

**Cross-Phylum Functional Equivalence of *Otx* Genes
and the Origin of Brain Patterning**

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Abbreviations

AEL,	after egg laying
<i>bFGF</i> ,	<i>basic fibroblast growth factor</i>
<i>bmp</i> ,	<i>bone morphogenetic protein</i>
BSH,	brain-specific homeobox
CNS,	central nervous system
DNS,	diffuse nervous system
<i>ems</i> ,	<i>empty spiracles</i>
<i>en</i> ,	<i>engrailed</i>
FITC,	fluorescein isothiocyanate
FM7,	first multiple 7
GL,	glass
<i>hh</i> ,	<i>hedgehog</i>
HOM,	homeotic complex
HRP,	horseradish peroxidase
<i>hsp</i> ,	<i>heat shock protein</i>
<i>oc</i> ,	<i>ocelliless</i>
ocb,	ocellar bristles
<i>otd</i> ,	<i>orthodenticle</i>
PNS,	peripheral nervous system
<i>poxn</i> ,	<i>pox neuro</i>
pvb,	postvertical bristles
SXL,	Sex lethal
VNC,	ventral nerve cord
<i>wg</i> ,	<i>wingless</i>

Abstract

Molecular mechanisms of cephalic development is an intriguing question in evolutionary and developmental biology. *Otx* gene plays important roles in animal brain and head development and *Otx* genes are found in all major animal groups: cnidarians, lophotrochozoans, ecdysozoans, and deuterostomes. Ascidians, positioned near the origin of the phylum Chordata, share a conserved set of anteroposterior patterning genes with that of vertebrates. Here I report the cross-phylum regulatory potential of the ascidian *Otx* gene in the development of the *Drosophila* brain and the head vertex structures. The ascidian *Otx* gene rescued the embryonic brain defect of *Drosophila* caused by null mutation of the *orthodenticle* (*otd*) gene and enhanced rostral brain development while it suppressed trunk nerve cord formation. Furthermore, the ascidian *Otx* gene restored the head vertex defects caused by a viable *otd* mutation, *ocelliless*, via specific activation and repression of downstream regulatory genes. The cross-phylum regulatory potentials of the ascidian *Otx* gene are equivalent to the activities of the *Drosophila* and human *otd/Otx* genes in these developmental processes. In contrast to these results, cnidarian *Otx* gene showed little functional equivalence in *Drosophila*. In addition, planarian *Otx* gene had failed to rescue *Drosophila* embryonic brain development.

The results with ascidian *Otx* gene function support the notion that basal chordates such as ascidians have the similar molecular patterning

mechanism for the anterior structures found in higher chordates, and suggest a common genetic program of cephalic development among invertebrate, protochordate and vertebrate. On the other hand, I discuss possible reasons of the failure of rescue with the planarian and cnidarian *Otx* genes referring functionally important domains in *Otx* genes.

Introduction

The natural patterns and diversity of animal bodies provide the anatomical basis that has allowed zoologists for more than a century to systematically separate the various phyla and reconstruct evolutionary trees (Brusca and Brusca 1990). Whereas differences among animal body plans have been emphasized by traditional zoologists, recent advances in embryology uncover the underlying unity of the construction principles of animal bodies at the molecular level. Pattern formation along the anteroposterior axis is the best example for such conservation at the deep level. An animal can be defined as an organism that acquired a particular spatial pattern of *Hox* gene expression along the anteroposterior axis (Slack et al. 1993). The body plan of each phylum becomes characteristic at a temporal point called a phylotypic stage in its embryonic development. The spatial expression of the *HOM/Hox* genes is the most clearly manifested at this stage in remarkably conserved manners in diverse species.

As an intriguing part of the debate on animal axial patterning, the origin and evolution of cephalic structures has long been the subject of extensive investigation (reviewed in Jefferies 1986; Gee 1996). Whereas it traditionally has been accepted that cephalic structures including the brain of the vertebrates and the invertebrates were evolved independently, recent molecular studies have revealed a set of conserved regulatory genes that are commonly expressed in the head and brain of animals of diverse phyla

(Finkelstein and Boncinelli 1994; Thor 1995; Sharman and Brand 1998; Holland and Holland 1999; Galliot and Miller 2000). Most of these regulatory genes encode nuclear transcription factors setting up the anterior patterns very early in embryonic development. Later in development, many of these genes also function in the formation of brain primordia and in the regionalization of the developing brain.

Among the anterior patterning genes, homologs of the *orthodenticle* (*otd*) /*Otx* genes encoding *paired*-class homeodomain proteins have been isolated from organisms of a wide range of phyla, including vertebrates, lower chordates and invertebrates (Finkelstein et al. 1990; Simeone et al. 1993; reviewed in Acampora and Simeone 1999). Strikingly, a homolog of the *otd/Otx* gene has also been isolated in hydra, which has a very primitive body plan with diffuse nervous system (Smith et al. 1999). *CnOtx*, the hydra *Otx*, is expressed in the tentacle zone and developing buds and is thought to be involved in the formation of new axes (Smith et al. 1999). In bilateral animals, *Otx* genes are expressed in anterior precursor tissues and play pivotal roles in the development and specification of the anterior structures including the rostral regions of the developing brain.

Planarians, once thought to be the basal bilaterian as acoelomates that first acquired central nervous system and brain, have *Otx* genes with expression in its cephalic ganglion (Umesono et al. 1999). Present

phylogenetic study based on 18S rRNA has produced new animal taxa (Adoutte et al. 1999). In the new phylogeny, protostomes are divided into two major branches: the lophotrochozoans and the ecdysozoans. Planarians belong to the lophotrochozoans while insects belong to ecdysozoans.

Ascidians (sea squirts) belong to a Chordata subphylum, Urochordata, which is the sister group of cephalochordates and vertebrates, and exhibit characteristic features of the chordate body plan such as a notochord, a dorsally located nerve cord and pharyngeal gill slits (Satoh et al. 1996). In their early development, ascidian embryos undergo gastrulation, which begins at around the 110-cell stage, and develop into larvae that possess a simple yet common body plan as compared with vertebrate embryos. The larval ascidians possess a hollow central nervous system at their dorsal side. Contrary to the conventional assumptions that ascidians are headless, the anterior part of the neural tube bulges to form an anterior structure called the sensory vesicle that contains most of the neurons of the ascidian central nervous system (Katz 1983; Nicol and Meinertzhagen 1991). In striking similarity to the developing processes of the vertebrate nervous system, formation of the ascidian sensory vesicle depends on a set of inductive signals from the vegetal blastomeres (Rose 1939; Okado and Takahashi 1988; Nishida 1991; Wada et al. 1999) that trigger a series of morphogenetic movements leading to the formation of the hollow neural tube (Nicol and Meinertzhagen 1991).

In ascidian embryos, the expression of the *Otx* homolog starts at the 32-cell stage in the precursor blastomeres of the anterior neuroectoderm as well as the mesoendoderm (Wada et al. 1996). The expression of the *Otx* gene precedes the onset of other neural markers and persists in the anterior neuroectoderm during gastrulation and neurulation. As the embryos develop to the tailbud stage, *Otx* gene expression gradually becomes restricted to the sensory vesicle of the dorsal nerve cord. In addition, the *Otx* gene also is expressed in the anterior epidermis from the neural plate stage onward, whereas its expression in the mesoendoderm becomes confined to the endoderm, mesenchyme and trunk lateral cells before it disappears by the early neurula stage.

In this paper, I report the abilities of *Otx* genes from all major animal taxa; cnidarian, planaria (lophotrochozoa), and ascidian (deuterostome) in *Drosophila* (ectdysozoa) to compare *Otx* genes activity in brain development. I report the cross-phylum regulatory potential of the ascidian *Otx* gene in the development of the *Drosophila* brain and the head vertex structures. I show that the ascidian *Otx* gene rescues the embryonic brain defect caused by a null mutation of the *Drosophila otd* gene, and that it enhances rostral brain development while suppressing trunk nerve cord formation in *Drosophila* embryos. Furthermore, I also show that the ascidian *Otx* gene restores the head vertex defects caused by a viable *otd* mutation, *ocelliless* (*oc*), via specific activation and repression of

downstream regulatory genes. These cross-phylum regulatory potentials of the ascidian *Otx* gene are equivalent to the activities of the *Drosophila* and human *otd/Otx* genes in these developmental processes. These results support the notion that primitive chordates such as ascidians have the same molecular patterning mechanism for the anterior structures found in higher chordates, and suggest an ancient origin of the underlying genetic programs of cephalic development of bilateral animals. In contrast to ascidian *Otx*, cnidarian *Otx* gene does not show clear function in *Drosophila*. Similarly, planarian *Otx* does not rescue *Drosophila* brain development either. I discuss discrepancies of the *Otx* gene functions based on evolutionally changes of functional domains in *Otx* genes.

Materials and methods

Plasmid construction

The ascidian *Otx* gene (Fig. 1; Wada et al. 1996), planarian *DjotxA* gene (Fig. 1; Umesono et al., 1999), and cnidarian *CnOtx* gene (Fig. 1; Smith et al., 1999) were subcloned downstream of a *heat shock protein (hsp) 70* promoter in pNHT4 heat shock P-element vector and introduced into *ry*⁵⁰⁶ flies (Fig. 2; Leuzinger et al. 1998; Nagao et al. 1998) to generate *hsp-Hroth*, *hsp-DjotxA*, and *hsp-CnOtx* lines.

Fly stocks

The fly stocks used in this work are summarized in Table 1. The *hsp-otd* line 5A was described by Royet and Finkelstein (1995) and the human *Otx* lines were generated by Nagao et al. (1998) and Leuzinger et al. (1998). The *otd*^{JA101} null allele was balanced over an FM7-lacZ balancer. The viable *otd* mutant was *ocelliless (oc)*¹, which specifically eliminates *otd* expression in the primordium of the head vertex (Royet and Finkelstein 1995).

Rescue of the embryonic brain

In brain rescue experiments, male flies carrying the *hsp-Otx* gene were crossed with heterozygous *otd*^{JA101} female flies (Fig. 3). Embryos of the

crosses were collected at 1-h intervals and exposed to a series of 37°C heat pulses as described (Fig. 3; Leuzinger et al. 1998). After the last heat pulse, embryos were allowed to develop at 18°C overnight until they reached late embryonic stages (corresponding to stage 15/16). Male embryos hemizygous for the *otd* mutant chromosome were identified by the absence of the FM7- lacZ and the female-specific *Sex lethal* (*Sxl*) protein (Bopp et al. 1991). Brain rescue was dependent on the presence of functional *otd/Otx* genes as heat pulses without the transgene resulted in no restoration of the embryonic brain.

Dominant suppression of the trunk nervous systems

Embryos of transformed flies were collected at 1-h intervals, exposed to a single heat induction at 37°C for the indicated duration, and allowed to develop at 25°C overnight. The organization of the central and peripheral nervous systems were examined using anti-HRP (Jan and Jan 1982) and monoclonal antibody 22C10 (Zipursky et al. 1984). Weaker phenotypes were obtained when the embryos were allowed to develop at 18°C after heat shock.

Rescue of the adult head vertex structures

Transformed males carrying *hsp-Hroth* were crossed with *oc*¹ mutant females and the expression of the *hsp-otd/Otx* gene was induced with

multiple heat pulses at 37°C in the third instar larval stage (Fig. 7; Nagao et al. 1998). The treated larvae were raised at 25°C and the heads of the adult flies were mounted in glycerol. For examination of gene expression in the vertex primordia, heatshocked larvae were kept at 18°C for 36 h and the eye-antennal discs were dissected for signal detection.

Immunocytochemistry and in situ hybridization

Embryonic brains were immunolabeled and examined as described by Nagao et al. (2000) and Leuzinger et al. (1998). Primary antibodies were FITC-conjugated rabbit anti-HRP 1:100 (Jackson ImmunoResearch), mouse 22C10 (Zipursky et al. 1984) 1:5, rabbit anti-Brain specific homeobox (BSH; Jones and McGinnis 1993) 1:50, mouse anti-Glass (GL; 9B2.1; Ellis et al. 1993) 1:5, and mouse anti-Sex lethal (SXL; Bopp et al. 1991) 1:50. Cy3- or Cy5- conjugated secondary antibody (Jackson ImmunoResearch) was used according to the instructions of the supplier. Embryos were mounted in Vectashield (Vector) and examined using a Zeiss LSM410 confocal microscope. Expression of *engrailed* (*en*; Patel et al. 1989), *hedgehog* (*hh*; Tabata and Kornberg 1994), and *wingless* (*wg*; DiNardo et al. 1985) in the head vertex primordia was examined as previously described (Nagao et al. 1998).

Results

Rescue of the embryonic brain defect of the *otd* null mutant

The supraesophageal ganglia of the embryonic *Drosophila* brain consists of three neuromeres, b1, b2, and b3, that give rise to proto-, deuto- and tritocerebrans, respectively (Hirth et al. 1995; Nagao et al. 2000). Molecular genetic studies have shown that the initial formation of these brain neuromeres is controlled by the homeobox-containing genes *otd* and *empty spiracles* (*ems*; Dalton et al. 1989; Walldorf and Gehring 1992). The product of the *otd* gene is required for the formation of the anteriormost neuromere, b1, while the product of the *ems* gene is required for the formation of the posterior two neuromeres, b2 and b3 (Hirth et al. 1995; Younossi-Hartenstein et al. 1997). In *otd* null mutants, the protocerebral brain anlage fails to develop and the preoral brain commissure is not formed (Fig. 4C, D; Fig. 5B; Hirth et al. 1995). In accordance with these gross anatomical defects, most of the anterior set of protocerebral cells that express BSH (Jones and McGinnis 1993) are lacking in the mutant although BSH expression at the postero-ventral position is often retained. Previous work showed that controlled expression of *Drosophila otd* and human *Otx* genes complements the absence of *otd* gene activity and restores the anterior brain formation with rescue of the anterior BSH-expressing cells (Leuzinger et al. 1998; Fig. 4E-H; Table 2).

To examine anterior patterning activity of the *Otx* genes, transgenic flies carrying the ascidian *Otx* gene (Fig. 1; Wada et al. 1996), planarian *Otx* gene (Fig. 1; Umesono et al. 1999), and cnidarian *Otx* gene (Fig. 1; Smith et al. 1999) under the *hsp-70* gene promoter were constructed and crossed with *otd* null mutant flies. Ubiquitous expression of the *Otx* gene was then induced in the *otd* mutant background with the heatshock regime used in the brain rescue experiments with *Drosophila* and human *otd/Otx* genes (Fig. 3).

Ubiquitous expression of ascidian *Otx* gene in the *otd* null mutant background resulted in restoration of anterior brain morphology to various extents in a total of 68% of the treated embryos (Table 2). Although the restored brain is somewhat distorted, the overall morphology of the rescued brains was very similar to the embryos rescued by the *Drosophila* and human *otd/Otx* genes in that both the protocerebral anlage and the brain commissure were recovered (Fig. 4I, J). Furthermore, BSH expression was also recovered in anterior protocerebral cells in 21% of the embryos. Notably, the efficiency of brain rescue by the ascidian *Otx* gene was comparable to that of the *Drosophila otd* gene (68%) and the human *Otx2* gene (50%). Similar brain restoration was confirmed with another transgenic line, Hr45-cII (40%). Thus, these data demonstrate that the ascidian *Otx* gene is able to replace the *Drosophila otd* gene in the development of the anterior part of the embryonic brain allowing

development to an extent similar to that restored with the *Drosophila* and human *otd/Otx* homolog.

In contrast to the ascidian *Otx* gene, planarian *Otx* gene *DjotxA* have failed to rescue *otd*^{JA101} mutant (Fig. 5C). Neither could I detect brain patterning function of *CnOtx*, a cnidarian *Otx* gene, in rescue of mutant brain formation (Fig. 5D).

Dominant suppression of the trunk nervous systems

In contrast to the brain rescue protocol, continuous overexpression of the fly *otd* gene for 20 min at stage 7-8 leads to dramatic transformation of the central nervous system in a wild-type background (Fig. 6A, B; Leuzinger et al. 1998). Dominant suppression of the ventral nerve cord was induced at high rate (Table 3). Furthermore, immunological staining with the 22C10 antibody revealed severe disruption of the peripheral nervous system in the trunk region suggesting general segmentation defects in the posterior part of the embryos. On the other hand, the anterior nervous structures were still preserved or often enhanced in many cases. Compared to such robust alterations with the *Drosophila otd* gene, milder transformation was caused by the ectopic expression of the human *Otx2* gene; both the central and peripheral nervous systems were moderately disrupted in the trunk whereas the anterior part of the brain was largely unaffected (Fig. 6C).

Ubiquitous overexpression of the ascidian *Otx* gene resulted in

significant transformation of the central and peripheral nervous systems (Fig. 6D; Table 3). As in the cases of the *Drosophila otd* gene, overexpression of the ascidian *Otx* gene resulted in dramatic suppression of the ventral nerve cord as well as the peripheral nervous system in the trunk leaving the majority of the embryos without distinct trunk structures. Moreover, anterior neural structures were often enhanced at a rate comparable to the transformation by the *Drosophila otd* gene. Thus, these results reveal an additional functional conservation of the ascidian *Otx* gene as compared with the *Drosophila* and human *otd/Otx* genes in the anteroposterior patterning of the nervous systems. On the other hand, ubiquitous expression of planarian *DjotxA* gene caused only minor defects of central nervous system (Fig. 6E). These effects on the central nervous system were much milder than those caused by the human *Otx2* gene overexpression (Fig. 6C). In particular, the peripheral nervous system was intact (Fig. 6E). Similarly, *CnOtx* gene had little effect on the general shape of the central nervous system (Fig. 6F). The severe defects of the peripheral nervous system observed with *hsp-otd*, *hsp-Hroth*, and *hsp-Otx2* (Fig. 6B-D) were not caused by *CnOtx* gene (Fig. 6F).

Rescue of the adult vertex head structures

The dorsal region of the head is considered one of the most anterior structures of the adult fly (Jürgens and Hartenstein 1993). The medial

subdomain of the vertex contains the light-sensing organs, ocelli, and a set of characteristic sensory bristles [the large ocellar bristles (ocb), postvertical bristles (pvb), and the small interocellar bristles, see Fig. 8A]. During pupal development, the medial vertex is formed by the fusion of the dorsomedial domains of the eye-antennal discs (Royet and Finkelstein 1995). The development of this region is controlled by the *otd* gene and severely affected in *oc*¹ mutant flies, in which *otd* expression in the primordium of the head vertex is specifically eliminated (Royet and Finkelstein 1995, 1996). Flies homozygous or hemizygous for *oc*¹ mutations lack the ocelli and most of the associated bristles (Fig. 8B).

The vertex defects of the *Drosophila oc*¹ mutation can be rescued by the *Drosophila otd* gene as well as the human *Otx* genes (Nagao et al. 1998). This complementation effect specifically requires the *otd/Otx* gene activity as no rescue is obtained with an *hsp* promoter-driven *ems* gene, which is another anteriorly expressed homeobox gene (Dalton et al. 1989; Walldorf and Gehring 1992). Furthermore, the restoration of vertex defects by the introduced *Otx* genes is not mediated by the activation of the endogenous *Drosophila otd* gene, providing a unique system to examine regulatory potentials of transgenic *Otx* homologs of other species (Nagao et al. 1998).

Previous results indicate the functional conservation of the ascidian *Hroth* gene, human *Otx* genes, and *Drosophila otd* gene but not

planarian *DjotxA* gene and hydra *CnOtx* gene. To further examine functional conservation of the ascidian *Otx* gene in cephalic development, I induced ascidian *Otx* gene expression in *oc*¹ background by heat shock pulses in larval stages. Similar to the *Drosophila* and human *otd/Otx* homologs, the ascidian *Otx* gene partially complemented the *oc*¹ defect, generating ocellar lenses (arrows in Fig. 8E) although the ascidian *Otx* gene was unable to restore the vertex bristles (summarized in Table 4).

The primordium of the vertex is situated near the dorsomedial edge of the eye-antennal disc (Fig. 9; Royet and Finkelstein 1995). A network of cross-regulatory segment polarity gene interactions is involved in the development of the vertex primordium: *en* (Patel et al. 1989) and *hh* (Tabata and Kornberg 1994) are expressed while *wg* (DiNardo et al. 1985) is suppressed in a medial patch of cells in the late third instar stage (Fig. 9F, K, P; Royet and Finkelstein 1996). These genetic interactions are unique to the vertex primordium and different from those in trunk development, where *wg* acts to maintain *en* and *hh*, and *en* and *hh* act to maintain *wg* (Hammerschmidt et al. 1997). The *oc*¹ mutation causes specific loss of expression of *en* and *hh* in this region (Fig. 9G, L) whereas expression of *wg* is maintained in a continuous crescent-like pattern in the dorsomedial region of the eye-antennal disc (Fig. 9Q). These genetic interactions can be restored by the larval heat-shock induction of the *Drosophila* and human *otd/Otx* genes as reported previously (Fig. 9; Nagao et al. 1998). Despite

the ubiquitous induction of the *otd/Otx* genes over the eye-antennal disc, the regulatory effects on the downstream genes was restricted to the vertex primordium suggesting that cell type-specific cofactor(s) are involved in the gene regulation.

To determine whether the morphological partial complementation by the ascidian *Otx* gene was a result of correct genetic interactions, we examined the expression of *en*, *hh* and *wg* in the vertex primordium cells following heat induction in *oc*¹ background. I also examined the expression of the GL protein (Ellis et al. 1993), which is expressed in the ocellar photoreceptor cells and missing in *oc*¹ ocellar primordia (Fig. 9B). The GL expression was partially restored by the *Drosophila otd* gene (Fig. 9C) but not by the human *Otx* gene (Fig. 9D). Induction of the ascidian *Otx* gene resulted in specific activation of *gl*, *en*, and *hh* in the vertex primordial cells (Fig. 9E, J, O; Table 5). Moreover, the ascidian *Otx* gene suppressed *wg* expression in the vertex primordial cells though its effect was somewhat weaker than those of *Drosophila* and human *otd/Otx* genes (Fig. 9T; Table 5). Notably, these regulatory effects by the ascidian *Otx* gene were regionally specific to the vertex primordial cells as was the case for the *Drosophila* and human *otd/Otx* homologs suggesting that the induced ascidian OTX protein functions in collaboration with the localized vertex cofactors.

In addition to the conserved functions in the development of the

embryonic nervous systems, these cross-phylum activities on the restoration of the vertex structures and gene expression patterns provide further examples of the functional equivalence of the *Drosophila*, human and ascidian *otd/Otx* homologs in patterning anterior structures.

Discussion

The origin of the anterior brain and the cephalic structures has been debated extensively since the last century for both invertebrates and vertebrates. By introducing the ascidian *Otx* gene into *Drosophila*, I have examined the conserved patterning activities of the ascidian *Otx* gene in the development of the embryonic brain and the central nervous system in *Drosophila*. Moreover, I have also examined the regulatory activities of the ascidian *Otx* gene in the formation of the head vertex structures of adult flies at both morphological and molecular levels. Notably, in all of these developmental processes, the ascidian *Otx* gene exhibits striking cross-phylum regulatory potentials that are equivalent to *Drosophila* and human *otd/Otx* homologs. In contrast to this striking conservation of *Otx* genes of fruitfly and chordates, there were no or little functions of planarian *DjotxA* and Cnidarian *CnOtx* genes in the *Drosophila* nervous system formation.

Patterning the central nervous system in ascidians

In contrast to the conventional anatomical notion that head structures including the anterior brain lobes are difficult to identify in ascidians, recent molecular studies reveal an increasing number of regulatory genes in common that are involved in the early development of the ascidian and vertebrate embryos (Williams and Holland 1998). In particular, regulatory genes containing homeobox genes exhibit similar and conserved expression

patterns along the body axis of chordate embryos (Fig. 11). While the *labial* homolog *HrHox-1* (Katsuyama et al. 1995), and the *caudal* homolog *Hrcad* (Katsuyama et al. 1999) are expressed in the middle and more posterior regions, respectively, in the ascidian embryos, the ascidian *Hroth* gene is expressed in the most anterior region, which corresponds to the sensory vesicle (Wada et al. 1996, 1999). Intriguingly, *HrPax-258*, which is the single known ascidian homologue of the vertebrate *Pax2*, *Pax5* and *Pax8* genes, is expressed in the region between the *Hroth* and the *HrHox-1* genes in the developing central nervous system (Fig. 11B; Wada et al. 1998). Comparative studies of the expression patterns of the ascidian *Hroth*, *Pax-258*, and *labial* genes suggest that the ascidian neural tube is subdivided into three regions: the sensory vesicle, which is marked by *Hroth*; the neck region, which is marked by *HrPax-258*; and the visceral ganglion/tail nerve cord, the anterior limit of which is marked by *HrHox1* (Fig. 11B; Wada et al. 1998). These subdivisions are proposed to correspond to the homologous vertebrate subdivisions: the forebrain/midbrain, the anterior hindbrain, and the hindbrain/ spinal cord, respectively (See Fig. 11A, B).

In addition to these genetic programs, the inductive processes of the central nervous system of ascidian embryos also resemble those of vertebrates. At the onset of gastrulation, the sensory vesicle of the ascidian embryo is induced by the vegetal hemisphere cells (Rose 1939; Reverberi

et al. 1960; Okado and Takahashi 1988; Nishida and Satoh 1989; Nishida 1991). Formation of neural tissues of the sensory vesicle is inhibited by overexpression of the ascidian homologue of *bone morphogenetic protein*, *Bmp-2/Bmp-4* (Miya et al. 1997) whereas the neural tissues of the sensory vesicle are induced by bovine recombinant *basic fibroblast growth factor* (*bFGF*, Inazawa et al. 1998). Although details of neural-inducing mechanisms in ascidian embryos remain to be elucidated, these data suggest that cellular mechanisms involved in the induction and patterning of the central nervous system are also conserved between vertebrates and ascidians.

Mutant phenotypes of the *otd/Otx* genes in axial patterning

Despite the apparent morphological differences, embryonic studies on vertebrates and invertebrates have demonstrated conserved genetic programs of brain development (reviewed in Thor 1995; Sharman and Brand 1998). In *Drosophila* the initial formation of the anterior brain is controlled by the *otd* and *ems* genes (Hirth et al. 1995; Younossi-Hartenstein et al. 1997; Nagao et al. 2000). Vertebrate homologs of the *Otx* and *Emx* genes also are required for the formation of anterior brain structures (reviewed in Acampora and Simeone 1999). In the *Otx* mutant mouse embryos, formation of mesodermal cells is disturbed as the embryos undergo gastrulation. At later stages, mutant embryos fail to develop

forebrain and midbrain as well as a part of the hindbrain anterior to rhombomere 3.

In addition to these loss-of-function studies, analyses of gain-of-function phenotypes of the *otd/Otx* genes have been carried out by ectopic overexpression in several animal species including *Xenopus*, *Drosophila* and ascidians (Blitz and Cho 1995; Pannese et al. 1995; Leuzinger et al. 1998; Wada and Saiga 1999). Microinjection of *Xotx2* mRNA into *Xenopus* embryos causes reduction of the trunk and the tail, formation of a partial secondary axis, and ectopic anterior structures such as cement glands and neural tissues (Blitz and Cho 1995; Pannese et al. 1995). In *Drosophila*, ubiquitous expression of the *otd* gene leads to suppression of the trunk nerve cord development and enhancement of anterior neural structures (this study and Leuzinger et al. 1998). Ectopic *otd* expression also causes duplication of anterior sensory structures in the epidermis and activation of *wg* and *en* expression in the anterior segment (Gallitano-Mendel and Finkelstein 1998). Also noteworthy is that microinjection of synthetic ascidian *Hroth* mRNA into fertilized eggs leads to expansion of the ascidian trunk, which may correspond to the anterior region in vertebrates, and reduction of tail structures. Intriguingly, ectopic expression of the ascidian *Otx* gene causes formation of anterior neuroectoderm at the expense of epidermal tissues via suppression of specific gene expression (Wada and Saiga 1999).

Combined with the data of loss-of-function studies, these gain-of-function phenotypes further argue for conserved genetic functions of the *otd/Otx* genes in embryonic axial specification in that the *otd/Otx* genes promote the formation of the brain and other anterior structures while suppressing the formation of more posterior structures. This idea is indeed supported by functional gene replacement studies (Acampora et al. 1998; Leuzinger et al. 1998; Nagao et al. 1998); in these studies, human *Otx1* and *Otx2* genes were introduced and expressed in *Drosophila* development to show that the human *Otx* homologs complement the *Drosophila otd* mutation leading to restoration of the anterior brain and head vertex structures (Leuzinger et al. 1998; Nagao et al. 1998). Similarly, the *Drosophila otd* gene successfully restores the brain patterning defects in *Otx1* mutant mice (Acampora et al. 1998). Thus, the conserved cross-phylum activities of the ascidian *Otx* gene presented in this work further supports the notion that patterning activities of the *otd/Otx* genes are functionally conserved across the diverse phyla of bilateral animals, including invertebrate, protochordate and vertebrate.

Conserved genetic programs of neuraxial patterning

Based on the similarities in gross anatomical organization and homeobox gene expression along the neuraxis, I propose putative homologous regions between the vertebrate, ascidian and invertebrate central nervous systems.

In all of these animals of diverse phyla, homologs of the *otd/Otx* gene are expressed in the most anterior part of the central nervous system (Fig. 11; Simeone et al. 1993; Hirth et al. 1995; Wada et al. 1996; Nagao et al. 2000). The posterior expression boundary of the *otd/Otx* gene marks the posterior limit of the midbrain in vertebrates (Simeone 2000), the posterior limit of the sensory vesicle in ascidians (Wada et al. 1996), and the posterior boundary of the supraesophageal ganglia in *Drosophila* (Nagao et al. 2000).

A characteristic boundary region with inductive activity follows the anterior *Otx* expression region in the vertebrate brain. In early mouse embryo, *Pax-2*, *Pax-5*, and *Pax-8* are expressed around the boundary region of the midbrain and hindbrain (Holland and Holland 1999). The ascidian homologue, *HrPax-258*, is expressed in the neck, which is immediately posterior to the *Otx* expression domain of the sensory vesicle. In *Drosophila*, two *Pax2/5/8* orthologues, *Pox neuro (Poxn)* and *Pax2* (Noll 1993; Fu and Noll 1997) are reported. Precise analysis of the *Drosophila Pax2/5/8* class genes expression pattern revealed that expression of *Pax2* and *Poxn* does not occur at a comparable anteroposterior position along the neuraxis, with one exception (Hirth et al. 2003). This exception is in the posterior b2 neuromere, immediately anterior to b3 brain neuromere, where *Pax2* and *Poxn* are co-expressed (Fig. 11D; Hirth et al. 2003). Thus, a *Drosophila* brain displays developmental genetic features similar to those

observed for the midbrain/hindbrain boundary region in vertebrate brain development.

Similar to these anterior regions, the posterior region of the nervous system is characterized by the expression of the *HOM/Hox* genes. The hindbrain and the spinal cord of vertebrates are likely to be homologous to the ascidian visceral ganglion and tail nerve cord. It is noteworthy that the anterior limit of the ascidian *Hox* expression domain reaches the posterior limit of the *HrPax-258* expression domain, whereas the anterior limit of *Hox* expression is posteriorly recessed in vertebrates with a gap behind the midbrain-hindbrain boundary. Interestingly, the anterior limit of the *Drosophila HOM* genes invades the supraesophageal region in partial overlap with the *otd* gene (Hirth et al. 1998; Nagao et al. 2000).

In contrast to these gene expressing domain similarity, there is no *Emx* expression in anterior CNS of ascidian embryogenesis (Fig. 11B; Oda and Saiga 2001). Although partial functional equivalence of *Drosophila ems* and murine *Emx2* genes are reported (Hartmann et al. 2000), Oda and Saiga suggest the possibility that the function of *ems/Emx* genes in the patterning of the anterior CNS in *Drosophila* and vertebrate embryos might have been acquired independently in the lineages to *Drosophila* and vertebrates (Oda and Saiga 2001). In the recent analysis of hemichordate, one of the closest invertebrate group to chordates, Lowe et al. (2003)

examined 22 orthologous genes that are involved in patterning the chordate central nervous system. Hemichordate *Saccoglossus kowalevskii* has tripartite body plan consists of prosome, mesosome, and metasome, from anterior to posterior, respectively (Fig. 11C). In that study, *Otx* homologue of hemichordate is expressed weakly in the prosome, mesosome, and anterior metasome, while the *Emx* orthologue expression domain is a single ring in the anterior mesosome plus an additional domain in the posterior metasome. Moreover, *Hox* cluster genes of *S. kowalevskii* are expressed in posterior to the *Otx* domain (Fig. 11C; Lowe et al. 2003). Thus, *Otx/Emx/Hox* genes expression domain topography is conserved in chordates, hemichordates, and *Drosophila* (Fig. 11). It is likely that the lack of *Emx* expression in ascidian CNS development was later characterized during the evolution of the urochordate lineage.

Origin of anterior brain patterning

Based on the 18S rRNA sequences, a novel phylogenetic tree has recently been proposed, in which bilateral metazoans are classified into three major groups of phyla: the ecdysozoans, lophotrochozoans, and deuterostomes (Fig. 10; reviewed in Adoutte et al. 1999). Intriguingly, homologs of the *Otx* genes have been cloned from diverse animals of all three groups and are expressed at the most anterior region of the central nervous system in all the cases. In this phylogeny, flatworms and annelids fall within

Lophotrochozoa, which is a sister group to the Ecdysozoa and the deuterostomes. Anterior neural expression of the *Otx* genes has also been reported for these animals (Bruce and Shankland 1998; Umesono et al. 1999). Combined with the functional conservation of the *otd/Otx* genes, these molecular taxonomical data suggest a common origin of the underlying molecular mechanisms of brain patterning, and support a tight link between the acquisition of bilateral symmetry and the anterior patterning of the central nervous system. Consistent with this view, it is noteworthy that the cnidarian *Otx* homolog (Smith et al. 1999) barely retains anterior patterning activity when examined in transgenic flies (Fig. 5D; Table 2,3).

Planarian *Otx* gene also shows little ability of anterior patterning in transgenic *Drosophila* (Fig. 5C; Tables 2, 3). There might be some possible accounts for this. The homology of homeodomain is lower in planaria (Fig. 1). And I speculate the incompatibility of planarian *DjotxA* gene and *Drosophila otd* gene could come from the peculiarity of planarian lineage in evolution. Once planarians were thought to be a basal animal of bilateria but recent molecular phylogenetic analysis does not necessarily support this notion (Adoutte et al. 1999). Planarians may have acquired its special molecular base of brain development in its evolutionary pathway. Knockdown of planarian *Otx* gene activities does not affect brain but eyes (Kobayashi and Agata, personal communication). The function of *Djotx*

genes in planarian brain formation is still unclear. Further studies on *Djotx* functions are needed. In addition to this, the broader functional and comparative study of *Otx* genes from other phyla, such as leech (Bruce and Shankland 1998), *C. elegans* (Satterlee et al. 2001), sea urchin (Kiyama et al. 1998), or hemichordate (Lowe et al. 2003), will be required to unveil the *Otx* function and brain evolution.

Contrary to these molecular commonalities, anatomical structures of the rostral region of the nervous system still show apparent morphological divergence among bilateral animals. Such neuroanatomical divergence is particularly substantial between protostomes and deuterostomes, leaving the salient structural properties of the putative ancestral brain with a number of possibilities. First, in the chordate evolution, the *Otx* gene of 'headless' ascidian has ability of anterior formation as this study have shown. This indicates that origin of the molecular base of vertebrate brain has established at very early stage of evolution (Fig. 12). Second, recent developmental study of hemichordate (Lowe et al. 2003) has suggested that this animal, having diffuse nerve net in its epidermis and lacking central nervous system, has similar gene expression patterns to the vertebrates brain (Fig. 11). With this discovery, Holland has proposed two alternative scenarios for central nervous system evolution (Holland 2003). One is that urbilateria (De robertis and Sasai 1996), the protostome/deuterostome ancestor, had 'skin brain', a

basiepidermal nerve net with gene expression patterns like present animals. Another scenario is that the ancestor of protostome and deuterostome had localized and anatomically complex central nervous system. For understanding of central nervous system evolution, comparative studies of the regulatory network of the *Otx* genes in brain development across diverse phyla, including deuterostomes, ecdysozoans, and especially lophotrochozoans, which are left from present molecular genetic studies, are eagerly needed (Tessmar-Raible and Arendt 2003).

In this regard, it is also noteworthy that, in addition to long recognized neuroanatomical and physiological similarities, recent studies focus on a conserved set of regulatory genes that play important roles in the early development of the olfactory perception centers in both the protostome and deuterostome brains (Strausfeld and Hildebrand 1999; Kurusu et al. 2000).

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Tables

Table 1**Summary of the fly stocks used in this study**

Gene	Stock	Chromosome	Balancing	Viability	Source
Ascidian <i>Otx</i>	Hr45-aIII	III	TM3	viable	this work
	Hr45-cII	II	CyO	lethal	this work
<i>Drosophila otd</i>	5A	III	homozygous	viable	ref. 1
Human <i>Otx1</i>	3.19.1	III	TM3	viable	ref. 2 and 3
Human <i>Otx2</i>	8.13.6	II	CyO	viable	ref. 2 and 3
Planaria <i>Otx</i>	Dj11-aII	II	CyO	viable	this work
	Dj15-bIII	III	TM3	viable	this work
Cnidaria <i>Otx</i>	Cn56II	II	CyO	viable	this work
	Cn125II	II	CyO	viable	this work

Refs. 1) Royet & Finkelstein., 1995. 2) Nagao et al., 1998. 3) Leuzinger et al., 1998.

Table 2

Embryonic brain rescue by *otd/Otx* genes

Flies	Efficiency of brain rescue,%(n)¹				Total embryos examined
	+++	++	+	-	
<i>otd</i> ^{JA101} / <i>Y</i> ; <i>hsp-otd</i> /+					
Line 5A	28.0 (7)	12.0 (3)	28.0 (7)	32.0 (8)	25
<i>otd</i> ^{JA101} / <i>Y</i> ; <i>hsp-Otx2</i> /+					
Line 8.13.6	13.3 (4)	3.3 (1)	33.3 (10)	50.0 (15)	30
<i>otd</i> ^{JA101} / <i>Y</i> ; <i>hsp-Hroth</i> /+					
Line Hr45-aIII	21.0 (8)	23.7 (9)	23.7 (9)	31.6 (12)	38
<i>otd</i> ^{JA101} / <i>Y</i> ; <i>hsp-DjotxA</i> /+					
Line Dj11-aII	0 (0)	0 (0)	0 (0)	100 (9)	9
<i>otd</i> ^{JA101} / <i>Y</i> ; <i>hsp-CnOtx</i> /+					
Line Cn50II	0 (0)	0 (0)	8.3 (1)	91.7 (11)	12
<i>otd</i> ^{JA101} / <i>Y</i> ; + /+ ²	0 (0)	0 (0)	0 (0)	100 (32)	32

1. +++: b1 neuromere, preoral commissure, and anterior protocerebral bsh expressing cells recovered.

++: b1 neuromere and preoral commissure recovered. +: b1 brain region enlarged. -: no rescue

observed. 2. Heat induction without the transgene.

Table 3

Transformation of the central nervous system by *otd/Otx* genes

	Categories of Transformation,%(n)					Total examined
	Brain only ¹	Brain + VNC ²	VNC only ³	others ⁴	Intact CNS	
<i>Drosophila otd</i>						
Line 5A	80.0 (32)	12.5 (5)	0 (0)	7.5 (3)	0 (0)	40
Human <i>Otx2</i>						
Line 8.13.6	0 (0)	20.8 (14)	0 (0)	3.0 (2)	76.1 (51)	67
Ascidian <i>Otx</i>						
Line Hr45-aIII	54.1 (46)	23.5 (20)	0 (0)	20.0 (17)	2.4 (2)	85
Line Hr45-cII	78.3 (65)	9.6 (8)	0 (0)	8.4 (7)	3.6 (3)	83
Planaria <i>Otx</i> *						
Line Dj15-b	0 (0)	69.2 (18)	0 (0)	0 (0)	30.8(8)	26
Cnidaria <i>Otx</i> *						
Line Cn125II	0 (0)	57.1(20)	0 (0)	0 (0)	42.9(15)	35
Oregon R	0 (0)	0 (0)	0 (0)	0 (0)	100 (84)	84

Gene expression was induced in wild-type background by heat shock at 37°C for 20 min at 3.5 h after egg laying.

Categories:

1. Only rostral brain is left including those with enhanced brain lobes.
2. Rostral brain attached degenerated partial VNC.

3. Partial VNC without rostral brain.
4. HRP positive staining of uncertain identity.

* Embryos carrying Planaria or Cnidaria *Otx* gene were heat shocked for 60 minutes.

Table 4

Developmental rescue of the vertex structures with chordate *Otx* genes

Flies	Ocelli rescue,%(n)		Macrochetae rescue, n		Total heads examined, n
	Lens	Pigment	ocb	pvb	
<i>oc</i> ¹ / <i>Y</i> ; <i>hsp-otd</i> /+					
Line 5A	26.0 (18)	0.0 (0)	1.7 ± 0.9	1.5 ± 0.8	69
<i>oc</i> ¹ / <i>Y</i> ; <i>hsp-Otx1</i> /+					
Line 3.19.1	16.1 (19)	3.4 (4)	1.5 ± 1.3	0.1 ± 0.4	118
<i>oc</i> ¹ / <i>Y</i> ; <i>hsp-Hroth</i> /+					
Line Hr45-aIII	20.7 (18)	6.9 (6)	0.6 ± 1.0	0.1 ± 0.3	87
Line Hr45-cII	9.2 (8)	3.4 (3)	0.6 ± 0.9	0.1 ± 0.3	87
<i>oc</i> ¹ / <i>Y</i> ; + / +*	6.8 (3)	0.0 (0)	0.5 ± 0.7	0.4 ± 0.7	44

For all samples, heat pulses were applied four times between 72 and 85 h AEL. Fly heads with the ocelli-like structures were counted. Ocb, ocellar bristles; pvb, postvertical bristles. Wild-type vertex has two ocellar and two postvertical bristles.

* *oc*¹ larvae were heat treated without crossing to the transgenic flies. Similar results were obtained with untreated *oc*¹ larvae.

Table 5

Control of vertex regulatory genes by chordate *Otx* genes

Flies	GL,% (n)	EN,% (n)	<i>hh</i>,% (n)	<i>wg</i>,% (n)*
Wild type	91.3 (23)	100.0 (6)	84.3 (32)	100.0 (19)
<i>oc¹/Y ; +/+</i>	0.0 (26)	0.0 (32)	0.0 (13)	0.0 (7)
<i>oc¹/Y ; hsp-otd/+</i>	11.9 (42)	42.8 (7)	43.5 (23)	66.7 (36)
<i>oc¹/Y ; hsp-Otx1/+</i>	0.0 (26)	37.5 (16)	35.7 (28)	58.6 (29)
<i>oc¹/Y ; hsp-Hroth/+</i>	21.2 (33)	10.4 (48)	48.3 (29)	20.0 (20)

Numbers of discs examined are shown in parentheses. Fly stocks used were *Drosophila otd* (5A), human *Otx1* (3.19.1) and ascidian *Otx* (Hr45-aIII).

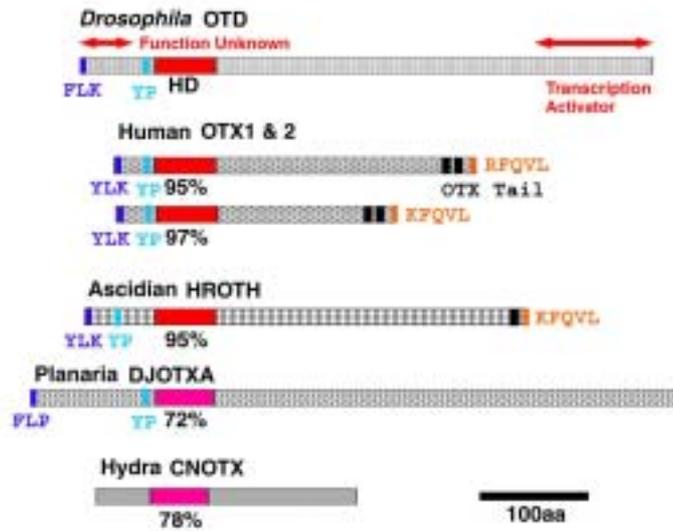
* Discs showing activation in the vertex primordium were counted for the GL and EN proteins and the *hh* transcripts. Discs with gene suppression in the vertex primordium were counted for the *wg* transcripts. Heat pulses were applied four times between 72 and 85 h after egg laying.

Figures

Fig. 1. (A) Structure comparison of the *Drosophila* Orthodenticle (OTD), human OTX2, ascidian OTX, planarian OTX, and cnidarian OTX proteins. Protein size is indicated by black bar (100 amino acids). All six proteins have the conserved homeodomain near the amino terminus and carry a long carboxy-terminus. In *Drosophila* OTD, there are two functional domains outside homeodomain. Carboxy-terminal region acts as transcription activator and amino-terminal region has unknown function (Wimmer, personal communication). In addition to the homeodomain conservation, the five OTD/OTX proteins excluding cnidarian OTX share a short hydrophobic Tyr-Pro stretch (*light blue*, YP) upstream of the homeodomain as well as a tri-peptide, Phe/Tyr-Leu-Lys (Phe-Leu-Pro in planaria), at the amino terminus (*dark blue*). Chordate OTX proteins share conserved OTX tail (Furukawa et al. 1997; *black*). In the most carboxy-terminal, there also is a conserved stretch, Lys-Phe-Gln-Val-Leu (*orange*). This stretch is also conserved in leech OTX (Bluce and Shankland 1998) and *C. elegans* OTX (Satterlee et al. 2001), indicating that this stretch is shared by all three bilateral animal taxa. Except for these short stretches and the homeodomain, little homology exists between the OTD/OTX proteins. The human OTX1 and OTX2 proteins show similarity to each other over the entire amino acid sequences, especially for carboxy terminal OTX tail (Furukawa et al. 1997). Hydra OTX has no such conserved short stretches, showing that the

emergence of such stretches might have added new function to OTX proteins in evolution. **(B)** Amino acid residues of the OTD/OTX homeodomains. *Dashes* indicate identical residues compared to the *Drosophila* OTD homeodomain. Homology is also indicated as percentage under homeodomain in **(A)**.

(A)



(B)

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Drosophila OTD GRHERITPTRAQLDVLLEALFGKTRYPDI PMREIVALKINLPESRVGVWFKRRRAKCRQQL
Human OTX1 -----S-----A-----D
Human OTX2 -----A-----Q
Ascidian OTX -----I-----Q
Planaria DjotxA T--D-----Q--EI--LS-E-S-----L-D-ISS-----E--SN
Hydra Cnotx R-----K-----DH-----E--V-----K--A--A-----F--SR
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Fig. 2. (A) Plasmid construction and making of transgenic flies. pNHT4 vector carries *heat shock protein 70* promoter (*hsp70*, blue) and *Drosophila rosy* gene (red). Each *Otx* cDNA was subcloned down stream of *hsp70*. **(B)** *Otx* plasmids were injected into *rosy*⁵⁰⁶ strain embryos stage 5 with helper plasmids coding transposase at the indicated concentration. Transformants of generation 1 (G1) showed wild type eye color. These transformants were crossed to balancer strains and transgenic flies carrying out source-*Otx* gene were established (Table 1).

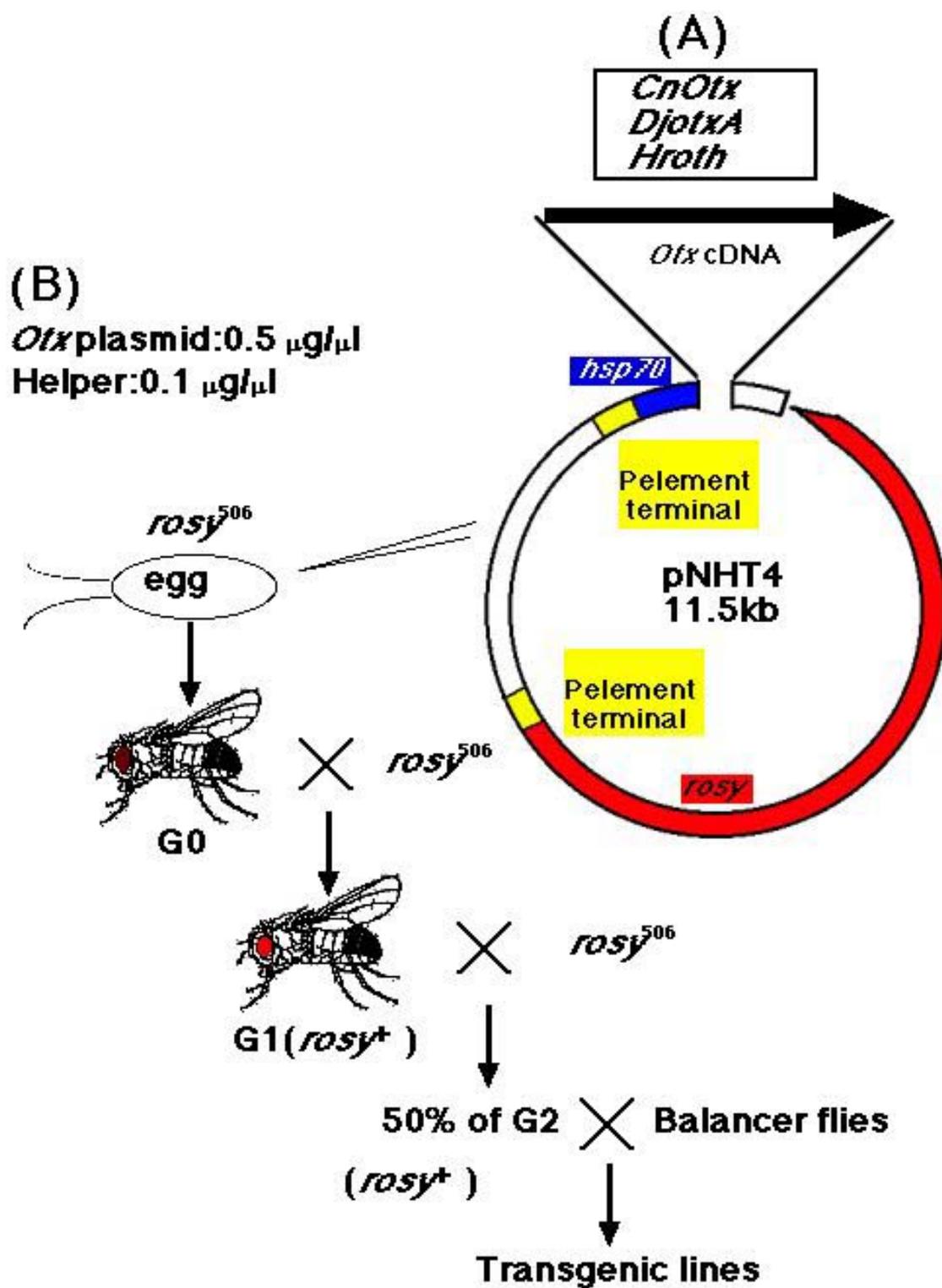
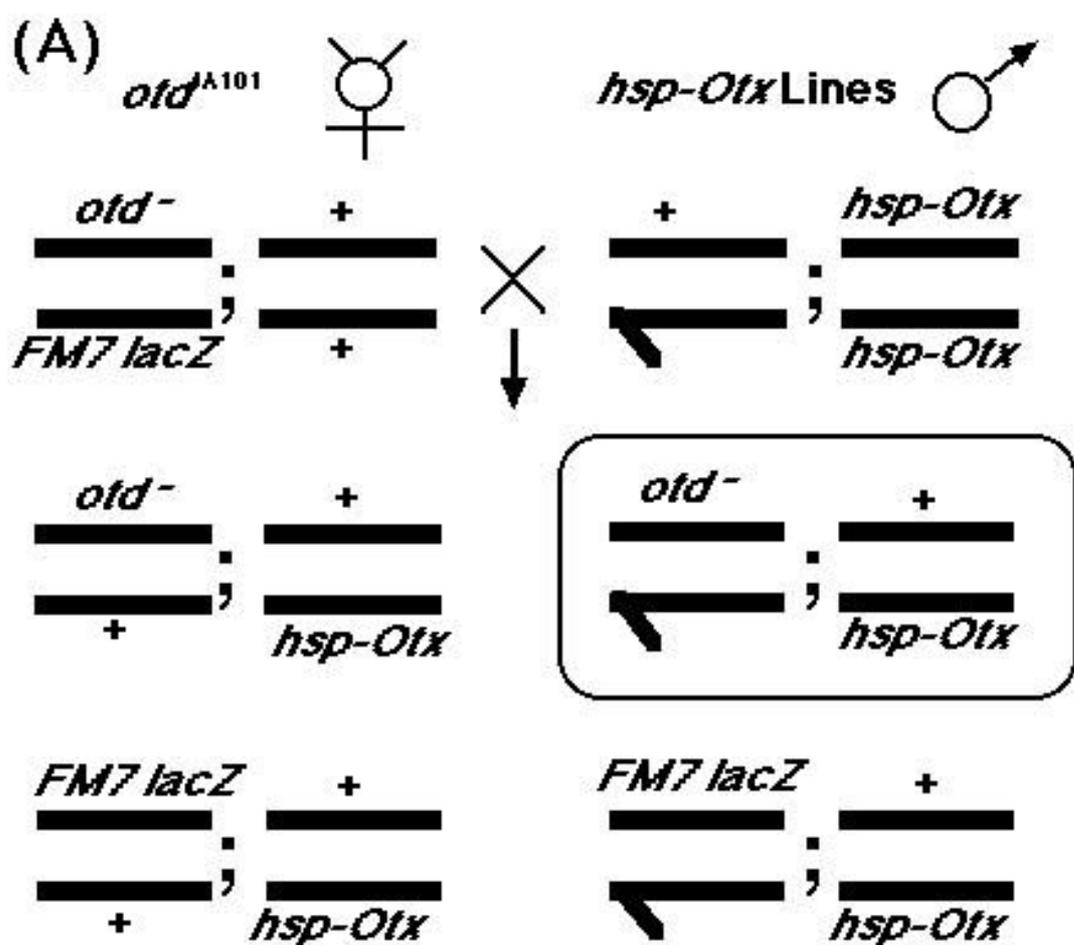


Fig. 3. (A) Genetic scheme of the embryonic brain rescue. Male transgenic flies were crossed to otdJA101 virgin females. Desired F1 embryos were distinguished by female specific Sex lethal (SXL) protein expression and LacZ expression (not shown). **(B)** Heat shock paradigm for rescue of the embryonic brain. 37°C Heat pulses of 5', 5', 7' and 10' with 25°C brakes of 5', 5' and 15' were given to the embryos after egg laying (AEL) 3.5h.



(B)

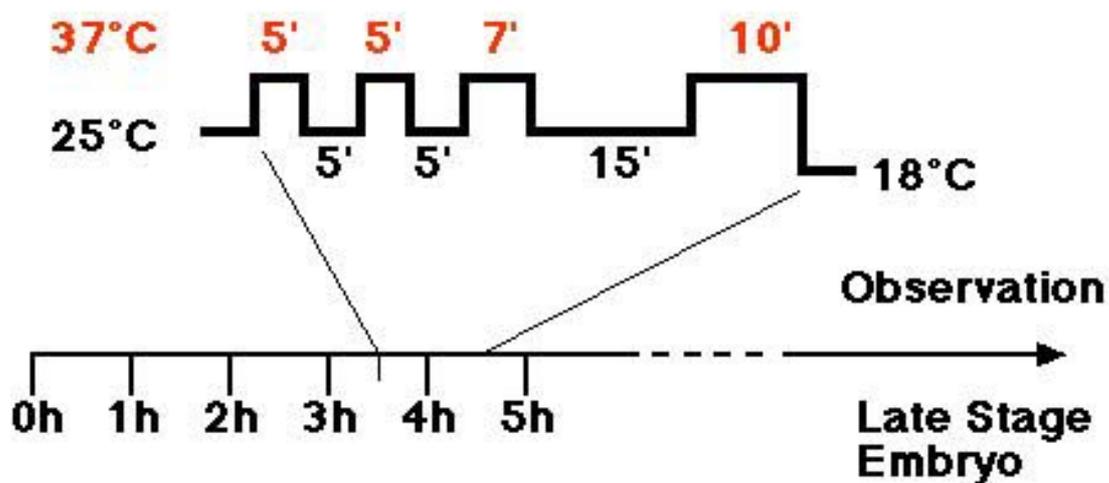


Fig. 4. A–J Embryonic brain rescue by chordate *Otx* genes. Laser confocal microscopy of embryos corresponding to stage 15/16. **A, C, E, G, I** Frontal views, **B, D, F, H, J** lateral views. The brain neuromeres were labeled with neuron-specific anti-HRP antibody (*green*) and with an anti-Brain specific homeobox (BSH) antibody (*magenta*). **A, B** Wild-type brain, **C, D** *otd* null mutant brain. In the wild type, the brain commissure (*arrow*) connects the two hemispheres at the posterior border of the protocerebral neuromere; the tritocerebral commissure is indicated by an *asterisk*. *Arrowheads* indicate the anterior BSH cells in the protocerebral neuromeres. In *otd* null mutant, most of the anterior BSH cells are lost, though some BSH cells are retained at the ventral and more posterior region (*open arrowhead* in **C**). **E, F** Restored brain with the *Drosophila otd* gene (line 5A). Expression of the *otd* gene was induced by moderate heat shock in transgenic mutant *otd*^{JA101} /*Y*; *hsp-otd* / +. **G, H** Restored brain with the human *Otx2* gene (line 8.13.6). Expression of the *Otx2* gene was induced in transgenic mutant *otd*^{JA101} /*Y*; *hsp-Otx2* / +. **I, J** Restored brain with the ascidian *Otx* gene (line Hr45-aIII). Expression of the *Otx* gene was induced in transgenic mutant *otd*^{JA101} /*Y*; *hsp-Hroth* / +. *Arrow* restored brain commissure, *arrowheads* restored anterior BSH cells, *asterisk* tritocerebral commissure. Anterior (in neuraxis) is *up* for the frontal images, and to the *right* and *up* for the lateral images. As did the *Drosophila* and human *otd/Otx* genes, the ascidian *Otx* gene restored the protocerebral neuromeres and the brain commissure at its

normal position. Cells expressing BSH also were recovered at the anterior end of the protocerebral neuromeres. *Scale bar* (10 μm) is the same in all panels.

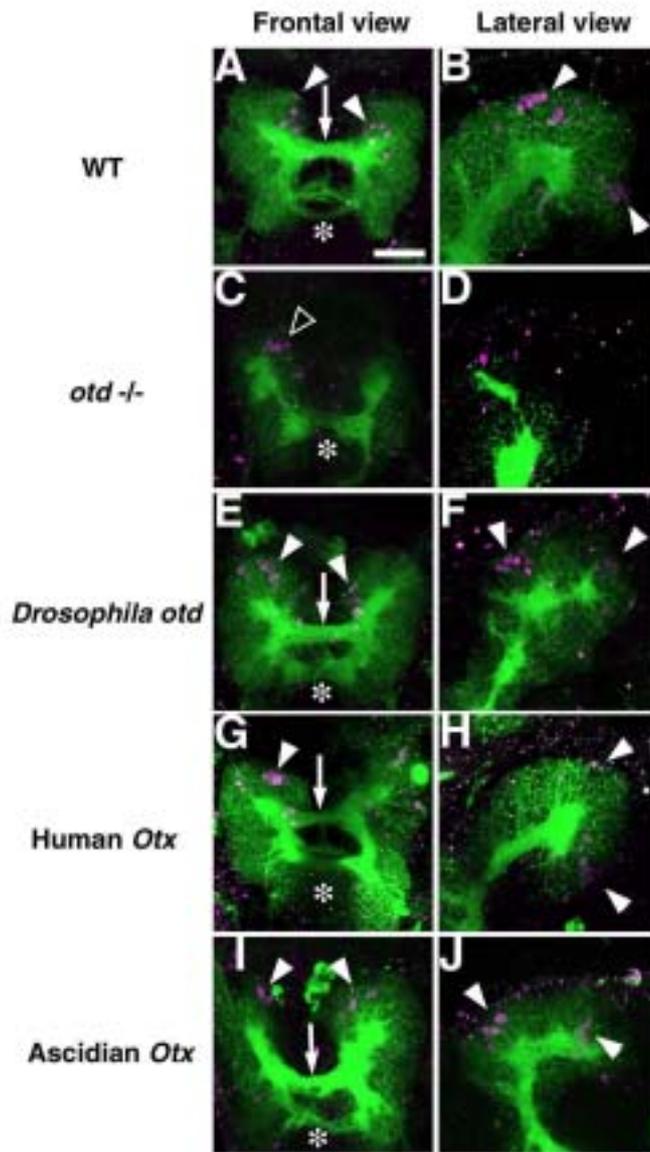


Fig. 5. Embryonic brain rescue by planarian *DjotxA* gene and Hydra *CnOtx* gene. The brain neuromeres were labeled with neuron-specific anti-HRP antibody. **A** Wild-type brain, **B** *otd* null mutant brain. **C** Brain rescue with planarian *DjotxA* gene did not succeed. **D** Cnidarian *Otx* gene *CnOtx* did not show the anterior brain forming ability in *Drosophila*. Asterisks indicate b1 region. *Scale bar* (10 μ m) is the same in all panels.

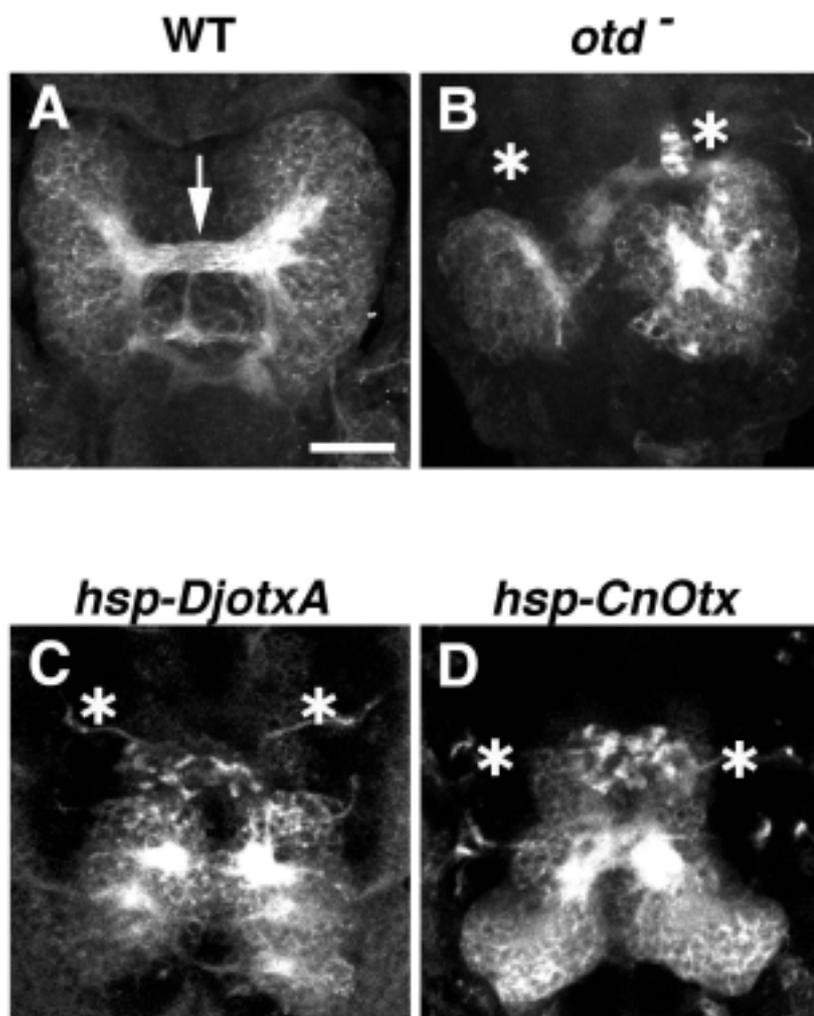


Fig. 6. A–D Dominant suppression of the trunk nervous system by chordate *Otx* genes. Reconstructions of optical sections from confocal microscopy. *Green* anti-HRP immunostaining revealing the central and peripheral nervous systems. *Magenta* 22C10 immunostaining revealing the peripheral nervous system (PNS). **A** Wild-type embryos without transgene but heat shocked at 37°C for 20 min. Both the central and peripheral nervous systems developed normally. **B** Embryos with ubiquitous overexpression of the *Drosophila otd* gene (line 5A), **C** the human *Otx2* gene (line 8.13.6), and **D** the ascidian *Hroth* gene (line Hr45-aIII). Expression of the *otd/Otx* genes were induced at stage 7–8 in wild-type genetic background. Both the central and peripheral nervous systems are lost in the trunk by the *Drosophila otd* and the ascidian *Hroth* genes while more anterior structures are retained or often enhanced. Milder transformation was caused by the human *Otx2* gene, though disruption of the trunk structures is evident for both the central and peripheral nervous systems; the anterior brain is still preserved in **C** (out of focus). **E** the planarian *DjotxA* gene (line Dj15-b) had less effect on central and peripheral nervous systems. **F** the cnidarian *CnOtx* gene (line Cn125II) did not suppressed ventral nerve cord or peripheral nervous system. Although slight nervous defects are observed in the central nervous system, the shape of peripheral nervous system generally unaffected. *Scale bar* (100 μm) is the same in all panels.

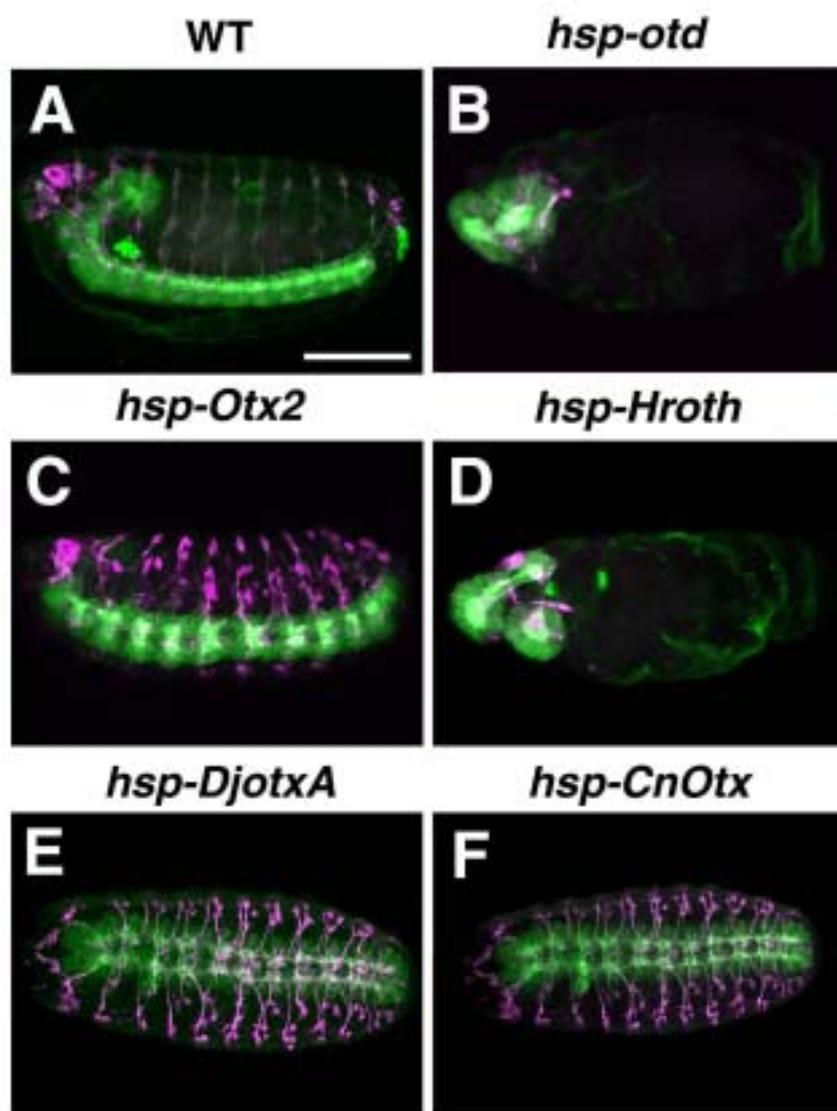


Fig. 7. (A) Genetic scheme of vertex structures rescue. Transgenic males were crossed with *oc*¹ females. Desired F1 males were distinguished in the larval stage by the shape of gonads (FM7 carrying males were embryonic lethal). **(B)** Heat shock scheme for rescue of vertex formation. One hour heat pulses with two hours of intervals at 25°C were given to the early third instar larvae AEL 72h. The rescue results were observed in the late third instar larval stage and early adult stage.

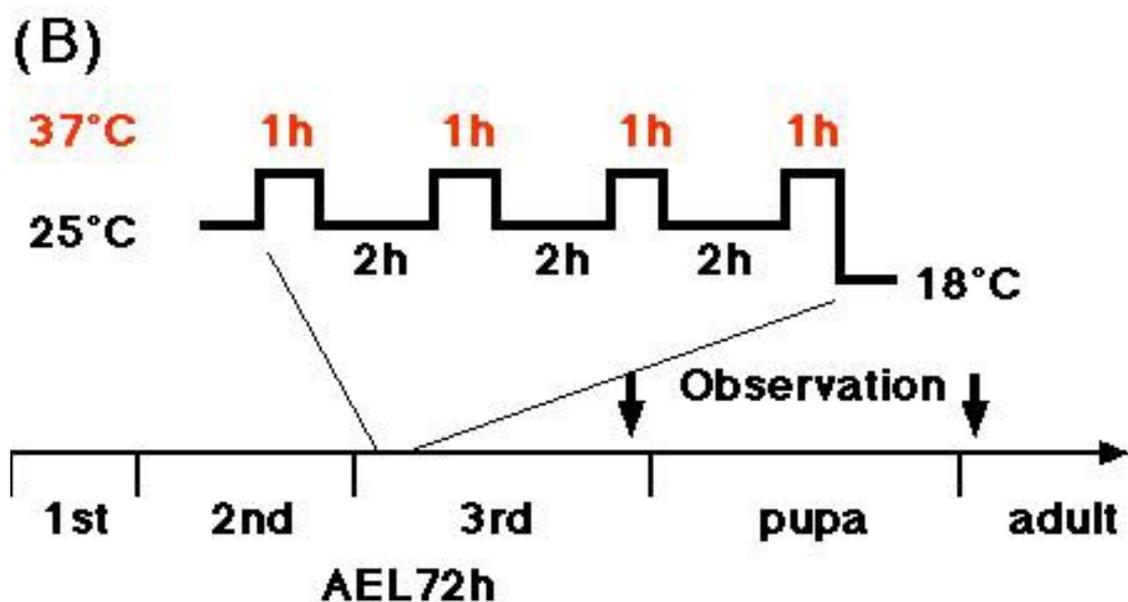
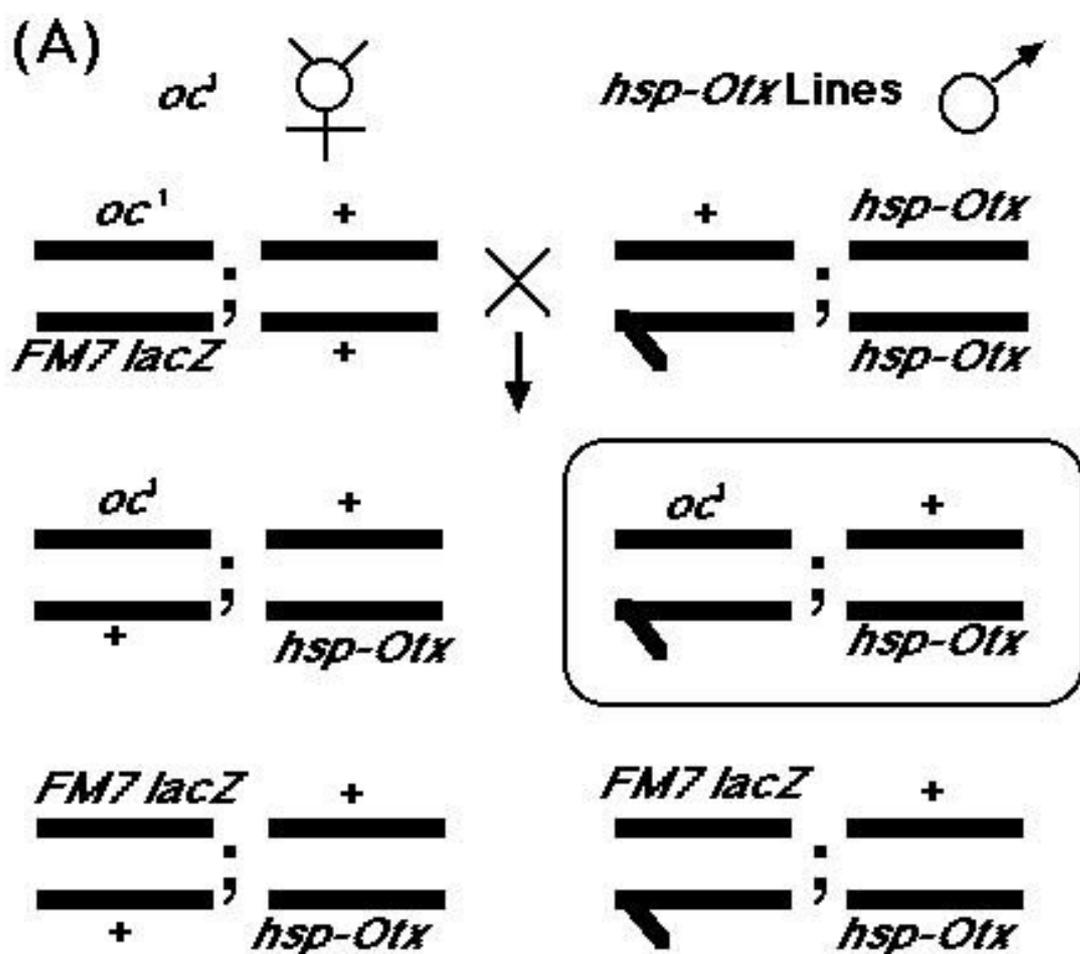


Fig. 8. A–E Developmental rescue of the vertex structures with chordate *Otx* genes. **A** Wild-type (Oregon R) vertex. Positions of ocelli (*oc*), ocellar bristles (*ocb*) and postvertical bristles (*pvb*) are indicated. Three to four pairs of smaller inter ocellar bristles are also located in the medial region. **B** *oc*¹ vertex. **C** *hsp-otd* (line 5A) vertex in *oc*¹ background. **D** *hsp-Otx1* (line 3.19.1) vertex in *oc*¹ background. **E** *hsp-Hroth* (line Hr45-aIII) vertex in *oc*¹ background. *Arrows* indicate rescued lens-like structures. *Arrowheads* indicate bristles at the postvertical positions. *Scale bar* (50 μm) is the same in all panels.

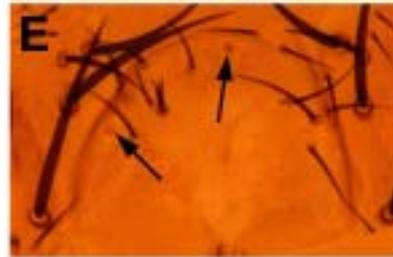
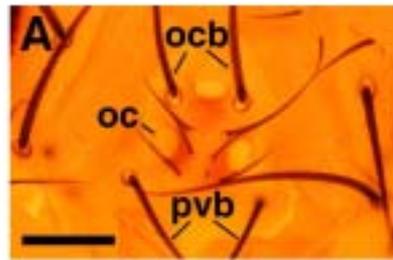


Fig. 9. A–T Control of vertex regulatory genes by chordate *Otx* genes. **A–E** Glass (GL) protein expression detected with the monoclonal antibody 9B2.1. **F–J** Engrailed (EN) protein expression detected with the monoclonal antibody 4D9. **K–O** *hedgehog* (*hh*) RNA expression and, **P–T** *wingless* (*wg*) RNA expression detected with in situ hybridization with specific RNA probes. **A, F, K, P** Wild type. **B, G, L, Q** *oc*¹. **C, H, M, R** *hsp-otd* line 5A in *oc*¹ background. **D, I, N, S** *hsp-Otx1* line 3.19.1 in *oc*¹ background. **E, J, O, T** *hsp-Hroth* line Hr45-aIII with *oc*¹ background. *Arrows* indicate the position of the vertex primordium. Heat pulses were applied four times in the 72–85 h after egg laying. Larvae were kept at 18°C for 36 h after the last heat pulse; the discs were then dissected for signal detection. *Scale bar* 50 μm. The illustration below the pictures indicates the shape and the position of eye-antennal discs in the third instar larva.

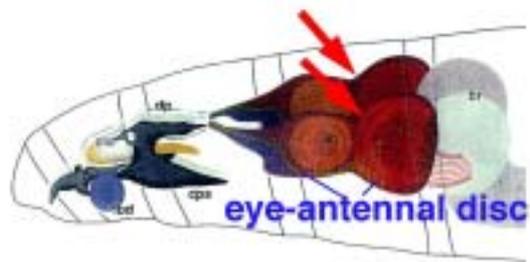
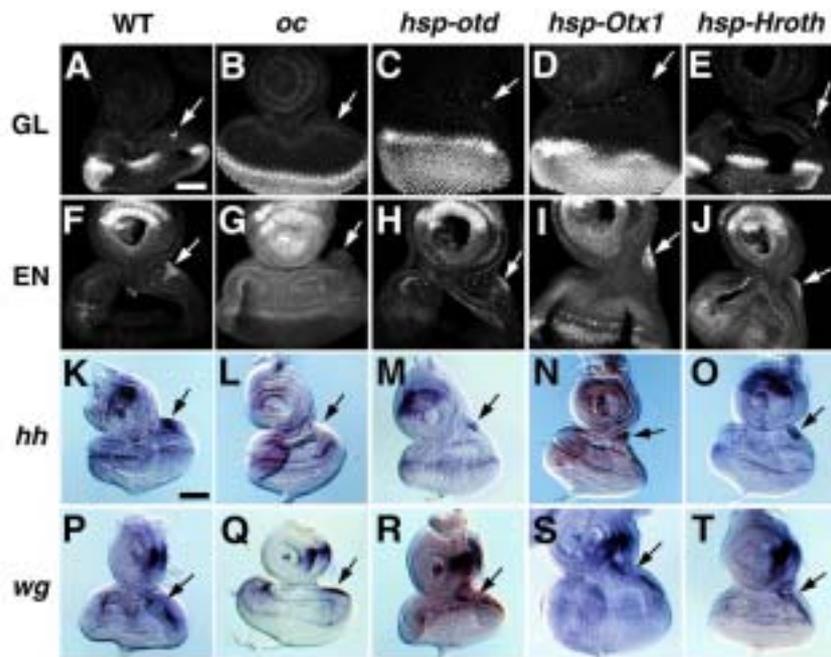


Fig. 10. Molecular phylogeny and the origin of brain. Based on 18S rRNA sequences, bilaterians are divided into three major groups: deuterostomes, lophotrochozoans, and ecdysozoans. Representative animal phyla in which *Otx* genes are described and show anterior brain expression are indicated. Triangle indicates the origin of *Otx* gene. Black star designates the putative ancestor that acquired anterior patterning of the central nervous system with an ancestral primitive brain. An *Otx* homolog is described in hydra (cnidaria) but does not have a direct role in head formation suggesting that appearance of the *otd/Otx* gene family preceded the acquisition of the anterior patterning mechanism in metazoan evolution.

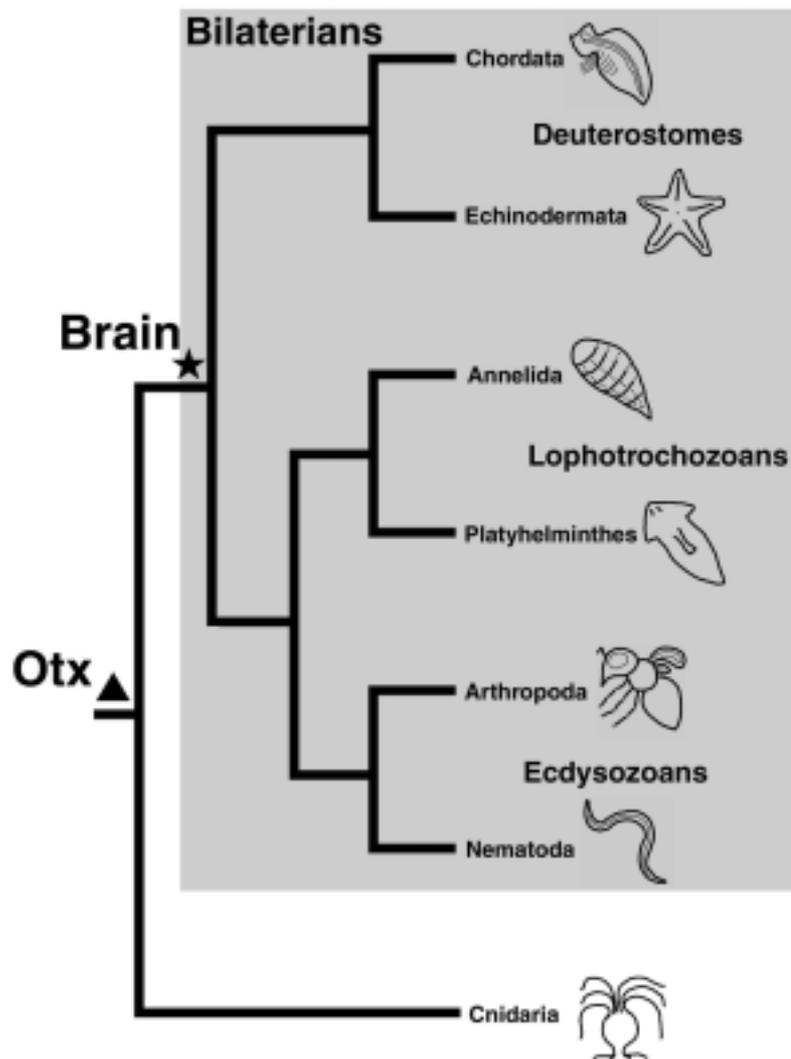
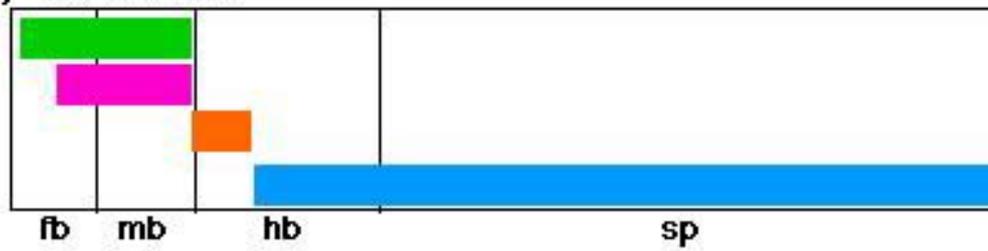
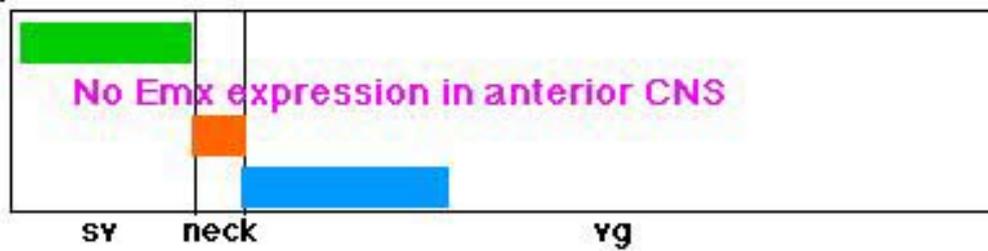


Fig. 11. Comparison of central nervous system (CNS) or diffuse nervous system (DNS) organization and gene expression in mouse(A), ascidian (B), acorn worm (C), and fruitfly (D). The expression of *otd/Otx*, *ems/Emx*, *Pax2/5/8* and *Hox* cluster gene orthologues in the developing CNS of mouse embryo, ascidian embryo, acorn worm embryo, and *Drosophila* embryo. Anterior is to the left. Thick bars show antero-posterior domains of widespread expression. Thin bars show narrow expression. Green bars indicate *otd/Otx* genes expression, magenta bars indicate *ems/Emx* genes expression, which is not observed in ascidian embryonic central nervous system. Orange bars indicate Pax2/5/8 class gene orthologues expression that has not been reported in hemichordates. Blue bars show the Hox gene cluster(s) expressing domain. Mouse CNS: fb, forebrain; mb, midbrain; hb, hindbrain; sp, spinal cord. Ascidian CNS: sv, sensory vesicle; vg, visceral ganglion. Acorn worm DNS: pro, prosome; meso, mesosome; meta, metasome. *Drosophila* CNS: seg, subesophageal ganglia; th, thorax; ab, abdomen.

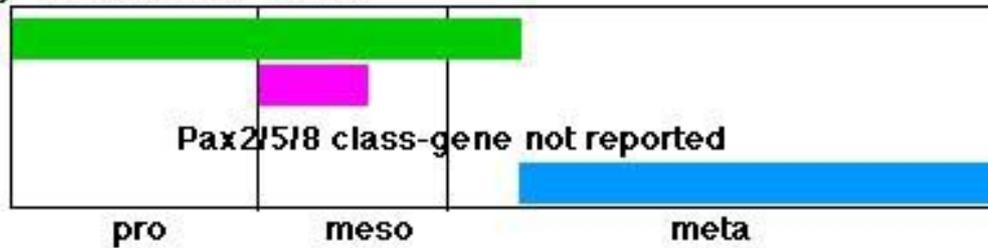
(A) Mouse CNS



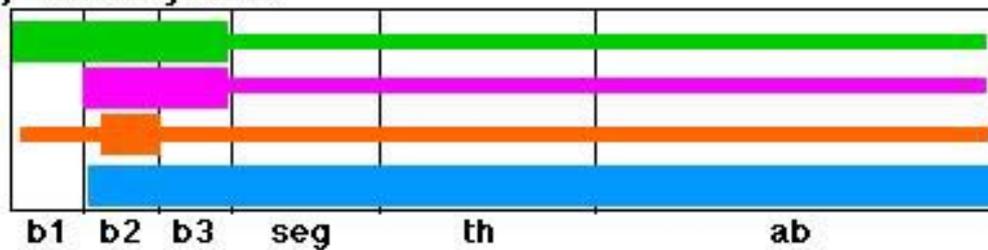
(B) Ascidian CNS



(C) Acorn worm DNS



(D) Fruitfly CNS



- OTD/OTX
- EMS/EMX
- PAX2/5/8
- HOM/Hox

Fig. 12. *Otx* gene and Chordate brain evolution. Vertebrates, cephalochordates (Williams and Holland 1996), and urochordates have *Otx* expression in their anterior central nervous systems. *Haikouella lanceolata*, a fossil chordate from the early Cambrian, has relatively large brain (Chen et al. 1999). The existence of this fossil animal also indicates that the origin of chordate brain should be older than previously thought.

