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Equivalence of the fly *orthodenticle* gene and the human *OTX* genes in embryonic brain development of *Drosophila*

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SUMMARY

Members of the *orthodenticle* gene family are essential for embryonic brain development in animals as diverse as insects and mammals. In *Drosophila,* mutational inactivation of the *orthodenticle* gene results in deletions in anterior parts of the embryonic brain and in defects in the ventral nerve cord. In the mouse, targeted elimination of the homologous *Otx2 or Otx1* genes causes defects in forebrain and/or midbrain development. To determine the morphogenetic properties and the extent of evolutionary conservation of the *orthodenticle* gene family in embryonic brain development, genetic rescue experiments were carried out in *Drosophila*. Ubiquitous overexpression of the *orthodenticle* gene rescues both the brain defects and the ventral nerve cord defects in *orthodenticle* mutant embryos; morphology and nervous system-specific gene expression are restored. Two different time windows exist for the rescue of the brain versus the ventral nerve cord. Ubiquitous overexpression of the human *OTX1* or *OTX2* genes also rescues the brain and ventral nerve cord phenotypes in *orthodenticle* mutant embryos; in the brain, the efficiency of morphological rescue is lower than that obtained with overexpression of *orthodenticle*. Overexpression of either *orthodenticle* or the human *OTX* gene homologs in the wild-type embryo results in ectopic neural structures. The rescue of highly complex brain structures in *Drosophila* by either fly or human *orthodenticle* gene homologs indicates that these genes are interchangeable between vertebrates and invertebrates and provides further evidence for an evolutionarily conserved role of the *orthodenticle* gene family in brain development.

Key words: *orthodenticle*, *OTX1*, *OTX2*, Gap gene, Brain development, Genetic rescue, Evolution

INTRODUCTION

The cephalic gap gene *orthodenticle (otd)* is required for head development and segmental patterning in *Drosophila*. At early blastoderm stages, the *otd* gene is expressed in a broad circumferential stripe in the anterior region of the embryo. This early domain of expression includes the precursors of the antennal and preantennal procephalic regions of the head; in *otd* null mutants pattern perturbations and deletions occur in the cuticular structures and the peripheral nervous system of these head regions (Finkelstein and Perrimon, 1990; Cohen and Jürgens, 1990; Finkelstein et al., 1990; Wieschaus et al., 1992; Grossniklaus et al., 1994; Schmidt-Ott et al., 1994; Gao et al., 1996). In addition to expression in the head region, *otd* is expressed in ventral mesectodermal structures of the embryo; *otd* null mutations cause the elimination of specific ventromedial ectodermal cells (Finkelstein et al., 1990; Klämbt et al., 1991; Wieschaus et al., 1992).

The *otd* gene is also required for the formation of the embryonic central nervous system (CNS) in *Drosophila*. During neurogenesis, *otd* expression is observed throughout most of the protocerebral anlage as well as in the anterior part of the deutocerebral anlage of the brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997) and *otd* is expressed along the midline of the developing ventral nerve cord (Finkelstein et al., 1990; Wieschaus et al., 1992). In the developing brain, mutation of *otd* results in the deletion of the protocerebral anlage due to defective neuroblast formation in these regions (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In the developing ventral nerve cord, mutational inactivation of *otd* causes CNS differentiation defects in specific midline neurons and glia and results in deranged or missing commissures (Finkelstein et al., 1990; Klämbt et al., 1991).

Recent studies demonstrate that genes belonging to the *orthodenticle* gene family play comparable roles in anterior brain development in vertebrates. Thus, in mice, the two vertebrate *otd* homologs, *Otx1* and *Otx2*, are expressed in overlapping domains of the developing forebrain and midbrain,

and the developing cerebral cortex and cerebellum (Simeone et al., 1992; 1993; Frantz et al., 1994). Mutation of *Otx2* leads to deletion of forebrain and midbrain regions due to a defective anterior neuroectoderm specification during gastrulation (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Suda et al., 1996). Mutation of *Otx1* affects telencephalic, mesencephalic and cerebellar brain structures, and causes epilepsy (Acampora et al., 1996). In addition, *Otx1* and *Otx2* cooperate to specify correct brain development in a dosage-dependent manner (Acampora et al., 1997).

The similarities between fly *otd* and mammalian *Otx* gene action during brain development suggest an evolutionary conservation of the *otd/Otx* genes that extends beyond gene structure to patterned expression and function, and have led to the proposal that the genetic programs controlling the development of the animal brain are highly conserved (Finkelstein and Boncinelli, 1994; Thor, 1995; Reichert and Boyan, 1997). In order to investigate the extent of evolutionary conservation among the members of the *otd/Otx* gene family, we used transgenic flies carrying either the *Drosophila otd* gene, the human *OTX1* gene, or the human *OTX2* gene under control of a heat-inducible promoter in genetic rescue experiments. Here, we report that heat-shock-induced overexpression of either *otd* or its human homologs *OTX1* and *OTX2* is able to rescue the brain and ventral nerve cord defects in *otd* mutant embryos. Furthermore, we demonstrate the existence of two separate time windows for the rescue of the brain versus the ventral nerve cord phenotype by *otd/OTX* overexpression in these mutants. Finally, we present evidence that ubiquitous overexpression of either the fly *otd* or the human *OTX1/OTX2* genes is able to induce ectopic neural structures in wild-type embryos.

MATERIALS AND METHODS

Heat-shock expression constructs and transgenic lines

For overexpression of OTD protein in flies, we used the *hsp-otd* line 5A generated by Royet and Finkelstein (1995). To overexpress human OTX1 and OTX2 proteins in flies, we cloned the corresponding full-coding cDNAs (a 1.6 kb *Sma*I-*Nae*I and a 0.95 kb *Pvu*II-*Xba*I filled-in fragment, respectively) into the *Not*I filled-in site of pNHT4, a modified version of the pHT4 plasmid (Schneuwly et al., 1987) carrying *Not*I as cloning site. The constructs were introduced into a ry^{506} recipient strain along with the pΔ2-3wc helper plasmid according to standard procedures (Rubin and Spradling, 1982). Eclosing adults were crossed to ry^{506} flies. From this cross, ry^{+} transformant progeny were used to establish several independent lines carrying the construct on either the second or third chromosome. The results presented here are based on the analyses of two representative lines, 4.11.8C (*hsp-OTX2*) and 7.5.6C (*hsp-OTX1*), which both contain the P-element insertion on the third chromosome maintained over a TM3 balancer chromosome. Similar results were obtained with other independent lines.

To test for functionality of the transgenes, embryos were collected overnight and exposed to a single heat shock at 37°C for 20 minutes. The embryos were fixed 1 hour after heat shock and stained with an antibody against *Drosophila* OTD or an antibody against the human OTX2 protein (Mallamaci et al., 1996), which also recognizes the OTX1 protein.

Rescue of the *otd* null mutant phenotype

The otd^{JA101} amorph allele (Wieschaus et al., 1984) was balanced over a *FM7* balancer carrying a *lacZ* insertion. Transgenes were introduced into the *otd* null mutant background by crossing males homozygous for the heat-shock constructs *hsp-otd* (Royet and Finkelstein, 1995) or *hsp-OTX1* or *hsp-OTX2* to otd^{JA101} virgin females heterozygous for the *otd* null allele otd^{JA101}. Additional lines transgenic for *hsp-Dfd* (Kuziora and McGinnis, 1988) or with no insertion (wild type Oregon R) crossed to females heterozygous for otd^{JA101} served as positive and negative controls, respectively. Heat shock applied to *hsp-Dfd* or wild type resulted neither in a rescue of the *otd* mutant phenotype nor in ectopic neural structures.

Offspring of the crosses was collected in 1 hour intervals and exposed to a series of heat pulses at 37°C as follows. For brain rescue, the applied pulses lasted 5, 5, 7 and 10 minutes at 37°C, respectively, interrupted by intervals of 5, 5 and 15 minutes at 25°C, respectively. For VNC rescue, the series of heat pulses included 5, 5, 7 and 10 minutes at 37°C, interrupted by intervals of 15, 15 and 23 minutes at 25°C. After the last heat pulse, embryos were allowed to develop at 25°C until they reached 18-20 hours after egg laying (stage 16/17). Only embryos hemizygous for the mutation show the *otd* mutant phenotype and were distinguished by the absence of *lacZ* and *Sxl* (Parkhurst et al., 1990) expression. Embryos were staged according to Campos-Ortega and Hartenstein (1985) and Wieschaus and Nüsslein-Vollhard (1986).

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), mouse anti-en 1:2 (Patel et al., 1989) (Developmental Studies Hybridoma Bank), rabbit anti-β-gal 1:400 (Milan Analytika), mouse anti-β-gal 1:100 (DSHB), mouse anti-fasciclin II 1:5 (van Vactor et al., 1993), rabbit anti-bsh 1:50 (Jones and McGinnis, 1993), rat anti-OTD (Wieschaus et al., 1992) 1:250, rabbit anti-Otx2 (Mallamaci et al., 1996) 1:200, and rabbit anti-Sxl (Parkhurst et al., 1990) 1:500. Secondary antibodies were Cy3-conjugated goat anti-mouse, Cy3-conjugated goat anti-rabbit, Cy3-conjugated goat anti-rat, FITC-conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit, FITC-conjugated goat anti-rat, DTAF-conjugated goat anti-mouse, DTAF-conjugated goat anti-rabbit, and DTAF-conjugated goat anti-rat (all Jackson Immunoresearch), all 1:250. Embryos were mounted in Vectashield H-1000 (Vector).

Laser confocal microscopy

For laser confocal microscopy a Leica TCS 4D was used. Optical sections ranged from 0.8 to 2 μm recorded in line average mode with picture size of 512×512 pixels. Captured images from optical sections were arranged and processed using IMARIS (Bitplane). Figures were arranged and labeled using Adobe Photoshop.

RESULTS

Rescue of the *otd* mutant brain phenotype by the *Drosophila orthodenticle* gene

To determine if the CNS phenotypes of *otd* mutants can be rescued by ubiquitous overexpression of *otd*, a number of heat-shock regimes was tested on *otd* mutant embryos carrying an *hsp-otd* transgene (Royet and Finkelstein, 1995). When conventional heat-shock regimes involving a continuous heat-shock pulse in excess of 20 minutes at 37°C were delivered to *otd* mutant embryos or to wild-type controls, ectopic neural structures were induced in the CNS (see below). To avoid these effects, a pulsed heat-shock regime was developed, which made ubiquitous overexpression of *otd* possible without inducing ectopic neural structures in *otd* mutant embryos and which had no effect on CNS development in the wild-type

Table 1. Rescue of the *otd* brain phenotype

Lines	Efficiency of rescue ++	+	–	Total embryos examined
otd^{JA101}/Y; hsp-otd/+	13 (39.4%)	6 (18.2%)	14 (42.4%)	33
otd^{JA101}/Y; hsp-OTX1/+	4 (12.9%)	3 (9.7%)	24 (77.4%)	31
otd^{JA101}/Y; hsp-OTX2/+	12 (27.3%)	8 (18.2%)	24 (54.5%)	44

Efficiency of rescue subdivided into three categories: (++) protocerebral anlage, preoral commissure and anterior protocerebral *bsh* expressing cells recovered. (+) protocerebral anlage and preoral commissure recovered. (–) no rescue observed, embryo shows mutant phenotype.

Table 2. Rescue of the *otd* ventral nerve cord phenotype

Lines	Efficiency of rescue ++	+	–	Total embryos examined
otd^{JA101}/Y; hsp-otd/+	55 (92.0%)	5 (8.0%)	0 (0.0%)	60
otd^{JA101}/Y; hsp-OTX1/+	31 (37.4%)	47 (56.6%)	5 (6.0%)	83
otd^{JA101}/Y; hsp-OTX2/+	46 (80.7%)	11 (19.3%)	0 (0.0%)	57

Efficiency of rescue subdivided into three categories: (++) commissures and connectives separated, *en* expressing cells appear at the midline. (+) commissures and connectives separated. (–) no rescue observed, embryo shows mutant phenotype.

controls. This regime was used subsequently in experiments designed to rescue the embryonic brain and ventral nerve cord defects of *otd* mutants (see Materials and Methods).

In *otd* null mutants, the protocerebral brain anlage fails to develop and the preoral brain commissure is missing or severely reduced (Fig. 1A,C; Hirth et al., 1995). In accordance with these gross anatomical defects, the anterior set of protocerebral cells that express the *brain specific homeobox (bsh)* gene (Jones and McGinnis, 1993) in the wild type are lacking in the mutant (Fig. 1B,D). Ubiquitous overexpression of the *otd* transgene in the *otd* null mutant background carried out at stage 7-8 resulted in restoration of anterior brain morphology in over half of the treated embryos (Table 1). In these embryos, the protocerebral anlage, the preoral commissure as well as anterior protocerebral *bsh*-expressing cells were recovered (Fig. 1E,F). This suggests that the existence of a functional *otd* gene product before stage 7 is not necessary for anterior brain development in the embryo and implies that a limited period of *otd* expression at stage 7-8 is sufficient to allow normal embryonic development of the brain.

In contrast to the successful rescue achieved by ubiquitous overexpression of *otd* at stage 7-8, a rescue of the *otd* brain phenotype was not possible in embryos after stage 8. This suggests that the developing procephalic neuroectoderm in *otd* mutants is competent to respond to ubiquitous *otd* overexpression at stage 7-8, but not thereafter.

We did not attempt to carry out and interpret experiments involving ubiquitous overexpression of *otd* or other transgenes on embryos that were younger than stage 7. This is because heat shock applied to embryos up to stage 6 produced phenocopies and lethality in wild-type controls (Walter et al., 1990).

Rescue of the *otd* mutant ventral nerve cord phenotype by the *Drosophila otd* gene

In addition to defects in the anterior brain, mutational inactivation of *otd* causes midline defects in the ventral nerve cord (VNC) resulting in deranged connectives and fused commissural axon tracts as well as in the absence of *engrailed (en)*-expressing midline cells (Fig. 2A-F; Finkelstein et al., 1990; Klämbt et al., 1991). Ubiquitous overexpression of the *otd* transgene in the *otd* null mutant background carried out on embryos at stage 10-11 resulted in restoration of VNC morphology in all of the treated embryos (Table 2). In these cases, connectives and commissures were separated again and *en*-expressing cells appeared at the midline (Fig. 2G-I). This indicates that *otd* overexpression at stage 10-11 is sufficient to allow normal development of the embryonic VNC in *otd* mutants, implying that the existence of a functional *otd* gene product prior to stage 10 is not necessary for embryonic VNC development.

Before stage 10 or after stage 11, a rescue of the VNC defects was not observed. This suggests that there is a restricted time period in embryogenesis during which *otd* action can result in the generation of normal VNC morphology in *otd* mutant embryos. Taken together with the data on rescue of brain morphology by ubiquitous overexpression of *otd*, this demonstrates that separate developmental time windows exist for the rescue of anterior brain versus VNC phenotypes in *otd* mutants.

Rescue of the *otd* mutant brain phenotype by human *OTX1* and *OTX2* genes

OTX1 and *OTX2* are the human gene homologs of the *Drosophila otd* gene. To determine if these human genes are capable of restoring the CNS phenotypes of *Drosophila otd* mutants, heat-shock promoter driven human *OTX1* and *OTX2* transgenes were generated and introduced into the *otd* null mutant background (see Material and Methods). Ubiquitous overexpression experiments with the human transgenes were then carried out in the *otd* mutant flies with heat-shock regime and embryonic stages identical to those used in the *otd* overexpression experiments described above.

Ubiquitous overexpression of human *OTX2* in the *otd* null mutant background resulted in restoration of anterior brain morphology in 45% of the treated embryos (Table 1). The morphology of the rescued anterior brains was very similar to that in embryos overexpressing *Drosophila otd* in that the protocerebral anlage, the preoral commissure and anterior protocerebral *bsh*-expressing cells were recovered (Fig. 3A,B; compare to Fig. 1E,F).

Ubiquitous overexpression of human *OTX1* in the *otd* null mutant background was less efficient in rescuing anterior brain morphology (Table 1). Moreover, ectopic projections of commissural axons from the brain hemispheres along the frontal commissure occurred in the anterior brain of some of the treated embryos (Fig. 3C, asterisk). Nevertheless, ubiquitous overexpression of human *OTX1* did result in the restoration of anterior brain structures in over one fifth of the treated embryos; in these embryos the protocerebral anlagen, the preoral commissure and some of the anterior protocerebral *bsh*-expressing cells were recovered (Fig. 3C,D).

For both *OTX2* and *OTX1*, a rescue of the anterior brain phenotype in *otd* mutants was only possible if ubiquitous overexpression of the human gene was carried out at stage 7-8; rescue of the brain phenotype was not possible at later embryonic stages. These data suggest that both the human *OTX1* gene and the human *OTX2* gene have the capability to

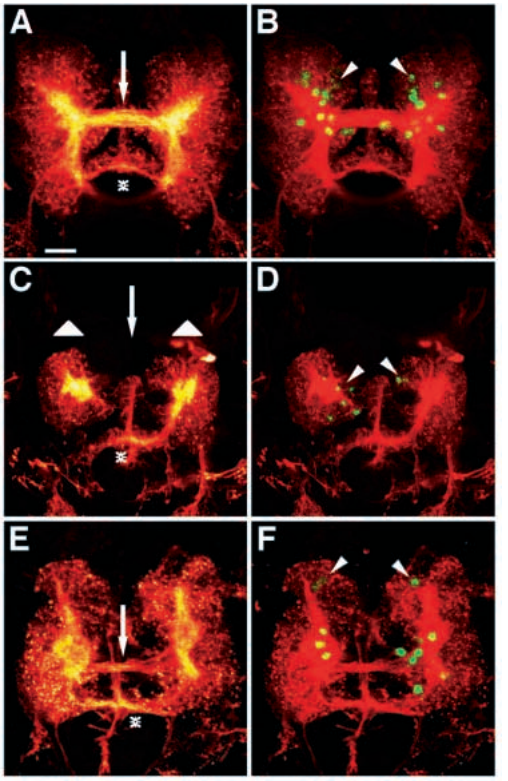

Fig. 1. Rescue of the *otd* mutant brain phenotype by *otd* overexpression. Comparison of the embryonic brain in the wild type (A,B), in the *otd* null mutant otd^{JA101} (C,D), and in the *otd*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-otd/+* (E,F). Laser confocal microscopy of stage 15 embryos, frontal views; images are reconstructions of optical sections showing the protocerebral and deutocerebral parts of the embryonic brain of *Drosophila*. According to neuraxis anterior is upwards in this and all subsequent figures. (A,C,E) Neuron-specific anti-HRP immunostaining reveals the structure of the embryonic brain. (B,D,F) Double labeling with an anti-bsh antibody (yellow/green) and the neuron-specific anti-HRP antibody (red/orange). In the wild type (A,B), the preoral commissure (A, arrow) connects the two brain hemispheres at the posterior border of the protocerebral neuromere; the frontal commissure is indicated by an asterisk, and cells expressing *bsh* are restricted to the protocerebral neuromere (B, arrowheads). In the *otd* null mutant, the protocerebral neuromere is almost completely deleted (C, indicated by triangles), the preoral commissure is absent (C, arrow), and the frontal commissure is unaffected (C, asterisk); only the most posterior *bsh*-expressing cells are present (D, arrowheads). In the *otd*-overexpressing transgenic mutant (E,F), the protocerebral neuromere is almost completely restored (E; compare to C), anterior cells again express *bsh* (F, arrowheads) and the preoral commissure is established at its normal position (E, arrow); the frontal commissure is indicated by an asterisk. Scale bar 10 μm.

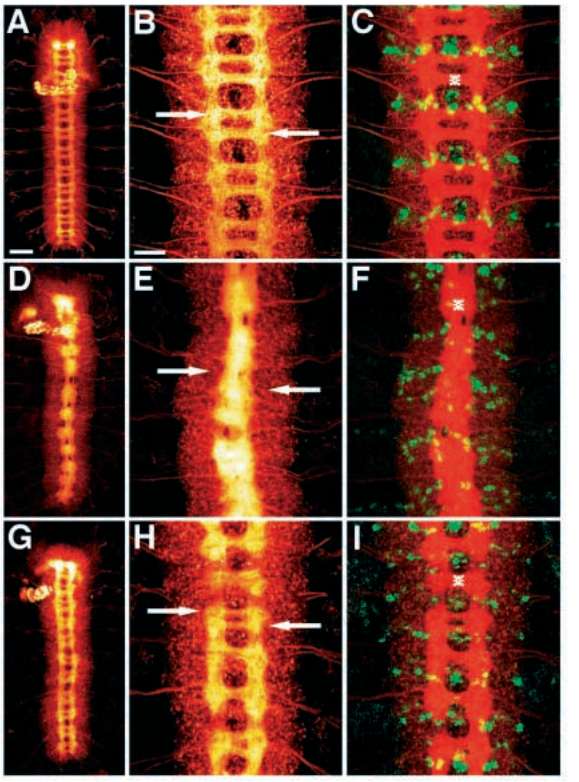

Fig. 2. Rescue of the *otd* mutant VNC phenotype by *otd* overexpression. Comparison of the embryonic VNC in the wild type (A-C), in the *otd* null mutant otd^{JA101} (D-F) and in the *otd*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-otd/+* (G-I). Laser confocal microscopy of stage 15 embryos, ventral views. Images are reconstructions of optical sections. (A,D,G) Neuron-specific anti-HRP immunostaining reveals the structure of the embryonic ventral nervous system. (B,E,H) Higher magnification of the ventral nerve cord revealed by neuron-specific anti-HRP immunostaining. (C,F,I) Double labeling with an anti-engrailed antibody (yellow/green) and the neuron-specific anti-HRP antibody (red/orange). The wild-type embryonic VNC (A-C) shows ladder-like axonal tracts with anterior and posterior commissures (B, arrows) and longitudinal connectives; specific midline cells express *engrailed* (C, asterisk). In the *otd* null mutant (D-F), the VNC is perturbed; separate anterior and posterior commissures do not appear (E, arrows; compare to B); *engrailed*-expressing cells along the midline are missing (F, asterisk; compare to C). In the *otd*-overexpressing transgenic mutant (G-I), the VNC is restored; separate anterior and posterior commissures appear (H, arrows) and the longitudinal axon tracts are located at their normal position; *engrailed*-expressing cells associated with the midline appear (I, asterisk). Scale bars: A, 20 μm; B, 10 μm.

replace the *otd* gene in the development of the anterior part of the embryonic *Drosophila* brain.

Rescue of the *otd* mutant ventral nerve cord phenotype by human *OTX1* and *OTX2* genes

Restoration of VNC morphology in transgenic *otd* mutants was observed in over 90% of the cases of ubiquitous overexpression of human *OTX1* and in 100% of the cases of ubiquitous overexpression of human *OTX2* (Table 2). In these embryos, the connectives and commissures in the embryonic VNC were separated again and *en*-expressing cells appeared at the midline (Fig. 4A-F). Again, as in *otd* overexpression experiments, a rescue of the VNC phenotype in *otd* mutants was only possible if human *OTX* gene overexpression was carried out at stage 10-11 and not at earlier or later embryonic stages.

These data show that the morphological extent of VNC restoration, the efficiency of VNC rescue and the developmental time window in which this rescue can occur are very similar for the human *OTX1* and *OTX2* genes and for the fly *otd* gene. This, in turn, indicates that the human *OTX1* and

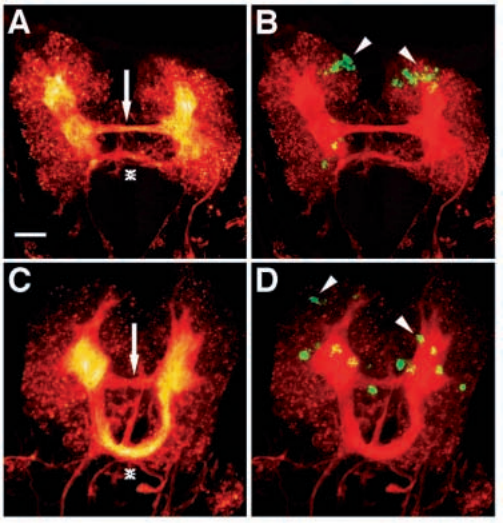

Fig. 3. Rescue of the *otd* mutant brain phenotype by human *OTX1* and *OTX2* overexpression. Comparison of the embryonic brain in the *OTX2*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-OTX2/+* (A,B), and in the *OTX1*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-OTX1/+* (C,D). Laser confocal microscopy of stage 15 embryos, frontal views; images are reconstructions of optical sections showing the protocerebral and deutocerebral parts of the embryonic brain of *Drosophila*. (A,C) Neuron-specific anti-HRP immunostaining reveals the structure of the embryonic brain. (B,D) Double labeling with an anti-bsh antibody (yellow/green) and the neuron-specific anti-HRP antibody (red/orange). In the *OTX2*-overexpressing transgenic mutant, the protocerebral neuromere is restored (A), and cells again express *bsh* (B, arrowheads); the preoral commissure is established at its normal position (A, arrow) and the frontal commissure is unaffected (A, asterisk). In the *OTX1*-overexpressing transgenic mutant, the protocerebral neuromere is restored (C) and cells again express *bsh* (D, arrowheads). The preoral commissure is established at its normal position (C, arrow); some axons ectopically project along the frontal commissure (C, asterisk). Scale bar 10 μm.

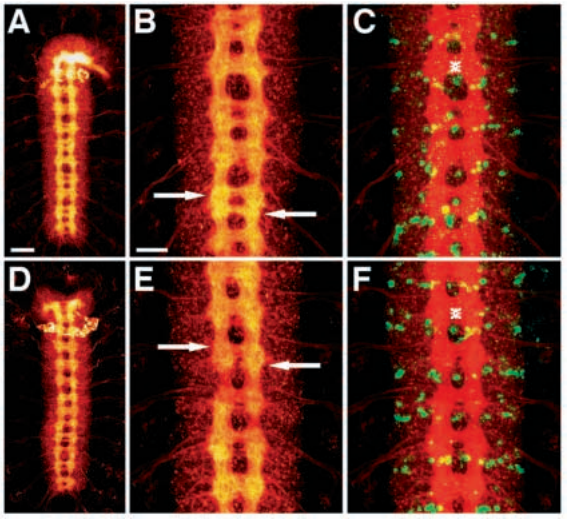

Fig. 4. Rescue of the *otd* mutant VNC phenotype by human *OTX1* and *OTX2* overexpression. Comparison of the embryonic ventral nerve cord in the *OTX2*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-OTX2/+* (A-C), and in the *OTX1*-overexpressing transgenic mutant otd^{JA101} */Y;hsp-OTX1/+* (D-F). Laser confocal microscopy of stage 15 embryos, ventral views. Images are reconstructions of optical sections. (A,D) Neuron-specific anti-HRP immunostaining reveals the structure of the embryonic ventral nervous system. (B,E) Higher magnification of the ventral nerve cord revealed by neuron-specific anti-HRP immunostaining. (C,F) Double labeling with an anti-engrailed antibody (yellow/green) and the neuron-specific anti-HRP antibody (red/orange). In the *OTX2*-overexpressing transgenic mutant (A-C), the VNC is restored; separate anterior and posterior commissures appear (B, arrows) and the longitudinal axon tracts are located at their normal position; *engrailed*-expressing cells associated with the midline appear (C, asterisk). In the *OTX1*-overexpressing transgenic mutant (D-F), the VNC is restored; separate anterior and posterior commissures appear (E, arrows) and the longitudinal axon tracts are located at their normal position; *engrailed*-expressing cells associated with the midline appear (F, asterisk). Scale bars: A, 20 μm; B, 10 μm.

OTX2 genes can replace the *otd* gene in the development of the VNC in *Drosophila*.

Induction of ectopic neural structures by overexpression of *otd* or *OTX1/2* genes in transgenic wild-type embryos

The pulsed heat-shock regime used to rescue the brain and VNC defects in transgenic *otd* mutant embryos had no effect on the development of the wild-type embryonic CNS after stage 6. In contrast, dramatic effects of ubiquitous overexpression of either the fly *otd* or human *OTX1/2* genes in a wild-type background were observed if a continuous heat shock in excess of 20 minutes at 37°C was applied to transgenic embryos.

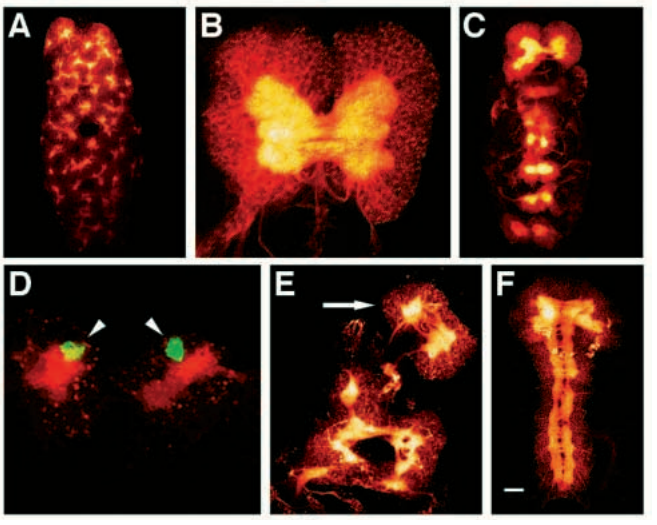

Fig. 5. Formation of ectopic neural structures by fly *otd* or human *OTX1/2* overexpression in transgenic wild-type embryos. Laser confocal microscopy; images are reconstructions of optical sections. Neuron-specific anti-HRP immunostaining reveals CNS structures. Overexpression of *otd* or *OTX1/2* in stage 7-8 transgenic wild-type embryos results in ectopic or transformed embryonic CNS structures such as neuralized embryos (A), dramatically enlarged brain lobes (B), enlarged brain lobes and fused, enlarged ventral ganglia (C), which in some cases show ectopic expression of the *brain specific homeobox (bsh)* gene (D, arrowheads), as well as ectopic head ganglionic structures (E, arrow). Continuous overexpression of *otd* or *OTX1/2* in stage 9-10 transgenic wild-type embryos results in less pronounced pattern perturbations in the CNS (F). Scale bar 50 μm.

If this type of continuous heat-shock regime was used to overexpress *otd* in a wild-type background at stage 7-8, ectopic or transformed CNS structures developed in virtually all embryos. The observed ectopic or transformed structures included neuralized embryos, dramatically increased brain lobes and ectopic head ganglionic structures, as well as fused and enlarged ventral ganglia (Fig. 5A-E). Interestingly, some of these ectopic head ganglia and the fused and enlarged VNC ganglia showed ectopic expression of the *bsh* gene, suggesting a partial protocerebral identity of the ectopic structures. If the continuous heat-shock regime was used to overexpress *OTX1* or *OTX2* in a wild-type background at stage 7-8, similar ectopic or transformed CNS structures were also observed, but at a markedly lower rate (10% of the treated embryos).

If overexpression of *otd (OTX1/2)* in a wild-type background was carried out with the continuous heat-shock regime at stage 9-10, the observed ectopic embryonic CNS transformations were much less pronounced (Fig. 5F). Ectopic transformations of the embryonic CNS were not observed if *otd (OTX1/2)* overexpression was performed later than stage 12.

DISCUSSION

The early blastoderm expression of *otd* is not required for brain development

The *Drosophila otd* gene has been classified as a head gap gene for two reasons. First, because its expression at the early cellular blastoderm stage is under the control of maternal positional information in a manner similar to that of (non-cephalic) gap genes (Finkelstein and Perrimon, 1990; Grossniklaus et al., 1994; Gao et al., 1996). Second, because mutation of *otd* leads to a gap-like phenotype in the anterior head, which includes deletions in cuticular structures, the absence of the antennal and preantennal expression of *engrailed* and *wingless*, the loss of several cephalic sensory structures, and the deletion of the protocerebral anlage (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Schmidt-Ott et al., 1994; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Fate map studies relate the regionalized cephalic defects seen in *otd* mutants to the broad anterior region of *otd* expression in the early cellular blastoderm stage (Jürgens et al., 1986; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Schmidt-Ott et al., 1994). It is, therefore, conceivable that the gap-like *otd* mutant phenotype is due to the absence of a functional *otd* gene at the cellular blastoderm stage. While this may apply to some of the non-CNS defects, our experiments indicate that this is not the case for the embryonic brain defects observed in *otd* mutants.

Genetic rescue experiments through ubiquitous overexpression of an *otd* transgene in *otd* mutant embryos indicate that the existence of a functional OTD gene product before embryonic stage 7 is not required for proper development of the anterior embryonic brain. This is because the gap-like brain defects in the *otd* mutant can be restored by overexpressing *otd* at stages 7-8. This, in turn, implies that the cells of the blastoderm embryo, which express *otd* in the wild type and are fated to give rise to the protocerebrum, are not deleted in the *otd* mutant at least up to stage 7-8. It is possible that other head gap genes with partially redundant function can compensate for the loss of *otd* in the cellular blastoderm embryo.

Although the existence of a functional *otd* gene product before stage 7 is not required for embryonic brain development, we cannot rule out that ubiquitous overexpression of *otd* at earlier stages might also rescue the *otd* mutant brain defects, since phenocopy effects did not allow this type of experimental manipulation. Our rescue experiments do, however, indicate that ubiquitous overexpression of *otd* at embryonic stage 9 or later cannot restore the *otd* mutant brain defects. Given that brain neuroblasts segregate from the procephalic neuroectoderm during embryonic stages 9-11 (Younossi-Hartenstein et al., 1996), this suggests that a functional *otd* gene product is required in the anlage of the anterior brain before neuroblast formation occurs.

Based on the observation that mutation of *otd* leads to a loss of expression of the proneural gene *lethal-of-scute* in the embryonic brain, it has been proposed that *otd* might control brain neuroblast formation by triggering proneural gene expression (Younossi-Hartenstein et al., 1997). The results of our rescue experiments on *otd* mutant embryos as well as of our ubiquitous *otd* overexpression experiments on transgenic wild-type embryos are in accordance with this notion.

Distinct *otd* functions during anterior brain and VNC development

The anterior brain of *Drosophila* is subdivided into protocerebrum, deutocerebrum and tritocerebrum, and develops from the procephalic neuroectoderm. The embryonic anlagen of several modified head segments contribute to the procephalic neuroectoderm (see Younossi-Hartenstein et al., 1996). Neither pair rule genes nor homeotic selector genes are thought to be involved in the embryogenesis of these head segment anlagen (reviewed in Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991; Jürgens and Hartenstein, 1993). It has, therefore, been proposed that the overlapping expression domains of head gap genes such as *orthodenticle*, *empty spiracles* and *buttonhead* mediate anterior head and anterior brain metamerization in a dual function as gap-like and homeotic genes (e.g. Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Grossniklaus et al., 1994; Hirth et al., 1995; Younossi-Hartenstein et al., 1997).

The posterior *Drosophila* brain is subdivided into mandibular, maxillar and labial neuromeres, and develops from the ventral neuroectoderm of the three gnathal head segments (Younossi-Hartenstein et al., 1996). In contrast to the anterior head region, embryonic patterning in this region is controlled in the same way as in the segments of the trunk region, which give rise to the VNC. There, gap genes serve as a positional reference system for the subsequent expression of their direct target genes including both the pair-rule genes and the homeotic genes which, in turn, act to specify segment identity (reviewed in McGinnis and Krumlauf, 1992; Pankratz and Jäckle, 1993).

The two different embryonic expression domains and mutational phenotypes of *otd* in the anterior brain and in the VNC (and posterior brain neuromeres) are in accordance with this difference in embryonic patterning (Finkelstein et al., 1990; Klämbt et al., 1991; Wieschaus et al., 1992; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In the anterior embryo, a first domain of *otd* expression appears as early at stage 5 and, following gastrulation, persists in the procephalic region which gives rise to the anterior brain. At the ventral midline, a second domain of *otd* expression appears at stage 9-10 in a longitudinal stripe that corresponds to the mesectoderm and will generate a

mixed population of VNC neurons and glia. In the anterior brain, mutational inactivation of *otd* results in a gap-like deletion of the protocerebral anlage. In the VNC of *otd* mutants, specific cells derived from the ventral mesectoderm fail to differentiate and deranged axon tracts result.

The experiments on *otd* mutants reported here support the notion that *otd* has distinct and separate functions during anterior brain and VNC development. This is because separate developmental time windows exist for the rescue of anterior brain versus VNC mutant phenotypes. Rescue of the anterior brain phenotype in *otd* mutants is not possible after stage 8; rescue of the VNC phenotype in *otd* mutants is not possible before stage 10.

Equivalence of homologous fly and human *otd/OTX* genes in CNS development

Several studies have demonstrated conserved transcriptional regulation of homologous fly and vertebrate genes in embryogenesis. For example, transcriptional regulatory elements of the human homeotic gene *HOXB-4* are able to evoke head-specific reporter gene expression in *Drosophila* comparable to the endogenous expression pattern of its *Drosophila* homolog *Deformed* (Malicki et al., 1992; Awgulewitsch and Jacobs, 1992). Equivalence of homologous fly and vertebrate genes in the development of embryonic cuticular structures and appendages has also been demonstrated in *Drosophila*. Thus, the avian *Hoxb-1* gene is able to rescue the cuticular head phenotype caused in *Drosophila* by mutational inactivation of the homologous *labial* gene (Lutz et al., 1996). Ectopic expression of the *Antennapedia* homolog *Hoxb-6* is able to induce homeotic transformations in *Drosophila* that are similar to those caused by ectopic expression of *Antennapedia* itself, such as thoracic denticle belts in place of head structures and (in adults) thoracic leg structures in place of antennae (Malicki et al., 1990). In addition to these studies, recent work on eye development shows that the equivalence of homologous fly and vertebrate genes also applies to the development of complex sensory structures. For example, ectopic expression of either the *Drosophila eyeless* gene or its homologous mouse gene *Pax6* is able to induce ectopic eyes in *Drosophila* (Halder et al., 1995).

In this report, we extend the notion of equivalence of vertebrate and invertebrate regulatory control genes to the development of the most complex organ in the animal, the brain. Our genetic rescue experiments demonstrate remarkable and extensive similarities in the results of overexpression of the fly *otd* gene and the human *OTX1* and *OTX2* genes in transgenic *Drosophila* embryos. The ubiquitous overexpression of all three genes can *(i)* rescue the *otd* mutant brain phenotype, *(ii)* rescue the *otd* mutant VNC phenotype, *(iii)* act in the same distinct developmental time windows which are different for embryonic brain versus embryonic VNC, and *(iv)* result in the formation of ectopic and transformed neural structures in transgenic wild-type embryos. The only major difference among the three genes in genetic rescue experiments in *Drosophila* is the lower efficiency of the human *OTX1* gene in rescuing brain morphology. The human *OTX1* gene rescues embryonic brain morphology in only one fourth of the cases. Moreover, in *OTX1* rescue experiments some of the restored anterior brain structures have ectopic commissural projections. The reason for this difference in *OTX1* action is not understood; it may reflect different efficiency in translation and/or transcriptional activating abilities between OTX2 and OTX1 (Simeone et al., 1993).

The human OTX1 and OTX2 gene products differ significantly from the *Drosophila* OTD gene product both in overall size and in amino acid sequence (see Simeone et al., 1993). The Drosophila OTD gene product is much larger (548 amino acid residues) than either the OTX1 gene product (354 amino acid residues) or the OTX2 gene product (289 amino acid residues). Moreover, the Drosophila OTD protein shares little amino acid sequence similarity with the human OTX proteins outside of the 60 amino acid residues of the homeodomain. Thus, with the exception of the homeodomain sequence, sequence homology among the three gene products is found only in a short Tyr-Pro-----Arg-Lys stretch immediately upstream of the homeodomain and in a tri-peptide region (Phe/Tyr-Leu-Lys) at the amino terminus (Simeone et al., 1993). In contrast, the homeodomains of the human OTX1 and OTX2 gene products are highly conserved and differ from the homeodomain of the fly OTD gene product in only three and two amino acids, respectively. The remarkable rescue of the embryonic CNS defects in *otd* mutants of *Drosophila* by the human *OTX* genes underscores the importance of the highly conserved homeodomain sequence of the *otd/OTX* genes. Indeed, it seems likely that developmental equivalence of the *otd/OTX* homologs in embryonic CNS development is mediated primarily by the evolutionarily conserved homeodomain. If this is the case, it will be interesting to determine the functional role of the long stretch of amino acids in the C-terminal region of the large fly OTD protein that is completely lacking in the smaller human OTX proteins.

In summary, our results, together with complementary results obtained in the mouse (Acampora et al., 1998), demonstrate a remarkable and extended conservation of the *otd/OTX* genes in brain development of both invertebrates and vertebrates. This provides further evidence for the idea that an extensive region of the anterior-posterior axis of the insect and mammalian body plan is homologous in a developmental genetic sense. It will now be important to identify and compare the regulatory cascade required to form an insect brain with that required to form a mammalian brain to determine what the differences are and which new genes have been recruited into these developmental pathways in the course of evolution.

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