Murine *Otx1* and *Drosophila otd* genes share conserved genetic functions required in invertebrate and vertebrate brain development

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SUMMARY

Despite the obvious differences in anatomy between invertebrate and vertebrate brains, several genes involved in the development of both brain types belong to the same family and share similarities in expression patterns. *Drosophila orthodenticle* (*otd*) and murine *Otx* genes exemplify this, both in terms of expression patterns and mutant phenotypes. In contrast, sequence comparison of OTD and OTX gene products indicates that homology is restricted to the homeodomain suggesting that protein divergence outside the homeodomain might account for functional differences acquired during brain evolution. In order to gain insight into this possibility, we replaced the murine *Otx1* gene with a *Drosophila otd* cDNA. Strikingly, epilepsy and corticogenesis defects due to the absence of *Otx1* were fully rescued in homozygous *otd* mice. A partial rescue was also observed for the impairments of mesencephalon, eye and lachrymal gland. In contrast, defects of the inner ear were not improved suggesting a vertebrate *Otx1*-specific function involved in morphogenesis of this structure. Furthermore, *otd*, like *Otx1*, was able to cooperate genetically with *Otx2* in brain patterning, although with reduced efficiency. These data favour an extended functional conservation between *Drosophila otd* and murine *Otx1* genes and support the idea that conserved genetic functions required in mammalian brain development evolved in a primitive ancestor of both flies and mice.

Key words: *orthodenticle* (*otd*), *Otx1*, Evolution, Brain, Conserved function, *Drosophila*

INTRODUCTION

Despite the enormous morphological diversity between invertebrates and vertebrates, several genetic programs for the control of regional specification are highly conserved. Striking examples are the specification of axial patterning by the invertebrate *HOM-C* and vertebrate *HOX* genes (reviewed in Lewis, 1978; McGinnis and Krumlauf, 1992; Krumlauf, 1994) and the control of eye morphogenesis by the invertebrate *eyeless* and vertebrate *Pax6* genes (reviewed in Callaerts et al., 1997).

In vertebrate brain development, the *HOX* genes are involved in hindbrain patterning, but are not expressed in the forebrain, midbrain and rostralmost hindbrain, where a different set of genes specify regional identity.

Based on sequence homology between highly conserved domains, the vertebrate homologs of *Drosophila* genes controlling head development have been isolated (Price et al., 1991; Simeone et al., 1992; Rubenstein et al., 1994; Thor, 1995; Joyner, 1996).

Most of these are homeobox-containing genes and, among them, the murine *Otx* and *Drosophila otd* genes represent a remarkable example of similarity in homeodomain sequence, embryonic expression pattern and mutant phenotype (Cohen and Jürgens, 1991; Holland et al., 1992; Finkelstein and Boncinelli, 1994; Acampora et al., 1995, 1996, 1997; Hirth et al., 1995; Matsuo et al., 1995; Thor, 1995; Ang et al., 1996).

In *Drosophila,* the *otd* gene is expressed at the anterior pole of the blastoderm embryo and later predominantly in the developing rostralmost brain neuromere (protocerebrum) (Finkelstein and Perrimon, 1990a; Cohen and Jürgens, 1991; Hirth et al., 1995). In *otd* mutants, most protocerebral neuroblasts and some deuterocerebral neuroblasts do not form, giving rise to a dramatically reduced brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). *otd* mutants also have pattern deletions in cephalic structures. For example, in *ocelliless,* a viable *otd* allele, expression in the vertex primordium is abolished and the ocelli (light-sensing organs) and associated sensory bristles (Finkelstein et al., 1990b) are lost. Finally, in cephalic development, different levels of OTD protein are required for the formation of

specific subdomains of the adult head (Royet and Finkelstein, 1995).

In mouse, *Otx1* and *Otx2* genes are activated sequentially during embryonic development. *Otx1* expression is first detected at the 1- to 3-somite stage throughout the forebrain and midbrain neuroepithelium. *Otx2* is already transcribed before the onset of gastrulation; at the end of gastrulation, *Otx2* is expressed in the rostral neuroectoderm fated to give forebrain and midbrain (Simeone et al., 1992, 1993). During brain regionalization, *Otx1* and *Otx2* show largely overlapping expression domains with a posterior border coincident with the mesencephalic side of the isthmic constriction (Simeone et al., 1992; Acampora et al., 1997). Additionally, *Otx1* is transcribed in neurons of deep layers of the adult cerebral cortex (Frantz et al., 1994) and both *Otx1* and *Otx2* are expressed in the olfactory, ocular and acoustic sense organs (Simeone et al., 1993). *Otx1* null mice mutants show spontaneous epileptic seizures and multiple abnormalities affecting the telencephalic dorsal cortex, the mesencephalon, the cerebellum and components of the acoustic and visual sense organs (Acampora et al., 1996). *Otx2* null mice mutants are early embryonic lethal and lack the rostral neuroectoderm fated to become forebrain, midbrain and rostral hindbrain (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Moreover, *Otx* genes may cooperate in brain morphogenesis and a threshold level of OTX proteins is required to specify early regional diversity between adjacent mesencephalic/metencephalic territories and to allow the correct positioning of the isthmic organizer (Acampora et al., 1997).

In contrast to the extensive similarities in expression and mutant phenotype of the *Drosophila otd* and the murine *Otx* genes, that of the *OTD* and *OTX* gene products is quite restricted; sequence homology is confined to the homeodomain and a few flanking aminoacids. Thus, although the ability to recognize the same target sequence might be evolutionarily conserved, murine *Otx* genes might have also acquired, outside the homeodomain, additional functional features that are different from those encoded by the *Drosophila otd* gene. This suggests that some conserved features of the invertebrate *OTD* gene product might now coexist in *Otx* genes together with additional new functions required for specific mammalian developmental processes.

Comparative analyses of the evolution of *otd* and *Otx* coding sequences and expression patterns demonstrate the existence of *otd*-related genes in all chordates (Simeone et al., 1992; Bally-Cuif et al., 1995; Mercier et al., 1995; Pannese et al., 1995), including urochordates (Wada et al., 1996) and cephalochordates (Williams and Holland, 1996), where they are expressed in the rostralmost CNS. However, unambiguous *Otx1*- and *Otx2*-related genes have so far been identified only in gnathostomes. To analyse the conserved versus newly established functions of *Otx1*- and *Otx2*-related genes further, in vivo genetic manipulation experiments are required. Here, we describe a first example of such an in vivo approach, performed by replacing the murine *Otx1* gene with the *Drosophila otd* cDNA.

Our results indicate that the majority of the defects due to the absence of *Otx1* are rescued in homozygous *otd* mice. Defects affecting the inner ear are not improved. Moreover, *otd*, like *Otx1*, is also able to cooperate genetically with *Otx2* in patterning the developing mouse brain albeit at a reduced efficiency. These data favour the idea of an extended functional conservation between the *Drosophila otd* and the murine *Otx1*

genes and suggest that genetic functions required for development of the mammalian brain originated in a primitive ancestor of both flies and mice.

MATERIALS AND METHODS

Construction of the targeting vector, transfection of ES cells and selection of targeted clones

The gene replacement vector was generated using the same plasmid (pGN) and arms for homologous recombination used to produce *Otx1*$^{-/-}$ mice (Acampora et al., 1996) but with the *Bsu*36I/*Xmn*I fragment of the *otd* cDNA (Finkelstein et al., 1990b) in place of the *lacZ* gene. As in the *Otx1* knock-out vector, a SV40 polyadenylation signal was present downstream of the cDNA to ensure transcription termination. 15 µg of the targeting vector were linearized by *Kpn*I digestion and electroporated into 2×10⁷ HM-1 ES cells. Homologous recombinant clones were identified using the same primers as previously described (Acampora et al., 1996) (filled arrows in Fig. 1A) and confirmed by hybridizing *Hin*dIII-digested genomic DNA with probes A and D (Acampora et al., 1996) (Fig. 1A).

Mouse production and genotyping

Two independent positive clones were injected into C57BL/6 blastocysts and the resulting chimaeric males back-crossed to B6/D2 F$_1$ females. Genotyping was performed by PCR using two primers specific for the wild-type allele and located in the *Otx1*-deleted sequence (sense primer, AGCAGACACATCGAAACCTTC; antisense primer, CACTTGGGATTTTGCACCCTC) (filled arrowheads in Fig. 1A) and two primers specific for the *otd* cDNA (sense primer, ATCAAGACGCACCACAGTTCCT; antisense primer, TCCTTTAGCTGATCATAGGGCG) (open arrowheads in Fig. 1A).

Western blot analysis

Crude extracts of 12.5 dpc (days post coitum) heads were obtained by lysis in 8 M urea in the presence of 5 mM Tris pH 8 and 0.5% β-mercaptoethanol. 80 µg of these extracts and 10 µg of nuclear extracts of HeLa cells transfected with plasmids overexpressing human *OTX1*, *OTX2* and *otd* cDNAs under a CMV enhancer-promoter (Simeone et al., 1993) were electrophoresed and transferred to nitrocellulose in a standard western blot assay and probed with anti-*OTD* antibody diluted 1:250.

Electroencephalographic recordings

The electroencephalographic activity recordings were performed as previously described (Acampora et al., 1996). Correct position of electrodes was confirmed by anatomical analysis.

Histological analysis of brains and sense organs

Dissected brains, eyes and inner ears were prepared as previously described (Acampora et al., 1996). Histological sections (10 µm) were stained with Cresyl-violet (brains) or haematoxylin-eosin (eyes). Lachrymal glands were analyzed during the eye dissection. For the fine histological analysis of the cortex, comparable groups of sections centered on the rostral hippocampus (four sections) and posteriorly on the presubicular area (six sections) were selected from 5 wild-type, 8 *Otx1*$^{-/-}$, 8 *otd^l/Otx1* and 12 *otd^l/otd^l* brains (1- to 2-month-old); histology was as previously reported (Acampora et al., 1996). Cell number was determined by counting cell bodies along a cortical area defined by the thickness of the cortex and by a unit length of 200 µm on the ventricular side (an area similar to that reported in Fig. 4A,B). Mean value ± s.e.m. of the different areas was expressed as a percentage of wild-type cell number.

BrdU labeling and detection of apoptotic cells

Pregnant mice at 9.75, 13.5 and 15.5 dpc were injected intraperitoneally with BrdU solution (50 mg/kg body weight) and

killed after 1 hour. After embryo genotyping, BrdU detection was performed according to Xuan et al. (1995). Three embryos for each genotype were scored at 9.75, 13.5 and 15.5 dpc. Four comparable sections for each embryo were analyzed at 9.75 dpc, while every fourth serial coronal section was selected for a total number of 8 sections at 13.5 and 15.5 dpc. The fraction of BrdU-positive cells was determined by dividing the number of BrdU-positive nuclei by the total number of nuclei identified in units of neuroepithelium 100 μm in length (Xuan et al., 1995). The proportion of BrdU-positive cells in wild-type embryos was considered 100%.

To detect apoptotic cells, the sections were processed by the TUNEL method as described (Gavrieli et al., 1992).

Generation and genotyping of double mutant mice

Otx1^{−/−}*; Otx2*^{+/−} embryos were generated as previously described (Acampora et al., 1997). *otd*[1]*/−; Otx2*^{+/−} embryos were generated by crossing *otd*[1]*/Otx1; Otx2*^{+/−} males with *Otx1*^{+/−}*; Otx2*^{+/+} females. *otd*[1]*/otd*[1]*; Otx2*^{+/−} embryos were generated by crossing *otd*[1]*/Otx1; Otx2*^{+/−} males with *otd*[1]*/Otx1; Otx2*^{+/+} females.

Genotypes were identified by PCR as described previously (Acampora et al., 1995, 1996, 1997 and see above).

Probes and in situ hybridization

The *Otx1*-deleted exons 1 and 2 (probe B) (Fig. 1A) and the region spanning from aa 1 to aa 133 of the *Drosophila otd* cDNA (probe C) (Fig. 1A) were used as specific probes for the two alleles. The *Otx2* functional allele was monitored using the *Otx2*-deleted probe (Acampora et al., 1995). The *Fgf-8, Gbx2, Wnt-1* and *En-2* probes were as previously described (Acampora et al., 1997). In situ hybridization on sections was performed as previously described (Hogan et al., 1994).

RESULTS

Generation of mice with the *Otx1* gene replaced by *Drosophila otd* cDNA

To assess the functional conservation between *Drosophila otd* and murine *Otx1* genes, we introduced a full coding *otd* cDNA-SV40 poly(A) cassette into a disrupted *Otx1* locus by homologous recombination in embryonic stem (ES) cells. The *Otx1* deletion corresponded to a 2.3 kb fragment including the coding region of exons 1 and 2 (Fig. 1A) (Acampora et al.,
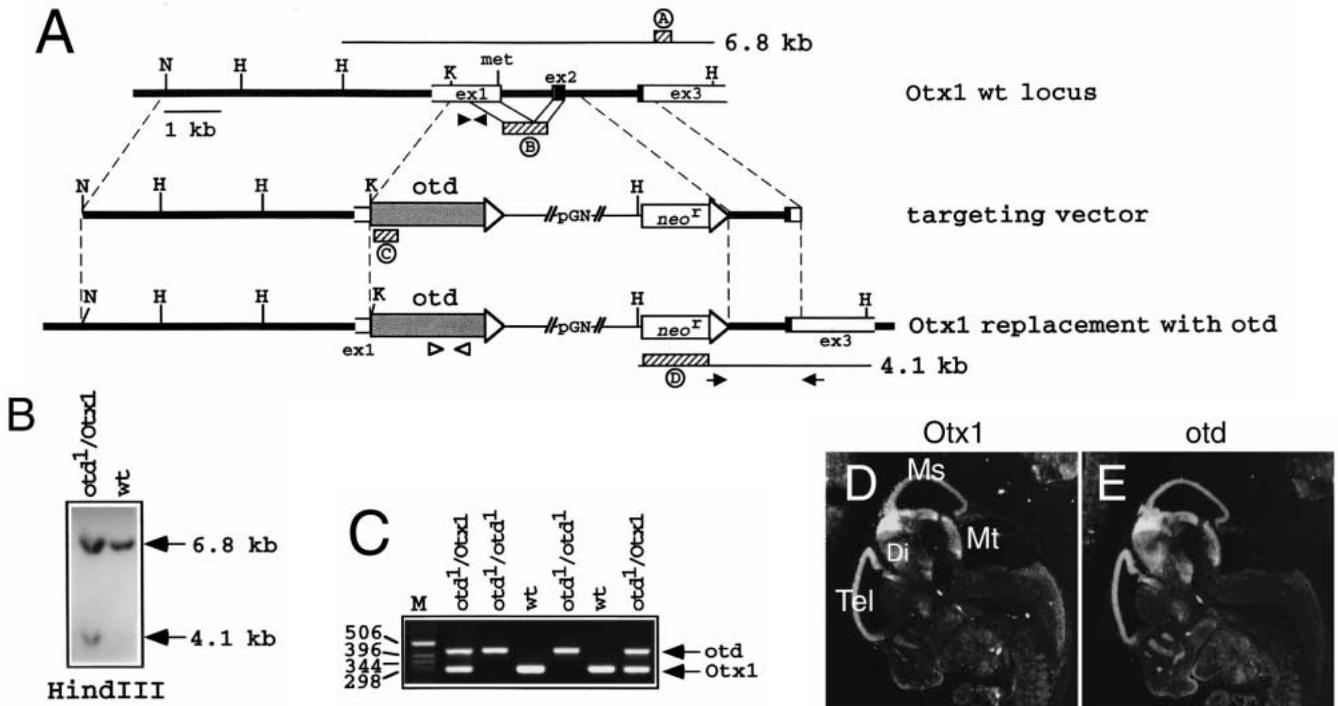

Fig. 1. Targeted replacement of *Otx1* with *otd* cDNA, expression of *otd* in *otd*[1]*/Otx1* embryos and detection of OTD protein. (A) Targeting vector shown in third line. Fourth line illustrates recombined locus. First and last lines show *Hin*dIII fragments (6.8 and 4.1 kb) detected by Southern blot using probes (hatched boxes) external to targeting vector or within the *neomycin* gene. N, H, K stands for *Nsi*I, *Hin*dIII and *Kpn*I. (B) Southern blot analysis of one targeted cell line (*otd*[1]*/Otx1*) and wild-type (wt) HM-1 ES cells showing expected hybridization pattern of *Hin*dIII-digested genomic DNA samples with probe A (A). Only the 4.1 kb fragment is detected with a neo-specific probe (probe D) (data not shown). (C) Genotyping of a litter from the mating of two heterozygotes by PCR reaction amplifying fragments specific for deleted region of *Otx1* (302 bp) and/or *otd* (443 bp), using the primers positioned in the second and fourth lines of A (filled and open arrowheads). (D,E) By using allele-specific probes B and C (A), the expression pattern of *Otx1* (D) is compared to that of *otd* (E) in adjacent sagittal sections of a 12.5 d.p.c. *otd*[1]*/Otx1* embryo showing that they are expressed with the same distribution. (F) Detection of OTD, OTX1 and OTX2 gene products in western blot analysis of total and nuclear extracts from wt, *otd*[1]*/Otx1* and *otd*[1]*/otd*[1] 12.5 d.p.c. embryonic heads and from HeLa cells untransfected and transfected with plasmids overexpressing human OTX1, OTX2 and *otd* cDNA, respectively. Abbreviations: Tel, telencephalon; Di, diencephalon; Ms, mesencephalon; Mt, metencephalon.
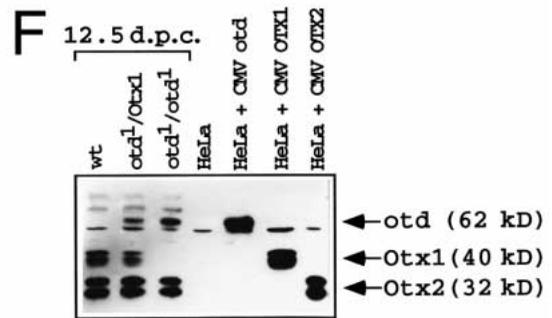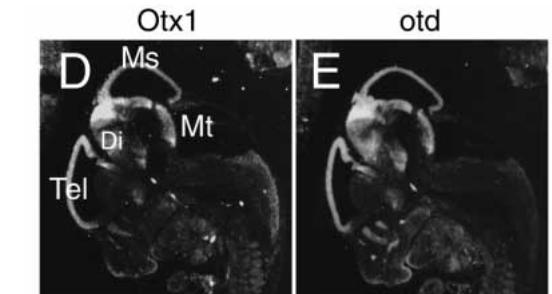
1996). The targeting vector (Fig. 1A) was constructed with the same DNA fragments previously used for *Otx1* knock-out strategy (Acampora et al., 1996), but with the *otd* cassette in place of the *E. coli lacZ* gene. This vector was introduced into HM-1 ES cells and 8 homologous recombinant clones were identified by PCR and Southern blot analyses (Fig. 1B and see Materials and Methods). Two independent positive clones were injected into C57BL/6 blastocysts to produce chimaeric mice. Male chimaeras were mated with B6D2 F_1 females to obtain heterozygotes *(otd¹/Otx1)*. The resulting heterozygotes were healthy and fertile; their genotypes were determined by allele-specific PCR reactions (Fig. 1C). Correct expression of *otd* under *Otx1* transcriptional control was verified by comparing the *Otx1* and *otd* expression patterns in *otd¹/Otx1* embryos at 12.5 dpc (Fig. 1D,E). No signal was detected by using the *otd* probe on wild-type embryos. Translation of the *otd* transcripts was monitored by using a *Drosophila* OTD polyclonal antibody which also recognized human OTX1 and OTX2 proteins, as shown in HeLa cell extracts transfected with expression vectors for each of the three genes (Fig. 1F). As expected, the OTD protein was only detected in 12.5 dpc head extracts from *otd¹/Otx1* and *otd¹/otd¹* genotypes, while the murine OTX1 protein was only detected in wild-type and *otd¹/Otx1* embryos (Fig. 1F). In *otd¹/Otx1* embryos at 12.5 dpc, the amount of OTD protein was ~30% lower than that of OTX1 (Fig. 1F), as revealed by densitometric scanning of three 12.5 dpc *otd¹/Otx1* embryos (data not shown).

The *Drosophila otd* gene rescues epilepsy and anatomical abnormalities seen in the telencephalic cortex of *Otx1⁻/⁻* mice

otd¹/otd¹ mice were generated at the expected frequency and postnatal lethality was significantly lower (~5%) than in *Otx1⁻/⁻* mice (~30%) (Acampora et al., 1996). All *Otx1⁻/⁻* mice exhibited epilepsy and aberrant high-speed-turning behaviours.
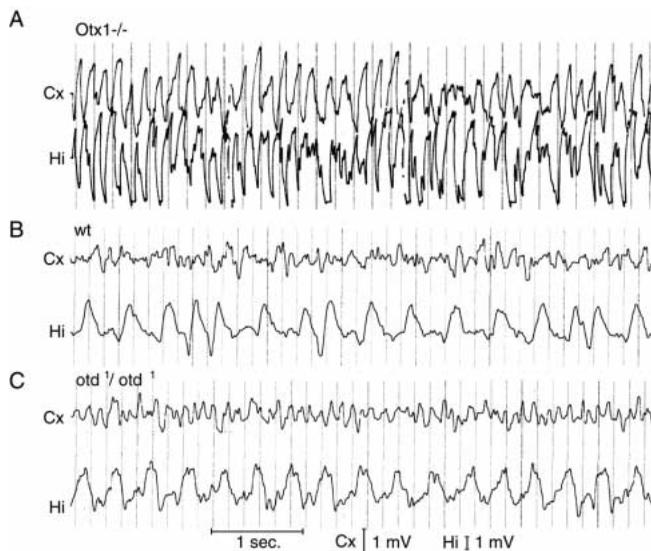
Fig. 2. *otd¹/otd¹* mice recover from epilepsy. (A-C) Representative electroencephalogram recordings showing a typical *Otx1⁻/⁻* seizure (A) with high voltage spikes in hippocampus (Hi) and cortex (Cx) while, in wild-type (B) and *otd¹/otd¹* (C) mice, a normal electrical activity was always recorded. Scale bars are indicated.
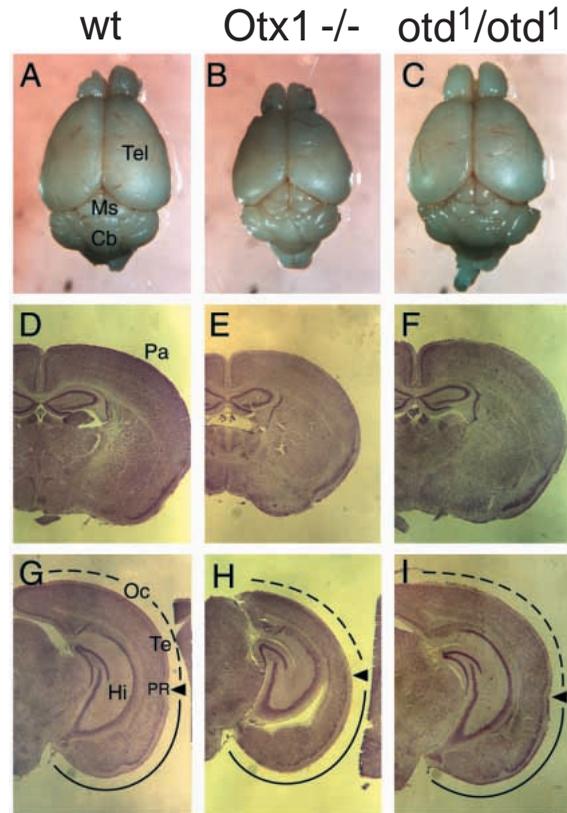
Fig. 3. Comparison of wild type, *Otx1⁻/⁻* and *otd¹/otd¹* brains. (A-C) Dorsal view showing that *otd¹/otd¹* brains are of normal size (C) compared to wild type (A) and *Otx1⁻/⁻* (B). (D-I) Frontal sections of wild-type (D,G), *Otx1⁻/⁻* (E,H) and *otd¹/otd¹* (F,I) adult (2 month) brains showing that all the *Otx1⁻/⁻* cortical abnormalities are largely recovered in *otd¹/otd¹* brain (F,I). Abbreviations as in previous figure, plus: Cb, cerebellum; Hi, hippocampus; Pa, Oc, Te and PR are parietal, occipital, temporal and perirhinal areas, respectively; arrowheads point to presumptive sulcus rhinalis; dashed and solid lines define cortical regions located dorsally and ventrally to the sulcus rhinalis, respectively.

otd¹/otd¹ mice retained only a moderate-speed-turning behaviour and lacked the phenotypic characteristics of both focal and generalized epileptic seizures. To assess the rescue of epilepsy electrophysiologically, electroencephalograms were performed on 15 *otd¹/otd¹*, 5 wild-type, 5 *otd¹/Otx1* (data not shown) and 5 *Otx1⁻/⁻* mice (two rounds of 1 hour recordings). These electroencephalograms showed that only the *Otx1⁻/⁻* mice exhibited prolonged seizures in hippocampus and cortex (Fig. 2A). In *otd¹/otd¹* mice, normal electric activity was always recorded (Fig. 2B,C).

To determine whether the *otd* gene also restored the morphological abnormalities in the dorsal telencephalon caused by the absence of *Otx1* (Acampora et al., 1996), anatomical and histological analyses of brains from 1- to 2-month-old mice were carried out. The genotypes studied were *otd¹/otd¹* (*n*=12), *otd¹/Otx1* (*n*=8, data not shown), *Otx1⁻/⁻* (*n*=8), and wild type (*n*=5).

Otx1⁻/⁻ brains were reduced in size and weight by about 25% compared to wild type (Fig. 3A,B). Histological sections showed an overall reduction of the dorsal telencephalic cortex

that was most evident in the temporal and perirhinal areas (Figs 3G,H, 4A-C). There, in addition to the marked reduction in thickness and cell number (up to 40-50%), a disorganization of cortical cell layers (Fig. 4A,B) and a barely visible sulcus rhinalis (Fig. 3H) were observed. Furthermore, when compared to wild type (Fig. 3G), the relative extent of the cortical regions located dorsal (dashed line in Fig. 3H) versus ventral (filled line in Fig. 3H) to the presumptive sulcus rhinalis (arrowhead in Fig. 3H) was abnormal in the $Otx1^{-/-}$ brain and a clear

reduction of the dorsal region was seen in the mutant (Fig. 3H). Additionally, in the $Otx1^{-/-}$ brain, the hippocampus was contracted (Fig. 3H), the superior and inferior colliculi of the mesencephalon were increased in volume (Fig. 4E) and, in approximately 70% of the cases, the cerebellum showed an additional lobule (Acampora et al., 1996).

The brains of otd^1/otd^1 mice (Fig. 3C,F,I) showed an increased size and weight compared to $Otx1^{-/-}$ brains (Fig. 3B,E,H) and were generally very similar to the wild type (Fig. 3A,D,G). Only a small percentage (~17%; n=2) of the otd^1/otd^1 brains were slightly reduced in size and weight by about 10% as compared to the wild type (data not shown). As compared to $Otx1^{-/-}$ brains, histological analysis revealed a marked increase in cell number and thickness of the telencephalic cortex in all otd^1/otd^1 brains (compare Figs 3F,I to E,H and 4A,B) whether fully recovered or slightly reduced in size (Fig. 4A,B). Indeed, the number of cell bodies detected in all dorsal telencephalic areas of otd^1/otd^1 brains was comparable to that seen in wild type (Fig. 4C). The increase in cell number in otd^1/otd^1 brains, as compared to $Otx1^{-/-}$ mutants, was particularly evident for the temporal and perirhinal areas (Fig. 4A-C). In these cortical areas, the histological disorganization seen in $Otx1^{-/-}$ brains was also recovered in otd^1/otd^1 brains and a normal hexalaminar organization resulted (Fig. 4A,B). Additional telencephalic abnormalities related to the sulcus rhinalis and hippocampus were also recovered (compare Fig. 3H to I).

In contrast to the recovery of telencephalic structures, the mesencephalic defects were only partially rescued in otd^1/otd^1 brains (n=22). Anatomical analysis showed that approximately 30% (n=7) of the superior and inferior colliculi in otd^1/otd^1 mice were like those of $Otx1^{-/-}$ mice (data not shown), approximately 15% (n=3) had a normal size (data not shown), and about one half (n=11) were intermediate in size (Fig. 4D-F). The abnormal cerebellar foliation seen in the majority of $Otx1^{-/-}$ mice was also not noticeably rescued in otd^1/otd^1 mice (~75% for $Otx1^{-/-}$ and ~65% for otd^1/otd^1 cerebella) (data not shown).

The *Drosophila otd* gene rescues the reduction of proliferation in the $Otx1^{-/-}$ telencephalic neuroepithelium

Failure of the dorsal cortex to grow normally in $Otx1^{-/-}$ mice could be due to developmental defects such as a reduction in proliferation or an increase in cell death in the developing telencephalon. To investigate this and identify the developmental process recovered in the otd^1/otd^1 adult cortex, we studied cell proliferation and apoptotic cell death in the embryonic telencephalon of wild-type, $Otx1^{-/-}$ and otd^1/otd^1 embryos at 9.75, 13.5 and 15.5 dpc.

Apoptotic cell death was studied by the TUNEL method (Gavrieli et al., 1992). No significant differences were observed in the dorsal and ventral telencephalon among the three genotypes at 9.75 dpc or at later stages. In the three genotypes, only sporadic apoptotic cells were identified at 9.75 dpc throughout the dorsal telencephalic neuroepithelium (data not shown).

Cell proliferation was investigated by a short pulse of bromodeoxyuridine (BrdU) incorporation and subsequent quantification of BrdU-positive cells in the dorsal and ventral telencephalic neuroepithelium. At 9.75 dpc, the percentage of
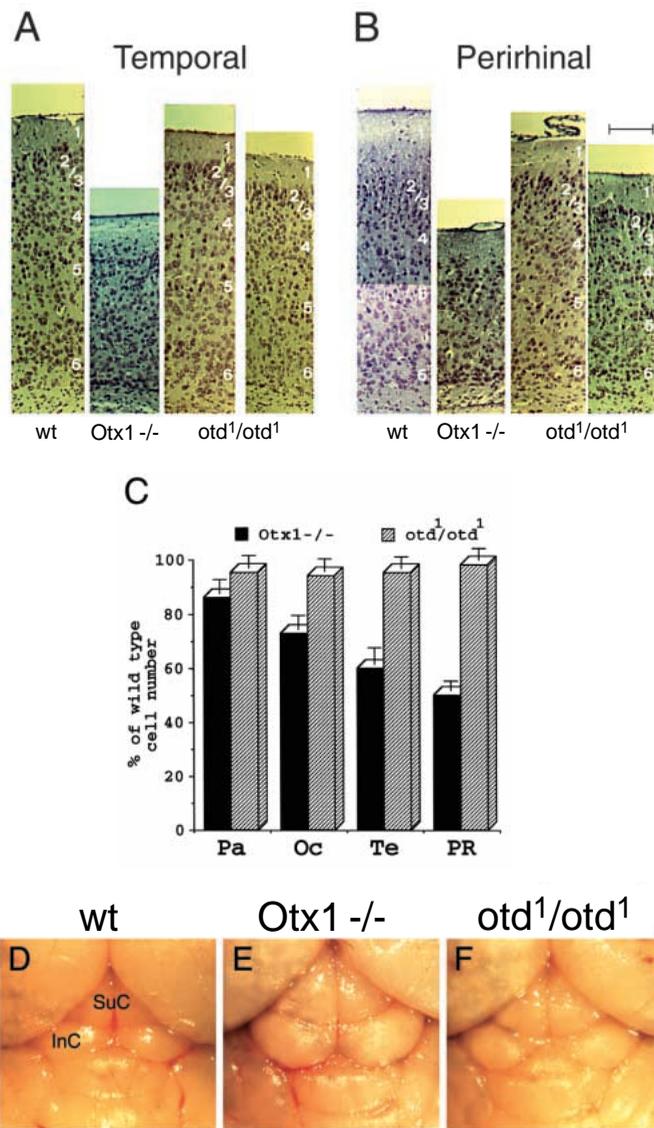

Fig. 4. Histology and cell number of cortex and morphology of mesencephalon of wild-type, $Otx1^{-/-}$ and otd^1/otd^1 brains. (A,B) Comparison of wild-type, $Otx1^{-/-}$ and two otd^1/otd^1 temporal (A) and perirhinal (B) cortices shows the recovery of all the otd^1/otd^1 brains either those with a normal size or those with a slight reduction. Scale bar, 100 µm. (C) $Otx1^{-/-}$ and otd^1/otd^1 cell number in parietal (Pa), occipital (Oc), temporal (Te) and perirhinal (PR) areas. Cell number throughout cortex is reported as percentage of wild type. Percentages are mean ± s.e.m. (D-F) Dorsal view of mesencephalon showing intermediate size of superior (SuC) and inferior (InC) colliculi of otd^1/otd^1 (F) mice as compared to wild type (D) and $Otx1^{-/-}$ (E).

proliferating cells in the dorsal telencephalic neuroepithelium of $Otx1^{-/-}$ embryos was reduced by approximately 25% as compared to otd^1/otd^1 and wild-type embryos (Fig. 5A-D). Proliferation was similar in the ventral telencephalic neuroepithelium in all cases. In $Otx1^{-/-}$ mice, proliferative activity in the dorsal telencephalon was only slightly affected (~10%) at 13.5 dpc (Fig. 5E-H) and 15.5 dpc (Fig. 5I-L). Therefore, proliferation in otd^1/otd^1 and wild-type embryos was similar at all stages.

Normally the telencephalon expands dramatically between 9.5 and 12.5 dpc to generate the dorsal telencephalic vesicle and the ganglionic eminence. A reduction in proliferation at the early stage should, therefore, affect the size of the telencephalon. Accordingly, $Otx1^{-/-}$ embryos showed a strong reduction of the telencephalon that was already evident by 13.5 dpc (Fig. 5F).

Thus, although the number of $Otx1^{-/-}$ proliferating cells/unit of neuroepithelium at 13.5 dpc (Fig. 5F,H) was similar to that of wild-type (Fig. 5E,H) and otd^1/otd^1 (Fig. 5G,H) brains, the total cell number in the $Otx1^{-/-}$ developing telecephalon was already strongly reduced. We conclude that an early embryonic defect in the rate of proliferation and/or in the number of neuronal progenitor cells is likely to contribute to the reduction in cell number and size of the $Otx1^{-/-}$ adult cortex, and that this impairment is rescued in otd^1/otd^1 brains.

Sense organ defects in otd^1/otd^1 mice

Several defects associated with sensory organs of the head are seen in $Otx1^{-/-}$ mice. The thickness of the iris is reduced, the ciliary process in the eye, the lachrymal and Harderian glands and the lateral semicircular duct of the inner ear are absent (Acampora et al., 1996).

A comparison of the affected structures in wild-type (Fig. 6A,D,G), $Otx1^{-/-}$ (Fig. 6B,E,H) and otd^1/otd^1 (Fig. 6C,F,I) mice is shown. In otd^1/otd^1 mice, a thickened iris and a (slightly reduced) ciliary process were present in 80% of the eyes ($n=50$) (Fig. 6I). Moreover, lachrymal and Harderian glands were found in approximately one third of the cases (34%; $n=45$) (Fig. 6F). In contrast, the lateral semicircular duct of the inner ear was never restored in otd^1/otd^1 mice (Fig. 6C).

The fact that the *Drosophila otd* gene cannot restore the missing lateral semicircular duct in *Otx1*-deficient mice is noteworthy. Since the lateral semicircular duct is only found in the

inner ear of gnathostomes (Kelly, 1985; Haddon and Lewis, 1991), this suggests that the ability to specify this structure might be an *Otx1*-specific function acquired for a specialized role in higher vertebrates.

Drosophila OTD and murine OTX2 gene products can cooperate in brain patterning

Recent findings show that *Otx1* and *Otx2* genes can cooperate in brain morphogenesis and that a minimal level of OTX proteins is required for proper regionalization of the developing brain (Acampora et al., 1997). Thus, while the $Otx1^{+/-}; Otx2^{+/-}$ brain is normal (Fig. 7A,E,I), the $Otx1^{-/-}; Otx2^{+/-}$ newborn brain lacks the Ammon's horn, dorsal thalamus, pretectum and mesencephalon, which is substituted by an enlarged metencephalon (cerebellum and pons) (Fig. 7B,F,J) (Acampora
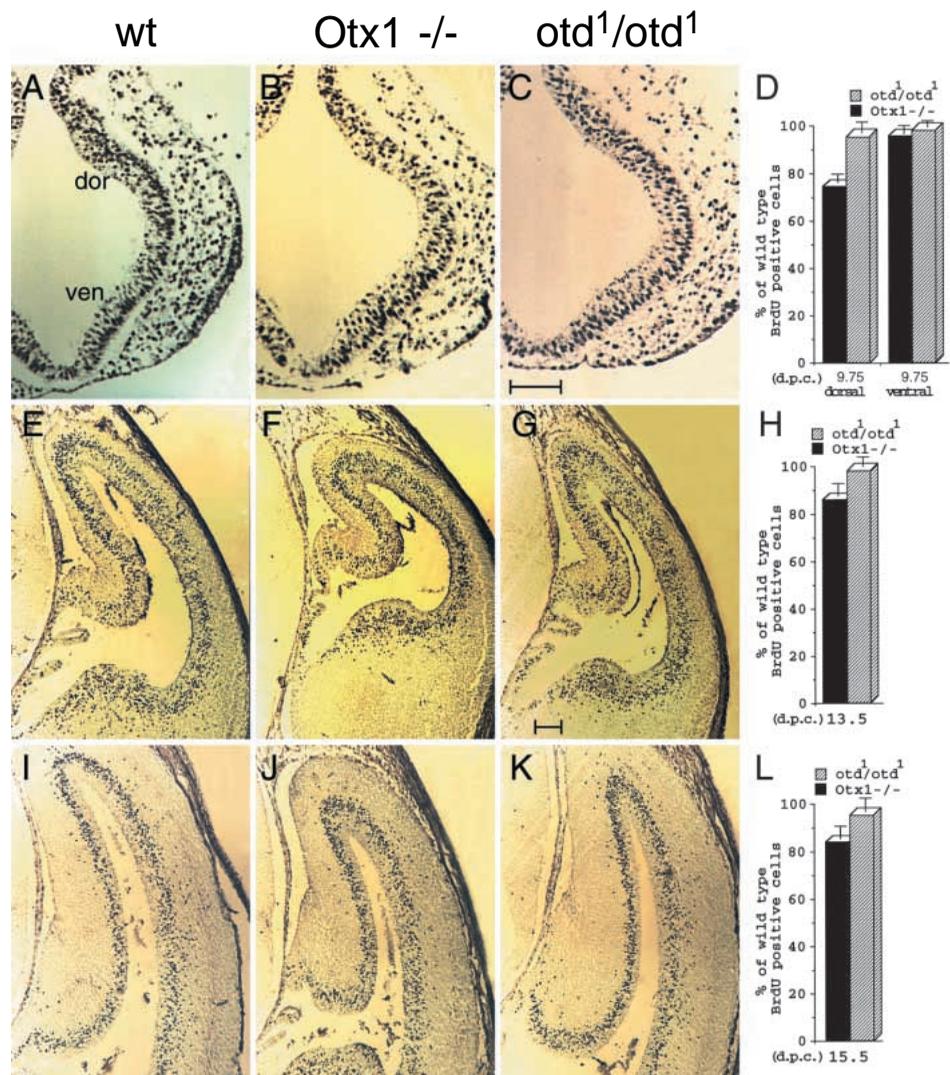

Fig. 5. Proliferation in telencephalon of wild-type, $Otx1^{-/-}$ and otd^1/otd^1 embryos. (A-D) The percentage of BrdU-positive cells in wt (A,D) and otd^1/otd^1 (C,D) do not show significant differences at 9.75 d.p.c. while a remarkable decrease is detected in the dorsal telencephalon of $Otx1^{-/-}$ embryos (B,D). (E-L) At 13.5 d.p.c. (E-H) and 15.5 d.p.c. (I-L) the BrdU-positive cells detected per unit of neuroepithelium (see Materials and Methods) of wild type (E,H,I,L), $Otx1^{-/-}$ (F,H,J,L) and otd^1/otd^1 (G,H,K,L) are similar, but the size of $Otx1^{-/-}$ telencephalon is heavily reduced. The number of BrdU-positive cells is reported as percentage of wild type. Percentages are mean ± s.e.m. Scale bar, 100 μm.
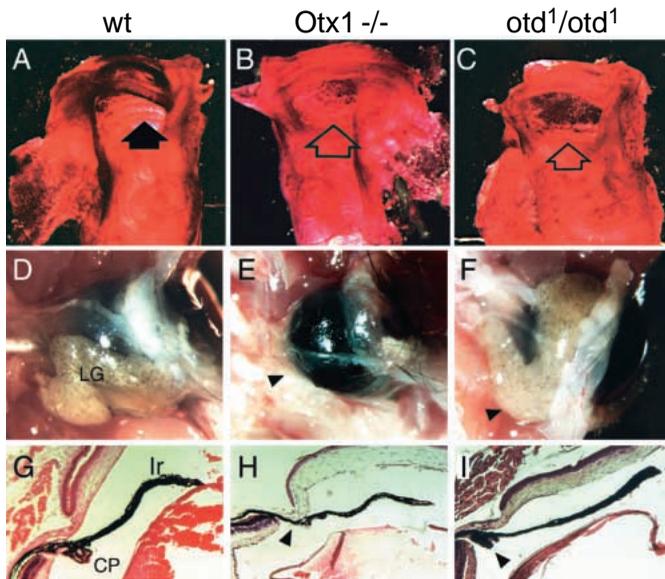
wt   Otx1 -/-   otd¹/otd¹

Fig. 6. Morphology and histology of lachrymal and Harderian gland complex, eye and inner ear. As compared to wild type (wt) (A,D,G), *Otx1⁻/⁻* mice lack the lateral semicircular duct of the inner ear (open arrow in B), the lachrymal and Harderian gland (LG in D) complex (arrowhead in E) and the ciliary process (CP in G) (arrowhead in H), while the iris (Ir) is thinner (H); in *otd¹/otd¹* mice, the lateral semicircular duct is never recovered (open arrow in C), the lachrymal and Harderian gland complex (arrowhead in F) is rescued in approximately one third of the eyes scored, the ciliary process is present (arrowhead in I) and the iris is thicker (I).

et al., 1997). Therefore, we performed genetic experiments aimed to gain insight into a possible *otd*-mediated rescue of these macroscopic defects.

otd¹/–; Otx2⁺/⁻ mice (see Materials and Methods) died at birth and had abnormal brain morphology. An anatomical analysis of the embryonic brain of these mice was carried out at 19 dpc. In all the *otd¹/–; Otx2⁺/⁻* brains (*n*=5) the Ammon's horn and the area corresponding to the dorsal thalamus and pretectum were restored (Fig. 7G,K). A presumptive mesencephalic area and a presumptive cerebellum were also always present (Fig. 7C,G). Mesencephalic morphology was, however, severely perturbed due to a marked reduction in thickness of the alar region; cerebellar morphology was less severely affected (Fig. 7G). In the wild type, *Gbx2,* is regionally expressed in the dorsal thalamus (Bulfone et al., 1993). Correct expression of *Gbx2* in the dorsal thalamus was observed in *Otx1⁺/⁻; Otx2⁺/⁻* and *otd¹/–; Otx2⁺/⁻* brains (Fig. 7M,O), but not in *Otx1⁻/⁻; Otx2⁺/⁻* brains (Fig. 7N), suggesting that the presumptive dorsal thalamus had indeed acquired its correct regional identity in *otd¹/–; Otx2⁺/⁻* brains.

The abnormalities of *Otx1⁻/⁻; Otx2⁺/⁻* embryos were likely caused by a reduced level of OTX gene products. Moreover, the amount of the OTD protein in *otd¹/Otx1* embryos was estimated to be ~30% lower than that of OTX1 (Fig. 1F). It, therefore, seemed likely that a more complete rescue of brain morphology might be possible if the level of OTD protein were higher than in *otd¹/–; Otx2⁺/⁻* embryos. To investigate this, *otd¹/otd¹; Otx2⁺/⁻* mice were generated. Compared to *otd¹/–; Otx2⁺/⁻* brains (Fig. 7C,G,K,O), the embryonic brains of these

animals (Fig. 7D,H,L,P) did show significant improvement in the morphology of dorsal thalamus, pretectal area and anterior mesencephalon (Fig. 7H,L,P). The posterior mesencephalon remained severely perturbed (Fig. 7H).

We reported that, in 10.5 dpc *Otx1⁻/⁻; Otx2⁺/⁻* embryos, the isthmus and the expression pattern of genes controlling the development of mes-metencephalic regions were coordinately shifted forwards in the area corresponding to the caudal diencephalon (Acampora et al., 1997) (Fig. 8F,J,N,R,V).

As compared to wild type (Fig. 8A) and *Otx1⁻/⁻; Otx2⁺/⁻* (Fig 8B), the morphology of *otd¹/–; Otx2⁺/⁻* (Fig. 8C) and *otd¹/otd¹; Otx2⁺/⁻* (Fig. 8D) embryos was improved mainly in the telencephalic and diencephalic territories, while the presumptive isthmic constriction was shifted forwards only slightly as compared to *Otx1⁻/⁻; Otx2⁺/⁻* embryos.

It is worth to note that this anterior morphological displacement became less evident as the *otd* copy number increased (compare arrow in Fig. 8C,D to A,B and G′,H′ to I′,F′). To correlate morphological changes of *otd¹/–; Otx2⁺/⁻* and *otd¹/otd¹; Otx2⁺/⁻* embryos to molecular events, the expression patterns of *Otx2, Fgf-8, Wnt-1, En-2* and *Gbx2* were determined (McMahon et al., 1992; Crossley and Martin, 1995; Joyner, 1996; Acampora et al., 1997; Wassarman et al., 1997).

Their expression was coordinately displaced forwards either in *otd¹/–; Otx2⁺/⁻* (Fig. 8G,K,O,S,W) or in *otd¹/otd¹; Otx2⁺/⁻* (Fig. 8H,L,P,T,X) embryos.

Nevertheless, this anterior shift was less severe than that observed in *Otx1⁻/⁻; Otx2⁺/⁻* (Fig. 8F,J,N,R,V). In particular, comparing the boundary between mesencephalic *(Wnt-1, Otx2)* and metencephalic *(Fgf-8, Gbx2)* markers (compare arrow in Fig. 8 from E to H), we found that it resulted more rostrally displaced in the sequence *Otx1⁻/⁻; Otx2⁺/⁻ > otd¹/–; Otx2⁺/⁻ > otd¹/otd¹; Otx2⁺/⁻ >* wild type.

It is noteworthy that the anterior border of the broad *Fgf-8* expression domain in *otd¹/–; Otx2⁺/⁻* and *otd¹/otd¹; Otx2⁺/⁻* embryos did not coincide with the morphological position of the presumptive isthmic constriction (compare arrow in Fig. 8G,H to G′,H′), suggesting that the OTD/OTX2 protein level reaches the threshold sufficient to antagonize the repatterning of mesencephalon in metencephalon but is not sufficient to position *Fgf-8* expression correctly. This molecular abnormality may contribute to the abnormal mesencephalic development.

At 12.5 d.p.c. in *otd¹/–; Otx2⁺/⁻* (Fig. 9B,E,H) and *otd¹/otd¹; Otx2⁺/⁻* (Fig. 9C,F,I) embryos, the expression patterns of *Fgf-8, Otx2* and *En-2* were stably retained in a more posterior position as compared to their more rostral location in *Otx1⁻/⁻; Otx2⁺/⁻* (Fig. 9A,D,G) embryos and only in *otd¹/–; Otx2⁺/⁻* embryos was the *Fgf-8* still distributed in a broader domain (Fig. 9B). Moreover, the expression of *Wnt-1* in the telencephalic commissural plate and of *En-2* throughout the neuroepithelium in *Otx1⁻/⁻; Otx2⁺/⁻* embryos at 12.5 d.p.c. was no longer seen (for *Wnt-1*) (data not shown) or markedly reduced (for *En-2*) in *otd¹/–; Otx2⁺/⁻* and *otd¹/otd¹; Otx2⁺/⁻* embryos (Fig. 9H,I), indicating that *otd* can successfully contribute to confer the territorial identity to the telencephalon.

Taken together these results show that the *Drosophila otd* gene can rescue the anatomical and molecular *Otx1⁻/⁻; Otx2⁺/⁻* brain phenotypes in a dose-dependent manner. The efficiency of rescue is high for the telencephalic structures, intermediate for the dorsal thalamus and pretectum, and relatively poor for the mesencephalon. This suggests a differential requirement of
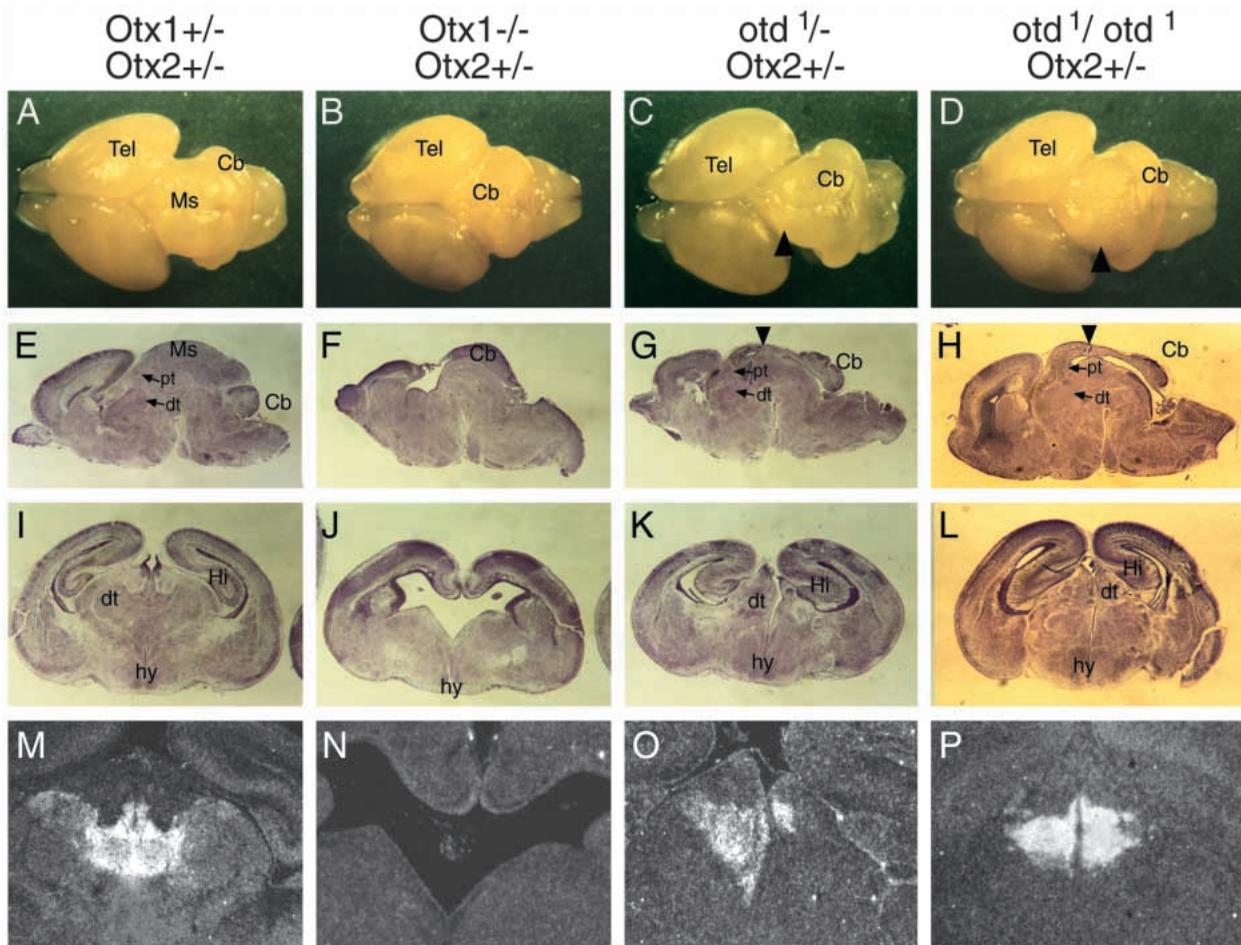
Fig. 7. Comparison among $Otx1^{+/-}$; $Otx2^{+/-}$, $Otx1^{-/-}$; $Otx2^{+/-}$, $otd^1/-$; $Otx2^{+/-}$ and otd^1/otd^1; $Otx2^{+/-}$ brains at 19 d.p.c. (A-L) Dorsal view (A-D), sagittal (E-H) and frontal (I-L) sections of $Otx1^{+/-}$; $Otx2^{+/-}$ (A,E,I), $Otx1^{-/-}$; $Otx2^{+/-}$ (B,F,J), $otd^1/-$; $Otx2^{+/-}$ (C,G,K) and otd^1/otd^1; $Otx2^{+/-}$ (D,H,L) showing that as compared to $Otx1^{-/-}$; $Otx2^{+/-}$ brain, in $otd^1/-$; $Otx2^{+/-}$ and otd^1/otd^1; $Otx2^{+/-}$ brains, the Ammon's horn, dorsal thalamus and pretectum are identified (G,K,H,L) while the territory corresponding to the presumptive mesencephalon begins to be defined (arrowhead in C,G,D,H) and the cerebellum confined posteriorly close to its natural position (C,G,D,H). Note that $otd^1/-$; $Otx2^{+/-}$ brains show abnormal cytoarchitecture and reduction in thickness of presumptive dorsal mesencephalic area (C,G) and abnormal histology of pretectum and dorsal thalamus (G,K) while in otd^1/otd^1; $Otx2^{+/-}$ brains, recovery of dorsal thalamus, pretectum (H,L) and mesencephalon (D,H) is even more improved but abnormalities are still retained in thickness of posterior mesencephalon. (M-P) $Gbx2$ expression in $Otx1^{+/-}$; $Otx2^{+/-}$ (M), $Otx1^{-/-}$; $Otx2^{+/-}$ (N), $otd^1/-$; $Otx2^{+/-}$ (O) and otd^1/otd^1; $Otx2^{+/-}$ (P) brains. Abbreviations as in previous figures plus: dt, dorsal thalamus; pt, pretectum; hy, hypothalamus.

OTX protein levels in specifying regional identities along the anteroposterior brain axis, which appear to be low in the telencephalon, intermediate in the posterior diencephalon and high in the posterior mesencephalon.

DISCUSSION

Evolutionary conservation of developmental control genes

Several examples for the evolutionary conservation of the regulatory genes that control vertebrate development are now known. For the *HOM/HOX* genes, this evolutionary conservation is manifest in genomic organization, expression and functional features (reviewed in Krumlauf, 1994). Sequence analysis indicates that the *HOM/HOX* clusters arose by duplication from a common ancestral cluster. The physical

order of the HOM/HOX genes along the chromosome is colinear with their expression along the anteroposterior axis of the embryo (Lewis, 1978; Duboule and Dollé, 1989; Graham et al., 1989, van der Hoeven et al., 1996). Transgenic mouse mutants exhibit homeotic transformation due to loss or gain of function and, finally, conservation of transcriptional regulatory mechanisms and common functional properties between corresponding *HOM/HOX* genes have been demonstrated in *Drosophila* and mouse (reviewed in Krumlauf, 1994; Bachiller et al., 1994; Pöpperl et al., 1995). For the *ey/Pax6* genes, evolutionary conservation is seen in their impressive homology and striking functional equivalence; vertebrate and invertebrate *ey/Pax6* genes can activate eye developmental program in *Drosophila* cells normally fated to give non-eye structures (reviewed in Callaerts et al., 1997).

Based on sequence homology, candidate genes likely to have

a conserved functional role in brain morphogenesis have been isolated. This assumption comes from striking similarities in their expression patterns and mutant phenotypes in *Drosophila* and in mouse (Finkelstein and Boncinelli, 1994; Rubenstein et al., 1994; Acampora et al., 1995, 1996, 1997; Hirth et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Joyner, 1996). For example, the *wg* and *en* genes are implicated in boundary formation between adjacent brain neuromeres in *Drosophila* and, similarly, *Wnt-1* and *En* genes control the mes-metencephalic region in mammals (reviewed in Thor, 1995; Joyner, 1996). In *Drosophila*, *otd* and *ems* genes are involved in the establishment of different head segments as well as in the specification of the proto-, deuto- and tritocerebral brain neuromeres, and in the development of visual and mechanosensory structures (Finkelstein et al., 1990b; Schmidt-Ott et al., 1994; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In mouse, *Otx* genes are required in early specification of the neuroectoderm fated to become forebrain, midbrain and rostralmost hindbrain *(Otx2),* in regionalization and patterning of the brain *(Otx1* and *Otx2),* in corticogenesis and proliferation of early telencephalic neuroblasts *(Otx1),* and in development of visual and acoustic sense organs *(Otx1)* (Acampora et al., 1995, 1996, 1997; Matsuo et al., 1995; Ang et al., 1996).

Nevertheless, several aspects are still unclear. For example, although vertebrate and *Drosophila* genes share some structural homology, these homologies are in general confined to specific, highly conserved domains such as the homeodomain. The finding that homeodomains of a specific type such as the *otd* type are highly conserved might imply that they are crucial in selecting, at a very high stringency, the same target sequence(s). In this connection, expression data as well as mutant phenotypes in *Drosophila* and mouse, support the possibility that they control genetic hierarchies sharing, at least in part, common functional features. In contrast, the role of coding sequences outside these conserved domains is only poorly understood and it is important to determine whether these regions code for new functions, whether they are evolved versions of an old function, or whether they represent a combination of old and new functions.

Otx genes and the evolution of vertebrate brain morphology

The basic organization of both vertebrate

and invertebrate brains is the subject of much debate. The evolutionary and anatomical correspondence among *otd/Otx*-expressing brain territories in mouse and *Drosophila* has important implications for this issue (Cohen and Jürgens, 1991;
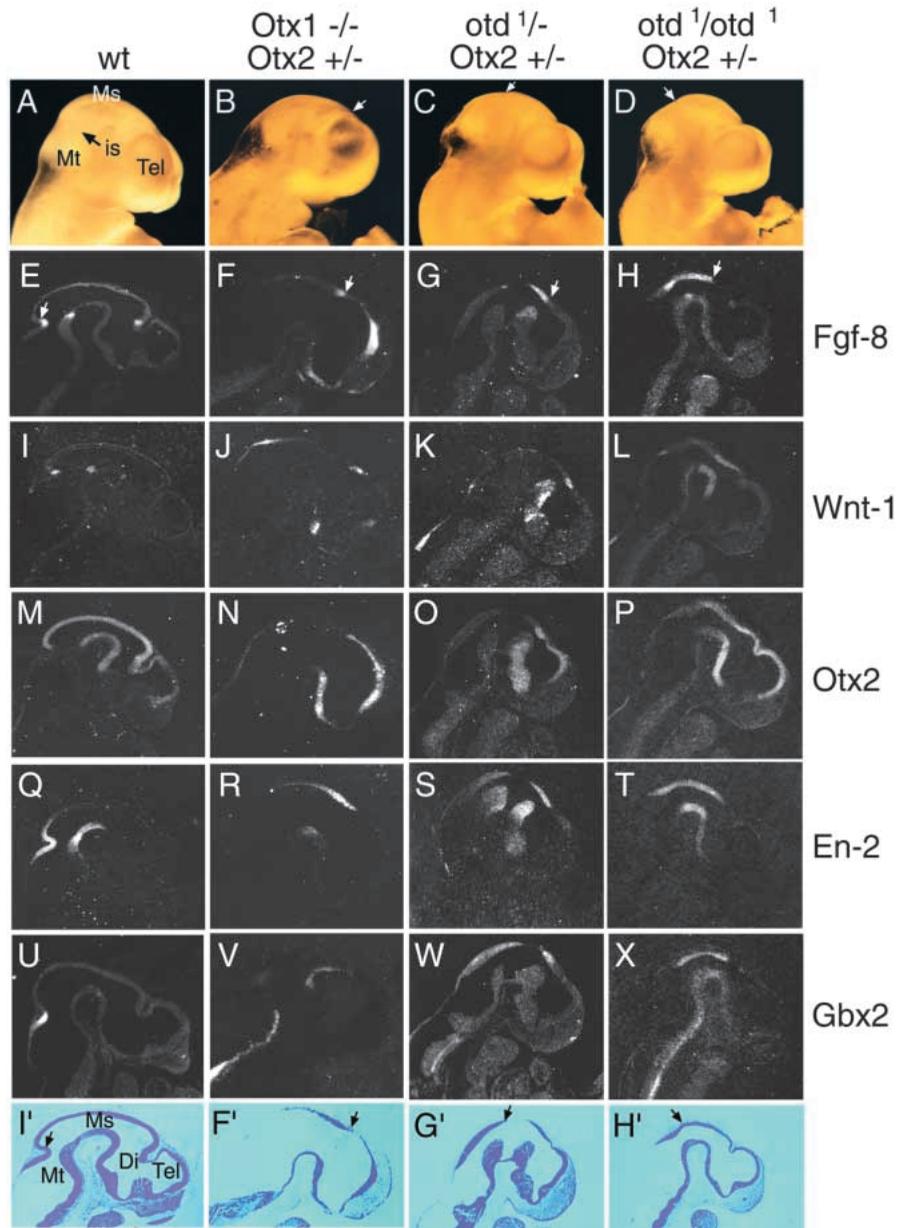

Fig. 8. Head morphology and expression patterns of *Fgf-8, Wnt-1, Otx2, En-2* and *Gbx2* at 10.5 d.p.c. (A-D) Comparing wild type (A), *Otx1^{−/−}; Otx2^{+/−}* (B), *otd^{1}/−; Otx2^{+/−}* (C) and *otd^{1}/otd^{1}; Otx2^{+/−}* (D) head morphology, the abnormalities detected along the brain of *Otx1^{−/−}; Otx2^{+/−}* (B) embryos are gradually recovered in *otd^{1}/−; Otx2^{+/−}* (C) and *otd^{1}/otd^{1}; Otx2^{+/−}* (D) embryos but a wild-type (A) phenotype is never restored. (E-X) *Fgf-8* (E-H), *Wnt-1* (I-L), *Otx2* (M-P), *En-2* (Q-T) and *Gbx2* (U-X) expression patterns in wild-type (E,I,M,Q,U), *Otx1^{−/−}; Otx2^{+/−}* (F,J,N,R,V), *otd^{1}/−; Otx2^{+/−}* (G,K,O,S,W), and *otd^{1}/otd^{1}; Otx2^{+/−}* (H,L,P,T,X) embryos showing that, according to morphology, their expression patterns become gradually more similar to wild type as the *otd* copy number increases. Note the boundary between mesencephalic and metencephalic markers (arrows in E-H), and the morphological position of the presumptive isthmic constriction (arrows in A-D and I′,F′,G′,H′). F′-I′ are bright-field views of F-I. The *Gbx2* expression in (U,V) is on embryos different from those of the same genotype reported for the other genes. Abbreviations as in previous figures plus: is, isthmus.

Finkelstein and Boncinelli 1994; Thor, 1995; Reichert and Boyan, 1977).

In the mouse, the *Otx2* gene is directly involved in specifying the rostral CNS anterior to rhombomere 2 (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). The *Otx1* gene is involved in cortical neurogenesis and in sense organ development, and may also cooperate with *Otx2* in positioning of the isthmic organizer (Acampora et al., 1997). The *Drosophila otd* gene is able to replace the mouse *Otx1* gene and fully rescue corticogenesis and epilepsy and also partially rescue eye defects and other brain patterning abnormalities of *Otx1*$^{-/-}$ mice. In contrast, the inner ear defect of *Otx1*$^{-/-}$ mice is never recovered by the *otd* gene. It is noteworthy that the affected inner ear structure first appeared during evolution in gnathostomes and was absent in agnatha (Kuhlenbeck, 1973; Kelly, 1985; Haddon and Lewis, 1991). The ability to specify this structure may, therefore, represent an *Otx1*-specific function that evolved for a specific role in gnathostomes. Similar considerations may also hold for the partial rescue observed for brain patterning defects in *otd*^{1}/$-$; *Otx2*$^{+/-}$ and *otd*^{1}/*otd*^{1}; *Otx2*$^{+/-}$ embryos. However, the gene dosage effect previously demonstrated for the *Otx* genes may also operate here and might be responsible for the failure of a complete rescue even in *otd*^{1}/*otd*^{1}; *Otx2*$^{+/-}$ animals.

The rostral architectural components of the vertebrate brain, the telencephalon, diencephalon and mesencephalon, are clearly recognizable in gnathostomes; their existence is less clear in agnatha (Kuhlenbeck, 1973). Comparative analyses show that *Otx* gene expression is always associated with the rostralmost CNS independently from the morphological complexity acquired by this area during evolution. During the phylogenetic development of the gnathostome-type brain, the architecture of this rostral *Otx*-expressing region of the CNS might have been greatly modified on the basis of new genetic instruction(s). A posterior displacement of the mesencephalic-metencephalic boundary as well as differential proliferative properties of the rostral neuroectoderm (forebrain, midbrain) versus the more posterior neuroectoderm (hindbrain and spinal cord) might have contributed to this gnathostome-type brain respecification. In this context, it is noteworthy that unambiguous *Otx1*-related genes have been identified only in gnathostomes, that an anterior displacement of the mesencephalic-metencephalic boundary is seen in *Otx2* heterozygous mice lacking *Otx1* and that *Otx1*$^{-/-}$ mice have reduced proliferative activity in the rostral neuroepithelium.

Conservation and diversity of *otd/Otx* gene action

The homeodomain is the only highly conserved structural feature between *Drosophila otd* and murine *Otx* genes even though it is worth noting that, in *Tribolium*, two *otd*-related

genes, namely *Tc otd-1* and *Tc otd-2*, have been isolated and that, surprisingly, while *Tc otd-1* is more related to *Drosophila otd, Tc otd-2* is more related to murine *Otx* genes and expressed only in a subset of cells in the anterior brain (Li et al., 1996).

This finding emphasizes the rescue observed with the *Drosophila otd* gene and suggests that the homeodomain-mediated ability to recognize the same target sequence(s) might have been retained in evolution. Nevertheless, the shared *otd/Otx1* homeodomain is probably not sufficient to mediate all of the actions of *Otx1*. Our findings suggest the existence of *otd/Otx1*-common (e.g. corticogenesis and sense organ defects) and *Otx1*-specific (e.g. inner ear defect) functional features that are unlikely to be defined by the homeodomain alone.

The requirement of *Otx2* for the specification of rostral CNS in forebrain, midbrain and rostral hindbrain probably represents one of the most important functions of *Otx* genes. It has been postulated that the origin of the vertebrate head was associated with a shift from a passive to an active mode of predation and that this was acquired quite recently by a modification of preexisting embryonic tissues in protochordates (Gans and Northcutt, 1983). It will be
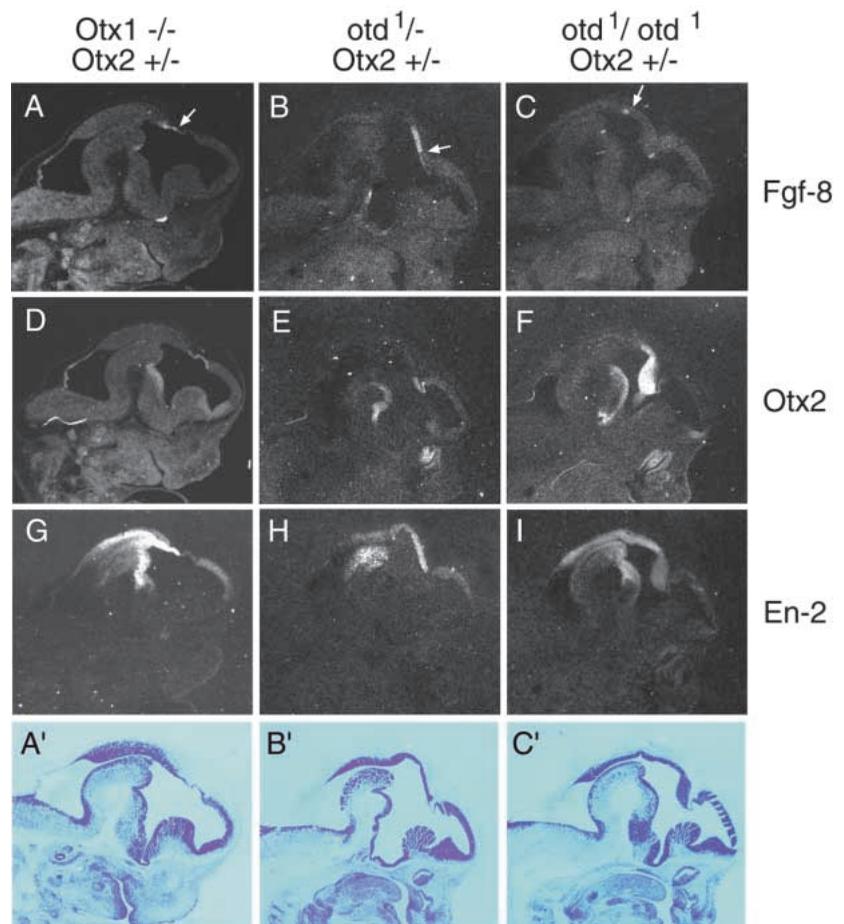

Fig. 9. *Fgf-8* (A-C), *Otx2* (D-F), *En-2* (G-I) expression patterns in 12.5 d.p.c. *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ (A,D,G), *otd*^{1}/$-$; *Otx2*$^{+/-}$ (B,E,H) and *otd*^{1}/*otd*^{1}; *Otx2*$^{+/-}$ (C,F,I) embryos showing the relative position of mes-metencephalic markers. Note that telencephalic expression of *En-2* is heavily reduced in *otd*^{1}/$-$; *Otx2*$^{+/-}$ (H) and almost undetectable in *otd*^{1}/*otd*^{1}; *Otx2*$^{+/-}$ (I) embryos. The arrows in A-C point to boundary between *Fgf-8* and *Otx2* expression. A′-C′ are bright-field views of A-C.

interesting to test whether *otd* can also rescue the *Otx2* phenotype. Preliminary results from *Otx2* replacement with *Otx1*, which is much more homologous to *Otx2* than *otd*, indicate that gastrulation impairments but not head specification due to the absence of *Otx2* are rescued by *Otx1*. It is conceivable, that the *otd/Otx1* common functional features arose independently in the two phyla. However, in view of the multitude of *Otx1* roles in corticogenesis, sense organ development and early brain patterning that are rescued by the *Drosophila otd* gene and vice versa (Leuzinger et al., 1998), it is unlikely that they have been adopted independently in the two phyla. Therefore, it can be speculated that during evolution the functional property of the *otd* homeodomain has been retained together with the ability of sequences outside the homeodomain to activate and/or repress target genes. Furthermore, this general ability might be inseparable from the specificity in interacting with additional transcription factors. In this basal scenario, new function(s) possibly corresponding to new domain(s) might be evolved and positively selected.

However to investigate this, further comparative analyses and genetic manipulations involving the replacement of murine *Otx* genes with those from urochordate, cephalochordate, cyclostome and cartilaginous fishes as well as replacement of sequences outside the homeodomain with unrelated transcription activating and/or repressing domains are needed.

Taken together, our data argue in favour of an extended evolutionary conservation between the murine *Otx1* and the *Drosophila otd* genes and support the idea that conserved genetic functions required in mammalian brain development evolved in a primitive ancestor of flies and mice more than 500 million years ago (Wray et al., 1996). It will now be important to dissect and compare *Drosophila otd* and mouse *Otx* gene products as well as their regulatory cascades in order to identify those functional domains and genetic elements that were recruited in evolution to specify the greater complexity of the mammalian brain.

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