The role of Otx and Otp genes in brain development

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ABSTRACT Over the last ten years, many genes involved in the induction, specification and regionalization of the brain have been identified and characterized at the functional level through a series of animal models. Among these genes, both Otx1 and Otx2, two murine homologues of the Drosophila orthodenticle (otd) gene which encode transcription factors, play a pivotal role in the morphogenesis of the rostral brain. Classical knock-out studies have revealed that Otx2 is fundamental for the early specification and subsequent maintenance of the anterior neural plate, whereas Otx1 is mainly necessary for both normal corticogenesis and sense organ development. A minimal threshold of both gene products is required for correct patterning of the fore-midbrain and positioning of the isthmic organizer. A third gene, Orthopedia (Otp) is a key element of the genetic pathway controlling development of the neuroendocrine hypothalamus. This review deals with a comprehensive analysis of the Otx1, Otx2 and Otp functions, and with the possible evolutionary implications suggested by the models in which the Otx genes are reciprocally replaced or substituted by the Drosophila homologue, otd.

KEY WORDS: Brain patterning, brain evolution, visceral endoderm, neuroendocrine hypothalamus.

Introduction

Morphogenesis of the central nervous system (CNS) and differentiation of the neural structures are highly complex processes. The first event is characterized by the induction of the neural tissue by an early organizer (Spemann and Mangold, 1924). The induced rostral neuroectoderm becomes regionalized in the forebrain, midbrain and hindbrain (Gallera, 1971; Storey et al., 1992; Rubenstein et al., 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).

In vertebrates, a remarkable amount of data has been collected on the role of genes which are candidates for the control of developmental programs underlying brain morphogenesis. Most of these genes are the vertebrate homologues of Drosophila genes coding for signal molecules or transcription factors (Lemaire and Kodjabachian, 1996; Tam and Behringer, 1997; Rubenstein et al., 1998). Among these, Otx1, Otx2 and Otp are the vertebrate homologues of the Drosophila orthodenticle (otd) and Orthopedia (Dm Otp) genes (Simeone et al., 1994; Simeone, 1998; Acampora and Simeone, 1999; Acampora et al., 1999c). The Drosophila otd gene is expressed at the anterior pole of the blastoderm embryo and later predominantly in the developing rostral-most brain neuromere (Cohen and Jürgens, 1990, 1991; Finkelstein and Perrimon, 1990; Finkelstein et al., 1995; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Mutations in the otd gene cause the loss of adjacent anterior head segments suggesting that it might act as a head gap gene.

In mouse Otx1 expression is first detected at the 1-3 somite stage throughout the fore- and midbrain neuroepithelium, while Otx2 is already transcribed before the onset of gastrulation in the epiblast and in the visceral endoderm (VE), and subsequently in the axial mesendoderm and rostral neural plate (Simeone et al., 1992, 1993). During brain regionalization, Otx1 and Otx2 are

Abbreviations used in this paper: CNS, central nervous system; otd, Drosophila orthodenticle; VE, visceral endoderm; Otp, Orthopedia; spv, supraoptic/paraventricular region; AH, anterior hypothalamus; rch, retrochiasmatic region; PVN, paraventricular nucleus; SON, supraoptic nucleus; aPV, anterior periventricular nucleus; ARN, arcuate nucleus; IZ, intermediate zone; CP, cortical plate; EEG, electroencephalographic recording; GH, growth hormone; βFSH, follicle-stimulating hormone; βLH, luteinizing hormone; αGSU, α-glycoprotein subunit; GRH, growth hormone releasing hormone; GnRH, gonadotropin releasing hormone; AVE, anterior visceral endoderm; A/P, antero-posterior; mes-met, mesencephalic-metencephalic; ZLI, zona limitans intrathalamica; OT, oxytocin; AVP, arginine vasopressin; ME, median eminence; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; SS, somatostatin.

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expressed in largely overlapping domains with a posterior border coincident with the mesencephalic side of the isthmic constriction (Simeone et al., 1992; Millet et al., 1996; Acampora et al., 1997). Furthermore, 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).

Orthopedia (Otp), a homeobox-containing gene, exemplifies in its name the homology shared by its homeodomain with both the orthodenticle and the Antennapedia homeodomains. Otp is highly conserved in evolution. In Drosophila, Dm otp first appears at gastrulation in the ectodermal proctodeum and, later on, in the hindgut, anal plate and along the CNS. Unfortunately, no mutant for the Dm Otp gene has been so far identified (Simeone et al., 1994). In the mouse, Otp expression is first detected at 9.5 days post coitum (d.p.c.) along the spinal cord, hindbrain and in restricted domains within the forebrain. At 12.5 d.p.c. Otp transcripts are regionally restricted to the supraoptic, paraventricular and suprachiasmatic regions (rch). The paraventricular (PVN), supraoptic (SON), anterior periventricular (aPV) and arcuate (ARN) nuclei, which constitute most of the neuroendocrine hypothalamus, originate from these areas. Further interest in this gene is also provided by its segmented-like expression domain that results complementary to that of Dlx genes. This observation suggests that Otp and Dlx genes could contribute to specify proper identity of different subregionally restricted areas within the hypothalamus (Simeone et al., 1994). The potential roles of Otx and Otp have been the object of intense study and are now being elucidated by genetic analyses.

**Otx1 plays a role in 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 later give rise to the typical layered organization of the adult cortex (Rakic, 1974). Otx1 represents a molecular correlate of deep layer neurogenesis and its expression is confined to neurons of layers 5 and 6 (Frantz et al., 1994). At mid-late gestation, 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 postmitotically born neurons of layer 5 and 6. Otx1 is absent in later differentiated neurons of upper layers 1-4 (Frantz et al., 1994).

Thus, the progressive down-regulation of Otx1 in the ventricular cells suggests that Otx1 may confer deep-layer identity to young neurons (Frantz and McConnell, 1996). 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). From the birthday onwards, Otx1 is also transcribed at relatively low level in the anterior lobe of the pituitary gland. In order to study its role, Otx1 null mice have been generated (Acampora et al., 1996). The major phenotypes are summarized in Table 1.

Otx1-/- mice exhibited both spontaneous high speed turning behavior and epileptic behavior. The latter consisted of the combination of focal seizures with electroencephalographic (EEG) recording of spikes in hippocampus and generalized seizures characterized by convulsions and high voltage synchronized EEG activity in hippocampus and cortex (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, mainly at the level of the temporal and perirhinal areas, where a 40% reduction in cell number and a disorganization of cortical layers were detected.

To assess whether the overall reduction of the Otx1-/- brains was due to reduced proliferation or an increase in cell death, the number of apoptotic and proliferating cells in Otx1-/- developing brain was determined. While abnormal apoptosis was not observed, by contrast, a reduction of proliferating cells (by about 25%) in the dorsal telencephalic neuroepithelium of 9.75 d.p.c. Otx1-/- embryos suggested that impaired cell proliferation may contribute to the cortical abnormalities. Further morphological defects were detected in the eye and inner ear. Lachrymal and Harderian glands were also absent. As regarding the inner ear abnormalities of Otx1-/-, these are consistent with the Otx1 expression pattern in the lateral canal ampulla and in a part of the utricle as well as 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 results in the absence of the lateral semicircular canal (Acampora et al., 1996; Morsli et al., 1999). In the eye and annexed structures of Otx1-/- mice the ciliary process is absent and the iris is thinner.

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), luteinizing hormone (LH), and α- and β-glycoprotein subunit (coSSU) 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 GH, FSH and LH pituitary hormones with restored growth and gonadal function at 4 months of age. Expression patterns of corresponding hypothalamic hormones such as the growth hormone releasing hormone (GRH), gonadotropin releasing hormone (GnRH), and their pituitary receptors (GRHR and GnRHRI) suggest that, in Otx1-/- mice, hypothalamic and pituitary cells of the somatotropic and gonadotropic lineages appear unaltered and that the ability to synthesize GH, FSH, and LH, rather than the number of cells producing these hormones, is affected (Acampora et al., 1998c).

An unprecedented feature of this study is the fact that most of the impaired functions described here had recovered by the adult stage (4 months). Although we are so far 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.

**Otx2 is required for early specification of the anterior neural plate**

A large body of evidence indicates that the anterior region of the primitive visceral endoderm in mouse as well as the leading edge of the involuting endoderm in Xenopus play a crucial role in head organizer activity (Bouwmeester et al., 1996; Thomas and Beddington, 1996; Varlet et al., 1997; Thomas et al., 1998). In this
context, Otx2 is transcribed in the cells that are believed to emit signals in early specification and patterning of the neural plate (the anterior visceral endoderm 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).

Otx2 null embryos die early in embryogenesis, lack the rostral neuroectoderm fated to become the 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 otocphalic phenotypes (Matsuo et al., 1995). The headless phenotype of Otx2−/− embryos could be due to abnormalities in tissues with inducing properties, such as the anterior visceral endoderm (AVE) (Thomas and Beddington, 1996; Varlet et al., 1997; Beddington and Robertson, 1998) and the prechordal mesendoderm (Lemaire and Kodjabachian, 1996), or in responding tissues such as the epiblast and anterior neuroectoderm. However, in homozygous embryos in which Otx2 was replaced by a lacZ reporter gene (Acampora et al., 1995), 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 in Otx2−/− embryos only in the VE. Therefore, at the onset of gastrulation, Otx2 is required in the VE to maintain its transcription in the epiblast and to mediate Otx2-dependent signals directed from the VE to the epiblast. Embryos lacking Otx2 fail to generate this signal in the VE, and display an abnormal mesoderm organization and the absence of the rostral neuroectoderm (see below).

The importance of Otx2 in the AVE has also been supported by the analysis of chimeric embryos containing Otx2−/− epiblast cells and wild-type VE, or vice versa (Rhinn et al., 1998). Indeed, only chimeric 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).

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 antero-posterior (A/P) body axis (Tam and Behringer, 1997; Rubenstein et al., 1998). Meinhardt (1983) proposed that the juxtaposition of differently-specified territories can generate organizing centers at their points of contact, where cellular interactions result in the production of signaling molecules with inducing properties. Elaborate transplantation experiments have demonstrated organizing properties of the isthmus at the mesencephalic-metencephalic (mes-met) junction and the existence of a different territorial competence between regions of the brain located rostrally and posteriorly to the zona limitans intrathalamica (ZLI) (Martinez et al., 1991; Marin and Puelles, 1994; Rubenstein et al., 1998).

According both to theory (Meinhardt, 1983) and embryological findings (Martinez et al., 1991; Marin and Puelles, 1994), FGF-8 inducing properties at the isthmic organizer of chick embryos have recently been demonstrated (Crossley et al., 1996). Indeed, the secreted factor FGF-8 that is expressed at the right time and in the right position to be involved in development of the isthmic organizer, has midbrain-inducing property being able to change the fate of the caudal diencephalon into mesencephalon (Crossley and Martin, 1995; Crossley et al., 1996). 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 organizer (e.g. isthmus). Otx genes are expressed when early regionalization takes place (Acampora et al., 1995) and the caudal limit of their expression domain identifies the mesencephalic side of the isthmic constriction (Simeone et al., 1992; Millet et al., 1996).
To test their possible involvement, 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+/- double mutant 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 prefrontal, dorsal thalamus and mesencephalon into an enlarged metencephalon (Fig. 1). Moreover, mesencephalic molecular features such as Wnt-1 and En-2 expression were also observed along the telencephalic commissural plate and the dorsal telencephalon of Otx1-/-; Otx2+/- brains, respectively. 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 (Acampora et al., 1997). The origin of the re-patterning process has been studied by monitoring the early expression pattern of genes involved in the establishment of the mes- and metregion such as Wnt-1, En-1 and Fgf-8 (Bally-Cuif and Wassef, 1995; Joyner, 1996; Rubenstein et al., 1998). This analysis suggested that the re-patterning process was probably triggered by the early misexpression of Fgf-8 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. Altogether these findings support the existence of a previously unsuspected mechanism depending on a precise threshold of OTX proteins that is strictly required to distinguish adjacent territories rather than in their early establishment. Thus, in Otx1+/-; Otx2+/- or in Otx1-/-; Otx2+/- embryos the threshold of Otx gene products is able to confer to the mesencephalic field a sufficient level of specification for allowing the correct positioning of the Fgf-8 inducing properties at the isthmic organizer. In contrast, in Otx1-/-; Otx2+/- embryos, an insufficient level of Otx gene product is likely to be responsible for the Fgf-8 mis-expression that triggers the following re-patterning process. Further experiments performed in chick embryos have provided evidence that a negative feedback loop between Fgf-8 and Otx2 is required for conferring territorial identity to the midbrain and anterior hindbrain (Martinez et al., 1999). Moreover, the analysis of mice lacking Gbx2, a homeobox-containing gene expressed from the late gastrula stage with an anterior border adjacent to that posterior of Otx2, has revealed that Gbx2 is essential for proper development of both anterior hindbrain and isthmic organizer, thus suggesting possible morphogenetic interactions at the boundary between Otx2 and Gbx2 expressing territories (Wassermann et al., 1997). This latter suggestive aspect has been recently experimentally approached by transplantation experiments using the chick/quail model. In this study confrontation between Gbx2 and Otx2 positive territories represents an important requirement in order to properly position the isthmic organizer (Hidalgo-Sanchez et al., 1999).

**OTX1, OTX2 and OTD functional equivalence**

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-

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Otx1-/-</th>
<th>hOtx21/hOtx21</th>
<th>otd1/otd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early telencephalic abnormalities</td>
<td>Reduced by 25%</td>
<td>Similar to wild-type</td>
<td>Similar to wild-type</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole brain size</td>
<td>Reduced in weight by 25%</td>
<td>Similar to wild-type</td>
<td>Similar to wild-type</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cell number</td>
<td>Heavy reduction in thickness and 40% drop in cell number of temporal and perirhinal areas</td>
<td>Temporal and perirhinal areas similar to wild-type</td>
<td>Temporal and perirhinal areas similar to wild-type</td>
</tr>
<tr>
<td>Anatomy and histology</td>
<td>Difficult detection of sulcus rhinalis Shrinkage of hippocampus Disorganization of temporal and perirhinal cortical layers</td>
<td>Normal detection of sulcus rhinalis Normal hippocampus Hexalaminar organization</td>
<td>Normal detection of sulcus rhinalis Normal hippocampus Hexalaminar organization</td>
</tr>
<tr>
<td>Mesencephalon</td>
<td>Enlarged</td>
<td>Normal in 30% Intermediate in 45%</td>
<td>Normal in 15% Intermediate in 50%</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar foliation</td>
<td>Normal in 25%</td>
<td>Normal in 50%</td>
<td>Normal in 10%</td>
</tr>
<tr>
<td>Behaviour</td>
<td>Epileptic seizures High-speed turning behaviour</td>
<td>No seizures Moderate-speed turning behaviour</td>
<td>No seizures Moderate-speed turning behaviour</td>
</tr>
<tr>
<td>Ear (lateral semicircular duct)</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Lachrimal and Harderian glands</td>
<td>Absent</td>
<td>Normal in 75%</td>
<td>Normal in 34%</td>
</tr>
<tr>
<td>Eye</td>
<td>Ciliary process</td>
<td>Absent</td>
<td>Normal in 70% Normal in 80%</td>
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</table>
specific biochemical properties, we generated mice in which the Otx1 gene was replaced by a human Otx2 (hOtx2) full-coding cDNA (hOtx21/hOtx21) (Acampora et al., 1999a) or the Otx2 gene was replaced by a human Otx1 (hOtx1) full-coding cDNA (hOtx12/hOtx12) (Acampora et al., 1998b).

In homozygous mice in which Otx1 was replaced with the human Otx2 cDNA (hOtx21/hOtx21), despite a reduced expression of the replacing allele, cerebral cortical development appeared to be normal and epilepsy was rescued (Table 1). This is particularly relevant considering the different expression patterns of Otx1 and Otx2 genes in the dorsal telencephalon from the 9.5 d.p.c. stage 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 completely disappears 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 hOtx21/hOtx21 mice suggests that Otx1 and Otx2 might have interchangeable roles in the cortex. hOtx21/hOtx21 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 (Morsli et al., 1999; Acampora and Simeone, 1999; and see below).

Homozygous mutant mice replacing Otx2 with the human Otx1 (hOtx1) cDNA (hOtx12/hOtx12) 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 hOtx1 mRNA was detected in the VE and the epiblast, the hOTX1 protein was revealed only in VE. This VE-restricted translation of the hOtx1 RNA was sufficient to recover gastrulation defects and induction of an early anterior neural plate. From 8.5 d.p.c. onwards, however, hOtx12/hOtx12 embryos failed to maintain fore-midbrain identities, and at the end of gestation, displayed a headless phenotype (Acampora et al., 1998b) (Fig. 1 and Table 2). These results, indicate that at least in the AVE, OTX1 and OTX2 are functionally equivalent. Moreover, these findings indicate that Otx2 is necessary in the mesendoderm and/or the neuroectoderm at the late gastrulation stage, for the maintenance of anterior patterning of the neural plate. Overall, these data indicate that, with the exception of the inner ear phenotype, OTX1 and OTX2 gene products share an extended functional conservatism and suggest that Otx1 and Otx2 null mice contrasting phenotypes originate mostly from their divergent patterns of expression.

otd/Otx genes are also likely to have a conserved functional role in brain morphogenesis. This assumption is based on a remarkable similarity in homeodomain sequence, embryonic expression pattern and mutant phenotype (Cohen and Jürgens, 1991; Holland et al., 1992; Finkelstein and Boncinielli, 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 deuterocebral 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 mouse, Otx genes are required in early specification and patterning of anterior neuroectoderm, in corticogenesis as well as in visual and acoustic sense organ development (Acampora et al., 1995, 1996, 1997; Matsuo et al., 1995; Ang et al., 1996).

Nevertheless, in contrast to the extensive similarities in expression and mutant phenotype between 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 recognize the same target sequence might be evolutionarily conserved, murine Otx genes might have also 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, we produced mice replacing Otx1 with a full-coding Drosophila otd cDNA (Acampora et al., 1998a) (Table 1).

Interestingly, many of the abnormalities of Otx1-/- mice, such as impaired cell proliferation, corticogenesis and epilepsy are fully rescued by otd (Acampora et al., 1998a) regardless of a lower level of OTD (about 30% less) in otd/otd mice as compared to the Otx1 level in wild-type animals. To a lesser extent, Otx1-/- eye defects and brain patterning alterations detected in Otx1-/-; Otx2+/- embryos are also recovered. In contrast, the lateral semicircular canal of the inner ear of Otx1-/- mice is 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 OTD/OTX1 proteins, restricted essentially to the homeodomain. These two observations imply that otd and

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Otx2-/-</th>
<th>hOtx12/hOtx12</th>
</tr>
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<tbody>
<tr>
<td>Arrested development</td>
<td>100% at 9 d.p.c.</td>
<td>20% at 9 d.p.c.</td>
</tr>
<tr>
<td>Brain abnormalities</td>
<td>Lack of fore-mid and rostral hindbrain</td>
<td>Fore-midbrain re-patterning into anterior hindbrain</td>
</tr>
<tr>
<td>Visceral endoderm</td>
<td>Not anteriorized (distal)</td>
<td>Anteriorized</td>
</tr>
<tr>
<td>Primitive streak</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
<tr>
<td>Anterior mesendoderm</td>
<td>Absent or strongly impaired</td>
<td>Normal</td>
</tr>
<tr>
<td>Anterior patterning of early neural plate</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>Maintenance of anterior neural plate identity</td>
<td>-</td>
<td>Absent or strongly affected</td>
</tr>
<tr>
<td>Body plan</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* Note (see also the text) that in this knock-in model the OTX1 protein is expressed only in the visceral endoderm at early and mid-streak stages.
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 (Sharman and Brand, 1998). 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 (Acampora et al., 1998a; Sharman and Brand, 1998) confirms the existence of an Otx1-specific function acquired during evolution to specify this structure.

**Orthopedia** is required for development of the neuroendocrine hypothalamus

The hypothalamus and pituitary gland constitute the main axis of the neuroendocrine system (Simmons et al., 1990; Treier and Rosenfeld, 1996). By integrating signals from the periphery and brain this neuroendocrine system controls the synthesis and secretion of the hormones required for body growth, behavior, reproduction and metabolism (Felig et al., 1987; Wilson and Foster, 1992; Gass and Kaplan, 1996; Treier and Rosenfeld, 1996). The neuroendocrine hypothalamus consists of two distinct neuronal populations: the magnocellular neurons that are grouped in the PVN and SON nuclei, project their axons to the posterior pituitary, and release oxytocin (OT) and arginine vasopressin (AVP); and the parvocellular neurons that project to the median eminence (ME) where they release hypophysiotrophic hormones (Swanson, 1987; Sharp and Morgan, 1996). The parvocellular neurons located in the PVN nucleus release corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH). Hypophysiotrophic neurons synthesizing somatostatin (SS) or growth-hormone-releasing hormone (GHRH) which impart the principal inhibitory and stimulatory regulation of GH, are centered in the aPV and ARN. Parvocellular and magnocellular precursor neurons of the PVN and SON are generated in the mouse embryos between 10 and 12 d.p.c. in the supraoptic/paraventricular (spv) area (Karim and Shoper, 1980). A fraction of them remains in a medial position to give rise to the PVN, while the residual portion migrates laterally where they reach the final destination between 13.5 and 14.5 d.p.c. and form the SON (Karim and Shoper, 1980). Between 13.5 and 14.5 d.p.c. neuroendocrine neurons start to synthesize hormones and this event defines the terminal differentiation of the neuroendocrine hypothalamus.

Members of a large family of POU domain factors such as Pit1 and Brn2, have been extensively studied to clarify their role in the coordinate development of the hypothalamic-pituitary axis (Bodner et al., 1988; Ingraham et al., 1988; Li et al., 1990; Simmons et al., 1990; Nakai et al., 1995; Schonemann et al., 1995). Recently, it has been demonstrated that the bHLH-PAS transcription factor Sim1 plays a crucial role in the development of parvocellular and magnocellular neurons of the PVN, SON, and aPV as well as in the maintenance of Brn2 expression (Michaud et al., 1998). Consequently, neuroendocrine impairments detected in Brn2-/- mice (Nakai et al., 1995; Schonemann et al., 1995) are present also in mice lacking the Sim1 gene (Michaud et al., 1998). Moreover, mice lacking the homeodomain factor Gsh-1 do not synthesize GHRH in the ARN (Li et al., 1996) indicating that Gsh-1 is required for proper development of a specific neuroendocrine cell-lineage different from those requiring Sim1 and Brn2 (Li et al., 1996; Treier and Rosenfeld, 1996).

**Fig. 2. Schematic summary illustrating neuroendocrine impairments, abnormal developmental processes and presumptive genetic interactions deduced from the analysis of Otp/- mice.** (A) In wild-type mice, magnocellular and parvocellular neurons of the PVN, SON, aPV and ARN secrete OT, AVP, CRH, TRH, SS and GHRH neuropeptides. Magnocellular neurons located in the PVN and SON project their axons to the posterior lobe of the pituitary, while parvocellular neurons of the PVN, aPV and ARN project to the ME. In Otp/- mice, failure in terminal differentiation results in morphological disruption of the PVN and SON, impairment of the aPV and ARN, lack of CRH, TRH, AVP, OT and SS neuropeptide expression and impaired axonal outgrowth to the ME and posterior pituitary. (B) Developmental milestones of the neuroendocrine hypothalamus are represented by early commitment to the neuronal fate, neuroblast proliferation, lateral migration of postmitotic neurons to the area of nuclei formation and terminal differentiation including neuropeptide expression, axonal outgrowth and maintenance of cell viability. In Brn2/- and Sim1/- mice, terminal differentiation of cell lineages secreting CRH, AVP, OT (for Brn2 and CRH, AVP, OT, TRH and SS for Sim1) is disrupted. In Otp/- mice, besides the failure in terminal differentiation of CRH, AVP, OT, TRH and SS cell lineages also an impairment in both neuroblast proliferation and their subsequent migration are observed, indicating that Otp is an important requirement of most of the crucial steps necessary for proper development of the neuroendocrine hypothalamus. (C) Otp is required from 12 d.p.c. onwards for Brn2 and neuropeptide expression. Similar findings have been provided also in Sim1/- mice. Our data indicate that Sim1 and Otp are largely coexpressed and that Sim1 and Otp expression is unaffected in Otp/- and Sim1/- embryos, respectively, thus providing genetic evidence that they act in parallel and are both required for Brn2 expression. However, it cannot be argued from our data whether Sim1 and Otp require an additional genetic element in order to activate SS and TRH expression in the aPV and PVN, respectively. Finally, in the ARN, Otp is required only for SS but not for GHRH expression that is controlled by Gsh1. These data support the existence of a complex hierarchy of genetic interactions among transcription factors selectively required in specific cell lineages of the developing neuroendocrine hypothalamus.
*Otp* is a highly conserved homeodomain-containing factor that is expressed during murine embryonic development in a segment-like expression pattern including the AH, spv, rch and ventral tuberal areas (Simeone *et al.*, 1994; Avantaggiato *et al.*, 1995), that give rise to the aPV, PVN, SON, and ARN (Puelles and Rubenstein, 1993; Rubenstein *et al.*, 1994; Alvarez-Bolado *et al.*, 1995). In order to follow the fate of *Otp*-expressing cells, the *Otp* gene has been disrupted and replaced with the *lacZ* reporter gene whose expression results correctly driven by the *Otp* promoter elements (Acampora *et al.*, 1999d). *Otp* null mice die soon after birth and lack aPV, PVN and SON, while ARN is impaired but present.

The anatomo-histological analysis of *Otp*-/- newborn brains showed evident abnormalities in the hypothalamus. Indeed, the presumptive PVN and SON showed hypocellularity and absence of proper morphological features while the median eminence (ME) and the posterior lobe of the pituitary gland were extremely hypoplastic. In *Otp*-/- mice, TRH, CRH, OT, and AVP were not expressed in the PVN (TRH and CRH) or in the PVN and SON (AVP and OT); SS was absent in the aPV and in an extended area including the ARN and surrounding territory while GHRH was correctly transcribed (Fig. 2A). These findings prove that *Otp* codes for a critical function required for the correct development of the neurosecretory system and that terminal differentiation fails to be established in the PVN and SON, while it results partially impaired in the ARN.

Failure in terminal differentiation of specific neuroendocrine cell lineages has been reported in *Sim1* /-/-, *Brn2/-/-* and *Gsh1/-/-* mice (Schonemann *et al.*, 1995; Li *et al.*, 1996; Michaud *et al.*, 1998). In this context, the finding that *Otp* controls not only aPV, PVN and SON cell-types that require *Sim1* or *Sim1* and *Brn2*, but also SS-producing neurons of the ARN, provides further support to the concept that specific combinations of regulatory factors are necessary to confer differentiative information that underlies the acquisition of a specific cellular identity (Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Lamoniere *et al.*, 1996; Sharp and Morgan, 1996; Sormon *et al.*, 1996; Treier and Rosenfeld, 1996).

Interestingly, in 12.5 d.p.c. *Otp*-/- embryos, *Brn2* was not transcribed in the spv and the *Sim1* expression domain was narrowed in both the spv and AH primordia. At 14.5 d.p.c., the major abnormalities described at P1 were evident. Indeed, in *Otp*-/- embryos the *Sim1* expression disappeared from the presumptive PVN region. *Brn2* expression continued to be undetectable in the PVN and *lacZ* (*Otp*)/*Sim1* positive cells resulted remarkably reduced in number and abnormally positioned along the hypothalamus, thus, suggesting that besides terminal differentiation, also proper migration, proliferation and/or cell viability of *Otp* positive cells were impaired. *In situ* detection of apoptotic cells revealed no significant difference between *Otp*+/+ and *Otp*+/- embryos thus indicating that even though *Otp*+/- embryos were affected by increased cell death, this was not due to apoptosis.

On the other hand, by investigating the number of proliferating neuroblasts detected over a longer temporal window (between 10 and 12 d.p.c.), when neuronal precursors of PVN and SON are generated, it appeared that BrdU-positive cells amassed within the *lacZ* domain were remarkably decreased in *Otp*+/- embryos and their position abnormal. Therefore, these data indicate that, at least between 10.3 and 12 d.p.c., *Otp* is required for normal proliferating activity and that a decreased neuroblast proliferation may contribute to explain the reduction in *lacZ* positive cells (Fig. 2B).

In *Otp*-/- brain at P1, the *lacZ* expression domain is heavily affected, since it is abnormal in position and extent. Indeed, the residual *lacZ* positive cells instead of being localized in the presumptive PVN and SON, occupy a ventro-lateral domain where they are never found in normal embryos. This finding suggests that the absence of a single gene function, namely *Otp*, results in the abnormal positioning of cells normally fated to generate hypothalamic neuroendocrine nuclei and suggests that in these cells proper migration and differentiation are controlled by the same gene product. In summary, *Otp* plays a role in proliferation and/or survival as well as in migration and differentiation processes. Multiple requirements might be the consequence of different cell and stage-specific roles, or, alternatively, all the abnormalities identified might be the consequence of an early and unique role that appears first manifested with abnormal proliferation and reduced cell number and, later, with impaired migration and failure in terminal differentiation (Fig. 2B). This aspect is potentially of great interest since raises the question of whether events underlying neuroendocrine development are hierarchically independent or independent. Further experiments based on cell-restricted and stage-specific inactivation of *Otp* might provide insights into these aspects.

**Otp acts upstream of Brn2 and in parallel with Sim1**

In order to investigate whether *Otp* might be downstream of *Sim1*, *Otp* expression was studied in *Sim1* /-/- embryos. In these mutants *Otp* was stably transcribed in the territory expressing the *Sim1* null alleles indicating that the *Otp* expression does not require the *SIM1* gene product to be maintained. Analysis of *Brn2* mutant mice has revealed that *Brn2* acts relatively late in neuroendocrine development, being required for terminal differentiation events of CRH, AVP and OT cell lineages (Schonemann *et al.*, 1995; Sharp and Morgan, 1996; Treier and Rosenfeld, 1996).

*Sim1* mutant mice showed a more general effect, since they were impaired in terminal differentiation events leading to the activation of neuropeptides of the PVN and SON as well as of SS in the aPV (Michaud *et al.*, 1998). Interestingly, from E12.5 d.p.c. onwards, *Sim1* /-/- mutants gradually lack *Brn2* expression in the dorsal spv primordium, indicating that *Sim1* acts upstream of *Brn2* and is required for maintenance of its expression (Michaud *et al.*, 1998). Data on the *Otp* /-/- phenotype reveal a striking similarity with *Sim1* mutant phenotype. Indeed, except in the ARN, *Otp* is fully coexpressed in time and space with *Sim1*, and is required for both terminal differentiation of parvocellular and magnocellular neurons of aPV, PVN and SON and for maintenance of *Brn2* expression. Interestingly, in *Otp* /-/- embryos, *Sim1* expression is maintained in *lacZ* positive cells where *Brn2* is lost and, in *Sim1* /-/- embryos, *Otp* is expressed in the territory where *Brn2* disappears. These findings provide strong *in vivo* evidence that *Otp* and *Sim1* act in parallel and are both required for proper expression of *Brn2* in the spv and its derivatives, the PVN and SON (Fig. 2C).

Furthermore, in the ARN of *Otp* /-/- brains, SS but not GHRH expression is affected. Interestingly, in the ARN the homeobox-containing gene *Gsh1* is essential for GHRH expression (Li *et al.*, 1996). Therefore, summing up our data, *Otp* is required early for proper proliferating activity and subsequently for correct migration and terminal differentiation events that involve maintenance of *Brn2* expression, activation of parvocellular and magnocellular neuropeptide gene expression, axonal outgrowth and cell survival.
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References


Otx and Otp genes in brain development

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