Int. J. Dev. Biol. 44: 669-677 (2000)

The role of *Otx* and *Otp* genes in brain development

DARIO ACAMPORA, MARIA PIA POSTIGLIONE, VIRGINIA AVANTAGGIATO,
MARIA DI BONITO and ANTONIO SIMEONE*

International Institute of Genetics and Biophysics, Naples, Italy.

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.*, 1990; 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.

***Address correspondence to:** Antonio Simeone. International Institute of Genetics and Biophysics. CNR, Via G. Marconi, 12, 80125, Naples, Italy.
TEL: 39 081 593 4652. FAX: 39 081 593 6123 or 725 7202. e-mail: simeone@iigbna.iigb.na.cnr.it

0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

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 region (spv), the anterior hypothalamic area (AH) and the retrochiasmatic region (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 postmigratory 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 (βFSH), luteinizing 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, 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 GnRHR) 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

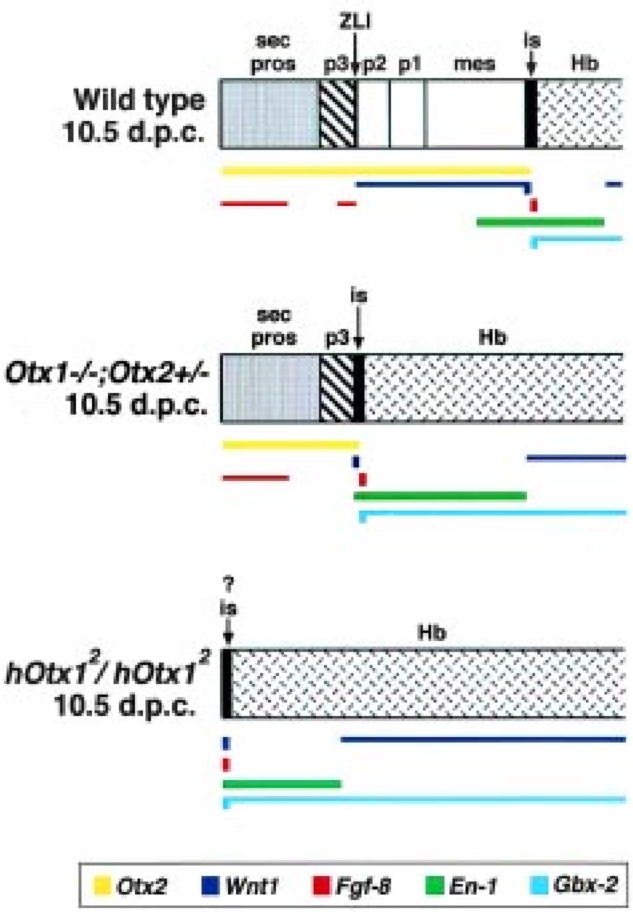

Fig. 1. *Otx1-/-; Otx2+/-* and *hOtx1^2/hOtx1^2* brain patterning abnormalities at 10.5 d.p.c. In wild-type embryos, the expression pattern of molecular markers, such as *Otx2, Wnt1, Fgf-8, En-1* and *Gbx-2* define the sharp molecular code of the isthmic organizer. In *Otx1-/-; Otx2+/-* mutants, this code is coordinately shifted rostrally, driving a re-patterning process that transforms posterior diencephalon and mesencephalon in an expanded rostral hindbrain. In *hOtx1^2/hOtx1^2* mutants, the re-patterning process is more dramatic due to the absence of any OTX proteins 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 organizer; Hb, hindbrain.

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 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 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. Elegant 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 pretectum, 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-met region 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 with different fates such as the mesencephalon and metencephalon, 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 (Wassarmann *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 1

COMPARISON OF MAJOR PHENOTYPES OF *Otx1-/-*, *hOtx2^1/hOtx2^1* AND *otd^1/otd^1* MUTANT MICE

Phenotype	*Otx1-/-*	*hOtx2^1/hOtx2^1*	*otd^1/otd^1*
Early telencephalic abnormalities			
Cell proliferation	Reduced by 25%	Similar to wild-type	Similar to wild-type
Whole brain size	Reduced in weight by 25%	Similar to wild-type	Similar to wild-type
Cerebral cortex			
Cell number	Heavy reduction in thickness and 40% drop in cell number of temporal and perirhinal areas	Temporal and perirhinal areas similar to wild-type	Temporal and perirhinal areas similar to wild-type
Anatomy and histology	Difficult detection of sulcus rhinalis Shrinking of hippocampus Disorganization of temporal and perirhinal cortical layers	Normal detection of sulcus rhinalis Normal hippocampus Hexalaminar organization	Normal detection of sulcus rhinalis Normal hippocampus Hexalaminar organization
Mesencephalon	Enlarged	Normal in 30% Intermediate in 45%	Normal in 15% Intermediate in 50%
Cerebellum			
Cerebellar foliation	Normal in 25%	Normal in 50%	Normal in 10%
Behaviour	Epileptic seizures High-speed turning behaviour	No seizures Moderate-speed turning behaviour	No seizures Moderate-speed turning behaviour
Ear (lateral semicircular duct)	Absent	Absent	Absent
Lachrimal and Harderian glands	Absent	Normal in 75%	Normal in 34%
Eye			
Ciliary process	Absent	Normal in 70%	Normal in 80%

TABLE 2

PHENOTYPES OF *Otx2-/-* AND *hOtx1*^{2}/*hOtx1*^{2} MUTANT MICE

Phenotype	*Otx2-/-*	*hOtx1*^{2}/*hOtx1*^{2*}
Arrested development	100% at 9 d.p.c.	20% at 9 d.p.c. 80% between E10 and P1
Brain abnormalities	Lack of fore-mid and rostral hindbrain	Fore-midbrain re-patterning into anterior hindbrain
Visceral endoderm	Not anteriorized (distal)	Anteriorized
Primitive streak	Abnormal	Normal
Anterior mesendoderm	Absent or strongly impaired	Normal
Anterior patterning of early neural plate	Absent	Normal
Maintenance of anterior neural plate identity	-	Absent or strongly affected
Body plan	Abnormal	Normal

* 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.

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 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 *hOtx2*^{1}/*hOtx2*^{1} mice suggests that *Otx1* and *Otx2* might have interchangeable roles in the cortex. *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 (Morsli *et al.,* 1999; Acampora and Simeone, 1999; and see below).

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 *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, *hOtx1*^{2}/*hOtx1*^{2} 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 conservation 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 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 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*^{1}/*otd*^{1} 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

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*^{1}/*otd*^{1} 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 Sloper, 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).

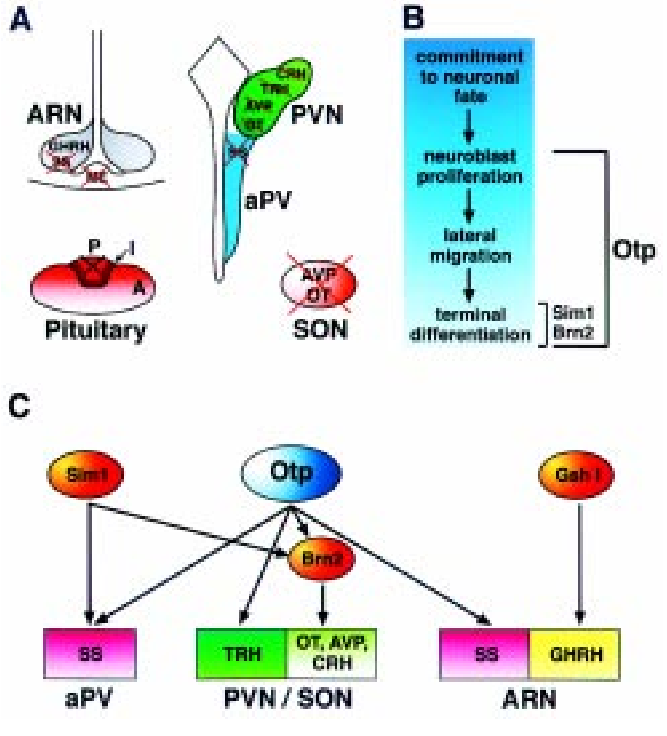

Fig. 2. Schematic summary illustrating neuroendocrine impairments, abnormal developmental processes and presumptive genetic interactions deduced from the analysis of *Otp-/-* mice. (A) In wild-type mice, magnocellular and parvocellular neurons of the PVN, SON, aPV and ARN secrete OT, AVP, CRH, TRH, SS 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 transcribed 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; Sornson *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 interdependent 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.

Acknowledgments

We would like to thank A Secondulfo for skillful secretarial assistance. This work was supported by the "MURST-CNR Biotechnology Programme Legge 95/95", the "CNR Target Project on Biotechnology" and the EC BIOTECH programme.

References

ACAMPORA, D. and SIMEONE, A. (1999). Understanding the roles of *Otx1* and *Otx2* in controlling brain morphogenesis. TINS 22*:* 116-122.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F. and SIMEONE, A. (1997). Genetic control of brain morphogenesis through *Otx* gene dosage requirement. *Development* 124: 3639-3650.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BARONE, P., REICHERT, H., FINKELSTEIN, R. and SIMEONE, A. (1998a). Murine *Otx1* and *Drosophila otd* genes share conserved genetic functions required in invertebrate and vertebrate brain development. *Development* 125: 1691-1702.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BRIATA, P., CORTE, G. and SIMEONE, A. (1998b). Visceral endoderm-restricted translation of *Otx1* mediates recovering of *Otx2* requirements for specification of anterior neural plate and proper gastrulation. *Development* 125: 5091-5104.

ACAMPORA, D., MAZAN, S., TUORTO, F., AVANTAGGIATO, V., TREMBLAY, J. J., LAZZARO, D., DI CARLO, A., MARIANO, A., MACCHIA, P. E., CORTE, G., MACCHIA, V., DROUIN, J., BRÛLET, P. and SIMEONE, A. (1998c). Transient dwarfism and hypogonadism in mice lacking *Otx1* reveal prepubescent stage-specific control of pituitary levels of GH, FSH and LH. *Development* 125: 1061-1072.

ACAMPORA, D., AVANTAGGIATO, V., TUORTO, F., BARONE, P., PERERA, M., CORTE, G. and SIMEONE, A. (1999a). Differential transcriptional control as the major molecular event in generating *Otx1-/-* and *Otx2-/-* divergent phenotypes. *Development* 126: 1417-1426.

ACAMPORA, D., BARONE, P. and SIMEONE, A. (1999b). *Otx* genes in corticogenesis and brain development. *Cerebral Cortex* 9: 533-542.

ACAMPORA, D., GULISANO, M. and SIMEONE, A. (1999c). *Otx* genes and the genetic control of brain morphogenesis. *Mol. Cell. Neuros.* 13: 1-8.

ACAMPORA, D., POSTIGLIONE, M.P., AVANTAGGIATO, V., DI BONITO, M., VACCARINO, F.M., MICHAUD, J. and SIMEONE, A. (1999d) Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the *Orthopedia* gene. *Genes Dev.* (13: 2787-2800)

ACAMPORA, D., MAZAN, S., AVANTAGGIATO, V., BARONE, P., TUORTO, F., LALLEMAND, Y., BRÛLET, P. and SIMEONE, A. (1996). Epilepsy and brain abnormalities in mice lacking *Otx1* gene. *Nature Genet.* 14: 218-222.

ACAMPORA, D., MAZAN, S., LALLEMAND, Y., AVANTAGGIATO, V., MAURY, M., SIMEONE, A. and BRÛLET, P. (1995). Forebrain and midbrain regions are deleted in *Otx2-/-* mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* 121: 3279-3290.

ALTMAN, J. and BAYER, S.A. (1988). Development of the rat thalamus: I. Mosaic organization of the thalamic neuroepithelium. *J. Comp. Neurol.* 275: 346-377.

ALVAREZ-BOLADO, G., ROSENFELD, M.G. and SWANSON, L.W (1995). Model of forebrain regionalization based on spatio-temporal patterns of POU-III homeobox gene expression, birthdates, and morphological features. *J. Comp. Neurol.* 355: 237-295.

ANG, S.-L., CONLON, R.A., JIN, O. and ROSSANT, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* 120: 2979-2989.

ANG, S.-L., JIN, O., RHINN, M., DAIGLE, N., STEVENSON, L. and ROSSANT, J. (1996). Targeted mouse *Otx2* mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* 122: 243-252.

AVANTAGGIATO, V., PANDOLFI, P.P., RUTHARDT, M., HAWE, N., ACAMPORA, D., PELICCI, P.G. and SIMEONE, A. (1995). Developmental analysis of the murine *Promyelocyte Leukaemia Zinc Finger (PLZF)* gene expression: implications for the neuromeric model of the forebrain organization. *J. Neurosci.* 15: 4927-4942.

BALLY-CUIF, L. and WASSEF, M. (1995). Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* 5: 450-458.

BEDDINGTON, R.S.P. and ROBERTSON, E.J. (1998). Anterior patterning in mouse. *Trends Genet.* 14: 277-283.

BODNER, M., CASTRILLO, J.L., THEILL, L.E., DEERINCK, T., ELLISMAN, M. and KARIN, M. (1988). The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* 55: 505-518.

BOUWMEESTER, T., KIM, S.H., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* 382: 595-601.

COHEN, S.M. and JÜRGENS, G. (1990). Mediation of *Drosophila* head development of gap-like segmentation genes. *Nature* 346: 482-488.

COHEN, S.M. and JÜRGENS, G. (1991). *Drosophila* headlines. *Trends Genet.* 7: 267-272.

CROSSLEY, P.H. and MARTIN, G.R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryos. *Development* 121: 439-451.

CROSSLEY, P.H., MARTINEZ, S. and MARTIN, G.R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* 380: 66-68.

FIGDOR, M.C. and STERN, C.D. (1993). Segmental organization of embryonic diencephalon. *Nature* 363: 630-634.

FINKELSTEIN, R. and BONCINELLI, E. (1994). From fly head to mammalian forebrain: the story of *Otd* and *Otx*. *Trends Genet.* 10: 310-315.

FINKELSTEIN, R. and PERRIMON, N. (1990). The *orthodenticle* gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development. *Nature* 346: 485-488.

FINKELSTEIN, R., SMOUSE, D., CAPACI, T.M., SPRADLING, A.C. and PERRIMON, N. (1990). The *orthodenticle* gene encodes a novel homeodomain protein involved in the development of the *Drosophila* nervous system and ocellar visual structures. *Genes Dev.* 4: 1516-1527.

FRANTZ, G.D. and McCONNELL, S.K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* 17: 55-61.

FRANTZ, G.D., WEIMANN, J.M., LEVIN, M.E. and McCONNELL, S.K. (1994). *Otx1* and *Otx2* define layers and regions in developing cerebral cortex and cerebellum. *J. Neurosci.* 14: 5725-5740.

GALLERA, J. (1971). Primary induction in birds. *Adv. Morph.* 9: 149-180.

GASS, G.H. and KAPLAN, H.M. (Eds.) (1996). *Handbook of Endocrinology.* 2nd edn. CRC Press, Inc., Boca Raton.

HIDALGO-SANCHEZ, M., SIMEONE, A. and ALVARADO-MALLART, R.M. (1999). *Fgf8* and *Gbx2* induction concomitant with *Otx2* repression is correlated with midbrain-hindbrain fate of caudal prosencephalon. *Development* 126: 3191-3203.

HIRTH, F., THERIANOS, S., LOOP, T., GEHRING, W.J., REICHERT, H. and FURUKUBO-TOKUNAGA, K. (1995). Developmental defects in brain segmentation caused by mutations of the homeobox gene *orthodenticle* and *empty spiracles* in Drosophila. *Neuron* 15: 1-20.

HOLLAND, P., INGHAM, P. and KRAUSS, S. (1992). Mice and flies head to head. *Nature* 358: 627-628.

INGRAHAM, H.A., CHEN, R.P., MANGALAM, H.J., ELSHOLTZ, H.P., FLYNN, S.E., LIN, C.R., SIMMONS, D.M., SWANSON, L. and ROSENFELD, M.G. (1988). A tissue-specific transcription factor containing a homeobox specifies a pituitary phenotype. *Cell* 55: 519-529.

JOYNER, A.L. (1996). *Engrailed, Wnt* and *Pax* genes regulate midbrain-hindbrain development. *Trends Genet.* 12: 15-20.

KARIM, M.A. and SLOPER, J.C. (1980). Histogenesis of the supraoptic and paraventricular neurosecretory cells of the mouse hypothalamus. *J. Anatom.* 130: 341-347.

LAMONERIE, T., TREMBLAY, J.J., LANCTÔT, C., THERRIEN, M., GAUTHIER, Y. and DROUIN, J. (1996). *Ptx1*, a bicoid-related homeobox transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.* 10: 1284-1294.

LEMAIRE, P. and KODJABACHIAN, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet.* 12: 525-531.

LEUZINGER, S., HIRTH, F., GERLICH, D., ACAMPORA, D., SIMEONE, A., GEHRING, W., FINKELSTEIN, R., FURUKUBO-TOKUNAGA, K. and REICHERT, H. (1998). Equivalence of the fly *orthodenticle* gene and the human *OTX* genes in embryonic brain development of Drosophila. *Development* 125: 1703-1710.

LI, H., ZEITLER P.S., VALERIUS, M.T., SMALL, K. and POTTER, S.S. (1996). *Gsh-1,* an orphan *Hox* gene, is required for normal pituitary development. *EMBO J.* 15:

714-724.

LI, S., CRENSHAW, E.B., RAWSON, E.J., SIMMONS, D.M., SWANSON, L.W. and ROSENFELD, M.G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* 347: 528-533.

MARIN, F. and PUELLES, L. (1994). Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. *Dev. Biol.* 163: 19-37.

MARTINEZ, S., CROSSLEY, P.H., COBOS, I., RUBENSTEIN, J.L. and MARTIN, G.R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. *Development* 126: 1189-1200.

MARTINEZ, S., WASSEF, M. and ALVARADO-MALLART, R.-M. (1991). Induction of a mesencephalic phenotype in the 2 day-old chick prosencephalon is preceded by the early expression of the homeobox gene *en. Neuron* 6: 971-981.

MATSUO, I., KURATANI, S., KIMURA, C., TAKEDA, N. and AIZAWA, S. (1995). Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev.* 9: 2646-2658.

MEINHARDT, H. (1983). Cell determination boundaries as organizing: regions for secondary embryonic fields. *Dev. Biol.* 96: 375-385.

MICHAUD, J.L., ROSENQUIST, T., MAY, N.R. and FAN, C.-M. (1998). Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. *Genes Dev.* 12: 3264-3275.

MILLET, S., BLOCH-GALLEGO, E., SIMEONE, A. and ALVARADO-MALLART, R.-M. (1996). Is the caudal limit of *Otx2* gene expression a marker of the midbrain/hindbrain boundary? A study using a chick-*Otx2* riboprobe and chick/quail homotopic grafts. *Development* 122: 3785-3797.

MORSLI, H., TUORTO, F., CHOO, D., POSTIGLIONE, M.P., SIMEONE, A. and WU, D. (1999). *Otx1* and *Otx2* activities are required for the normal development of the mouse inner ear. *Development* 126: 2335-2343.

NAGAO, T., LEUZINGER, S., ACAMPORA, D., SIMEONE, A., FINKELSTEIN, R., REICHERT, H. and FURUKUBO-TOKUNAGA, K. (1998). Developmental rescue of *Drosophila* cephalic defects by the human *Otx* genes. *Proc. Natl. Acad. Sci. USA* 95: 3737-3742.

NAKAI, S., KAWANO, H., YUDATE, T., NISHI, M., KUNO, J., NAGATA, A., JISHAGE, K., HAMADA, H., FUJII, H., KAWAMURA, K., SHIBA, K. and NODA, T. (1995). The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. *Genes Dev.* 9: 3109-3121.

PUELLES, L. and RUBENSTEIN, J.L.R. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *TINS.* 16: 472-479.

RAKIC, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* 145: 61-84.

RAKIC, P. (1974). Neurons in the rhesus monkey visual cortex: systematic relationship between time of origin and eventual disposition. *Science* 183: 425-427.

RHINN, M., DIERICH, A., SHAWLOT, W., BEHRINGER, R.R., LE MEUR, M. and ANG, S.-L. (1998). Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* 125: 845-856.

RUBENSTEIN, J.L.R., MARTINEZ, S., SHIMAMURA, K. and PUELLES, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* 266: 578-580.

RUBENSTEIN, J.L.R., SHIMAMURA, K., MARTINEZ, S. and PUELLES, L. (1998). Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* 21: 445-477.

SCHONEMANN, M.D., RYAN, A.K., MCEVILLY, R.J., O'CONNELL, S.M., ARIAS, C.A., KALLA, K.A., LI, P., SAWCHENKO, P.E. and ROSENFELD, M.G. (1995). Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2. *Genes Dev.* 9: 3122-3135.

SHARMAN, A.C. and BRAND, M. (1998). Evolution and homology of the nervous system: cross-phylum rescues of *otd/Otx* genes. *Trends Genet.* 14: 211-214.

SHARP, D.Z. and MORGAN, W.W. (1996). Brain POU-er. *BioEssays 18:* 347-350.

SIMEONE, A. (1998). *Otx1* and *Otx2* in the development and evolution of the mammalian brain. *EMBO J.* 17: 6970-6978.

SIMEONE, A., ACAMPORA, D., GULISANO, M., STORNAIUOLO, A. and BONCINELLI, E. (1992). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* 358: 687-690.

SIMEONE, A., ACAMPORA, D., MALLAMACI, A., STORNAIUOLO, A., D'APICE, M.R., NIGRO, V. and BONCINELLI, E. (1993). A vertebrate gene related to *orthodenticle* contains a homeodomain of the *bicoid* class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* 12: 2735-2747.

SIMEONE, A., D'APICE, M.R., NIGRO, V., CASANOVA, J., GRAZIANI, F., ACAMPORA, D. and AVANTAGGIATO, V. (1994). *Orthopedia,* a novel homeobox-containing gene expressed in the developing central nervous system of both mouse and Drosophila. *Neuron* 13: 83-101.

SIMMONS, D.M., VOSS, J.W., INGRAHAM, H.A., HALLOWAY, J.M., BROIDE, R.S., ROSENFELD, M.G. and SWANSON, L.W. (1990). Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev.* 4: 695-711.

SORNSON, M.W., WU, W., DASEN, J.S., FLYNN, S.E., NORMAN, D.J., O'CONNELL, S.M., GUKOVSKY, I., CARRIÈRE, C., RYAN, A.K., MILLER, A.P., ZUO, L., GLEIBERMAN, A.S., ANDERSEN, B., BEAMER, W. and ROSENFELD, M.G. (1996). Pituitary lineage determination by the *Prophet* of *Pit-1* homeodomain factor defective in Ames dwarfism. *Nature* 384: 327-333.

SPEMANN, H. and MANGOLD, H. (1924). Über induktion von Embryonanlagen durch Implantation artfremder Organisatoren. *Wilhelm Roux Arch. Entw. Mech. Organ.* 100: 599-638.

STOREY, K.G., CROSSLEY, J.M., DE ROBERTIS, E.M., NORRIS, W.E. and STERN, C.D. (1992). Neural induction and regionalization in the chick embryo. *Development* 114: 729-741.

SUDA, Y., MATSUO, I. and AIZAWA, S. (1997). Cooperation between *Otx1* and *Otx2* genes in developmental patterning of rostral brain. *Mech. Dev.* 69: 125-141.

SWANSON, L.W. (1987). The hypothalamus. In *Handbook of chemical neuroanatomy. Part I.* (Eds. A. Bjorklun, T. Hokfelt, and L.W. Swanson). Elsevier Publishing Company, Amsterdam, pp. 1-124.

TAM, P.P.L. and BEHRINGER, R.R. (1997). Mouse gastrulation: the formation of a mammalian body plan. *Mech. Dev.* 68: 3-25.

THOMAS, P. and BEDDINGTON, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr. Biol.* 6: 1487-1496.

THOMAS, P.Q., BROWN, A. and BEDDINGTON, R.S. (1998). *Hex*: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* 125: 85-94.

THOR, S. (1995). The genetics of brain development: conserved programs in flies and mice. *Neuron* 15: 975-977.

TREIER, M. and ROSENFELD, M.G. (1996). The hypothalamic-pituitary axis: co-development of two organs. *Curr. Opin. Cell Biol.* 8: 833-843.

VAAGE, S. (1969). The segmentation of the primitive neural tube in chick embryos (*Gallus domesticus*). *Adv. Anat. Embryol. Cell Biol.* 41: 1-87.

VARLET, I., COLLIGNON, J. and ROBERTSON, E.J. (1997). *nodal* expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* 124: 1033-1044.

WASSARMAN, K.M., LEWANDOSKI, M., CAMPBELL, K., JOYNER, A.L., RUBENSTEIN, J.L.R., MARTINEZ, S. and MARTIN, G.R. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* 124: 2923-2934.

WILSON, J.D. and FOSTER, D.W. (1992). *Williams Textbook of Endocrinology.* 8th edn., W. B. Saunders Company, Philadelphia.

YOUNOSSI-HARTENSTEIN, A., GREEN, P., LIAW, G.J., RUDOLPH, K., LENGYEL, J. and HARTENSTEIN, V. (1997). Control of early neurogenesis of the *Drosophila* brain by the head gap genes *tll, otd, ems* and *btd. Dev. Biol.* 182: 270-283.