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. © 2000 Elsevier Science B.V. All rights reserved.

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, 0378-1119/00 - see front matter © 2000 Elsevier Science B.V. All rights reserved.
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Among these, the orthodenticle group is defined by the Drosophila orthodenticle (otd) and the vertebrate Otx1, Otx2 and Otx3 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 rostral-most brain neuroepithelium (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 and lateral cortex but absent in the frontal, insular and orbital cortices, while Otx1 expression in layer 6 is more prominent in the posterior cerebral cortex. The expression of Otx1 is required for corticogenesis, sense organ development and pituitary functions.

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 forebrain in monortery. Later in gestation, when the generation of first postmitotic neurons starts in the dorsal telencephalon, high level transcription of Otx1 occurs only in ventricular layers, 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 postmitigatory 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 differentiation 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 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 a relatively low level in the anterior lobe of the pituitary gland (Acampora et al., 1996). 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 sulci rhinalis appeared dorsally displaced and the hippocampus was shrunken with a variated dentate gyrus (Table 1). The cortex was particularly affected at the level of the
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 apoptotic cells 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. 

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 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 cochlcoacoustic 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 (FSH), luteinising hormone (LH), 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, FSH and LH 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, FSH, and LH, rather than the number of cells producing these hormones, is affected (Acampora et al., 1996c).

An intriguing aspect of this study is the fact that transcription factors of the Ptx and Otx subfamilies recognise similar DNA target sequences (Simonne et al., 1993; Lamoneze et al., 1996; Sze 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 hOtx12/hOtx12

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<tr>
<th>Major phenotypes</th>
<th>Otx2−/−</th>
<th>hOtx12a</th>
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<tr>
<td>Embryo lethality</td>
<td>100% at E9</td>
<td>20% at E9</td>
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<td></td>
<td></td>
<td>80% between E10 and P1</td>
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<tr>
<td>Gastrulation</td>
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<tr>
<td>Visceral endoderm</td>
<td>not anteriorised</td>
<td>anteriorised</td>
</tr>
<tr>
<td>Anterior mesendoderm and node</td>
<td>strongly impaired or absent</td>
<td>normal</td>
</tr>
<tr>
<td>Neural plate</td>
<td></td>
<td></td>
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<tr>
<td>Anterior patterning at late gastrula</td>
<td>absent</td>
<td>normal</td>
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<td>Maintenance of anterior identity</td>
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<td>repatterning of fore- and midbrain into rostral hindbrain</td>
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a The OTX1 protein has been detected only in the visceral endoderm cells of hOtx12/hOtx12 embryos (see Fig. 1).
b Maintenance of anterior patterning cannot be analysed due to the failure in the establishment of the rostral neural plate.

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) (Simone et al., 1993; Ang et al., 1994) (Table 2). 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; Thomas et al., 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).
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<sup>12</sup>/hOtx1<sup>12</sup> 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 VV; and vertical signalling from AME →) result in impairment and lead to a headless phenotype. Abbreviations: Epi, epiblast; ANE, anterior neuroectoderm; AME, anterior mesendoderm; VE, visceral endoderm; rVE, residual visceral endoderm.
Fig. 2. Otx1−/−; Otx2+/− and hOtx1+/− hOtx1−/− 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+/− Otx1−/− 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...
epiblast cells displayed the Otx2 least two embryonic regions operating at di
idea that in mouse the organiser might be split into at ceral endoderm. Together, these findings reinforce the OTX gene products stage are also previously expressed in the anterior vis-
or in the axial mesendoderm cells at mid–late streak observation that most of the genes expressed in the node and represents a potent head inducer (Bouwmeester logical 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 gastru-
lation 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

phological landmarks, and gain of midbrain molecular markers in the most anterior neuroectoderm (Simone 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.
V
do not develop neuroectoderm rostral to the rhombomere 3 (Acampora et al., 1995); (iii) the causal limit of Otx2 expression identifies the midhindbrain boundary at the isthmic constriction (Millet et al., 1996; Wassermann et al., 1997); (iv) Otx genes are both expressed in close proximity to the ZLI (Simone et al., 1993); (v) retinoic acid-induced phenocopies show an early ordered AP repatterning of the brain, correlating with the posterior repression of Otx2 (Simone 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 prepectum, 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; Jouyer, 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 posteriorizing 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 (Foxe, Fgf8) was shifted caudally into rh3, 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 (Wasarman 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 Otx1 (hOtx1) full-coding cDNA (hOtx1/−) (Acampora et al., 1999a) or the Otx2 gene was replaced by a human Otx2 (hOtx2) full-coding cDNA (hOtx2/−) (Acampora et al., 1998b). In homozygous mice in which Otx1 was replaced with the human Otx2 cDNA (hOtx2/−), 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 mid–hindbrain area and in the basal neuroepithelium and disappears completely from the mid–hindbrain 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/− 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/− 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 suggest when the Otx1–type gene appeared in evolution. The inner ear of lower agnates such as mixinoidea shows only one semicircular canal, cyclostomes 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/−) recovered
the anterior neural plate induction and a normal gastrulation but showed a headless phenotype from 9.5 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 anterior early neural plate. From 8.5 d.p.c. onwards, however, hOtx1+/hOtx1+ 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 organizing 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 HOX/HOM complexes (Lewis, 1978; Duboule and Dollé, 1989; Krumlauf, 1994; van der Hoeven et al., 1996) and small Pax/Pax 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 deuterocephalic 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-neuronal structures (Finkelstein et al., 1998).

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 (Royer 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+/otd− 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 cephalic development through conserved genetic interactions, possibly involving a homeobox-mediated choice of the same target sequence and, probably, the same target genes (Sharmain 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+/otd− 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 embryonic 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 regenerative capacities 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 genetic regulatory 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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