GENETIC MECHANISMS UNDERLYING NEUROMERE SPECIFICATION DURING EMBRYONIC BRAIN DEVELOPMENT OF *DROSOPHILA*

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1. SUMMARY

In Drosophila, a set of evolutionarily conserved transcription factors are required for the specification of neuronal identity along the anteroposterior (AP) and dorsoventral (DV) axes, such as the Hox genes for AP, or the columnar genes for DV axis patterning. The results presented in this thesis analyse the expression and function of the Hox genes and the columnar gene ventral nervous system defective (vnd) during embryonic brain development of Drosophila. These results provide evidence that the Hox gene labial (lab) is required for the regionalized specification of the tritocerebral neuromere. Misexpression of posterior Hox genes in the embryonic neuroectoderm results in a lab loss-of function phenotype and a corresponding lack of Labial protein expression in the tritocerebrum. This is due to repression of labial gene transcription operating on a 3.65kb brain-specific lab-enhancer element. A functional analysis of Antennapedia and Ultrabithorax protein domains shows that the transcriptional repression of labial requires homeodomain-DNA interactions but is not dependent on a functional hexapeptide. The repressive activity of a Hox protein on labial expression in the tritocerebrum can, however, be abolished by concomitant misexpression of a Hox protein and the co-factors Homothorax (HTH) and nuclear-targeted Extradenticle (EXD) , suggesting that specification of tritocerebral neuronal identity requires equilibrated levels of a Hox protein and Hth and n-Exd cofactors. Moreover, evidence is presented that mutational inactivation of the columnar gene vnd results in regionalized axonal patterning defects which are similar to the brain phenotype caused by mutation of the Hox gene lab. However, in contrast to lab, vnd is required for precursor cell development and neuronal progeny maintenance during tritocerebral neuromere formation. In vnd mutant embryos, a subset of identified tritocerebral neuroblasts which normally express lab do not form. During later stages, programmed cell death leads to reduced or absent neuronal tissue which is normally specified by lab. The resulting vnd mutant brain phenotype is characterized by the lack of the tritocerebral neuromere, which can be rescued by targeted inactivation of the apoptotic program. Thus, in contrast to its DV patterning function in the VNC, vnd is required for AP patterning during embryonic brain development of Drosophila. These results indicate that the activity of the columnar gene vnd is integrated into pattern formation along the anteroposterior neuraxis by generating and maintaining cells which subsequently become specified by the activity of the Hox gene lab.

2. INTRODUCTION

2.1. Primary anteroposterior axis formation in the *Drosophila* embryo

Anteroposterior axis formation of *Drosophila melanogaster* occurs as early as in the developing oocyte controlled by the maternal genes which function at the top of a genetic hierarchy. Interactions among maternally encoded gene products lead to the graded expression of regulatory molecules in the embryo. Along the anteroposterior body axis, gap genes activate pair rule genes in repetitive patterns, which in turn act on the metameric expression of segment polarity genes (Pankraz, 1993). The combination of gap gene and pair rule gene products define the spatial domains of the homeotic selector genes which are necessary to provide correct segmental identity (Caroll, 1995; McGinnis and Krumlauf, 1992). Homologs of these genes have been found throughout the animal kingdom from basally assigned invertebrates to higher vertebrates, including man. It is now generally accepted that similar molecular circuits guide the formation of the basic body plan (Callerts et al., 1997; Caroll, 1995; McGinnis and Krumlauf, 1992). During Gastrulation the Drosophila embryo gets subdivided as all Triploblasts into the three germlayers, the mesoderm, endoderm and ectoderm. The ectoderm gets further subdivided into a neurectodermal part, which will contribute to the nervous system, and a non-neuronal part, which will mainly give rise to epidermal structures (Rusch and Levine, 1996). The maternally distribution of the NFkappaB family transcription factor Dorsal is initially provided throughout the cytoplasm of the developing oocyte where it becomes localized into nuclei shortly after fertilization. By activating and repressing zygotic genes in a concentration dependent manner along the dorsoventral axis Dorsal initiates the differentiation of three embryonic tissues, the mesoderm, neurectoderm and dorsal ectoderm

2.2. Development of the central nervous system (CNS)

2.2.1. Neurectoderm formation

Early patterning of the dorsoventral axis is a fundamental step for the formation of the ventral neurectoderm, but also for the establishment for the mesodermal germlayer and the ectoderm.

A combination of two signaling pathways has been assigned to play a major role in the dorsoventral patterning of the ventral neuroepithelium: first *dorsal* (*dl*) signaling being necessary for ventral mesoderm and neurectoderm formation, and second *decapentapletic* (*dpp*)/ *short gastrulation* (*sog*) signaling defining the dorsal border of the neurogenic region. At the ventral side of the embryo, the high nuclear Dorsal concentration induces expression of the mesodermal genes *twist* and *snail*, which in turn repress neurectoderm formation (Rushlow, 1989, Steward, 1989; and Roth, 1989). One of the genes which are regulated by high Dorsal concentration in the neurepithelium seems *short gastrulation* (*sog*). Conversely, at the dorsal side of the embryo, Dorsal acts in a context-dependent manner as a repressor which restricts the expression of genes like *dpp* to dorsal regions (Stathopoulos and Levine, 2002). *dpp* expression defines the dorsal border of the presumptive neurectoderm and also has an essential role in establishing dorsal embryonic tissues, such as the dorsal ectoderm and the amnioserosa, an extra-embryonic tissue. A marked expansion of the neurogenic ectoderm to the expense of dorsal ectoderm occurs in *dpp* mutants. Conversely, if *dpp* is ectopically expressed in more ventral regions it can induce dorsal structures and inhibits neurectoderm formation (Ferguson and Anderson, 1992; Wharton et al., 1993). *sog* is expressed in two broad lateral stripes and is activated by a distinct level of nuclear Dorsal concentration along its graded distribution. SOG is a secreted protein and its initial expression domain seems to coincide with the limits of the presumptive neurogenic ectoderm, at the ventral side. The morphogenic gradient of SOG antagonizes the dorsalization factor DPP, thereby preventing the neurectoderm to become dorsal epidermis (Ferguson, 1996). Loss of *sog* function in turn results in a reduction of the neurectoderm and a expansion of the dorsal epidermis (Holley et al., 1995; Biehs et al., 1996).

The molecular interplay between DPP/BMP4 and SOG/Chordin represents an evolutionary conserved mechanism in neurectoderm formation which was not only described in insects but also in vertebrates. The two groups of interacting signaling molecules, DPP/BMP-4 and SOG/Chordin act from opposing dorsoventral poles in both insects and vertebrate embryos (Holley et al., 1995). Interestingly, in *Drosophila* DPP, exerts its activity on dorsal cells and SOG on ventral cells, whereas in vertebrates, BMP-4 acts on ventral cells and Chordin activity is found in dorsal cells. In both cases it is the region of the embryo that attains neurogenic potential and forms the neuroepithelim in which SOG/Chordin is expressed and inhibits the action of invading DPP/BMP-4 signals. This functional conservation of the

SOG/Chordin and the DPP/BMP-4 morphogens suggests an evolutionarily conserved, homologous mechanism of dorsoventral patterning.

2.2.2. Formation of columnar domains

In addition to the signaling system, which initially induces neurogenic potential, a further set of genetic elements involved in early dorsoventral patterning of the CNS appears to be evolutionarily conserved (Chan and Jan, 1999; Cornell and Ohlen, 2000). These genetic regulatory elements are three sets of homeobox genes that control the formation of columnar dorsoventral domains in the ventral neurectoderm of *Drosophila*; their homologues may act in a similar fashion in dorsoventral patterning in the neural plate of vertebrates. In *Drosophila*, the homeobox genes *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*) and *muscle specific homeobox* (*msh*) and they are expressed in longitudinal stripes along the ventral (*vnd*), intermediate (*ind*) and dorsal (*msh*) columns in the neurectoderm (Isshiki et al., 1997; McDonald et al., 1998; Chu et al., 1998; Weiss et al., 1998). In each column, expression of the appropriate homeobox gene is required for neuroblast formation and/or for the specification of columnar identity. Comparable expression patterns have recently been reported for the beetle *Tribolium* (Wheeler et al., 2005). In the developing neural plate of vertebrates, the homologous genes of the *Nkx2* (*vnd*), *Gsh* (*ind*) and *Msx* (*msh*) families are similarly involved in dorsoventral patterning (Invagination of the vertebrate neural plate to form the neural tube results in translocation of lateromedial into dorsoventral position.). In vertebrates, several *Nkx* family members are expressed in ventral regions of the neural tube and at least one of these is expressed earlier in the corresponding medial region of the neural plate (Qiu et al., 1998; Pera et al., 1998; Pabst et al., 1998; Shimamura et al., 1995). Similarly, expression of vertebrate *Msx* family members is seen in the lateral neural plate, which later forms the dorsal neural tube (Wang et al., 1996). Finally, vertebrate *Gsh* family genes are expressed at dorsoventrally intermediate levels in the neural tube (Valerius et al., 1995; Hsieh-Li et al., 1995). Functional studies suggest that some of these genes are involved in controlling regional identity along the dorsoventral axis of the neural tube (Briscoe et al., 1999; Sussel et al., 1999). In addition, *Epidermal growth factor receptor* (*Egfr*) signaling in *Drosophila* is crucial for ventral and intermediate neurectoderm specification. Active *Egfr* signaling occurs in the medial and intermediate columns prior to the first wave of NB formation and persists in the medial column throughout neurogenesis.

Egfr activates *ind* expression in the intermediate column, whereas in the medial column *Egfr* signaling in combination with *vnd* acts in neuroblast formation (Yagi et al., 1998; Skeath, 1998). In the lateral column proper EGFR signaling is required to specify the expression boundary of *msh* (D'Alessio and Frasch, 1996). Cells of the ventral midline provide extrinsic positional information via *Egfr* signaling that maintains the initial subdivision of the ventral neurectoderm into three dorsoventral columns during early neurogenesis (Kim et al., 2005). Furthermore, there seems to be a genetic hierarchy of transcriptional repression among columnar genes in that the more ventral genes repress the more dorsal ones in the domain where they are expressed (Weiss et al., 1998).

2.2.3. Neuroblast formation

In the development of the CNS of *Drosophila*, the first visible sign of neurogenesis is delamination of stem-cell like neuronal progenitors, termed neuroblasts (NB) from the neurectoderm. NBs of the ventral nerve cord (VNC) delaminate in a highly stereotyped manner (Doe, 1992). It is generally accepted that an individual NB acquires a singular fate based on both the time and the exact location it forms. Genetic studies have provided evidence that a small number of proneural genes, which encode basic helix-loop-helix (bHLH) transcription factors are necessary and sufficient to initiate neural differentiation in the neurepithelium. Molecular studies identified four proneural genes belonging to the acheate-scute complex (ASC), namely *acheate* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) (Ghysen and Dambly-Chaudiere, 1989; Campuzano and Modolell, 1992). More recently a further proneural gene, *atonal* (*ato*) was isolated, which together with two other *ato* related genes (*amos* and *cato*), comprises the *ato* family (Bertrand et al., 2002). By interactions among cells that make up proneural equivalence groups of five to six cells, single cells are selected to acquire the NB cell fate. This is achieved by the process of lateral inhibition and is based on a molecular regulatory loop between adjacent cells. As a result, proneural genes inhibit their own expression in adjacent cells thereby preventing these neighboring cells from adopting a neuroblast fate (Skeath and Carroll, 1994). Lateral inhibition is mediated through *Delta/Notch* signaling by its ability to repress expression of the proneural genes. Notch signaling is initiated by Delta binding to Notch on apposing cells (Artavanis-Tsakonas, et al., 1999; Kopan, 2002). The Delta/Notch interaction leads to a series

of intramembranous cleavages of Notch, which result in the nuclear translocation of the Notch intracellular domain, $Notch^{intra}$. In the nucleus, $Notch^{intra}$ interacts with Su(H) and Mastermind, a complex that activates transcription of the Enhancer of Split complex genes. These genes encode bHLH transcriptional repressors that directly down-regulate expression of the proneural genes directly.

2.2.4 Primary lineage formation

Studies of NB delamination and primary lineage formation have so far largely been concentrating on the relatively simple VNC. The genetic program that distinguishes NBs is already evident in specific gene expression patterns in the proneural clusters. The selected NB enlarges and delaminates into the interior of the embryo, whereas remaining cells of each proneural cluster either retain an undifferentiated state or adopt an alternative epidermal fate. Subsequent to delamination, each NB begins to divide asymmetrically in a stem cell-like manner along the apical-basal axis. In each division the NB renews itself and buds off a smaller daughter cell, the ganglion mother cell (GMC). NB and GMC have different gene expression profiles. Neural precursor genes such as *ase* and *deadpean* (*dpn*) are expressed in NBs but are repressed in GMCs (Bier et al., 1992; Brand et al., 1993), whereas genes such as *even-skipped* (*eve*) and *fushi tarazu* (*ftz*) are expressed in GMCs, where they may help to confer GMC identity (Doe et al., 1988a; Doe et al., 1988b). The two best characterized cell fate determinants in the GMC are *prospero* (*pros*) and *Numb*. The Prospero transcription factor is transcribed and translated in the NB but the mRNA and the protein are asymmetrically distributed and inherited only by the GMC (Knoblich et al., 1995). Nuclear Prospero activates GMC-specific gene expression and represses NB-specific genes (Buescher et al., 1998; Skeath and Doe, 1998). Subsequently, the GMC divides once more to produce two postmitotic neurons that start their terminal differentiation program and are characterised by differentiation marker gene expression, such as *embryonic lethal, abnormal vision* (*elav*).

In the early embryonic brain, studies on the procephalic NB pattern, which applied morphological criteria in wholemount embryos uncovered a population of 70–80 brain NBs per hemisphere (Hartenstein and Campos-Ortega, 1984). Based on the expression of the molecular markers *l'sc*, *ase* and *seven-up* (*svp*), these were subdivided into 23 groups of one to five NBs each (Younossi-Hartenstein et al., 1996). Recently, using a different preparation

technique, the development of the brain NB pattern has been described at higher resolution at the level of individually identified NBs. About 100 brain NBs were identified, and, based on their segmental assignment and positional relationships, they were subjected to a new systematic nomenclature (Urbach et al., 2003). This NB map presumably represents the complete population of embryonic brain NBs. Conversely the VNC displays a much simpler NB delamination pattern (Doe, 1992). Each NB acquires a unique fate based on where and when it forms, giving rise to a stereotyped primary lineage (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). Genetic and cell transplantation analyses indicate that NBs inherit their identity from the cluster of neurectodermal cells from which they delaminate (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Udolph et al., 1995).

A novel set of genes has recently been assigned to provide temporal identity to the NB, in addition to the spatial identity. These observations give rise to a model where the temporal expression of *Hb*, *Kr*, *Pdm1*, *Cas*, *Grh* transcription factors specify the sequentially generated offspring of defined NB lineages. Loss- and gain-of function experiments suggest extensive cross-regulation among these transcription factors such that the earlier expressed transcription factor activates the next gene in the pathway and concomitantly represses the "next plus one" gene (Isshiki et al, 2001). The precise timing of HB -> KR -> PDM1 -> CAS expression in the NB is critical for proper CNS development. In an early phase after delamination, the NBs express the transcription factors encoding the genes *hunchback* (*hb*) and *Krüppel* (*Kr*), which seem to be necessary and sufficient for specification of the early generated progeny. When *hb* or *Kr* is misexpressed later during the NB lineage, presumptive later-born neurons acquire markers and morphology of early-born neurons (Isshiki et al., 2001; Novotny et al., 2002). During a brief window following the normal down-regulation of HB, the neuroblast remains competent to respond to a pulse of HB by making extra early-born neurons. However, as the HB pulse is given progressively later, the neuroblast gradually loses competence to respond Therefore the NB is progressively restricted in its ability to respond to HB (Pearson and Doe, 2003). Misexpression experiments show that HB and KR can activate the next gene in the series, raising the possibility of a positive transcriptional cascade; however, *hb* or *Kr* mutants have little effect on the timing of later gene expression (Issiki et al., 2001). Therefore the simple model of a linear positive transcriptional cascade must be ruled out. Instead, it has been proposed that there is an independent "temporal identity timer" that regulates HB -> KR -> PDM1 -> CAS expression in neuroblasts (Isshiki et al., 2001).

2.3. The embryonic brain of *Drosophila*

The embryonic brain of Drosophila consists of an anterior supraoesophageal ganglion and a posterior suboesophageal ganglion. The supraoesophageal ganglion develops from the procephalic neurectoderm, therefore also referred to as the procephalic brain. The supraoesophageal ganglion consists of three neuromeric structures: the protocerebrum, the deuterocerebrum and the tritocerebrum; it is often also termed "brain". The suboesophageal ganglion develops from the anteriormost part of the ventral neurectoderm, more precisely from the neurectoderm which can be assigned to the gnathal segments; therefore it is also named as gnathal brain and displays a number of similarities to the VNC. It consists of three neuromeres: the mandibular neuromere, the maxillary neuromere and the labial neuromere. The NBs from the procephalic neurectodermal domain, which can be assigned to individual head segments, were subdivided and assigned to the neuromeres of the supraoesophageal ganglion (Urbach et al., 2003). The intercalary segment gives rise to the tritocerebral NBs, whereas the antennal segment gives rise to deuterocerebral NBs. It has been proposed that protocerebral NBs can be assigned to the ocular and labral segments. The exact segmental subdivision of the pregnathal neurectoderm is still largely under debate (Urbach and Technau, 2003; Urbach and Technau 2004).

Subsequently when all NBs have delaminated, a relatively simple primary axon scaffold of commissural and descending pathways is established by a small number of pioneering axons. This initial set of *Fasciclin II* (*FasII*) expressing axon is used for guidance and fasciculation by later outgrowing axons. Thereby a grid of midline-crossing commissures and longitudinal connectives is set up in the embryonic brain and VNC. In the supraoesophageal ganglion at the level of the protocerebrum and tritocerebrum, the two bilaterally symmetric hemispheres are interconnected by the prominent preoral commissure (also protocerebral commissure), and by the tritocerebral commissure, respectively. In the posterior brain and VNC each hemi-neuromere is interconnected by one or two transverse commissures (Therianos et al.1995; Nassiv et al., 1998). Segmental boundaries in the embryonic brain are defined by the marked expression of *engrailed* (*en*) demarcating the posteriormost cells in each neuromere, as the protocerebral *en*-b1, the deuterocerebral *en*-b2, and the tritocerebral *en*-b3 stripes (Hirth et al., 1995).

2.4. Anteroposterior patterning of the embryonic brain

Cephalic gap genes, for example *orthodenticle* (*otd*) and *empty spiracles* (*ems*), have been implicated to be essential for proper embryonic brain development (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). The earliest expression of these genes is observed in the blastodermal stage in circumferential stripes at the anterior pole of the embryo; these domains include the head anlagen of several head segments as judged by blastoderm fate mapping (Hartmann and Reichert, 1998). Mutations in the cephalic gap genes lead to specific deletions of the embryonic brain indicating that these genes are required in early patterning and specification of the anterior brain anlage. For example, *otd* mutants show that otd plays a key role in the establishment of the anterior brain. In homozygous otd mutant embryos most of the protocerebral and deuterocerebral anlage is deleted. Comparably in *ems* mutants large parts of the deuterocerebral and tritocerebral anlage are deleted (Hirth et al., 1995).

The homeotic or Hox genes were originally discovered in *Drosophila* through the homeotic transformation that resulted from their mutation and subsequently were found in a variety of metazoan organisms (reviewed in Lewis, 1978; McGinnis and Krumlauf, 1992). In *Drosophila*, the homeotic genes are arranged in one cluster, but map to the separated Antennapedia (ANT-C) and Bithorax (BX-C) complexes, which are collectively referred to as the Homeotic complex (HOM-C). The ANT-C includes the genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*). The BX-C contains the genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Akam, 1989). The homeotic genes show a spatial co-linearity in their chromosomal arrangement and their expression patterns, in that more 3' located genes are expressed more anteriorly along the body axis of the embryo, whereas more 5' located genes are expressed more posteriorly. Furthermore, there appears to be a functional hierarchy among *Hox* gene products in that more posteriorly expressed *Hox* genes are functionally dominant over more anteriorly expressed *Hox* genes; a phenomenon termed "posterior prevalence" (Duboule and Morata, 1994).

In the embryonic CNS of *Drosophila*, homeotic gene expression is not observed in the most anterior regions where *orthodenticle* (*otd*) and *empty spiracles* (*ems*) and other cephalic gap genes are required for the formation of the supraoesophageal neuromere (Finkelstein et al., 1990; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). The homeotic gene with the most defined anterior expression domain in the embryonic brain is *lab*, which is expressed in

the posterior tritocerebrum. *lab* expression is followed by non-overlapping domains of *Dfd*, *Scr* and *Antp* expression in the mandibular, maxillary and labial neuromeres, respectively (Fig.4). The BX-C genes are expressed in the more posterior thoracic and abdominal neuromeres (Hirth et al., 1998). Neuroanatomical analyses have shown that loss-of-function mutations for two *Hox* genes, *lab* and *Dfd,* result in severe defects in the embryonic brain. In *lab* null mutants, the neural progenitor cells that give rise to the tritocerebrum are present in the mutant domain and express neuroblast markers, such as *hunchback* (*hb*) and *ase*. Similarly, cells that have the characteristic position of GMCs and express *pros* as well as their postmitotic progeny are correctly positioned in the tritocerebral mutant domain. However, their postmitotic progeny does not express the neuron-specific markers that positionally equivalent neuronal cells express in the wildtype and these cells do not extend axons or dendrites and are not contacted by axons from other parts of the brain. In conclusion, the *lab* mutant cells fail to adopt a neuronal identity and seem to remain in an undifferentiated yet postmitotic state. This results in severe cell-autonomous and cell-non-autonomous axonal patterning defects, including loss of the tritocerebral commissure and reduced or absent longitudinal axonal pathways. Interestingly, glia cell differentiation appears to be unaffected in the mutant domain since the number of REPO-positive cells is similar compared to the wildtype. It also indicates that the *lab* mutant cells have not acquired a glial identity. This suggests that the *lab* gene is necessary for the establishment of correct neuronal cell fate, but not glia cell fate, in the part of the developing brain giving rise to the posterior tritocerebrum (Hirth et al., 1998).

It is likely that transcription factors such as LAB mediate neuronal identity by regulating a battery of downstream genes, which are involved in cell adhesion, cell cycle regulation, and cell differentiation. Using genome-wide oligonucleotide arrays a large number of genes, which are potentially involved in the genetic network downstream of *lab,* have been identified (Leemans et al., 2001). Genetic rescue experiments of all of the *Drosophila* Hox genes in their potential to specify the neuronal identity in the tritocerebral neuromere showed that the *lab* mutant brain phenotype can be rescued by targeted expression of the LAB protein under the control of CNS-specific *lab* regulatory elements. Most of the other *Drosophila* Hox gene products are also able to replace the LAB protein in the specification of the tritocerebral neuromere, with the exception of the Abdominal-B protein, which does not efficiently rescue the *lab* mutant phenotype in the brain. For the other Hox

proteins a correlation between their efficiency of rescue the *lab* mutant brain phenotype and the chromosomal arrangement of their encoding loci was described (Hirth et al., 2001). Most Hox proteins are functionally equivalent in their ability to replace LAB in the specification of neuronal identity in the brain, therefore Hox gene action in brain development may mainly rely on *cis* acting regulatory elements and not on Hox protein specificity. Further support for the hypothesis of replaceability of Hox gene products in tritocerebral development comes from the functional analysis of the *Drosophila* Hox cofactors *homothorax* (*hth*) and *extradenicle* (*exd*) in embryonic brain development (Nagao et al., 2001). The homeodomain proteins EXD and its mammalian homologues *Pbx* contributes to Hox protein specificity by cooperatively binding to DNA together with HOX proteins. For example, the Hox protein LAB cooperatively binds with the EXD protein to a 20 base pairs sequence that is sufficient to direct a *labial*-like expression pattern in *Drosophila* embryos (Chan and Mann, 1996). *hth* was previously shown to be indirectly required for Hox function because, in *hth* mutant embryos, EXD is found exclusively in the cytoplasm, and therefore cannot act as a Hox cofactor (Rieckhof et al., 1997; Kurant et al., 1998). *Hth* encodes a homeodomain protein that has very similar relatives in vertebrates called the MEIS and PREP proteins (Moskow et al., 1995; Nakamura et al., 1996; Steelman et al., 1997). HTH and EXD proteins directly interact with each other, and the nuclear localization of EXD depends on this protein-protein interaction (Rieckhof et al., 1997; Abu-Shaar et al., 1999; Berthelsen et al., 1999).Therefore it has been suggested that, for many Hox target genes, Hox proteins bind to DNA as a HTH/Hox/EXD trimeric complex. Indeed, in addition to importing EXD into nuclei, HTH is part of an essential DNA-bound HTH/Hox/EXD trimeric complex (Ryoo et al., 1999).

In addition to their homeotic regulatory functions in trunk development, *exd* and *hth* have important functions in patterning the primary axonal scaffolds and primary lineages in the developing brain. *exd* and *hth* genes are co-expressed in many of the neurons of the fiber tract founder clusters, suggesting that the activities of these genes are intrinsically required for axonal programming of the tract founder cluster neurons. Mutations in the *exd* and *hth* genes result in gross anatomical defects in the developing brain, such as abnormal positioning of the preoral commissure and an alteration of molecular neuroanatomical marker expression. The anterior HOM-C genes *lab*, *Dfd* and *Scr* are significantly suppressed. *exd* and *hth* mutation leads to loss of neuronal structures, including the embryonic tritocerebral neuromere, suggesting a combinatorial influence of Hox proteins and Hox-cofactors (Nagao et al., 2001).

Therefore it can be assumed that Hox proteins and their co-factor interact together to specify tritocerebral identity. Indeed, the aminoacid residues which are necessary for the interaction of a Hox protein and its partner EXD, termed hexapeptide or YPWM motif, seems to be essential for a Hox protein for proper tritocerebral development. The Abdominal-B protein, which does not efficiently rescue the *lab* mutant phenotype in the brain, does not contain a functional hexapeptide (Hirth et al., 2001). This may indicate that in embryonic brain development, the differences of homeotic gene action mainly rely on cis-acting regulatory elements, and not Hox protein specificity. In addition it indicates that the combination of Hox protein and their co-factors might be essential for proper neuronal development.

2.5. Dorsoventral patterning of the embryonic brain

The columnar genes *vnd*, *ind* and *msh* are expressed in specific domains in the developing ventral neurectoderm and subsequently in delaminating NBs of the VNC in a ventral to dorsal order. A recent study shows that columnar genes are also expressed in the procephalic neuroectoderm and in subsets of neuroblasts in the developing brain. (Urbach et al., 2003). The typical column-like expression domains observed in the VNC are also observed in the developing tritocerebral neuromere, but become obscure towards more anterior sites. The anterior extent of expression is specific of *msh* and *vnd*: *msh* is confined to more posterior regions, and *vnd* expression extends into anterior regions of the brain. Surprisingly, the DV patterning genes *vnd* and *msh* endorse a separation of brain neuromeres along the AP axis. *vnd* expression demarcates the ventral part of the posterior border of the tritocerebrum, deutocerebrum and protocerebral neuromere, and *msh* demarcates the dorsal anterior border of the deutocerebrum. Integrated expression data of columnar patterning genes and pair-rule genes, such as *hedgehog* and *wingless*, have provided a grid for neuromeric subdivision of the neurectoderm and the early brain (Urbach and Technau, 2003; Urbach and Technau, 2004). The specific expression of columnar patterning genes, which are involved in the process of neuronal development at different levels in the ventral neurectoderm lead to speculate, that these genes might also play a role in embryonic brain development. Despite the detailed knowledge of columnar gene expression during early procephalic neurectoderm and brain neuroblast formation, nothing is known about the later expression or the function of these genes during embryonic brain development of *Drosophila*.

2.5 This Thesis

The molecular mechanisms that integrate anteroposterior and dorsoventral positional information in the developing nervous system remain elusive. An increasing number of conserved patterning genes are being described to act at different levels during embryonic CNS development.

In the first part of this thesis, results are presented that further investigate the role of Hox genes in tritocerebral development using a gain-of-function approach, thereby analyzing the molecular and genetic basis of cross-regulatory interactions between *lab* and other more posterior Hox genes. Misexpression of posterior Hox genes in the embryonic neurectoderm results in a *lab* loss-of function phenotype, due to repression of *lab* gene transcription. These results suggest that equilibrated levels of a Hox protein and HTH and n-EXD cofactors are required for the specification of tritocerebral neuronal identity.

In the second part of this thesis, results are presented that further investigate the role of the dorsoventral patterning gene *vnd* in embryonic brain development. We describe its expression and mutant phenotype during different stages of brain development. *vnd* mutants display a severe loss of neuronal tissue together with axonal patterning defects in the tritocerebrum; this loss of neuronal tissue is associated with increased apoptotic activity. VND is required for the formation and maintenance of neuroblasts as well as neuronal progeny, whereas the Hox gene *lab* appears to be independently required for the specification of neuronal identity within the same territory during later stages. This indicates that the activity of the DV columnar gene *vnd* is integrated into pattern formation along the anteroposterior neuraxis by generating and maintaining cells which subsequently become specified by the activity of the Hox gene *lab.*

Hox gene cross-regulatory interactions in the embryonic brain of *Drosophila*

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ABSTRACT

During embryonic development of the *Drosophila* brain, the Hox gene *labial* is required for the regionalized specification of the tritocerebral neuromere. In order to gain further insight into the mechanisms of Hox gene action in the CNS, we have studied the molecular and genetic basis of cross-regulatory interactions between *labial* and other more posterior Hox genes using the GAL4-UAS system for targeted misexpression. Misexpression of posterior Hox genes in the embryonic neuroectoderm results in a *labial* loss-of function phenotype and a corresponding lack of Labial protein expression in the tritocerebrum. This is due to repression of *labial* gene transcription in the embryonic brain. Enhancer analysis suggests that this transcriptional repression operates on a 3.65kb brain-specific *labial*-enhancer element. A functional analysis of Antennapedia and Ultrabithorax protein domains shows that the transcriptional repression of *labial* requires homeodomain-DNA interactions but is not dependent on a functional hexapeptide. The repressive activity of a Hox protein on *labial* expression in the tritocerebrum can, however, be abolished by concomitant misexpression of a Hox protein and the cofactors Homothorax and nuclear-targeted Extradenticle. Taken together, these results provide novel and detailed insight into the cross-regulatory interactions of Hox genes in embryonic brain development and suggest that specification of tritocerebral neuronal identity requires equilibrated levels of a Hox protein and Hth and n-Exd cofactors.

Key words: *Drosophila*; Embryo; Brain development; Neurogenesis; Hox genes; *labial*; *Ultrabithorax*; Gal4/UAS; Gain of function, Cross-regulation.

INTRODUCTION

The homeotic or Hox genes encode a network of conserved transcription factors that are involved in specifying segmental identity along the anteroposterior body axis of animals as diverse as insects and vertebrates (McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Carroll, 1995). Their functional role in insect development has been studied in detail in *Drosophila*, where the genes are arranged along the chromosome in two gene clusters known as the Antennapedia and Bithorax complexes. There is a correlation between the relative position of the Hox genes in the clusters and their spatial and temporal expression patterns in the embryo in that genes located towards the 3' end of their complexes are expressed more anteriorly and earlier than genes towards the 5' end; this is referred to as spatial and temporal colinearity. Furthermore, Hox genes have been shown to interact both genetically and molecularly, and the term posterior dominance has been proposed to describe the cross-regulation of these genes and the phenotypic consequences of their expression (Duboule and Morata, 1994; Graba et al., 1997; Mann and Morata, 2000). Hox gene transcription factors often bind to DNA as a heterodimer with another homeodomain protein encoded by the *extradenticle* (*exd*) gene. When the Exd cofactor binds together with Hox proteins, it increases their DNA binding specificity and affinity, and also modifies their transcriptional regulatory properties (Mann and Chan, 1996; Pinsonneault et al., 1997). A further homeodomain protein that is thought to interact with Exd/Hox heterodimers is encoded by the *homothorax (hth)* gene. Hth and Exd proteins directly interact with each other and the nuclear localization of Exd depends on this interaction (Mann and Affolter, 1998; Ryoo et al., 1999).

In the developing central nervous system (CNS) of *Drosophila*, Hox genes are expressed in an anteroposterior ordered set of domains, and in the embryonic brain, specific Hox genes are expressed in the tritocerebrum, the posterior neuromere of the supraesophageal ganglion, and in the three subesophageal neuromeres (reviewed in Hirth and Reichert, 1999). Initial loss- and gain-of-function studies revealed that cross-regulatory interactions among Hox genes also occur in the developing CNS. Thus, a regulatory hierarchy of transcriptional repression appears to act on the genes *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*), in that Hox genes expressed more posteriorly act as negative regulators of Hox genes that are expressed in more anterior regions of the embryonic CNS (reviewed in Doe and Scott, 1988). However a lack of

phenotypic consequences challenged the view that these interactions appear to play a role in determining segmental identity in the embryonic CNS of *Drosophila*.

Subsequent detailed loss-of-function analyses showed that only the Hox genes *labial* (*lab*) and *Deformed* (*Dfd*) are involved in regionalization of the embryonic brain, whereas mutations in other Hox genes do not lead to any obvious defects during this process of brain development (Hirth et al., 1998). Thus, *lab* null mutants show marked defects in the tritocerebral neuromere where Lab is normally expressed. In these mutants, the tritocerebral commissure is missing and the longitudinal connectives that interconnect the tritocerebrum with posterior parts of the brain, are absent or reduced. Moreover, the cells in the *lab* mutant domain do not acquire a neuronal identity indicating that *lab* is required for the specification of neuronal identity in the tritocerebrum (Hirth et al., 1998). In addition, mutational inactivation of *exd* or *hth* encoded cofactors correlate with the absence of *lab* expression (Nagao et al., 2000), implying that interactions between *lab* and these two cofactors occur in the developing tritocerebrum.

More recently, misexpression studies using the Gal4/UAS-system (Brand and Perrimon, 1993) revealed that ectopic Hox genes repress only *lab* and *Sex combs reduced* (*Scr*) in the CNS in a timing dependent manner (Miller et al., 2001), suggesting that expression of these genes in the developing CNS is subject to posterior dominance cross-regulatory interactions. Since *lab* (in contrast to *Scr*) appears to be involved in the specification of tritocerebral neuronal identity, an investigation of these interactions presents an excellent opportunity to analyse the mechanisms underlying cross regulatory interactions during embryonic brain development of *Drosophila*.

In this report we analyse the molecular and genetic basis of cross-regulatory interactions between *lab* and other more posterior Hox genes using the GAL4-UAS system (Brand and Perrimon, 1993) for targeted misexpression of Hox genes. We find that misexpression of posterior Hox genes such as *Antp, Ubx* and *abd-A* results in a *lab* loss-of function phenotype in the developing tritocerebrum, and that this is correlated with a lack of Lab protein expression in the tritocerebrum. Moreover, we show that this lack of Lab protein is due to transcriptional repression of the *lab* gene in the embryonic brain during the time period at

which Lab is normally required to specify tritocerebral identity. A functional analysis of protein domains involved in Hox specificity shows that transcriptional repression of *lab* requires homeodomain-DNA interactions. Moreover, the repressive activity that underlies this posterior dominance effect on *lab* expression can be abolished by the concomitant targeted misexpression of a Hox gene and the cofactors Hth and nuclear-targeted Exd (n-Exd).

EXPERIMENTAL PROCEDURES

Fly strains and genetics

The Yeast Gal4 transcriptional activation system (Brand and Perrimon, 1993) was utilized in order to ectopically express UAS/Gal4 responder constructs at various time points during central nervous system development of *Drosophila*. This was accomplished with three different Gal4 driver lines: *sca::Gal4* (Klaes et al., 1994), an enhancer trap line which expressed Gal4 during neuroectoderm specification and neuroblast formation; *1407::Gal4* (Broadie et al., 1995. courtesy of J. Urban), an enhancer trap line that expresses Gal4 during neuroblast and ganglion mother cell formation; and *C155 elav::Gal4* (Lin and Goodman 1994), an enhancer trap line that expresses Gal4 in postmitotic neurons.

For targeted ectopic expression of Hox genes, the following responder lines were used: *P(w^{+mC}, UAS::lab) 2.4a* (Miller et al., 2001); *P(w^{+}, UAS::pb) 49.1* (Aplin and Kaufman, 1997); *P(w^{+mC}, UAS::Dfd) pC41* (Brown et al.,1999); *P(w^{+mC}, UAS::Scr) EE2* (Miller et al., 2001); *P(w^{+mC}, UAS::Antp) W2* (Miller et al., 2001); *P(w^{+mC}, UAS::Ubx) M2A* (Ia isoform of Ubx) (Miller et al., 2001); *P{w^{+mC}=UAS-Ubx.Ia.C}36.2* (supplied by M. Akam); *P(w^{+}, UAS::abdA) 21.6* (Greig and Akam, 1993); *w^{1118}; P(w^{+}, UAS::AbdBm) 1.1 c23* (morphogenetic isoform of AbdB) (Castelli-Gair et al., 1994). In addition, we used the following UAS responder lines: *P(w^{+mC}, UAS::δAntp)* (Plaza et al., 2001); *P(w^{+mC}, UAS::Antp.A50,51)* (Plaza et al., 2001); *P(w^{+mC}, UAS::Antp.Q50K.C)* (Capovilla et al., 2001); *P(w^{+mC}, UAS::Ubx YAAA)* (Galant et al., 2002); *P(UAS::hth)* (Pai et al., 1998); *P(UAS::FLAG::NLS::EXD)* (Jaw et al., 2000). *UAS::responder* transgene activity was confirmed by immunoreactivity.

For a detailed analysis and comparison of the tritocerebral phenotype resulting in *sca::Gal4xUAS::Hox* lines, we used the *lab*^{vd1} null mutant allele (Merrill et al., 1989; Hirth et al., 1998), balanced over TM3, Ubx-lacZ. Homozygous null mutants were identified by the absence of Ubx-lacZ. To identify former *lab* expressing tritocerebral cells in the *sca::Gal4xUAS::Hox* background, we used line P{w^{+} 3.65 *lab-lacZ*} (Chouinard and Kaufman, 1996; Hirth et al., 2001). P{w^{+} 3.65 *lab-lacZ*} shows nuclear distribution of *βgal* and reflects endogenous *lab* expression in the embryonic head ectoderm, tritocerebrum, and posterior midgut.

In order to rescue the tritocerebral brain phenotype obtained by *sca::Gal4xUAS::Hox*, the following genotypes were generated and analysed:

sca::Gal4/+; P(UAS::hth), P{w^{+mC}=UAS-Ubx.Ia.C}36.2
sca::Gal4/ P(w^{+mC}, UAS::Ubx) M2A; P(UAS::FLAG::NLS::EXD)/+
sca::Gal4/+; P(UAS::hth), P{w^{+mC}=UAS-Ubx.Ia.C}36.2/ P(UAS::FLAG-NLS-EXD)
sca::Gal4/+; P(w^{+mC}, UAS::Ubx YAAA)/+; P(UAS::FLAG-NLS-EXD), P(UAS::hth),

UAS::responder transgene activity for these genotypes was confirmed by immunoreactivity, except for the recombinant chromosomes *P(UAS::hth), P{w^{+mC}=UAS-Ubx.Ia.C}36.2 and P(UAS::FLAG-NLS-EXD), P(UAS::hth),* where immunoreactivity was carried out only for one of the designated UAS responders (either Hth or Ubx, or either Exd or Hth). All experiments reported here were carried out at 25°C; no significant differences were obtained when experiments were carried out at 28°C. Embryos were staged according to Campos-Ortega and Hartenstein (1997).

Immunocytochemistry

Embryos were dechorionated, fixed and labeled according to Therianos et al. (1995). Primary antibodies were rabbit anti-HRP (FITC-conjugated) 1:100 (Jan and Jan, 1982) (Jackson Immunoresearch), rabbit anti-LAB (F. Hirth and H. Reichert, unpublished) at 1:100, rat anti-LAB (F. Hirth and H. Reichert, unpublished) at 1:500, rabbit anti-PB (Pultz et al., 1988) at 1:200, guinea pig anti-DFD (Kuziora and McGinnis, 1988) at 1:200, rabbit anti-SCR

(LeMotte et al., 1989) at 1:200; mouse anti-ANTP 1:100 (Condie et al., 1991), mouse anti-UBX 1:5 and mouse anti-ABD-A 1:100 (A. Macias and G. Morata, unpublished), mouse anti-ABD-B 1:1 (Celniker et al., 1989), rabbit anti-βGAL 1:400 (Milan Analytika), mouse anti-βGAL 1:100 (DSHB), mouse anti-Fasciclin II 1:5 (Lin and Goodman, 1994), rat anti-ELAV 1:30 (DSHB), mouse anti-REPO 1:20 (DSHB), rabbit anti-HTH (Pai et al., 1998) at 1:200, and monoclonal mouse anti-EXD at 1:2 (Aspland and White, 1997). As secondary antibodies we used the respective Alexa-488, Alexa-568, and Alexa-647 antibodies generated in goat (Molecular probes), all 1:150. Embryos were mounted in Vectashield H-1000 (Vector).

Laser confocal microscopy

For laser confocal microscopy, a Leica TCS SP was used. Optical sections ranged from 0.4 to 2 μm recorded in line average mode with picture size of 512 x 512 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Figures were arranged and labeled using Adobe Photoshop and Power Point.

RESULTS

To reveal potential cross-regulatory effects of posterior Hox genes on *lab* expression in the embryonic brain, the GAL4-UAS system was used for targeted misexpression of Hox proteins during embryonic development. Three separate neuronal lineage-specific Gal4 drivers with distinct spatial and temporal expression patterns were used to stimulate transcription from the various Hox responders in the developing embryonic CNS. These were *sca::*Gal4 (Klaes et al., 1994), which expresses Gal4 during neuroectoderm specification and neuroblast formation; *1407::*Gal4 (Broadie et al., 1995), which expresses Gal4 during neuroblast and ganglion mother cell formation, and *C155 elav::*Gal4 (Lin and Goodman 1994), which expresses Gal4 in postmitotic neurons (figure 1).

Targeted misexpression of Hox genes using the *1407::*Gal4 driver or the *C155 elav::*Gal4 driver did not result in an obvious mutant phenotype in the developing tritocerebrum. In contrast, misexpression of Hox genes using the *sca::*Gal4 driver resulted in a robust and

reproducible mutant phenotype in the embryonic tritocerebrum. This indicates that early, neuroectoderm-specific misexpression of Hox genes, but not later misexpression of Hox genes in neuroblasts, ganglion mother cells or differentiated neurons, leads to the mutant brain phenotype. The mutant phenotype caused by early Hox gene misexpression was observed at high penetrance for all of the Hox genes with the exception of the *lab* gene. In terms of mutant phenotype penetrance, all Bithorax-complex genes had values above 95% ($n > 60$), whereas all Antp-complex genes (excepting *lab*) had values of 70-80% ($n > 60$), and *lab* had a value of 25% ($n = 75$) (data not shown). The mutant phenotype caused by early Hox gene misexpression is shown in figure 2 exemplary for targeted misexpression of the *Ubx* gene.

A detailed analysis of the mutant phenotype produced in the developing tritocerebrum by misexpression of posterior Hox genes using the *sca::*Gal4 driver revealed defects that phenocopied the *lab* loss-of-function mutation (figure 3). Thus, marked defects in axonal patterning associated with the tritocerebral neuromere were seen which were identical to those found in *lab* null mutants (Hirth et al., 1998). The longitudinal connectives that normally run through this neuromere were missing or reduced and the tritocerebral commissure which interconnects the brain hemispheres at the level of the tritocerebrum was completely absent. Also, the frontal connectives no longer projected into the tritocerebral neuromere but rather grew ectopically into the more anterior brain neuromeres. In *lab* null mutants, the cells in the mutant domain of the tritocerebrum no longer express the neuron-specific RNA-binding protein Elav due to a lack of neuronal identity (Hirth et al., 1998). This was also the case when posterior Hox genes such as *Ubx* were misexpressed using the *sca::*Gal4 driver (figure 3); anti-ELAV immunostaining was no longer seen in any of the cells in the tritocerebral *lab* domain, but continued to be expressed in all other neuronal cells of the embryonic brain. An alternative glial fate does not appear to be adopted by the affected cells, since expression of the glial-specific *repo* gene is seen in cells in the affected part of the tritocerebrum. The *repo*-expressing glial cells are, however, reduced in number and/or misplaced, but they are not totally absent.

The similarity of the phenotype observed in *sca::*Gal4/UAS::*Hox* embryonic brains to that seen in *lab* null mutants suggests that this phenotype may be due to a suppression of Lab protein in the affected domain. To investigate this, we studied expression of Lab protein in

the tritocerebrum by immunocytochemistry in wildtype and in *sca::*Gal4/UAS::*Hox* embryos. In contrast to the pronounced expression of Lab in the posterior tritocerebral domain of wildtype embryos, a total absence of Lab immunoreactivity was observed in the corresponding domain as exemplified by *sca::*Gal4/UAS::*Ubx* embryos (figure 4). (Endoderm-specific, expression of *lab* in the midgut was ectopically expanded anteriorly in these embryos.) Similar effects on Lab protein expression were seen for misexpression of all other posterior Hox genes under the control of the *sca::*Gal4 driver (data not shown). These results are in accordance with findings on the regulation of Lab protein expression by ectopic Hox proteins reported by Miller et al. (2001).

The suppression of Lab protein expression in the tritocerebral domain of *sca::*Gal4/UAS::*Hox* embryonic brains could act at the level of translation or transcription. To investigate this, we carried out in situ hybridization studies using a *lab*-specific RNA probe (figure 5). In the wildtype, *lab* transcription is first observed at stage 9 in the neurogenic region of the intercalary segment that gives rise to the tritocerebrum. Subsequently, *lab* expressing neuroblasts delaminate from this region and generate neuronal progeny (Urbach and Technau, 2003), some of which continue to express the *lab* gene throughout embryogenesis (Hirth et al., 1998). In *sca::*Gal4/UAS::*Ubx* embryos, initiation of *lab* transcription is seen at stage 9 in the neurogenic region of the intercalary segment that gives rise to the tritocerebrum. However, subsequently, *lab* transcripts disappear in the developing tritocerebrum and by stage 10/11 are completely absent in the developing brain. This absence of *lab* transcript in the developing brain of *sca::*Gal4/UAS::*Ubx* embryos continues throughout embryogenesis. (Endoderm-specific transcription of *lab* in the midgut is expanded anteriorly in these embryos.)

The tritocerebrum-specific expression of the *lab* gene has been shown to be controlled by a 3.65kb enhancer element upstream of the *lab* gene transcriptional start site (Chouinard and Kaufman, 1996; Hirth et al., 2001). Given that early misexpression of a posterior Hox gene like *Ubx* results in a loss of *lab* transcripts in the tritocerebrum, we wanted to know if this repression of *lab* might be acting on the 3.65kb enhancer element. To study this we utilized a transgene in which *β-gal* reporter gene expression was driven by the 3.65 enhancer element, and which mimics endogenous *lab* expression in the intercalary segment, tritocerebral

neuromere and anterior midgut (figure 6a, c). This transgene was crossed into a *sca::*Gal4/UAS::*Ubx* genetic background and subsequently reporter gene activity in this line was studied. Initiation of reporter gene expression was seen at stage 9 in the neurogenic region of the intercalary segment that gives rise to the tritocerebrum (figure 6b). However, starting at stage 10/11, reporter gene expression disappeared in the developing tritocerebrum (figure 6d). This loss of *lab*-specific reporter gene expression in the developing brain of *sca::*Gal4/UAS::*Ubx* embryos continued throughout embryogenesis.

From these experiments we conclude that the effect of early misexpression of a posterior Hox gene like Ubx on the developing tritocerebrum is due to cross-regulatory interactions which cause a transcriptional repression of the *lab* gene in the brain. This, in turn, results in an absence of Lab protein in the developing tritocerebrum from stage 10/11 onward and, correspondingly, gives rise to a *lab* loss-of-function brain phenotype since this time period coincides with the temporal requirement of Lab for the specification of tritocerebral identity (Page, 2000; Hirth et al., 2001).

In order to gain further insight into the molecular mechanisms that underlie this posterior dominance, we carried out a set of *sca*::Gal4/UAS::*Hox* misexpression experiments involving posterior Hox genes such as *Antp* and *Ubx* with mutated protein motifs. To study the role of the homeodomain in the posterior dominance phenomenon, we focused on Antp and analysed the effect of *sca*::Gal4 driven misexpression of four different UAS constructs in which the DNA-binding activity of the *Antp* homeobox was perturbed. These were UAS::*AntpΔHD* in which the homeodomain was deleted, UAS::*AntpK50* in which the Antp DNA-binding specificity was changed to that of Bicoid, as well as UAS::*AntpR5A* and UAS::*AntpA50A51* in which residues involved in DNA contacts were mutated in order to abolish binding to DNA (Plaza et al., 2001). In all four cases, *sca*::Gal4 driven misexpression resulted in a wildtype-like embryonic brain (data not shown). This indicates a lack of repressive activity of Antp in the absence of a functional homeodomain.

To study the role of the conserved stretch of aminoacids termed the hexapeptide that is found in many Hox proteins and is involved in interactions between Hox proteins and Exd cofactor (Mann and Chan, 1996; Merabet et al., 2003), we analyzed the effect of *sca*::Gal4 driven

misexpression of a UAS::*UbxYAAA* transgene in which the critical YPWM motif of Ubx was mutated to the sequence YAAA (Galant et al., 2002). Misexpression of the mutated *Ubx* gene under *sca*::Gal4 control resulted in a *lab* loss-of-function phenotype in the embryonic brain (data not shown), suggesting that the hexapeptide is not necessary for the suppressive effect of *Ubx* on *lab* action in the developing brain.

An analysis of the effects of interactions between Hox genes and Hth and Exd cofactors in anterior body segmentation has given rise to a model in which these cofactors are dispensable for Hox protein transcriptional repression functions, but are required for Hox protein transcriptional activation functions (Pinsonneault et al., 1997). This model implies that Exd can convert Hox protein action from one functional state into another. Accordingly, we next investigated whether concomitant misexpression of Exd and Hth cofactors and a Hox protein like Ubx might be able to convert or cancel the repressive effect of Ubx misexpression on *lab* transcription.

To investigate this, we used the *sca*::Gal4 driver in combination with UAS::*Ubx*, UAS::*exd*, UAS::*n-exd* and UAS::*hth* responders to misexpress Ubx and these cofactors together. Combined misexpression of the Hox gene *Ubx* with either *exd*, or nuclear *exd* (n-*exd*), or *hth* did not alter the repressive effect of the Hox gene on the developing tritocerebrum (data not shown). (Control experiments in which either *exd*, n-*exd* or *hth* were misexpressed under the control of *sca*::Gal4 but without concomitant misexpression of a Hox gene resulted in a wildtype-like brain.) In contrast, when *Ubx* was misexpressed together with both n-*exd* and *hth*, a complete phenotypic rescue of the tritocerebral defect was observed (figure 7). Thus, the tritocerebral neuromere developed normally and both commissural and longitudinal pathways were restored. Moreover, normal expression of the Lab protein was seen in the appropriate tritocerebral domain. This indicates, that early CNS-specific misexpression of n-*exd* and *hth* encoded cofactors combined with early CNS-specific misexpression of Ubx cancels the repressive effect of Ubx on *lab* expression in the tritocerebral domain. This phenotypic rescue was not observed when a mutated UbxYAAA was misexpressed together with n-Exd and Hth (data not shown), suggesting that Ubx with a functional hexapeptide and the cofactors n-Exd and Hth are required to convert or cancel the repressive effect of *Ubx* misexpression on *lab* transcription.

DISCUSSION

The main function of Hox genes is to assign positional identities along the embryonic body axis in animals ranging from arthropods to vertebrates (McGinnis and Krumlauf, 1992; Manak and Scott, 1994; Carroll, 1995). Several mechanistic paradigms have been proposed to describe Hox gene action, two of which are the concepts of cross-regulation among Hox genes, and of co-operative interactions between Hox genes and protein cofactors (Duboule and Morata, 1994; Graba et al., 1997; Mann and Morata, 2000). In the developing CNS of *Drosophila*, loss-of-function studies have shown that Hox genes expressed in more posterior regions act as negative regulators of Hox genes that are expressed in more anterior regions of the CNS. For example *Antp* is primarily expressed in Parasegment (PS) 4 and PS5 of the CNS, but it is also expressed at lower levels in PS6-13 (Levine et al., 1983; Hafen et al., 1984; Carroll et al., 1986; Hirth et al., 1998). In embryos that lack the Bithorax-Complex genes, *Antp* expression is high in PS4-13 (Hafen et al., 1984; Harding et al., 1985; Carroll et al., 1986), suggesting that BX-C gene action keeps *Antp* expression low in PS6-13. Similarly, BX-C genes that are expressed and function in more posterior abdominal segments keep *Ubx* expression low in PS7-13. In the absence of the abdominal BX-C genes, *Ubx* products are found at high levels in PS6-13 (Struhl and White, 1985; White and Wilcox, 1985). In addition, recent gain-of-function experiments have shown that ectopic Ubx and Abd-A are able to repress *lab* and *Scr* in the CNS in a timing dependent manner while otherwise overlapping expression of other Hox genes is tolerated (Miller et al., 2001).

In our analysis, we have focused on *lab*, the Hox gene specifically expressed in the tritocerebral neuromere. Genetic analyses have shown that *lab* is essential for the acquisition of neuronal identity in its tritocerebral expression domain, and *lab* loss-of-function mutations lead to severe defects in the establishment of the tritocerebral neuromere (Hirth et al., 1998). The action of *lab* in this domain can be eliminated by targeted misexpression of posterior Hox genes through the *sca*::Gal4 driver, resulting in a *lab* loss-of-function phenotype in the brain. This suppression of *lab* action has a number of features that are characteristic of the type of cross-regulatory Hox gene interactions that have been demonstrated in developing epidermal structures (Miller et al., 2001). First, the suppression of *lab* in the tritocerebrum appears to be time dependent. While early misexpression of posterior Hox genes during neuroectoderm specification and neuroblast formation at embryonic stage 9 reliably results in *lab* suppression

in the tritocerebrum, later misexpression, after embryonic stage 10/11, does not. Second, *lab* suppression by misexpression of posterior Hox genes is tissue specific. Thus, while Hox gene misexpression via the *sca*::Gal4 driver suppresses *lab* expression in the tritocerebrum, it augments *lab* expression in the endodermal cells of the midgut. Third, misexpression of posterior Hox genes leads to a loss of Lab protein in the affected domain, and this lack of Lab is in accordance with the observed phenocopy of a *lab* loss-of-function mutation observed in this domain.

In several respects these experiments extend our insight into cross-regulatory interactions beyond the observations made on developing epidermal structures. We provide evidence that the suppression of *lab* by a posterior Hox gene like *Ubx* is due to transcriptional repression. Thus, in *sca*::Gal4/UAS::*Ubx* embryos, *lab* transcripts disappear and are absent in the developing tritocerebrum from stage 10/11 onward. This tritocerebrum-specific repression appears to be mediated through a 3.65kb enhancer element upstream of the *lab* gene transcriptional start site. Moreover, our results imply that suppression of *lab* in the developing tritocerebrum by posterior Hox genes requires a functional homeodomain; mutations of the homeodomain in the *Antp* gene abolish the repressive activity of this Hox gene. In addition, our findings indicate that the suppressive cross-regulatory action of a posterior Hox gene like *Ubx* is not dependent on a functional hexapeptide. Thus, misexpression of a UAS::*UbxYAAA* transgene in which the critical YPWM motif of Ubx was mutated to the sequence YAAA (Galant et al., 2002), still results in complete suppression of *lab* in the developing tritocerebrum. Finally, we provide evidence that concomitant misexpression of *Ubx*, nuclear-targeted Exd and Hth is able to completely rescue the *lab* loss-of-function mutant phenotype. This implies that the Exd and Hth cofactors can switch Ubx protein action between different functional states in which Exd and Hth are required for Hox protein transcriptional activation functions whereas they are dispensable for Hox transcriptional repression functions (Pinsonneault et al., 1997; Li et al., 1999). Moreover, our findings can be explained by models in which the hexapeptide is involved in the regulation of Hox protein activity (Merabet et al., 2003; In der Rieden et al., 2004), and may also reflect a requirement for equilibrated levels of a Hox gene product and the Hth and n-Exd cofactors in the specification of tritocerebral identity.

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FIGURES

Figure. 1. Expression patterns of Gal4 drivers. Antibodies to Ubx were used to detect the responder UAS::*Ubx* under the control of three different Gal4 drivers. (A) *sca::*Gal4 expresses Gal4 during neuroectoderm specification and neuroblast formation starting from stage 9. (B) *1407::*Gal4 expresses Gal4 during neuroblast and ganglion mother cell formation and strong UAS activity is apparent by stage 12. (C) *C155 elav::*Gal4 expresses Gal4 in postmitotic neurons and strong UAS activity is apparent by stage 14. A-C; embryos double-immunolabeled with a neuron-specific anti-HRP antibody (red) and an anti-Ubx antibody (green, yellow). Reconstructions of optical sections obtained by laser confocal microscopy; lateral views, anterior to the left.

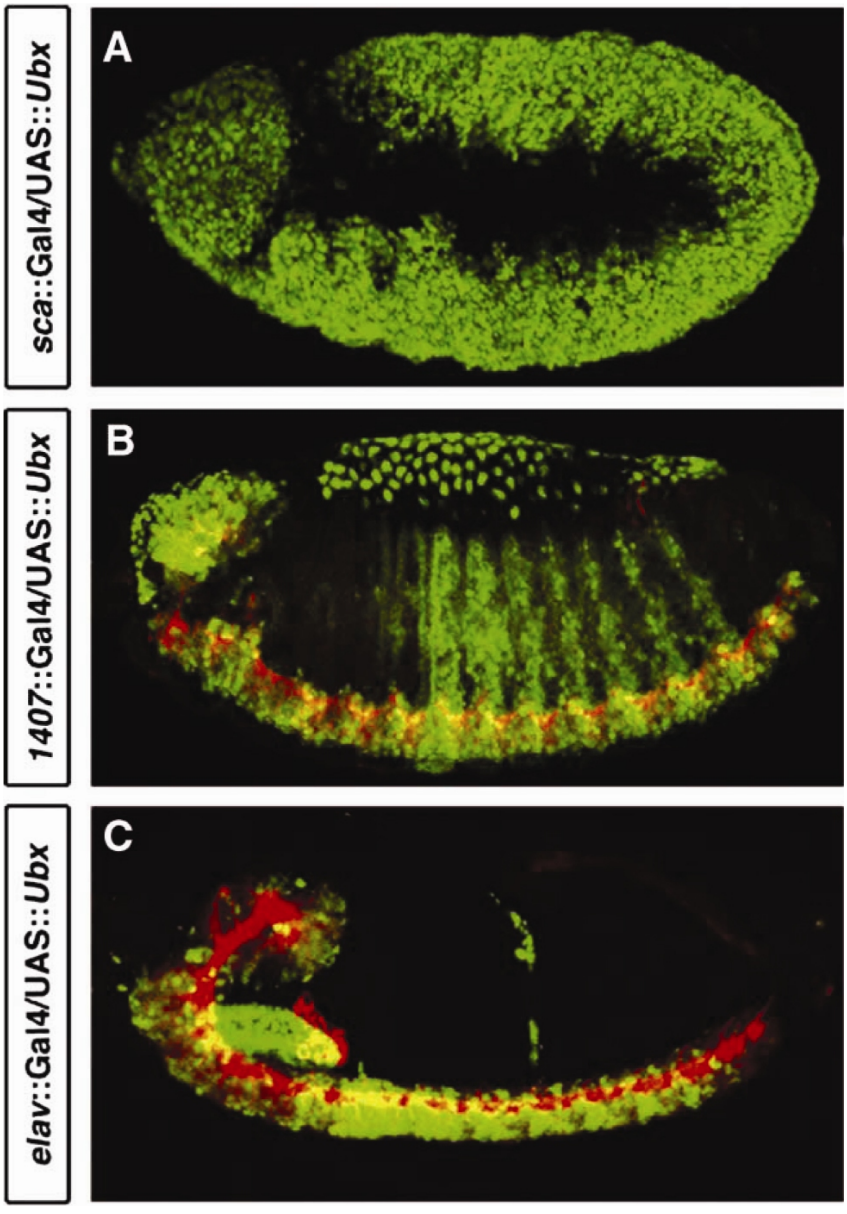

sca::Gal4/UAS::Ubx
A
1407::Gal4/UAS::Ubx
B
elav::Gal4/UAS::Ubx
C

Figure. 2. Early, neuroectoderm-specific misexpression of Ubx, but not later misexpression of Ubx in the nervous system leads to a mutant brain phenotype. Different Gal4 drivers are used to misexpress Ubx. (A,B) Wildtype; (C,D) *sca*::Gal4/UAS::*Ubx*; (E,F) *1407*::Gal4/UAS::*Ubx*; (G,H) C155 *elav*::Gal4/UAS::*Ubx*. Only in the case of *sca*::Gal4/UAS::*Ubx* a brain patterning defect is observed in the tritocerebral domain (arrow in D, compare to wildtype in B). A,C,E,G; embryos double-immunolabeled with a neuron-specific anti-HRP antibody (red) and an anti-Ubx antibody (green, yellow); B,D,F,H; embryos immunolabeled with a neuron-specific anti-HRP antibody (red). Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections, lateral views.

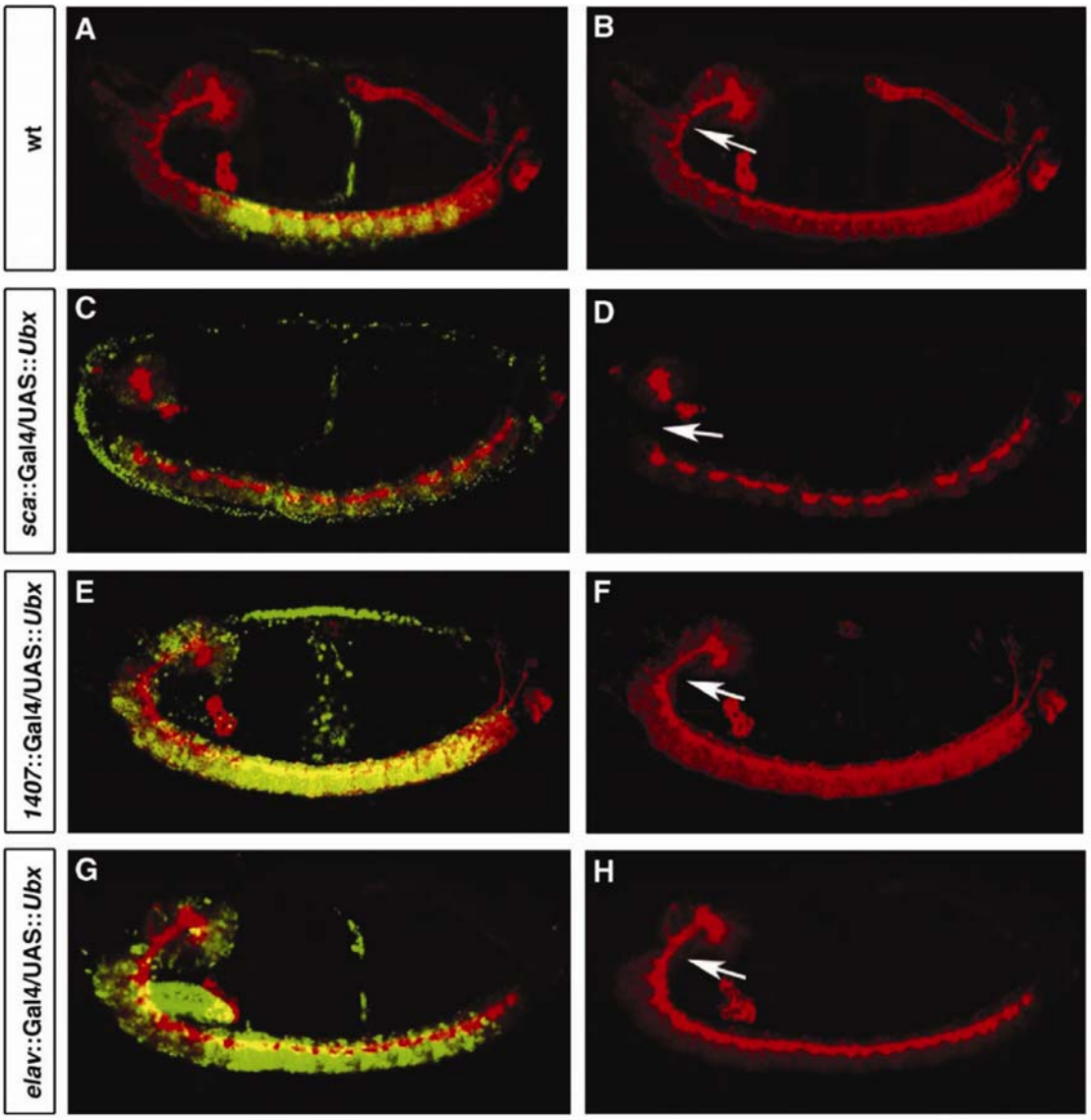

wt
A
B
sca::Gal4/UAS::Ubx
C
D
1407::Gal4/UAS::Ubx
E
F
elav::Gal4/UAS::Ubx
G
H

Figure. 3. Mutant brain phenotype caused by misexpression of Ubx using the *sca*::Gal4 driver. (A,B) Immunolabeling with anti-ELAV (green); (C,D) Double-immunolabeling with anti-HRP (red) and anti-REPO (yellow/green); (E,F) Double-immunolabeling with anti-HRP (red) and anti-Fasciclin II (yellow/green). In contrast to the wildtype situation (A), the neuron specific marker ELAV is missing in the tritocerebral domain of *sca*::Gal4/UAS::*Ubx* transgenic embryos (B, arrow). As in the wildtype (C), the glia-specific marker REPO is present in the tritocerebral domain of *sca*::Gal4/UAS::*Ubx* transgenic embryos, however repo-expressing cells appear reduced in number and/or misplaced (D, arrow). Fasciclin II, which in the wildtype is expressed in the tritocerebral domain by a subset of neurons and their axons (E) is absent in *sca*::Gal4/UAS::*Hox* transgenic embryos (F, arrow). Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections, lateral views.

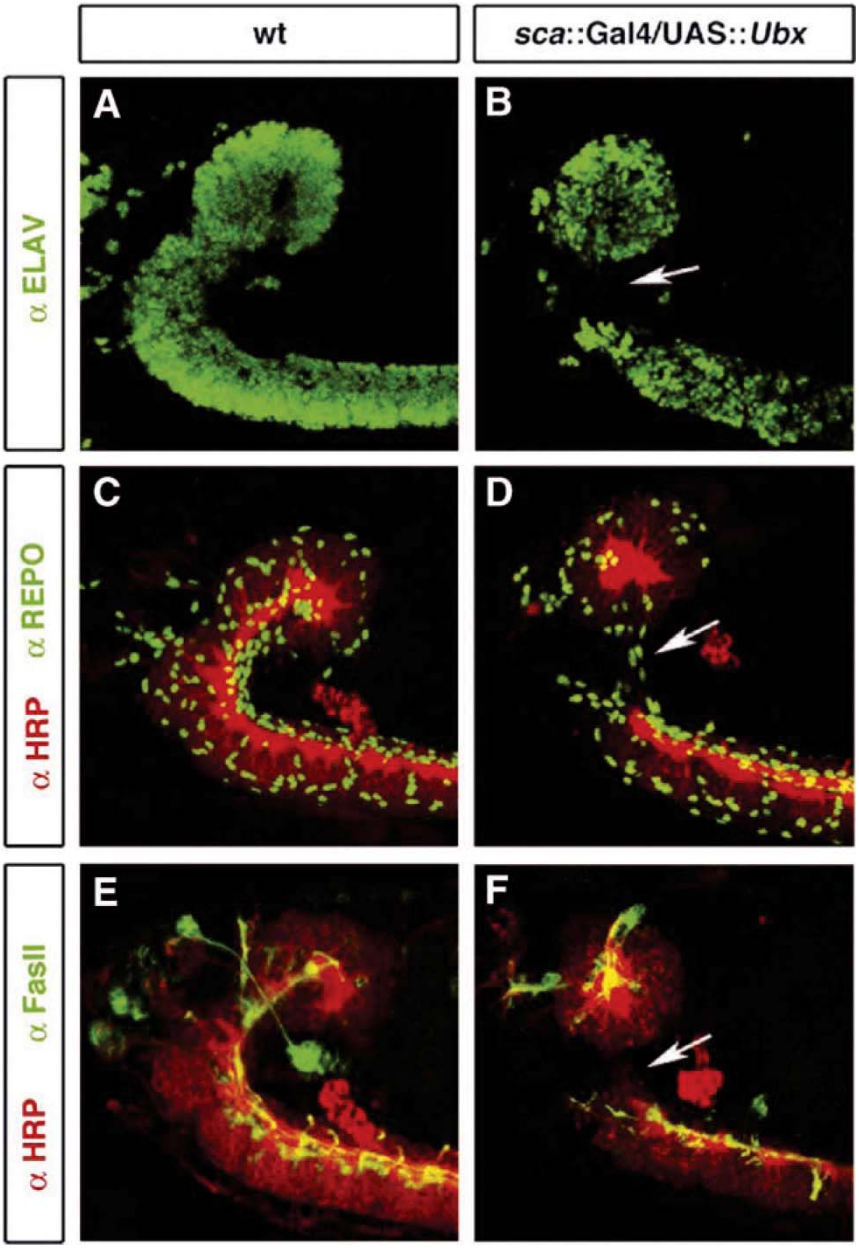

wt
sca::Gal4/UAS::Ubx
A
B
α ELAV
C
D
α HRP
α REPO
E
F
α HRP
α FasII

Figure. 4. Absence of Labial protein results from early, neuroectoderm-specific misexpression of Ubx. (A) Wildtype, (B) *sca*::Gal4/UAS::*Ubx*. Double-immunolabeling with anti-LAB (red) and anti-ELAV (green) antibodies. In *sca*::Gal4/UAS::*Ubx* transgenic embryos, Labial protein as well as the neuron specific marker ELAV are missing in the tritocerebral domain (arrow in B; compare to arrow in A), whereas endoderm-specific expression of Lab in the midgut is ectopically expanded anteriorly (bracket in B, compare to A). Laser confocal microscopy of stage 13/14 embryos, reconstructions of optical sections, lateral views.

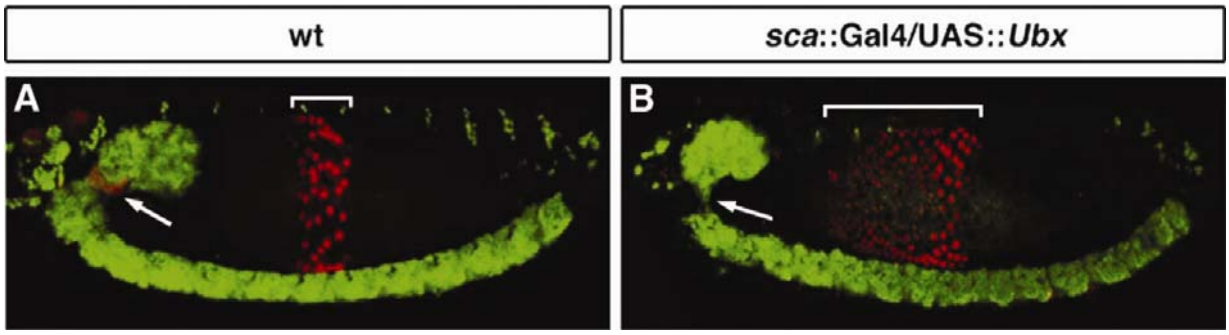

wt
sca::Gal4/UAS::Ubx
A
B

Figure. 5. Transcriptional repression of *lab* in the developing intercalary segment and tritocerebrum caused by *sca*::Gal4 driven UAS::*Ubx* misexpression. Whole mount *in situ* hybridization of *labial* transcripts in wildtype (A-C) and in *sca*::Gal4/UAS::*Ubx* (D-F) embryos; lateral views; anterior is to the left. At stage 9, *lab* expression is detectable in both wildtype (A) and *sca*::Gal4/UAS::*Ubx* (D) embryos in the neurogenic region of the intercalary segment (arrow) that gives rise to the tritocerebrum as well as in the developing midgut. Subsequently, in *sca*::Gal4/UAS::*Ubx* embryos, *lab* transcripts disappear in the developing tritocerebrum and from stage 10/11 onwards are completely absent in the developing brain (arrows in E, F; compare to B,C). (Note that endoderm-specific expression of *lab* in the midgut is ectopically expanded anteriorly; bracket in F, compare to C).

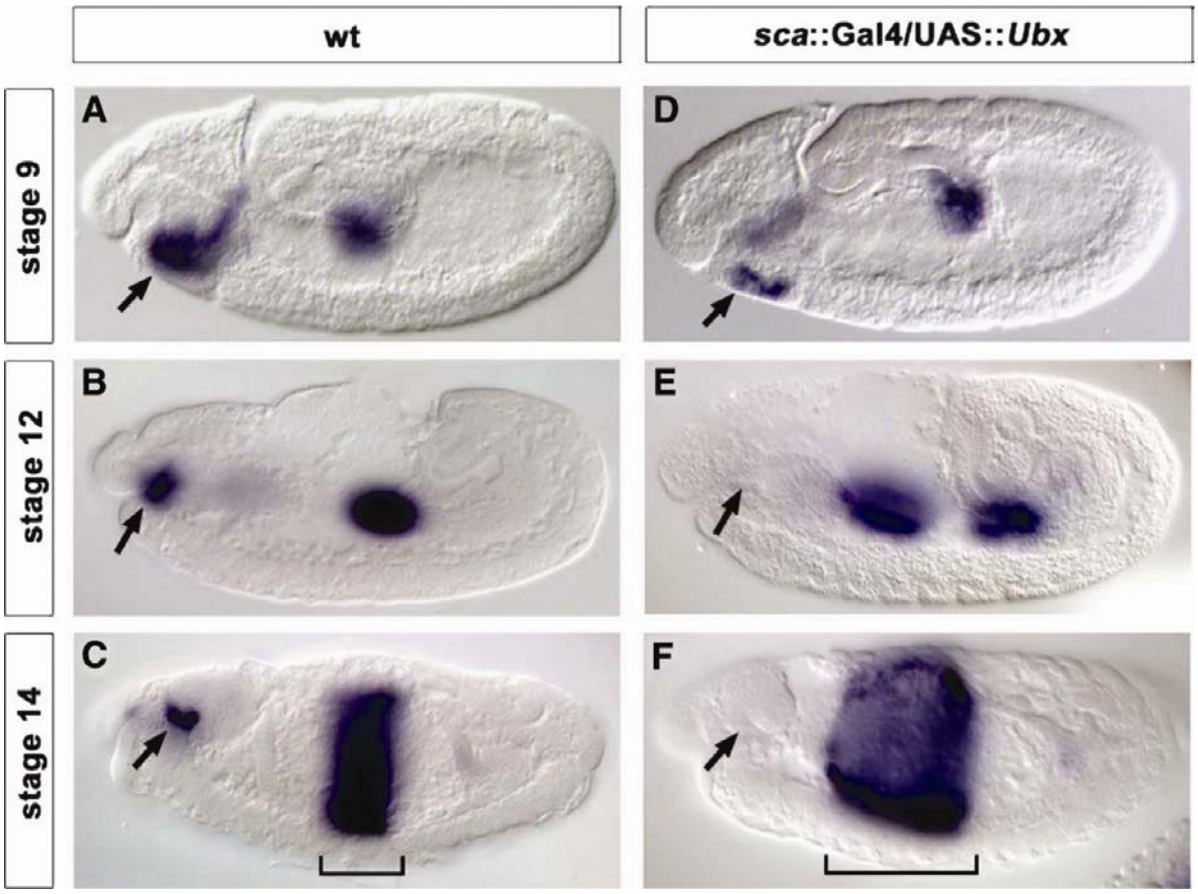

wt
sca::Gal4/UAS::Ubx
stage 9
stage 12
stage 14
A
B
C
D
E
F

Figure. 6. Transcriptional repression of a 3.65kb *lab*-specific enhancer element due to *sca*::Gal4 driven UAS::*Ubx* misexpression. Laser confocal microscopy of stage 9/10 (A,B) and stage 13/14 (C,D) embryos, reconstructions of optical sections, lateral views. (A,C) P{w$^+$ 3.65 *lab-lacZ*}, (B,D) P{w$^+$ 3.65 *lab-lacZ*}; *sca*::Gal4/UAS::*Ubx*. (A,B) Double-immunolabeling with anti-UBX (red) and anti-βgal (green/yellow). (C,D) Double-immunolabeling with anti-HRP (red) and anti-βgal (green/yellow). At embryonic stage 9/10, reporter gene expression is seen in the neurogenic region of the intercalary segment (arrow) that gives rise to the tritocerebrum in both P{w$^+$ 3.65 *lab-lacZ*} (A) and P{w$^+$ 3.65 *lab-lacZ*}; *sca*::Gal4/UAS::*Ubx* (B) embryos. At embryonic stage 13/14, *βgal* expression is seen in the tritocerebrum of P{w$^+$ 3.65 *lab-lacZ*}embryos (C, arrow), but disappears in P{w$^+$ 3.65 *lab-lacZ*}; *sca*::Gal4/UAS::*Ubx* embryos due to *Ubx* misexpression, (D, arrow). (Note that midgut-specific expression of *βgal* is ectopically expanded anteriorly; bracket in D, compare to C).

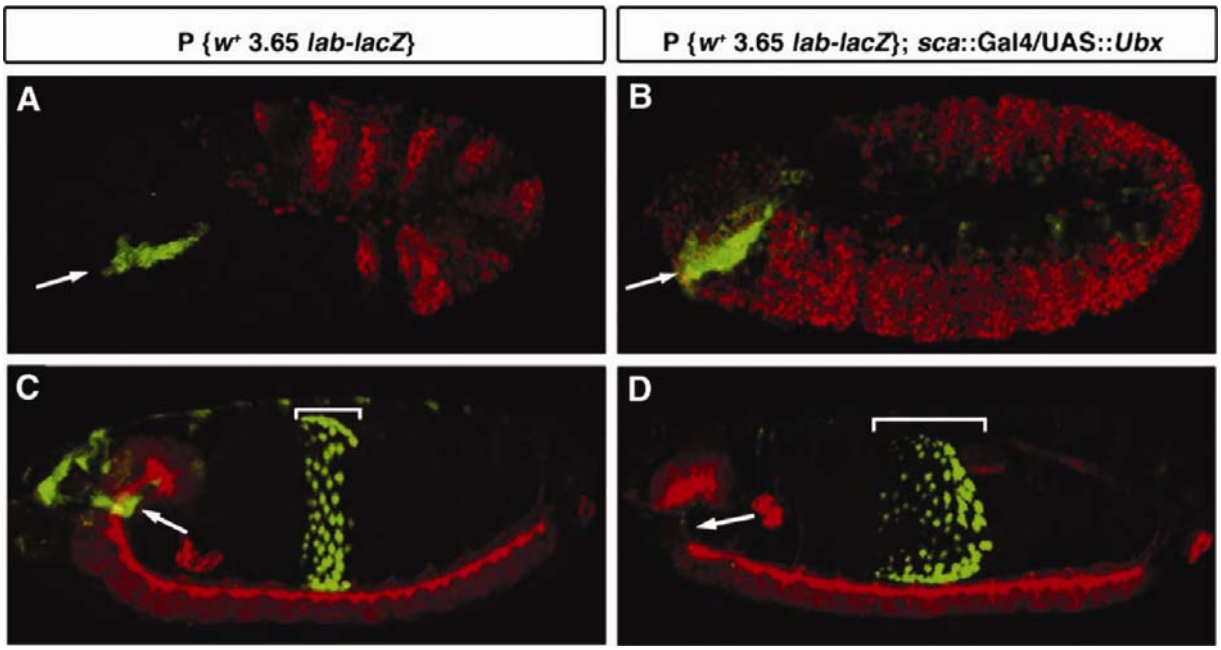
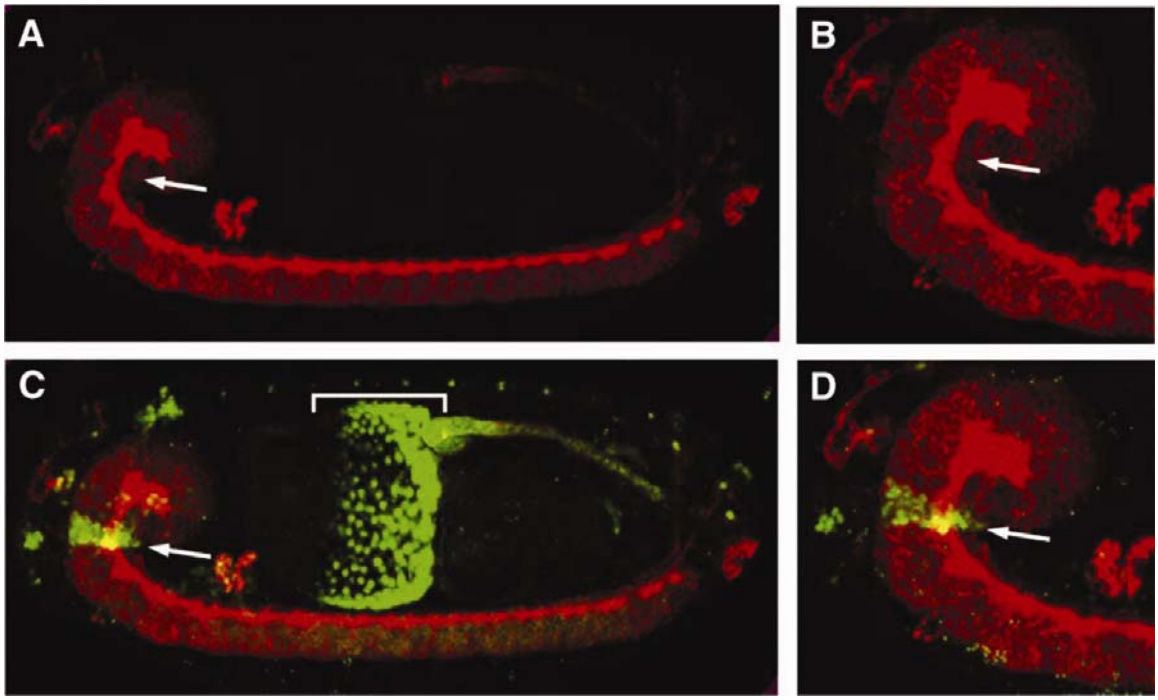

P {w+ 3.65 lab-lacZ}
P {w+ 3.65 lab-lacZ}; sca::Gal4/UAS::Ubx
A
B
C
D

Figure. 7. Rescue of the tritocerebral lab mutant brain phenotype by concomitant misexpression of UAS::*Ubx*, UAS::*hth*, UAS::*nls-exd*. (A-D) *sca::*Gal4*/+; P(*UAS*::hth), P{w*$^{+mC}$=UAS::*Ubx.Ia.C}36.2/ P(UAS::FLAG-NLS-EXD)*. (A,B) Immunolabeling with a neuron-specific anti-HRP antibody (red). (C,D) Double-immunolabeling with anti-HRP (red) and anti-LAB (green/yellow) antibodies. *sca*::Gal4 driven misexpression of UAS::*Ubx*, UAS::*hth*, and UAS::*nls-exd* results in normal development of the tritocerebral neuromere including commissural and longitudinal pathways (arrow in A, higher magnification in B), and normal expression of the Lab protein is seen in the appropriate tritocerebral domain (arrow in C, higher magnification in D). (Note that midgut-specific expression of *Lab* is still ectopically expanded anteriorly; bracket in C). Laser confocal microscopy of stage 13/14 (C,D) embryo, reconstructions of optical sections, lateral views.


A
B
C
D

The columnar gene *vnd* is required for tritocerebral neuromere formation during embryonic brain development of *Drosophila*

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To be submitted

ABSTRACT

In *Drosophila*, a set of evolutionarily conserved transcription factors are required for the specification of neuronal identity along the anteroposterior (AP) and dorsoventral (DV) axes, such as the *Hox* genes for AP, or the columnar genes for DV axis patterning. In this report we focus on the role of the columnar patterning gene *ventral nervous system defective* (*vnd*) during embryonic brain development. Expression of *vnd* is observed in each developing neuromere and subsequently becomes restricted to posterior boundary regions. Mutational inactivation of *vnd* results in regionalized axonal patterning defects which are similar to the brain phenotype caused by mutation of the *Hox* gene *labial* (*lab*). However, in contrast to *lab*, *vnd* is required for precursor cell development and neuronal progeny maintenance during tritocerebral neuromere formation. Thus, in *vnd* mutant embryos, a subset of identified tritocerebral neuroblasts which normally express *lab* do not form. During later stages, programmed cell death leads to reduced or absent neuronal tissue which is normally specified by *lab*. The resulting *vnd* mutant brain phenotype is characterized by the lack of the tritocerebral neuromere, which can be rescued by targeted inactivation of the apoptotic program. Thus, in contrast to its DV patterning function in the VNC, *vnd* is required for AP patterning during embryonic brain development. Our results indicate that the activity of the columnar gene *vnd* is integrated into pattern formation along the anteroposterior neuraxis by generating and maintaining cells which subsequently become specified by the activity of the *Hox* gene *lab*.

Key Words: Brain development, anteroposterior/dorsoventral patterning, neuromere, *vnd*, *lab*, *Drosophila melanogaster*

INTRODUCTION

In *Drosophila*, the spatial integration of two independent gene networks controls the patterning of the ventral nervous system along the anteriorposterior (AP) and dorsalventral (DV) axes. The sequential action of the maternal AP coordinate, gap and pair-rule genes defines the location of each AP stripe of segment-polarity gene expression in a segment (Akam, 1987). The segment-polarity genes in turn control gene expression along the AP axis and enable neuroblasts (neuroblasts) that form in different AP rows to acquire different fates (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Bhat, 1996; Bhat and Schedl, 1997; Bhat, 1998). Simultaneously, the graded DV action of the nuclear factor NF-kappaB, bone morphogenetic protein (BMP) and epidermal growth factor receptor (EGFR) signaling pathways determines the DV borders of the neuroectoderm and further establishes the tripartite DV subdivision of the neuroectoderm into longitudinal stripes (Skeath, 1998; von Ohlen and Doe, 2000; Skeath and Thor, 2003).

The restricted expression domains of the three homeobox genes, *ventral nervous system defective* (*vnd*), *intermediate neuroblasts defective* (*ind*), and *muscle specific homeobox* (*msh*), in precise bilateral columns of neuroectodermal cells (Skeath et al., 1994; Isshiki et al., 1997; Buescher and Chia, 1997; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998) correspond to the DV columnar subdivision of the *Drosophila* neuroectoderm. *vnd* is expressed in ventral neuroectodermal cells (Jimenez et al., 1995; Mellerick and Nirenberg, 1995), while *ind* expression is restricted to intermediate neuroectodermal cells (Weiss et al., 1998), and *msh* is expressed in lateral neuroectodermal cells (D'Alessio and Frasch, 1996; Isshiki et al., 1997). These DV stripes of neuroectodermal cells in turn give rise to the three columns of neuroblasts—medial or ventral, intermediate, and lateral, respectively. The mutually exclusive expression patterns of *vnd*, *ind*, and *msh* are maintained by negative regulatory interactions in which *vnd* represses *ind* expression in the medial column and *ind* represses *msh* expression in the intermediate column (reviewed by Skeath, 1999; Skeath and Thor, 2003). Thus, the activities of the columnar genes subdivide the neuroectoderm into three DV columns, promote neural precursor formation in their respective expression domains, and ensure that neural precursors that arise in different columns acquire different fates.

The *Nkx2*-type homeobox gene, *vnd,* is unique amongst the columnar genes, because it is continuously expressed, from cellularization until the completion of embryonic development, within the developing VNC. *vnd* is essential both for the formation and identity of ventral MP2 and NB 7-1 neuroblasts which are generally not formed, while other surviving early ventral neuroblasts are mis-specified in *vnd* loss-of-function mutants. The absence or mis-specification of ventral neuroblasts correlate with the loss or mis-specification of neuronal progeny, axonal pathfinding defects and a reduced number of cells in the developing VNC of *vnd* mutant embryos. Thus, commissures are fused, VUM neurons show pathfinding defects, and midline glia is reduced in number in *vnd* loss-of-function mutants. Conversely, over-expression of *vnd* can lead to transformations in the identity of intermediate and lateral neuroblasts (Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002).

In contrast to the developing VNC, less is know about the expression and function of the columnar gene *vnd* in the developing head and brain of *Drosophila*. A recent study shows that *vnd* is expressed in the procephalic neuroectoderm and in subsets of neuroblasts in the developing brain. Thus, *vnd* is expression is seen in identified neuroblasts of the developing protocerebrum, deutocerebrum and tritocerebrum which demarcate the posterior boundaries of these brain neuromeres. This brain-specific expression of *vnd* appears to differ from the expression in trunk neuromeres in that *vnd* expression is dynamic and from stage 9 onwards is downregulated in parts of the antennal neuroectoderm and deutocerebral neuroblasts (Urbach and Technau, 2003a and 2003b). Despite the detailed knowledge of *vnd* expression during early neurectoderm and brain neuroblast formation, nothing is known about the later expression or the function of *vnd* during embryonic brain development of *Drosophila*.

Here we analyse the role of the columnar patterning gene *vnd* during embryonic brain development of *Drosophila*. Using molecular neuroanatomical techniques, we map the expression of *vnd* in the embryonic brain, and show that it is confined to neuromeric boundaries. We then carry out a mutant analysis and demonstrate that, comparable but distinct to the Hox gene *labial, vnd* is essential for tritocerebral neuromere formation in the developing brain. Furthermore we show that the patterning defects in the *vnd* mutant brain are due to defective neuroblast formation and the subsequent loss of neuronal tissue in the

mutant domain. Moreover, we show that this loss of neuronal tissue is associated with increased apoptotic activity, resulting in the loss of the tritocerebral commissure and the longitudinal connectives that normally run through this neuromere. Finally we show that blocking apoptosis in *vnd* null mutant embryos results in the restoration of tritocerebral axon tracts and the wildtype-like expression domain of the Hox gene *labial*. Our results indicate that *vnd* action in ventral precursor cells and subsequent neuronal progeny within the developing tritocerebral neuromere is apparently conveyed into patterning along the anteroposterior neuraxis. We propose that this is achieved by *vnd* acting on cells that express the Hox gene *labial*.

MATERIAL AND METHODS

Drosophila strains and genetics

The wildtype was Oregon-R. For *vnd* mutant analysis vnd^{6} (Jimenez and Campos-Ortega, 1990) and $vnd^{\Delta 38}$ (Ashraf and Ip; 2001) were used**.** Mutant alleles were balanced over FM7, ftz-lacZ. Homozygous null mutants were identified by the absence of *ftz-lacZ*. To analyse the development of the tritocerebral *lab* expression territory in the *vnd* mutant background, we used the line *7.31 lab-lacZ/7.31 lab-lacZ* (Tremml and Bienz, 1992) crossed into vnd^{6}. To identify *vnd* expression in former *lab* expressing tritocerebral cells in the *labial* mutant background, we used line *7.31 lab-lacZ/7.31 lab-lacZ;* lab^{vd1}*/TM3, hb-lacZ* (Tremml and Bienz, 1992). Homozygous null mutants were identified by the absence of *hb-lacZ*. For comparison with the wildtype situation, *7.31 lab-lacZ* was crossed back to wildtype. *7.31 lab-lacZ* shows cytoplasmic distribution of *βgal* and reflects endogenous *lab* expression with additional ectopic expression patterns in the deutocerebral anlage (Hirth et al., 1998; 2001). The UAS/Gal4 transcriptional activation system (Brand and Perrimon, 1993) was utilized in order to perform rescue experiments in vnd^{6} mutant embryos. For the ubiquitous block of apoptosis within neural lineages, we used the: *sca::*Gal4 driver line (Klaes et al., 1994; Sprecher et al., 2004) in order to activate UAS::*p35* transcription (Mergliano and Minden, 2003) from neurectodermal stage to primary neural lineage generation. All experiments were carried out at 25°C. Embryos were staged according to Campos-Ortega and Hartenstein (1997).

Immunocytochemistry and TUNEL assay

Embryos were dechorionated, fixed, immunostained, flattened and stage according to according to previously published protocols (Patel, 1994; Therianos et al. 1995; Urbach et al., 2003). Primary antibodies were rabbit anti-Deadpan (1:300, Bier et al., 1992; kindly provided by H. Vaessin), rabbit anti-HRP (FITC-conjugated) 1:100 (Jan and Jan, 1982) (Jackson Immunoresearch), rabbit anti-LAB at 1:100 (F. Hirth and H. Reichert, unpublished), rat anti-LAB at 1:500 (F. Hirth and H. Reichert, unpublished), mouse anti-NRT at 1:20 (BP106 antibody, DSHB), rabbit anti VND (Mc Donald et al., 1998; kindly provided by C.Q. Doe), rabbit anti-βGAL 1:200-1:400 (Milan Analytika), mouse anti-βGAL 1:50 (DSHB), mouse anti-Fasciclin II 1:5 (Lin and Goodman, 1994), rat anti-ELAV 1:30 (DSHB), mouse anti-PROS 1:4 (Spana and Doe, 1995), mouse anti-REPO 1:20 (DSHB), mouse anti-Engrailed (4D9,1:6, Patel et al., 1989; DSHB). Secondary antibodies used for confocal microscopic analysis were Alexa-488, Alexa-568, and Alexa-647 antibodies generated in goat (Molecular probes), all at 1:150 dilution. Secondary antibodies used for flat mount preparations analyzed using Nomarski optics were either biotinylated or alkaline phosphatase-conjugated antibodies generated in goat all at 1:500 (Dianova). Apoptotic activity was assayed by TUNEL analysis using a commercial TUNEL kit (ApoTag, Oncor) as previously described (Richter et al., 1998) with the following modifications: After fixation, embryos were washed in PBT for 2x5min, then washed in Equilibration Buffer (from the ApoTaq kit) for 2min; Embryos were incubated in the working strength TdT mixture (from the ApoTaq kit) for 1h at 37°C. After incubation, supernatant was removed and embryos were washed 2x2min with Stop/Wash solution (from the ApoTaq kit), and subsequently washed 3x2min, 2x30min in PBT before starting Immunolabelling. Embryos were mounted in Vectashield H-1000 (Vector).

Laser confocal microscopy and generation of 3D digital models

For laser confocal microscopy, a Leica TCS SP was used. Optical sections ranged from 0.2 – 1.5 μm recorded in line average mode with picture size of 512 x 512 pixels, or 1024 x 1024 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Complete series of optical sections were imported and processed using ImageJ. For the generation of 3D digital models, raw tiff stacks (stacks of optical sections) were imported into AMIRA (Mercury Computer Systems). User-defined materials were drawn

manually around the labeled structures (immunoreactivity of a given antibody) in each layer of a given tiff stack, which were to be included in the model. Subsequently, the program synthesizes a surface by triangulation around the defined materials, which was further processed by increasing the number of triangles per material and smoothening of the surface. For 2D representations in Figures, screen shots were generated showing the model in the appropriate angle, virtual lighting and transparency of individual material surfaces (For details see Pereanu and Hartenstein, 2004). Figures were arranged and labeled using Adobe Photoshop.

RESULTS

Neuromere-specific *vnd* expression during embryonic brain development

During the initial phase of embryonic neurogenesis, *vnd* expression is seen in the procephalic neurectoderm and delaminating neuroblasts (neuroblasts) in all three neuromeres of the developing anterior brain (Urbach and Technau, 2004; for a detailed description of *vnd* expression during the early phase of neurogenesis see accompanying paper). At late stage 12, a large expression domain is seen in the protocerebral neuromere (Fig 1C, D), which extends towards the protocerebral-deutocerebral boundary region. A second smaller expression domain is observed in the deutocerebrum; these *vnd* expressing cells are located near and within the deuterocerebral-tritocerebral boundary region (DTB). A third *vnd* expression domain is located in the tritocerebrum. Although the tritocerebral and deuterocerebral *vnd* expression clusters are in close proximity to each other, they do not overlap. Towards the end of embryogenesis, at embryonic stage 15, expression of *vnd* is still visible in these three neuromeric domains (Fig. 1E, F). All three domains are composed of tightly packed clusters of cells and are clearly separated from each. The protocerebral cell cluster extends from the surface of the brain to the neuropile and is composed of approximately 50 cells. The deutocerebral and tritocerebral cell clusters are markedly smaller and are composed of approximately 20 to 25 cells each. During these developmental stages we find *vnd* expression in neuroblasts, GMCs and neurons as judged by immunolabeling with neuron-specific anti-ELAV and anti-PROS antibodies. Immunolabeling with glia-specific anti-REPO antibody indicates that none of the glia cells of the embryonic brain express *vnd* (data not shown). *vnd*

expressing cells are also seen in the neuromeres of the suboesophageal ganglion and the VNC, as well as in peripheral sense organs; these *vnd* expressing cells will not be considered further in this report.

Brain patterning defects in *vnd* loss-of-function mutants

During early neurectoderm development, pattern formation of brain neuroblasts is affected in *vnd* loss-of-function mutants (see below and accompanying paper). Likewise, a pronounced *vnd* loss-of-function phenotype is observed in the late embryonic brain. In *vnd* null mutants axonal patterning defects are associated with the deutocerebral and tritocerebral neuromere. Immunolabeling with a neuron specific anti-HRP antibody reveals that the longitudinal connectives that normally run through these neuromeres (Fig. 2A) are missing or reduced (Fig. 2B, D, F arrow). The tritocerebral commissure which interconnects the brain hemispheres at the level of the tritocerebrum is completely absent and the frontal connectives no longer project into the tritocerebral neuromere. Moreover, only a thin strand of neuronal cells interconnects the protocerebrum and the suboesophageal ganglion (Fig. 2D, F), and in severe cases even these cells are missing (Fig. 2B).

To determine which regions of the late embryonic brain are affected by loss of *vnd* function, we studied the expression of *engrailed* (*en*), which in the wildtype embryonic brain is located in several small clusters of cells at the posterior boundary of each neuromere. The b1 *en*-stripe (or *en* head spot) delimits the posterior protocerebrum (several *en* cells are also seen more anteriorly in the protocerebrum as the secondary head spot), the b2 *en*-stripe (or *en* antennal stripe) delimits the posterior deuterocerebrum, and the b3 *en*-stripe delimits the posterior tritocerebrum (Fig 2A). In the *vnd* mutant brain, only the b1 *en*-stripe and the secondary head spot are visible; neither the b2 *en*-stripe nor the b3 *en*-stripe (or *en* intercalary stripe) can be identified. This suggests that parts of the embryonic tritocerebrum and deutocerebrum are lacking in the *vnd* mutant (Fig 2B). Immunostaining with the neuron-specific anti-ELAV antibody indicates that this phenotype is due to an absence of neurons in the affected regions (Fig 2C, D). Glia-specific anti-REPO immunoreactivity reveals that glial cells are present but fail to be correctly localized in the affected region, most likely due to the absence of neuronal tissue (Fig 2E, F). In addition to this patterning defect in the tritocerebral/deutocerebral brain region, a less marked reduction in overall size of the

protocerebrum is also seen in *vnd* mutant embryos. Moreover the organization of the suboesophageal ganglion and the VNC is affected in the mutant (see also Mellerick and Modica, 2001). These latter two phenomena were not further studied.

Defective tritocerebral neuroblast formation in *vnd*

Previous analyses of *vnd* action demonstrated that this gene is involved in dorsoventral patterning of the VNC (Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002). Our findings indicate that *vnd* acts along the anteroposterior axis during embryonic brain development. At the gross morphological level, the late *vnd* mutant brain phenotype described at the level outlined above is reminiscent of the late mutant brain phenotype observed for the anterior Hox gene *labial (lab)*. In *lab* null mutants, tritocerebral cells are generated and positioned correctly, however these cells fail to express neuronal markers (like HRP and Elav) and marked axogenesis defects occur (Hirth et al., 1998; Page, 2000; Hirth et al., 2001). Moreover, *lab* and *vnd* show overlapping expression in the Tv1-5 neuroblasts of the developing tritocerebrum (Urbach and Technau, 2003a). This may indicate that *lab* expressing tritocerebral neuroblasts are affected in *vnd* mutant embryos. Thus, we first investigated whether loss of *vnd* function has any defect on *lab* expressing tritocerebral neuroblast formation.

During the phase of neuroblast formation (developmental stages 8-11), the domain of *lab* expression principally demarcates the intercalary segment (Urbach and Technau, 2003b). As confirmed by antibody double labelings against Lab and the general neuroblast marker Deadpan (Dpn) in wildtype embryos, about 15 neuroblasts have developed by late stage 11 from the (ventral half) of the Lab domain encompassing all neuroblasts of the tritocerebrum (TC) and two adjacent ventral neuroblasts of the deutocerebrum (DC) (Dv2, Dv4; Urbach and Technau, 2003; for nomenclature of brain neuroblasts see Urbach et al., 2003) (Fig. 3A, B). The most ventral part of the Lab domain, from which the two deutocerebral neuroblasts and the ventral neuroblasts of the tritocerebrum (Tv1-5) originate, dynamically coexpresses *vnd* between stages 8-11 (Urbach and Technau, 2003a; and accompanying paper). In *vnd* mutants the extension of the Lab domain from which neuroblasts delaminate appears to be reduced at its ventral sites (Fig.3C, D). Accordingly, we observed that the corresponding set of neuroblasts descending from the Lab domain is diminished. Only about 4-6 Dpn expressing

neuroblasts can be found, although their final number might be slightly higher (about 6-8 neuroblasts), since a few neuroblast-like (enlarged, rounded cells in subectodermal position), but Dpn-negative cells can be found (Fig. 3D). This reduction appears to preferentially affect the ventral but not the dorsal neuroblasts of the TC and adjacent part of the DC. This is supported by the expression of molecular markers indicative for dorsal neuroblasts (e.g. *ladybird early*, *empty spiracles*, *wingless*; see Urbach and Technau, 2003b) in *vnd* mutants (see accompanying paper).

These data suggest that *vnd* is required for the formation of a subset of *lab* expressing neuroblasts in the developing tritocerebrum. However, the late *vnd* mutant brain phenotype shows that neuronal cells interconnecting the protocerebrum and the suboesophageal ganglion are strongly reduced in number or even lacking (Fig. 2B, D). Thus, defective neuroblast formation cannot fully account for the late *vnd* mutant brain phenotype, especially since the majority of tritocerebral *lab* expressing cells are detectable by late stage 11 (Fig. 3C, compare to 3A). Accordingly, in addition to defective neuroblast formation, other mechanisms must account for the late *vnd* mutant brain phenotype. Given that both the late *vnd* mutant brain phenotype and the late *lab* mutant brain phenotype are characterized by the lack of neuronal marker expression, this may indicate that *vnd* is required also later in development for the proper formation of the tritocerebral neuromere - either by acting directly on *lab* expression or by a *lab*-independent requirement.

vnd and _lab_ act independently in tritocerebral neuromere formation

Thus, we determined whether *vnd* and *lab* show overlapping expression also at later stages during the development of the tritocerebrum. Using double-immunolabeling of anti-LAB and anti-VND antibodies, a partial overlap of *vnd* and *lab* expression was detected throughout late embryogenesis. At stage 15, this overlap is most prominent in the ventral portion (according to neuraxis) of the developing tritocerebrum (Fig. 4B). Next, we analysed the expression of the *lab* gene in late *vnd* loss-of-function mutant brains. Figure 4D shows a stage 15 *vnd* mutant brain stained with a neuron specific anti-HRP antibody. As expected, a marked reduction is seen in the tritocerebral region, longitudinal connectives are absent and only a thin strand of cells (arrow) interconnects the protocerebrum with the suboesophageal ganglion. As shown in Figure 4D, expression of *lab* in the same *vnd* mutant is confined to

thin residual strand of cells. This thin strand of cells located at the dorsal-most position of the developing tritocerebrum might correspond to the non-overlapping expression domain of *vnd* and *lab* (see Fig. 4B, arrow).

In order to determine whether normal expression of *vnd* occurs in the absence of *lab*, we also studied the expression of *vnd* in *lab* loss-of-function mutants. In *lab* null mutants, cells in the tritocerebral mutant domain are generated and can be visualized by a *lab*-specific reporter construct (Hirth et al., 1998). Surprisingly, despite the lack of expression of neuronal differentiation markers in the cells of the *lab* mutant domain (Fig. 4E), *vnd* is expressed normally and shows partial overlap with the *lab* mutant cells as visualized by the 7.31 *lab-LacZ* reporter construct (Tremml and Bienz, 1992; Fig. 3F). This suggests that the expression of *vnd* is not affected by the absence of *lab* during embryonic brain development. Moreover, the presence of Lab immunoreactivity in the developing intercalary segment and tritocerebral neuromere of *vnd* mutants (Fig. 3C, D; Fig. 4D) suggests that *vnd* acts independently to *lab* in the specification of the tritocerebrum. Taken together these findings suggest that the dorsoventral patterning gene *vnd* and the anterior-posterior patterning gene *lab* act in a genetically independent manner in tritocerebral neuromere formation during embryonic brain development.

Increased apoptosis in *vnd* mutant tritocerebrum

In addition to defective neuroblast formation, our data suggest that the late patterning defect in the tritocerebrum observed in *vnd* mutants might be caused by the reduction or absence of neuronal tissue that normally develops in this territory. To determine if the patterning defects in the late *vnd* mutant brains are due to late patterning defects in the mutant domain, we analysed transgenic flies in which the 7.31 *lab-LacZ* reporter construct was introduced into a *vnd* null mutant background. At stage 12, expression of this reporter construct in a wildtype background reflects endogenous *lab* expression in the tritocerebrum (Fig. 5B, G, compare to A, F). A comparison of endogenous *lab* and 7.31 *lab-LacZ* reporter gene expression in the wildtype (Fig. 5A, B) with 7.31 *lab-LacZ* reporter gene expression in *vnd* null mutants at early stage 12 (Fig. 5C) shows that the *lab*-expressing tritocerebral domain is slightly reduced in size (see also Fig. 3C, D) but not deleted in the *vnd* null mutant. However, by late stage 12, comparison of endogenous *lab* and 7.31 *lab-LacZ* reporter gene expression in the wildtype

(Fig. 5F, G) with 7.31 *lab-LacZ* reporter gene expression in *vnd* null mutants shows that the *lab*-expressing tritocerebral domain is markedly reduced in the *vnd* null mutant (Fig. 5H). This suggests that in addition to defective neuroblast formation, loss of *vnd* affects the maintenance of neuronal cells in the *lab*-expressing tritocerebral domain.

Analysis of programmed cell death (apoptosis) reveals that a significant increase in apoptotic activity can be observed between early and late stage 12 in *vnd* mutant embryos as compared to wildtype. Figure 5D shows that at early stage 12, a low level of apoptotic activity as assayed by TUNEL staining is associated with the tritocerebral *lab* expressing domain, as is the case for other cells of the developing procephalic region (Nassif et al., 1998, Abrams et al., 1993). However, TUNEL staining together with endogenous *lab* expression in early stage 12 *vnd* mutants reveal a decrease in the number of anti-LAB positive cells and an increase in the number of TUNEL-positive apoptotic cells in this region as compared to the wildtype situation (Fig. 5D, E, see also accompanying paper). By late stage 12, a significant reduction of the number of anti-LAB positive cells is observed in the *vnd* mutant as compared to wildtype. Moreover, a low level of apoptotic activity is associated with the *lab* expressing tritocerebral cells in the *vnd* null mutant and no obvious difference to the wildtype can be detected (Fig. 5 I, J). This suggests that the marked reduction of *lab* expressing cells in the tritocerebrum occurs as early as embryonic stage 12 and that this reduction is caused by defective NB formation and increased apoptosis of neuronal tissue in this region.

In order to further substantiate these results, we wondered whether blocking apoptosis might prevent the loss of cells in the *vnd* mutant and correspondingly might rescue the late *vnd* mutant brain phenotype. Thus, the GAL4-UAS system was used for targeted inhibition of apoptosis during embryonic brain development. For this, a transgenic fly line carrying a Gal4 transcriptional activator under the control of the *scabrous* regulatory elements (Klaes et al., 1994; Sprecher et al., 2004) was used to misexpress P35, an inhibitor of cell-death effector caspases (Mergliano and Minden, 2003), in a *vnd* null mutant background. Figure 6A shows the resulting stage 15 *vnd* mutant brain immunolabeled with a neuron specific anti-HRP antibody. Remarkably, due to the block of apoptosis, a virtually complete rescue of the affected region is obtained (Fig. 6A, arrow). Both, the cells of the deutocerebrum and tritocerebrum as well as the descending longitudinal connectives are restored and are

comparable to the wildtype situation (see Fig. 4A). Moreover, using anti-LAB immunolabeling, *lab* expression appears largely normal in the tritocerebrum and positioned correctly both in anterior-posterior and dorsoventral extent (compare Fig 6B with Fig. 4B). This suggests that the late expression of *lab* is not affected by the absence of *vnd* in the embryonic brain as long as apoptosis is prevented. Furthermore, these observations suggest that the late tritocerebral patterning defects observed in *vnd* mutant embryos are primarily due to increased apoptotic activity and the subsequent loss of neuronal tissue in this domain.

DISCUSSION

Previous analyses have demonstrated that the genes *vnd*, *ind* and *msh* are required for the columnar subdivision of the neuroectoderm and the subsequent formation and determination of neuroblasts along the dorsoventral axis during *Drosophila* embryogenesis (reviewed by Skeath and Thor, 2003). In the case of *vnd*, detailed studies have shown that *vnd* is required for the specification of the ventral neuroectodermal column and specific neuroblasts, and that the absence or misspecification of ventral neuroblasts correlate with the loss or mis-specification of neuronal progeny. For example, the aCC/pCC and dMP2/vMP2 neurons are lost and the RP2 neuron is frequently absent in *vnd* mutant embryos, resulting in axonal pathfinding defects and defective commissure formation in the developing VNC (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002).

In contrast to the developing VNC, expression of the DV patterning genes in the procephalic neuroectoderm region and during brain neuroblast formation is confined to restricted domains along the anteroposterior axis. *vnd* expression demarcates the ventral part of the posterior border of the tritocerebrum, deutocerebrum and ocular neuromere, and *msh* demarcates the dorsal anterior border of the deutocerebrum, implying that these genes might be required for providing positional information in the procephalic neuroectoderm and for subsequent specification of individual brain neuroblasts along the AP axis (Urbach and Technau, 2003b; Urbach and Technau, 2004). Our results presented here provide genetic evidence that the DV columnar gene *vnd* is involved in anteroposterior patterning both at the level of neural

precursor formation as well as neuronal progeny maintenance during embryonic brain development of *Drosophila*.

vnd is required for tritocerebral neuromere formation

Starting from neuroectoderm and brain neuroblast formation, *vnd* expression is seen in each developing neuromere (see also accompanying paper) and subsequently becomes restricted to the posterior boundary regions of each brain segment. We have focused our study on the role of *vnd* in the formation of the tritocerebral neuromere, and our mutant analysis suggests that *vnd* acts at least during two important steps in its development: precursor cell development and neuronal progeny maintenance. Thus, in *vnd* loss of function mutants, the *lab* expressing neuroblasts Tv1-5 are not detectable, suggesting that *vnd* is required for the formation of ventral neuroblasts of the developing tritocerebrum. Later in development, *vnd* mutants display a severe loss of neuronal tissue together with axonal patterning defects in the tritocerebrum. This loss of neuronal tissue is associated with increased apoptotic activity, suggesting that *vnd* is required for the maintenance of neuronal cells that are involved in the establishment of the tritocerebral commissure and the longitudinal connectives that normally run through this neuromere. This suggestion is further supported by the fact that blocking apoptosis in *vnd* null mutant embryos results in the restoration of tritocerebral axon tracts and the wildtype-like expression domain of the Hox gene *labial*.

Taken together, these functional roles of *vnd* in embryonic brain development are reminiscent of its role during VNC development: the formation of specific neuroblasts and their progeny (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002). However, in contrast to its role in DV patterning of the developing VNC, the resulting brain phenotype of *vnd* mutants suggests that *vnd* is required for brain patterning along the anteroposterior axis. This is surprising especially since *vnd* expression is confined to the ventral portion of the developing tritocerebrum (Urbach and Technau, 2003b; Urbach and Technau, 2004). Thus, *vnd* action in ventral precursor cells and subsequent neuronal progeny within the developing tritocerebral neuromere is apparently conveyed into patterning along the anteroposterior neuraxis. We propose that this is achieved by *vnd* acting on cells that express the Hox gene *labial.*

Brain neuromere formation by integrated activity of *vnd* and *lab*

Expression of the *Drosophila* Hox gene *labial* is seen in identified intercalary neuroblasts (Urbach and Technau, 2003a; Urbach and Technau, 2004) and subsequently becomes restricted to the posterior tritocerebrum (Hirth et al., 1998; Page, 2000; Sprecher et al., 2004). In *lab* loss-of-function mutants, mutant cells are generated and positioned correctly in the tritocerebral domain, but these cells do not extend axons and defective commissural and longitudinal axon pathways occur: the tritocerebral commissure is absent and the longitudinal pathways between the supraesophageal and subesophageal ganglia are reduced or absent. Immunocytochemical analyses demonstrated that cells in the mutant domain do not express neuronal markers such as HRP and ELAV, suggesting that *lab* is involved in the specification of tritocerebral neuronal identity in the *Drosophila* brain, (Hirth et al., 1998; Page, 2000; Hirth et al., 2001; Sprecher et al., 2004).

Our results on the expression and function of *vnd* during embryonic brain development suggest that *lab* and *vnd* act in an integrated manner but independently in the formation and specification of the tritocerebral neuromere. Although *vnd* and *lab* show overlapping expression in tritocerebral neuroblasts (Urbach and Technau, 2003b; Urbach and Technau, 2004) and subsequently in neuronal cells of the posterior tritocerebrum (this study), expression of *vnd* appears unaffected in *lab* mutant cells. Conversely, *vnd* does not act on *lab* expression since the complete absence of *lab* expression in *vnd* mutants (with the exception of a rare thin strand of neuronal cells) reflects a secondary defect because of the absence of cells that normally express *lab*. This independent genetic activity of *vnd* and *lab* is further supported by the fact that blocking apoptosis in *vnd* null mutant embryos results in the wildtype-like expression of *lab.* Thus, although the *lab* and *vnd* mutant brain phenotypes result in comparable axonal patterning defects (loss of the tritocerebral commissure as well as longitudinal connectives that normally run through this neuromere), their mode of action within the developing tritocerebrum is different. Our results suggest that *vnd* is required for the formation and maintenance of neural precursor cells as well as neuronal progeny within the developing tritocerebral neuromere, whereas the Hox gene *lab* appears to be independently required for the specification of neuronal identity within the same territory during later stages (Hirth et al., 1998; Page, 2000; Hirth et al., 2001; Sprecher et al., 2004). This indicates that the activity of the columnar gene *vnd* is integrated into pattern formation

along the anteroposterior neuraxis by generating and maintaining cells which subsequently become specified by the activity of the Hox gene *lab*.

vnd/*Nkx2* genes in brain development and evolution

The *Drosophila* columnar gene *vnd* belongs to the highly conserved *Nkx2* class of transcription factors that have been found in various animals including mammals (Harvey, 1996; Cornell and von Ohlen, 2000). Notably, the *vnd/Nkx2* family of genes is exceptionally well conserved, in terms of both expression and function. Thus, the vertebrate homologs of *vnd* are expressed in the neural plate, or tube, in topologically similar positions as is *vnd* in the *Drosophila* ventral neuroectoderm and in the absence of *vnd/Nkx2* genes, the fates of the ventral-most cells in the spinal cord and the Drosophila VNC are transformed (Cornell and von Ohlen, 2000; Rallu et al., 2002). Moreover, this evolutionary conservation in expression and function of *vnd/Nkx2* genes appears to apply to some extend to brain development as well. A comparison of the anteroposterior sequence of *vnd/Nkx2* gene expression in the early brain of *Drosophila* with that published for the early mouse brain reveals striking similarities (Urbach and Technau 2003b; 2004).

In terms of function, genetic knockouts in mice have shown that *Nkx2* genes appear to play a crucial role in patterning and neuronal specification during embryonic development of the telencephalon and hindbrain. *Nkx2.1* mutant mice display patterning defects in that the entire pituitary is missing (Kimura et al., 1996), a twofold reduction in the number of cortical interneurons, as well as a complete absence of TrkA-expressing cells in the developing telencephalon is observed, and the ventral-most aspect of the telencephalon – the medial ganglionic eminence – becomes trans-fated to that of the adjacent, more dorsal lateral ganglionic eminence (Sussel et al., 1999). Thus, comparable to the role of *vnd* during *Drosophila* brain development (this study and accompanying paper), Nkx2.1 is involved in pattern formation and in cell fate determination during embryonic brain development in mice (Rallu et al., 2002)

In addition, recent studies have shown that *Nkx2.2* is involved in motor neuron specification in the developing hindbrain. Thus, the sequential generation of visceral motor neurons and

serotonergic neurons from a common pool of neural progenitors located in the ventral hindbrain critically depends on the integrated activities of Nkx2.2- and Hox1/2-class homeodomain proteins (Pattyn et al., 2003a; 2003b). A primary function of these proteins is to coordinate the spatial and temporal activation of the homeodomain protein Phox2b, which in turn acts as a binary switch in the selection of motor neuron or serotonergic neuronal fate (Pattyn et al., 2003a; Samad et al., 2004). These data suggest that comparable to the integrated activity of *vnd* and *lab* in *Drosophila* brain neuromere specification, integrated activity of the Nkx2.2 and Hox1/2 proteins is involved in the specification of segmental neuronal identity (Samad et al., 2004). This indicates that the integration of AP and DV patterning systems by homeodomain transcription factors of the *Hox* and *vnd/Nkx2* genes might represent an ancestral feature of insect and mammalian brain development (Hirth et al., 2003). It will be interesting to determine whether these similarities in homologous gene action also extend to an integrated activity of *vnd* and *Hox* genes in motor neuron subtype specification during embryonic brain development of *Drosophila*.

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FIGURES:

Figure. 1.

Spatio-temporal expression of *vnd* during embryonic brain development. Neuronal expression domains and a corresponding 3D reconstructed model of the head region are shown for embryonic late stage 12 (A, B), and stage 15 (C, D). (A) embryo double immunolabeled with an anti-Neurotactin (NRT) antibody (red) and an anti-VND antibody (green/yellow); (C) Double immunolabeling with a neuron-specific anti-HRP antibody (red) and anti-VND antibody (green, yellow). (A) At stage late 12, three *vnd* expression domains become apparent. (C) At stage 15 these three domains are still observable. (B, D) 3D reconstructed models show the relative location of these domains within the developing brain (blue, protocerebral *vnd* expression domain blue; green, deuterocerebral *vnd* expression domain; red, tritocerebral *vnd* expression domain. (A, C) laser confocal microscopy, reconstructions of optical sections, lateral views. (B, D) 3D reconstructed models of the confocal microscopic stacks, covering the corresponding optical sections.

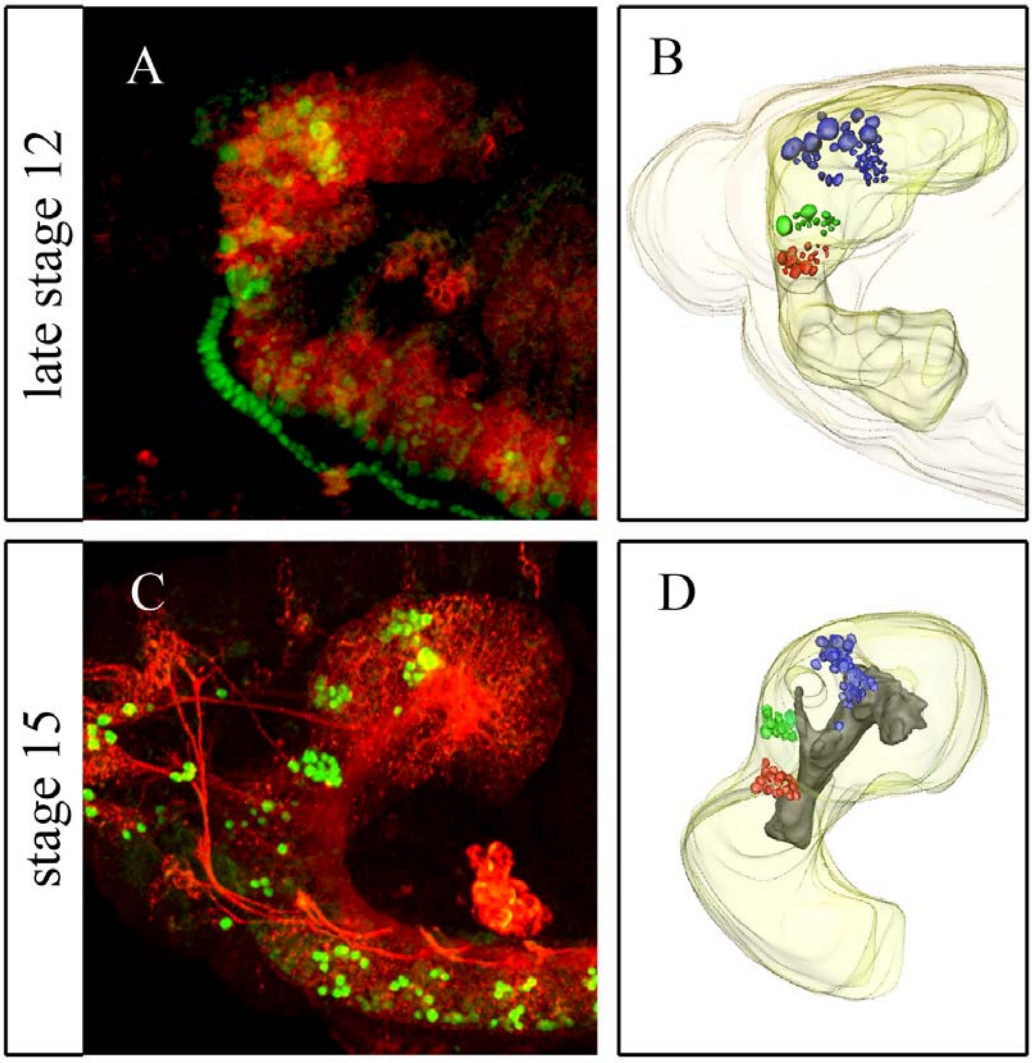

late stage 12
A
B
stage 15
C
D

Figure. 2.

Mutant brain phenotype observed in *vnd* null mutant embryos at embryonic stage 15. Laser confocal microscopy reconstructions of optical sections, lateral views. (A, B) Double immunolabeling with anti-HRP antibody (red) and an anti-EN antibody (yellow/green). (C, D) Double immunolabeling with an anti-HRP antibody (red) and a neuron-specific anti-ELAV antibody (yellow/green). (E, F) Double immunolabeling with an anti-HRP antibody (red) and a glial-specific anti-REPO antibody (yellow/green). Arrows indicate the region of the tritocerebrum and deutocerebrum. (A) In the wildtype situation, the protocerebral b1 *en*-stripe (b1), the deuterocerebral b2 *en*-stripe (b2), the tritocerebral b3 *en*-stripe and the anteriormost *en* expressing secondary head spot (shs) are visible (arrowheads). (B) In contrast, in *vnd* null mutant embryos only the b1 *en*-stripe and the *en* expressing secondary head spot are present (arrowheads), and the neuron-specific HRP marker reveals a cellular gap in the region of the tritocerebrum and deuterocerebrum (arrow). (C) In the wildtype the neuron-specific marker ELAV reveals all neural cell bodies. (D) In contrast, in *vnd* null mutants a large gap is seen in the area of the tritocerebral/deuterocerebral region (arrow). (E) The glia-specifc marker REPO reveals the localization of the glial cell bodies in the embryonic wildtype brain. (F) In *vnd* mutant embryos, the REPO expressing cells in the residual tritocerebral/deuterocerebral region appear to be present but are severely misplaced (arrow).

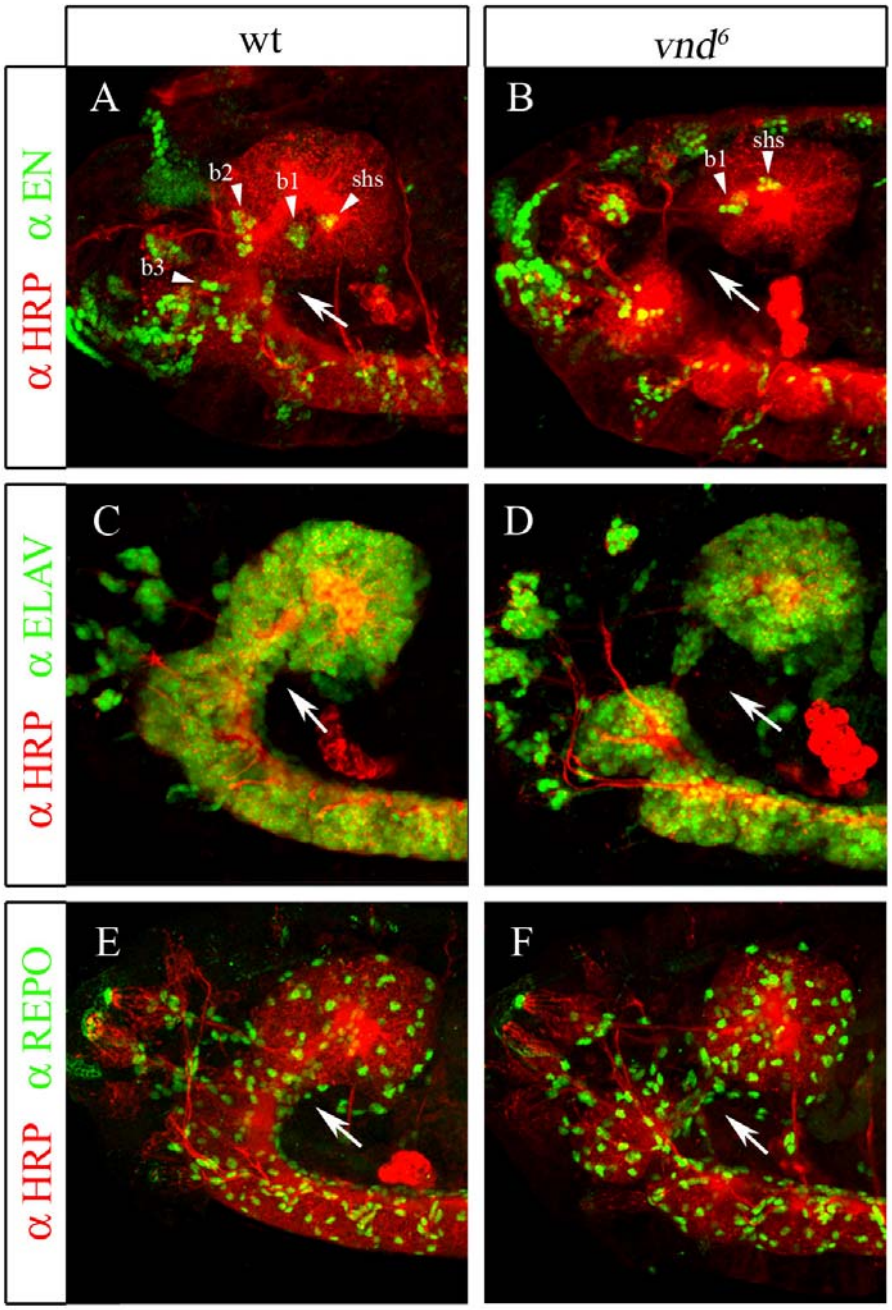

wt
vnd6
A
B
C
D
E
F
α HRP α EN
α HRP α ELAV
α HRP α REPO
b2
b1
shs
b3
b1
shs

Figure. 3.

Defective neuroblast formation in the *lab*-expressing tritocerebral domain of *vnd* mutants. (A-D) Antibody double labelling against the neuroblast-specific marker Deadpan (DPN), in combination with Labial (LAB), at embryonic stage 11, in wildtype (WT) (A,B) and *vnd* null mutants (C, D). (A-D) display ventral views of flat preparations. (A,C) focus on the peripheral head ectoderm, (B,D) depict close-ups of regions indicated in (A, C) by black frames, at the level of brain neuroblasts. (A) In wildtype embryos, all brain neuroblasts have developed by stage 11. (B) Two deutocerebral and the complete set of tritocerebral neuroblasts developing from the LAB domain are indicated (according to the nomenclature of Urbach et al., 2003.: Tv1-5, ventral tritocerebral neuroblasts, Td1-8, dorsal tritocerebral neuroblasts; Dv2, Dv4,4 ventral deutocerebral neuroblasts. The dotted line encircles a group of dorsal neuroblasts which are assumed to be retained in *vnd* null mutants (compare with (D)). (C) In *vnd* null mutants, the overall expansion of the LAB domain appears to be reduced as compared to wildtype (A), and the invagination of the foregut (Fg) is affected (compare the lateral extension of the foregut invagination as marked by red arrowheads in (A) and (C)). (D) The number of DPN-positive neuroblasts (white asterisks) is diminished, as compared to (B). Note, that one neuroblast (white dot) does not express DPN at detectable levels; similarly in WT a neuroblast expressing DPN at significantly lower levels is found in the same relative position (see Td5 in cluster of neuroblasts encircled by dotted line, (B)). Abbreviations: Fg, foregut; Lr, labrum; Md, Mx, mandibular and maxillary segment, respectively.

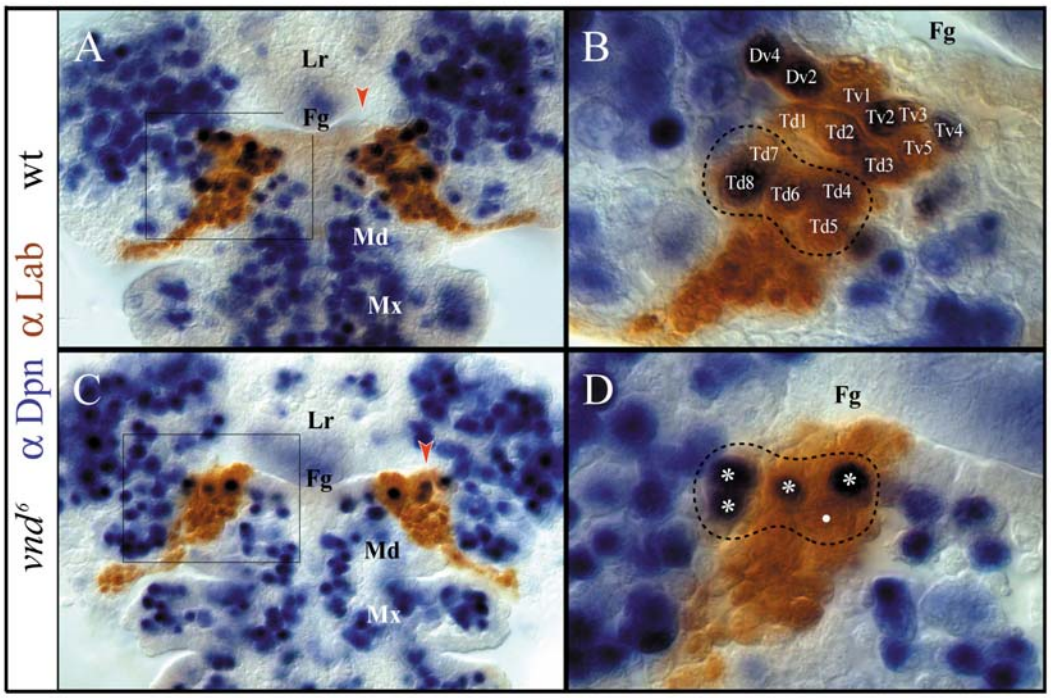

wt
α Lab
α Dpn
vnd⁶
A
Lr
Fg
Md
Mx
B
Fg
Dv4
Dv2
Tv1
Td1
Tv2
Tv3
Td2
Tv4
Tv5
Td7
Td3
Td8
Td6
Td4
Td5
C
Lr
Fg
Md
Mx
D
Fg

Figure. 4.

vnd and the anterior Hox gene *labial* act independently in the development of the tritocerebrum. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections, lateral views. Arrows indicate the tritocerebral region. (A) Wildtype embryonic brain immunolabeled with a neuron-specific anti-HRP antibody (red). (B) Wildtype embryonic brain triple immunolabeled with a neuron-specific anti-HRP antibody (red), an anti-LAB antibody (green), and an anti-VND antibody (blue); co-expression of *vnd* and *lab* is seen in a part of the *lab* expressing tritocerebral domain (arrow). A and B are from the same section. (C) *vnd* mutant embryonic brain immunolabeled with a neuron-specific anti-HRP antibody (red). (D) *vnd* mutant embryonic brain double immunolabeled with a neuron-specific anti-HRP antibody (red) and an anti-LAB antibody (green); only few cells remain in the tritocerebrum and express *lab.* A and B are from the same section. (E) P{ry^{+} 7.31 *lab*-LacZ} in a *lab* null mutant background; embryonic brain immunolabeled with a neuron-specific anti-HRP antibody (red); no anti-HRP immunoreactivity is detected in the tritocerebral domain. (F) P{ry^{+} 7.31 *lab*-LacZ} in a *lab* null mutant background; embryonic brain triple immunolabeled with a neuron-specific anti-HRP antibody (red), an anti-VND antibody and an anti-beta GAL antibody revealing the 7.31 *lab-LacZ* reporter (green). *vnd* expression is seen in a part of tritocerebral domain mutant for *lab* and expression overlaps with *lab-lacZ* specific reporter gene expression. C and D are from the same section.

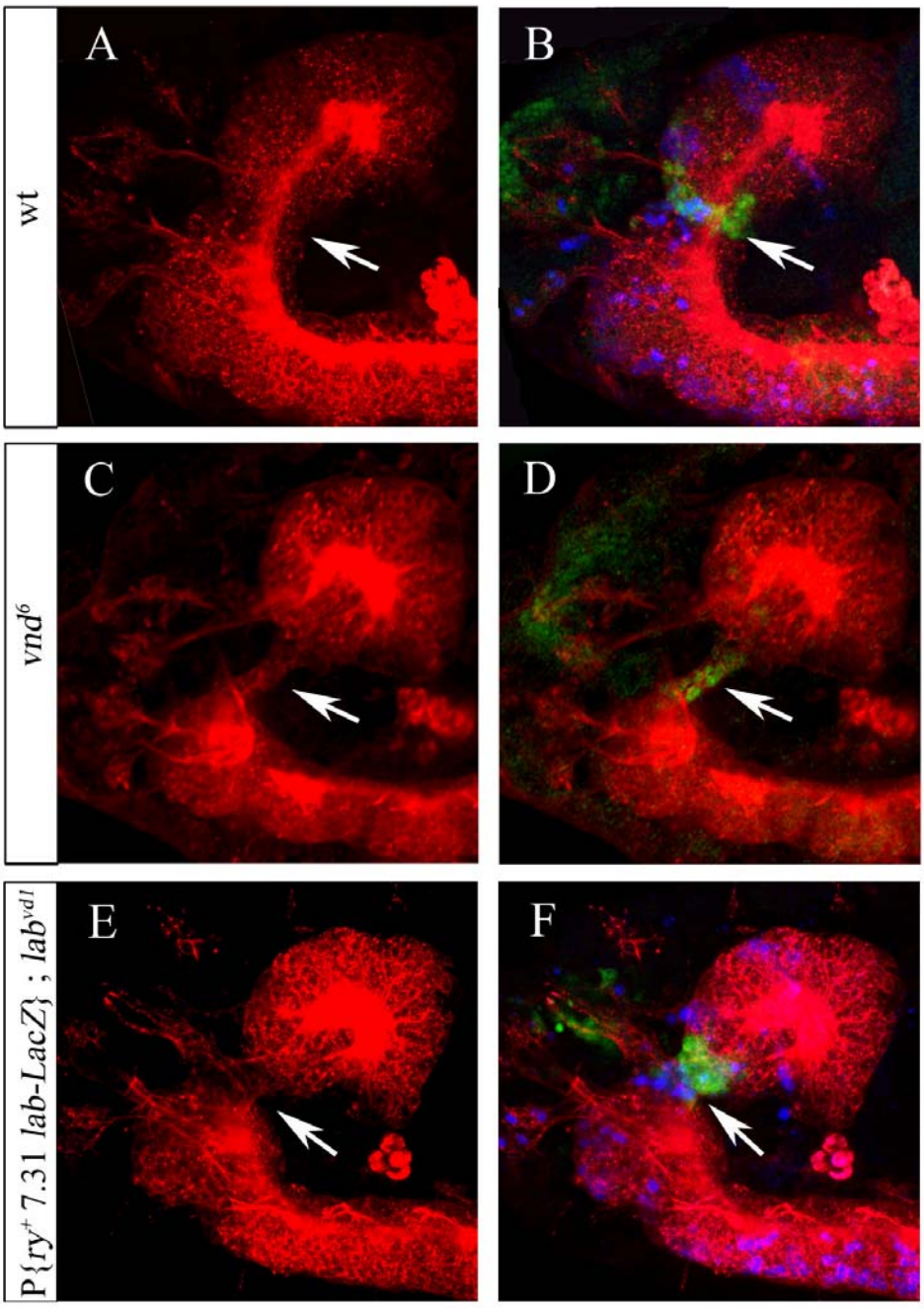

wt
A
B
vnd6
C
D
P{ry+ 7.31 lab-LacZ} ; labvd1
E
F

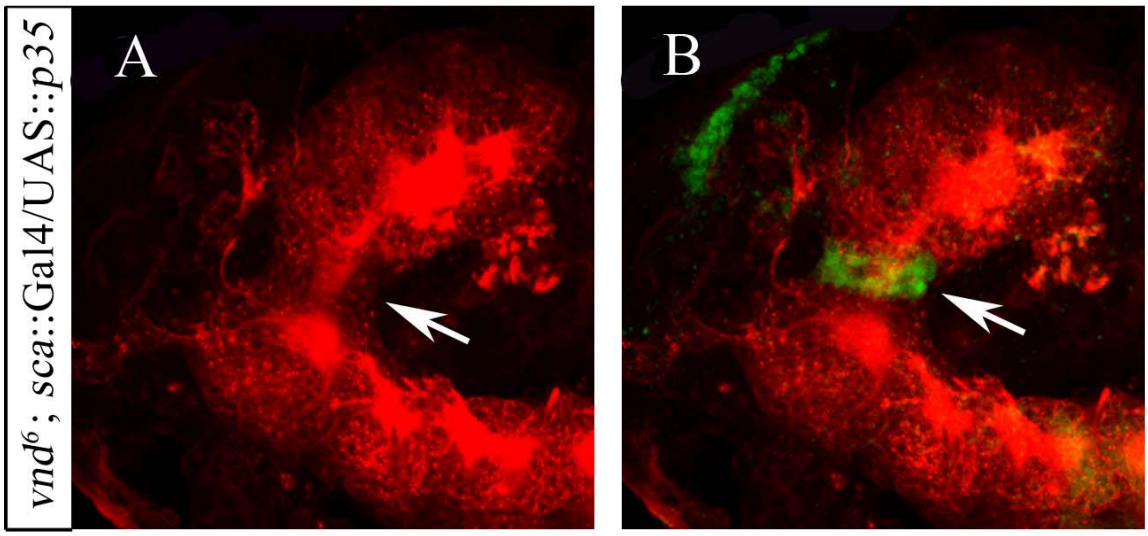
Figure. 5.

Analysis of the brain-deletion phenotype in *vnd* null mutant embryos at embryonic stage 12. Laser confocal microscopy, reconstructions of optical sections, lateral views. In (A-E) embryos at early stage 12 are shown; the extent of the *lab* expressing domain in wildtype embryos at this stage is outlined (white line, arrow) and projected onto each figure in the top row. In (F-J) embryos at late stage 12 are shown; the extent of the *lab* expressing domain in wildtype embryos at this stage is outlined (white line, arrow) and projected onto each figure in the bottom row. (A, F) Wildtype, double immunolabeled with an anti-NRT antibody (green) and an anti-LAB antibody (red) showing the *lab* expression domain (arrow). (B, G) P{ry^{+} 7.31 *lab*-LacZ} in a wildtype background. Double immunolabeling using an anti-NRT antibody (green) and anti-beta GAL antibody shows that the 7.31 *lab-LacZ* reporter construct mimics the endogenous *lab* expression. (C, H) P{ry^{+} 7.31 *lab*-LacZ} in a *vnd* null background. Double immunolabeling using an anti-NRT antibody (green) and anti-betaGAL antibody reveals the extent of the *lab* expression domain as assayed by the 7.31 *lab-LacZ* reporter construct. (D, I) Wildtype double immunolabeled with an anti-LAB antibody (red) and TUNEL staining (green) showing the low level of apoptotic activity in the *lab* domain. (E, J) *vnd* null mutant. Anti-LAB antibody immunolabeling (red) and TUNEL staining (green) shows the increased level of apoptotic activity in the *lab* expression domain at early stage 12.

early stage 12
late stage 12
wt
P{ry+ 7.31 lab-LacZ}
vnd6 ; P{ry+ 7.31 lab-LacZ}
wt
vnd6
A
B
C
D
E
F
G
H
I
J
α NRT α LAB
α NRT α βGal
α NRT α βGal
TUNEL α LAB
TUNEL α LAB

Figure. 6.

Genetic rescue of *lab* expression in *vnd* mutant embryos by blocking apoptosis. Laser confocal microscopy of stage 15 embryos, reconstructions of optical sections, lateral views. Arrows indicate tritocerebral region. (A, B) *sca*::Gal4/UAS::*p35* in a *vnd* null mutant background. (A) Embryonic brain immunolabeled with a neuron-specific anti-HRP antibody (red). *p35*-mediated block of apoptosis restores a wildtype-like tritocerebral region and longitudinal connectives. (B) Embryonic brain double immunolabeled with a neuron-specific anti-HRP antibody (red) and an anti-LAB antibody (green). *p35*-mediated block of apoptosis results in a wildtype-like *lab* expression domain in the tritocerebrum. C and D are from the same section.


vnd6; sca::Gal4/UAS::p35
A
B

The urbilaterian brain: Developmental insights into the evolutionary origin of the brain in insects and vertebrates

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ABSTRACT

Classical phylogenetic, neuroanatomical and neuroembryological studies propose an independent evolutionary origin of the brains of insects and vertebrates. Contrasting with this, data from three sets of molecular and genetic analyses indicate that the developmental program of brains of insects and vertebrates might be highly conserved and suggest a monophyletic origin of the brain of protostomes and deuterostomes. First, recent results of molecular phylogeny imply that none of the currently living animals correspond to evolutionary intermediates between protostomes and deuterostomes, thus making it impossible to infer the morphological organization of an ancestral bilaterian brain from living specimens. Second, recent molecular genetic evidence provides support for the body axis inversion hypothesis, which implies that a dorsoventral inversion of the body axis occurred in protostomes *versus* deuterostomes, leading to the inverted location of neurogenic regions in these animal groups. Third, recent developmental genetic analyses are uncovering the existence of structurally and functionally homologous genes that have comparable and interchangeable functions in early brain development in insect and vertebrate model systems. Thus, development of the anteriormost part of the embryonic brain in both insects and vertebrates depends upon the *otd/Otx* and *ems/Emx* genes; development of the posterior part of the embryonic brain in both insects and vertebrates involves homologous control genes of the *Hox* cluster. These findings, which demonstrate the conserved expression and function of key patterning genes involved in embryonic brain development in insects and vertebrates support the hypothesis that the brains of protostomes and deuterostomes are of monophyletic, urbilaterian origin.

Keywords: *Hox* genes, dorsoventral inversion, brain development, brain evolution, *Drosophila melanogaster*

INTRODUCTION

The brains of insects and vertebrates are highly complex organs. Both types of brains are composed of a large number of differentiated neuronal and glial cell types, which are organized into complex neural networks, and which are capable of orchestrating complex behavioural responses. Comparative neuroanatomical studies carried out on insect and vertebrate brains in their mature, adult stage have, in some cases, revealed structures, which appear to be similarly organized. For example, the relative position of motor and sensory neuropiles are comparable. For the most part, however, the neuroanatomical features of the adult brains of insects and vertebrates, appear to be strikingly different, thus, suggesting that these two brain types are based on different ground plans that derived independently in evolutionary time. This notion of a different “Bauplan” in both animal groups receives support from descriptive neuroembryological studies of early brain development in the two animal groups. Consider, for example the different modes of embryogenesis of the brain in the insect *Drosophila melanogaster* Meigen, 1830 (Diptera, Drosophilidae), as compared to that of the vertebrate mouse (figure 1).

In *D. melanogaster*, the neurons of the embryonic CNS are generated by proliferation of neurogenic stem cells, called neuroblasts, which delaminate from a single-layered ventral ectodermal epithelium, the neuroectoderm. These neurons differentiate and form complex ganglionic arrangements of neuropil and cell body regions. The anterior part of the embryonic brain, the supraesophageal ganglion, is generated by neuroblasts in the region of the procephalic neuroectoderm (Younossi-Hartenstein et al., 1996, 1997). The posterior part of the embryonic brain, the subesophageal ganglion, derives from delaminating neuroblasts of the anteriormost part of the ventral neuroectoderm (Bossing et al., 1996; Hartmann and Reichert, 1998). The procephalic neuroectoderm is specified through genetic interactions during gastrulation (Campos-Ortega and Hartenstein, 1997). Neuromeres of the subesophageal ganglion are under the control of a regulatory gene network, which also acts in the ventral nervous system (ventral nerve cord, VNC) of the trunk segments (Cohen and Jürgens, 1991; Doe, 1996). Both the supraesophageal and the subesophageal ganglion are thought to comprise 3 neuromeres (Younossi-Hartenstein et al., 1996).

In the mouse, the brain derives primarily from neural progenitors localized in a neural tube that invaginates from the dorsal neuroectoderm. Initial induction and patterning of the anterior dorsal neuroectoderm, which gives rise to the presumptive brain, are due to organizer-like actions of adjacent tissue and involve, among other structures, the anterior visceral endoderm (AVE), a component of the extraembryonic tissue, and the dorsal axial mesoderm (Brewster and Dahmane, 1999; Beddington and Robertson, 1999; Rallu et al., 2002). The process of early regionalization of the anterior neuroepithelium results in a tripartite organization of the embryonic brain consisting of the developing forebrain, (prosencephalon; telencephalon/dienecephalon), midbrain (mesencephalon), and hindbrain, (rhombencephalon; metencephalon/myelencephalon). The embryonic vertebrate hindbrain has a basic neuromeric organization and is subdivided into eight lineage-restricted cellular compartments or rhombomeres (Lumsden and Krumlauf, 1996). The embryonic vertebrate forebrain may also have a basic neuromeric organization and be composed of six distinct transverse metameric regions termed prosomeres (Rubenstein et al., 1994, 1998).

Contrasting with the different neuroanatomical and neuroembryological characteristics of insect and vertebrate brains, which argue for an independent evolutionary origin of the two brain types, are remarkable similarities in the molecular and genetic control mechanisms, which govern their embryonic development. Indeed, a number of evolutionarily conserved control genes implicated in embryonic brain development have been identified recently, and the expression domains and mutant phenotypes of these control genes reveal a high degree of conservation from vertebrates to insects. Thus, *Otx* and *Emx* gene families are essential for proper development of the anterior brain; 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. Moreover, the genes of the homeotic *(Hox)* complex are important for patterning of the developing posterior brain and CNS in insects and vertebrates; similar *Hox* gene mutant phenotypes are indicative of comparable function of homologous genes in embryonic development of the two brain types.

Here we review the notions of different *versus* common evolutionary origins of the insect and vertebrate brains in the light of recent molecular genetic evidence derived from several different areas. We reconsider the phylogeny-based and embryology-based evidence for

different *versus* common origins of basic organ system organisation in insects and vertebrates in view of the results of molecular phylogeny and molecular embryology. Subsequently, we review in more detail the implications of the studies on expression and function of key developmental control genes that direct the early embryogenesis of the brain. For this, we focus on the remarkably similar roles of *Otx, Emx,* and *Hox* gene families in *D. melanogaster* and mouse brain development.

Implications on the origin of the brain from animal phylogeny

Many classical animal phylogenies place specific clades, whose members are characterized by apparently simple morphological complexity, near the ancestral urbilaterian. For example, in the classical animal phylogeny shown in simplified form in figure 2a, the platyhelminths might considered to be most representative of basal ancestral bilaterians (Brusca and Brusca, 1990). Since the CNSs of extant representatives of clades such as the platyhelminths are often regarded as simple neuronal networks with a low degree of cephalisation, it would follow from this phylogeny that the CNS of the last common ancestor of bilaterians, the urbilaterian, might also have been characterized by simple CNS structures which most probably did not include cephalized brain ganglia. This could imply that the Bauplan for brains evolved only in the lineages that gave rise to protostomes and deuterostomes. Moreover, in the deuterostome lineage, phyla such as echinoderms and lophophorates, which are taken to be representative of ancestral deuterostomes, have CNS structures that are also often simple and rather diffuse in organization, and in some cases lack cephalized ganglia completely. This would imply that the types of brains that characterize chordates might have evolved during the evolution of deuterostome animals. Thus, on the basis of classic phylogeny, it has been proposed that the basic organisation of brains of protostome insects and deuterostome vertebrates evolved independently.

This assumption of an independent evolutionary origin of vertebrate and invertebrate brains must be reconsidered in view of recent findings of molecular systematics, which result in a new version of animal phylogeny that contrasts markedly with that of classical phylogeny. This new molecular phylogeny is based on small ribosomal subunit RNA analysis as well as on homeotic *Hox* gene analysis, and presents a deeply changed phylogenetic tree (for review

see Adoutte et al., 2000). A simplified version of one of these molecular phylogenies of animals is shown in figure 2b. The most striking change that results from this analysis is that there are no longer any living animals that can be considered as evolutionarily ancestral basal bilateria. Thus several invertebrate phyla that were previously thought to be ancestral, such as platyhelminths, which are considered as having simple and diffuse CNS structures, have now been placed within the protostomes. Indeed, the platyhelminths are now placed among the lophotrochozoan protostomes at the same level with phyla such as molluscs. Since the apparently "advanced" phyla such as arthropods and molluscs include groups with a high degree of complexity in brain structures and body morphology, this revised phylogeny implies that the apparently ancestral clades such as extant platyhelminths and nematodes might have body and brain morphologies that are simplified structures. Similarly striking changes characterize the molecular phylogeny of deuterostomes. Thus, according to the new molecular systematics, the hemichordates and echinoderms are considered to be sister groups of chordates (Adoutte et al., 2000). Furthermore, the morphologically ancestral lophophorate clades have been removed from the deuterostome lineage altogether and have been placed firmly within the lophotrochozoan protostomes (Halanych et al., 1995).

These changes in animal phylogeny leave no example of an extant basal group located between the protostome and the deuterostome lineages. This leads to an information gap and prohibits reconstruction of the features of the urbilaterian animal, and its urbilaterian brain, based on comparative anatomical and neuroanatomical information alone (Adoutte et al., 1999). Put in different terms, the new molecular phylogeny of animals indicates that there are no living ancestral bilaterians, which could give us neuroanatomically-based insight into the organization of an ancestral urbilaterian brain. Thus, the urbilaterian brain could have been a rather complex neuronal structure compared, for example, with the relatively simple CNS of extant platyhelminth planarians. Indeed, the general morphological organization of the urbilaterian might not have been as simple as has been generally assumed. It has been proposed that the genome of the urbilaterian might have consisted of 12,000 to 18,000 genes, comparable to the complexity of the genome of *D. melanogaster* (Rubin et al., 2000). This suggests that the complex genetic organization that controls morphological development in extant animals such as *D. melanogaster* might already have evolved before the deuterostome and protostome lineages separated. The recent discovery of homologies in the molecular

genetic control of body axis determination of insects and vertebrates indicates that this is, in fact, the case, and provides further evidence for a monophyletic origin of complex organ systems such as the brain in bilaterians.

Dorsoventral body axis inversion between protostomes and deuterostomes

In classical embryology, the morphologically opposite location of the nerve cord in insects (ventral) and the spinal cord (dorsal) in vertebrates has been taken as evidence, that these two types of nervous systems evolved independently. Accordingly, invertebrates such as insects were grouped as Gastroneuralia and chordates were assigned to Notoneuralia (Hatschek, 1891). The Gastroneuralia including protostomes such as arthropods, annelids and molluscs, shared the common feature of a ventral nerve cord, whereas the Notoneuralia, including vertebrates, cephalochordates, and urochordates, were characterized by a dorsal nerve cord. Contrasting with the notion that the nervous systems of Gastroneuralia and Notoneuralia evolved independently is the hypothesis that the dorsoventral body axis might be inverted in these two animal groups. The idea that the dorsal side of vertebrates might correspond to the ventral side of insects has been put forward repeatedly, beginning with the work of Geoffroy St.Hilaire in the early nineteenth century, and classically was based on morphological considerations. Recently, a wealth of new findings deriving from developmental, genetic and molecular experiments in *D. melanogaster* and several vertebrate model systems such as frog, zebrafish and mouse, have provided strong support for the idea that the Bauplan of protostomes, such as insects, represents a dorsoventral inversion of the Bauplan of deuterostomes, such as vertebrates, due to an evolutionary body axis inversion between these two groups (Arendt and Nübler-Jung, 1994; De Robertis and Sasai, 1996). This molecular embryonic evidence is summarized in figure 3. If this hypothesis is correct and an ancestral body axis inversion did occur, this would imply that evolutionarily equivalent body sides give rise to the CNS of both protostomes and deuterostomes, and, therefore, that the CNS of these two animal groups might indeed be homologous.

One line of experimental support for the body axis inversion hypothesis is provided by the analysis of the two antagonistically acting signalling systems that control dorsoventral axis establishment in vertebrates and insects. One of these signalling systems is represented by

genes of the Transforming Growth Factor β (TGFβ) family. A member of this gene family in the insect *D. melanogaster* is the *dpp* (*decapentaplegic*) gene; its vertebrate homolog is the *BMP4 (Bone Morphogenetic Protein 4)* gene. In both organism types the Dpp/BMP4 proteins are involved in establishing embryonic dorsoventral polarity, however, their site of action is localized to the dorsal side of insects and to the ventral side of vertebrates. The antagonistically acting extracellular signalling proteins are encoded by the *sog* (*short gastrulation*) gene in *D. melanogaster* and a homologous *Chordin* gene in vertebrates; Sog protein acts from the ventral side in insects whereas Chordin protein acts from the dorsal side in vertebrates (Holley et al., 1995; De Robertis and Sasai, 1996). Interestingly, in both insects and vertebrates, it is the region of the embryo where *sog*/*Chordin* is expressed and thus inhibits *dpp*/*BMP4* signalling, which later adopts neurogenic potential and gives rise to the neuroectoderm. The fact that homologous signalling proteins, engaged in similar molecular interactions, lead to the initial induction of the ventral neurogenic region in insects and the dorsal neurogenic region in deuterostomes strongly supports the idea of an homology of the early embryonic CNS in these animal groups.

In addition to the signalling systems, which initially induce neurogenic potential, a set of further genetic interactions implicated in establishing early dorsoventral patterning in the CNS appears to be evolutionarily conserved (Chan and Jan, 1999; Cornell and Von Ohlen, 2000). The genes that control these interactions also manifest a dorsoventral inversion in their relative expression domains in protostomes and deuterostomes. In *D. melanogaster*, the key genetic players are the homeobox genes *vnd* (*ventral nerve cord defective*), *ind* (*intermediate neuroblasts defective*), *msh* (*muscle specific homeobox*). These genes are involved in the formation of columnar dorsoventral domains in the ventral neuroectoderm and are essential for neuroblast formation and specification in the ventral (*vnd*), intermediate (*ind*) and dorsal (*msh*) columns of the neuroectoderm (McDonald et al., 1998; Chu et al., 1998; Weiss et al, 1998). In the developing neural plate of vertebrates, the homologous genes of the *Nkx2* (*vnd*), *Gsh* (*ind*) and *Msx* (*msh*) families are similarly involved in dorsoventral patterning. For example, in the mouse, the *Nkx2.2* gene is required for cell fate specification in the column in which it is expressed, indicating the functional equivalence of the *Nkx2.2* gene to the *vnd* gene of *D. melanogaster* (Briscoe et al., 1999); (Interestingly, neurons deriving from precursor cells within a given neurogenic column in vertebrates and insects are also sometimes similar.

For example precursors of the medial column give rise to interneurons that pioneer the medial longitudinal fascicles, and to motorneurons that exit via lateral nerve roots and then project peripherally, in both animal groups; Arendt and Nübler-Jung, 1999). Importantly in the context of the dorsoventral body axis inversion hypothesis, the relative position of the expression domains of these columnar specification genes in the CNS of vertebrates is inverted in dorsoventral polarity as compared to the CNS expression domains of the homologous *D. melanogaster* genes.

The most reasonable explanation for the strikingly similar functional properties and relative expression pattern of these control genes in the developing CNS of protostomes and deuterostomes is that of a common evolutionary origin of the nerve cords in these two animal groups. It is, thus, likely that the ventral CNS of insects and the dorsal CNS of vertebrates are homologous and that their opposite location relative to the body axis is due to an ancestral dorsoventral inversion between protostomes and deuterostomes. It should, however, be mentioned that several alternative explanations to the dorsoventral inversion hypothesis have been proposed (reviewed in Gerhart, 2000). These hypotheses consider movements of ciliary rows (and the associated nerves), condensation of multiple nerve cords or nerve nets, differential evolution of a two-part nervous system and ambiguous dorsoventral organisations of potential evolutionary intermediates as basic explanations for the origins of nerve cords in vertebrates *versus* invertebrates (Nielsen, 1999; Lacalli, 1996; Salvini-Plawen, 1998; Gerhart; 2000). Currently, neither none of these alternative explanations, nor an extreme case of evolutionary convergence can be ruled out as explanations for these findings. However, a consideration of several key control genes involved in anterior-posterior patterning of the developing brain and nerve cord of insects and vertebrates provides additional and independent support for a common Bauplan and, concomitantly, for a common evolutionary origin of the bilaterian CNS.

Cephalic gap genes pattern the anterior brain in insects and vertebrates

In *D. melanogaster*, the cephalic gap genes, also termed head gap-like genes, share characteristics with the "classical" gap genes involved in development of the trunk. First, their initial expression is under the control of maternal effect genes; their transcripts are found in

specific domains where they transform maternal positional information into the anteroposterior patterning system of early embryogenesis (Dalton et al., 1989; Finkelstein and Perrimon, 1990a; Walldorf and Gehring, 1992). Second, loss-of-function phenotypes of cephalic gap genes such as *otd (orthodenticle), ems (empty spiracles), btd* (*buttonhead*), *tll* (*tailless*) and *slp* (*sloppy paired*) lead to gap-like phenotypes, affecting structures of several head segments (Cohen and Jürgens, 1991; Grossniklaus et al., 1994). However, cephalic gap genes also manifest unique features that are not characteristic of gap gene expression in the trunk. Thus, the expression domains of cephalic gap genes show a strong overlap, whereas the expression domains of gap genes in the trunk are arranged in non-overlapping stripes (Pankratz and Jäckle, 1993). Additionally, cephalic gap genes act in the anterior segments of the head in the absence of pair-rule and *Hox* genes, whereas in more posterior head segments pair-rule and *Hox* genes action is involved.

For several cephalic gap genes, namely *tll, otd, ems* and *btd*, a critical role in early embryogenesis of the brain has been demonstrated. The earliest expression of these genes is observed in the blastodermal stage in circumferential stripes at the anterior pole of the embryo; these domains include the head anlagen of several head segments as judged by blastoderm fate mapping (Hartmann and Reichert, 1998). Later, expression of these cephalic gap genes is observed in patterned subsets of delaminating neuroblasts in the anterior brain primordia (Younossi-Hartenstein et al., 1997). Loss-of-function mutations in the cephalic gap genes lead to specific deletions of the embryonic brain indicating that these genes are required in early patterning and specification of the anterior brain anlage.

The homologs of the *D. melanogaster* cephalic gap genes have also been shown to be involved in fundamental processes of embryonic vertebrate brain development. The two vertebrate homologs of the *D. melanogaster otd* gene, *Otx1* and *Otx2*, are involved in fundamental processes of anterior neuroepithelium patterning (Acampora et al., 2001a; Boyl et al., 2001; Simeone, 1998). Similarly *Emx2* and *Emx1*, the two vertebrate homologs of the *D. melanogaster ems* gene are expressed embryonically in the presumptive cerebral cortex and have been shown to play a role in the establishment of the cerebral cortex (Cecchi, 2002; Shinozaki et al., 2002). A number of descriptive and mechanism-oriented studies on the roles of the genes of the *otd*/*Otx* and *ems/Emx* families, carried out on the genetic model systems *D.*

melanogaster and mouse, indicate that expression and function of these two sets of genes in brain embryogenesis are remarkably conserved. In the following, we consider some of the genetic evidence for an evolutionary conservation of the *otd*/*Otx* and *ems/Emx* gene action obtained in these two model systems.

The *otd/Otx* genes in embryonic brain development of *D. melanogaster* and mouse

A simplified summary scheme of the expression patterns and null mutant phenotypes of *otd* and *Otx2* in the embryonic brains of *D. melanogaster* and mouse is shown in figure 4. In *D. melanogaster*, embryonic expression of the *otd* gene becomes restricted to the protocerebral primordium and the anterior deuterocerebral primordium (Younossi-Hartenstein et al., 1997). The *otd* gene is expressed throughout most of the embryonic protocerebral neuromere; only the anteriormost region (according to neuraxis) of the protocerebral neuromere is devoid of *otd* expression. Similarly most of the adjacent anterior part of the deuterocerebral neuromere expresses *otd*; (Additionally a segmental repetitive pattern of cells expressing *otd* is found in the VNC). Mutational analysis shows that *otd* plays a key role in the establishment of the anterior brain. In homozygous *otd* mutant embryos most of the protocerebral and deutocerebral anlage is deleted, and the brain of these embryos is dramatically decreased (Hirth et al., 1995). This phenotype is due to defective specification of the neuroectoderm in that area resulting in the absence of most of the neuroblasts in the protocerebrum and the anterior deuterocerebrum. This absence of anterior brain neuroblasts correlates with the absence of the proneural gene *l'sc (lethal of scute),* which is thought to be required for neuroectodermal cells to adopt the competence to become a neuroblast (Younossi-Hartenstein et al., 1997). Genetic rescue experiments show that overexpression of the *otd* gene in *otd* null mutant embryos is able to restore the establishment of otherwise missing brain tissue. These experiments also show that the *otd* gene seems to be required specifically at embryonic stage 7-8 for correct specification of the procephalic neuroectoderm; earlier expression of *otd* at the blastoderm stage is not necessary for genetic rescue of the brain phenotype. Ubiquitous overexpression of *otd* in wildtype embryos can lead to the induction of ectopic or transformed ganglionic structures. Interestingly these ectopic ganglia express the protocerebrum-specific gene *bsh* (*brain-specific homeobox*), which indicates that *otd* is able to provide a partial

protocerebral identity to these neuronal cells (Leuzinger et al., 1998).

In the mouse, earliest expression of *Otx2* occurs prior to the onset of gastrulation in the epiblast and the visceral endoderm. During gastrulation *Otx2* is expressed in cells that are involved in early specification and patterning of the anterior neural plate (anterior visceral endoderm and axial mesoderm), as well as in the responding cells in the anterior neuroectoderm (reviewed in Simeone, 1998; Acampora and Simeone, 1999). Later, during regionalization of the brain, the expression domain of *Otx2* extends throughout most of the prosencephalic and mesencephalic neuroectoderm and has a posterior border at the mesencephalic side of the midbrain-hindbrain boundary (Simeone et al., 1992; Millet et al., 1996). Accordingly, it has been proposed that *Otx2* has two distinct functions in brain development, first the induction of the rostral neural plate and second the regional specification of forebrain and midbrain areas. This has been demonstrated in chimeric mouse models. In embryos containing an *Otx2*-/- epiblast and a wildtype visceral endoderm, the induction of the rostral neural plate is rescued, whereas the epiblast-derived tissue fails to regionalize forebrain and midbrain structures. On the other hand, in chimeras consisting of an *Otx2*-/- visceral endoderm and an *Otx2*+/+ epiblast none of the phenotypic features were restored, revealing important functional inductive properties of *Otx2* in the visceral endoderm (Rhinn et al., 1998). In homozygous mutant *Otx2-/-* mouse embryos, the rostral neuroectoderm, which normally gives rise to the forebrain, midbrain and rostral hindbrain, is not specified and, in consequence, these mutants die early in embryogenesis (Acampora et al., 2001a).

Expression of *Otx1* occurs later than that of *Otx2* in a large region of the anterior neural tube that includes the presumptive neuroepithelium of the telencephalon, diencephalon and mesencephalon (Simeone et al., 1992). During corticogenesis, *Otx1* expression is initially maintained uniformly across the ventricular zone of the cortical anlage. Subsequently, *Otx1* expression becomes progressively dominant in the cortical plate, notably in postmigratory neurons of layers 5 and 6, whereas it is absent in the differentiated neurons of layer 1-4 (Franz, 1994). *Otx1* null mutant mice are viable, but 30% of them die within the first postnatal month. These mice show a reduced brain size, due to a reduced thickness of the telencephalic cortex. This is thought to be due to a lack of proliferation in the early telencephalic

neuroepithelium (Acampora et al., 1996; Acampora et al., 1998a). Additionally, *Otx1* null mutant mice display dramatic epileptic behaviour (Acampora, 1996).

Experiments replacing *Otx2* with *Otx1* and vice versa were carried out to distinguish their functional properties. In *Otx1* mutant mice, the human *Otx*2 gene can fully restore corticogenesis abnormalities and epilepsy phenotypes. Introducing the human *Otx1* gene in *Otx2* null mutant mice restored the anterior neural plate induction but later resulted in a headless phenotype. This indicates that *Otx1* can replace the inductive function of *Otx2*, however, the later requirement for *Otx2* for specification of forebrain and midbrain areas is not rescued by *Otx1* (Acampora et al., 1998a).

Taken together, these data point to remarkable similarities in brain-specific expression of the *D. melanogaster otd* gene and the murine *Otx* genes. Moreover, they uncover similar mutant phenotypes that are observed in the embryonic brains of *D. melanogaster* and mouse when the *otd/Otx* gene homologs are functionally eliminated in null mutants. The most reasonable explanation for these findings is that expression and function of these developmental control genes in brain embryogenesis are evolutionarily conserved.

The *ems/Emx* genes in embryonic brain development of *D. melanogaster* and mouse

A simplified summary scheme of the expression patterns and null mutant phenotypes of *ems* and *Emx2* in the embryonic brains of *D. melanogaster* and mouse is shown in figure 5. In the cephalic neuroectoderm of *D. melanogaster*, expression of the *ems* gene is found in two stripe-like domains; first, in the anterior deuterocerebral primordium and second, in the anterior part of the tritocerebral primordium of the procephalic neuroectoderm (Younossi-Hartenstein et al., 1997). Subsequently, in the early embryonic brain of *D. melanogaster*, *ems* expression is seen in the anterior part of the deuterocerebral neuromere as well as in the anterior tritocerebrum; (Additionally a segmental repetitive pattern of cells expressing *ems* is found in the VNC). Mutant analysis shows that an *ems* loss-of-function mutation leads to severe deletions of the deuterocerebral and tritocerebral anlage (Hartmann et al., 2000). This is due to defective specification of the neuroectoderm in these anlagen resulting in the absence of neuroblasts, and this, in turn results in the loss of most of the deuterocerebrum and

the tritocerebrum. As in the case of *otd* null mutants, the absence of neuroblasts in the *ems* mutant domains correlates with the loss of expression of the proneural gene *l'sc* (Younossi-Hartenstein et al., 1997). Rescue of the *ems* null mutant phenotype can be achieved by ubiquitously overexpressing *ems* at stage 11. This overexpression results in a restoration of brain morphology, indicating that *ems* expression at stage 11 is sufficient for proper brain development; earlier *ems* expression does not seem to be necessary for this specific developmental function (Hartmann et al., 2000).

In the mouse, *Emx2* and *Emx1* are expressed in the embryonic forebrain in similar, but not completely overlapping domains. *Emx2* expression is first seen at embryonic day 8.5 and precedes that of *Emx1*, which is first seen at embryonic day 9. Around embryonic day 9.5, the cerebral cortex starts to become apparent as a layer of proliferating neuroblasts comprising the germinative neuroepithelium, and *Emx2* is expressed in this area. The anteriormost expression boundary of *Emx*2 corresponds to the olfactory placodes, the posterior boundary localizes in the roof of the presumptive diencephalon. Expression of *Emx2* during corticogenesis is restricted to the ventricular zone, where neurons are generated (Gulisano et al., 1996). Later in corticogenesis the Cajal-Retzius cells as well as most marginal cortical plate neurons also express *Emx2* (Mallamaci et al., 2000). In the developing neocortex, *Emx2* is expressed in a gradient, with low levels in rostrolateral regions and high levels in caudomedial regions. *Emx2* null mutant mice die within a few hours after birth. They lack kidneys and reproductive organs, and have a severe reduction of cerebral hemispheres and olfactory bulb. The dentate gyrus is missing and the hippocampus and medial limbic cortex are severely reduced in size. Mutant analysis of neocortical regionalization suggests that *Emx2* interacts with the *Pax6* gene, which is expressed in the developing neocortex in a gradient opposite to that of *Emx2*. Thus, loss of function of *Emx2* causes an expansion of rostrolateral neocortical areas (motor and somatosensory) and a reduction of caudomedial areas (visual), whereas the opposite effects are seen in *Pax6* deficient mice (Bishop et al., 2002; O'Leary and Nakagawa, 2002). Interestingly, the *Pax6* homologous genes in *D. melanogaster*, *ey* (*eyeless*) and *toy* (*twin of eyeless*), are expressed in the anterior part of the embryonic fly brain, however it is not know whether there are similar opposing mechanisms of *ems* and *ey/toy* action involved in regionalization of the developing insect brain (Kammermeier et al., 2001b). More recent findings have shown that *Emx2* is also involved in proliferation of stem cells in the adult

mammalian CNS; altering levels of *Emx2* expression increased or decreased the proliferation rate of stem cells. Moreover, when *Emx2* expression is abolished, the frequency of symmetric cell division generating two stem cells increases, where as it decreases when *Emx2* is overexpressed (Galli et al., 2002).

As mentioned above, early embryonic *Emx1* expression coincides largely with the expression domain of *Emx2* (Simeone et al., 1992). In the developing neocortex, *Emx1*, like *Emx2*, is expressed in a gradient, with low levels in rostrolateral regions and high levels in caudomedial regions. Postnatal *Emx1* null mice are viable and fertile and display more or less subtle neuroanatomical phenotypes, restricted to the forebrain. Among the mutant phenotypes observed in embryonic forebrain development of *Emx1* null mice are disorganized fasciculation, defects of the corpus callosum, and poor differentiation of the cerebral cortex, as well as reduction of cortical plate and subplate (Qiu et al., 1996; Yoshida et al., 1997). Lineage analysis of *Emx1* expressing progenitors show that a variety of cells of most palladial structures originate from *Emx1* lineages. These include radial glia, Cajal-Retzius cells, glutaminergic neurons, astrocytes and oligodendrocytes (Gorski et al., 2002). In the adult cerebral cortex, *Emx1* expression is found in pyramidal neurons (Chan et al., 2001).

As in the case of the *otd/Otx* gene family, expression of the *D. melanogaster ems* gene and the murine *Emx* genes during early embryogenesis of the brain are remarkably similar. Furthermore, mutant analysis reveals that the *D. melanogaster ems* gene and the murine *Emx* genes might be performing a comparable function in the control of early development of the anterior brain in both insects and vertebrates.

The *otd/Otx* and *ems/Emx* genes: crossphylum rescue experiments in *D. melanogaster* and mouse

As mentioned above, there are striking similarities in the brain-specific topology of expression of the *D. melanogaster otd* gene and the murine *Otx* genes, as well as of the *D. melanogaster ems* gene and the murine *Emx* genes. In addition, comparable, and indeed in some cases remarkably similar, mutant phenotypes are observed in the embryonic brain of *D. melanogaster* and mouse when these gene homologs are functionally eliminated in null

mutants. This, in turn, suggests that expression and function of these two sets of developmental control genes in brain embryogenesis are evolutionarily conserved. To obtain a deeper insight into the evolutionary conservation of these sets of key developmental control genes in brain development, crossphylum rescue experiments were carried out in *D. melanogaster* and mouse in which the mutated endogenous gene of interest is replaced by a homologous vertebrate or insect gene (figure 6).

In crossphylum rescue experiments carried out in *D. melanogaster*, mammalian *Otx1* or *Otx2* genes were placed in an *otd* mutant background under the control of an inducible promoter (Leuzinger et al., 1998). Ubiquitous overexpression of the human *Otx2* gene in mutant fly embryos was able to restore the phenotypic features of the *otd* null mutants. Most importantly, the lack of an anterior brain anlage and the resulting gap-like anterior brain deletion in these mutants could be restored. The human *Otx1* transgene also rescued the brain defects in *otd* null mutants, albeit somewhat less efficiently. Flies that are homozygous for *ocelliless*, a viable *otd* allele, lack ocelli and associated sensory bristles of the vertex (Finkelstein et al., 1990b). Interestingly these phenotypic features were also rescued by overexpression of the human *Otx2* gene (Nagao et al., 1998); (The *otd* homolog of the urochordate ascidian *Halocynthia roretzi* was also shown to rescue the brain defects in an *otd* mutant; Adachi et al., 2001).

Crossphylum rescue experiments carried out in the mouse demonstrated that the *D. melanogaster otd* gene can restore many brain defects caused by an *Otx1* null mutation; a full rescue of corticogenesis and epilepsy and a partial rescue of eye defects were observed. Interestingly the inner ear defects seen in *Otx1* null mutants were not rescued by the fly *otd* gene suggesting a more diverged function of *Otx1* in inner ear development. The cooperative interaction of *Otx1* and *Otx2* in brain formation was also partially restored by *otd* misexpression. In *Otx1-/-*; *Otx2-/+* embryos, the fly *otd* rescued the observed CNS abnormalities in a dosage-dependent manner; thus two copies of the *otd* gene lead to stronger restoration of the defects than one (Acampora et al., 1998b). The potential of the *D. melanogaster otd* gene to rescue the mouse *Otx2* mutant phenotype is more limited. Similar to the replacement of *Otx2* by *Otx1*, the fly *otd* gene was able to take over the function of the *Otx2* gene in the anterior visceral endoderm, therefore restoring the absence of the anterior

neural plate otherwise seen in *Otx2* null mutants. However, expression of the fly *otd* transgene did not occur in the embryonic mouse epiblast, and defects in the maintenance of anterior brain patterning were not rescued. Interestingly, a more detailed analysis of the genomic region of *Otx2* revealed important aspects of the gene's 3' and 5' UTRs in the spatial control of expression (Boyl et al., 2001). Thus, when the coding sequence of the fly *otd* gene was fused to the intact 5' and 3' UTRs of the murine *Otx2* gene and then used to rescue *Otx2* null mutants, translation of *otd* mRNA did occur in the epiblast and anterior neuroectoderm. Moreover, this epiblast and neuroectoderm translation of *otd* rescued the maintenance of anterior forebrain and midbrain patterning in *Otx2* null mutants (Acampora et al., 2001b).

Conserved functional equivalence of head gap genes in brain development of fly and mouse is not restricted to the *otd*/*Otx* gene family. Crossphylum rescue experiments were also carried out successfully for the *ems* and *Emx2* genes. Thus, the defects in brain morphology observed in *ems* null mutants of *D. melanogaster* could be rescued by transgenic expression of the murine *Emx2* gene. Ubiquitous overexpression of the *Emx2* gene at the embryonic stage at which the endogenous *ems* gene is required for embryonic brain patterning, rescued the brain phenotype of *ems* null mutant flies; neuronal specification defects were restored resulting in the presence of the deuterocerebrum and tritocerebrum (Hartmann et al., 2000). To date, corresponding crossphylum rescue experiments have not yet been carried out in the mouse, therefore it is not known if the *D. melanogaster ems* gene is able to restore the defects observed in *Emx2* mutants. Despite the incomplete information on the crossphylum rescue potential of the *ems/Emx* genes, the overall result of the crossphylum rescue experiments performed so far is a clear indication that the function of *otd/Otx* and *ems*/*Emx* genes in brain development are to a large degree evolutionary conserved between protostomes and deuterostomes.

Hox genes pattern the posterior brain in insects and vertebrates

Homeotic genes, or *Hox* genes, are found throughout the animal kingdom and are essential for patterning the anterior-posterior body axis (Hughes and Kaufman, 2002; Carpenter, 2002; Prince 2002; Vervoort, 2002; Ferrier et al., 2001; Schilling and Knight, 2001). *Hox* genes are organized in clusters along the chromosome, and they show a spatial co-linearity in their

expression patterns, in that more 3' located genes are expressed more anteriorly along the body axis of the embryo, whereas more 5' located genes are expressed more posteriorly. In some cases, *Hox* genes located more 3' in the cluster are also expressed earlier in the developing embryo, whereas the expression of more 5' located genes along the chromosome occurs later in embryogenesis (Mann, 1997). This is called “temporal co-linearity". Furthermore, there appears to be a functional hierarchy of *Hox* gene products in that more posteriorly expressed *Hox* genes are functionally dominant over more anteriorly expressed *Hox* genes; this is termed "posterior prevalence" (Duboule and Morata, 1994). Mutations of *Hox* genes generally lead to a change of segmental identity in the affected structures. Interestingly both gain-of-function and loss-of-function mutations can result in a transformation of segmental features. In general, loss-of-function mutations in a given *Hox* gene lead to a transformation of posterior structures into more anterior ones (anterior transformation). On the other hand, gain-of-function mutations lead to transformation of more anterior structures into more posterior ones (posterior transformation).

Although *Hox* genes in insects and vertebrates are organized in a co-linear fashion along chromosome, there are several differences in their genomic makeup (Carroll, 1995; Mann, 1997). Most striking are the differences in number and arrangement of *Hox* genes. For example, while the *D. melanogaster* homeotic complex only comprises 8 genes, there are 39 *Hox* genes in the mouse. In *D. melanogaster*, the homeotic complex is split into two chromosomal clusters, 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 three more posteriorly expressed genes *Ultrabithorax* (*Ubx), abdominal-A* (*abd-A*) and *Abdominal-B (Abd-B)* are ordered in the BX-C. Mammalian *Hox* genes are organized in four chromosomal complexes of 9 to 11 *Hox* genes which can be aligned into 13 sets, termed paralogous groups, based upon their organization, sequence and homology to their *D. melanogaster* orthologs. Interestingly, not all mammalian *Hox* genes are represented in all clusters, and some genes within a cluster seem to have undergone an evolutionary duplication. For example, mammalian *Hox* genes related to the *D. melanogaster Abd-B* gene have been duplicated several times and exist in multiple copies in three of the four clusters of murine *Hox* genes. Differences in *Hox* gene size in insects and vertebrates can be remarkable. Thus, a single *Hox*

gene of *D. melanogaster* such as *Ubx* spans approximately 100kb, which is comparable in size to an entire *Hox* complex in the mouse; (as an explanation for this, it has been proposed that the regulatory sequences must be more compactly organized in vertebrates than in insects).

The relative anteroposterior order of *Hox* gene expression domains in the CNS of insects and vertebrates, including man, are remarkably similar (figure 7). In the embryonic CNS of *D. melanogaster, Hox* genes are expressed in specific domains of the posterior brain and the VNC. Their expression domains do not strictly follow the rule of spatial co-linearity, the exception being *pb*, which is expressed more posteriorly than *lab*, whereas the rule of spatial co-linearity of *Hox* gene expression in the epidermis is perfectly fulfilled (Hirth et al., 1998; Kaufman et al., 1990). In the developing CNS of vertebrates, *Hox* genes are expressed in the hindbrain and spinal cord (Lumsden and Krumlauf, 1996). *Hox* gene expression precedes the formation of rhombomeres and becomes restricted to certain domains during embryogenesis. Patterning domains follow the rule of spatial-temporal co-linearity (interestingly also with the exception of the *pb* homolog *Hoxb2*) and show a two-rhombomere periodicity of expression starting at rhombomere 3 (r3).

In *D. melanogaster*, neuroanatomical analyses has shown that loss-of-function mutations for two *Hox* genes, *lab* and *Dfd*, result in severe defects of the embryonic brain (Hirth et al., 1998). In *lab* null mutants, axonal projection defects are observed in the posterior part of the tritocerebral neuromere, which in the wildtype is the normal expression domain for *lab*. Interestingly the affected neuromere is not deleted; neuronal progenitors giving rise to the posterior tritocerebrum are present and correctly located in the mutant domain. However, the postmitotic progeny of these cells do not assume their correct cell fate. Although these mutant cells do not undergo apoptosis and remain at their proper position, they do not form axonal or dentritic extensions. Rather, they seem to stay in an undifferentiated state and fail to express the appropriate neuronal markers indicating that these cells fail to adopt a neuronal cell fate. Comparable defects are observed in *Dfd* null mutants for the mandibular neuromere, which in the wildtype is the normal expression domain for *Dfd*. Thus, in the *Dfd* mutant domain, proper delamination of putative neuronal progenitors occurs, but their progeny fail to adopt a proper neuronal cell fate.

Genetic rescue experiments carried out in *D. melanogaster* reveal that other genes of the *Hox* cluster can functionally replace *lab* in the control of tritocerebral brain development (Hirth et al., 2001). By targeted misexpression of other *D. melanogaster Hox* genes in the *lab* mutant domain, it has been shown that all *Hox* genes can functionally rescue the *lab* null mutant phenotype, with the exception of *Abd-B*. Most strikingly there is a correlation between the rescue efficiency and the chromosomal location within the homeotic complex, in that more 3' located *Hox* genes have a higher rescue efficiency than more 5' located ones. These findings indicate that even though the amino acid sequence of the *D. melanogaster Hox* proteins have diverged considerably during evolution, most of the *Hox* proteins can still functionally replace the *lab* protein. This, in turn, suggests, that correct spatiotemporal expression of most *Hox* proteins through cis-acting regulatory regions might be of more importance in functional terms than differences in *Hox* protein sequence.

In the mouse, neuroanatomical analysis of *Hox* gene mutants reveals that these genes act in specific domains along the anteroposterior neuraxis (Carpenter, 2002; Glover, 2001; Maconochie et al., 1996; Rijli et al., 1998). Loss-of-function mutations of the *lab* orthologs *Hoxa1* and *Hoxb1* have given a deeper insight into this process of *Hox* gene action on brain development. *Hoxa1* null mutations result in segmentation defects in the mutant domain of the hindbrain, such as a reduced size of rhombomere 4 (r4) and rhombomere 5 (r5) as well as axonal projection defects of motor neurons and defects of the trigeminal and facial/vestibuloacoustic nerve. Nevertheless, the normal identity of r4 is not altered (Rijli et al., 1998; Studer et al., 1998; Gavalas et al., 1998). Functional inactivation of *Hoxb1* does not change the actual size of r4, but results in a loss of identity of this area leading to a partial transformation of r4 identity to r2 identity (Goddart et al., 1996; Studer et al., 1996). Double knock-out of *Hoxa1* and *Hoxb1* causes a reduced size of r4 and additionally a loss of expression of r4-specific markers, resulting in the formation of a domain of unknown identity between r3 and r5 (Gavalas et al., 1998; Studer et al., 1998). These findings suggest that the identity of r4 is achieved by the synergistic action of *Hoxa1* and *Hoxb1*, and point to a functional role of these *Hox* genes which is surprisingly similarly to the mode of action of the orthologous *Hox* gene *lab* in the posterior tritocerebrum of *D. melanogaster*.

Thus, in the case of these *Hox* genes, as in the case of the cephalic gap genes, we are again confronted with homologous developmental control genes that have strikingly similar expression patterns and functional roles in brain development in insects and mammals. Taken together, these data imply that these basic molecular genetic features of brain development are evolutionary conserved.

Concluding remarks

The results reviewed in this work show that a number of the molecular genetic control elements involved in embryonic brain development are remarkably similar in insects and vertebrates. Genetic master regulatory programs in vertebrates and insects are highly evolutionarily conserved. Thus, homologous sets of interacting genes responsible for the establishment of dorsoventral polarity are expressed and function on opposing sides of the embryos of insects and vertebrates, most likely due to a dorsoventral body axis inversion after the separation of protostome and deuterostome lineages. Significantly, the signalling proteins that are encoded by these gene homologs, lead to the induction of the ventral neurogenic region in insects and the dorsal neurogenic region in vertebrates. Furthermore, in both insects and vertebrates, genes of the *otd/Otx* family and *ems/Emx* family are responsible for the development of the anterior brain, whereas the *Hox* genes are essential for posterior brain patterning. Thus, homologous developmental control genes are involved in anteroposterior patterning of the brain as well in dorsoventral patterning of the nervous systems in these two animal groups. The striking conservation of genetic regulatory networks responsible for early developmental steps in brain development in insects and mammals, and the interchangeability of some of these key developmental control genes, further supports the hypothesis that a common genetic Bauplan underlies brain development in protostomes and deuterostomes. In consequence, it seems likely that the seemingly divergent brain types found in living animals as diverse as insects and vertebrates have a common evolutionary origin in a brain-like structure that existed before the protostome-deuterostome split occurred over 570 million years ago (Valentine et al., 1999; Arendt and Nübler-Jung, 1996; DeRobertis and Sasai, 1996; Kammermeier and Reichert, 2001a). Moreover, key elements of the ancestral molecular genetic program that controlled the development of this urbilaterian brain are likely to be

conserved and common in the development of the brains of all bilaterian animals including our own.

Acknowledgements:

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FIGURES:

Figure. 1.

Simplified summary scheme of the two different modes of neurogenesis of the CNS in insects (left) and vertebrates (right). (**A**) During early embryogenesis a part of the ectodermal germ layer (E) becomes specified as neuroectoderm (NE). (**B**) In insects the neuronal stem cells, neuroblasts (NB), delaminate from the neuroectoderm, whereas in vertebrates the neuroectoderm invaginates to form the neural tube (delamination of neurogenic stem cells also occurs in some deuterostomes); (InvNT, invaginating neural tube). (**C**) As a consequence of the progress of delamination in insects and invagination in vertebrates, two opposing located nerve cords are formed, the ventral nerve cord (VNC) and the dorsal neural tube (NT).

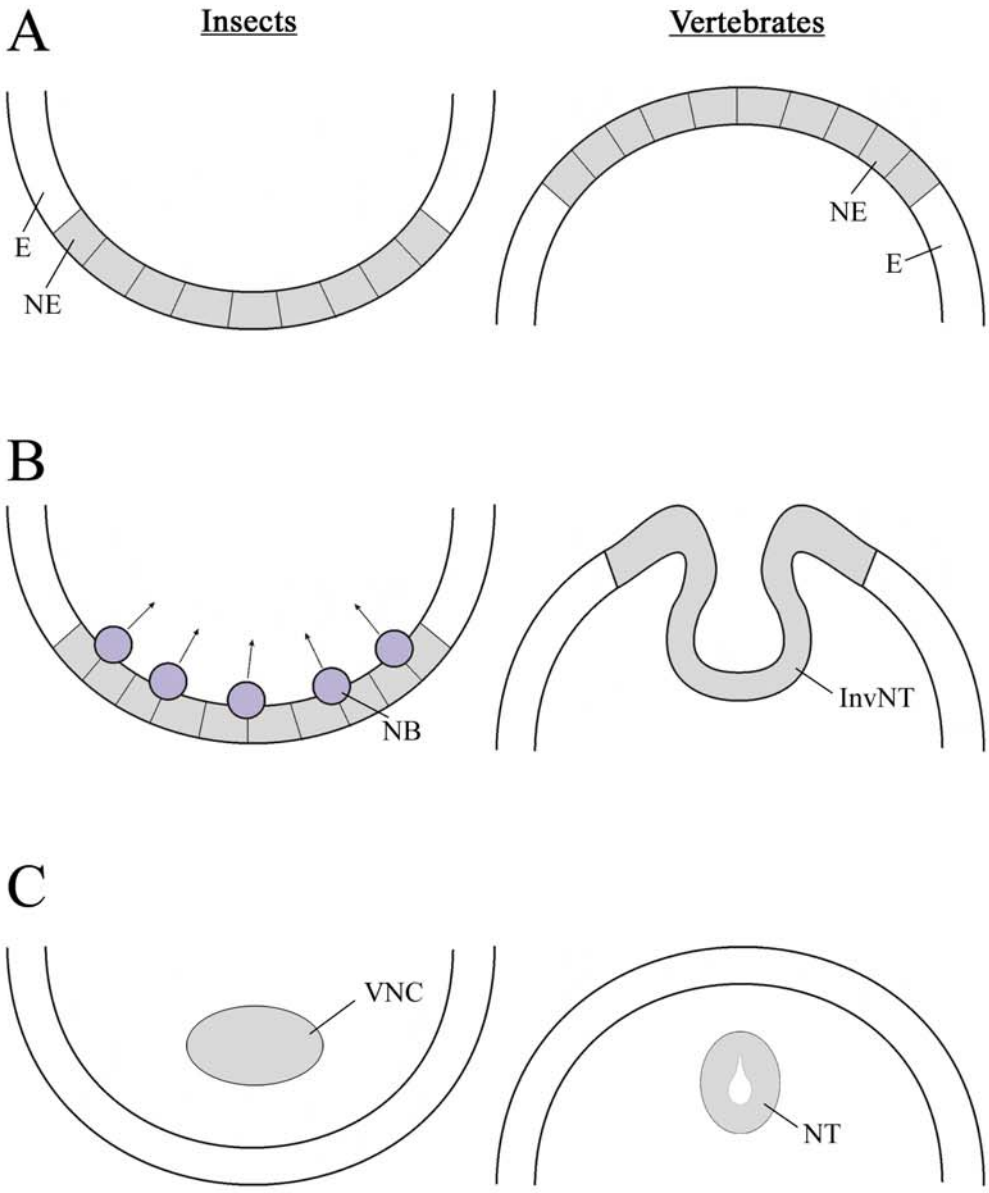

A
Insects
Vertebrates
E
NE
NE
E
B
NB
InvNT
C
VNC
NT

Figure. 2.

Simplified versions of bilaterian phylogenies; only a subset of all bilaterian phyla is represented. (**A**) A classical phylogeny based mainly on embryological and morphological studies. In this phylogeny, extant metazoan groups such as the platyhelminths are considered as representatives of ancestral bilaterian lineages (the phyla of nematoda are thought to be placed near the protostome lineage); (modified after Brusca and Brusca, 1990). (**B**) One of several new molecular phylogenies based on rRNA and Hox sequence analyses. Several bilaterian lineages, such as platyhelminths that were considered classically as “primitive” have been placed among protostome phyla, which have a high degree of complexity in morphology, (modified after Adoutte et al., 2000). Chordata and Arthropoda, which are considered in more detail in this review, are highlighted.

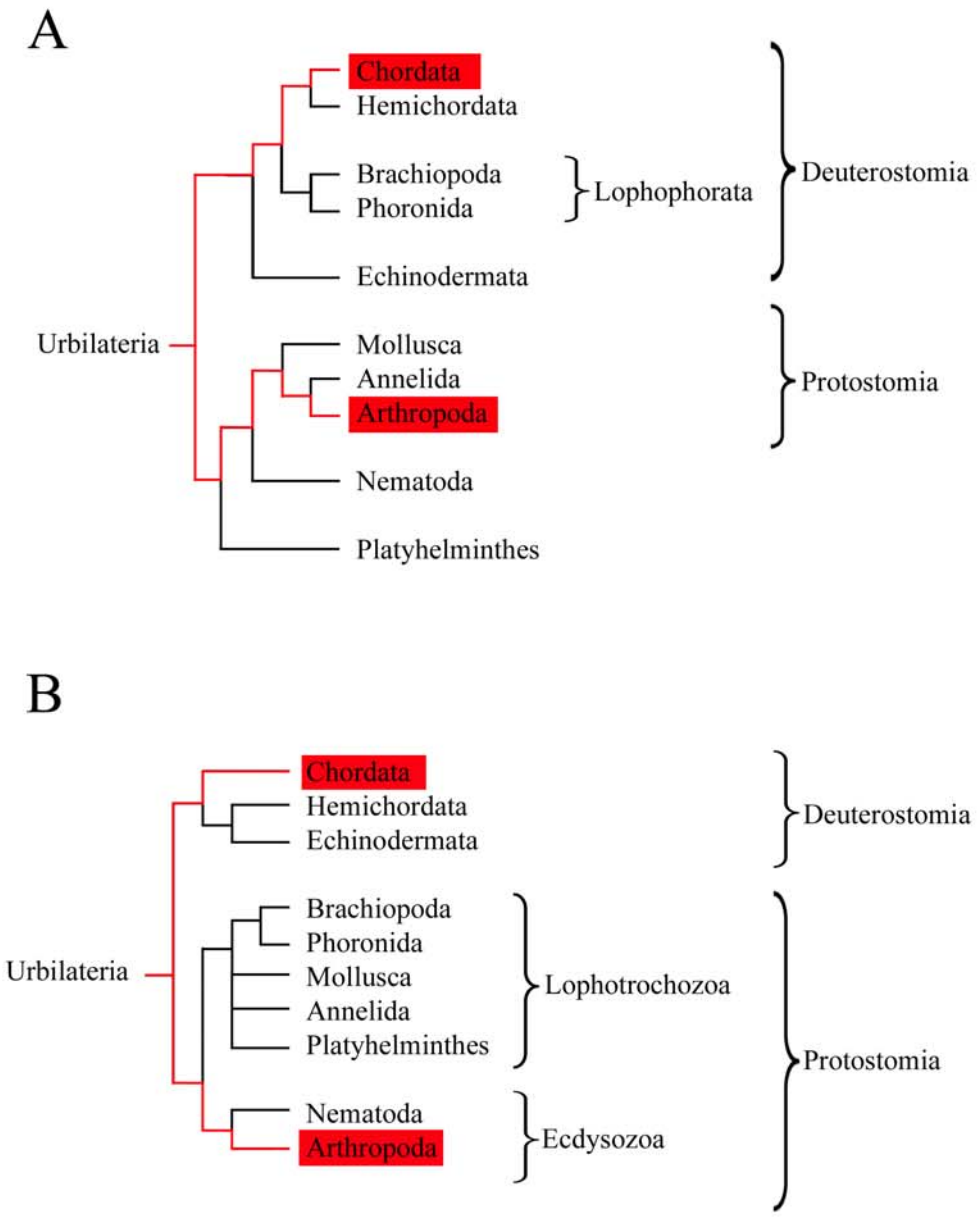
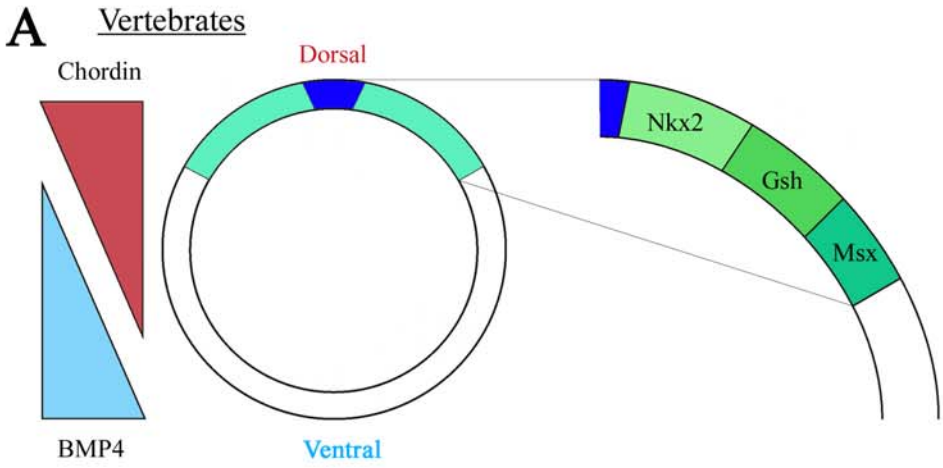

A
Chordata
Hemichordata
Brachiopoda
Phoronida
Lophophorata
Deuterostomia
Echinodermata
Urbilateria
Mollusca
Annelida
Arthropoda
Protostomia
Nematoda
Platyhelminthes
B
Chordata
Hemichordata
Echinodermata
Deuterostomia
Urbilateria
Brachiopoda
Phoronida
Mollusca
Annelida
Platyhelminthes
Lophotrochozoa
Protostomia
Nematoda
Arthropoda
Ecdysozoa

Figure. 3.

Schematic representation of the molecular and genetic evidence for the dorsoventral body axis inversion hypothesis between (**A**) vertebrates and (**B**) insects. Two groups of molecules with opposing action, *dpp/BMP4* and *sog/Chrodin*, are expressed in vertebrates in a fashion which is dorsoventrally reversed as compared to insects. In consequence the neuroepithelium (neuroectoderm green and midline blue) is located dorsally in vertebrates, whereas it is located ventrally in insects. The developing CNS in both animal groups is characterized by three longitudinally running columns. A set of similar homeobox genes is expressed in the same medial-lateral order in insects and vertebrates (*vnd/Nkx2* ventromedially/dorsomedially, *ind/Gsh* in intermediate domains and *msh/Msx* dorsolaterally/ventrolaterally).


A
Vertebrates
Chordin
Dorsal
Nkx2
Gsh
Msx
BMP4
Ventral

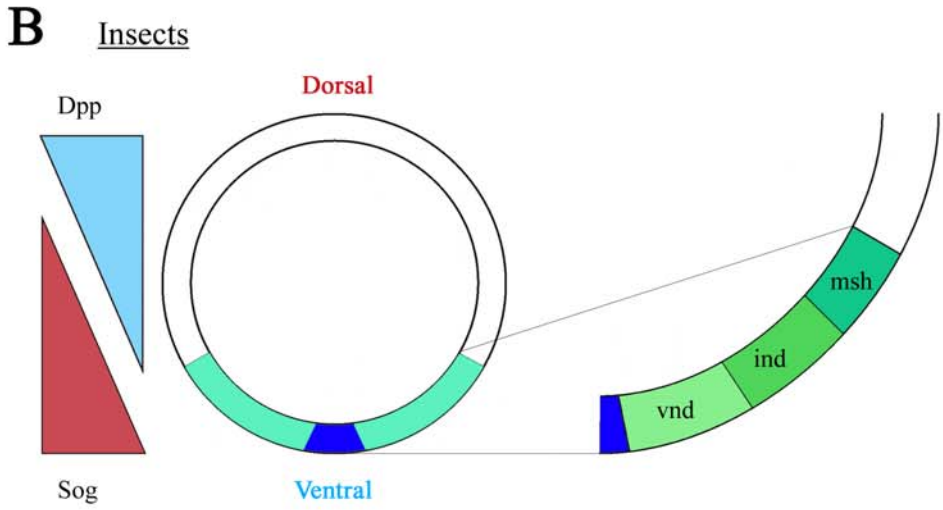

B
Insects
Dpp
Dorsal
msh
ind
vnd
Sog
Ventral

Figure. 4.

Summary scheme of expression domains and null mutant phenotypes of the *otd/Otx2* genes in insects (*D. melanogaster*) and vertebrates (mouse). (**A**) In insects the *otd* gene is expressed throughout most of the protocerebral and the anterior part of the deuterocerebral neuromeres. In *otd* mutant embryos the protocerebrum and the anterior part of the deuterocerebrum are missing. (**B**) In vertebrates the *Otx2* gene is expressed in the anterior part of the embryonic brain including the presumptive telencephalon (T), diencephalon (D) and mesencephalon (M), excepting the anteriormost part of the telencephalon. In *Otx2* null mutants the entire forebrain and midbrain (as well as rhombomeres 1 and 2) are absent. Abbreviations: b1, protocerebrum; b2, deuterocerebrum; b3, tritocerebrum; s1, mandibular neuromere; s2, maxillary neuromere; s3, labial neuromere; VNC, ventral nerve cord; r1-r8, rhombomeres 1-8; Sc, spinal cord.

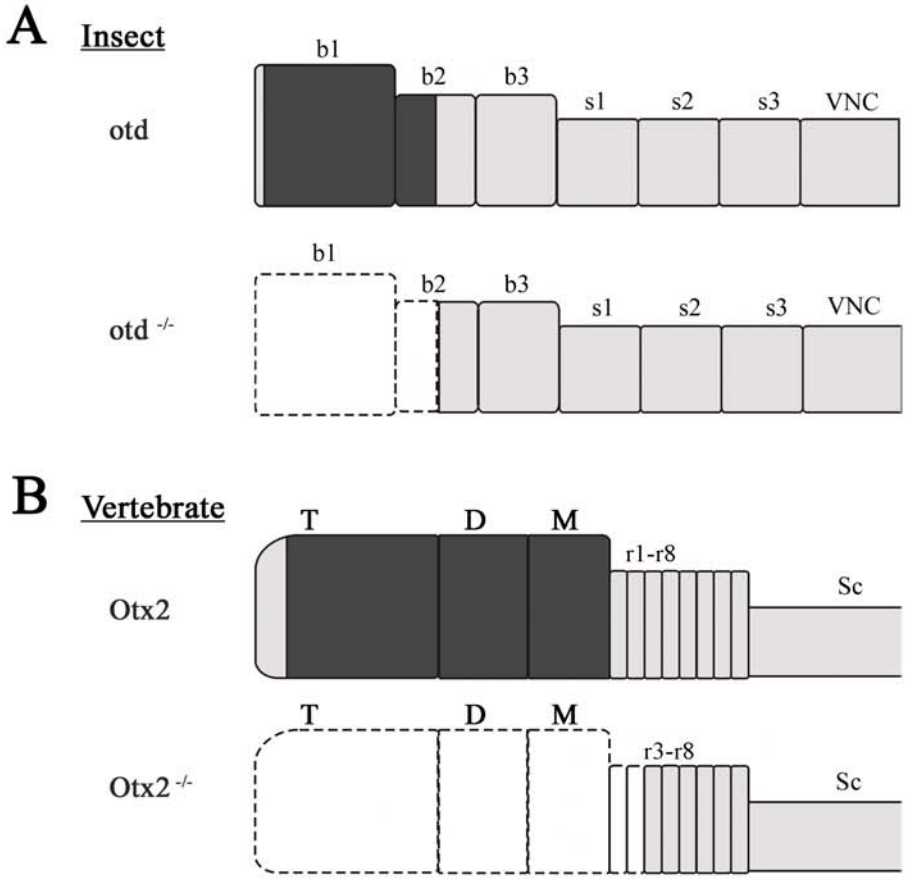

A
Insect
otd
b1
b2
b3
s1
s2
s3
VNC
otd -/-
b1
b2
b3
s1
s2
s3
VNC
B
Vertebrate
Otx2
T
D
M
r1-r8
Sc
Otx2 -/-
T
D
M
r3-r8
Sc

Figure. 5.

Summary scheme of expression patterns and null mutant phenotypes of the *ems/Emx2* genes in insects (*D. melanogaster*) and vertebrates (mouse). (**A**) In insects the *ems* gene is expressed in the anterior part of the deuterocerebrum and the anterior part of the tritocerebrum. Loss-of-function mutation of *ems* leads to the absence of the deuterocerebrum and the tritocerebrum. (**B**) In the developing mammalian neocortex *Emx*2 is expressed in a gradient, with high caudomedial and low rostrolateral expression levels. In *Emx2* null mutants the anterior motor (M) and sensory (S) cortical areas are expanded, whereas the posterior visual (V) cortical areas are reduced in size. (Abbreviations see Figure 4); (modified after Hartmann et al., 2000; O'Leary and Nakagawa, 2002).

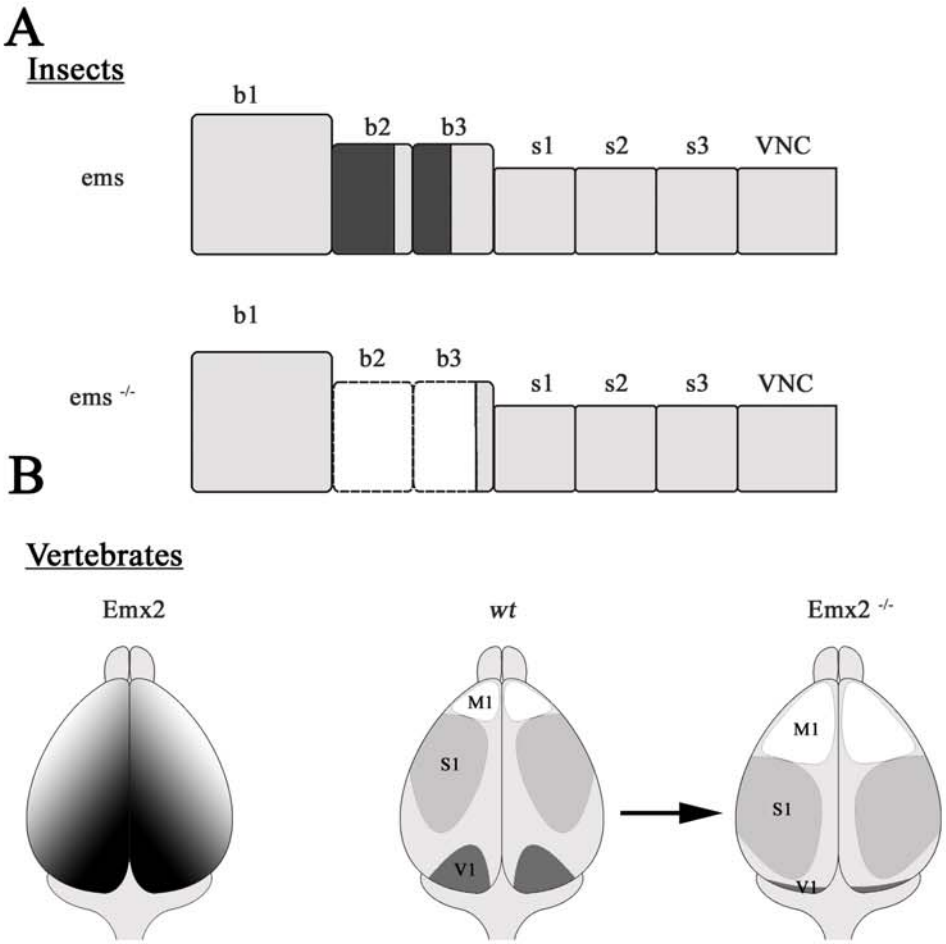

A
Insects
b1
b2
b3
s1
s2
s3
VNC
ems
b1
b2
b3
s1
s2
s3
VNC
ems -/-
B
Vertebrates
Emx2
wt
Emx2 -/-
M1
S1
V1
M1
S1
V1

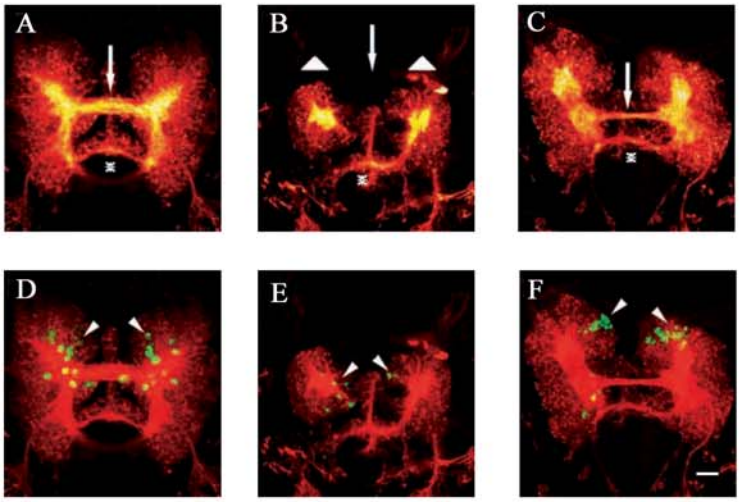
Figure. 6.

Cross-phylum rescue experiments in insects (*D. melanogaster*) and vertebrates (mouse), replacing the insect *otd* gene by the mammalian *Otx2* gene and vice versa. In *D. melanogaster* embryos the anterior brain is interconnected by a commissure (arrow, the frontal connective is indicated by asterisk), (A); expression of the *brain-specific homeobox* gene (green) is restricted to the anterior brain (D). In *otd* null mutants the anterior brain is lost (indicated by triangles) including the protocerebrum and anterior deuterocerebrum, and the preoral commissure is absent (arrow) (B); most cells expressing *bsh* are also missing (E). Overexpression of the vertebrate *Otx2* gene in *otd* mutant embryos results in a rescue of the anterior brain, and the preoral commissure (arrow) is established in the normal position (C); cells of the anterior brain also express *bsh* normally (F). In embryonic mouse brain the major brain regions are the forebrain (fb), midbrain (mb) and hindbrain (hb) (G, embryonic day 10.5); normal brain development correlates with normal development of the head (K, embryonic day 16). In homozygous mutant *Otx2* mouse embryos in which the *otd* coding sequence has replaced the Otx2 gene (*Otx2*-/-; *otd*2/*otd*2*)*, the rostral neuroectoderm, which normally gives rise to the forebrain, midbrain and rostral hindbrain, is not specified and the forebrain, midbrain and parts of the hindbrain are lacking (H); this correlates with headless embryos that die in embryogenesis (L). In homozygous mutant *Otx2* mice the *D. melanogaster otd* coding sequence, flanked by 5' and 3' UTR of the *Otx2* gene, (*Otx2*-/-; *otd*2FL/*otd*2FL) is able to replace the function of the *Otx2* gene in embryonic brain development, therefore restoring the absence of the anterior neuroectoderm (I); this correlates with a rescued development of the head (M); (modified after Leuzinger et al., 1998; Acampora et al., 2001b). Immunostaining: A-C: anti-HRP (orange); D-F: anti HRP (red), anti BSH (green). Scale bars: 10μm (A-F); 250μm (G-I); 1mm (K-M).

Crossphylum rescue:

Insect

wt | otd$^{-/-}$ | otd$^{-/-}$ hs-Otx2



Mouse

wt | Otx2$^{-/-}$ otd^{2}/otd^{2} | Otx2$^{-/-}$ otd^{2FL}/otd^{2FL}

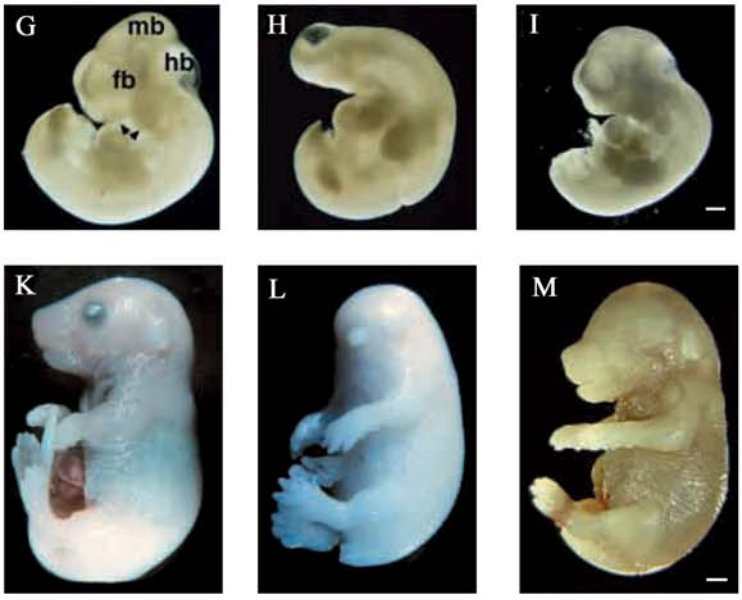
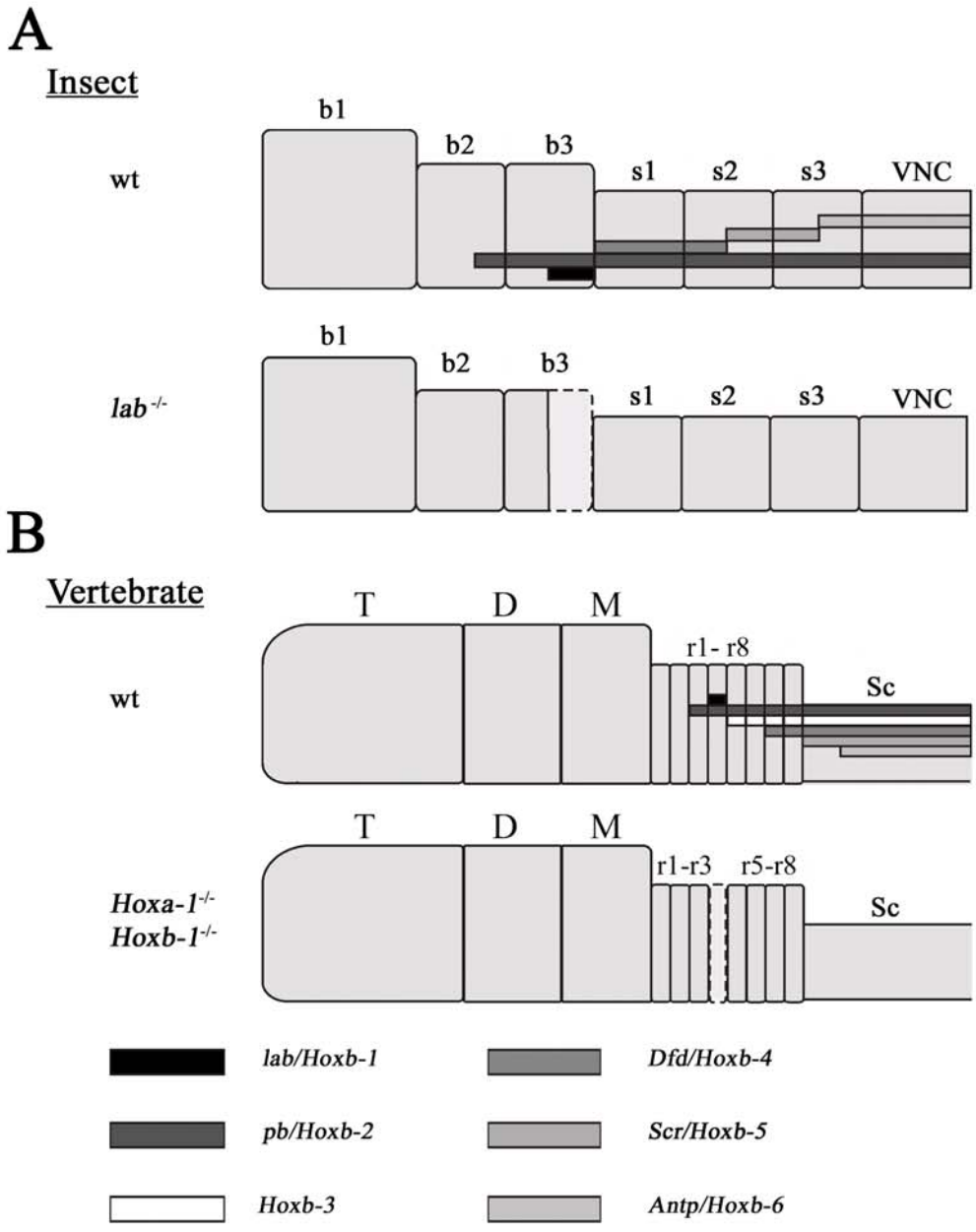


Figure. 7.

Simplified schematic comparison of *Hox* gene expression domains and mutant phenotypes in the CNS of insects (*D. melanogaster*) and vertebrates (mouse). (**A**) Expression domains of the homeotic genes *lab* (*labial*), *pb* (*proboscipedia*), *Dfd* (*Deformed*), *Scr* (*Sex combs reduced*) and *Antp* (*Antennapedia*) in the embryonic brain and anterior VNC. In *lab* null mutants (*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. (**B**) Expression of the homeotic genes *Hoxb-1*, *Hoxb-2*, *Hoxb-3*, *Hoxb-4*, *Hoxb-5* and *Hoxb-6* in the embryonic CNS of mouse. Double mutant embryos of *Hoxa1* and *Hoxb1* (*Hoxa-1-/-; Hoxb-1-/-*) result in a reduced size of r4 and additionally a loss of expression of r4-specific markers. The identity of r4 is may be provided by the synergistic action of *Hoxa-1* and *Hoxb-1*, which together therefore resemble the action of the orthologous *Hox* gene *lab* in the posterior tritocerebrum of *D. melanogaster*; (Abbreviations see Figure 4).


A
Insect
wt
b1
b2
b3
s1
s2
s3
VNC
lab -/-
b1
b2
b3
s1
s2
s3
VNC
B
Vertebrate
wt
T
D
M
r1- r8
Sc
Hoxa-1 -/-
Hoxb-1 -/-
T
D
M
r1-r3
r5-r8
Sc
lab/Hoxb-1
Dfd/Hoxb-4
pb/Hoxb-2
Scr/Hoxb-5
Hoxb-3
Antp/Hoxb-6

6. DISCUSSION

Labial and the co-factors Extradenticle and Homothorax are required for development of the tritocerebrum

The homeotic genes are expressed in a virtually co-linear anteroposterior pattern in the developing posterior brain of insects and mammals, where they are required for the specification of segmental neuronal identity. In the embryonic brain of Drosophila, the Hox gene with the most defined anterior expression domain is *lab*, which is expressed in the posterior tritocerebrum. Neuroanatomical analyses have shown that loss-of-function mutations of the *Hox* gene *lab* result in severe defects in the embryonic brain. In *lab* null mutants cells of the tritocerebrum seem to develop at the right place, however, they do not express the neuron-specific markers, and these cells do not extend axons or dendrites and are not contacted by axons from other parts of the brain. Therefore *lab* mutant cells fail to adopt a neuronal identity and seem to remain in an undifferentiated yet postmitotic state. This results in severe cell-autonomous and cell-non-autonomous axonal patterning defects, including loss of the tritocerebral commissure and reduced or absent longitudinal axonal pathways (Hirth et al., 1998).

Mutant analysis of homeotic genes in the VNC in more posterior regions of the CNS have shown that Hox genes expressed in posterior regions act as negative regulators on Hox genes that are expressed in more anteriorly. For example *Antp* is primarily expressed in Parasegment (PS) 4 and PS5 of the CNS, but it is also expressed at lower levels in PS6-13 (Levine et al., 1983; Hafen et al., 1984; Carroll et al., 1986; Hirth et al., 1998). In embryos that lack the BX-C genes, *Antp* expression is high in PS4-13 (Hafen et al., 1984; Harding et al., 1985; Carroll et al., 1986), suggesting that BX-C gene action keeps *Antp* expression low in PS6-13. Similarly, BX-C genes that are expressed and function in more posterior abdominal segments keep *Ubx* expression low in PS7-13. In the absence of the abdominal BX-C genes, *Ubx* products are found at high levels in PS6-13 (Struhl and White, 1985; White and Wilcox, 1985).

Gain-of-function experiments have shown that ectopic UBX and ABD-A are able to repress *lab* and *Scr* expression in the CNS in a timing dependent manner while otherwise overlapping expression of other Hox genes is tolerated (Miller et al., 2001). The action of *lab* in the developing tritocerebral neuromere can be eliminated by targeted misexpression of

posterior Hox genes through the *sca*::Gal4 driver, resulting in a *lab* loss-of-function phenotype in the brain. This suppression of *lab* has a number of features that are characteristic of the type of cross-regulatory Hox gene interactions that have been demonstrated in developing epidermal structures (Miller et al., 2001). The suppression of *lab* in the tritocerebrum appears to be time dependent. Early misexpression of posterior Hox genes during neurectoderm specification and neuroblast formation reliably results in *lab* suppression in the tritocerebrum, whereas later misexpression does not. Misexpression of posterior Hox genes leads to a loss of LAB protein in the affected domain, and this lack of LAB is in accordance with the observed phenocopy of a *lab* loss-of-function mutation observed in this domain. These experiments extend our insight into cross-regulatory interactions beyond the observations made on developing epidermal structures. The suppression of *lab* by a posterior Hox gene like *Ubx* is due to transcriptional repression. Thus, in *sca*::Gal4/UAS::*Ubx* embryos, *lab* transcripts disappear and are absent in the developing tritocerebrum from stage 10/11 onward. This tritocerebrum-specific repression appears to be mediated through a 3.65kb enhancer element upstream of the *lab* gene transcriptional start site. Moreover, our results imply that suppression of *lab* in the developing tritocerebrum by posterior Hox genes requires a functional homeodomain; mutations of the homeodomain in the *Antp* gene abolish the repressive activity of this Hox gene. In addition, our findings indicate that the suppressive cross-regulatory action of a posterior Hox gene like *Ubx* is not dependent on a functional hexapeptide. Thus, misexpression of a UAS::*UbxYAAA* transgene in which the critical YPWM motif of Ubx was mutated to the sequence YAAA (Galant et al., 2002), still results in complete suppression of *lab* in the developing tritocerebrum. Concomitant misexpression of *Ubx*, nuclear-targeted EXD and HTH is able to completely rescue the *lab* loss-of-function mutant phenotype. This implies that the EXD and HTH co-factors can switch Ubx protein action between different functional states in which EXD and HTH are required for Hox protein transcriptional activation functions whereas they are dispensable for Hox transcriptional repression functions (Pinsonneault et al., 1997; Li et al., 1999). Moreover, our findings can be explained by models in which the hexapeptide is involved in the regulation of Hox protein activity (Merabet et al., 2003; In der Rieden et al., 2004), and may also reflect a requirement for equilibrated levels of a Hox gene product and the HTH and n-EXD co-factors in the specification of tritocerebral identity. This further suggests that in embryonic brain development, proper interaction with co-factors could also

play a pivotal role. Indeed, embryos mutant for *exd* and *hth* display apparent defects in the primary axonal scaffolds, and misregulation of homeotic genes in the brain (Nagao et al., 2001). Therefore it can be assumed that Hox proteins and their co-factor interact together to specify tritocerebral identity. Further, genetic rescue experiments investigate the functional equivalence of all of the *Drosophila* Hox genes in specifying the neuronal identity in the tritocerebral neuromere provide a novel few on the role of Hox proteins and Hox co-factors. Most of the other *Drosophila* Hox gene products are also able to replace the LAB protein in the specification of the tritocerebral neuromere. Only the Abdominal-B protein does not efficiently rescue the *lab* mutant phenotype in the brain (Hirth et al., 2001). Interestingly *Abd-B* is the only member of the Hox complex, which does not belong to the Hexapeptide-superclass. This lack of the YPWM-motive might be the cause of the failure of ABD-B to functionally replace LAB in tritocerebral development. This in turn further supports the notion that Hox protein co-factor interaction plays an essential role in the specification of neuronal identity in the developing tritocerebrum.

vnd is required for tritocerebral neuromere formation

In the development of the VNC, genetic analyses have demonstrated that the genes *vnd*, *ind* and *msh* are required for the columnar subdivision of the neuroectoderm and the subsequent formation and determination of neuroblasts along the dorsoventral axis during *Drosophila* embryogenesis (reviewed by Skeath and Thor, 2003). In the case of *vnd*, detailed studies have shown that *vnd* is required for the specification of the ventral neuroectodermal column and specific neuroblasts. The absence or mis-specification of ventral neuroblasts correlates with the loss or mis-specification of neuronal progeny. For example, the aCC/pCC and dMP2/vMP2 neurons are lost and the RP2 neuron is frequently absent in *vnd* mutant embryos, resulting in axonal pathfinding defects and defective commissure formation in the developing VNC (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002). In contrast to the developing VNC, expression of the columnar patterning genes in the procephalic neuroectoderm region and during brain neuroblast formation is confined to restricted domains along the anteroposterior axis. *vnd* expression demarcates the ventral part of the posterior border of the tritocerebrum, deutocerebrum and ocular neuromere, implying that *vnd* might be required for providing positional information in the procephalic

neuroectoderm and for subsequent specification of individual brain neuroblasts along the AP axis (Urbach and Technau, 2003b; Urbach and Technau, 2004). Further, *vnd* mutant analysis suggests that *vnd* acts at least during two important steps in the formation of the tritocerebral neuromere: precursor cell development and neuronal progeny maintenance. In *vnd* loss of function mutants, the *lab* expressing neuroblasts Tv1-5 are not detectable, suggesting that *vnd* is required for the formation of ventral neuroblasts of the developing tritocerebrum. Later in development, *vnd* mutants display a severe loss of neuronal tissue together with axonal patterning defects in the tritocerebrum. This loss of neuronal tissue is associated with increased apoptotic activity, suggesting that *vnd* is required for the maintenance of neuronal cells that are involved in the establishment of the tritocerebral commissure and the longitudinal connectives that normally run through this neuromere. This suggestion is further supported by the fact that blocking apoptosis in *vnd* null mutant embryos results in the restoration of tritocerebral axon tracts and the wildtype-like expression domain of the Hox gene *labial*. Together, these functional roles of *vnd* in embryonic brain development are reminiscent of its role during VNC development: the formation of specific neuroblasts and their progeny (Jimenez et al., 1995; Chu et al., 1998; McDonald et al., 1998; Mellerick and Modica, 2002). However, in contrast to its role in DV patterning of the developing VNC, the resulting brain phenotype of *vnd* mutants indicates that *vnd* is required for brain patterning along the anteroposterior axis. This is surprising especially since *vnd* expression is confined to the ventral portion of the developing tritocerebrum (Urbach and Technau, 2003b; Urbach and Technau, 2004). Thus, *vnd* action in ventral precursor cells and subsequent neuronal progeny within the developing tritocerebral neuromere is apparently conveyed into patterning along the anteroposterior neuraxis.

Integration of anteroposterior and dorsoventral patterning systems in embryonic brain development of *Drosophila*

The molecular mechanisms that integrate anteroposterior and dorsoventral positional information in the neurepithelium or in neural progenitors that specify distinct neuronal types remain largely unknown. For example in the vertebrate hindbrain it has been shown that the homeotic genes *Hoxb1* and *Hoxb2* together with the *vnd* homolog, the *Nkx2.2* gene, act upon the *Phox2b* gene to specify cranial motoneuron cell fate (Samad et al., 2004). Therefore synergistic action of correctly expressed AP and DV genes are essential to proper spatial

pattern formation the vertebrate hindbrain. Weather similar interactions also exist in the *Drosophila* brain has not yet been shown. The DV gene *vnd* and the homeotic gene *lab* act genetically independent in brain neuromere development. In *lab* mutant embryos, tritocerebral cells develop but fail to adopt proper neuronal cell fate (Hirth et al., 1998). Interestingly the ventral portion of the tritocerebral cells still correctly expresses *vnd*, which represents the first regionalized molecular neuronal marker which remains unchanged in *lab* mutant cells. Therefore *vnd* expression does neither depend upon proper Hox gene expression nor on correct neuronal specification, but remains unchanged. In *vnd* mutant embryos the tritocerebrum is largely absent, due to increased apoptosis, mainly in the neurectoderm, and failure of NB formation. In rare cases only a few cells in that region still develop, forming a thin strand. These cells do express *lab*, additionally also express neuronal specific neuronal makers. If apoptosis is prevented cells in the tritocerebrum express *lab* in a wildtype-like manner, and additionally axonal projections cross that territory. Interestingly in this rescue, albeit the tritocerebrum does not express *vnd*, the cells which are restored do express *lab*. This argues that not only *vnd* expression is independent of the homeotic gene *lab*, but also that *lab* expression does not require *vnd* expression. Therefore the two genes and their role in tritocerebral development seem largely independent. Furthermore, the combination of both patterning systems, homeotic genes and columnar genes, are required at different steps during embryonic brain development for proper neuromeric organization. *vnd* is required for the formation and maintenance of neural precursor cells as well as neuronal progeny within the developing tritocerebral neuromere, whereas the Hox gene *lab* appears to be independently required for the specification of neuronal identity within the same territory during later stages. This indicates that the activity of the DV columnar gene *vnd* is integrated into pattern formation along the anteroposterior neuraxis by generating and maintaining cells which subsequently become specified by the activity of the Hox gene *lab*.

Conserved usage of developmental control genes in embryonic brain development of insects and vertebrates

In the development of the embryonic *Drosophila* brain, the homeotic genes, are responsible for the specification of segmental neuronal identity. Loss-of-function of *lab* results in severe axonal patterning defects, these defects are not due to deletions in the affected neuromere,

since the neuronal progenitor cells and their postmitotic progeny are present in the affected domains, but the cells never adopt neuronal identity (Hirth et al., 1998). In the mouse, neuroanatomical analysis of *Hox* gene mutants reveals that these genes act in specific domains along the anteroposterior neuraxis (Carpenter, 2002; Glover, 2001, Maconochie et al., 1996; Rijli et al., 1998). Loss-of-function of mutations of the *lab* orthologous genes *Hoxa1* and *Hoxb1* provided further insight into the process of *Hox* gene action on brain development. *Hoxa1* null mutation result in segmentation defects in the mutant domain of the hindbrain, whereas *Hoxb1* mutation results in a loss of identity of the affected area in the hindbrain (Goddart et al., 1996; Studer et al., 1996; Rijli et al., 1998; Studer et al., 1998; Gavalas et al., 1998). Double knock-out of *Hoxa1* and *Hoxb1* causes a reduced size and additionally a loss of expression of specific markers in the mutant domain of the hindbrain, resulting in the formation of a domain of unknown identity between (Gavalas et al., 1998; Studer et al., 1998). The *Hoxa1* and *Hoxb1* double knock-out points to a functional role of these *Hox* genes which is surprisingly similarly to the mode of action of the orthologous *Hox* gene *lab* in the posterior tritocerebrum of *Drosophila.*

Interestingly also co-factors of the Hox proteins have been found to be important for proper brain development in both invertebrates and vertebrates. In *Drosophila*, embryos mutant for *exd* and *hth* display apparent defects in the primary axonal scaffolds, and misregulation of homeotic genes in the brain (Nagao et al., 2001). *exd* and *hth* mutation leads to loss of neuronal structures, including the embryonic tritocerebral neuromere, suggesting a combinatorial influence of Hox proteins and Hox co-factors (Nagao et al., 2001). Therefore it can be assumed that Hox proteins and their co-factor interact together to specify tritocerebral identity. The finding that ectopic expression of a Hox protein during brain development, leads to a phenocopy of the *lab* mutant brain phenotype, which can be rescued by concomitantly mis-express a Hox protein with both co-factors, indicates that equilibrated levels of Hox proteins and both co-factors are essential for proper tritocerebral development in *Drosophila*. The homologous genes of the the Drosophila *hth* and *exd* genes are *Meis*/*Prep*, and *Pbx*, respectively. Genetic knockout experiments of *Meis* and *Pbx* families in zebrafish demonstrate that in vertebrates these genes are essential for hindbrain development. In *Pbx4* mutants that have been injected with a *Pbx2* morpholino (to achieve a total block of early Pbx function) the hindbrain is not segmented and r2-r7 acquire an r1-like identity, referred to as the hindbrain ground state (Waskiewicz et al., 2002). Likewise, when the function of *Meis* is

blocked, using two dominant-negative constructs, a similar, nonsegmented hindbrain is produced (Choe and Sagerstrom, 2004). Therefore as in *Drosophila* loss-of-function of the Hox co-factors display brain patterning defects. This further suggests that the synergistic action of Hox proteins and co-factor proteins, as indicated by tritocerebral development in *Drosophila* might also be a valid mechanism in vertebrates for proper hindbrain development.

In *Drosophila* little is know about columnar genes in embryonic brain development despite their detailed expression pattern in the procephalic neuroectoderm and in subsets of neuroblasts. So far genetic analysis of columnar patterning genes have largely been concentrating on the development of the VNC. The *Drosophila* columnar gene *vnd* belongs to the highly conserved *Nkx2* class of transcription factors that have been found in various animals including mammals (Harvey, 1996; Cornell and von Ohlen, 2000). Notably, the *vnd/Nkx2* family of genes is exceptionally well conserved, in terms of both expression and function. Vertebrate homologs of *vnd* are expressed in the neural plate, or tube, in topologically similar positions as is *vnd* in the *Drosophila* ventral neuroectoderm and in the absence of *vnd/Nkx2* genes, the fates of the ventral-most cells in the spinal cord and the Drosophila VNC are transformed (D'Alessio and Frasch, 1996; Cornell and von Ohlen, 2000). Moreover, this evolutionary conservation in expression and function of *vnd/Nkx2* genes appears to apply to some extend to brain development as well. A comparison of the anteroposterior sequence of *vnd/Nkx2* gene expression in the early brain of *Drosophila* with that published for the early mouse brain reveals striking similarities (Urbach and Technau 2003b; 2004). Mutant analysis in mice have shown that *Nkx2* genes appear to play a crucial role in patterning and neuronal specification during embryonic development of the telencephalon and hindbrain. *Nkx2.1* mutant mice display patterning defects in that the entire pituitary is missing (Kimura et al., 1996), a twofold reduction in the number of cortical interneurons, as well as a complete absence of TrkA-expressing cells in the developing telencephalon is observed. Furthermore, the ventral-most aspect of the telencephalon – the medial ganglionic eminence – becomes trans-fated to that of the adjacent, more dorsal lateral ganglionic eminence (Sussel et al., 1999). Thus, comparable to the role of *vnd* during *Drosophila* brain development (this study and accompanying paper), Nkx2.1 is involved in pattern formation and in cell fate determination during embryonic brain development in mice (Rallu et al., 2002) Recent studies have shown that *Nkx2.2* is involved in motor neuron specification in the developing hindbrain. Thus, the sequential generation of visceral motor

neurons and serotonergic neurons from a common pool of neural progenitors located in the ventral hindbrain critically depends on the integrated activities of Nkx2.2- and Hox1/2-class homeodomain proteins (Pattyn et al., 2003a; 2003b). A primary function of these proteins is to coordinate the spatial and temporal activation of the homeodomain protein Phox2b, which in turn acts as a binary switch in the selection of motor neuron or serotonergic neuronal fate (Pattyn et al., 2003a; Samad et al., 2004). These data suggest that comparable to the integrated activity of *vnd* and *lab* in *Drosophila* brain neuromere specification, integrated activity of the Nkx2.2 and Hox1/2 proteins is involved in the specification of segmental neuronal identity (Samad et al., 2004). This indicates that the integration of AP and DV patterning systems by homeodomain transcription factors of the *Hox* and *vnd/Nkx2* genes might represent an ancestral feature of insect and mammalian brain development.

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8. APPENDIX:

Substrate specificity and Faviid juvenile predominance in coral recolonization of Maldivian reef zones

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Key words: Coral reefs, coral recolonization, coral substrates, juvenile corals

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INTRODUCTION

During the coral bleaching event of 1998 the majority of hermatypic corals in the Indopacific showed extensive mortality (Naeem et al. 1998; Berkelsmans et al. 1999; Edwards et al. 2001; McClanahan et al. 2001; Mumby et al. 2001). For example, observations on the North Male Atoll of the Maldives Islands indicated that up to 90% of hermatypic corals died subsequent to the coral bleaching event; before this mass mortality, 42% of the reef surface was covered with hermatypic corals, after the coral bleaching only 2% remained (Naeem et al. 1998; Edwards et al. 2001). Due to the period of mass coral mortality, two new substrate types became available in great quantity in 1998 which could be used for coral recolonization. These substrates were first, the numerous coral fragments that resulted from disintegration of dead Acroporidae and other branching corals, and second, the solid surface of dead compact coral stocks. In this report, we characterise the recolonization of these two substrate types by juvenile corals three years after the massive mortality event. Our study was carried out on five locations on the outer reef of the Rasdhoo Atoll of the Maldives. We found that the solid substrate of compact coral material showed a high degree of recolonization, whereas the fragmented coral substrate was only rarely colonised. Moreover, the contribution of different coral families to coral recolonization showed a strong dominance of the family of the Faviidae, which represented 78% of the reestablished colonies. Based on the size of the juvenile coral colonies, we postulate that the recolonization must have started shortly after the coral bleaching.

METHODS

Data collection was carried out at five different survey sites on the outer reef of the Rasdhoo Atoll during May 13-19, 2001. The location of these sites is shown in figure 1A. At each site, several sample reef surface areas of 250 cm^2 each were chosen randomly at depths of 3-6 m and studied during SCUBA-diving. The surface areas sampled could be divided into two distinct groups based on substrate type. The first was solid substrate consisting of entire dead coral blocks, which generally had a smooth and hard surface (Fig. 1B). The second was a

fragmented substrate consisting of coral debris and parts of broken branched corals (Fig. 1C). At each survey site, both solid substrate and fragmented substrate sample areas were investigated; 15 sample surface areas of each substrate type (30 sites in total) were studied.

Both the number of coral colonies and the average diameter of each colony were determined in each sample surface area. (Preliminary observations indicated that all of the coral colonies involved in recolonization had the round or oval form characteristic of juvenile corals; this made it possible to characterise coral colony size in terms of average diameter.) A total of 375 coral colonies were found on solid substrate and a total of 16 coral colonies were found on fragmented substrate. All 391 colonies were further characterised according to the families to which they belong. The majority of the coral colonies belonged to the families Faviidae, Poritidae, Pocilloporidae or Acroporidae; corals belonging to other families (7% of the colonies studied) were grouped as “others".

RESULTS AND DISCUSSION

During the coral bleaching event of 1998 on the Maldive Islands up to 90% of all coral colonies down to 15 m depth were destroyed (Naeem et al. 1998). Given that this mass mortality did not affect all of the coral colonies, some large-size coral colonies were expected to have survived in the Maldivian reef zones. Indeed, rare examples of surviving large coral colonies were observed at the survey sites, however none of these were located within any of the sample surface areas studied. Thus, all living coral observed within the 15 sample surface areas were small juvenile colonies.

The degree of juvenile coral recolonization appeared strikingly different on solid versus fragmented substrate (Fig. 2A). A relatively high number of juvenile corals was observed on the solid substrate of dead coral blocks. The average number of recolonizing madreporaria colonies per sample surface area (250 cm^2) of solid substrate was 25. This value ranged from 14 to 75 coral colonies in the 15 areas studied. In contrast, there was almost no recolonization observed on the fragmented substrate of broken dead coral debris. On average, only one coral colony was observed per sample surface area (250 cm^2) of fragmented substrate. This value

ranged from 0 to 3 coral colonies per sample surface area; in 5 of the 15 surface areas of fragmented substrate studied no coral colonies were seen.

The average size of the juvenile coral colonies was also different on solid versus fragmented substrate. On fragmented substrate the average diameter of coral colonies was approximately 2.5 cm (range 1-6 cm). In contrast, the average diameter of the coral colonies on solid substrate was 5 cm (range 1-20 cm). Further analysis of the juvnile coral colonies on solid substrate showed that Pocilloporidae colonies had the largest average size (10.2 cm) and that Faviidae colonies had the smallest average size (4cm); Poritidae, Acroporidae and other colonies had intermediate average sizes (range 5-6.2 cm) (Fig. 2B).

An analysis of the relative abundance of different families of juvenile corals revealed a dominant presence of the Faviidae (Fig. 2C). Taken together, the 15 sample areas of solid substrate had 294 colonies of Faviidae, 43 colonies of Poritidae, 10 colonies of Pocilloporidae, 3 colonies of Acroporidae, and 25 colonies of other families. Thus, on average 78% of the coral colonies observed belonged to the the Faviidae. The relative abundance of Faviidae coral colonies observed in each of the 15 sample areas ranged from a minimum of 43% to a maximum of 96%. All other coral families were much less abundant; on average 11% of the coral colonies observed belonged to the Poritidae with all remaining families also making up 11% of the observed colonies. A comparable dominant presence of the Faviidae among the different families of juvenile corals was also observed in the 15 sample areas of fragmented substrate, where 8 colonies of Faviidae, 2 colonies of Poritidae, 1 colony of Pocilloporidae and 5 colonies belonging to other families were seen.

Neither the number of colonies found in each sample area, nor the average size of the colonies showed significant site-specific differences at the five different sites of the Rasdhoo Atoll surveyed (data not shown). Moreover, the relative abundance of coral colonies belonging to the different families was also similar at all five survey sites. This suggests that recolonization had taken place in a comparable manner in all outer reef areas of the Rasdhoo Atoll.

The findings reported here indicate that coral recolonization in Maldivian reef zones following the mass coral mortality of the 1998 bleaching event was strikingly substrate

dependent and dominated by members of the Faviidae family. The two types of coral-derived substrate types that became available due to the mass mortality event, namely coral fragment debris and solid compact coral blocks, showed a very different degree of recolonization three years later. In 2001, the solid coral substrate had a high degree of overall recolonization, whereas the fragmented coral substrate was only rarely colonized. Moreover, the contribution of different coral families to coral recolonization showed a strong dominance of the family of the Faviidae which represented more than three fourths of the juvenile colonies on hard coral-derived substrate. Neither the Faviidae, nor any of the other coral families that were involved in reef recolonization showed a preference for the fragmented coral-derived substrate. It is conceivable that the fragmented substrate is less suitable for recolonization since it is subject to displacement due to wave and current influence. While the reasons for this substrate-dependence are not yet known, the relatively high number of juvenile madreporaria, and notably Faviidae, colonies on hard substrate does demonstrate that considerable recolonization has already occurred within three years. Considering the fact that the coral-derived substrate has only become free for recolonization after the bleaching event, and in view of the published annual growth rate of coral colonies of only a few cm per year (Bak et al. 1979; Lough et al. 2000), we postulate that intensive recolonization of the solid coral-derived reef substrate must have begun shortly after the coral bleaching in 1998.

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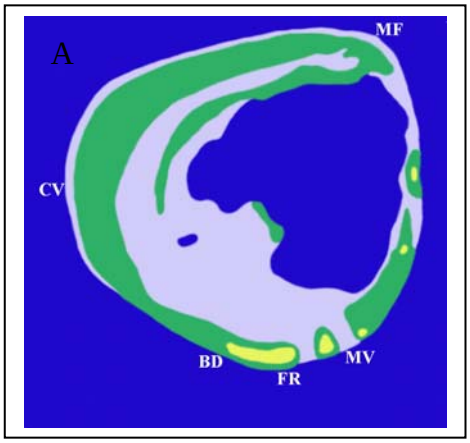
FIGURES:

Figure. 1.

A Location of the five survey sites around the Rasdhoo Atoll: Caves (CV), Fan Reef (FR), Madivaru (MV) Boduga (BD) and Miyaru Faru (MF). (**B)** Example of solid substrate. (**C**) Example of fragmented substrate.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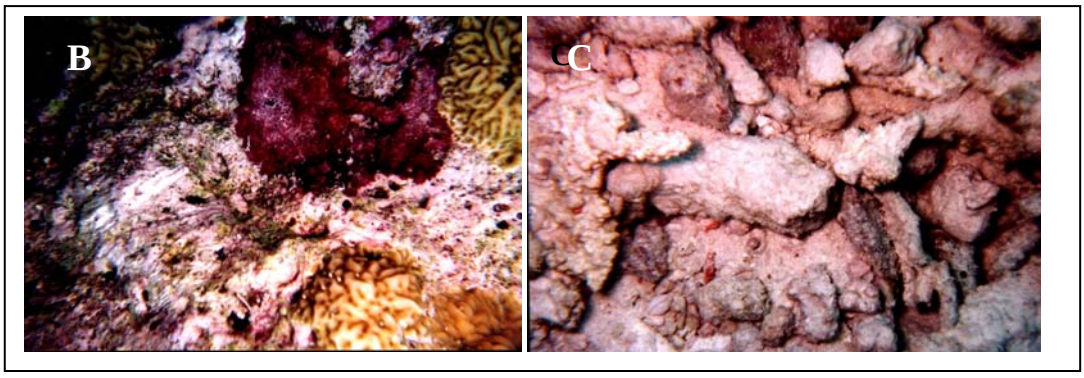
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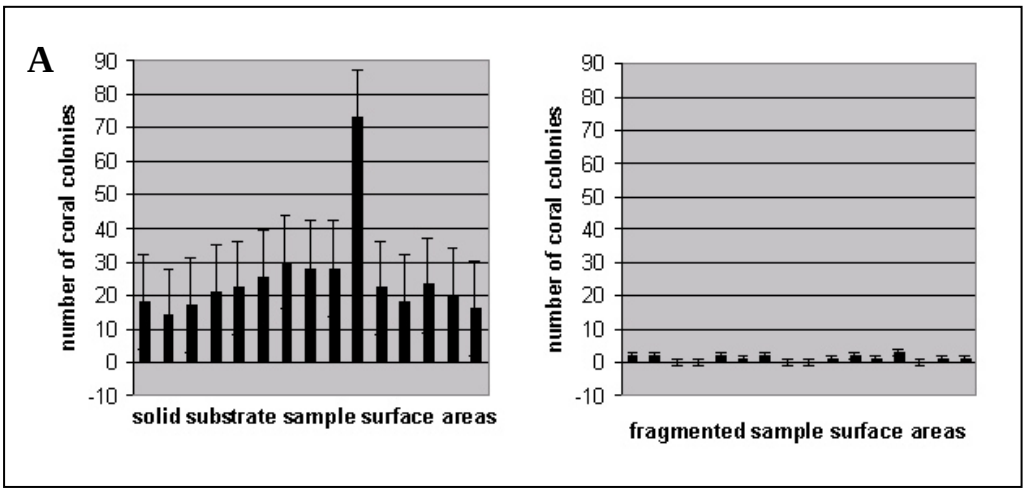
Figure. 2.

(A) Number of juvenile coral colonies found on each sample surface area of solid substrate (right) compared to fragmented substrate (left). Almost no juvenile coral colonies were found on fragmented substrate, where as the solid substrate was recolonized on average by 25 juvenile coral colonies. (B) Average size of juvenile coral colonies on solid substrate. According to coral families Pocilloporidae colonies had the largest average size (10.2 cm) Faviidae colonies had the smallest average size (4cm); Poritidae, Acroporidae and other colonies had intermediate average sizes (range 5-6.2 cm). (C) Relative abundance of different familes of juvenile corals. An average of 78% of all juvenile coral colonies found on solid substrate were Faviids.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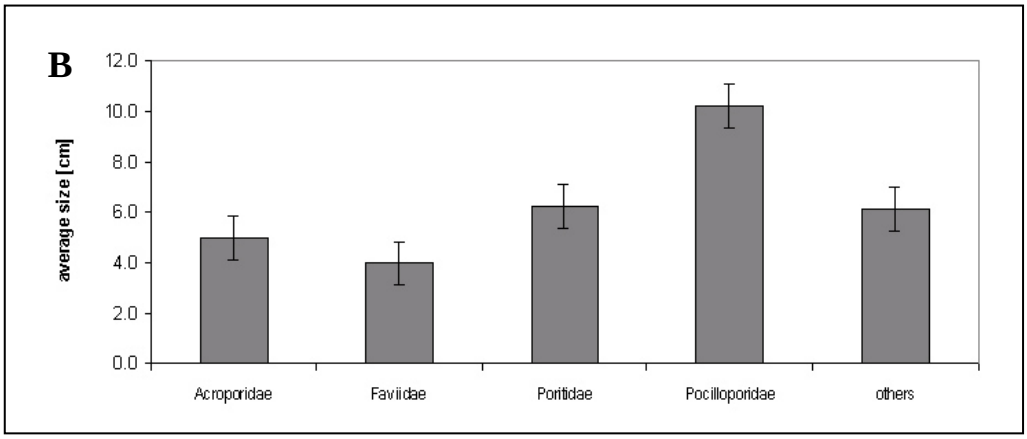
solid substrate sample surface areas
number of coral colonies
fragmented sample surface areas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Poritidae
Pocilloporidae
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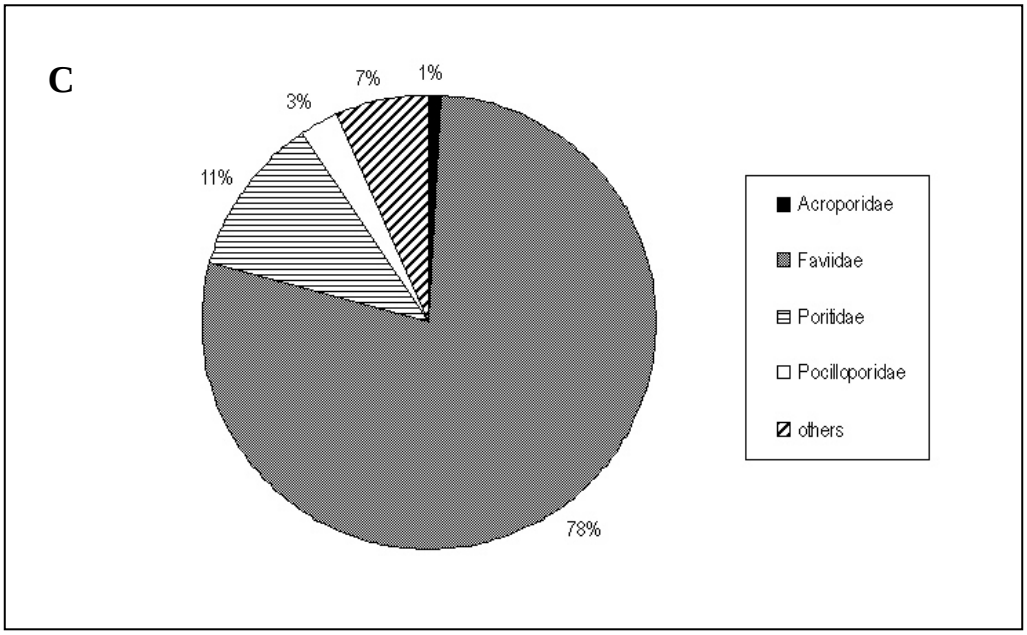
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Acroporidae
Faviidae
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- 15th Biennal Meeting of the "International Society for Developmental Neuroscience". 2004, Edinburgh, Scotland.
- 10th European Symposium on Drosophila Neurobiology. 2004, Neuchâtel, Switzerland.
- 46th Annual Drosophila Research Conference, 2005, San Diego, USA
- invited by Prof. V. Hartenstein, oral presentation title:"Hox gene crossregulatory interactions in the embryonic brain of Drosophila", March, UCLA, Los Angeles, USA.
- invited by Prof. F. Rijli, oral presentation title: "Hox gene crossregulatory interactions in the embryonic brain of Drosophila". June 2004 IGBMC, Illkirch, France.
- invited by Prof. G.M. Technau, oral presentation title: "dorsoventral patterning genes in embryonic brain development of Drosophila". July, 2004, Inst. of Genetics, Mainz, Germany.

List of Publications

Sprecher SG, Galle S, Reichert H
Substrate specificity and juvenile Faviid predominance of coral colonization at the Maldive Islands following the 1998 bleaching event
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Sprecher SG, Reichert H
The urbilaterian brain: developmental insights into the evolutionary origin of the brain in insects and vertebrates
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Sprecher SG, Urbach R, Technau GM, Reichert H, Hirth F
vnd function in embryonic brain development of Drosphila melanogaster
To be submitted

ERKLÄRUNG

Ich erkläre, dass ich die Dissertation

GENETIC MECHANISMS UNDERLYING NEUROMERE SPECIFICATION DURING EMBRYONIC BRAIN DEVELOPMENT OF *DROSOPHILA*

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

Basel, den 14. Juni 2005

Simon Sprecher