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Review

# Genetic and molecular roles of Otx homeodomain proteins in head development

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**Abstract**

Insights into the molecular mechanisms underlying neural development in vertebrates come from the cloning and the functional analysis of genes which are involved in the molecular pathways leading to neural induction, tissue specification and regionalisation of the brain. Among them, transcription factors belonging to the *orthodenticle* family (*Otx1*, *Otx2*) play an important role during early and later events required for proper brain development. To better understand their functions, several mouse mutants have been generated by homologous recombination. Their analysis clearly indicates that *Otx1* is involved in corticogenesis, sense organ development and pituitary functions, while *Otx2* is necessary earlier in development, for the correct anterior neural plate specification and organisation of the primitive streak. A molecular mechanism depending on a precise threshold of OTX proteins is necessary for the correct positioning of the isthmic region and for anterior brain patterning. Finally, vertebrate *Otx* genes share functional equivalence with the *Drosophila* homologue *otd*, indicating that the genetic mechanisms underlying pattern formation in insect and mammalian brain development are evolutionarily conserved. 

*Keywords:* Brain patterning; CNS; Gastrulation; Isthmus; Morphogenesis; Mouse; Neural induction; Organiser

## 1. Introduction

The vertebrate central nervous system (CNS) is a very complex structure derived from sequential molecular and morphogenetic events that pattern the epiblast first and neural plate later. When induced by an organiser (Spemann and Mangold, 1924), the most anterior ectoderm tissue responds to diffusible molecules undergoing morphogenetic changes and becomes subdivided into broad regions corresponding to the forebrain, midbrain and hindbrain (Gallera, 1971; Storey et al., 1992; Ruiz I Altaba, 1994; Shimamura and Rubenstein, 1997; Rubenstein and Beachy, 1998).

Anatomical and histological studies postulate the existence of genetic fate determinants which subdivide the large neural regions into smaller longitudinal and transverse domains (Vaage, 1969; Altman and Bayer, 1988; Figdor and Stern, 1993; Rubenstein et al., 1994). Some of the patterning events along the anterior–posterior (A/P) axis may require the presence of transverse rings of neuroepithelia that possess inductive and boundary properties (Rubenstein et al., 1998; Ruiz I Altaba, 1998).

In vertebrates, several genes controlling developmental programs underlying brain morphogenesis have been isolated and their role studied in detail. Most of them are the vertebrate homologues of *Drosophila* genes coding for signal molecules or transcription factors (Lemaire and Kodjabachian, 1996; Tam and Behringer,

Abbreviations: A/P, antero-posterior; αGSU, α-glycoprotein subunit; AVE, anterior visceral endoderm; AVP, arginine vasopressin; βFSH, follicle-stimulating hormone; βLH, luteinising hormone; CDGA, constitutional delay in growth and abdolescence; CNS, central nervous system; CP, cortical plate; CRH, corticotropin-releasing hormone; EEG, electroencephalographic recording; GH, growth hormone; GnRH, gonadotropin releasing hormone; GnRHR, gonadotropin releasing hormone receptor; GRH, growth hormone releasing hormone; GRHR, growth hormone releasing hormone receptor; IGF1, insulin growth factor 1; IZ, intermediate zone; ME, median eminence; mes–met, mesencephalic–metencephalic; OT, oxytocin; *otd*, *Drosophila orthodenticle*; SS, somatostatin; TRH, thyrotropin-releasing hormone; VE, visceral endoderm; VZ, ventricular zone; ZLI, zona limitans intrathalamica.

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1997; Rubenstein et al., 1998). Among these, the *orthodenticle* group is defined by the *Drosophila orthodenticle* (*otd*) and the vertebrate *Otx1*, *Otx2* and *Crx* genes which contain a bicoid-like homeodomain (Finkelstein and Boncinelli, 1994; Chen et al., 1997; Freud et al., 1997; Simeone, 1998). The *Drosophila otd* gene is expressed at the anterior pole of the blastoderm embryo and, later on, predominantly in the developing rostralmost brain neuromeres (Cohen and Jürgens, 1990, 1991; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Mutations in the *otd* gene cause the loss of anterior head segments where it is expressed, suggesting that it might act as a gap gene.

In the mouse, two cognates for *otd* (*Otx1* and *Otx2*) were identified by virtue of the high conservation of their homeobox sequences (Simeone et al., 1992, 1993). *Otx1* and *Otx2* are activated sequentially during embryonic development. *Otx2* is already transcribed before the onset of gastrulation in the epiblast and visceral endoderm, and at the end of gastrulation is expressed in the axial mesendoderm and rostral neural plate. *Otx1* expression is first detected at the 1–3 somite stage [8 days post-coitum (d.p.c.)] throughout the fore- and midbrain neuroepithelium (Simeone et al., 1993). During brain regionalisation, *Otx1* and *Otx2* are transcribed in largely overlapping expression domains with a posterior border coincident with the mesencephalic side of the isthmic constriction (Simeone et al., 1992, 1993; Millet et al., 1996; Acampora et al., 1997). Since 11 d.p.c., only *Otx1* is expressed along the dorsal telencephalon, and subsequently it is restricted to neuronal cells fated to form the deep layers of the adult cortex (Frantz et al., 1994). Both *Otx1* and *Otx2* are expressed in the olfactory, ocular and acoustic sense organs (Simeone et al., 1993).

Expression pattern analysis of *Otx* genes have suggested that these transcription factors might play an important role during brain morphogenesis in vertebrates. A systematic genetic approach using transgenic mice has contributed to elucidate some of their roles in the molecular mechanisms underlying the major events occurring during brain morphogenesis.

## 2. *Otx1* is required for corticogenesis, sense organ development and pituitary functions

During corticogenesis, postmitotic neurons migrate along radial glial cells (Rakic, 1972), through the overlying intermediate zone (IZ), to the cortical plate (CP), which will give rise to the hexalaminar adult cerebral cortex. The cortical layers are generated in an inside-out pattern, in which cells of the deepest layers (6 and 5) are born first in the ventricular zone (VZ), and those of the upper layers (4, 3 and 2) progressively later (Rakic, 1974).

During murine embryonic development, *Otx1* begins to be expressed at 8 d.p.c. in the anterior neuroectoderm, corresponding to the presumptive fore–midbrain territory. Later in gestation, when the generation of first postmitotic neurons starts in the dorsal telencephalon, high level transcription of *Otx1* occurs only in ventricular cells, which at these stages are precursors of deep layer neurons. By the time upper layer neurons are generated, *Otx1* expression decreases in the VZ and becomes progressively prominent in the cortical plate which consists of postmigratory neurons of layer 5 and 6. *Otx1* is absent in later differentiated neurons of upper layers 1–4 (Frantz et al., 1994). In the adult cortex its expression is specifically confined to neurons of layers 5 and 6 (Frantz et al., 1994).

Heterochronic transplantation experiments have demonstrated that during cortex development the broad differentiative potentials of the early neuronal progenitors (McConnell and Kaznowski, 1991) become progressively restricted over time (Frantz and McConnell, 1996). Thus, the progressive down-regulation of *Otx1* in the ventricular cells suggests that *Otx1* may confer deep-layer identity to young neurons.

Indeed, *Otx1* expression is heterogeneous across the regions of the adult cortex, suggesting that it might also be involved in the forming of the cortical areas. Its expression in layer 5 is more prominent in the posterior and lateral cortex but absent in the frontal, insular and orbital cortices, while *Otx1* expression in layer 6 is more uniform throughout the neocortex (Frantz et al., 1994).

*Otx1* is also expressed at early stages in precursor structures of sense organs corresponding to the olfactory placode, otic and optic vesicles (Simeone et al., 1993). Later on, *Otx1* is transcribed in the olfactory epithelium, sacculus, cochlea and semicircular canals of the inner ear as well as in the iris and ciliary process in the eye and in the lachrymal glands (Simeone et al., 1993). From the birthday onwards, *Otx1* is also transcribed at relatively low level in the anterior lobe of the pituitary gland (Acampora et al., 1998c).

*Otx1*−/− mice exhibited both spontaneous high speed turning behaviour and epileptic behaviour (Acampora et al., 1996). The latter consisted of the combination of: (1) focal seizures characterised by automatisms (head bobbing and teeth chattering) and electroencephalographic (EEG) recording of spikes in hippocampus; (2) generalised seizures characterised by convulsions and high voltage synchronised EEG activity in hippocampus and cortex. Occasionally, convulsions were followed by status epilepticus and exitus (Acampora et al., 1996, 1999b).

Adult brains were reduced in weight and size and histological analysis revealed that the dorsal telencephalic cortex was reduced in thickness, the sulcus rhinalis appeared dorsally displaced and the hippocampus was shrunken with a divaricated dentate gyrus (Table 1). The cortex was particularly affected at the level of the

Table 1
Major phenotypes observed in *Otx1*−/−, *hOtx2*$^1$/*hOtx2*$^1$ and *otd*$^1$/*otd*$^1$ mutant mice

| Major phenotypes | *Otx1*−/− | *hOtx2*$^1$/*hOtx2*$^1$ | *otd*$^1$/*otd*$^1$ |
|---|---|---|---|
| *Early dorsal telencephalon* | | | |
| Cell proliferation | reduced by 25% | normal | normal |
| *Cerebral cortex* | | | |
| Cell number | reduced | normal | normal |
| Layer organisation | abnormal | normal | normal |
| Temporal cortex | reduced by 40% | normal | normal |
| Perirhinal cortex | reduced by 40% | normal | normal |
| Hippocampus | shrunken | normal | normal |
| *Mesencephalon size* | enlarged | normal in 30%<br>intermediate in 45% | normal in 15%<br>intermediate in 50% |
| *Cerebellum* | abnormal foliation | normal in 50% | normal in 10% |
| *Ear* | | | |
| Lateral semicircular duct | absent | absent | absent |
| *Eye* | | | |
| Iris | reduced | normal in 70% | normal in 80% |
| Ciliary process | absent | normal in 70% | normal in 80% |
| Lachrymal and Harderian gland | absent | normal in 75% | normal in 34% |
| *Behaviour* | | | |
| Turning behaviour | high-speed | moderate-speed | moderate-speed |
| Epileptic seizures | present | absent | absent |

temporal and perirhinal areas, where a 40% reduction in cell number was detected. Furthermore, in these same areas, cortical organisation was lost and cortical layers were not identifiable (Table 1) (Acampora et al., 1996).

While no differences in apoptosis were observed between mutant and wild-type embryos, bromodeoxyuridine (BrdU) labelling experiments revealed a reduction of proliferating cells (by about 25%) in the dorsal telencephalic neuroepithelium of 9.75 d.p.c. *Otx1*−/− embryos. A defective proliferation of neuronal progenitors at these early stages may contribute to the adult phenotype of the *Otx1* mutant mice.

As regarding the inner ear abnormalities of *Otx1*−/− mutants, these are consistent with the expression pattern of *Otx1*. Indeed, *Otx1* is expressed in the lateral canal and ampulla, as well as part of the utricle and in the saccule and cochlea. Interestingly, *Otx2* is coexpressed with *Otx1* in the saccule and cochlea but not in the components of the pars superior. Lack of *Otx1* always results in the absence of the lateral semicircular canal, while defects in the lateral ampulla, utriculosaccular duct and cochleosaccular duct are less penetrant (Acampora et al., 1996; Morsli et al., 1999).

In the eye and annexed structures *Otx1* transcripts are restricted to the iris, ciliary process and ectodermal cells migrating from the eyelid and included in the mesenchymal component of the lachrymal gland. These ectodermal cells are believed to induce differentiation of mesenchymal cells into a glandular exocrine cell type. In *Otx1*−/− mice the ciliary process is absent, the iris is thinner and the lachrymal glands fail to develop. Interestingly, the ectodermal cells embedded within the mesenchymal components are not identified in *Otx1*−/− mice, thus indicating that failure in development of the lachrymal glands is a consequence of the impaired migration of the ectodermal cells from the eyelid to the mesenchymal primordium of the lachrymal gland, that in turn is not induced to differentiate into the exocrine glandular phenotype (Acampora et al., 1996).

Finally, as previously mentioned, *Otx1* is postnatally transcribed and translated in the pituitary gland. Cell culture experiments indicate that *Otx1* may activate transcription of the growth hormone (GH), follicle-stimulating hormone (βFSH), luteinising hormone (βLH), and α-glycoprotein subunit (αGSU) genes. Analysis of *Otx1* null mice indicates that, at the prepubescent stage, they exhibit transient dwarfism and hypogonadism due to low levels of pituitary GH, βFSH and βLH hormones which, in turn, dramatically affect downstream molecular and organ targets. Nevertheless, *Otx1*−/− mice gradually recover from most of these abnormalities, showing normal levels of pituitary hormones with restored growth and gonadal functions at 4 months of age. Expression patterns of the hypothalamic growth hormone-releasing hormone (GRH), gonadotropin-releasing hormone (GnRH), and their pituitary receptors (GRHR and GnRHR) suggest that, in *Otx1*−/− mice, hypothalamic and pituitary cells of the somatotropic and gonadotropic lineages appear unaltered and the ability to synthesise GH, βFSH, and βLH, rather than the number of cells producing these hormones, is affected (Acampora et al., 1998c).

An intriguing aspect of this study is the fact that transcription factors of the *Ptx* and *Otx* subfamilies recognise similar DNA target sequences (Simeone et al., 1993; Lamonerie et al., 1996; Szeto et al., 1996; Tremblay et al., 1998), and that *Ptx1* and *Ptx2* are expressed in pituitary somatotropic and gonadotropic cells

Table 2
Major phenotypes observed in *Otx2*−/− and *hOtx1*$^2$/*hOtx1*$^2$

| Major phenotypes | *Otx2*−/− | *hOtx1*$^{2a}$ |
|---|---|---|
| *Embryo lethality* | 100% at E9 | 20% at E9<br>80% between E10 and P1 |
| *Gastrulation* | | |
| Visceral endoderm | not anteriorised | anteriorised |
| Primitive streak | disorganised | normal |
| Anterior mesendoderm and node | strongly impaired or absent | normal |
| *Neural plate* | | |
| Anterior patterning at late gastrula | absent | normal |
| Maintenance of anterior identity | [b] | absent or strongly affected |
| *Brain abnormalities* | lack of fore-, mid- and rostral hindbrain | repatterning of fore- and midbrain into rostral hindbrain |
| *Body plan* | abnormal | normal |

[a] The OTX1 protein has been detected only in the visceral endoderm cells of *hOtx1*$^2$/*hOtx1*$^2$ embryos (see Fig. 1).
[b] Maintenance of anterior patterning cannot be analysed due to the failure in the establishment of the rostral neural plate.

(Tremblay et al., 1998). *Ptx1* is the most highly expressed of these genes followed by *Ptx2* and then *Otx1* (Tremblay et al., 1998). Yet, the *Otx1* knock-out has a dramatic effect during the prepuberal period. The unique activity of *Otx1* during this period might reflect a specific interaction of *Otx1*, but not of the related *Ptx* factor(s) with a coregulator of transcription in the somatotrophic and gonadotrophic cells.

Taken together with other reports, these observations support the existence of complex regulatory mechanisms defining combinatorial cell and stage-specific interactions between transcription factors belonging to the same or different gene families for the establishment/maintenance of pituitary functions.

A novel feature of this study is the fact that most of the impaired functions described here had recovered by the adult stage (4 months). Indeed, after the prepubescent stage, *Otx1*−/− mice begin to gradually recover from their abnormalities, showing at 4 months of age normal levels of GH, βFSH and βLH, which parallel the restored body weight, differentiation and size of both testis and ovary, as confirmed also by their sexual fertility, and normal levels of downstream molecular targets such as testosterone and IGF1. Although we are unable to explain the mechanism underlying this recovery, this observation might represent a possible example of temporal-restricted competence in hormonal regulation of specific cell-lineages by the *Otx1* transcription factor. This recovery appears similar to the 'catch-up growth' (Boersma and Wit, 1997) described in children with delayed growth and puberty, also called constitutional delay in growth and abdolescence, CDGA (Horner et al., 1978).

## 3. *Otx2* is required during gastrulation for anterior neural plate specification

Specification and early patterning of the CNS promordium are controlled by distinct mechanisms involving vertical signals directed from axial mesendoderm to the surrounding neural plate and planar signals acting through the neuroectodermal plane (Doniach, 1993; Ruiz I Altaba, 1993, 1994 and see also Figs. 1 and 2). In this context, it has recently been shown that in *zebrafish* a small group of ectodermal cells located in the prospective head region is required for the patterning and survival of the anterior brain (Houart et al., 1998; Ruiz I Altaba, 1998). However, a large body of evidence indicates that the anterior region of the primitive visceral endoderm (AVE) in mouse, as well as the leading edge of the involuting endoderm in *Xenopus*, also play a crucial role in head organiser activity (Bouwmeester et al., 1996; Thomas and Beddington, 1996; Varlet et al., 1997; Thomas et al., 1998).

*Otx2* is transcribed in the cells that are believed to emit signals in early specification and patterning of the neural plate (the AVE and axial mesendoderm) as well as in those responding to these instructing signals (the epiblast and anterior neuroectoderm) (Simeone et al., 1993; Ang et al., 1994) (Fig. 1).

In order to assess its role, mice lacking *Otx2* have been generated. *Otx2* null embryos die early in embryogenesis, lack the rostral neuroectoderm fated to become forebrain, midbrain and rostral hindbrain, and show major abnormalities in their body plan (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996) (Table 2). Heterozygous *Otx2*+/− embryos, depending on their genetic background, show head abnormalities that are reminiscent of otocephalic phenotypes (Matsuo et al., 1995).

The headless phenotype of *Otx2*−/− embryos could be due to abnormalities in tissues with inducing properties, such as the AVE (Thomas and Beddington, 1996; Varlet et al., 1997; Beddington and Robertson, 1998) and the axial mesendoderm (Lemaire and Kodjabachian, 1996), or in responding tissues such as the epiblast and anterior neuroectoderm. In homozygous embryos in which *Otx2* was replaced by a *lacZ* reporter

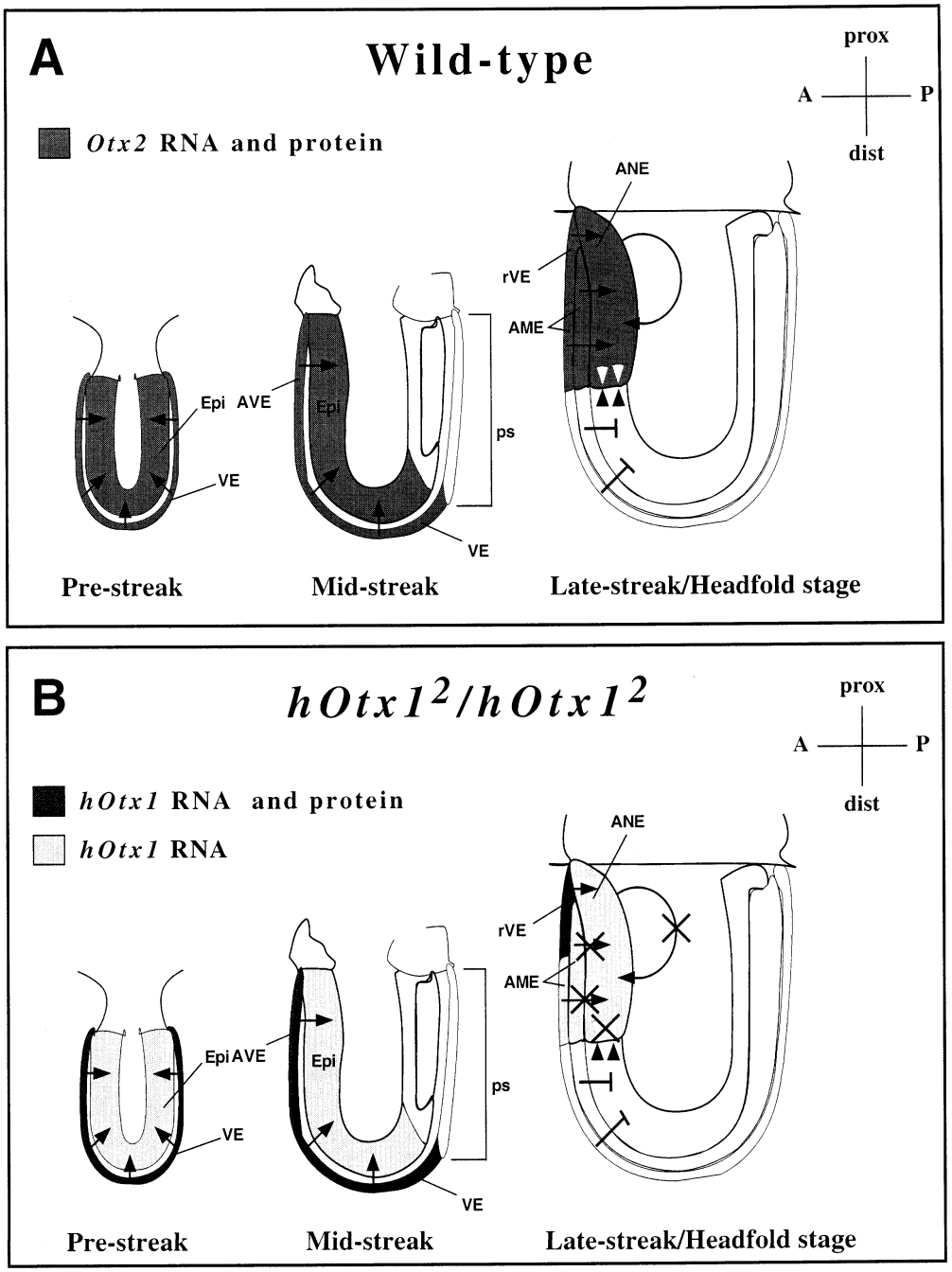


Fig. 1. *Otx2* expression and hypothetical *Otx2*-mediated tissue interactions during murine gastrulation. (**A**) Wild-type embryos. At the pre-streak stage *Otx2* is transcribed in the entire visceral endoderm and epiblast. As the primitive streak progresses, *Otx2* expression is gradually restricted to the anterior third of the embryo and at late streak/headfold stage includes all three germ layers. At this stage the anterior neuroectoderm is underlined by node-derived axial mesendoderm and, in the most anterior region, by residual visceral endoderm cells intermingled with definitive endoderm cells. Tissue recombination experiments, chimaeric embryos and *Otx2* null embryos lead us to hypothesise the existence of an early streak *Otx2*-dependent signal(s) (arrows) emanating from the visceral endoderm, directed to the epiblast and required for early neural plate specification and primitive streak organisation. At late streak/headfold stage a positive vertical signal (arrows) from anterior node-derived axial mesendoderm may act to maintain *Otx2* expression in the surrounding neuroectoderm and coexist with that coming from residual visceral endoderm. Similarly, a negative signal (T), mimicked by retinoic acid and deriving from posterior axial mesendoderm, might contribute to defining the posterior border of *Otx2* expression together with planar interactions throughout the neuroectodermal plane (arrowheads) between different gene products (e.g. *Otx2* and *Gbx2*). Finally, from the headfold stage onwards (0–8 somite stage), an *Otx2*-autonomous function (circular arrow) might be required for maintenance of fore–midbrain regional identities (Acampora et al., 1997; Rhinn et al., 1998; AS, unpublished results). (**B**) $hOtx1^2/hOtx1^2$ embryos. In the absence of OTX protein in epiblast and its derivatives, all the *Otx2* functions that are required for maintenance of anterior identity along the neural plate (cell autonomous; planar interaction at posterior border ∇∇; and vertical signalling from AME →) result in impairment and lead to a headless phenotype. Abbreviations: Epi, epiblast; ANE, anterior neurectoderm; AME, anterior mesendoderm; VE, visceral endoderm; rVE, residual visceral endoderm.

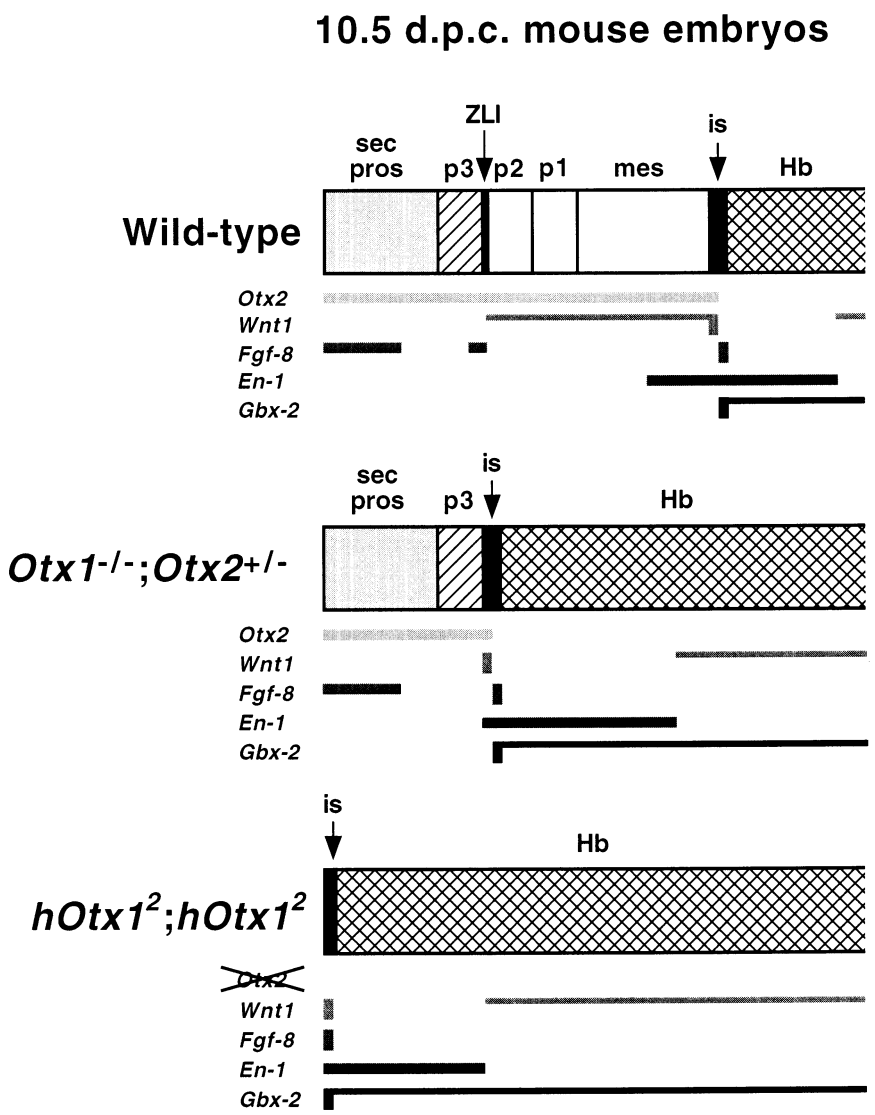


Fig. 2. *Otx1*−/−; *Otx2*+/− and $hOtx1^2/hOtx1^2$ brain patterning abnormalities at 10.5 d.p.c. In wild-type embryos the expression pattern of molecular markers, such as *Otx2*, *Wnt1*, *Fgf8*, *En1* and *Gbx2*, defines the sharp molecular code of the isthmic organiser. In *Otx1*−/−; *Otx2*+/− mutants, this code is coordinately shifted rostrally, driving a repatterning process that transforms posterior diencephalon and mesencephalon in an expanded rostral hindbrain. In $Otx1^2/Otx12$ mutants the repatterning process is more dramatic due to the absence of any OTX protein in the rostral neural plate. The initial anterior specification of the neural plate is not maintained, thus determining the transformation of the presumptive fore–midbrain neuroectoderm into a hindbrain territory. Abbreviations: sec pros, secondary prosencephalon; p1, p2, p3, prosomeres 1, 2 and 3; ZLI, zona limitans intrathalamica; mes, mesencephalon; is, isthmic organiser; Hb, hindbrain.

gene (Acampora et al., 1995), however, the first abnormality was detected at the pre-early streak stage. Indeed, at this stage, *lacZ* transcripts are detected in both the VE and the epiblast of *Otx2*+/− embryos, but only in the VE of *Otx2*−/− embryos. Therefore, at the onset of gastrulation, *Otx2* is required in the visceral endoderm to maintain its transcription in the epiblast and to mediate *Otx2*-dependent signals directed from the visceral endoderm to the epiblast. Embryos lacking *Otx2* fail to generate this signal in the visceral endoderm, and display an abnormal mesoderm organisation and the absence of the rostral neuroectoderm (see below).

These results support the possibility that abnormal primitive streak organisation and the headless phenotype might be determined very early at the pre-early streak stages by an impairment of visceral endoderm properties. These visceral endoderm properties could correspond to *Otx2*-dependent signal(s) having the epiblast cells as target (Fig. 1). In this context, an increasing amount of data strongly supports a role for the anterior visceral endoderm in head organiser activity: (i) removal of a patch of anterior visceral endoderm cells expressing the *Rpx/Hesx1* gene prevents the subsequent expression of the gene in the rostral headfolds which become reduced and abnormally patterned (Thomas and Beddington, 1996; Dattani et al., 1998); (ii) chimaeric embryos

composed of wild-type epiblast and *nodal*−/− visceral endoderm are found to be heavily impaired in rostral CNS development (Varlet et al., 1997); (iii) transplantation of axial mesoderm in mouse induces a secondary axis lacking the most anterior neural tissues (Beddington, 1994); (iv) in *Xenopus*, the expression of the secreted molecule encoded by the *cerberus* gene is restricted to the leading edge of the involuting endoderm and represents a potent head inducer (Bouwmeester et al., 1996; Bouwmeester and Leyns, 1997); and (v) the observation that most of the genes expressed in the node or in the axial mesendoderm cells at mid–late streak stage are also previously expressed in the anterior visceral endoderm. Together, these findings reinforce the idea that in mouse the organiser might be split into at least two embryonic regions operating at different stages to specify head and trunk organiser signals (Thomas and Beddington, 1996; Belo et al., 1997; Ruiz I Altaba, 1998).

The importance of *Otx2* in the AVE has also been supported by the analysis of chimaeric embryos containing *Otx2*−/− epiblast cells and wild-type VE and vice versa (Rhinn et al., 1998). Indeed, only chimaeric embryos containing wild-type VE and *Otx2*−/− epiblast cells were able to rescue an early neural plate, while embryos containing *Otx2*−/− VE and wild-type epiblast cells displayed the *Otx2*−/− phenotype.

Further proof of the relevance of *Otx2* in the AVE and in the epiblast cells has been provided by the in vivo replacement of *Otx2* with *Otx1* (Acampora et al., 1998b and see below). Additional evidence indicating that *Otx2* is responsive to inductive interactions between ectoderm and mesendoderm comes from explant-recombination experiments in gastrulating mouse embryos. These experiments have demonstrated the existence of a positive signal from the anterior mesendoderm of headfold stage embryos able to maintain *Otx2* expression in the anterior ectoderm of early streak embryos, and a negative signal from the posterior mesendoderm, mimicked by exogenous retinoic acid, able to repress *Otx2* expression in the anterior ectoderm of late streak embryos (Ang et al., 1994). Similar interactions have also been demonstrated in *Xenopus* (Blitz and Cho, 1995).

The possibility that retinoic acid might indeed play a role in vivo by controlling *Otx2* expression to distinguish fore–midbrain from hindbrain territory at an early stage is supported by the finding that the administration of exogenous retinoic acid at mid–late streak stage represses early *Otx2* expression in both the axial mesendoderm and posterior neural plate (Ang et al., 1994; Simeone et al., 1995; Avantaggiato et al., 1996). This repression correlates with the appearance of microcephalic embryos showing early anteriorisation of *Hoxb1* expression, hindbrain expansion (Sive and Cheng, 1991; Conlon and Rossant, 1992; Marshall et al., 1992; Krumlauf, 1994), loss of forebrain molecular and morphological landmarks, and gain of midbrain molecular markers in the most anterior neuroectoderm (Simeone et al., 1995; Avantaggiato et al., 1996). Moreover, *Otx2* responsiveness to retinoic acid application is a common feature in different species including *Xenopus* and chick (Bally-Cuif et al., 1995; Pannese et al., 1995). Nevertheless, the question of whether the interaction between endogenous retinoic acid and *Otx2* is a physiological event in rostral CNS demarcation still remains unsolved.

## 4. Brain patterning depends on a minimal threshold of OTX gene products

Events underlying the antero-posterior patterning of the CNS begin to be established during the early gastrulation stage and lead to the generation of distinct transverse domains along the A/P body axis (Tam and Behringer, 1997; Rubenstein et al., 1998). Meinhardt (1983) proposed that the juxtaposition of differently-specified territories can generate organising centres at their points of contact, where cellular interactions result in the production of signalling molecules with inducing properties.

An inductive signal may be generated either at the boundary between adjacent transverse domains or in a restricted longitudinal domain running all along the A/P axis. In both cases, target tissues activate specific differentiating programs depending on their ability to respond to the inductive signal. Territorial competence and inductive signals produced by organising centres are the main contributors towards the establishment of the morphogenetic fate of distinct brain areas.

Elegant transplantation experiments have demonstrated organising properties of the isthmus at the mesencephalic–metencephalic junction and the existence of a different territorial competence between regions of the brain located rostrally (prosomeres 3 to 6) and caudally (mesencephalon and prosomeres 1 and 2) to the zona limitans intrathalamica (ZLI) (Martinez et al., 1991; Marin and Puelles, 1994; Rubenstein et al., 1998).

While rostral midbrain tissue grafted in a more anterior region is influenced by adjacent territory to acquire host fate, transplants of the mes–met junction are able to maintain their identity and to induce surrounding tissue to acquire a mes–met fate. On the other hand, forebrain territories rostral to the ZLI (ventral thalamus and secondary prosencephalon) never change their fate in response to signal(s) emitted by the mes–met junction. Interestingly, the host response to the morphogenetic signal is restricted to a single prosomere. Therefore, there is a specific response of different brain regions to the same signal, indicating that neuromeric boundaries might act either as morphogenetic sources or as morphogenetic barriers that block the spreading of the signal (Martinez et al., 1995). Alternatively, the

different response of territories anterior or posterior to the ZLI might underlie a different competence of the signals. This may be due to the different origin of pre- and post-ZLI territories, deriving from prechordal and epichordal regions, respectively.

The secreted diffusible factor, FGF8, is expressed at the metencephalic side of the mes–met boundary at the right time to be involved in the development of the isthmic organiser. According to both theory (Meinhardt, 1983) and previous embriological findings (Martinez et al., 1991; Marin and Puelles, 1994), Crossley et al. (1996) have demonstated that FGF8 possesses midbrain-inducing properties. Indeed, FGF8-soaked beads implanted into the caudal diencephalon induce a midbrain by changing the fate of the host tissue (Crossley and Martin, 1995; Crossley et al., 1996; Martinez et al., 1999). A mouse model carrying an *Fgf8* hypomorphic allele (Meyers et al., 1998) and acerebellar (ace), a zebrafish mutant lacking *Fgf8* function (Reifers et al., 1998) confirm the crucial role played by this molecule for the correct patterning of the mes–met regions. In the forebrain the signalling molecule, *Shh*, is similarly expressed along the ZLI, the boundary separating dorsal (p2) from ventral (p3) thalamus (Echelard et al., 1993; Puelles and Rubenstein, 1993; Bally-Cuif and Wassef, 1995).

Although never demonstrated, *Shh* expression and other circumstantial evidence led us to hypothesise that the ZLI might potentially have morphogenetic property-(ies), thereby playing an organising role of prosencephalic territories and/or having an antagonistic effect on the rostral transmission of inductive signals, thus representing a sort of morphogenetic barrier (Martinez et al., 1991; Figdor and Stern, 1993; Marin and Puelles, 1994; Rubenstein et al., 1994; Bally-Cuif and Wassef, 1995; Crossley et al., 1996). In this context, an essential point is to determine the molecular mechanism(s) defining the regional diversity necessary to specify adjacent territories with different identity (e.g. midbrain and hindbrain), and in turn to allow the correct positioning and/or the establishment of an organiser (e.g. isthmus). Some evidence suggests that *Otx* genes might contribute to the specification of regional diversity between adjacent territories, as well as in the positioning and/or establishment of morphogenetic boundaries.

In fact, (i) *Otx* genes are expressed when early regionalisation takes place (Simeone et al., 1992); (ii) *Otx2* null mice do not develop neuroectoderm rostral to the rhombomere 3 (Acampora et al., 1995); (iii) the caudal limit of *Otx2* expression identifies the mid–hindbrain boundary at the isthmic constriction (Millet et al., 1996; Wassarmann et al., 1997); (iv) *Otx* genes are both expressed in close proximity to the ZLI (Simeone et al., 1993); (v) retinoic acid-induced phenocopies show an early ordered A/P repatterning of the brain, correlating with the posterior repression of *Otx2* (Simeone et al., 1995; Avantaggiato et al., 1996).

In order to verify this hypothesis, the level of OTX proteins was modified by altering the *Otx* gene dosage in vivo (Acampora et al., 1997; Suda et al., 1997). Only *Otx1*−/−; *Otx2*+/− embryos showed 100% penetrance of gross brain malformations that included a remarkable reduction of the Ammon's horn, as well as a morphological and molecular transformation of the pretectum, dorsal thalamus and mesencephalon into an enlarged metencephalon. Mesencephalic molecular features within the dorsal telencephalon of *Otx1*−/−; *Otx2*+/− brains were also observed. Indeed, *Wnt1* was expressed along the telencephalic commissural plate, and *En2* along the dorsal telencephalon. The rescue of the abnormal phenotype observed in the presence of an additional copy either of *Otx2* or *Otx1* indicated that *Otx* genes might cooperate in brain patterning through a gene dosage requirement. The origin of the repatterning process has been studied by monitoring the early expression of genes involved in the establishment of the mes–met region such as *Wnt1*, *En1* and *Fgf8* (Fig. 2) (Bally-Cuif and Wassef, 1995; Joyner, 1996; Rubenstein et al., 1998). This analysis suggested that the repatterning process was probably triggered by the early mis-expression of *Fgf8* in response to a critically low level of OTX gene products (Acampora et al., 1997).

Suda et al. (1997) presented similar results in their analysis of double heterozygous embryos (*Otx1*+/−; *Otx2*+/−) from a different genetic background.

Furthermore, the fact that the dorsal telencephalon in *Otx1*−/−; *Otx2*+/− embryos acquires mesencephalic molecular features correlates with the absence of the ZLI, as revealed by anatomical inspection and loss of *Shh* expression, thus suggesting that it might act as a morphogenetic barrier to the spreading of posteriorising signals (Crossley et al., 1996; Martinez et al., 1999). The incomplete transformation of the telencephalon observed in the absence of ZLI could be a consequence of the reduced ability of the telencephalic territory to respond over time to the mesencephalic inductive signals coming from the anteriorised isthmic-like structure.

Altogether these findings support the existence of a previously unsuspected mechanism depending on a precise threshold of OTX proteins that is strictly required to diversify adjacent territories fated to become the mesencephalon and metencephalon.

Thus, in *Otx1*−/−; *Otx2*+/− embryos, an insufficient level of OTX gene products is responsible for the *Fgf8* misexpression that triggers the following repatterning process.

Further experiments performed in chick embryos by implanting beads soaked in *FGF8* (Martinez et al., 1999) have provided evidence that a negative feedback loop between *Fgf8* and *Otx2* is required to confer territorial identities to midbrain and anterior hindbrain.

## 5. The interaction between *Otx2* and *Gbx2* expressing territories positions the isthmic organiser region

To determine whether the caudal limit of *Otx2* domain and its adjacent expression to that of *Gbx2* at the mid–hindbrain border was critical for the positioning of the isthmic organiser, the caudal limit of *Otx2* expression was either shifted across the mid–hindbrain border into the anterior hindbrain (Broccoli et al., 1999) or *Gbx2* was ectopically expressed in the rostral midbrain (Millet et al., 1999). As a consequence of the ectopic *Otx2* expression, at early embryonic stages, the expression of all the midbrain- (*Ephrin-A5*, *Wnt1*) and isthmic-specific markers (*Pax2*, *Fgf8*) was shifted caudally into rh1, whereas the hindbrain-specific marker *Gbx2* was repressed. As a result, the isthmic organiser was repositioned in the anterior hindbrain (Broccoli et al., 1999). On the other hand, analysis of mutant mice in which *Gbx2* was transiently expressed more anteriorly under the control of a *Wnt1* enhancer showed that the *Otx2* caudal limit was shifted rostrally in the mesencephalon (Millet et al., 1999). The exact location of the new *Otx2* limit varied among transgenics, but it was always sharp and located within the rostral mesencephalon. Noteworthy, in *Wnt1-Gbx2* transgenic mice the *Otx2*-positive domain stopped abruptly within a territory weakly expressing *Gbx2*, thus indicating that there may be a minimal threshold of *Gbx2* required to repress *Otx2* expression. However, the crucial role of *Gbx2* and *Otx2* in defining the proper positioning and specification of the isthmic organiser was also suggested by previous studies.

In particular, in *Gbx2*−/− embryos, the *Otx2* domain was posteriorly expanded within the rostral hindbrain, the *Otx2* limit did not sharpen in the mutant embryos and genes defining the isthmic molecular code lacked their proper spatial relationships, thus indicating that *Gbx2* and *Otx2* interaction played an important role in this event (Wassarman et al., 1997; Millet et al., 1999). Moreover, transplantation experiments indicated that the confrontation of *Otx2* and *Gbx2* positive territories is required to activate *Fgf8* expression in the *Gbx2* territory adjacent to that expressing *Otx2* (Hidalgo-Sanchez et al., 1999).

Therefore, these data indicate that the posterior border of *Otx2* is instrumental in positioning the isthmic organiser and that *Gbx2* is required for its sharpening.

## 6. Comparison of OTX1 and OTX2 functional properties

Even though mammalian OTX1 and OTX2 proteins share extensive similarities in their sequences, downstream of the OTX1 homeodomain, the regions of homology to OTX2 are separated by stretches of additional amino acids (Simeone et al., 1993). To determine whether these differences code for OTX1- and OTX2-specific biochemical properties, we generated mice in which the *Otx1* gene was replaced by a human *Otx2* (*hOtx2*) full-coding cDNA ($hOtx2^1/hOtx2^1$) (Acampora et al., 1999a) or the *Otx2* gene was replaced by a human *Otx1* (*hOtx1*) full-coding cDNA ($hOtx1^2/hOtx1^2$) (Acampora et al., 1998b).

In homozygous mice in which *Otx1* was replaced with the human *Otx2* cDNA ($hOtx2^1/hOtx2^1$), despite a reduced expression of the transgenic alleles, cerebral cortex development appeared normal, epilepsy was absent (Table 1) and a normal cell proliferation in the dorsal neuroepithelium was restored (Acampora et al., 1999a). This is particularly relevant considering the different expression patterns of *Otx1* and *Otx2* in the dorsal telencephalon from 9.5 d.p.c. onwards. Indeed, at this stage, while *Otx1* is expressed throughout the entire dorsal telencephalon, *Otx2* is expressed only in the mediodorsal area and in the basal neuroepithelium and disappears completely from the mediodorsal area at 11 d.p.c. Considering the absence of the OTX1 gene product and a reduced level of the hOTX2 protein in regions that normally would not express *Otx2*, the rescue observed in $hOtx2^1/hOtx2^1$ mice suggests that *Otx1* and *Otx2* have interchangeable roles in neuroblast proliferation. This finding also suggests that the differential transcriptional control of *Otx1* and *Otx2*, rather than their amino acid divergence, is responsible for the contrasting phenotypes of *Otx1*−/− and *Otx2*−/− mutants.

$hOtx2^1/hOtx2^1$ mice also showed a significant improvement in mesencephalon, eye and lachrymal gland defects. In contrast, the lateral semicircular canal of the inner ear was never restored, suggesting that the ability to specify this structure may be an *Otx1*-specific property (Acampora and Simeone, 1999). The inner ear phenotype hints at possible evolutionary implications. In fact, the absence of this structure in *Otx1*−/− mice might represent a back evolutionary mutation and suggests when the *Otx1*-type gene appeared in evolution. The inner ear of lower agnates such as mixinoides shows only one semicircular canal, ciclostomes two and gnathostomes three. The last to be created is the lateral semicircular canal in gnathostomes (Fritzsch et al., 1986; Torres and Giraldez, 1998). Only one *Otx* gene has been identified so far in protochordates (urochordates and cephalochordates) (Wada et al., 1996; Williams and Holland, 1996), while at least two *Otx* genes in lamprey (agnates) (Ueki et al., 1998). However, even though one of these two genes is clearly related to *Otx2*, the other cannot be unambiguously related to the murine *Otx1* (Ueki et al., 1998). Therefore, it may be speculated that in lamprey the *Otx1* ancestor was not yet duplicated or, alternatively, that it was still evolutionary unstable.

Homozygous mutant mice replacing *Otx2* with the human *Otx1* (*hOtx1*) cDNA ($hOtx1^2/hOtx1^2$) recovered

the anterior neural plate induction and a normal gastrulation but showed a headless phenotype from 9 d.p.c. onwards (Table 2). A combined analysis of both *hOtx1* RNA and protein distribution during early gastrulation has revealed that while the *hOtx1* mRNA was detected in the VE and epiblast, the hOTX1 protein was revealed only in VE (Fig. 2). Nevertheless, this VE-restricted hOTX1 protein translation was sufficient to recover gastrulation defects and induction of an early anterior neural plate. From 8.5 d.p.c. onwards, however, $hOtx1^2/hOtx1^2$ embryos failed to maintain fore–midbrain identities and, at the end of gestation, displayed a headless phenotype in which the body plan showed no detectable defects (Acampora et al., 1998b) (Fig. 2).

These results, besides reinforcing the head organising activity of the VE (Beddington and Robertson, 1998), indicate that at least in this tissue, *Otx1* and *Otx2* are functionally equivalent. Moreover, these findings indicate that at the late gastrulation stage, *Otx2* is necessary in the axial mesendoderm or in the neuroectoderm or in both for the maintenance of anterior patterning of the neural plate.

These data support the idea of an extended functional conservation between OTX1 and OTX2, and suggest that *Otx1* and *Otx2* null mice contrasting phenotypes originate mostly from their divergent expression patterns.

## 7. Functional equivalence between *Drosophila otd* and murine *Otx* genes

Striking evolutionary conservation of regulatory genes that control vertebrate development is exhibited by *HOM/HOX* complexes (Lewis, 1978; Duboule and Dollé, 1989; Krumlauf, 1994; van der Hoeven et al., 1996) and *small eye/Pax6* genes (Callaerts et al., 1997).

*otd/Otx* genes are also likely to have a conserved functional role in brain morphogenesis. This assumption derives from a remarkable 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 mutant flies lacking *otd* function, the protocerebral anlage is deleted and some deuterocerebral neuroblasts do not form, giving rise to a dramatically reduced brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Other defects are also observed in the ventral nerve cord and in non-neural structures (Finkelstein et al., 1990).

In *ocelliless*, a viable *otd* allele, expression in the vertex primordium is abolished and the ocelli (light sensing organs) and associated sensory bristles are lost (Finkelstein et al., 1990). 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 contrast to the extensive similarities in expression and mutant phenotype shared by the *Drosophila otd* and the murine *Otx* genes, the homology between OTD and OTX gene products is quite restricted. Indeed, sequence homology is confined to the homeodomain and a few flanking amino acids (Simeone et al., 1993). Thus, although the ability to recognise the same target sequence might be evolutionarily conserved, murine *Otx* genes might also have acquired additional functional features, outside the homeodomain, 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.

To verify this hypothesis, mice replacing *Otx1* with a full-coding *Drosophila otd* cDNA have been generated (Acampora et al., 1998a).

Interestingly, many of the abnormalities of *Otx1*−/− mice, such as impaired cell proliferation, corticogenesis and epilepsy, were fully rescued by *otd* (Acampora et al., 1998a) regardless of a lower level of OTD (about 30% less) in $otd^1/otd^1$ mice compared with the OTX1 level in wild-type animals. To a lesser extent, *Otx1*−/− eye defects and brain patterning alterations detected in *Otx1*−/−; *Otx2*+/− embryos were also partially recovered. On the contrary, the lateral semicircular canal of the inner ear of *Otx1*−/− mice was never restored (Table 1).

In a complementary experiment performed in *Drosophila*, overexpression of human *Otx1* and *Otx2* genes rescued the brain and ventral nerve cord phenotypes of *otd* mutants (Leuzinger et al., 1998) as well as the cephalic defects of adult flies carrying the *ocelliless* mutation (Nagao et al., 1998). Moreover, ubiquitous overexpression of *Otx1* and *Otx2* genes in a *Drosophila* wild-type background was able to induce ectopic neural structures (Leuzinger et al., 1998).

These cross-phylum rescues are surprising not only because of the different anatomy and complexity of insect and mammalian brains, but also because of the very limited region of homology shared by the proteins and restricted essentially to the homeodomain. These two observations imply that *otd* and *Otx* genes can trigger a basic program of cefalic development through conserved genetic interactions, possibly involving a homeobox-mediated choice of the same target sequence and, probably, the same target genes (Sharman and Brand, 1998; Hirth and Reichert, 1999; Reichert and Simeone, 1999). The incomplete rescue mediated by the *Drosophila otd* gene may reflect both quantitative (higher level of *otd* expression) and qualitative (*Otx*-specific) requirements. In particular, failure to recover the lateral semicircular canal of the inner ear in $otd^1/otd^1$ mice

(Reichert and Simeone, 1999; Sharman and Brand, 1998) strongly supports the existence of an *Otx1*-specific function required to specify this structure and acquired during evolution.

## 8. Conclusions

Brain patterning results from a genetic and morphogenetic cascade of events that starts very early during embryo development in vertebrates. The molecular basis of this cascade has not yet been elucidated. The identification of a growing number of genes encoding transcription factors, inducing molecules, membrane ligands and receptors, has provided great support for a better understanding of the mechanisms underlying brain regionalisation.

Results presented in this review strongly support the relevance of *Otx1* and *Otx2* within this cascade, and indicate *Otx2* as a master gene for the correct induction and positioning of the region that will give rise to the adult brain. The two genes share a high level of homology in the protein sequence, and are functionally interchangeable for many of their roles. Thus, divergence between *Otx1* and *Otx2* functions mainly resides in their regolative regions that distinguish their role during development. Amazingly enough, also the *Drosophila* homologue gene *otd*, which shares homology only in the homeodomain region, shows functional equivalence to their mouse cognates, suggesting that the extensive functional equivalence of the *otd*/*Otx* genes may be due to conserved developmental genetic circuits with common functional features that are controlled by the homeodomain. Further investigations focused on the identification of functional domains of the OTX proteins and regulatory control elements will allow us to improve our knowledge of the molecular mechanism(s), leading to specification and patterning of the mammalian brain.

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