoxb-1* double loss-of-function mutant results in a territory of unknown identity and reduced size between rhombomeres 3 and 5 suggesting a synergistic action of the two genes in rhombomere 4 specification (Fig. 3 B) (Gavalas et al., 1998; Studer et al., 1998). Thus, *Hoxa-1* and *Hoxb-1* have a very similar role in the specification of neuronal identity during embryonic brain development as their orthologue *lab* in *D. melanogaster*. The similar functions and expression domains of the homologous *Hox* genes in the developing hindbrains and posterior brains of fly and mouse support the idea of a common bauplan of the CNS.

4.6 THE EMS/EMX GENES ARE INVOLVED IN ANTERIOR BRAIN DEVELOPMENT OF D. MELANOGASTER AND MOUSE

The *D. melanogaster ems* gene belongs to the cephalic gap genes together with *tailless (tll), orthodenticle (otd), buttonhead (btd)* and *sloppy paired (slp)*. At the early blastoderm stage of embryogenesis the cephalic gap genes are broadly expressed in overlapping anterior stripes where their expression is initially regulated by maternal effect genes (Dalton *et al.*, 1989; Finkelstein and Perrimon, 1990; Walldorf and Gehring, 1992). The functional inactivation of any of these genes results in gap-like phenotypes where structures of several head segments are missing (Cohen and Jurgens, 1990; Grossniklaus et al., 1994). The cephalic gap genes *tll, otd, ems* and *btd* have been shown to be essential in early brain development. By the time of neuroblast delamination in the anterior brain their expression domains becomes restricted to specific subsets of neural progenitors (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). Mutational inactivation of a given cephalic gap gene results in the deletion of a specific brain area indicating the requirement of these genes in early specification of the anterior brain primordium (Hirth et al., 1995; Younossi-Hartenstein et al., 1997).

The expression domain of the homeodomain transcription factor *ems* in the procephalic neuroectoderm and in the subsequently formed early embryonic brain of *D. melanogaster* comprises two stripes in the anterior parts of the deutocerebral (b2) und tritocerebral (b3) neuromeres (Fig. 4-4 A). A reiterated segmental expression pattern is also seen in the ventral nerve cord at later embryonic stages (not shown in Fig. 4-4). Loss-of-function of the *ems* gene results in a gap-like phenotype in the brain due to the absence of cells in the deutocerebral and anterior tritocerebral neuromeres (Hartmann et al., 2000; Hirth et al., 1995). In the *ems* mutant domain the expression of the proneural gene *lethal of scute (l'sc)* is lost and neuroblasts fail to form (Younossi-Hartenstein et al., 1997). This phenotype can be rescued by ubiquitous overexpression of *ems*, which results in proper brain development (Hartmann et al., 2000). Thus, *ems* function is required for the specification and formation of the anterior embryonic brain in *D. melanogaster*.

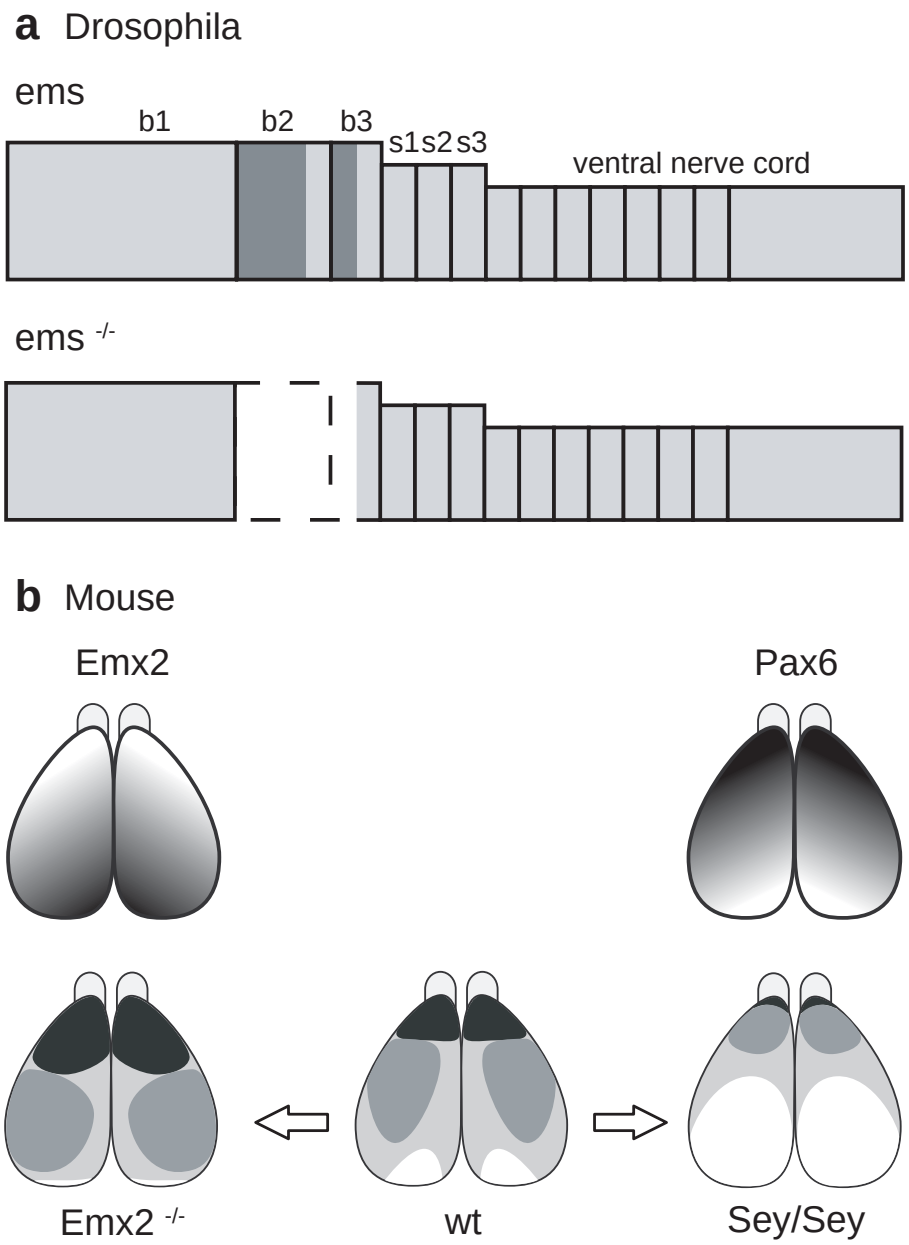

Figure 4-4 Schematic representation of expression patterns and loss-of-function mutant phenotypes of *ems* in *D. melanogaster* and *Emx2* and *Pax6* in mouse. (A) In insects the *ems* gene is expressed in the anterior part of the deutocerebrum and the anterior part of the tritocerebrum. Mutational inactivation of *ems* (*ems*-/-) results in the absence of the deutocerebrum and anterior part of the tritocerebrum. (B) In the developing mouse neocortex *Emx2* is expressed in a gradient, with high caudomedial and low rostrolateral expression levels. In *Emx2* null mutants (*Emx2*-/-) the anterior motor (black) and sensory (dark grey) cortical areas are expanded caudally, whereas the posterior visual cortical areas (white) are reduced in size. *Pax6* is expressed in a gradient opposite to that of *Emx2* expression in the developing neocortex. An opposite expansion of the cortical areas with respect to *Emx2* mutants is observed in the *Pax6* mutant *Small eye* (*Sey/Sey)*, which indicates the interaction of *Emx2* and *Pax6* in regionalizing the neocortex. (Abbreviations see Fig. 4-3); (modified after Hartmann *et al*., 2000 and Bishop *et al*., 2002).

The two mouse orthologues, *Emx1* and *Emx2*, of the *D. melanogaster* cephalic gene *ems*, show largely overlapping expression domains in the developing brain. Whereas *Emx1* mutant mice are postnatal viable and show rather subtle phenotypes restricted to the forebrain, *Emx2* mutant mice die immediately after birth (Pellegrini et al., 1996; Qiu et al., 1996; Yoshida et al., 1997). *Emx2* expression is seen in the germinative neuroepithelium of the presumptive cerebral cortex in the developing forebrain at around embryonic day 9.5. During early corticogenesis *Emx2* is restricted to the germinative layer in the ventricular zone where it is expressed in proliferating neuroblasts. Subsequently, *Emx2* expression is also found in Cajal-Retzius cells and the most marginal cortical plate neurons in the marginal zone (Gulisano et al., 1996; Mallamaci et al., 2000; Pellegrini et al., 1996; Qiu et al., 1996; Yoshida et al., 1997). The anteriormost expression of *Emx2* in the brain is found in the olfactory epithelium, whereas posteriorly the expression domain extends into the roof plate of the diencephalon. *Emx2* is expressed throughout the developing neocortex in a graded manner with high levels at caudomedial and low levels at rostrolateral regions (Fig. 4-4 B). An opposed gradient is built up by the *Pax6* gene that has been shown to interact with *Emx2* in the regionalization of the neocortex. Mutational inactivation of *Emx2* results in an expansion of the rostrolateral motor and somatosensory areas at the expense of the caudomedial neocortical areas, such as the visual area. An opposite shift in regional identity is seen in the *Pax6* loss-of-function mutant, while in the *Emx2* and *Pax6* double mutant the cerebral cortex seems to acquire the identity of basal ganglia (Bishop et al., 2002; Muzio et al., 2002; O'Leary and Nakagawa, 2002). Interestingly, two orthologues of *Pax6*, *ey (eyeless)* and *toy (twin of eyeless)* are expressed in the anterior brain of the *D. melanogaster* embryo (Kammermeier et al., 2001). This raises the question whether they interact with *ems* in the regional specification of the embryonic fly brain.

The expression of the *D. melanogaster ems* and the mouse *Emx* genes in the developing embryonic brain are similar as is their ability to confer regional identity to the cells of a specific domain in the brain. Further evidence for the functional equivalence of the *ems* and *Emx2* gene products comes from a cross-phylum rescue experiment carried out in *D. melanogaster* embryos. Ubiquitous overexpression of a mouse *Emx2* transgene in an *ems* null mutant background rescues the brain phenotype of the mutant fly embryos (Hartmann et al., 2000).

4.7 FUNCTIONAL CONSERVATION OF *OTD/OTX* GENES IN EMBRYONIC BRAIN DEVELOPMENT OF *D. MELANOGASTER* AND MOUSE

The *D. melanogaster* cephalic gap gene *otd* encodes a transcription factor with a *bicoid*-like homeodomain and is required for head development and segmental patterning in the fly embryo. The first *otd* transcripts appear in the anterior region of the early blastoderm stage embryo where they are expressed in a broad circumferential stripe. During gastrulation this anterior expression domain becomes more and more restricted to the procephalic neuroectoderm, and *otd* is expressed in most delaminating neuroblast of the presumptive protocerebrum and anterior deutocerebrum. This corresponds largely to the domain where *otd* is expressed at later embryonic stages in the brain (Hirth et al., 1995; Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). Interestingly, *otd* expression is not detected in the anteriormost part of the brain (Fig 4-5 A). A second *otd* expressing domain is found at the ventral midline of the fly embryo in mesectodermal cells that will give rise to neurons and glia of the ventral nerve cord (not shown in Fig. 4-5). Mutational inactivation of *otd* results in a striking phenotype of the fly embryo in which the entire anterior part of the brain is lacking (Hirth et al., 1995). Mutant analysis has shown that most protocerebral and part of the adjacent deutocerebral neuroblasts are absent in the *otd* mutant, a fact that correlates with the loss in the expression of the *l'sc* gene, which is thought to be required for neuroectodermal cells to adopt a neuroblast fate (Younossi-Hartenstein et al., 1997). In addition to the gap phenotype in the anterior brain, *otd* loss-of-function flies exhibit impairments in the development of visual structures as well as midline defects in the ventral nerve cord (Finkelstein et al., 1990). Ubiquitous overexpression of *otd* in a null mutant background at stages preceding neuroblast formation is able to restore anterior brain structures and ventral nerve cord defects. In a wild type background, ubiquitous overexpression of *otd* results in the generation of ectopic neuronal structures, such as enlarged ganglia. Interestingly, some of the ectopic cells express the protocerebrum-specific gene *bsh* (*brain-specific homeobox*) indicating that *otd* expression might induce a partial protocerebral identity in these neuronal structures (Leuzinger et al., 1998).

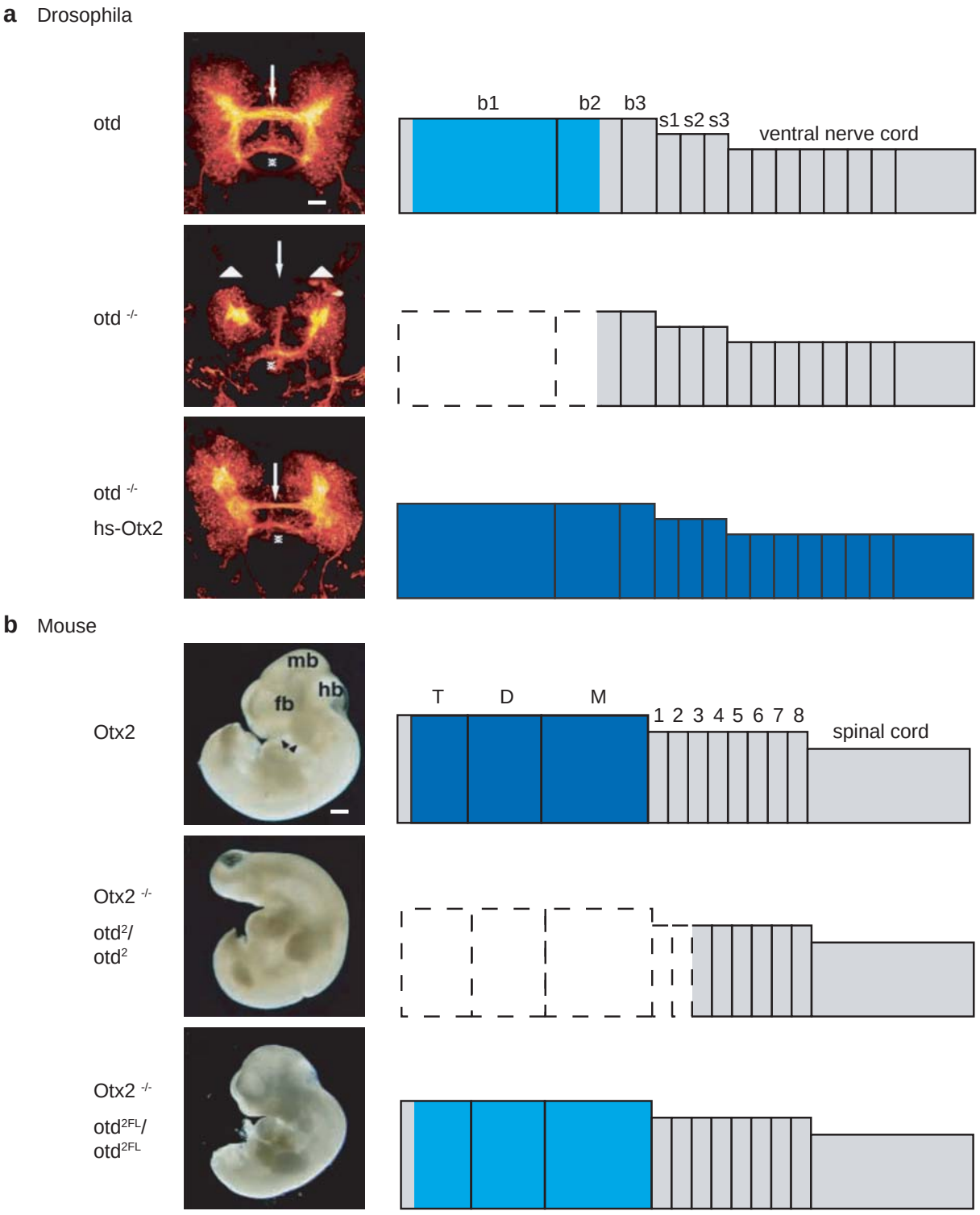

Figure 4-5 Summary scheme of expression domains, null mutant phenotypes and cross-phylum rescue experiments of the *otd/Otx2* genes in *D. melanogaster* and mouse. Genotypic indications on the left of the corresponding rows are indicated in (A) and (B). The photographs show frontal views of *D. melanogaster* embryonic brains (anti HRP immunostaining; scale bar: 10µm) in (A) and lateral views of whole mount mouse embryos (embryonic day 10.5; scale bar: 250µm) in (B). The column on the right hand side shows schematic representations of expression domains and phenotypes in the brain of the corresponding animal and genotype. (A) In the fly the *otd* gene is expressed throughout most of the protocerebrum and the anterior part of the deutocerebrum (light blue in the scheme). In the frontal view of the embryonic *D. melanogaster* wild type brain

the preoral commissure interconnecting the two anterior brain hemispheres is indicated by an arrow (the frontal connective is marked with an asterisk). In *otd* mutant embryos (*otd-/-*) the protocerebrum including the preoral commissure and the anterior deutocerebrum are absent (indicated by triangles in the picture and by dashed lines in the scheme). Overexpression of human *Otx2* gene (dark blue in the scheme) in *otd* mutant embryos (*otd-/-*; *hs-Otx2*) results in a rescue of the anterior brain including the preoral commissure. (B) In mouse the *Otx2* gene is expressed in the anterior part of the embryonic brain including the presumptive telencephalon (except for anteriormost part), diencephalon and mesencephalon (dark blue in scheme). In the lateral view of the mouse embryo the major brain regions are labelled as forebrain (fb), midbrain (mb) and hindbrain (hb). In *Otx2* null mutants in which the *D. melanogaster otd* replaces the *Otx2* gene (*Otx2-/-*; *otd2*/ *otd2*) the entire forebrain and midbrain (as well as rhombomeres 1 and 2) are absent. In *Otx2* mutants, in which the *otd* coding sequence has been fused to the 3' and 5' UTRs of *Otx2* gene (*Otx2-/-*; *otd2FL*/ *otd2FL*) the hybrid transcript is translated in the anterior neuroectoderm of the mouse embryo (light blue in the scheme) and the rostral forebrain is restored. (modified after Leuzinger *et al*., 1998; Acampora *et al*., 2001).

The two mouse orthologues, *Otx1* and *Otx2*, of the *otd* gene are expressed in nested domains of the developing brain. *Otx1* expression is first observed at approximately 8 dpc (days post coitum) in the neuroepithelium of the presumptive telencephalon, diencephalon and mesencephalon (Simeone et al., 1992a). During corticogenesis, *Otx1* expression is maintained in the ventricular zone of the cortical anlage but decreases as upper layer neurons are generated. By this time, postmigratory neurons of layers 5 and 6 progressively start to express *Otx1* whereas later differentiated neurons of upper layers 1-4 remain devoid of *Otx1* expression (Frantz et al., 1994). *Otx1* is also expressed at early embryonic stages in precursor structures of sense organs, such as the olfactory epithelium and the inner ear (Simeone et al., 1993).

Otx1 null mice are viable but suffer from spontaneous epileptic seizures and exhibit a smaller brain size, mainly due to a reduced thickness of the telencephalic cortex. In addition, the development of the acoustic sense organs are impaired, as the lateral semicircular duct of the inner ear is lost .

The earliest expression of *Otx2* is found in the epiblast and in the visceral endoderm (VE) prior to the onset of gastrulation. During gastrulation, *Otx2* expression is observed in the epiblast and anterior neuroectoderm as well as in the underlaying anterior visceral endoderm (AVE) and the node-derived axial mesendoderm (ame). The AVE and ame are believed to generate *Otx2*-mediated instructive signals that are required in the early specification and

patterning of the overlaying anterior neuroectoderm (Acampora and Simeone, 1999; Simeone, 1998). *Otx2* expression in the anterior neuroectoderm is maintained during brain regionalization and extends from the telencephalon to the posterior border of the mesencephalon, anterior of the midbrain-hindbrain boundary (MHB) (Fig. 4-5 B). Interestingly, the domain of *Otx2* expression does not include the most anterior brain region, which is similar to the expression pattern of *otd* in the embryonic fly brain (Simeone et al., 1992a).

Otx2 null mice die early in embryogenesis and lack the rostral neuroectoderm fated to become forebrain, midbrain and rostral hindbrain as a result of an impairment in early specification of the anterior neuroectoderm by the visceral endoderm (Acampora et al., 1995; Rhinn et al., 1998). This has been demonstrated in chimeric mouse embryos containing *Otx2-/-* epiblast and wild type VE, where the early induction of the anterior neural plate was transiently rescued but subsequent brain development remained impaired. No rescue was obtained in chimeras containing a wild type epiblast and a *Otx2-/-* visceral endoderm (Rhinn et al., 1998).

Cross-phylum rescue experiments between fly *otd* and mammalian *Otx1* and *Otx2* genes were carried out in order to assess the functional equivalence or diverged properties of the gene homologues. Ubiquitous overexpression of either human *Otx1* or human *Otx2* in a *otd* mutant fly in both cases restored the anterior brain structures absent in the *otd* null mutant (Fig 4-5 A) (Leuzinger et al., 1998).

Similar cross-phylum experiments were carried out in mouse with *otd* replacing the vertebrate *Otx* orthologues. In an *Otx1* null mutant background, *otd* is able to fully rescue epilepsy and corticogenesis abnormalities restoring wild type brain size. However, the lateral semicircular duct of the inner ear is never restored (Acampora et al., 1998a). A similar rescue potential is also observed in homozygous mutant mouse embryos, where *Otx1* was replaced with human (Acampora et al., 1999; Morsli et al., 1999). Thus, the ability to specify the lateral semicircular duct of the inner ear may be an *Otx1* specific property (Acampora and Simeone, 1999). Gene replacement experiments where different portions of the *Otx2* locus were exchanged with the cDNA of the fly *otd* or human *Otx1* genes revealed a crucial role of regulatory control mechanisms in *Otx2* specific action during anterior neuroectoderm specification. Two different replacement strategies were utilized. A first mouse model (otd^2/otd^2) was generated in which an *Otx2* region including 5’ and 3’ untranslated regions

(UTRs) was replaced with the fly *otd* cDNA whereas in a second mutant (otd^{2FL}/otd^{2FL}) the *otd* coding sequence was directly fused to the intact 5' and 3' UTRs of *Otx2*. In the otd^{2}/otd^{2} mouse model, *otd* is able to take over the early function of the *Otx2* gene in the AVE leading to a transient restoration of the anterior neural plate absent in *Otx2* mutants. However, otd^{2}/otd^{2} mutants fail to maintain the anterior identities of the neuroectoderm giving rise to a headless phenotype (Fig. 4-5 B). Mutant analysis revealed that *D. melanogaster otd* transcripts were present in both AVE and presumptive anterior neuroectoderm, whereas translation only occurred in the AVE. Additional evidence from similar experiments where *Otx2* including UTRs was replaced with human *Otx1* favored the view that post-transcriptional control was involved in the cell type-specific translation of *Otx2* mRNA in the epiblast and anterior neuroectoderm (Acampora et al., 1998b; Boyl et al., 2001). This was confirmed in the second mouse model otd^{2FL}/otd^{2FL} where translation of the hybrid transcript consisting of the fly *otd* fused to the 5' and 3' UTRs of *Otx2* occured in the epiblast and anterior neuroectoderm. Moreover, the correct translation of *otd* in the epiblast and anterior neuroectoderm restored the maintenance of anterior brain pattening in *Otx2* null mutants including the normal positioning of the midbrain-hindbrain boundary (Fig. 4-5 B) (Acampora et al., 2001b). This was also shown by a similar hybrid mouse model where human *Otx1* was fused to the 5' and 3' UTRs in the mouse *Otx2* locus (Acampora et al., 2003). Taken together, *Otx1*, *Otx2* and *otd* genes show a high degree of functional equivalence in the regions of the developing organism where they are normally expressed. This supports the idea that *otd/Otx* functions were originally established in a common ancestor of fly and mouse and conserved throughout evolution. On the other hand, their regulatory control mechanisms appear to have been modified during evolution, thus, generating the specific properties of the genes.

4.8 EVIDENCE FOR A COMMON TRIPARTITE GROUND-PLAN OF THE BILATERIAN BRAIN

A detailed comparison of gene expression patterns and developmental neuroanatomy in vertebrates and urochordates (ascidians) has uncovered a common tripartite ground-plan along the anteroposterior axis for the embryonic CNS. In all cases studied, a rostral brain region expressing *Otx* family genes (corresponding to the vertebrate forebrain and midbrain) is followed by a central region expressing *Pax2/5/8* genes (delimiting the midbrain-hindbrain boundary of vertebrates), and subsequently a *Hox* gene expressing caudal region (hindbrain and spinal cord of vertebrates) (Holland and Holland, 1999; Wada et al., 1998; Wada and Satoh, 2001). Recently, a similar tripartite pattern of gene expression has been reported for arthropods (see below) and hemichordates suggesting an evolutionarily more ancient origin of the tripartite organization of brains than chordates (Fig. 4-6 A - E) (Hirth et al., 2003; Lowe et al., 2003). (Interestingly, no *Pax2/5/8* expression can be detected between the anterior *Otx* and posterior *Hox* expression domains in the neural tube of the cephalochordate *Amphioxus*; the most parsimonious explanation for this is the secondary loss of the tripartite pattern in the *Amphioxus* CNS (Kozmik et al., 1999; Takahashi and Holland, 2004).)

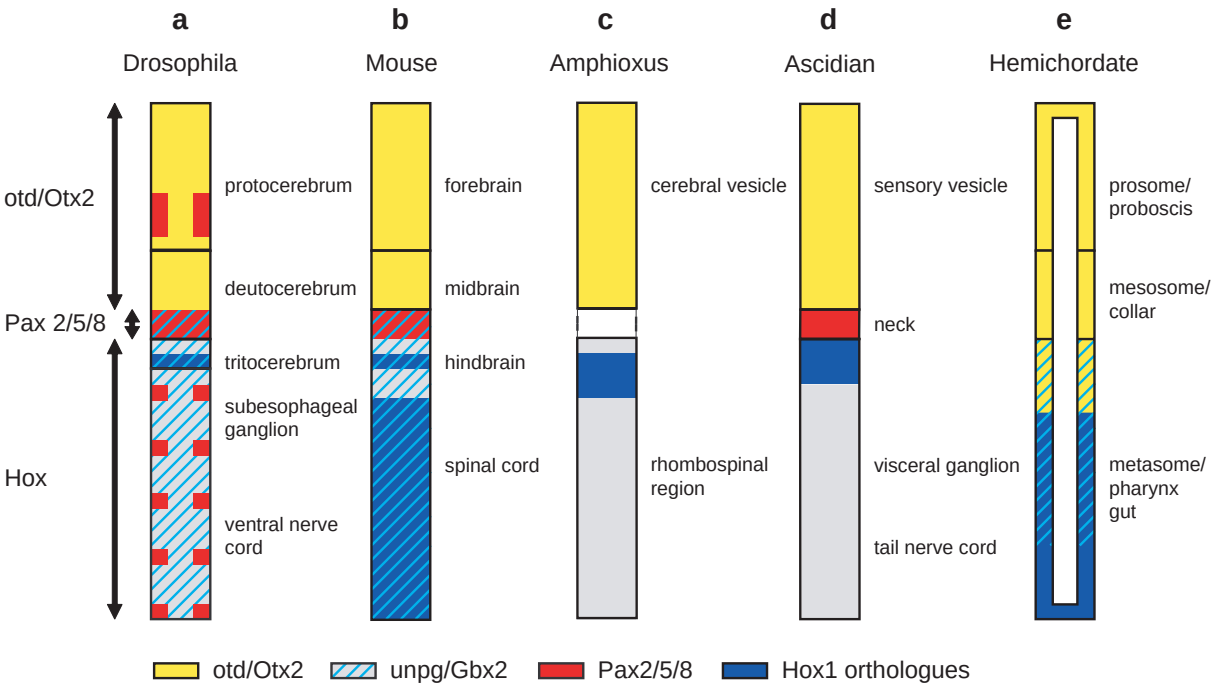

Figure 4-6 Tripartite ground-plan of the bilaterian nervous system based on expression patterns of orthologous genes in *Drosophila*, mouse, *Amphioxus*, ascidian and hemichordate. The expression of *otd/Otx2*, *unpg/Gbx2*, *Pax2/5/8* and *Hox1* gene orthlogues in the developing nervous systems of (A) stage 13/14 *D. melanogaster* embryo (Hirth *et al*., 2003), (B) embryonic day 10 mouse embryo (Wurst and Bally-Cuif, 2001), (C) 10 somite stage *Amphioxus* embryo (Wada and Satoh, 2001), (D) neurula ascidian (Wada *et al*., 1998) and (E) 1 gill slit stage hemichordate embryo (Lowe *et al*., 2003). In all cases an *otd/Otx2* expressing region is located anterior to a *Hox*-expressing region in the posterior nervous system. In *D. melanogaster* and mouse a *Pax2/5/8*-expressing domain is positioned at the interface between the anterior *otd/Otx2* and the posteriorly abutting *unpg/Gbx2* expression domains (up to date, no *unpg/Gbx2* orthologues have been isolated in *Amphioxus* and Ascidians). In contrast, the expression domains of the hemichordate *otd/Otx2* and *unpg/Gbx2* orthologues show no sharp boundary but overlap in an intermediate region of the basiepithelial nerve net. Nevertheless, the expression of the hemichordate *Pax2/5/8* orthologue is consistent with its relative location in chordates (C.J. Lowe, personal communication). No *Pax2/5/8* expression can be found between the *otd/Otx2* domain and the *Hox1* domain in *Amphioxus*, which is thought to be due to a secondary reduction.

In vertebrate brain development, the *Pax2/5/8* domain at the midbrain-hindbrain boundary is an early marker for the isthmic organizer (IsO), which controls both the growth and the ordered rostrocaudal specification of mesencephalic and metencephalic territories (reviewed by Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). The IsO was first identified through transplantation experiments, in which MHB tissue grafts were transplanted to more rostral or caudal neural locations resulting in the induction of mesencephalic-metencephalic fate in the host tissue surrounding the graft (Marin and Puelles, 1994; Martinez et al., 1991). This organizer-like activity on the surrounding neural tissue is

thought to be mediated by Fgf8 (fibroblast growth factor 8) and Wnt1 proteins which are secreted from the MHB tissue. During late gastrulation and early neural plate stages of the vertebrate embryo, the two homeodomain transcription factors *Otx2* and *Gbx2* are expressed in a complementary, mutually exclusive fashion anterior and posterior to the MHB. Whereas *Otx2* null mutant mice lack the brain rostral to rhombomere 3 (see above), mice of the genotype *Otx1-/-*; *Otx2+/-* show a rostral extension of metencephalic tissue and the absence of the mesencephalon and caudal diencephalon. Furthermore, the expression of MHB specific markers, such as *Fgf8*, *Gbx2* and *Wnt1*, align in a domain that is shifted rostrally to the corresponding position of prosomere 2 (Acampora et al., 1997). Conversely, a caudal shift of MHB markers can be observed in *Gbx2* null mutants, where isthmic nuclei, cerebellum and rhombomeres 1-3 of the hindbrain are absent (Fig. 4-7 A) (Millet et al., 1999; Wassarman et al., 1997). Together with evidence from misexpression experiments, these results suggest that an antagonistic interaction between *Gbx2* and *Otx2* during early embryonic stages is responsible for the correct positioning of the MHB at their common interface.

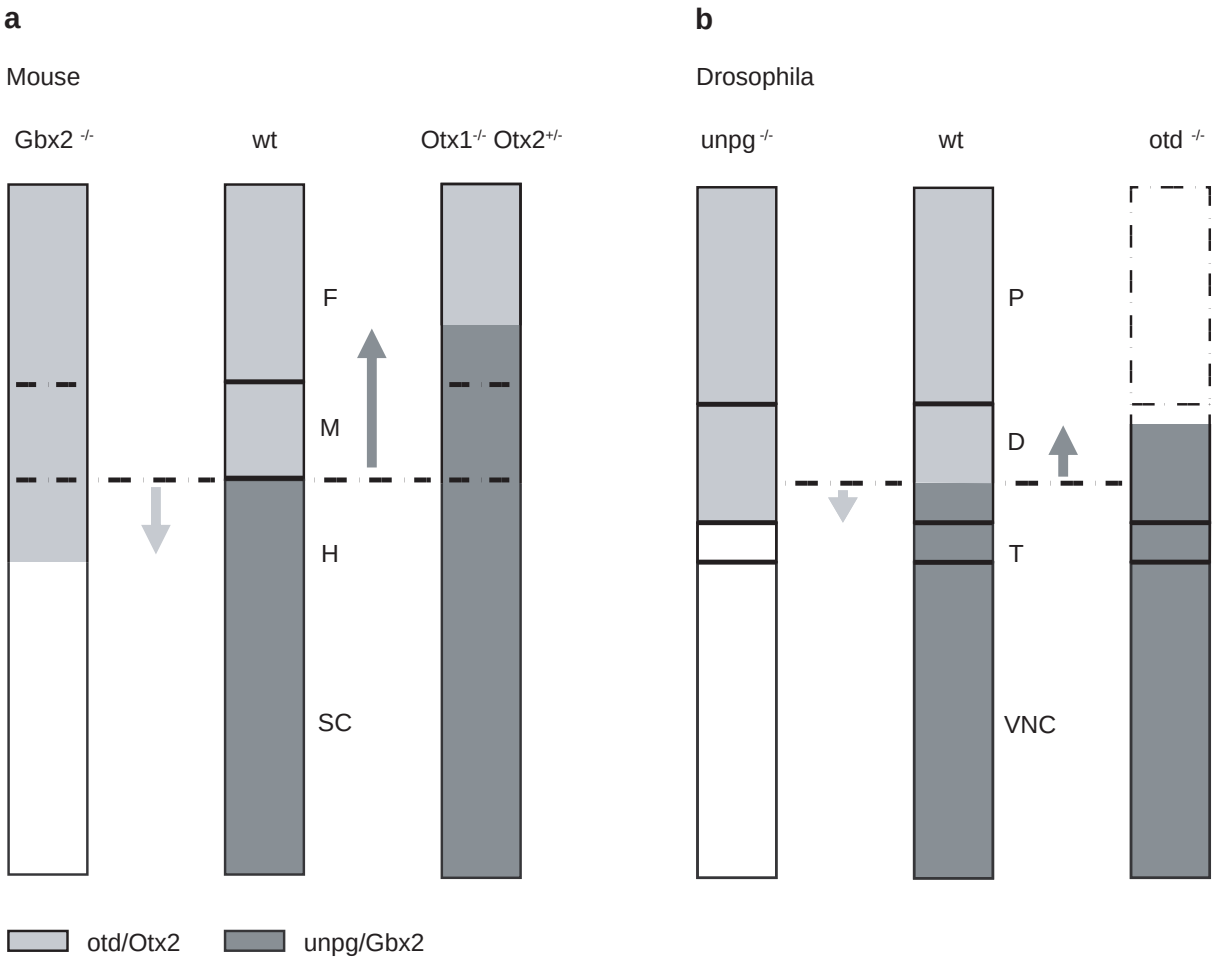

Figure 4-7 Antagonistic interactions of the *otd/Otx* and *unpg/Gbx2* genes in the positioning of their common interface. (A) Expression domains of *Otx2* and *Gbx2* in the developing mouse CNS corresponding to the six-somite stage in the *Gbx2* null mutant (*Gbx2-/-*), wild type (wt) and *Otx1-/-*; *Otx2+/-* (*Otx1-/-*; *Otx2+/-*) genetic background. In the wild type mouse embryo *Otx2* is expressed with a sharp limit at the posterior mesencephalon and *Gbx2* expression abuts the *Otx2* expression domain, creating a common interface. In mice homozygous mutant for *Otx1* and heterozygous mutant for *Otx2* (*Otx1-/-*; *Otx2+/-*) the common interface is shifted anteriorly into the forebrain (dark grey arrow). A posterior expansion of the *Otx2* expression into the hindbrain is observed in *Gbx2* null mutant (*Gbx2-/-*) brains. (B) Expression domains of *otd* and *unpg* in the developing CNS of *D. melanogaster* in the *unpg* null mutant (*unpg-/-*), wild type (wt) and *otd* null mutant (*otd-/-*) genetic background. The expression domains of *otd* and *unpg* in the wild type *D. melanogaster* CNS form a sharp common boundary in the posterior deutocerebrum. In the *otd* null mutant embryo (*otd-/-*) the protocerebrum and the anterior deutocerebrum are absent (dashed lines). In addition, the *unpg* expression is shifted anteriorly (dark grey arrow). In the brain of the *unpg* null mutant embryo (*unpg-/-*) the *otd*-expressing domain expands posteriorly (light grey arrow). Abbreviations: P, protocerebrum; D, deutocerebrum; T, tritocerebrum; VNC, ventral nerve cord; F, forebrain; M, midbrain; H, hindbrain; SC, spinal cord (modified after, Hirth *et al*., 2003; Joyner and Millet, 2000).

Gene expression studies indicate that embryonic anteroposterior patterning of the *D. melanogaster* brain is strikingly similar to the tripartite ground-plan of the vertebrate brain.

The two *D. melanogaster Pax2/5/8* orthologues, *Pox neuro* (*Poxn*) and *Pax2*, are both expressed at the interface of *otd* and the *Gbx2* orthologue *unplugged* (*unpg*), anterior to a *Hox*-expressing region (Fu and Noll, 1997; Hirth et al., 2003; Noll, 1993). The intermediate domain where the two *Pax2/5/8* orthologues are coexpressed in the developing brain coincides with the deutocerebral-tritocerebral boundary (DTB) of the insect brain. Additional *Poxn* and *Pax2* expression is observed in a segmentally reiterated pattern in the fly CNS (not shown in Fig. 4-6). Mutational inactivation of *otd* results in the deletion of the anterior brain of the fly embryo (see above) as well as in the rostral extension of the *unpg* expression domain. In *unpg* loss-of-function mutants, the posterior limit of the anterior brain-specific *otd* expression shifts caudally (Fig. 4-7 B). Thus, in both *D. melanogaster* and mouse, mutational inactivation of *otd/Otx2* and *unpg/Gbx2* genes results in the loss or misplacement of an intermediate brain domain characterized by the *otd/Otx2* and *unpg/Gbx2* interface and by the expression of *Pax2/5/8* genes. Moreover, *otd/Otx2* and *unpg/Gbx2* appear to negatively regulate each other at the interface of their expression domains in insects and vertebrates. (Interestingly, *D. melanogaster otd* is able to replace *Otx* gene function in the correct positioning of the MHB during mouse brain development as demonstrated in cross-phylum rescue experiments (see above) (Acampora et al., 2001b).) Taken together, these results reveal remarkable similarities in gene expression and functional interactions involved in establishing the insect DTB and mouse MHB. However, not all functional interactions among genes involved in MHB formation in the mouse appear to be conserved at the DTB of *D. melanogaster*. Although expression of patterning genes that characterize the vertebrate MHB region, such as *engrailed* (*en*), *Pax2*, *Poxn* or the fly Fgf orthologue *branchless* (*bnl*) can be found at the DTB, no brain patterning defects are observed in the corresponding null mutant embryos in the fly (Hirth et al., 2003). Moreover, even though *D. melanogaster* has a tripartite ground-plan for the developing brain and a boundary region genetically corresponding to the vertebrate MHB, evidence for organizer activity of the fly DTB has not been obtained.

In summary, current comparative data suggest that a tripartite ground-plan for the developing brain was already present in the common ancestor of bilateria. To date, organizer activity of the intermediate boundary region has only been demonstrated in vertebrates (Takahashi and Holland, 2004). As proposed by Wada and Satoh (2001), it may be useful to distinguish between the homology of two characteristics of the vertebrate MHB: homology as a developmental genetic region of the brain and homology as an organizer. In this sense, the *D. melanogaster* DTB can be considered as a region homologous to the vertebrate MHB.

4.9 CONCLUSIONS

Recent investigations on mechanisms controlling insect and vertebrate brain development have revealed an expanding number of homologous genes with similar expression patterns and comparable functions. The expression and interactions of homologous dorsoventral patterning genes show comparable relative patterning and orientation with respect to the presumptive neurogenic region. Genes of the *otd/Otx* and *ems/Emx* families are required for correct formation and specification of the developing anterior brain, and *Hox* genes are involved in patterning and specification of the developing posterior brain. Moreover, *otd/Otx* genes and *unpg/Gbx2* genes position an intermediate domain between an anterior and a posterior brain region and thus contribute to the tripartite ground-plan of the insect and vertebrate brain.

Taken together these results imply the evolutionary conservation of genetic programs underlying embryonic brain development in insects and vertebrates and provide further evidence for the idea that the protostome and deuterostome brain is homologous in a developmental genetic sense (Arendt and Nubler-Jung, 1999; De Robertis and Sasai, 1996; Hirth and Reichert, 1999). Specific gene functions that are not shared between orthologous control genes in fly and mouse, such as the posttranscriptional control of the *Otx2* gene in the mouse epiblast, appear primarily to involve modifications of regulatory control elements but not the coding sequence of the gene. This suggests that genes involved in essential mechanisms of brain development could exert additional, novel functions by modification of their spatial or temporal regulatory control (Acampora et al., 2001b; Acampora and Simeone, 1999).

In the mouse midbrain, recent studies have revealed a regulative role of *Otx2* in the integration of anteroposterior and dorsoventral body axis patterning. *Otx2* interacts with the dorsoventral regionalization gene *Nkx2.2* in the developing ventral midbrain regulating the extent and identity of neuronal progenitor domains (Puelles *et al.*, 2003; Puelles *et al.*, 2004). It is unknown to date, whether this interaction constitutes an evolutionarily ancient mechanism for the integration of the positional information from two different body axis in the brain. It will be important to test if a similar interaction between *otd* and *vnd* (the *D. melanogaster* orthologue of *Nkx2.2*) is involved in regionalization of the developing insect

brain. The identification of downstream targets of conserved developmental control genes as well as the analysis of genetic mechanisms at more advanced stages of development should also reveal more insight into the degree of conservation of genetic programs between insects and vertebrates.

A recent gene expression study on hemichordates has lead to the view that the deuterostome ancestor might have been characterized by a diffuse basiepithelial nervous system and that a centralized brain could have evolved independently in the deuterostome and protostome lineages (Holland, 2003; Lacalli, 2003; Lowe et al., 2003). Homologies in embryonic brain development of vertebrates and insects would therefore derive from axis patterning mechanisms or the correlating gene expression patterns, which were present in the circumferential nerve net of the last common ancestor. Other similarities that are not inherited from a common ancestor characterized by a well patterned nerve net would therefore be a product of convergent or parallel evolution (Gould, 2002). An alternative explanation for the absence of a centralized nervous system in hemichordates is a secondary reduction of a CNS and the retention of a peripherally located nerve net during evolution. The expression patterns of the hemichordate orthologues of dorsoventral patterning genes of vertebrates and *D. melanogaster* should nurture the discussion on the urbilaterian nervous system.

5 Anteroposterior regionalization of the brain: genetic and comparative aspects

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5.1 SUMMARY

Developmental genetic analyses of embryonic CNS development in *Drosophila* have uncovered the role of key, high-order developmental control genes in anteroposterior regionalization of the brain. The gene families that have been characterized include the *otd/Otx* and *ems/Emx* genes which are involved in specification of the anterior brain, the *Hox* genes which are involved in the differentiation of the posterior brain, and the *Pax* genes which are involved in the development of the anterior/posterior brain boundary zone. Taken together with work on the genetic control of mammalian CNS development, these findings indicate that all three gene sets have evolutionarily conserved roles in brain development, revealing a surprising evolutionary conservation in the molecular mechanisms of brain regionalization.

5.2 INTRODUCTION

In most animals, the central nervous system (CNS) is characterized by bilateral symmetry and by an elongated anteroposterior axis, both of which are established very early in embryonic development. During embryogenesis, regionalized anatomical subdivisions appear along the anteroposterior axis, also referred to as the neuraxis. These subdivisions are most prominent near the anterior pole, where the complex structures that comprise the brain are generated. As the brain differentiates, the neuraxis often bends and species-specific flexures arise, which in later stages tend to distort the original anteroposterior coordinates of the CNS. However, when this is taken into account and the neuraxis is reconstructed, remarkable similarities in anteroposterior regionalization of the CNS in animals as diverse as arthropods and vertebrates become apparent. A full appreciation of these similarities comes from combined comparative neuroanatomical and molecular genetic studies carried out in *Drosophila* and mouse, which reveal that comparable, evolutionarily conserved developmental patterning mechanisms operate in regionalization of the embryonic CNS (Arendt and Nubler-Jung, 1999; Reichert and Simeone, 1999).

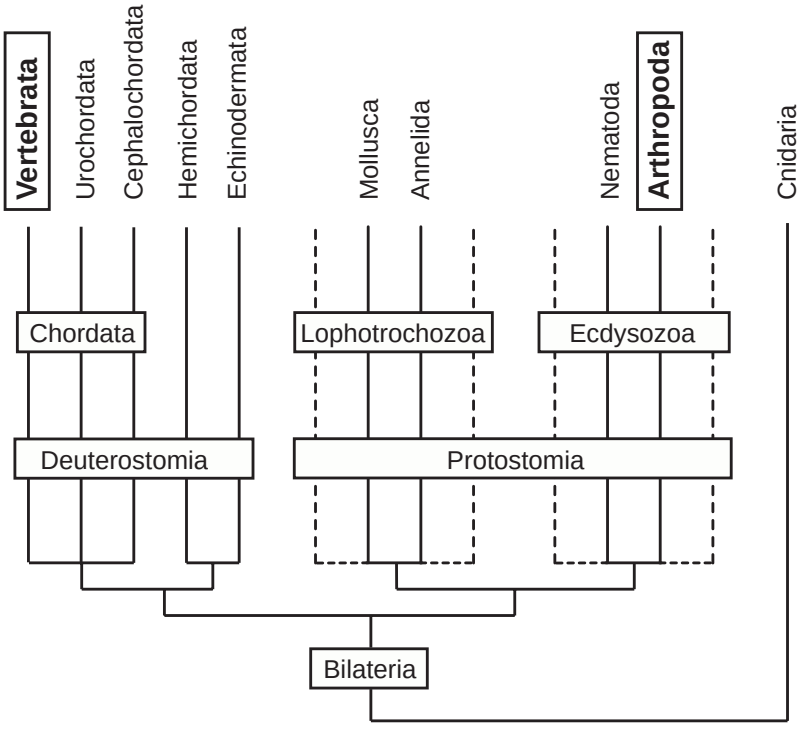

Figure 5-1 Phylogenetic relationship of Bilateria. Simplified version of the new molecular-based phylogeny showing a selection of bilaterian phyla with the Cnidaria as outgroup. Bilaterian phyla are grouped according to major cladistic classifications. The phylogenetic tree suggests that evolutionarily conserved, homologous features of mouse and *D. melanogaster* already existed in the common ancestor of all bilaterian animals.

Here we review recent findings on the developmental genetic control of anteroposterior regionalization in the embryonic CNS in *Drosophila* and compare these findings with investigations carried out on regionalization of the embryonic murine CNS. The similarities in the expression patterns of key developmental control genes together with the comparable functions of these genes during CNS development in flies and mice suggest a common evolutionary origin of the mechanism of embryonic CNS regionalization. Given the current molecular-based phylogeny of bilaterian animals, it seems likely that these features of brain development in arthropods and vertebrates were already present in the common bilaterian ancestor from which protostomes and deuterostomes evolved (Fig. 5-1) (Adoutte et al., 2000). This, in turn, challenges the classical view of an independent origin of protostome and deuterostome brains.

The early embryonic CNS of both insects and vertebrates is composed of longitudinally arranged subdivisions that can be grouped into two major parts, an anterior cephalized brain which rapidly forms prominent morphological specializations, and a posterior nerve cord-like structure. In insects, the embryonic brain consists of a supraesophageal ganglion that can be subdivided into the protocerebral (b1), deutocerebral (b2), and tritocerebral (b3) neuromeres and a subesophageal ganglion that is subdivided into the mandibular (s1), maxillary (s2), and labial (s3) neuromeres (Fig. 5-2A). The neuromeres of the developing ventral nerve cord extend posteriorly from the subesophageal ganglion into the body trunk (Younossi-Hartenstein et al., 1996). In vertebrates, the anterior CNS develops three embryological brain regions; the prosencephalon or forebrain (presumptive telencephalon and diencephalon), the mesencephalon or midbrain and the rhombencephalon or hindbrain. The developing hindbrain reveals a metameric organization based on eight rhombomeres, and parts of the developing forebrain may also be metamerically organized (Lumsden and Krumlauf, 1996; Puelles and Rubenstein, 2003)**.** The developing spinal cord extends posteriorly from the hindbrain into the body trunk.

The topology of these embryonic neuroanatomical regions is reflected in the regionalized expression along the neuraxis of key developmental control genes which appears to be largely conserved between insects and vertebrates. Thus, the anterior CNS of *Drosophila* and mouse is characterized by the expression of the genes *orthodenticle* (*otd*/*Otx*) and *empty spiracles*

(*ems*/*Emx*). Similarly, the posterior CNS of both species exhibits a conserved and highly ordered expression pattern of the homeotic (*Hox*) gene family. Finally, expression of the *Pax2/5/8* genes defines a third CNS region between the anterior *otd*/*Otx* and the posterior *Hox* domains, thus revealing a tripartite ground plan of embryonic CNS development in both vertebrates and insects. In the following we consider the roles of each of these three sets of developmental control genes in anteroposterior regionalization of the CNS.

5.3 THE CEPHALIC GAP GENES OTD/OTX AND EMS/EMX CONTROL ANTERIOR BRAIN DEVELOPMENT

The *orthodenticle* (*otd*) and *empty spiracles* (*ems*) homeobox genes belong to the cephalic gap genes in *Drosophila* together with *tailless* (*tll*), *buttonhead* (*btd*) and *sloppy paired* (*slp*). At the early blastoderm stage of embryogenesis, the cephalic gap genes are broadly expressed in overlapping anterior domains under the control of maternal genes (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Walldorf and Gehring, 1992). The functional inactivation of any of these transcription factors results in gap-like phenotypes where structures of several head segments are missing (Cohen and Jurgens, 1990; Schmidt-Ott et al., 1994). In addition, the cephalic gap genes *tll*, *otd*, *ems* and *btd* have been shown to play essential roles early brain development. By the time of neuroblast delamination, their expression domains become restricted to specific subsets of neural progenitors in the anterior procephalic neuroectoderm (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). Mutational inactivation of a given cephalic gap gene results in the deletion of a specific brain area, indicating the requirement of these genes in early specification of the anterior brain primordium (Hirth et al., 1995; Younossi-Hartenstein et al., 1997).

The cephalic gap gene *otd* encodes a transcription factor with a *bicoid*-like homeodomain and is required for head development and segmental patterning in the fly embryo. In the early blastoderm stage embryo, *otd* is first expressed in a broad circumferential stripe in the anterior region. During gastrulation, however, expression becomes more and more restricted to the anterior procephalic neuroectoderm, where *otd* is expressed in most delaminating neuroblasts of the presumptive protocerebrum (b1) and anterior deutocerebrum (b2) (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). This expression domain corresponds largely to the *otd* expression pattern detected at later embryonic stages in the brain (Hirth et al., 1995) (Fig. 5-2B). Interestingly, *otd* expression is not observed in the anterior most protocerebral region. An additional, segmentally reiterated expression pattern of *otd* is found at the ventral midline of the fly embryo in mesectodermal cells that will give rise to neurons and glia of the ventral nerve cord (not shown in Fig. 5-2B). Comparable to *otd*, the homeobox gene *ems* is first expressed in a broad stripe posterior and adjacent to *otd* in the early blastoderm stage embryo. In the procephalic neuroectoderm and in the subsequently formed early embryonic brain *ems* expression becomes restricted to two stripes in the anterior parts of

the deutocerebral (b2) and tritocerebral (b3) neuromeres (Fig. 5-2B). In the ventral nerve cord *ems* expression is also found in a segmentally repeated pattern (not shown in Fig. 5-2B) (Hartmann et al., 2000; Hirth et al., 1995).

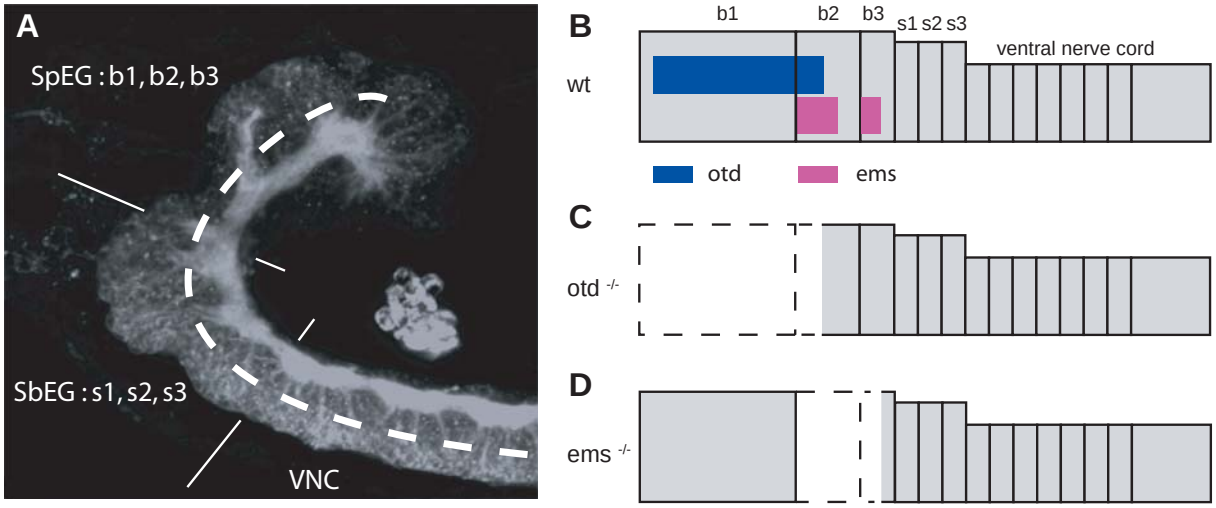

Figure 5-2 Schematic representation of expression patterns and mutant phenotypes of *otd* and *ems* in the embryonic CNS of *Drosophila*. (A) Lateral view of the anterior portion of the embryonic CNS. Because of morphogenetic processes, such as the beginning of head involution, the neuraxis (dashed line) of the embryonic brain curves dorsoposteriorly withing the embryo. Accordingly, in the following, anteroposterior coordinates refer to the neuraxis rather than the embryonic body axis. The major anteroposterior CNS regions are subdivided by white lines. (B – D) Schematic representations of the embryonic brain with anterior towards the left and posterior towards the right. (B) In the wild type (wt) brain the *otd* gene is expressed throughout most of the protocerebrum (b1) and the anterior part of the deutocerebrum (b2). Expression of *ems* in the brain is restricted to the anterior part of the deutocerebrum and the anterior part of the tritocerebrum (b3). The segmentally reiterated expression patterns of both *otd* and *ems* are omitted for clarity in this schematic. (C) In *otd* mutant embryos (*otd*$^{-/-}$) the protocerebrum and the anterior deutocerebrum are absent (indicated by dashed lines). (D) Mutational inactivation of *ems* (*ems*$^{-/-}$) results in the absence of the deutocerebrum and anterior part of the tritocerebrum. Abbreviations: b1, protocerebrum; b2, deutocerebrum; b3, tritocerebrum; s1, mandibular neuromere; s2, maxillary neuromere; s3, labial neuromere; SbEG, subesophageal ganglion; SpEG, supraesophageal ganglion; VNC, ventral nerve cord.

Mutational inactivation of either *otd* or *ems* results in striking embryonic brain phenotypes in which large brain regions are absent. In the *otd* mutant the entire anterior part of the brain is lacking (Fig. 5-2C) and mutant analysis has shown that most protocerebral neuroblasts and part of the adjacent deutocerebral neuroblasts are absent in the procephalic neuroectoderm (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In addition to the gap phenotype in the anterior brain, *otd* mutant flies exhibit impairments in the development of visual structures as

well as midline defects in the ventral nerve cord (Finkelstein and Perrimon, 1990). Ubiquitous overexpression of *otd* in a null mutant background at specific stages preceding neuroblast formation is able to restore anterior brain structures and ventral nerve cord defects (Leuzinger et al., 1998). Similarly, loss-of-function of the *ems* gene results in a gap-like phenotype in the embryonic brain due to the absence of cells in the deutocerebral and tritocerebral neuromeres (Fig. 5-2D). Additionally, axon pathfinding defects can be observed in the ventral nerve cord of *ems* mutant embryos. These phenotypes are rescued by ubiquitous overexpression of *ems* during specific early embryonic stages (Hartmann et al., 2000). Mutant analysis for both *otd* and *ems* shows that the absence of cephalic gap gene expression in the procephalic neuroectoderm correlates with the loss in the expression of the proneural gene *lethal of scute* (*l'sc*) and the ability to form neuroblasts in the mutant domain (Younossi-Hartenstein et al., 1997). In summary, *otd* and *ems* are expressed in adjacent and slightly overlapping domains in the anterior embryonic fly brain. The function of these cephalic gap genes is required for the formation of specific regions of the anterior brain primordium.

Based on homology between homeobox sequences, orthologs of the *Drosophila otd* and *ems* genes have been isolated in various vertebrates including zebrafish, mouse and humans (Acampora et al., 2005; Cecchi, 2002). In mouse, the two vertebrate orthologs of the *otd* gene, *Otx1* and *Otx2*, are expressed in nested domains of the developing head and brain. *Otx1* transcripts first appear at approximately 8 days post coitum (dpc), whereas *Otx*2 expression is detectable earlier at the prestreak stage (5.5 dpc) within the entire epiblast and visceral endoderm prior to the onset of gastrulation. Subsequently, the domain of *Otx2* expression becomes restricted to the anterior region of the embryo, which includes a territory fated to give rise to forebrain and midbrain, defining a sharp boundary at the future midbrain-hindbrain boundary. *Otx1* expression is nested within this *Otx2* domain and subsequently becomes spatially and temporally restricted to the developing cortex and cerebellum. Interestingly, the domain of *Otx2* expression does not include the most anterior brain region, which is similar to the expression pattern of *otd* in the embryonic fly brain (Acampora et al., 2005; Simeone et al., 1992a). Analysis of *Otx1* mutants does not reveal any apparent defects in early brain development. However, later in development loss of *Otx1* function affects cortical neurogenesis and causes epilepsy. In addition, the development of eye and inner ear is impaired (Acampora et al., 2005; Acampora et al., 1996). In contrast to *Otx1* mutant mice, *Otx2* null mice die early in embryogenesis and lack the rostral brain regions including

forebrain, midbrain and rostral hindbrain due to defective anterior neuroectoderm specification (Acampora et al., 2005; Acampora et al., 1995).

A comparison of the role of the *otd*/*Otx* genes in early brain patterning in *Drosophila* and mouse reveals striking similarities suggesting an evolutionary conservation of *otd*/*Otx* gene function. An interesting confirmation of the functional conservation in patterning the rostral brain can be carried out in cross-phylum rescue experiments. Ubiquitous overexpression of either human *Otx1* or human *Otx2* in an *otd* mutant fly embryo restores the anterior brain structures absent in the *otd* null mutant (Leuzinger et al., 1998). Similarly, overexpression of *Drosophila otd* in an *Otx1* null mouse embryo fully rescues epilepsy and corticogenesis abnormalities (but not inner ear defects) (Acampora et al., 2005; Acampora et al., 1998a). Moreover, overexpression of a hybrid transcript consisting of the fly *otd* coding region fused to the 5' and 3' UTRs of *Otx2* restores the anterior brain patterning in *Otx2* null mutant mice including the normal positioning of the midbrain-hindbrain boundary (Acampora et al., 2001a).

As is the case for the *otd*/*Otx* genes, two vertebrate orthologs of the *Drosophila ems* gene, *Emx1* and *Emx2*, have been identified. *Emx1* and *Emx2* expression in the mouse CNS is restricted to the forebrain, where largely overlapping expression patterns are seen. Whereas, *Emx1* expression only begins after neurulation, *Emx2* is already detectable around 8.5 dpc in the rostral neural plate (Gulisano et al., 1996; Simeone et al., 1992a; Simeone et al., 1992b). Within the developing neocortex, *Emx2* is expressed in a high caudomedial to low rostrolateral gradient, which is contrasted by an opposed gradient of *Pax6* gene expression. Mutational inactivation of *Emx2* results in an expansion of the rostrolateral brain areas at the expense of the caudomedial neocortical areas. An opposite shift in regional identity is seen in the *Pax6* loss-of-function mutant. In the *Emx2* and *Pax6* double mutant, the cerebral cortex completely loses its identity and instead acquires characteristics of basal ganglia (Bishop et al., 2002; Muzio et al., 2002). Whereas *Emx2* mutant mice die immediately after birth, *Emx1* mutant animals are postnatal viable and show rather subtle phenotypes that are restricted to the forebrain (Qiu et al., 1996; Yoshida et al., 1997). The regionalized expression patterns of the *ems*/*Emx* genes in the developing brain of *Drosophila* and mouse are remarkably similar, as is their ability to confer regional identity to the cells of a specific domain in the brain. Moreover, overexpression of a mouse *Emx2* transgene in an *ems* mutant background can

rescue the brain phenotype of fly embryos (Hartmann et al., 2000). Taken together, the similar spatiotemporal expression patterns and the high degree of functional equivalence between *Drosophila* and mouse suggest an evolutionarily conserved role of the *ems*/*Emx* and *otd*/*Otx* genes in anterior brain development.

5.4 THE HOX GENES PATTERN THE POSTERIOR BRAIN

The homeotic or *Hox* genes, encoding homeodomain transcription factors, were first discovered as crucial regulators of anteroposterior segment identity in the ectoderm of *Drosophila melanogaster*. Subsequently, *Hox* genes were found in a wide range of species where they have essential roles in many aspects of anteroposterior body axis patterning (Ferrier and Holland, 2001; Hughes and Kaufman, 2002). In *Drosophila*, the *Hox* genes are arranged along the chromosome in two gene clusters known as the *Antennapedia* (*ANT-C*) and *Bithorax* (*BX-C*) complexes. The *ANT-C* contains the five more anteriorly expressed *Hox* genes: *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*). The *BX-C* contains the three posteriorly expressed genes: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*). Interestingly, there exists a correlation between the relative position of the genes within the cluster and their spatial and temporal expression pattern along the body axis; genes located towards the 3’ end of the cluster are expressed more anteriorly and earlier in the embryo than are genes located towards the 5’ end. This correlation has been termed spatial and temporal colinearity (Mann, 1997). In mammals, *Hox* genes are arranged into four chromosomal clusters, termed *Hox A–D*, which contain between 9 and 11 *Hox* genes that can be assigned to 13 paralogous groups. Only the *Hox B* cluster comprises orthologs of all *Drosophila* homeotic genes. As in *Drosophila*, spatial and temporal colinearity is also observed among vertebrate *Hox* genes and more posterior acting genes impose their developmental specificities upon anterior acting genes (Duboule and Morata, 1994; Mann, 1997).

Hox gene expression in the developing CNS is a shared feature of a wide range of bilaterian animals, including protostomes such as insects or annelids, and deuterostomes, such as hemichordates or vertebrates (Hirth et al., 1998; Kourakis et al., 1997; Lowe et al., 2003; Wilkinson et al., 1989). Remarkably, throughout the Bilateria, *Hox* gene orthologs are expressed in a similar anteroposterior order. In *Drosophila*, the expressions of *Hox* cluster genes delineate discrete domains in the embryonic brain and ventral nerve cord (Fig. 5-3A). Their anterior expression boundaries often coincide with morphologically defined neuromere compartment boundaries. Although the anteroposterior order of *Hox* gene expression domains largely follows the spatial colinearity rule known from ectodermal structures, one important difference is noteworthy: expression of the two 3’-most *Hox* genes of the ANT-C is inverted, in that the anterior expression boundary of *lab* lies posterior to that of *pb* (Hirth et al., 1998).

Interestingly, this particularity of the *Hox* expression pattern in the CNS is common to fly and mouse. In vertebrates, *Hox* genes are expressed in the developing hindbrain and spinal cord. The relative anteroposterior order of *Hox* gene expression in the CNS of vertebrates is virtually identical to their arrangement in *Drosophila*, including the inverted order of the *lab* and *pb* orthologs, *Hoxb-1* and *Hoxb-2* (Fig. 5-3B) (Hunt and Krumlauf, 1991). As more expression data from different protostome and deuterostome species becomes available, the ordered expression of *Hox* genes along the anteroposterior axis of the developing nervous system is likely to consolidate as a common feature of bilaterian animals.

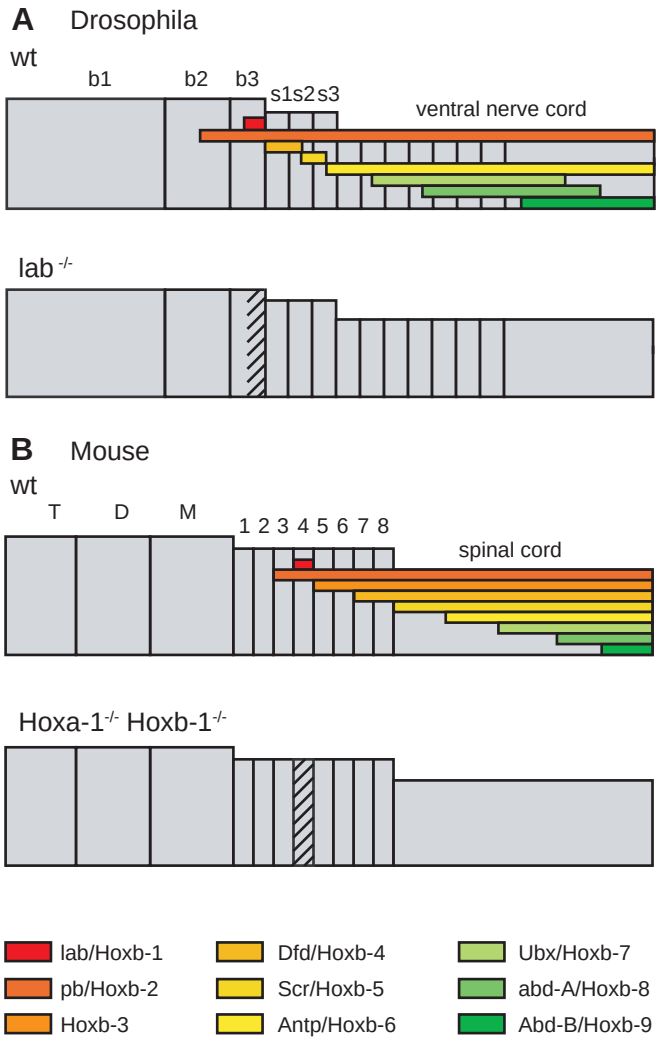

Figure 5-3 Simplified schematic comparison of *Hox* gene expression domains and mutant phenotypes in the CNS of *Drosophila* and mouse. Schematic representations of the embryonic brain with anterior towards the left and posterior towards the right. (A) Expression domains of the homeotic genes of the Antennapedia and Bithorax complexes in the CNS of *Drosophila* (see text for gene nomenclature). In *lab* null mutant embryos (*lab*$^{-/-}$), cells of the posterior part of the tritocerebrum (b3) are correctly located in the mutant domain, but fail to assume their correct neuronal cell fate (dashed lines). (B) Expression of the *Hox* genes *Hoxb-1* to *Hoxb-9* in the

developing mouse CNS. *Hoxa-1*$^{-/-}$ and *Hoxb-1*$^{-/-}$ double mutant embryos (*Hoxa-1*$^{-/-}$; *Hoxb-1*$^{-/-}$) lose rhombomere 4 identity (dashed lines). Abbreviations: T, telencephalon; D, diencephalon; M, mesencephalon; 1 – 8, rhombomeres 1 – 8; (for other abbreviations see Fig. 5-2). Modified and reprinted with permission from: Hirth F, Hartmann B, Reichert H. Development 1998; 125: 1579-1589. © The Company of Biologists Limited.

In *Drosophila*, mutational inactivation of either of the homeotic genes *lab* or *Dfd* causes severe axonal patterning defects in the embryonic brain (Hirth et al., 1998). In *lab* null mutants, axonal projection defects are observed in the posterior tritocerebrum where *lab* is expressed in the wild type brain. In the mutant, longitudinal pathways connecting supraesophageal and subesophageal ganglia as well as projections in the tritocerebral commissure are absent or reduced. These brain defects are not due to deletions in the affected neuromere; neuronal progenitors are present and give rise to progeny in the mutant domain. However, these postmitotic progeny fail to acquire a neuronal identity, as indicated by the absence of neuronal markers and the lack of axonal and dendritic extensions (Fig. 5-3A). Comparable defects are seen in *Dfd* mutants in the corresponding mandibular/anterior maxillary domain, where the gene is expressed in the wild type brain (Hirth et al., 1998). Thus, the activity of the homeotic genes *lab* and *Dfd* is necessary to establish regionalized neuronal identity in the brain of *Drosophila*.

The mouse *lab* orthologs, *Hoxa-1* and *Hoxb-1*, are expressed in overlapping domains with a sharp anterior boundary coinciding with the presumptive rhombomere 3/4 border. Functional inactivation of *Hoxa-1* results in segmentation defects leading to a reduced size of rhombomeres 4 and 5, and defects in motor neuron axonal projections but the normal identity of rhombomere 4 is not altered (Gavalas et al., 1998). In contrast, loss of *Hoxb-1* function has no influence on the size of rhombomere 4 but causes a partial transformation into a rhombomere 2 identity (Studer et al., 1996). The *Hoxa-1*, *Hoxb-1* double mutant results in a territory of unknown identity and reduced size between rhombomeres 3 and 5, suggesting a synergistic action of the two genes in rhombomere 4 specification (Fig. 5-3B) (Gavalas et al., 1998). Thus, the concerted activity of *Hoxa-1* and *Hoxb-1* has a similar role in the specification of the regionalized neuronal identity as does their ortholog *lab* in the CNS of *Drosophila*. This suggests a functional conservation of *Hox* genes, in addition to a similar mode of expression, during nervous system development of bilaterian animals and supports the idea of a common origin of the CNS.

5.5 EVIDENCE FOR A TRIPARTITE ORGANIZATION OF THE BRAIN

Comparative gene expression studies, as reviewed here for *Drosophila* and mouse, have been carried out in numerous protostome and deuterostome phyla (Bruce and Shankland, 1998; Canestro et al., 2005; Castro et al., 2006; Hirth et al., 2003; Lowe et al., 2003). The subdivision of the developing brain into an anterior region specified by genes of the *otd*/*Otx* family and a posterior region specified by genes of the *Hox* family appears to be a universal feature of bilaterian animals. In vertebrates and urochordates, a third embryonic domain along the anteroposterior neuraxis, characterized by overlapping expression of the *Pax2*, *Pax5* and *Pax8* genes, is located between the anterior *Otx* and the posterior *Hox* expressing regions of the embryonic brain (Liu and Joyner, 2001; Wada et al., 1998; Wurst and Bally-Cuif, 2001). In vertebrate brain development, this *Pax2/5/8* domain is located between the presumptive mesencephalon and metencephalon, where it plays a crucial role in development of the midbrain-hindbrain boundary (MHB) region or isthmus. Transplantation experiments, in which MHB tissue grafts are inserted to more rostral or caudal brain regions inducing ectopic mesencephalic-metencephalic structures, reveal an organizer function of the MHB. This organizer activity on the surrounding neural tissue is thought to be mediated by fibroblast growth factor 8 (Fgf8) and Wnt1 proteins, which are secreted by cells located in the MHB (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). In early embryonic development of the vertebrate CNS, the homeobox gene *Gbx2* is expressed in the anterior hindbrain just posterior to the *Otx2* domain in the forebrain and midbrain. During gastrulation and early neurulation the MHB is established at the *Otx2*/*Gbx2* interface, where subsequently the expression domains of other MHB markers including *Pax2/5/8*, Fgf8, Wnt1 and *En1/2* are positioned (Fig. 5-4C). The two homeobox genes *Otx2* and *Gbx2* mutually repress one another, and upregulation or downregulation of either gene shifts the position of the MHB accordingly (Liu and Joyner, 2001; Wurst and Bally-Cuif, 2001). Therefore, in vertebrates an antagonistic interaction between *Otx2* and *Gbx2* during early embryonic development is involved in the correct positioning of the MHB at their common interface.

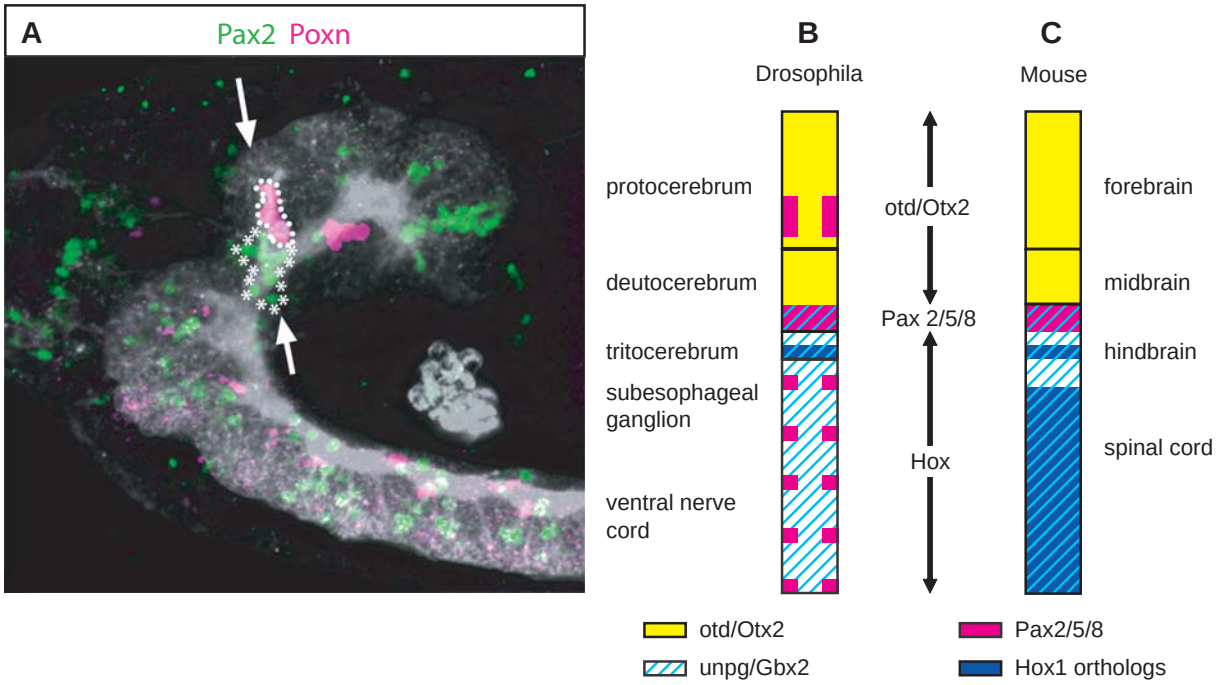

Figure 5-4 Tripartite organization of the embryonic CNS in *Drosophila* and mouse. (A) Expression of *Pax2* and *Poxn* in the brain of stage 13/14 embryos. At the deutocerebral-tritocerebral boundary (indicated by white arrows), *Pax2* (white dots) and *Poxn* (white asterisks) are expressed in adjacent domains forming a transversal line in the CNS (immunolabelled with anti-HRP and shown in grey). (B and C) The expression of *otd/Otx2*, *unpg/Gbx2*, *Pax2/5/8* and *Hox1* gene orthologs in the developing CNS of *Drosophila* (B) and mouse (C). (In this schematic, anterior towards the top and posterior is towards the bottom.) In both cases, *otd/Otx2* is expressed in the anterior nervous system rostral to a *Hox*-expressing region in the posterior nervous system. In addition, a *Pax2/5/8*-expressing domain positioned at the interface between the anterior *otd/Otx2* domain and the posteriorly abutting *unpg/Gbx2* expression domain is common to both nervous systems. Modified and reprinted with permission from: Hirth F, Kammermeier L, Frei E, et al. Development 2003; 130: 2365-2373. © The Company of Biologists Limited.

Gene expression studies indicate that a similar tripartite ground plan for anteroposterior regionalization of the embryonic brain is also present in *Drosophila*. The *Drosophila* genome contains two genes, *Pox neuro* (*Poxn*) and *Pax2*, which are together considered to be orthologs of the *Pax2/5/8* genes (Noll, 1993). Remarkably, expression of both orthologs is present at the interface of *otd* and the *Drosophila Gbx2* ortholog *unplugged* (*unpg*), anterior to a *Hox*-expressing region (Fig. 5-4A and B) (Hirth et al., 2003). Although *Poxn* and *Pax2* are expressed in a segmentally reiterated pattern along the entire embryonic CNS, their expression at the *otd*/*unpg* interface is exceptional in two ways. The two genes are expressed in adjacent domains delineating together a transversal stripe of the brain and this is the only position along the neuraxis where expression of both genes coincides with a brain neuromere boundary, the deutocerebral-tritocerebral boundary (DTB) (Fig. 5-4A and B) (Hirth et al.,

2003). Analyses of either *otd* or *unpg* mutants reveal a mutually repressive function of the two genes during early brain patterning. Thus, in *otd* mutant embryos a rostral extension of the *unpg* expression domain is observed (in addition to the deletion of the anterior brain). On the other hand, mutational inactivation of the *unpg* gene results in a caudal shift of the posterior limit of *otd* expression (Hirth et al., 2003). Therefore, in both *Drosophila* and mouse, the early interaction of *otd*/*Otx2* and *unpg*/*Gbx2* is essential for the correct positioning of an intermediate brain domain characterized by a sharply delimited *otd*/*Otx2* and *unpg*/*Gbx2* interface and the expression of *Pax2/5/8* genes. In contrast to vertebrates, mutational inactivation of the *Drosophila Pax2/5/8* orthologs *Poxn* or *Pax2* does not appear to result in brain patterning defects. Moreover, to date, there is no evidence of an organizer activity at the fly DTB, suggesting that the organizer function at the *otd*/*Otx2* and *unpg*/*Gbx2* interface might have emerged after the protostome/deuterostome divergence that separated insects and vertebrates. In fact, an organizer activity of the MHB region has so far only been demonstrated for vertebrate species within deuterostomes.

In summary, current comparative data indicates that similar genetic patterning mechanisms act in anteroposterior regionalization of the developing brain in *Drosophila* and vertebrate species and establish a common, evolutionarily conserved tripartite ground plan. This suggests that a corresponding tripartite organization of the developing brain was already present in the last common bilateral ancestor of insects and vertebrates.

6 DISCUSSION

The experimental data presented in chapter 2 and 3 of this thesis provides a first insight into the expression and function of the cephalic gap gene *empty spiracles* during larval and pupal development of the *Drosophila* brain. One interesting finding of this analysis is that *ems* can play different and multiple roles in diverse aspect of postembryonic brain development. Another issue that deserves further consideration is the question if and how the expression pattern of *ems* in the brain is related between different developmental stages including the adult. This, in turn, raises the question how general the observed specification of distinct lineages by single or multiple transcription factors is. Finally, our results provide new evidence for the evolutionary conservation of *ems*/*Emx* expression and function at later development stages in the brains of insects and vertebrates. In the following, these aspects will be discussed in more detail.

6.1 SUCCESSIVELY RESTRICTED EMS EXPRESSION DURING BRAIN DEVELOPMENT

In the early embryo approximately 100 bilaterally symmetrical brain Nbs segregate from the neuroepithelium in a stereotyped array. Out of these, 11 identified Nbs express *ems*. All 11 *ems* positive Nbs are located either within the future deutocerebral neuromere or in closely associated regions in the protocerebral and tritocerebral neuromeres (Urbach and Technau, 2003). During later embryonic stages *ems* expression is restricted to the deutocerebral and anterior tritocerebral neuromeres in the brain (Hirth et al., 1995). However, no expression data at single Nb and primary lineage resolution is available for embryonic stages. In the late larval brain approximately 100 secondary lineages have recently been identified in each hemisphere (Pereanu and Hartenstein, 2006). Here we have found that *ems* is exclusively expressed in 8 secondary lineages per hemisphere which have been attributed to the ventral deutocerebrum that surrounds the larval antennal neuropile compartment. Only 1 of the 8 lineages continues to express *ems* in the adult brain. There the cell bodies of the *ems*-positive lineage are located in the cortex between antennal lobes and suboesophageal ganglion. Although a clear anatomical distinction between tritocerebrum and deutocerebrum in this area seems problematic, the adult EM lineage most probably belongs to the tritocerebrum according to the *flybrain* atlas of adult neuromeres (URL: http://www.flybrain.org). This, however, is in contrast to the attribution of the same EM-lineage (BAmas2) to the ventral deutocerebrum in the digital atlas of secondary lineages in the late larval brain (Pereanu and Hartenstein, 2006; Lichtneckert *et al*., 2007). Thus, the attribution of the EM-lineage to either the deuto- or tritocerebral neuromere remains controversial. Nevertheless, a general image of the *ems* expression pattern in the developing *Drosophila* brain emerges. At all developmental stages studied *ems* expression is limited to the deutocerebrum and a narrow area surrounding it. Moreover, *ems* expression is never found in the anterior protocerebrum and in the posterior tritocerebrum. Thus, the early embryonic *ems* expression domain delimiting an intermediate region on the anteroposterior axis of the brain is preserved throughout development and into the adult. Is the preservation of *ems* expression also valid for single Nbs and their lineages produced during embryonic and larval life? To date, we can answer this question only for the transition between larval and adult stage. There, we have found that *ems* expression is preserved in 1 adult lineage, whereas it disappears from the other 7 *ems*-positive lineages of the larval brain. Furthermore, no *de novo* expression of *ems* in additional lineages could be observed in this time-frame. In order to answer this question also for the transition between

the 11 *ems*-positive Nbs observed at early embryonic stage and the 8 *ems*-expressing Nb lineages in the late larva, single Nbs need to be traced through intermediate developmental stages. The EM lineage with its characteristic feature of persistent *ems* expression in all secondary and most likely all primary neurons is a suitable candidate for tracing a single identified Nb lineage from the early embryo into the adult. Alternatively, the two *ems* expressing PN lineages could probably be followed back into the early embryo based on a combination of anatomical features characterizing them as differentiating PNs and *ems* expression. This presupposes, however, that *ems* is already expressed in PN lineages during embryonic development. Additional markers, which were used for the characterization of single delaminating Nb in the early embryo (Urbach and Technau, 2003) could probably be used, in terms of a combinatorial code, to identify distinct Nbs and their lineages during embryonic and larval development. A different promising approach for fate mapping of primary lineages into later developmental stages had been demonstrated for *atonal*-positive cells using an *atonal*-specific GAL4 line in combination with a special self-perpetuating UAS-GAL4 construct (Hassan et al., 2000). Specific GAL4 lines labeling single *ems*-positive Nb lineages in the early embryo would be required for lineage tracing into later developmental stages.

Taken together, a broad *ems* expression domain in the brain of *Drosophila* is laid down in the early embryo where 11 *ems* expressing Nbs per hemisphere are detected. At later developmental stages *ems* expression gets more and more restricted within this broad region to 8 lineages in late larval stage and to 1 single lineage in pupal and adult stage. The equivalence of single *ems*-positive Nbs in the early embryo to *ems* expressing secondary lineages awaits further analysis. Future clonal characterization of additional gene expression patterns during postembryonic brain development will be very informative on the question if successive spatial restriction reflects a general trend for transcription factors that act as patterning genes during early development.

6.2 DIVERSE ROLES OF EMS FUNCTION DURING BRAIN DEVELOPMENT

In our work, we have addressed the function of *ems* in postembryonic brain development applying a clonal mutant analysis. Thereby, we have focused on 3 out of 8 lineages that express *ems* during larval development. Our results demonstrate a cell-autonomous requirement of *ems* for at least 4 different developmental processes. Moreover, there is evidence for multiple roles of *ems* within the same lineage and even within the same cell. Thus, our results indicate that *ems* function is necessary for, (1) the survival of postmitotic cells, (2) correct formation of major nerve tracts, (3) correct dendritic targeting, and (4) correct axon terminal arborizations. An additional role in Nb proliferation could not yet be ascertained. In the embryo *ems* has been shown to play an essential role in the formation of Nbs within the *ems* expressing domain of the neuroectoderm (Hirth *et al*., 1995; Yonoussi-Hartenstein *et al*., 1997). In addition, *ems* is required for correct axonal pathfinding of specific interneurons in the embryonic ventral nerve cord (Hartmann *et al*., 2000). Thus, compared to the roles of *ems* during embryonic CNS development additional *ems* functions were revealed at later developmental stages. This provides further evidence for the general observation in invertebrates and vertebrates that classical developmental control genes implicated in early embryonic neurogenesis and neural patterning exert additional functions during later development (Callaerts et al., 2001; Martini and Davis, 2005; Martini et al., 2000; Salie et al., 2005; Zapala et al., 2005).

6.3 DIFFERENT IDENTITIES OF EMS-POSITIVE SECONDARY LINEAGES – A COMBINATORIAL CODE?

Expression of *ems* during larval brain development is specifically restricted to 8 Nb lineages. How is such a restricted expression pattern compatible with the essential roles of *ems* in neuronal differentiation like cell survival or correct connectivity? What other factors exert the same functions in lineages where *ems* is not expressed? The expression and function of a number of different transcription factors have been studied during postembryonic brain development. Similar, lineage restricted expression patterns have been found for Acj6, Drifter, Atonal, Engrailed, Sine oculis, Dachshund, Eyeless (Callaerts et al., 2001; Komiyama et al., 2003; Kurusu et al., 2000; Martini and Davis, 2005; Martini et al., 2000; Pereanu and Hartenstein, 2006; Zapala et al., 2005). Although, functional data is only available for a subset of these genes, mutants of some of these genes show axon branching and axon targeting defects, which are similar to those observed in *ems* mutants. This suggests, that different transcription factors may play comparable but distinct roles in different lineages translating lineage identity into wiring specificity. Moreover, the coincident expression of 2 or more of these transcription factors controlling different sub-aspects of neuronal differentiation within the same lineage could increase lineage diversity in a combinatorial way. For instance, co-expression of *acj6* or *Drifter* with *ems* in the adPN and lPN lineages, respectively, or co-expression of *engrailed* with *ems* in the secondary lineage DAlv2 could increase the diversity among the 7 transiently expressing *ems*-positive lineages (Komiyama *et al*., 2003; Pereanu and Hartenstein, 2006; Lichtneckert *et al*., 2006; R. Lichtneckert, unpublished observations).

In the embryonic ventral nerve cord the combinatorial expression of LIM- and POU-domain transcription factors has been shown to generate distinct motor-neuron identities (Certel and Thor, 2004; Thor et al., 1999). Therefore, it would be interesting to test whether a similar combinatorial code could contribute to the neuronal diversification at the lineage level during postembryonic development. Clonal analysis of different double mutants in combination with ectopic misexpression of selected genes in specific lineages could be used to test this model.

6.4 DIVERSE REPERTOIRE OF EMS FUNCTION IN SECONDARY LINEAGES

One obvious difference between the EM-lineage and the other 7 *ems*-positive larval lineages is the temporal expression dynamics of *ems* in postmitotic cells. Whereas, *ems* expression in secondary neurons of the EM lineage is continuous and persists in the adult brain, transient *ems* expression limited to the early differentiation steps of the neuronal progeny is observed in the other 7 lineages, including the adPN and lPN lineages. Does this dissimilarity in *ems* expression account for the differences of *ems* function observed in the EM lineage as opposed to the PN lineages?

The comparison of the various phenotypes observed in the 3 *ems* mutant lineages revealed no clear correlation between *ems* function and expression mode. Thus, formation of correct number of progeny was affected in the mutant EM lineage as well as in the lPN lineage, although the question for the origin of this phenotype in the lPN has not yet been solved. On the other hand, misprojections of *ems* mutant clones in neuropile compartment outside the target area have been observed in EM lineage as well as in the adPN lineage. However, a significant difference between the phenotypes can be noted. Whereas, the misprojections of mutant EM clones are randomly distributed in the neuropile compartment surrounding the cell bodies, misprojections of mutant adPN clones always extend through the antennal lobe into the adjacent suboesophageal ganglion. Thus, in contrast to EM cells, adPNs might still direct their dendrites to their wild-type target area but then overshoot into neighboring compartments.

Another argument against a link between *ems* expression dynamics and mutant phenotypes is produced by the comparison of *ems* mutant PN clones. Both lineages express *ems* in a transient way during early neuronal differentiation. Whereas the mutant adPN clone was not affected with respect to the clone size and gross projection pattern, hardly any *ems* mutant lPN could be detected, suggesting a defect in cell survival or Nb proliferation. Taken together, no obvious link could be found between the wild-type *ems* expression dynamics and mutant phenotypes in postmitotic cells of the EM and PN lineages.

A possible explanation for this could be that the phenotype observed in the mutant EM lineage reflects an early requirement of *ems* corresponding to the transient expression in the

PN lineages. The role of continuous *ems* expression during later differentiation steps and in the adult are probably masked by the early phenotype, and therefore, cannot be analyzed with mutant clones generated by mitotic recombination.

Although we could not address the significance of the maintenance of *ems* expression in the late pupal and adult EM lineage, we could gain some insight in the importance of down-regulation of *ems* expression in the differentiating PNs. Interestingly, *ems* misexpression in PN clones produced phenotypes of the same kind as their *ems* mutant counterparts in the adPN and lPN lineages, respectively. This suggests, that *ems* function could probably still interfere with the same cell biological process as during their endogenous expression and therefore timely down-regulation within the differentiating cell is necessary to obtain an optimal effect of *ems* activity.

In summary, *ems* can play different and multiple roles in developing Nb lineages of the larval and pupal brain. The dissimilarity in *ems* expression dynamics alone can not explain the different phenotypes obtained in 3 distinct *ems* mutant lineages. Nevertheless, timely down-regulation of *ems* expression in the adPN and lPN lineages is essential for the correct formation of PNs. Thus, differences in the cell-intrinsic and extrinsic context of distinct lineages may significantly contribute to the diversity of the functional repertoire of *ems*.

6.5 EVOLUTIONARY CONSERVED FUNCTIONS DURING LATER BRAIN DEVELOPMENT

Comparative molecular genetic analyses of early brain development in *Drosophila* and mouse revealed strikingly similar expression and function of various early patterning genes including *ems*. Together with another cephalic gap gene family, the *otd*/*Otx* genes¸ *ems* and its mouse homologs, *Emx1*/*Emx2*, are responsible for the correct regionalization of the anterior brain anlage in both species (Cecchi, 2002; Simeone, 1998). Interestingly, functional equivalence of the *Drosophila* Ems and the mouse Emx2 protein could be demonstrated in a cross-phylum gene-replacement experiment where misexpression of a mouse *Emx2* transgene could rescue the early brain phenotype of *ems* mutant fly embryos (Hartmann et al., 2000). Together with other independent lines of evidence it was suggested, that genetic programs underlying embryonic brain development in insects and vertebrates are conserved in evolution (Arendt and Nubler-Jung, 1999; Lichtneckert and Reichert, 2005; Reichert and Simeone, 1999). Thus, the common ancestor of protostomes and deuterostomes already had a complex centralized nervous system.

An alternative view was proposed based on the discovery that anteroposterior patterning genes, including *ems* and *otd*, were expressed in an ordered array along the basiepithelial diffuse nervous system of a hemichordate, a basal deuterostome species. From this perspective the common ancestor of protostomes and deuterostomes was characterized by a diffuse nerve net. Therefore, similar mechanisms in embryonic development of the centralized brains of *Drosophila* and mouse have their evolutionary origin in general axis patterning mechanisms (Holland, 2003; Lacalli, 2003; Lowe et al., 2003). It was previously proposed, that the analysis of genetic mechanisms involved in more advanced stages of brain development, when a certain level of complexity is already evident, should shed more light on the degree of conservation of genetic programs between insects and vertebrates (Lichtneckert and Reichert, 2005).

In our work, we have gained insight into the functions of *ems* during later brain development of *Drosophila*. In short, *ems* is required for the correct number of cells in distinct neuronal populations and for the proper formation of neurite tracts, as well as dendritic connections.

Moreover, we have found that *ems* expression and function is required in second order projection neurons of the *Drosophila* olfactory system. A comparison with the expression and function of the mouse *Emx* genes during later brain development revealed remarkable similarities to our results in *Drosophila*. The reduced cortical surface area as well as the reduced thickness of the preplate and cortical plate in *Emx1*/*Emx2* double mutant mice suggests that the *Emx* genes regulate the number of cortical neurons. In addition, many cortical neurons show major axonal pathfinding defects in *Emx1*/*Emx2* double mutant mice (Shinozaki *et al*., 2002; Bishop *et al*., 2003). Moreover, *Emx* genes have been shown to be expressed in second order projection neurons (mitral cells) during the development of the olfactory systems of mouse and *Xenopus*. Interestingly, the olfactory bulb of *Emx1/Emx2* double mutant mice is reduced in size and the mitral cell layer is disorganized, although at least part of the mitral cells is still produced (Bishop et al., 2003; Brox et al., 2004; Mallamaci et al., 1998; Yoshida et al., 1997). This indicates that expression and functions of *ems*/*Emx* genes in differentiating neurons and particularly in second order projection neurons of the olfactory system might be evolutionarily conserved between insects and vertebrates. These findings would favour the existence of conserved genetic mechanisms between protostomes and deuterostomes that were involved in the development of a centralized nervous system in the last common ancestor.

7 Experimental Procedures

7.1 FLY STRAINS AND GENETICS

Unless otherwise stated fly stocks were obtained from the Bloomington stock center. Wild-type was Oregon R. Two recombinant chromosomes were constructed: *FRT82B, ems*^{9Q64} (Jurgens et al., 1984) on chromosomal arm 3R and *tubP-GAL4, UAS-mCD8::GFP*^{LL5} on chromosome 2. For MARCM analysis (Lee and Luo, 1999) *+; UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B, ems*^{9Q64} or *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B* males were crossed to *hs-FLP; tubP-GAL4; FRT82B tubP-GAL80*^{LL3} females (Bello et al., 2003) resulting in *ems* mutant or wild-type clones. Ems or P35 rescue experiments were performed by combining *UAS-ems* (H. Reichert, unpublished) or UAS-P35^{BH1} on chromosome 2 with *FRT82B, ems*^{9Q64} and crossing them to the stock *hs-FLP; tubP-GAL4, UAS-mCD8::GFP*^{LL5}*; FRT82B tubP-GAL80*^{LL3} respectively to generate MARCM clones. For wild-type or mutant MARCM clones in the adult projection neurons *+; UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B, ems*^{9Q64} or *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B* males were crossed to *hs-FLP; GH146-GAL4; FRT82B tubP-GAL80*^{LL3} females (Stocker *et al*., 1997). For *ems* misexpression in adult projection neurons +;*UAS-ems*;*FRT82B* males were crossed to *hs-FLP; GH146-GAL4, UAS-mCD8::GFP*^{LL5}*; FRT82B tubP-GAL80*^{LL3}. The following genotype was used to generate the dual-expression-control MARCM tubP-lexA::GAD/+; FRTG13, GAL4-GH146, UAS-mCD8/FRTG13, hs-FLP, tubP-GAL80; lexAop-rCD2::GFP/+ (Lai and Lee, 2006).

For MARCM experiments, embryos of appropriate genotype were collected on standard medium over a 4 hour time window and raised at 25°C for 21 to 25 hours before 1 hour heat-shock treatment (except for GH146-GAL4 clones in larva as well as dual-expression-control clones 1 hour heat-shock was provided at 3 – 6 hours after egg laying).

7.2 IMMUNOLABELLING

Larval and adult brains were fixed and immunostained as previously described (Bello et al., 2003). The following antibodies were used: rat anti-Ems (1:200; gift of U. Walldorf), rabbit anti-Ems (1:500; gift of U. Walldorf), rabbit anti-Grh (1:200), rabbit anti-H3P (1:400; Upstate Biotechnology), rabbit anti-cleaved caspase 3 (1:75; Cell Signalling Technologies) rat anti-Elav Mab7E8A10 (1:30; DSHB), mouse anti-cycE (1:50; gift of H. Richardson), mouse anti-Pros MaMR1A (1:10; DSHB), mouse anti-βGAL (1:20; DSHB), mouse anti-Nrt BP106 (1:10; DSHB), mouse anti-BrdU (1:100; DSHB), mouse monoclonal nc82 (1:20; gift of A. Hofbauer), rabbit anti-Castor (Kambadur et al., 1998), rat anti-mCD8 (CALTAG Laboratories), rab anti-GFP (Invitrogen). Secondary antibodies were Alexa-488, Alexa-568, and Alexa-647 antibodies generated in goat (1:300; Molecular probes).

To estimate the number of dividing cells in wild-type or *ems* mutant clones induced at early first instar stage larvae were transferred to BrdU containing standard medium (final concentration: 1mg/ml) at 60 hours after hatching and raised for 12 hours before dissection (Truman and Bate, 1988). Brains were fixed immediately in 4% paraformaldehyde for 15 minutes at RT and incubated with the anti-Ems and anti-βGAL primary antibodies. Subsequently, an additional fixation step in 2% paraformaldehyde was applied and the brains were incubated in 2 N HCL for 30 minutes to denature BrdU-labelled DNA before incubation with the anti-BrdU antibody.

7.3 MICROSCOPY AND IMAGE PROCESSING

Fluorescent images were recorded using a Leica TCS SP scanning confocal microscope. Optical sections were taken at 1 µm intervals in line average mode with picture size of 512 x 512 pixels. Digital image stacks were processed using ImageJ (http://rsb.info.nih.gov/ij/). For visualizing particular MARCM clones, image stacks with few non-interfering clones were selected and stained processes and cell bodies from other clones were removed using the lasso tool in every single optical section. Digital 3D-models were generated using the AMIRA software by manually labelling structures of interest like cell bodies, processes, whole clones, or neuropile and subsequent automated 3D surface rendering.

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Appendix

Origin and Evolution of the First Nervous System

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Glossary:

Anterior class *Hox* genes: Group of *Hox* genes that are involved in specification of the anteriormost part of the anteroposterior body axis of bilaterians. The bilaterian Hox cluster genes are believed to be descended from an ancestral ProtoHox cluster which included four genes, the ancestor of the present day Hox classes (anterior, group-3, central, and posterior).

Basal Metazoa: Here used to refer to Porifera, Cnidaria, Ctenophora and Placozoa. Other authors include the Platyhelminthes (flatworms).

Bilateria: A monophyletic group of metazoan animals that is characterized by bilateral symmetry. Traditionally this group includes deuterostomes (e.g. chordates, echinoderms, hemichordates), and protostomes (e.g. arthoropods, nematodes, annelids, mollusks).

Coelenterata: Cnidaria and Ctenophora were traditionally joined together as Coelenterata based on the presence of a single gastrovascular system serving both nutrient supply and gas exchange.

Deuterostome: A bilaterian animal whose mouth forms embryonically as a secondary opening, separate from the blastopore. Deuterostomes include chordates, hemichordates and echinoderms.

Effector cell/organ: Single cells or group of specialized cells transducing external stimulation or neuronal signals into a specific response like contraction, secretion, bioluminescence, or electricity.

Eumetazoa: A monophyletic group of animals including all metazoans except the phylum Porifera.

Excitable epithelia: Epithelia which can conduct electrical signals over wide areas without decrement.

Expressed Sequence Tag (EST): A nucleic acid sequence that is derived from cDNA as part of sequencing projects.

Four-domain Na^+ channel: A single protein ion channel composed of four linked domains, each of which consists of six transmembrane segments. The whole protein folds-up into a

channel forming a pore that is selective for Na^+ ions. The four-domain Na^+ channels are believed to have evolved from structurally similar Ca^{2+} channels.

Gap junctions: Membrane protein complexes (connexons) that join the plasma membranes of two neighboring cells creating a communication between the cytoplasm of the two cells. This allows the exchange of molecules and the direct propagation of electrical signals.

Higher Metazoa: We use these terms as synonym of Bilateria.

Homolog: A gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (see ortholog) or to the relationship between genes separated by the event of genetic duplication (see paralog).

Hypostome: The terminal region of a polyp, on which the mouth is situated.

Low resistance pathway: A tract of multiple cells which are cytoplasmically connected through specialized pores in the cell membranes allowing the fast conduction of electrical signals.

Medusa: Mobile form (jellyfish) of life history in the cnidarian classes Hydrozoa, Scyphozoa, and Cubozoa (Medusozoa).

Medusozoa: Comprises three of the four cnidarian classes (Hydrozoa, Scyphozoa, and Cubozoa), which produce a sexually reproducing medusa (jellyfish) as part of the life cycle.

Mesenteries: Longitudinal sheets of tissues that extend radially from the body wall of polyps into the body cavity.

Mesogloea: (Also known as mesoglea). The body layer between ectoderm and endoderm in cnidarians, ctenophores and acoelomates, which is traditionally distinguished from mesoderm on the basis of the former being acellular and the latter cellular.

Myoepithelium: A single-layered tissue of contractile cells.

Ortholog: Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Orthologs often retain the same function in the course of evolution.

Pacemaker: Single cell or group of cells (neuronal or muscular) that spontaneously drive rhythmic activity in neighboring cells.

Paralog: Paralogs are genes related by duplication within a genome. Paralogs may evolve new functions.

Planula: The free-swimming, ciliated larva of a cnidarian.

Polyp: The sessile form of life history in cnidarians; for example, the freshwater *Hydra*.

Posterior class *Hox* gene: Group of *Hox* genes that is involved in specification of the posterior part of the anteroposterior body axis of bilaterians. The bilaterian Hox cluster genes are believed to be descended from an ancestral ProtoHox cluster which included four genes, the ancestor of the present day Hox classes (anterior, group-3, central, and posterior).

Protomyocyte: An evolutionary antecedent of muscle cells.

Protoneuron: Term coined by Parker (1919) for the type of nervous cell from which modern ganglionic neurons evolved.

Protostome: A bilaterian animal whose mouth and anus develop embryonically from the same invagination (the blastopore) during embryogenesis.

Radiata: Animals that are traditionally considered to have radial symmetry. This group includes the Ctenophora and the Cnidaria.

Siphonophora: Cnidarian order of marine colonial hydrozoans.

Statocyst: The statocyst is a balance organ and consists of a pouch lined with sensory hairs, within which sits a heavy granule called the statolith. The sensory hair cells are connected by nerve fibres to the animal's nervous system. The sensed motion of the statolith in response to gravity allows the animal to orientate itself.

SUMMARY

The first nervous systems emerged during the early evolution of metazoan animals. The lack of direct evidence from the fossil record has motivated scientists over the last 150 years to study nervous systems of modern representative of basal metazoan phyla in order to learn more about the first nervous system and its cellular origins. With the advent of molecular biology many of the features thought of as typically neuronal, such as ion channels, receptors, signaling molecules, have been found in non-nervous cells and unicellular organisms. Furthermore, the study of sponges and unicellular protists revealed that coordinated behavior does not necessarily require a nervous system. Cnidarians may be one of the oldest extant phyla having a nervous system. However, the complexity in nervous system organization found in modern cnidarians tends to reflect the life histories of single species more than the original state of the first evolved nervous system. It is therefore difficult to deduce features of the earliest nervous systems from morphological and physiological studies alone. The phylogenetic comparison of genes involved in neurogenesis and pattern formation during nervous system development in cnidarians and bilaterian animals may lead to insights into the mechanisms of nervous system development in the last common ancestor. Although conserved functions of homologous genes are beginning to emerge, the reconstruction of the ancestral mechanisms of nervous system development awaits further functional analyses of candidate genes in bilaterians and cnidarians.

I. INTRODUCTION

A. Tracing Back the First Nervous System

By definition, the first nervous system evolved after the evolutionary shift from unicellular to multicellular life forms. Complex, coordinated behavior controlled by a primitive nervous system in early metazoan animals must have conferred strong selective advantages and thus contributed significantly to the evolutionary success of nervous systems within metazoan animals. Ultimately, more advanced nervous systems, including our own, evolved into the most complex structures found in living matter. In order to learn more about the origins of complex nervous systems in highly evolved animal species, research on the more simple nervous systems that characterize basal metazoan phyla was initiated more than two centuries ago. Then, as today, understanding the origin and early evolution of these simple nervous systems may lead to more profound insight into fundamental principles of development, organization and function of modern nervous systems.

It is highly likely that the emergence of the first nervous system predated the evolutionary divergence of Bilateria and Radiata 600-630 million years ago (Peterson *et al.*, 2004) given the fact that neurons and nervous systems are present in both animal groups. However the independent evolution of the Bilateria and Radiata during this long period of time implies that most extant animals cannot be regarded as primitive in terms of the organization of their nervous systems. Moreover, for the Radiata, which are generally considered to be basal eumetazoan groups, the fossil record is poor and does not allow reconstruction of fossil nervous systems (Chen *et al.*, 2002). Thus, in the quest to understand the origin of the first nervous systems, it seems best to pursue a comparative approach, in which the structure, function and development of nervous systems in several basal metazoan phyla are considered and compared in terms of key molecular, cellular and morphological aspects.

In this review, we will begin by defining what neurons and nervous systems are and then present a current version of the phylogenetic relationships that characterize the systematic groups that are relevant for subsequent considerations. Following this, we will give a brief

historical overview of the ideas concerning the origin and evolution of the first nervous system. The main part of the review will then present a detailed comparative analysis of nervous systems in the basal metazoan phyla which may have participated in the origin of the nervous system. Here the main emphasis will be on Cnidaria, but Porifera, Ctenophora, and Placozoa will also be presented, and electrical conduction outside of the animal kingdom will be considered. Finally we will discuss the implications of recent molecular genetic findings on neurogenesis and axial patterning in cnidarians and bilaterians for our current understanding of the origin of the first nervous system.

B. Definition of the Nervous System

All living cells respond to stimuli and engage in signal processing. Thus, even in the absence of a nervous system, reactions to external stimuli do occur. In most metazoans however, a discrete subset of specialized somatic cells form an interconnected network, called the nervous system, in which multiple sensory stimuli can be processed and conducted to specific effector organs, achieving coordination of complex behaviors. A useful general definition of nervous systems has been given by Bullock and Horridge (1965): “A nervous system is an organized constellation of cells (neurons) specialized for the repeated conduction of an excited state from receptor sites or from other neurons to effectors or to other neurons”. An additional aspect was put forward by Passano (1963) who pointed out that the ability to generate activity endogenously is as much a part of the definition of a nervous system as is the ability to respond to stimulation. It follows from these considerations, that connectivity, specialization for propagating an excited state and spontaneous generation of activity are important anatomical and physiological criteria for a true nervous system.

The functional units of nervous systems are nervous cells or neurons, which are specialized for the reception of stimuli, conduction of excitation, and signal transmission to other cells. Neurons appear in the most simple animals as specialized conducting, secreting, and spontaneously active cells within epithelia which themselves may show sensory, conducting, and pacemaker features. Given their role in conduction, a key point about neurons is that they

are elongated, which enables them to transmit beyond their immediate neighbors without exciting all the interspersed cells (Horridge, 1968).

Some extant animals have a diffuse nerve net representing either an ancestral organization or a secondary loss of centralized structures as often observed in parasitic or sedentary life forms. A nerve net has been defined by Bullock and Horridge (1965), as “a system of functionally connected nerve cells and fibers anatomically dispersed through some considerable portion of an animal and so arranged as to permit diffuse conduction of nervous excitation, that is, in relatively direct paths between many points. The paths, as opposed to indirect routing through a distant ganglion or central structure, are multiple and confer a tolerance of incomplete cuts”.

C. Basal Metazoan Phylogeny

A comparative approach to nervous system structure, function and origin requires an understanding of the phylogeny that underlies the animal groups considered. It is now commonly agreed that all metazoan phyla including Porifera have a monophyletic origin (reviewed in Müller, 2001; Müller *et al.*, 2004). In this section the phylogenetic relationships of major extant taxonomic groups at the stem of bilaterian animals will be presented (Figure 1).

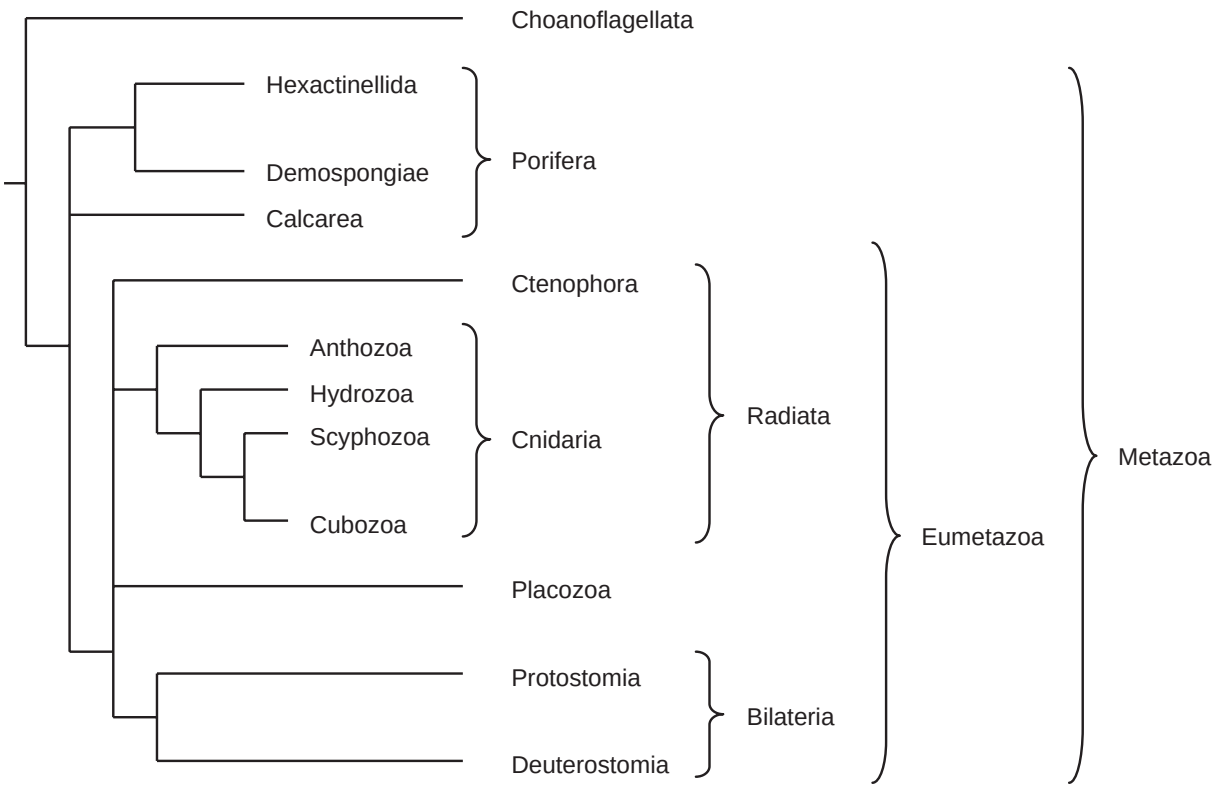

Figure 1 Phylogeny of metazoan animals at the stem of Bilateria. Choanoflagellata have been included as the closest unicellular relatives to the metazoans. The phylogeny is based on widely accepted molecular data and the currently uncertain relationships between the different sponge classes as well as among the potential bilaterian sister groups (Ctenophora, Cnidaria and Placozoa) have been left open. Terms used in the text for higher classification of animal phyla are indicated on the right hand side.

Choanoflagellata, which show a striking structural resemblance to the choanocytes found in sponges, have been hypothesized to be the closest relative to multicellular animals, and Porifera have been proposed to derive from a colonial form of choanoflagellates (James-Clark, 1867). Recent molecular phylogenetic data provides further support for this hypothesis indicating that choanoflagellates are indeed more closely related to animals than are fungi and, thus, form a monophyletic sister group of metazoans (Medina *et al.*, 2001; Brooke and Holland, 2003).

Porifera represent the earliest known metazoan phylum and consist of three major taxa: Hexactinellida, Demospongiae, and Calcarea. The molecular sequence analysis of key proteins from these three poriferan classes, suggest that Hexactinellida are the

phylogenetically oldest taxon, while Calcarea represent the class most closely related to higher metazoan phyla (Medina *et al.*, 2001; Müller *et al.*, 2004).

The relative positions of the potential sister groups to the bilaterians namely Cnidaria, Ctenophora and Placozoa are controversial. Classically the Cnidaria and Ctenophora have been grouped together as the sister group to bilaterians. Together, they are also referred to as the Radiata based on their radially symmetrical appearance (This term may be inappropriate given that biradial and even bilateral symmetry are also common among these animals). On morphological and embryological grounds, such as the presence of mesoderm as a third germ layer, multiciliated cells or a simplified through gut, Ctenophora have been suggested to be the closest relative to Bilateria (Nielsen, 1997; Martindale and Henry, 1999). However, recent molecular phylogenetic analyses support the notion that Cnidaria are more closely related to Bilateria than are Ctenophora, and Cnidaria are therefore often considered as the true sister group of Bilateria (Collins, 1998; Kim *et al.*, 1999; Medina *et al.*, 2001; Martindale *et al.*, 2002). Within Cnidaria recent molecular data based on ribosomal DNA sequence analysis and mitochondrial genome organization is in agreement with the view that the Anthozoa, which have only a polyp stage, are basal to the other three classes, Hydrozoa, Scyphozoa, and Cubozoa, that are characterized by an additional medusa stage in their life cycle (Medusozoa; Petersen, 1979).

The Placozoa, represented by a single known species, *Trichoplax adhaerens*, were long believed to be cnidarians with a simple organization as the result of secondary reduction (Bridge *et al.*, 1995). Analysis of molecular data, however, has shown that Placozoa are not derived cnidarians (Ender and Schierwater, 2003). Furthermore, Bilateria and Placozoa may have a more recent common ancestor than either does to Cnidaria (Collins, 2002). The rapidly increasing amount of molecular data from basal metazoans such as sponges, ctenophorans, cnidarians, placozoans is expected to further clarify the phylogenic relationships among these groups in the coming years. A robust phylogeny based on different sets of molecular data, and importantly, including a large number of representing species for each taxonomic group will be essential to understand early metazoan evolution and, thus, gain more insight into the origin of the first nervous system.

II. HISTORICAL CONCEPTS AND THEORIES ABOUT THE EVOLUTIONARY ORIGIN OF NERVOUS SYSTEMS

A. The Elementary Nervous System

The cornerstone for studies of the evolution of nervous systems at the cellular level was the application of the cell theory (Schleiden 1838; Schwann, 1839) to the anatomical units of the nervous system in the neuron doctrine which was put forward by Cajal, Kölliker, Waldeyer and others at the end of the 19th century (reviewed in Shepherd, 1991). Subsequently, with improved anatomical staining methods it became possible to specifically label nervous structures in basal metazoan organisms. With experimental access to the neurons and nervous systems of basal metazoans, it became conceivable to address the question of which cell lineages originally gave rise to nerve cells and how the first nervous system was organized at the cellular level. Hypothetical considerations were initially based on the conceptual model of an elementary nervous system, defined as “a group of nerve cells with the minimal number of specializations required to perform the basic functions of nervous tissue” (Lentz, 1968). However, Lentz pointed out that this simplified conceptual approach does not necessarily determine the actual characteristics of an evolutionarily early, simple system.

Nerve cells are likely to have arisen in multicellular organisms from epithelial cells that became able to transduce external information (pressure, light, chemicals, temperature) into chemical and electric signals, and then transmit these signals to neighboring cells (Mackie, 1970; Anderson, 1989). Assuming an epithelial layer of equivalent cells all having the potential of receiving stimuli and producing some form of effector response, different evolutionary theories on the origin of specialized sensory cells, nerve cells and muscle cells have been proposed. In the following a brief historical overview of the most influential theories about the evolution of the first nervous system will be given.

B. Proposals for the Evolution of the First Nervous System

One of the earliest theories on the origin of the nervous system was that of Kleinenberg (1872) which he based on the discovery of “neuromuscular cells” in the freshwater hydrozoan *Hydra*. He viewed this cell type as a combination of receptor, conductor, and effector cell. The apical ends of the described cells were exposed on the surface of the epithelium and were believed to act as nervous receptors. Their basal ends were drawn out into muscular extensions and supposedly served as effectors which received signals from the cell bodies. Kleinenberg postulated that comparable “neuromuscular cells” gave rise to nerve and muscle cells in the course of evolution. In 1878 the Hertwig brothers described sensory cells, ganglionic cells, and muscular cells in Cnidaria, and postulated that each element was differentiated from a separate epithelial cell but still in a physiologically interdependent way (Hertwig and Hertwig, 1878). In contrast to this notion, Claus (1878) and Chun (1880) suggested that nerve and muscle cells arose independently and became associated only secondarily.

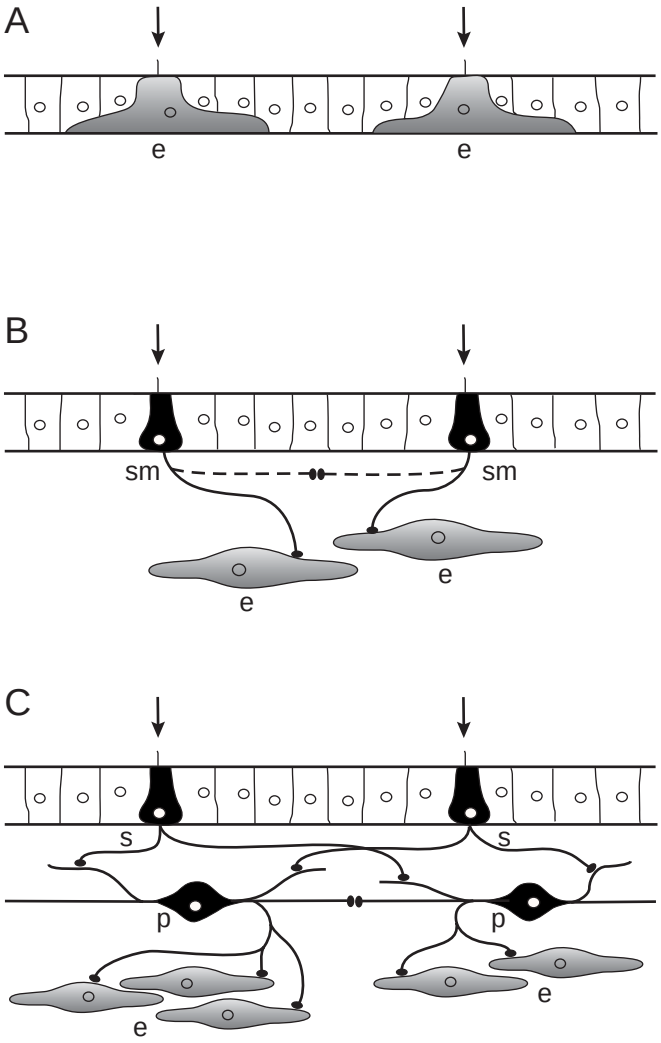

Figure 2 Succession of three evolutionary stages of neuromuscular organization according to Parker (1919). (A) "Independent effectors". Single contractile effector cells surrounded by epithelial cells are directly stimulated, which leads to a response in the cell. (B) Receptor-effector system. Sensory motor neurons directly conduct external stimuli to the underlying muscle cells. In a more complex form, sensory motor neurons can be interconnected among each other (dashed lines). (C) Nerve net. A second type of neuronal cell termed "protoneuron" by Parker intercalates between the sensory cells and the muscle cells and forms a highly interconnected neuronal network. Parker proposed that nerve cells of higher animals had their origin in "protoneurons". Abbreviations: e, effector/muscle cell; p, protoneuron; s, sensory cell; sm, sensory-motor neuron. Arrows indicate the site of stimulation.

The theory of the Hertwigs in which nerve and muscle were thought to have evolved simultaneously was generally accepted until Parker's publication of "The Elementary

Nervous System" in 1919. In this influential publication, Parker proposed a succession of three major evolutionary stages in the organization of the neuromuscular system (Figure 2; Parker, 1919). In sponges which Parker considered as extant representatives of the first evolutionary stage, muscle is present at the absence of nerve cells. This stage is characterized by the appearance of "independent effectors" such as the contractile cells of the oscula sphincters in sponges, which respond directly to environmental stimuli. Although sponges lack nerves, Parker pointed out that they do have a slow type of conduction due to elementary protoplasmic transmission, and he suggested that this "neuroid transmission" might be considered the forerunner of nervous activity. The second stage of evolution was postulated to be a receptor-effector system such as that believed to exist "in the tentacles of many cnidarians" (Parker, 1919). Receptors were thought to arise from epithelial cells that were in close proximity to the already differentiated muscle cells and, in its simplest form, directly connected to the subjacent muscle cells. However, the separate existence of this type of receptor-effector system has never been directly observed and even Parker admitted that this organizational level might frequently be complicated by the fact that receptor cells not only innervate muscle cells but are also interconnected among each other. In the final stage of early nervous system evolution, a third type of cell, termed "protoneuron" by Parker, was intercalated between the sensory and effector cells forming a true nerve net. This stage was thought to be represented by the nerve nets of extant Cnidaria, and Parker suggested that nerve cells of higher animals derived from this third type of protoneuronal cell. In a nutshell, Parker proposed that the first nervous system evolved as a consequence of the selective advantage obtained by coordinating independent effectors.

In the second half of the twentieth century, a number of alternative theories for the evolutionary origin of the nervous system were put forward. Based on morphological and physiological studies on sea anemone nerve nets, Pantin (1956) proposed that nervous systems functioned from the beginning to coordinate the behavior of the whole animal. He argued that the nervous system did not evolve on the basis of single cells, but rather originated as whole networks innervating multicellular motor units. Only later would specific conducting tracts have become associated with specific reflexes in the nerve net and given rise to the reflex arc, which according to this view, is not primitive. Pantin's major objection to Parker's theory was the lack of evidence for the independent existence of a receptor-effector system.

Based on studies of *Hydra* and scyphomedusae Passano (1963) postulated that the nervous system evolved from specialized pacemaker cells whose function was to generate contractions within groups of protomyocytes from which they derived. In this view, nerve cells would have derived from pacemaker cells, retaining rhythm generation as their primary function, and only later becoming specialized for conduction over long distances and as sensory receptors. Grundfest (1959, 1965) postulated that the ancestral neuron derived from a secretory cell that developed a conducting segment between its receptive and secretory poles. Accordingly, true neurons were originally formed when the secretory activity became confined to the terminations of the cells' processes. Thus, this theory is based on the notion that secretion is a primitive feature of the nervous system (Figure 3). A few years earlier, Haldane (1954) proposed that signaling by means of neurotransmitters and hormones had its origin in chemical signaling in protists exemplified by the chemical signals involved in the control of conjugation among different mating types in ciliates. Lentz (1968) noted that protists as well as many non-nervous cells have excitable and conductile properties, and furthermore, that "neurohumors" occur in protists, indicating that these substances could have evolved before the appearance of neurons. He therefore suggested, "that the nerve cell arose by the coupling of electrical activity with secretion of biologically active substances so that a chain of events in response to stimuli resulted in alteration of effector activity." In contrast to Grundfest's proposal that the ancestral neuron was a secretory cell which developed specialized receptive surfaces and a conductile intermediate component, Lentz proposed that both neuronal functions evolved simultaneously.

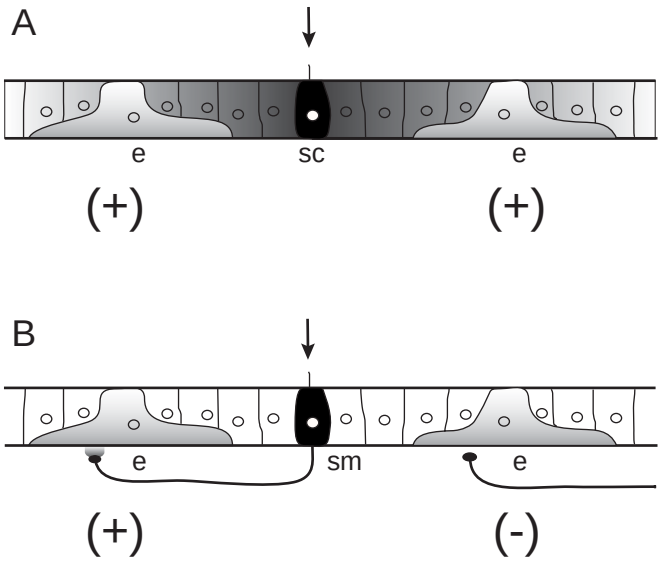

Figure 3 Cell signalling by diffuse secretion preceded synaptic innervation according to Grundfest (1959, 1965). (A) Ancestral state. Single cells secrete biologically active substances upon stimulation, which diffuse throughout the epithelium and activate all surrounding effector cells. (B) Emergence of neurons. Upon stimulation, sensory neurons specifically activate their target cells by local synaptic release of neurotransmitters. Abbreviations: e, effector cell; sc, secretory cell; sm, sensory-motor neuron. Arrows indicate the site of stimulation, (+) stands for an active state and (-) for an inactive state of the effector cell.

Horridge (1968) and Mackie (1970) described excitable epithelia in hydromedusae and siphonophores, which conduct action potentials and serve as pathways mediating certain types of behavior. Based on this discovery they proposed that nerves evolved from tissue whose cells were already interconnected by pathways for metabolic exchange and electrical current flow, thus making cell-to-cell propagation of action potentials possible. According to Horridge, the primary function of neurons was neurosecretory or growth regulatory and only later did their elongated axons become effective in impulse propagation. Nerve cells, with their elongated form and functional isolation from surrounding tissues, would have arisen in response to a need for a more selective type of excitation within conductile epithelia in which effector sub-groups could be controlled independently (Horridge, 1968). Mackie proposed that the starting point for a metazoan nervous conducting system resembled a myoepithelial tissue sheet in coelenterates. The cells in the tissue capable of reception, transmission and contraction were connected by cytoplasmic pathways, which also served for metabolic exchange among the cells (Figure 4). Specialized muscle cells arose by segregation from the primordial epithelium, whereas cells that lost their contractile component but retained their conducting ability gave rise to nerve cells (Mackie, 1970). Westfall propagated the idea that

receptive, electrogenic and neurosecretory functions coevolved in primitive protoneurons. This proposal was based on his demonstration with electron microscopical resolution that nerve cells in *Hydra* not only have receptor poles with a sensory cilium and basal neurites making synaptic contact with effectors but also contain neurosecretory material (Westfall, 1973; Westfall and Kinnamon, 1978). He further proposed that specialized neurons found in modern higher animals derived from multifunctional neuronal ancestors comparable to those found in *Hydra* (e.g. Grimmelikhuijzen, 1996).

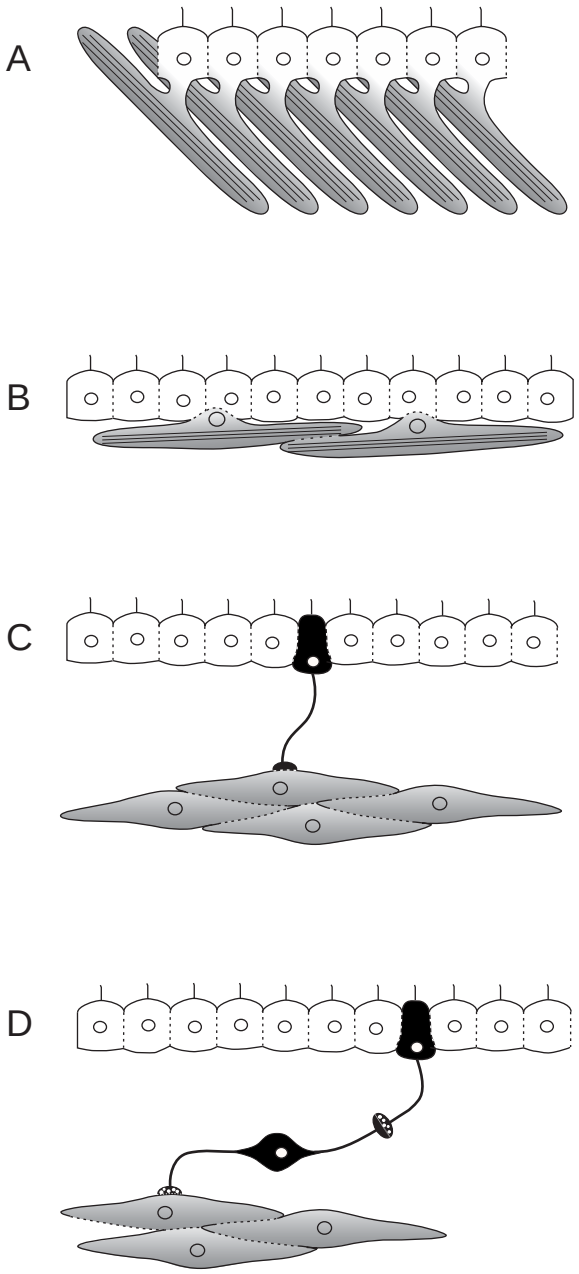

Figure 4 The evolution of nerve and muscle cells from electrically coupled myoepithelial cells according to Mackie (1970). (A) Primordial myoepithelium. (B) Protomyocytes start to leave the epithelium and move into the interior. (C) Protoneurons evolve, conveying excitation to the myocytes from the exterior. All cells are still shown as electrically coupled. (D) Neurosensory cells and neurons evolve. They are connected to one another and to the myocytes by chemically transmitting, polarized synapses. Electrical coupling persists in many epithelia and muscles. However, conduction of impulses becomes increasingly a property of the nervous system. Dashed lines at junctions between cells indicate low resistance pathways through which electrical currents can flow. Modified from The Quarterly Review Of Biology, Vol. 45, No. 4, Mackie GO, 'Neuroid conduction and the evolution of conducting tissues', pages 319-332, Copyright 1970, with permission from The University of Chicago Press.

In more recent studies, Seipel and colleagues (2004) working on the development of the hydrozoan *Podocoryne carnea* have found molecular evidence supporting the hypothesis that muscle and nerve cells derive from a common myoepithelial precursor. In bilaterian animals, neuronal determination and differentiation is controlled by genes encoding basic-helix-loop-helix transcription factors and among these are the genes of the Atonal gene family (reviewed in Lee, 1997; Dambly-Chaudiere and Vervoort, 1998). In *Podocoryne*, the cnidarian Atonal-like 1 (Atl1) gene is expressed in a subset of nerve cell precursors of the medusa and additionally in developing striated muscle cells. Simlarly, the neuronal marker gene coding for the cnidarian Rfamide neuropeptide is expressed not only in mature nerve cells but also transiently in the developing muscle of *Podocoryne* (Seipel *et al.*, 2004). Based on these developmental genetic similarities, the authors propose that nerve and muscle cells are likely to have been closely linked in evolution and share a common ancestor. In contrast, Miljkovic-Licina and coworkers (2004) studying regulatory genes involved in differentiation of neuronal cell lineages in *Hydra* have proposed a scenario in which mechanoreceptor cells would have preceded neuronal cell types in evolution. Their work shows that the nematocyte and neuronal cell differentiation pathways share regulatory genes that exhibit a high level of conservation during metazoan evolution (Miljkovic-Licina *et al.*, 2004). Nematocytes can sense chemical and mechanical stimuli, transduce these signals, and react to them through nematocyst discharge. The authors propose that this type of fast and cell-autonomous response was a hallmark of very primitive nerve cells and that nematocytes were a derived cnidarian by-product of these ancestral “neuro-epithelial” cells. In subsequent evolutionary steps, the “neuro-epithelial” cells could have differentiated into neuronal cells with elongated processes that began to establish connections with myoepithelial cells and involve them in the response to the stimulus. During later stages, neuronal cells would have become progressively more interconnected with each other in a nervous system allowing coordinated behavior.

In summary, a variety of alternative theories implying different origins of the nervous system have been suggested in the last 150 years. Most of these theories are based on extrapolations of observations made on extant protists, sponges and cnidarians. The origin of neurons is generally attributed to epithelial cells, however, the characteristics of these ancestral cells are variously considered to have been contractile, neurosecretory, conductile, chemoreceptive or mechanoreceptive, and each theory emphasizes one or several of these features as driving

force for the evolution of the nervous system. While many of these proposals appear plausible and inspiring for further discussion, it seems impossible to rate one of the theories as more relevant than the others. However, all of the proposed scenarios for the evolution of the nervous system do focus attention on the cell biology of excitable cells in the basal animal groups, and this focus will be explored in more depth in the following pages.

III. ORIGIN OF THE FIRST NERVOUS SYSTEM: A COMPARATIVE PHYLOGENETIC APPROACH

A. Introduction

Although the nervous system must have arisen in a multicellular organism, unicellular organisms such as protists show a variety of behavioral programs in response to their environment. In protists, behavioral responses to external stimuli are achieved at a subcellular level by organelles specialized for signal reception, signal conduction and effector response (Deitmer, 1989; Febvre-Chevalier *et al.*, 1989; Hennessey, 1989). Thus, molecular machineries capable of reception of chemical, mechanical or light stimuli, secretion of biologically active substances, propagation of electrical potentials along membranes and conversion of stimuli into effector responses, were probably already present in the ancestor of metazoans. Assuming colonial protists with equivalent cells as an intermediate form between unicellular protists and early metazoans, an increasing specialization of subgroups of cells must have occurred during evolution. Porifera represent the most basal extant metazoan phylum and are thought to have derived from a colonial form of choanoflagellates. Although a variety of different cell types can be found in sponges, no nerve cells could be identified so far (Jones, 1962; Pavans de Ceccatty, 1974; Mackie, 1979). Nevertheless, contractile cells encircling the oscular openings in sponges are able to react upon mechanical stimulation. In cnidarians and ctenophores, the closest metazoan relatives of sponges, nerve cells are present and can form sophisticated nervous systems capable of solving complex behavioral tasks. This evolutionary step from poriferan to cnidarian or ctenophoran organization may harbor the emergence of nerve cells and nervous systems.

B. Non-Nervous Conduction Outside of the Animal Kingdom

Many key characteristics of nerve cells can be found in non-nervous cells of metazoans, plants, fungi as well as in unicellular organisms like protists and even prokaryotic bacteria.

These characteristics include reception and transmission of signals to other cells, intercellular communication by secretion of biologically active substances, and the propagation of electrical potentials. Nevertheless, the combined appearance of these features in morphologically and functionally specialized nerve cells is unique to the nervous systems of metazoan animals.

Ion channels, which can be gated by ligands, voltage or mechanical forces and are permeable to specific ions, such as K^+, Ca^{2+}, Na^+, and Cl^-, play a major role in the generation of neuronal excitability in higher animals. Moreover, ionic fluxes across cellular membranes mediate a great variety of biological processes that are essential for viability of most life forms. A large number of genes presumably coding for ion channels have been identified in prokaryotes, but although structural or electrophysiological information has been obtained for some of these proteins, their biological roles are mostly unknown. Presumably, prokaryote channels are involved in metabolic function, osmoregulation, and motility (Ranganathan, 1994; Kung and Blount, 2004). In the bacterium *Escherichia coli*, genome sequencing suggests the presence of six putative mechanosensitive channels, one putative voltage-gated K^+ channel and two Cl^- channel-like structures. Three of the mechanically gated channels are involved in osmoregulation and release solutes upon osmotic down shock, whereas Cl^- channels apparently function in short-term acid tolerance. Although, the function of the K^+ channel is still unknown, its protein shares extensive topological and structural similarity with eukaryotic K^+ channels suggesting a common ancestral origin from which K^+ and later probably Ca^{2+} and Na^+ channels evolved (Milkman, 1994; Ranganathan, 1994; Kung and Blount, 2004). Voltage-dependent and stretch-activated ion channels have been found in the plasma membrane of yeast (Gustin *et al.*, 1986; Gustin *et al.*, 1988; Zhou *et al.*, 1995). In addition, the yeast genes involved in the pheromone response show high similarity to signal transduction genes of higher animals. For example, the mating factor receptor STE2 of *Saccharomyces cerevisiae* belongs to the rhodopsin/beta-adrenergic receptor gene family (Marsh and Herskowitz, 1988), and the alpha-type mating factor shows amino acid sequence similarities with the vertebrate reproductive hormone gonadotropin-releasing hormone (Loumaye *et al.*, 1982).

In addition to the transmission of information through substrate flux, plants have electrical and hormonal signaling systems. Action potentials in plants were described for the first time in 1873 by Burdon-Sanderson. He recorded electrical signals from a specimen of the Venus's flytrap, *Dionaea muscipula*, which he received from Charles Darwin (Burdon-Sanderson, 1873; Sibaoka, 1966). The leaves of *Dionaea* are divided into two lobes each of which carries three tactile sense hairs functioning as trigger for an all-or-nothing electrical signal that is followed by the fast closing of the lobes entrapping the prey. In plants like the Venus's flytrap, action potentials are part of a signaling system that responds to mechanical stimulation by changing cell turgor, which leads to relatively rapid movements. Propagation of action potentials from the site of stimulation to the effector cells has been studied in the seismonastic movements of the leaves of *Mimosa pudica* (Sibaoka, 1966; Simons, 1992). Non-nervous electrical conduction in plants involves low-resistance pathways (plasmodesmata) between the phloem cells, comparable with gap junctions that electrically couple cells in excitable epithelia and muscles in animals. Action potentials in plants have been studied in detail in the giant internodial cells of the freshwater algae *Chara* and *Nitella*. In these large cells, a motility system based on actin and myosin drives cytoplasmic streaming, which serves to equally distribute organelles and nutrients around the central vacuole. Upon mechanical or electrical stimulation, an action potential is generated, which spreads in both directions along the shoot and immediately stops the cytoplasmic streaming probably to avoid leakage of the cell in case of injury. In contrast to the action potentials of higher animals where the influx of Na^+ and Ca^{2+} support the depolarizing phase, in *Chara* and *Nitella* Ca^{2+} and Cl^- are the key components of depolarization, a situation which is typical for plant action potentials. A fast initial influx of Ca^{2+} ions is followed by the efflux of Cl^- through Ca^{2+} activated Cl^- channels across the vacuolar and plasma membranes. The falling phase of the action potential is due to an increase in K^+ permeability, similarly to what occurs in nervous cells of higher animals (Sibaoka, 1966; Simons, 1992; Wayne, 1994; Kikuyama, 2001). Although molecules that act as neurotransmitters in higher animals such as glycine, GABA, glutamate and acetylcholine have been isolated from plants, no chemical transmission of electrical signals between cells of plants has been observed. Rather, these substances are involved in a variety of functions related to metabolism, circadian rhythm or light response of plants (Simons, 1992; Mackie, 1990; Hille, 1984).

A number of neuroactive substances including adrenalin, noradrenalin, 5-HT, DOPA, dopamine and beta-endorphin as well as receptors for acetylcholine, catecholamines and opiates have been reported in protists (Zipser *et al.*, 1988; Carr *et al.*, 1989; Görtz *et al.*, 1999). Furthermore, receptor tyrosine kinase genes, known to be involved in cell-cell signaling in metazoans, have been recently isolated from choanoflagellates suggesting that this family of signal receptor molecules evolved before the origin of multicellular animals (King and Carroll, 2001; Brooke and Holland, 2003; King *et al.*, 2003). Some protists can respond to mechanical stimulation with depolarizing or hyperpolarizing membrane potentials. Their membranes are equipped with mechanically-, ligand- or voltage-gated ion channels, and in some cases, action potentials are elicited when the cell membrane is depolarized up to a threshold level by receptor potentials. In most protists, Ca^{2+} ions are responsible for carrying ionic currents and coupling membrane excitation to motile response or contractile activity (Febvre-Chevalier *et al.*, 1989). In some ciliates, ion channels are not distributed uniformly over the cell membrane; this is reminiscent of neuronal cell membranes that have distinct channel populations in dendrites, soma, axon and presynaptic terminals. For example, in *Paramecium* and *Stylonychia* different ion channels can be found at the front and back poles of the cell generating different ion currents, which lead to opposed escape behaviors away from the source of mechanical stimulation. (Kung, 1989; Deitmer, 1989; Kung and Blount, 2004). Behavioral responses in protists elicited by action potentials often involve changes of cell shape or alterations in the pattern of ciliary or flagellar beating (Febvre-Chevalier *et al.*, 1989; Hennessey, 1989). The complexity of effector responses driven by different types of electrical potentials within a unicellular organism is nicely illustrated by the dinoflagellate *Noctiluca*. Two different kinds of flagellar movements and a bioluminescent light response are controlled through different action potentials involving different ion currents across the cytoplasmic and vacuolar membrane. In this manner, multiple bioelectric activities in *Noctiluca* are able to control altered effector responses within a single cell (Oami, 2004). Thus, in the absence of a nervous system, protists exhibit complex behaviors which incorporate features of sensory receptors and effectors into a single, highly structured eukaryotic cell.

C. Porifera: Specialized Cells and Electrical Conduction

Sponges, the most basal extant metazoans, probably evolved from a colonial choanoflagellate. At this stage of phylogeny a number of specialized cell types including muscle-like contractile cells has made its appearance, however, nerve cells are lacking (Jones, 1962; Pavans de Ceccatty, 1974; Mackie, 1979). Some of the actin-containing contractile cells (myocytes) are concentrated as sphincters around the osculum and pore canals of sponges. To contract, the sphincters have to be directly stimulated and they thus represent "independent effectors" as proposed by Parker (1919). Slow contractile responses that spread over short distances have been described in several sponge species, but the responsible cells do not seem to be electrically excitable, and there is no evidence of associated changes in membrane potentials (Mackie, 1979). Thus some form of mechanical interaction between neighboring cells seems likely. The sponge epithelial cells that build the external and internal boundary of the mesenchyme are not joined together with occluding junctions and, therefore, the internal milieu may not be very well isolated from the external. Nevertheless, the mesenchyme provides an environment in which electrical and chemical gradients could be generated and nutrients and hormones diffuse without excessive leakage through the body wall (Mackie, 1990). Acetylcholinesterase, catecholamines and serotonin have been shown to be present in sponges by histochemical techniques (Lentz, 1968) and some neuroactive substances have been demonstrated to influence the water circulation in the sponge *Cliona celata* (Emson, 1966), but so far there is no clear evidence that they are involved in intercellular signaling processes. Interestingly, a recent finding has shown that cells isolated from the marine sponge *Geodia cydonium* (Demospongiae) react to the excitatory amino acid glutamate with an increase in intracellular calcium concentration (Perovic *et al.*, 1999). Extracellular agonists as well as antagonists known from metabotropic glutamate/GABA-like receptors in mammalian nerve cells were found to elicit similar effects in these sponge cells. In addition, a cDNA coding for a 7-transmembrane receptor was isolated from *Geodia*, which has high sequence similarity to metabotropic glutamate/GABA-like receptors in mammals. Although these findings suggest that Porifera possess a sophisticated intercellular communication and signaling system, there so far is no evidence for the type of specialized intercellular signal transmission in sponges that might foreshadow the evolutionary origin of nervous systems.

The tissue of glass sponges (Hexactinellida) is syncytial allowing the rapid propagation of electrical events, which is a fundamental difference between this class and the other two cellular sponge classes, Demospongiae and Calcarea (Müller, 2001). All-or-nothing electrical impulses were recorded from the glass sponge *Rhabdocalyptus dawsoni*. Tactile and electrical stimuli evoke impulses, which lead to the abrupt arrest of water flow through the body wall, presumably due to the coordinated cessation of beating of the flagella in the flagellated chambers. From the superficial pinacoderm, impulses are conducted through the trabecular reticulum, a multinucleate syncytial tissue draped around the spicules of the sponge skeleton, to the flagellated chambers. Impulses are propagated diffusely at 0.27 +/- 0.1 cm per second, a value that falls within the lower range of action potential conduction velocities in non-nervous tissues. It is assumed that signal propagation through the syncytium depends on Ca^{2+} influx and that Ca^{2+} channels may also mediate the flagellar arrest (Leys *et al.*, 1999). The trabecular syncytium seems to be a derived feature specific to the most ancient sponge class Hexactinellida. Since calcareous sponges and demosponges lack comparable syncytial tissue, they would require low-resistance pathways equivalent to eumetazoan gap junctions to conduct electrical signals from cell to cell, but no similar structures have been found so far (Leys *et al.*, 1999; Müller, 2001).

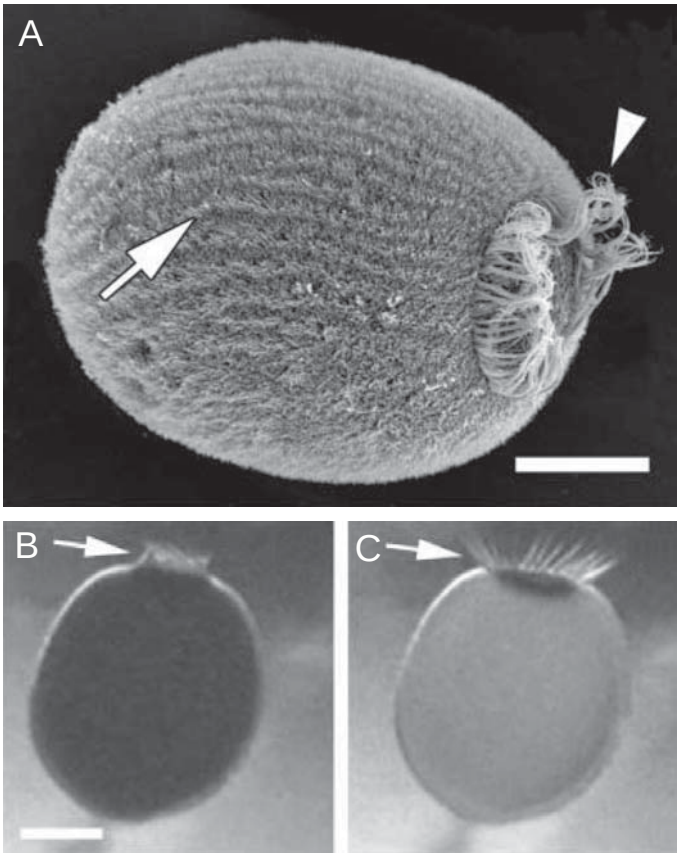

Figure 5 Photosensitive cells and ciliary light response of the sponge *Reneira* larva. (A) Scanning electron micrograph showing the structure of the demosponge larva. Monociliated epithelial cells form most of the outer layer (arrow). The posterior pole is circumscribed by a ring of long cilia (arrowhead). (B) Video recording of bending (B) and straightening (C) of the long posterior cilia (arrows) in response to shutting and opening of a shutter in front of the light source. Scale bars 100 µm. Reprinted from Journal of Comparative Physiology A, Vol. 188, 2002, pages 199-202, 'Spectral sensitivity in a sponge larva', Leys *et al.*, figures 1 A and 2 B (I + II). Copyright 2002. With kind permission from Springer Science and Business Media.

Larvae of many sponge species exhibit rapid responses to external stimuli including light, gravity and current (reviewed in Wapstra and van Soest, 1987). Demosponge larvae have a spheroid body shape and consist of an outer epithelial layer of monociliated cells and a solid center of amoeboid cells in an extra cellular matrix of collagen. The spheroid shaped body is polarized anteroposteriorly with respect to the swimming movement of the larvae, and a ring of pigmented cells that gives rise to long cilia is located at the posterior end. In the demosponge *Reneira* directional swimming is mediated by the long cilia of the posterior pigmented cells and incorporates an asymmetric response of these cells to different light intensities (Figure 5). Increased light intensity causes a bending of the cilia such that they shield the pigment vesicles, whereas decreased light intensity reverses this process. This

results in steering the larva away from bright light (Leys and Degnan, 2001). Interestingly, reanalysis of the action spectrum of the ciliary response to light suggests that the photoreceptive pigment in the sponge larva has the characteristics of rhodopsin similar to the situation in other metazoans that have a rhodopsin-like protein as their primary photoreceptive pigment (Leys *et al.*, 2002). In *Reneira* the light response of the posterior cells has been suggested to depend on the depolarization of the membrane potential and the influx of Ca^{2+} into the cilium. Since sponge larvae lack neurons or gap junctions that would allow coordination of signals among cells with long cilia, each posterior cell appears to respond independently to changes in light intensity. On the other hand, no intercellular coordination seems to be required, given the inherent photokinetic responses of each ciliated cell depending on its position relative to the light source (Leys and Degnan, 2001). Therefore, in some cases "independent effectors" in sponges may mediate coordinated behavior. Although sponges emerged at an early level in multicellular animal evolution when nervous systems had not yet evolved, they do represent the oldest extant metazoans with specialized cells responding to different stimuli and performing behavioral tasks.

D. Ctenophora and Cnidaria: The Oldest Extant Nervous Systems

Ctenophora and Cnidaria are the lowest animal phyla that have a nervous system. The two phyla were traditionally joined together in one group termed Coelenterata based on the presence of a single gastrovascular system serving both nutrient supply and gas exchange among the body parts. Molecular phylogenetic data, however, suggests an independent origin of the two phyla in the prebilaterian line, and their relative position in early metazoan phylogeny is controversial (Martindale and Henry, 1999; Medina *et al.*, 2001; Podar *et al.*, 2001; Ball *et al.*, 2004). Whereas most molecular data supports the more basal position of ctenophores with cnidarians forming the sister group to bilaterians, other evidence, including the presence of true subepithelial muscles and multiciliated cells, supports the view that ctenophores are more closely related to bilaterians than cnidarians (Nielsen, 1997). Thus, it is presently not clear whether Ctenophora or Cnidaria are the closest extant metazoan relatives

of Porifera. Nevertheless, it is likely that the first nervous system evolved at the evolutionary step from Porifera to either of the two coelenterate phyla.

Ctenophores are medusoid gelatinous animals, which generally have two tentacles for capturing prey and eight ciliary comb rows on their outer surface for locomotion. The nervous systems of ctenophores are organized into diffuse nerve nets, which show some local tract-like accumulations below the ciliary comb rows and around the mouth and pharynx. At the ultrastructural level, polarized as well as symmetrical chemical synapses have been shown to be present in these nerve nets. Sensory nerve cells are interspersed among the epithelial cells, except at the aboral pole where sensory and nerve cells constitute, together with a statocyst, the apical organ. Locomotory movements of ctenophores involve metachronal beating of eight comb plate rows radiating from the aboral region. The apical organ serves as pacemaker of the comb plate rows and coordinates geotactic responses (Satterlie and Spencer, 1987). Transmission of ciliary activity among comb plate cells is non-nervous by mechanical coupling (Tamm, 1982). In addition, comb cells are electrically coupled through gap junctions, probably allowing the synchronous response of neighbouring cells to modulatory synaptic input (Hernandez-Nicaise *et al.*, 1989). In *Pleurobrachia* different inhibitory and excitatory pathways coordinate the electromotor behavior of comb plates cells with tentacle movements during prey capture and ingestion (Moss and Tamm, 1993). In their basic elements the ctenophoran nervous systems already share many features with nervous systems of higher animals, thus, allowing well-coordinated behavioral programs in a basal metazoan animal.

E. Cnidarian Nervous Systems: Multiple Levels of Organization

It is often assumed that nervous systems probably evolved first in Cnidaria or a closely related ancestor, and their nervous systems are, thus, often considered to be among the simplest forms and reflect an early stage of evolution. This view prevailed until few decades ago and is still present in many textbooks (Brusca and Brusca, 1990; Ruppert and Barnes, 1994). However, cnidarians have been evolving independently for some 600-630 million years, and have

therefore had plenty of time to develop sophisticated solutions for comparable behavioral tasks and under similar conditions as have many higher animals. During this long evolutionary time period, a wide spectrum in nervous system complexity emerged within the cnidarian phylum ranging from the diffuse nerve nets of sessile polypoid species to the multiple ring shaped nerve tracts, giant axons and highly specialized sensory organs in actively swimming medusoid species. Thus in some cases, the complexity of nervous systems in modern cnidarians may reflect more the behavior tasks of the species considered than any ancestral organization. Many physiological and structural solutions found exclusively in the nervous systems of cnidarians deal with the problem of generating coordinated behavior in a radially symmetrical animal (Mackie, 1990). Ring shaped nerve nets or diffuse epithelial conduction may, therefore, represent adequate systems for specific behavioral functions rather than remnants of a primitive nervous system. Nevertheless, many basic features of bilaterian nervous systems can be found in cnidarian nervous systems and consequently are likely to have been present in their common ancestors in which the first nervous system probably evolved. These features, which have been the subject of considerable research, are considered in more detail below.

Different levels of nervous system organization are encountered in the phylum Cnidaria and often even in the same animal. The spectrum of levels ranges from independent effector cells, as already found in sponges, to the first trends of centralization of integrative and coordinative functions in the nerve rings of some medusae (Bullock and Horridge, 1965; Mackie, 2004). In many aspects the cnidarian nematocytes can be considered as “independent effectors” (Miljkovic-Licina *et al.*, 2004). Nematocytes are mechanoreceptor cells found in the ectodermal tissue of cnidarian tentacles that discharge the toxic content of a highly specialized capsule named the cnidocyst upon contact with the prey. Although most nematocytes are innervated, they are still able to discharge in the absence of nerve cells (Aerne *et al.*, 1991) and thus respond to direct stimulation. Another example of an “independent effector” in cnidarians are the photoreceptor cells of the cubozoan *Tripedalia* planula. These unicellular photoreceptors contain the photoreceptor and shielding pigment granules within the same cell, which in addition carries a motor cilium that enables the larva to perform phototactic behavior. Ultrastructural analysis further reveals that there is no nervous system to which these photosensitive cells transmit visual information. These cells are thus self-contained

sensory-motor entities that respond directly without a coordinating nervous system (Nordström *et al.*, 2003). The unicellular photoreceptors of the *Tripedalia* larva represents an interesting parallel to the photosensitive ciliated cells of sponge larvae in that each cell has a well-developed motor-cilium, which directly responds to light stimulation (Leys and Degnan, 2001; Leys *et al.*, 2002; Nordström *et al.*, 2003). However, since no similar autonomous photosensory motor cells have been described in more basal cnidarian larvae the homology of these two structures can be most likely excluded.

Excitable epithelia are another non-nervous element involved in signal conduction that can be found in Cnidaria side by side with highly specialized nervous conduction pathways. Excitable epithelia are present in the endodermal radial canals of hydrozoan medusae where they conduct signals involved in motor control of behavioral responses such as ‘crumpling’ (protective involution), feeding or swimming. In the pelagic jellyfish *Aglantha*, this epithelial pathway is preserved despite the presence of a highly complex nervous system consisting of several neuronal conduction systems that include diffuse nerve nets, nerve rings and giant axons (Mackie, 2004). Thus, relatively slow, non-nervous signal conduction of the type known from sponges and even plants can offer alternative pathways in parallel to highly specific, fast nervous conduction. Epithelial conduction consisting of electrically coupled equivalent cells, from which more specific pathways evolved with the emergence of elongated nerve cells, has been proposed as a characteristic of the hypothetical metazoan ancestor (Horridge, 1968; Mackie, 1970). Whether epithelial conduction is indeed an ancient feature or rather arose several times during evolution is unclear. Nevertheless, this mode of conduction can be found throughout the animal kingdom, from ctenophores to the early tadpole larvae of amphibians (Roberts, 1969; Mackie, 1970).

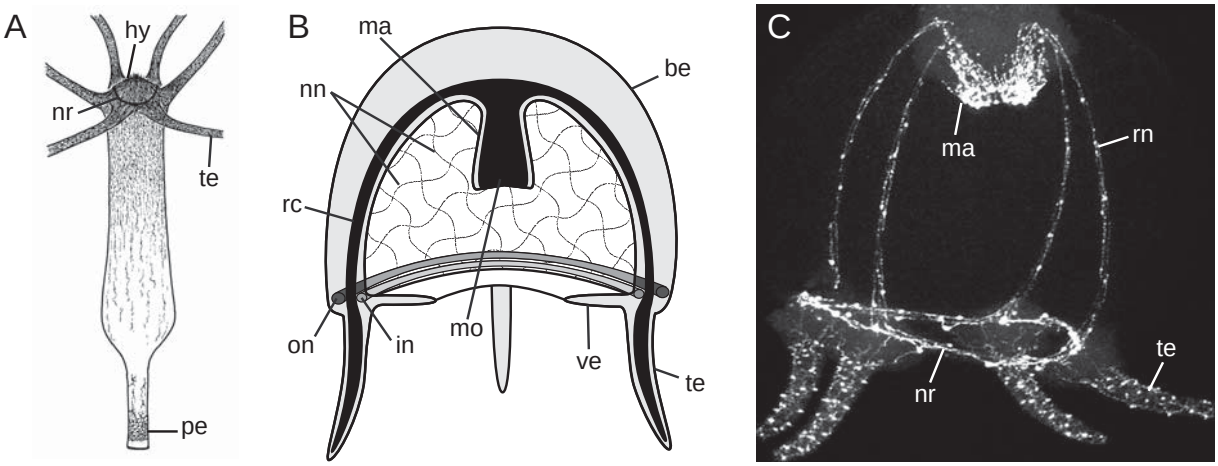

Figure 6 Nervous system organization of hydrozoan polyps and medusae. (A) Drawing showing the RFamide-positive nervous system in *Hydra oligactis*. This species has a dense plexus of immunoreactive neurites in the hypostome and a nerve ring between hypostome and tentacle bases. A collar of neurons can be found in the peduncle. (B) Nerve net and nerve rings in a hydromedusa. Nerve nets underlying the ectodermal and endodermal tissues span the inner surface of the bell. An inner and an outer nerve ring encircle the bell near the margin. These nerve rings connect with fibers innervating the tentacles, muscles, and sensory organs. (C) Fluorescent RFamide staining of the hydromedusa *Podocoryne carnea*. Nerve cells expressing RFamide can be detected in the nerve ring around the margin of the bell and the radial nerves which line the four radial canals. In addition many RFamide positive cells are found around the mouth opening at the tip of the manubrium and scattered over the surface of the tentacles. Abbreviations: be, bell; hy, hypostome; in, inner nerve ring; ma, manubrium; mo, mouth; nn, nerve net; nr, nerve ring; on, outer nerve ring; pe, peduncle; rc, radial canal; rn, radial nerve; te, tentacle; ve, velum. (A) modified from Cell and Tissue Research, Vol 241, 1985, pages 171-182, 'Antisera to the sequence Arg-Phe-amide visualize neuronal centralization in hydroid polyps', Grimmelikhuijzen, figure 9 B. Copyright 1985. With kind permission of Springer Science and Business Media. (C) courtesy of V. Schmid.

A diffuse, two-dimensional nerve net formed by bi- or multipolar neurons, is considered to be a simple form of nervous system organization. A classical example of this simple type of neural ground plan is found in *Hydra*. This cnidarian has a network of multifunctional nerve cells, which combine sensory and motor tasks and have processes that conduct impulses bidirectionally. Traditionally, the nervous system of *Hydra* has been illustrated with a simple meshwork of equally spaced neurons, as it is still the case in many textbooks (e.g. Brusca and Brusca, 1990). However, detailed neuroanatomical analysis of the *Hydra oligactis* nerve net shows that its neurons are not equally distributed throughout the polyp body wall but rather form a ring-shaped area between tentacles and mouth opening and local concentrations in the peduncle suggesting a level of regional specialization (Figure 6 A; Grimmelikhuijzen and

Graff, 1985). Furthermore, distinct neuronal subsets can be distinguished morphologically or neurocytochemically based on neuropeptide expression (Grimmelikhuijzen *et al.*, 1996). In *Hydra* new nerve cells are constantly generated by interstitial cells in a specific zone of the polyp body column and migrate towards the body extremities where old nerve cells are lost. As they migrate, nerve cells can undergo morphological and neurochemical transformations and give rise to the different neuronal subsets (Bode, 1988; Grimmelikhuijzen *et al.*, 1996). In addition to their roles in behavior, nerve cells in *Hydra* are directly involved in the regulation of growth and in the production of chemical morphogenetic gradients (Schaller *et al.*, 1996). Thus, the nervous system of *Hydra* is not a simple, diffuse meshwork of interconnected nerve cells and it is unlikely to represent an ancestral situation within the Cnidaria. In the sea pansy, *Renilla koellikeri*, belonging to the phylogenetically basal cnidarian class of Anthozoa, the nervous system is also found to consist of multiple interconnected nerve nets with local concentrations at specific organs involved in feeding or reproduction (Pernet *et al.*, 2004; Umbriaco *et al.*, 1990). Indeed, it appears that the simplest form of nervous system organization found in extant cnidarians is that of multiple interconnected nerve nets formed by different neuronal subtypes and showing local concentrations.

An important feature of nerve nets is diffuse conduction, characterized by the spreading of an impulse in all directions from the site of stimulation. Symmetric synapses are frequently seen in cnidarian nerve nets, especially in scyphomedusae, where they can transmit excitation bidirectionally (Anderson and Spencer, 1989). Although, bidirectionality can often account for diffuse conduction, symmetrical synapses are apparently not an absolute requirement for this and diffuse conduction can also be obtained by the distributed arrangement of many unidirectional pathways (Bullock and Horridge, 1965). Asymmetrical as well as symmetrical chemical synapses have been identified in all cnidarian classes whereas electrical synapses have been demonstrated only in hydrozoans by electrical and dye coupling and by the presence of conventional gap junctions (Anderson and Mackie, 1977; Spencer and Satterlie, 1980; Westfall *et al.*, 1980). In the multiple nerve net system of hydrozoans, neurons belonging to the same nerve net are generally electrically coupled by gap junctions or even represent true syncytia, whereas chemical synapses are restricted to the interfaces between different nerve nets or utilized for excitation of epithelia, including myoepithelia (Satterlie and Spencer, 1987; Mackie, 2004). The restriction of gap junctions within the phylum

Cnidaria to Hydrozoa raises the question of whether electrical signaling between neighboring cells via gap junctions could have preceded the evolution of true nervous conduction. If gap junctions evolved before neurons, the ancestors of Anthozoa and Scyphomedusae must have independently lost their gap junctions secondarily during evolution, which is rather unlikely. Alternatively, gap junctions arose *de novo* in the ancestor of Hydrozoa after nervous cells had already evolved (Mackie, 1990).

Cnidarian nerve rings and nerve tracts have been proposed to correspond to “compressed nerve nets” (Spencer and Schwab, 1982), although nerves consisting of parallel axon bundles, which are not interconnected by synapses have also been described (Mackie, 2004). A nerve ring, which has been taken as a simple example of neuronal centralization in Cnidaria, is located near the oral pole of the polyp *Hydra oligactis* (Figure 6 A; Grimmelikhuijzen and Graff, 1985). Even more obvious is the presence of nerve rings in medusae at the margin of the bell (Figure 6 B and C). These nerve rings are integrative centers, where different peripheral pathways from sensory organs converge and where activity patterns that result in coordinated behavior are generated. A further striking example is found in the two marginal nerve rings of *Aglantha*, a pelagic hydrozoan medusa. In these interconnected nerve rings, information from 14 conduction systems, including multiple nerve nets, giant axons, and two epithelial pathways are processed and result in the generation of complex behavioral patterns (Mackie, 2004). A ring-shaped central nervous system has been proposed to be appropriate for a radially symmetrical organism, where the term “central” is not meant morphologically but rather in terms of the functions carried on within it (Spencer and Arkett, 1984; Mackie, 1990). Cnidarian nerve rings may therefore represent the first integrating concentrations of nervous tissue in the animal kingdom (Bullock and Horridge, 1965).

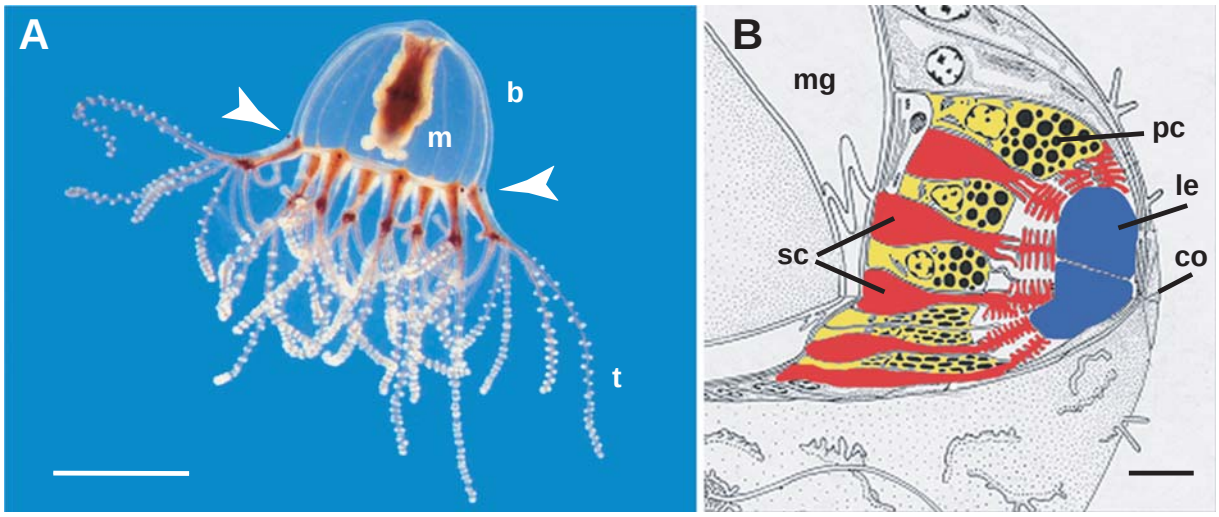

Figure 7 Photosensitive organs in the hydromedusa *Cladonema radiatum*. (A) Photograph of an adult medusa with lens eyes located at the base of the tentacles at the margin of the bell (arrowheads). (B) Structure of the lens eye in a schematic cross section. The lens eye consists of sensory cells, pigment cells and a tripartite lens that is covered by a cornea. Abbreviations: b, bell; co, cornea; le, lens; m, manubrium; mg, mesogloea; pc, pigment cell; sc, sensory cell; t, tentacle. Scale bar is 700 µm in (A) and 10 µm in (B). Reprinted from Developmental Biology, Vol 274, No1, Stierwald M *et al.*, 'The *Sine oculis/Six* family of homeobox genes in jellyfish with and without eyes: development and eye regeneration', Pages 70-81, Copyright 2004, with permission from Elsevier.

Ganglionic centers, which contain a variety of sensory structures including statocysts, ocelli or even, lens eyes, can be found spaced around the bell margin at the base of the tentacles of many medusae (Figure 7 A). The occurrence of photosensitive structures in Cnidaria includes a wide range of complexity and specializations. The sessile polyps of all cnidarian classes respond to light (Tardent and Frei, 1969) but until now no photoreceptive structures or specialized cells for light detection have been identified in polyps. The free-swimming medusa stage, however, can have differentiated photoreceptor organs, which range from simple ocelli to highly evolved lens eyes (Figure 7 B; Land and Fernald, 1992; Stierwald *et al.*, 2004; Piatigorsky and Kozmik, 2004; Gehring 2005). The diversity of photosensitive structures is illustrated by the cubozoan *Tripedalia cystophora* where the planula develops unicellular photoreceptors scattered over the posterior epidermis of the larva, whereas the adult jellyfish forms elaborate multicellular lens eyes (Nordström *et al.*, 2003).

The presence of giant axons is another feature of nervous systems that is common to cnidarians and higher invertebrates. Giant axons are distinguishable from normal axons by

their large diameter and relatively high speed of signal conduction. Indeed, the first intracellular neuronal recordings in Cnidaria have been carried out from the giant axons in the stem of the siphonophoran *Nanomia*, a colonial hydrozoan (Mackie, 1973). Giant axons may have evolved independently in different cnidarian groups, most probably by axonal fusion within nerve nets or endomitotic polyploidy (Mackie, 1989). In the hydromedusa *Aglantha*, several giant axons have been shown to be involved in rapid escape behavior. Interestingly, motor giant axons of *Aglantha*, which synapse onto swimming muscles, can conduct two types of action potentials. Rapidly conducted Na^+ dependent action potentials result in fast swimming associated with escape behavior, whereas slow swimming movements depend on low amplitude Ca^{2+} action potentials. Thus, two kinds of impulse propagation within the same giant axon subserve different behavioral responses in *Aglantha* (Mackie and Meech, 1985), showing that structural simplicity does not allow inference of functional simplicity in Cnidaria.

F. Cnidarian Nervous Systems: Ion Channels and Neuroactive Substances

Cnidarian nervous systems have electrophysiological properties which are similar to those of higher animals. Neurons exhibit conventional action potentials with Na^+ inward currents and K^+ outward currents, miniature end-plate potentials, Ca^{2+}-dependent quantal transmitter release, and with spatial and temporal synaptic summation and facilitation (Spencer, 1989). Typical four-domain Na^+ channels are found in Cnidaria, although these channels are not tetrodotoxin-sensitive as in higher metazoans (Mackie, 1990). Whereas most protists use Ca^{2+} as the inward charge carrier, purely Na^+-dependent action potentials are common to metazoans, including cnidarians. This prompted Hille (1984) to speculate that Na^+ channels evolved from Ca^{2+} channels in parallel with the evolution of the first nervous system. With the emergence of voltage gated Na^+-selective channels, neurons that generate action potentials at high frequency would have become possible; if Ca^{2+} were the only positive charge carrier, high frequency discharges would probably cause intracellular Ca^{2+} to accumulate to toxic levels (Anderson and Greenberg, 2001). Hille further suggested that oubain-sesitive Na^+-K^+

ATPase molecules, involved in maintaining the electroosmotic gradient of these two ions, evolved coincidentally with Na^+ channels (Hille, 1984).

Two different classes of neuroactive substances, classical neurotransmitters and neuropeptides, have been detected in cnidarian tissues. The major difference between these two classes is their mode of synthesis. While classical transmitters are synthesized in nerve terminals, neuropeptides are synthesized in neuronal cell bodies, processed within vesicles and then transported along the axons to the nerve terminals. A large percentage of cnidarian neurons show immunoreactivity with antisera against neuropeptides that have either an Arg-Phe-NH2 or Arg-Trp-NH2 carboxyterminus (LWamide, RFamide). Furthermore, from a single anthozoan species, *Anthopleura elegantissima*, 17 different neuropeptides have been isolated so far, some of which are specifically expressed in at least six identified neuronal subpopulations (Grimmelikhuijzen *et al.*, 1996). Cnidarian neuropeptides occur only in neurons and have been shown to have behavioral effects in several species. Interestingly, some of these neuropeptides also play an important role in growth regulation, morphogenesis and the induction of metamorphosis (Schaller *et al.*, 1996). This dual role is exemplified in the planula of the hydrozoan *Hydractinia echinata*, where LWamide and RFamide neuropeptides form an antagonistic system that influences both planula migratory behavior and initiation of larval metamorphosis in response to environmental cues (Katsukura *et al.*, 2003; Plickert *et al.*, 2003; Katsukura *et al.*, 2004). Although, the cnidarian nervous system is primarily peptidergic, there is growing evidence for the involvement of classical neurotransmitters in signal transmission. This is supported by presence of biogenic amines and acetylcholine in the tissues of several cnidarian species and the role of these substances in modulating behavior. Furthermore, serotonin-immunoreactive neurons have been described in the colonial anthozoan *Renilla*, and GABA and glutamate receptors mediate a modulatory function of pacemaker activity and feeding response in *Hydra* (Umbriaco *et al.*, 1990; Concas *et al.*, 1998; Kass-Simon *et al.*, 2003; Pierobon *et al.*, 2004). However, it remains controversial to what extent neuronal signal transmission in Cnidaria is accomplished by the use of classical transmitters since their action at the synaptic level has not yet been demonstrated (Mackie, 1990; Grimmelikhuijzen *et al.*, 1996; Anctil, 1989). Nevertheless, the presence of both aminergic and peptidergic neurotransmitters in cnidarians indicates a parallel evolution of the two transmitter systems (Prosser, 1989).

G. Placozoa versus Cnidaria

The phylogenetic position of Placozoa, which is currently represented by a single known species, *Trichoplax adhaerens*, is controversial. Recent evidence, however, favors the localization of Placozoa between Cnidaria and Bilateria, rather than within medusozoan cnidarians. Placozoa have a low level of tissue organization consisting of only four different somatic cell types arranged in a functional lower and upper side enfolding a number of intermediate cells (Grell and Ruthman, 1991). Although, *Trichoplax* apparently lacks nerve cells, some cells react with antibodies raised against the neuropeptide RFamide (Schuchert, 1993). The possible presence of neuropeptides in *Trichoplax* may indicate a secondary loss of a nervous system, in accordance with the notion that placozoans are reduced derivatives of an early metazoan. Alternatively, RFamides could have a primitive pre-nervous role in growth regulation or differentiation. Be that as it may, extant placozoans do not have neurons and do not have nervous systems. Thus we are left with the Cnidaria.

The analysis of signal conducting systems in cnidarians representing the most basal extant phyla with nervous systems, leads to the conclusion that many basic features characterizing nervous systems of higher animals were already present in the last common ancestor of cnidarians and bilaterians. Cnidarian neurons resemble structurally those of higher animals. Furthermore, the biophysical basis of electrogenesis in neurons is conventional, and chemical and electrical synapses are similar to those found in all higher metazoans, although the common use of bidirectional synapses in cnidarians is somewhat unusual. Therefore, the “simplicity” of the cnidarian nervous system does not lie at the level of individual neurons, but rather in the organization of such cells into conducting systems, such as nerve nets. The evolutionary origin of the neuron remains elusive.

IV. ORIGIN OF THE FIRST NERVOUS SYSTEM: A COMPARATIVE DEVELOPMENTAL GENETIC APPROACH

A. Conserved Genes in Neuronal Development

In 1990, Mackie relaunched Parker's discussion of the elementary nervous system and proposed that the evolutionary origin of the nervous system should be reconsidered in the light of recent results form molecular biology and developmental genetics (Mackie, 1990). Indeed, over 80 years after Parker first put forward his theoretical views it seems appropriate to consider not only the origin of the cell lineages that initially gave rise to neurons, but also the origin of the genes involved in neurogenesis and neuronal differentiation. Ideally this type of molecular evolutionary developmental approach should allow identification of a basal set of genes that are likely to have been involved in generating the first nervous system. Thus, a novel and promising approach to nervous system evolution is the comparative analysis of the genes that control neuronal proliferation and differentiation in key metazoan phyla. Which key phyla should be subjected to such a molecular genetic analysis? Although, impulse conduction and sensitivity to neuromodulatory substances have been shown in different Porifera, extant sponges lack nerve cells and a nervous system and are therefore not ideal for studies on the molecular genetics of neuronal development. In contrast, true neurons as well as different levels of nervous system organization can be found in the Cnidaria, and, in consequence, a comparative developmental genetic analysis of cnidarian versus bilaterian nervous systems is likely to be useful. In the following, the evolution and origin of the first nervous system will be considered in light of the molecular genetic control elements for neurogenesis, axial patterning and eye development that are conserved between Cnidaria and Bilateria. A caveat for all of these considerations is, however, the fact that functional analyses of key control genes are still lacking in the Cnidaria.

B. Genetic Control of Neurogenesis in Cnidaria and Bilateria

Key genetic regulators of neurogenesis have been studied in a number of vertebrate (mouse, chick, frog, zebrafish) and invertebrate (Drosophila, C. elegans) model organisms. Several transcription factors involved in early neurogenesis events have been identified that are structurally and functionally conserved among protostome and deuterostome phyla (Arendt and Nübler-Jung, 1999; Bertrand *et al.*, 2002; Reichert and Simeone, 2001). This suggests that similar transcription factors might already have been involved in neurogenesis of the common ancestor of all bilaterians. Different classes of regulatory genes involved in neurogenesis have been isolated and their expression patterns studied in the cnidarian model organism *Hydra*, and homologs of regulatory genes expressed during neurogenesis in deuterostomes and protostomes have been found.

Two homeobox genes *prdl-a* and *prdl-b* are expressed in nerve cell precursors and neurons in the body column of the *Hydra* polyp (Gauchat *et al.*, 1998; Gauchat *et al.* 2004; Miljkovic-Licina *et al.*, 2004). They are both related to the paired-like *aristaless* family, members of which have been shown to be important for normal forebrain development in vertebrates (Seufert *et al.*, 2005). The *COUP-TF* genes which encode orphan nuclear receptors are implicated both in neurogenesis and in CNS patterning during embryogenesis as well as in the adult nervous system of vertebrates and *Drosophila* (Gauchat *et al.*, 2004). The *Hydra* homolog *hyCOUP-TF*, was found to be expressed in a subset of neurons and in the nematocyte lineage (Miljkovic-Licina *et al.*, 2004). The basic-helix-loop-helix (bHLH) transcription factor *CnASH* is related to the *achaete-scute* gene family in *Drosophila*, which has proneural activity (Grens *et al.*, 1995). *CnASH* is expressed in the differentiation of sensory neurons in the tentacles of *Hydra* (Hayakawa *et al.*, 2004). Another bHLH transcription factor *Atonal-like1* (*Atl1*), which belongs to the Atonal gene family, has been isolated in the hydrozoan *Podocoryne*. Atonal homologs are responsible for the determination of neural fate in sense organs as well as in the peripheral and central nervous systems of bilaterian model organisms (reviewed in Hassan and Bellen, 2000). In the medusa of *Podocoryne*, *Atl1* is expressed in subsets of presumed nerve cells of the tentacle and the feeding organ (Seipel *et al.*, 2004). These findings suggest that some elements of the genetic

network underlying neuronal development may be conserved from cnidarians to vertebrates, implying that the molecular genetic control of neuronal development evolved only once.

C. Genetic Control of Anteroposterior Patterning in Cnidaria and Bilateria

The bilateral symmetry of bilaterian animals is achieved by the orthogonal intersection of an anteroposterior and a dorsoventral body axis. Different genetic mechanisms are responsible for patterning each axis and the underlying gene networks are widely conserved between Protostomia and Deuterostomia. Thus, *Hox* genes play an evolutionary conserved role in patterning the anteroposterior axis of all bilaterians studied to date (Slack *et al*., 1993). Interestingly, Hox genes are also responsible for the anteroposterior patterning of bilaterian nervous systems as has been shown in genetic experiments carried out for arthropods and vertebrate model systems. The anteroposterior expression pattern of the *Hox* genes during nervous system development largely reflects their pattern of expression in the embryonic body and corresponds to the spatial arrangement of the *Hox* genes in their chromosomal clusters (spatial colinearity). Similarly, the homeobox transcription factors of the *orthodenticle (otd/Otx)* and *empty spiracles (ems/Emx)* families have evolutionarily conserved expression domains in the anterior cephalic regions of all bilaterian animals studied to date. Moreover, both gene families are known to play an important role in the development of the most anterior part of the nervous system, the anterior brain, in arthropods and vertebrates. Mutations in these genes lead to severe brain phenotypes such as the absence of large neurogenic regions of the brains of both insects and vertebrates. Thus, bilaterian brains are universally characterized by a rostral region specified by genes of the *otd/Otx* and *ems/Emx* family and a caudal region specified by genes of the *Hox* family (Figure 8 A; Shankland and Bruce, 1998; Sharman and Brand, 1998; Arendt and Nübler-Jung, 1999; Hirth and Reichert, 1999; Reichert and Simeone, 2001; Lowe *et al.*, 2003; Lichtneckert and Reichert, 2005).

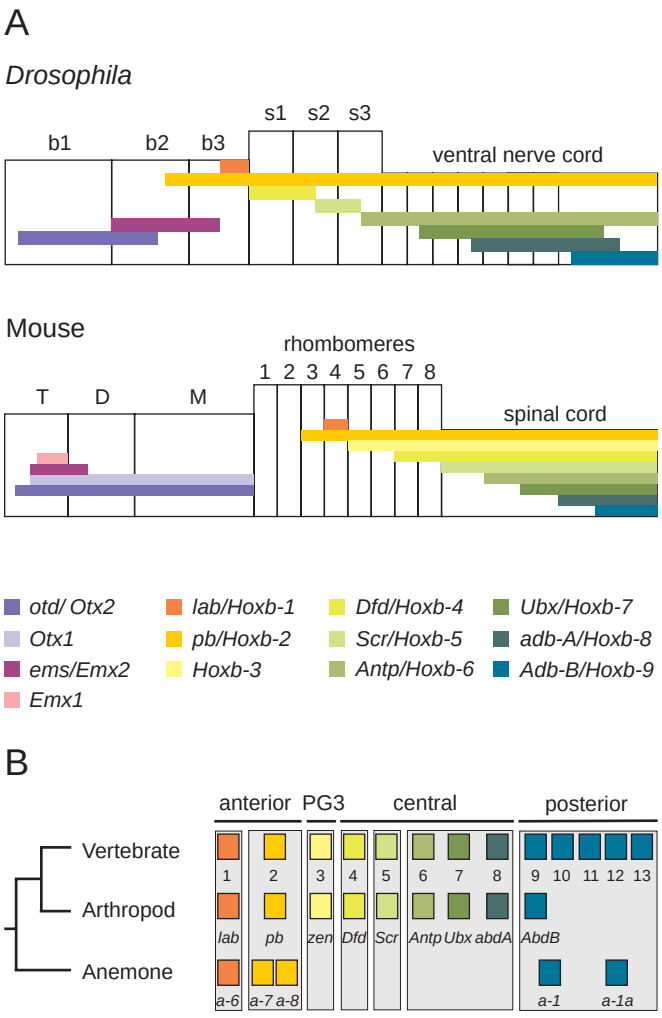

Figure 8 Conserved anteroposterior order of gene expression in embryonic central nervous system development of bilaterians and occurrence of Hox genes in bilaterians and anthozoans. (A) Schematic of *otd/Otx*, *ems/Emx* and *Hox* gene expression patterns in the developing CNS of *Drosophila* (stage 14 embryo) and mouse (stage 9.5-12.5 embryo). (B) Homology of *Nematostella vectensis Hox* genes to vertebrate and arthropod orthologs based on phylogenetic analysis of homeodomains. Vertebrate *Hox* paralogs are numbered from 1 to 13. Arthropod *Hox* paralogs are named with *Drosophila* terminology (*lab, labial; pb, proboscipedia; zen, zerknüllt; Dfd, Deformed; Scr, Sex combs reduced; Antp, Antennapedia; Ubx, Ultrabithorax; abd-A, abdominal-A; Abd-B, Abdominal-B*). Paralog groups are classified as anterior, paralog group 3 (PG3), central, and posterior *Hox* genes. Abbreviations: *a-1, anthox1; a-1a, anthox1a; a-6, anthox6; a-7, anthox7; a-8, anthox8;* b1-b3, segments in the *Drosophila* brain (proto-, deuto- and trito-cerebrum, respectively); s1-s3, mandibular, maxillary and labial segments, respectively, of the fly subesophageal ganglion; T, telencephalon; D, diencephalon; M, mesencephalon. (a), reprinted from Trends in Genetics, Vol 14, No 6, Sharman, A.C., and Brand, M., ‘ Evolution and homology of the nervous system: cross-phylum rescues of *otd/Otx* genes’, Pages 211-214, Copyright 1998, with permission from Elsevier. (b) reprinted with permission from Science, Vol 304, Finnerty, J.R. *et al.*, ‘Origins of Bilateral Symmetry: *Hox* and *Dpp* Expression in a Sea Anemone’, Pages 1335-1337. Copyright 2004 AAAS.

Homologous genes involved in anteroposterior patterning of the body wall and nervous systems of bilaterians have been isolated from different Cnidarian species. *Otd/Otx* family genes have been cloned from two hydrozoans, *Hydra* (Smith *et al.*, 1999) and *Podocoryne* (Müller *et al.*, 1999). Whereas *Podocoryne Otx* is only expressed in the striated muscle of the developing medusa, which seems unrelated to *otd/Otx* function in Bilateria, *Hydra Otx* expression can be found in ectodermal epithelial cells throughout the body column. In addition, *Hydra Otx* expression has been detected in nerve cells by cell-type Northern; however the *Otx*-positive neural subpopulation has not yet been identified. In gastrozooid polyps of the hydrozoan *Hydractinia symbiolongicarpus*, expression of *Emx* is detected at the oral "head" end of the oral-aboral axis, specifically in endodermal epithelial cells of the hypostome (Mokady *et al.*, 1998). No *Emx* expression in nervous systems of cnidarians has been described so far.

The question of whether true *Hox* genes are present in cnidarians is controversial (reviewed in Galliot, 2000; Finnerty, 2003; Ball *et al.*, 2004). Based on sequence analysis, several authors have argued for the presence of anterior class and posterior class *Hox* genes in cnidarians. The chromosomal linkage of these genes in clusters is still a matter of debate. The expression data from hydrozoans and anthozoans show that different *Hox* genes are expressed in specific regions along the oral-aboral body axis. Five *Hox* genes were recovered from the sea anemone *Nematostella vectensis*; their expression was studied during larval development (Finnerty *et al.*, 2004). Two cnidarian-specific gene duplications appear to have produced two pairs of sister genes *anthox1-anthox1a* which are homologous to bilaterian posterior group *Hox* genes, and *anthox7-anthox8* which are homologous to the anterior *pb/Hox2* genes in vertebrates and flies (Figure 8 B). Whereas expression of *anthox1* is restricted to the ectoderm at the aboral tip of the polyp, a nested expression of *anthox1a, anthox7* and *anthox8* is found in the endoderm layer all along the body column. The *lab/Hox1* homolog, *anthox6* is expressed in the endodermal body layer of the pharynx, the oral-most part of the polyp. Therefore, during development *Nematosella Hox* gene expression spans nearly the entire oral-aboral axis, which is similar to the situation in the body of bilaterian animals. Whether expression of anthozoan *Hox* genes is present in the nerve cells of *Nematosella* is currently unknown. Cnidarian *Hox* gene expression has also been reported in larval development of the hydrozoan *Podocoryne carnea* (Masuda-Nakagawa *et al.*, 2000; Yanze *et al.*, 2001). Three *Hox* genes, *cnox1-Pc*,

cnox2-Pc, and *cnox4-Pc* are expressed in restricted domains along the oral-aboral axis in ectodermal and endodermal germ layers of the planula larva. Although, an anteroposteriorly polarized nerve-net has been described in the planula larva of *Podocoryne* (Gröger and Schmid, 2001), the presence of the *Hox* genes in the cells of this nerve net has not been investigated yet. Interestingly, comparison of orthologous *Hox* genes between *Nematostella* and *Podocoryne* reveals that their axial expression patterns in the planula are reversed. For example, the anterior *Hox* gene, *cnox1-Pc* is expressed at the apical end of the planula in *Podocoryne*, while the *Nematostella* homolog, *anthox6* is expressed at the blastoporal end of the planula. This apparent contradiction may be attributed to a developmental reversal of spatial polarity that has been described for *Hox* expression in *Podocoryne* during metamorphosis (Masuda-Nakagawa *et al.*, 2000). Thus, while clear homologs of bilaterian anterior and posterior class *Hox* genes are present in cnidarians, the correlation between cnidarian and bilaterian *Hox* gene expression patterns remains ambiguous. Moreover, the expression and function of cnidarian *Hox* genes in nerve cells has not been explicitly investigated so far, leaving the question of their involvement in nervous system patterning unanswered.

D. Genetic Control of Dorsoventral Specification in Cnidaria and Bilateria

A hallmark of dorsoventral polarity in many bilaterians is the dorsoventral location of the central nervous system. Whereas in vertebrates the central nervous system is located dorsally, in arthropods the central nervous system is located ventrally. This reversal in the relative position of the CNS led Geoffroy Saint-Hilaire to propose that the dorsoventral axes of vertebrates and arthropods are inverted with respect to the position of their mouth openings (Geoffroy Saint-Hilaire, 1822). This “dorsoventral inversion” hypothesis has gained strong support in recent years, since homologous, but spatially inverted patterning mechanisms were found to be operating in vertebrates and insects (Holley *et al.*, 1995). The *transforming growth factor-β (TGF-β)* superfamily members *decapentaplegic/Bone Morphogenetic Protein 4 (dpp/BMP4)* are required for patterning the dorsal region in arthropods and for promoting

ventral fates in vertebrates (Figure 9 A and B). In both animal groups *dpp/BMP4* have strong anti-neurogenic properties, and therefore, the nerve cord can only develop where *dpp/BMP4* activity is inhibited or absent. In *Drosophila*, the ventral expression of the *dpp* antagonist *short gastrulation* (*sog*) allows the development of the ventral neuroectoderm, whereas in vertebrates, the same effect is achieved dorsally by the *sog*-related *Chordin* gene (Reichert and Simeone, 2001; Lichtneckert and Reichert, 2005).

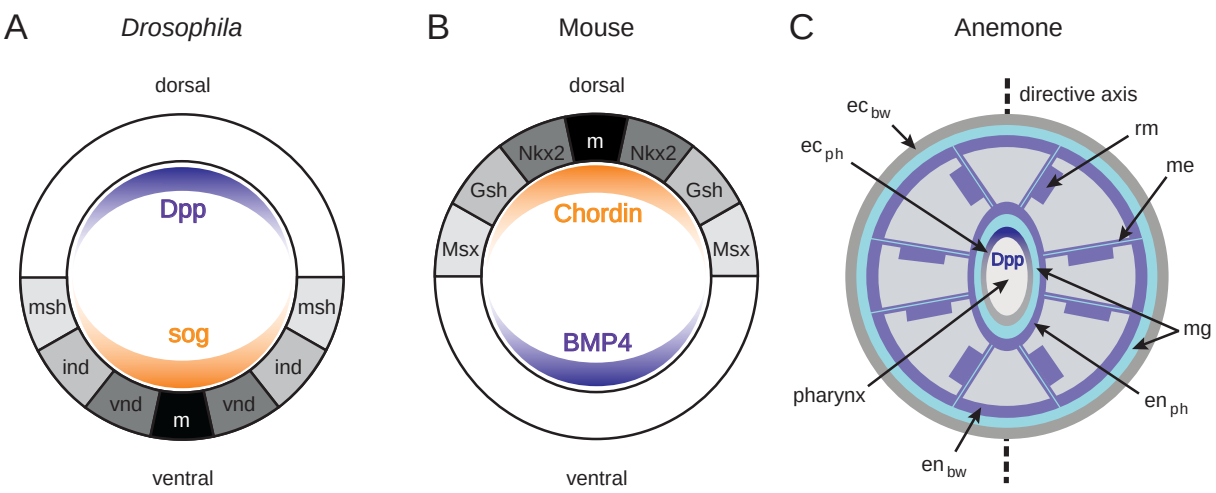

Figure 9 Asymmetric Dpp/BMP4 signaling along the dorsoventral and directive axis of bilaterians and anthozoans. (A and B) The secreted products of the homologous genes *dpp/Bmp4* form a dorsoventrally inverted gradient in mouse (Deuterostomia) with respect to *Drosophila* (Protostomia). Sog/Chordin act from opposing dorsoventral poles in both insect and vertebrate embryos antagonizing the antineurogenic effect of Dpp/BMP4. The neuroepithelium is further subdivided by a set of homeobox genes into medial (*vnd/Nkx2*), intermediate (*ind/Gsh*) and lateral (*msh/Msx*) neurogenic domains in *Drosophila* and mouse. © Cross section through the pharyngeal region of the anemone *Nematostella* reveals bilateral symmetry about the directive axis. The pharynx is attached to the outer body wall via eight endodermal mesenteries. Each mesentery bears a retractor muscle on one face. The only plane of mirror symmetry passes through the directive axis. During development, Dpp is expressed throughout the endoderm. In addition, Dpp expression is transiently found in the pharynx ectoderm in an asymmetric distribution relative to the directive axis. Abbreviations: ec_{bw}, body wall ectoderm; ec_{ph}, pharyngeal ectoderm; en_{bw}, body wall endoderm; en_{ph}, pharyngeal endoderm; m, midline; me, mesentery; mg, mesogloea; rm, retractor muscle. (a) and (b), modified from Phil. Trans. R. Soc. Lond. B, Vol 356, Reichert, H., and Simeone, A., 'Developmental genetic evidence for a monophyletic origin of the bilaterian brain', Pages 1533-1544. Copyright 2001, with permission from The Royal Society. (c) reprinted with permission from Science, Vol 304, Finnerty, J.R., *et al.*, 'Origins of Bilateral Symmetry: *Hox* and *Dpp* Expression in a Sea Anemone'. Pages 1335-1337, Copyright 2004 AAAS.

Although, textbooks usually characterize cnidarians as radially symmetrical (Brusca and Brusca, 2003; Campbell *et al.*, 2004; Johnson, 2003), it has long been recognized that many anthozoan cnidarians exhibit bilateral symmetry (Stephenson, 1926; Hyman, 1940). In many sea anemones a secondary body axis, referred to as the directive axis, crosses the pharynx orthogonally to the primary oral-aboral body axis. For example, a cross section through the sea anemone *Nematostella vectensis* reveals that the mesenteries and their associated retractor muscle fibers exhibit a bilateral symmetry in their orientation around the pharynx. Genes involved in specifying the dorsoventral axis in Bilateria have recently been found to be expressed asymmetrically along the directive axis of anthozoans. In the gastrulating embryo of *Acropora millepora*, expression of *bmp2/4-Am* (a *dpp/BMP4* homolog) is not symmetrical about the primary body axis, which runs through the blastopore. Rather *bmp2/4-Am* mRNA is concentrated in one quadrant of the surface ectoderm next to the blastopore (Hayward *et al.*, 2002). This suggests that the *bmp2/4-Am* expression domain defines a second polarized axis, in addition to the one defined by the blastopore. A similar distribution of *dpp/BMP* mRNA has been reported during early embryogenesis of the sea anemone *Nematostella vectensis* (Finnerty *et al.*, 2004); at later developmental stages of *Nematostella*, *dpp/Bmp4* is expressed in the pharynx and the mesenteries in a bilaterally symmetrical fashion relative to the directive axis (Figure 9 C). Within the Cnidaria, bilateral symmetry is a characteristic of anthozoans and thus probably represents an ancestral trait of the phylum that might have been lost secondarily in medusozoans due to the emergence of a clearly radial symmetric medusoid life stage. Although at least part of the dorsoventral patterning system that has antineural function in bilaterians is present in anthozoan polyps, no morphological regionalization of the nervous system along the directive axis of polyps has been observed yet.

In arthropods and vertebrates, initial regionalization of the dorsoventral axis by *dpp/Bmp4* and their antagonists is followed by further patterning of the neuroectoderm along its dorsoventral axis by a group of conserved homeobox genes. In *Drosophila*, *vnd* (*ventral neuroblasts defective*), *ind* (*intermediate neuroblasts defective*), and *msh* (*muscle segment homeobox*) are involved in the dorsoventral specification of a ventral, intermediate, and lateral column of neuroblasts in the developing ventral neuroectoderm (Figure 9 A and B). During vertebrate neurogenesis, genes closely related to *Drosophila msh* (*Msx*), *ind* (*Gsh)*, and *vnd* (*Nkx2*) are expressed in domains corresponding to those in *Drosophila* along the dorsoventral axis of the

developing CNS suggesting that this system was conserved throughout evolution (reviewed in Arendt and Nübler-Jung, 1999; Reichert and Simeone, 2001; Lichtneckert and Reichert, 2005).

All three of these dorsoventral patterning genes (*vnd*/*Nkx2*, *ind*/*Gsh*, *msh*/*Msx*) are present in cnidarians (Schummer *et al.*, 1992; Grens *et al.*, 1996; Hayward *et al.*, 2001). In the anthozoan *Acropora millepora*, *cnox-2Am*, the ortholog of the vertebrate *Gsh* gene, is expressed in scattered ectodermal cells of the larva with a restricted distribution along the oral-aboral body axis. Based on morphology, these cells have been characterized as transectodermal neurons (Hayward *et al.*, 2001). The expression of *cnox-2Am* in a subset of neurons is consistent with the restricted expression of *Gsh* orthologs in bilaterians. The presence of all three dorsoventral patterning homeobox genes in Cnidaria together with the spatially restricted neuronal expression of *cnox-2Am* along the anteroposterior axis of the planula larva, suggests that the *msh*/*ind*/*vnd* system may have had an ancient evolutionary origin that predated the Cnidaria/Bilateria split, and thus might represent an ancient nervous system patterning process. It remains to be shown, however, if the cnidarian orthologs of *vnd*/*Nkx2* and *msh*/*Msx* are also expressed in nerve cells and if their expression specifies different neuronal subsets located on a secondary body axis, as in bilaterians.

E. Genes Involved in Eye Development in Cnidaria and Bilateria

A conserved gene regulatory network including members of the *Pax6*, *six*, *dachshund*, and *eyesabsent* families has been shown to orchestrate eye development in a wide range of bilaterian animals. *Pax6* mutations in the mouse or fly cause a reduction or absence of eyes. On the other hand, ectopic expression of *Pax6* from various bilaterian species induces ectopic eyes in *Drosophila*, implying that *Pax6* might represent a “master control” gene for eye development (reviewed in Piatigorsky and Kozmik, 2004; Gehring, 2005). The fundamental, evolutionarily conserved role of the genetic network underlying eye development led to the suggestion of a monophyletic origin of the eye (Gehring and Ikeo, 1999). The *Pax2/5/8* family comprises one single *D-Pax2* gene in *Drosophila* (Fu and Noll, 1997), whereas in

mammals three genes, *Pax2, Pax5, and Pax8,* arose by duplications at the onset of the vertebrate lineage (Pfeffer *et al.*, 1998). The *Pax2/5/8* genes play an important role in brain patterning and are also implicated in eye development.

In cnidarians, eyes are found sporadically in some hydrozoan (see Figure 7 B) and cubozoan medusae, and it is not known whether other jellyfish have lost their eyes in the course of evolution or whether they never acquired them (Piatigorsky and Kozmik, 2004; Gehring, 2005). Four *Pax* genes (*PaxA, PaxB, PaxC,* and *PaxD*) have been isolated from anthozoans (Miller *et al.*, 2000) and a number of other cnidarian species (Sun *et al.*, 1997, 2001; Gröger *et al.*,2000; Kozmik *et al.*, 2003), but none of these have a protein domain structure that corresponds of bilaterian *Pax6*. In the cubomedusa *Tripedalia cystophora*, *PaxB* is expressed in the lens and the retina of the complex eyes as well as in the statocyst. Interestingly, it has been shown that *PaxB* is structurally a mosaic between *Pax2* and *Pax6*. This is further supported by functional studies in *Drosophila*, where *PaxB* complements *Pax2* mutants (*sparkling*) and also induces ectopic eyes like *Pax6* (Kozmik *et al.*, 2003). Therefore, *PaxB* of *Tripedalia* might resemble an ancestral gene of the *Pax6* and *Pax2/5/8* subfamilies, which arose by duplication of the ancestral form in the bilaterian line (Kozmik *et al.*, 2003; Piatigorsky and Kozmik, 2004). Thus, the competence to regulate eye development was either inherited from the ancestral *PaxB*-like gene by cnidarian *PaxB* and bilaterian *Pax6*, which would support the monophyletic origin of eyes (Gehring and Ikeo, 1999; Gehring, 2005), or it emerged parallely during the evolution of the two *Pax* genes following the cnidarian bilaterian split (Piatigorsky and Kozmik, 2004). Interestingly, a *PaxB* ortholog has been isolated from sponges (Hoshiyama *et al.*, 1998), however it is not known whether the expression of this gene is associated with the photoreceptive cells in sponge larva. Additional support for the monophyletic origin of the eyes was obtained from the hydrozoan *Cladonema*. Orthologs of two *Six* family members, which are known to control eye development in vertebrates and arthropods, are expressed in the lens eyes of the hydromedusa and are involved in eye regeneration (Stierwald *et al.*, 2004). This implies that the common ancestor of Cnidaria and Bilateria may already have possessed some kind of photoreceptive organ. Moreover, it suggests that at least part of the gene regulatory network used for the development of eyes by modern species, was already used by the eumetazoan ancestor. Taken together, the presence of photosensitive cells, probably autonomous receptor-effector cells, in multicellular animals,

as exemplified by certain sponge larvae, may have anticipated the emergence of a nervous system. If this were the case, then the sensory input from these photoreceptors might have had a strong influence on the early evolution of the nervous system.

V. CONCLUSIONS AND OUTLOOK

The origin and evolution of the first nervous system remains elusive. Over the last 150 years the evolution of the first nervous system has been a central issue in notions about the emergence of eumetazoan animals, and a variety of theories have been proposed. The main question has been the identification of the primordial cell lineage from which nerve cells might have been derived. During the last decade, however, advances in molecular genetic techniques have focussed our interest on the genes that might have been involved in the generation of the first nervous system. In terms of comparative developmental genetics, it appears that genes involved in patterning of the anteroposterior axis in bilaterians, such as the *Hox* genes, are also expressed in restricted domains along the main body axis during cnidarian larval development as well as in the adult polyp. However, the validity of comparing gene expression patterns along the oral-aboral axis of cnidarians to those found along the anteroposterior axis of bilaterians is questionable. Moreover in contrast to bilaterians, *Hox* gene expression in cnidarian nerve cells has not yet been unequivocally demonstrated. Similar considerations apply to most the genes involved in dorsoventral patterning in cnidarians and bilaterians. Thus, although there is morphological and genetic evidence for bilateral symmetry with respect to the directive axis in anthozoans, no regional restriction of neurogenesis in the cnidarian body has been reported to date. Does this mean that the restriction of nervous tissue to one side of the dorsoventral body axis by early genetic pattering mechanism evolved only in bilaterian animals?

One of the most intriguing findings to emerge from preliminary EST projects on several cnidarian species is that the gene sets of cnidarians and, by implication, the common metazoan ancestor, are surprisingly rich and complex (Kortschak *et al.*, 2003). A long held assumption is that fewer genes should be required to build a sea anemone than a fly, but this seems not to be true. This paradox is exemplified by the fact that, whereas anthozoan cnidarians have the simplest extant nervous systems, the *Acropora millepora* genome contains many of the genes known to specify and patterns the much more sophisticated nervous systems of vertebrates and insects. It has been proposed that the first major wave of gene duplications in metazoans predated the Parazoa and Eumetazoa split some 940 million years ago resulting in large genomes in basal metazoans (Nikoh *et al.*, 1997, Suga *et al.*, 1999).

Gene number seems to be a poor indicator of the sophistication of gene use; it is now widely accepted that alternative splicing and transcriptional regulation are generally more complex in mammals than in insects and that this difference accounts for the execution of more complex molecular programs in complex animals (Ball *et al.*, 2004).

A comparative genetic approach including Cnidaria and Ctenophora as well as different bilaterian groups may help to reconstruct different aspects of the nervous system of the last common ancestor, which might have resembled the first nervous system in evolution. Moreover, the availability of genomic data from Porifera in the near future (Leys, *et al.*, 2005), should pave the way for the identification and analysis of further sponge homologs to genes involved in neurogenesis or in sensory organ development in Eumetazoa, thus, providing more information about the origin and the evolution of the first nervous system.

Further Reading

Ball, E.E., Hayward, D.C., Saint, R., and Miller, D.J. (2004). A Simple Plan--Cnidarians and the Origins of Developmental Mechanisms. Nat. Rev. Genet. 5, 567-577.

Reviews developmental genetic mechanisms involved in mesoderm specification, body axis patterning and photoreceptor specification that are conserved between Cnidaria and Bilateria.

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ERKLÄRUNG

Ich erkläre, dass ich die Dissertation

Cell Lineage Specification during Postembryonic Brain Development in Drosophila:
Expression and Function of the Cephalic Gap Gene *empty spiracles*

nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Basel, den 30. Januar 2007

Robert Lichtneckert