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Otx and *Emx* homeobox genes in brain development

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ABSTRACT Over the last few years great progress has been made in the understanding of the formation and regionalisation of the mouse brain. In this review we will focus our attention on two families of homeobox-containing genes essentially coding for four transcription factors involved in brain and forebrain development: the two *Emx* and the two *Otx* genes. Here we describe the expression pattern of these genes in the developing mouse, as well as the characterisation of the corresponding knockout mice with special emphasis on *Emx2*. Whereas *Otx* genes are clearly involved in the formation and regionalisation of the whole rostral brain, comprised of forebrain and midbrain, our data suggest a role for *Emx2* in the specification of the cytoarchitecture of the cerebral cortex, achieved through the control of proliferation of neuronal precursors and of migration of newly-formed neurons to their final destination.

KEY WORDS: *Emx, Otx, mouse forebrain development, cerebral cortex formation.*

Introduction

The development and formation of forebrain and particularly of cerebral cortex is one of the most fascinating and intriguing fields of research in developmental neurobiology. Many are the questions still unanswered in this field, but the intense efforts of several laboratories working in this area have provided interesting and useful insights as to what happens in a developing mammalian cerebral cortex. Among the topics yet to be elucidated, some are particularly interesting: how neuroblasts proliferate and become neural cells, how they then migrate, either radially or tangentially from the proliferative area to the different districts of the brain, and how they differentiate in order to form and pattern the mature cerebral cortex.

Most of the results obtained in this field come from the analysis of mouse embryonic brain development. *In situ* and immunohistochemistry techniques, as well as the transgenesis and knock-out approaches have been essential tools for the elucidation of some functions of the genes thought to be involved in these processes. Mouse central nervous system (CNS), i.e. the brain and spinal cord, derives from the neural plate, and is first distinguishable at day 7.5 of embryonic life (E7.5), at the late primitive streak stage. The first and most evident process occurring in the developing nervous system from E8.5 of mouse development is regionalization along the antero-posterior axis. By E10.0 forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon) and spinal cord domains are formed. Over the last few years significant progress has been made in the understanding of the molecular mechanisms underlying the patterning of midbrain and hindbrain (Bally-Cuif and Wassef, 1995; Guthrie, 1995 for reviews), whereas the development of the forebrain remains relatively obscure.

In the mouse forebrain, the telencephalon vesicle appears at E9.0, whereas cerebral cortex can be seen from E9.5. Here, neurogenesis starts at E11.0 ventrolaterally and at E12.0 dorsomedially, peaks around E12.5-13.5, and continues at lower levels until postnatal day 17 (P17) (Bayer and Altman, 1991), whereas differentiation and migration of these cells continue after birth. The cerebral cortex is characterized by at least three major layers: the innermost germinative neuroepithelium or ventricular zone (VZ), where neuroblasts proliferate, the transitional field (TF), and the cortical plate (CP), which will subsequently form the cortical grey matter. From the beginning of corticogenesis and up to E12.5 the cortex entirely consists of VZ. Later on the TF differentiates into the subventricular and the intermediate zones, the latter one representing a transition area for differentiating cortical cells before they migrate to outer regions.

Later on during the development of the embryo, the proliferative area becomes thinner and thinner, whereas the thickness of the TF and the CP progressively increases. At this time, one can notice two major neurogenetic and morphogenetic gradients, one anterior to posterior and one ventrolateral to dorsomedial. At the end of this process, the CP will consist of six cortical layers originated according to an “inside-out” developing rule (Bayer and Altman, 1991). Neurons located deep in the cortex are generated first and

Abbreviations used in this paper: A/P, anterior-posterior; BrdU, Bromodeoxyuridine; CNS, Central Nervous System; CP, Cortical Plate; CR, Cajal-Retzious cells; E, Embryonic day; TF, Transitiona field; VZ, Ventricular Zone.

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neurons subsequently generated bypass them by active migration movements and settle above them in more external locations.

Homeobox genes in the developing mouse brain

An important contribution to the understanding of the development and formation of the forebrain has come from the characterization of several regulatory genes coding for transcription factors. A large part of these are homeobox genes, such as the members of the *Dlx*, *Emx*, *Lim*, *Otx* and (in part) *Pax* gene families. The patterning of this region is often associated with precise antero-posterior expression domains or gradients of certain genes. Among these genes, *Emx1* and *Emx2*, the mouse homologues of *Drosophila melanogaster ems* (*empty spiracles*) and *Otx1* and *Otx2*, homologous to *otd* (*orthodenticle*), play a primary role (Simeone *et al*., 1992a, 1992b, 1993; Finkelstein and Boncinelli, 1994; Boncinelli, 1999).

The onset of expression of the four genes during mouse development varies. *Otx2* is already expressed at the gastrula stage of E5.5 mouse embryos (Simeone *et al*., 1993), whereas *Otx1* and *Emx2* can be detected in E8-8.5 embryos. Finally, *Emx1* expression starts from E9.5 (Frantz *et al*., 1994; Gulisano *et al*., 1996). In fore- and mid-brain all four genes are expressed between day 9.5 and 10.5 of mouse development, in extended regions of the developing rostral brain, including the presumptive cerebral cortex and olfactory bulbs (Simeone *et al*., 1992a). The *Emx* genes are also expressed in the uro-genital apparatus from the very beginning of its development (Miyamoto *et al*., 1997).

In the forebrain, *Emx1* expression domain includes the dorsal telencephalon, the posterior boundary being slightly anterior to that between presumptive di- and telencephalon. *Emx2* expression seems to define the boundary between future dorsal and ventral thalamus. The gene is expressed in dorsal and ventral neuroectoderm, its anterior boundary lying slightly anterior to that of *Emx1* and the posterior one within the roof of the presumptive diencephalon. *Otx1* expression domain includes dorsal and basal telencephalon, di- and mesencephalon (Simeone *et al*., 1993; Frantz *et al*., 1994; Gulisano *et al*., 1996). Its anterior boundary almost overlaps that of *Emx2*. Finally, *Otx2* expression domain includes the entire fore- and mid-brain both dorsally and ventrally, approximately down to the rhombic isthmus (Simeone *et al*., 1993). *Otx1* and *Otx2* expression is also detected in restricted regions of diencephalon: epithalamus, dorsal thalamus and mammillary region of posterior hypothalamus, where the expression is almost exclusively restricted to cells of the ventricular zone. *Otx* transcripts are also present in mesencephalic regions of tectum and tegmentum, as well as in developing special sense organs, such as the olfactory epithelium, the developing inner ear and the developing eye, including the external sheath of the optic nerve (Simeone *et al*., 1993, Boncinelli *et al*., 1993). Focusing our attention on murine cerebral cortex development, it is important to stress that all four genes are expressed between E9.5 and E10.5 in this area. *Emx1, Emx2* and *Otx1* transcripts are present during a more extended period of time corresponding to major events in cortical neurogenesis (Simeone *et al*., 1993). On the other hand, starting from E10.75, *Otx2* expression progressively disappears from this region. This process is relatively quick and starts in the central areas of both hemispheres. These data suggest a possible role played by the four genes in establishing the identity of some embryonic brain regions.

Over the last few years much useful information concerning the pattern of expression and the potential role of the four *Emx* and *Otx* genes has been accumulated through the concerted efforts of different labs, including ours. The detailed expression pattern has been analyzed for most of them using both *in situ* and immunohistochemistry techniques; in particular, localization of OTX2 (Mallamaci *et al*., 1996), EMX1 (Briata *et al*., 1996), and EMX2 (Mallamaci *et al*., 1998) proteins has been investigated. On the other hand, knock-out mice have been produced for each of the four genes, as well as double knock-out mutants for some of them: the results obtained provide insights into their functional role.

Otx genes in early and midgestation development

The *Otx2* gene is the first of the four genes to be activated during development. In fact, it is already expressed in the E5.5 mouse blastocyst, more precisely in the entire epiblast, from which all embryonic tissues will originate. From E7 its domain of expression becomes restricted rostrally, contributing to the specification of the rostral brain (Simeone *et al*., 1993). The localization of OTX2 protein in mouse embryos parallels that of the corresponding transcript. An interesting feature is the gradient observed in the distribution of the protein, with a maximum in the anterior neuroectoderm of the headfold and a gradually decreased level of expression in the more posterior ectoderm (Mallamaci *et al*., 1996).

Interestingly, a detailed analysis of the distribution of OTX2 has shown the presence of the protein in migrating granule cells and their precursors in the cerebellum from E9.5. Here the signal can be seen until P15, when the majority of these cells have reached their final destination. OTX2 plays an essential role also in the olfactory area, where it has been suggested to determine the formation of the olfactory placode and its derivatives (Matsuo *et al*., 1995). In the olfactory epithelium of mid- to late gestation mouse embryos, expression of OTX2 is observed in post-mitotic as well as in differentiated cells. Like in the cerebellum, all migrating cells in the olfactory area do express OTX2. This suggests a possible involvement of these cells in fasciculating and guiding nerve fibers, as previously described for *Otx1* and *Otx2* transcripts (Nguyen *et al*., 1998). A further evidence for this role comes from heterozygous *Otx2*$^{+/-}$ embryos, frequently displaying abnormal fasciculation and dorsally directed axons of oculomotor and trochlear nerves (Matsuo *et al*, 1995).

Otx transcripts in di- and mesencephalon of E12.5-14.5 embryos colocalize with boundary regions and presumptive axon tracts, including anterior and posterior commissures (Boncinelli, 1994). Expression is detected in precursor cells surrounding these structures, which might represent a border for pioneer axon tracts. This is particularly visible in posterior commissure and along the zona limitans intrathalamica (Boncinelli *et al*., 1993). Both *Otx* genes are also expressed around the developing optic nerve. This localization, like that along the zona limitans intrathalamica, might be responsible for axon pathfinding and patterning. In this light, *Otx* genes are likely to provide a primary scaffold for specific axon pathways in the neuroepithelium of the developing forebrain. We suggest that *Otx* genes play at least two roles in head development, at two different stages. They first define territories or areas in rostral brain of E8-E10 mouse embryos, and provide later on a set of positional cues required for growing axons to follow specific pathways within the embryonic CNS. It is not clear whether the two functions are related.

Otx2$^{-/-}$ knockout embryos are early embryonic lethal, as they fail to gastrulate and stop developing at early midgestation (Matsuo *et al*., 1995; Acampora *et al*., 1995; Ang *et al*., 1996). By E8.25 the rostral part of the neural tube, corresponding to mid- and forebrain, is absent. This phenotype is consistent with a multi-functional role for *Otx2* in gastrulation and in the patterning of rostral brain in the mouse. On the other hand, frog embryos deriving from zygotes where *Otx2* has been overexpressed (Pannese *et al*., 1995) show severely reduced trunk and tail structures as well as an expansion of anterior head structures including pharynx and forebrain (Boncinelli and Mallamaci, 1995). We demonstrated (Morgan *et al*., 1999) that a protein of the calponin family is able to implement *Otx2*-mediated determination of head versus trunk cell identity in early frog embryos, through the control of cell migration movements, highly reduced in early head cells and very active in early trunk cells. Very recently, the caudal border of expression of *Otx2* has been analyzed as to its function in positioning the mid/hindbrain organizer (isthmic organizer). Two different experiments shifting the *Otx2* domain expression either more caudally (Broccoli *et al*, 1999) or rostrally (Millet *et al*., 1999), show that the caudal limit *Otx2* expression is sufficient for the correct positioning of the isthmic organizer.

Otx1 is expressed in the VZ, particularly in the deepest layers of telencephalic cortex since their birth. In the adult cortex *Otx1* is expressed in layer VI and in a subpopulation of neurons in layer V (Frantz, 1994; Gulisano, 1996). *Otx1*$^{-/-}$ mice are viable and show alterations in brain development as well as spontaneous epileptic seizures (Acampora *et al*., 1996). Particularly affected in these mice are the dorsal telencephalic cortex, mesencephalon and cerebellum. One can therefore attribute to *Otx1* a role in regional specification of the developing brain. Very recently, it has been shown that the *Drosophila otd* gene can rescue the phenotype in *Otx1* $^{-/-}$ mice, supporting the idea of evolutionary conservation for the function of *otd/Otx* genes in head development in flies and mice (Acampora *et al*., 1998). Recently, the hypothesis of the requirement of a specific threshold of OTX proteins for the correct regionalization and patterning of the rostral neurotube as well as for the correct positioning of the isthmic organizer has been demonstrated (Acampora *et al*., 1997).

Emx genes in the developing forebrain

Emx2 is one of the earliest markers for the developing cerebral cortex. *In situ* hybridization along with immunohistochemistry techniques have revealed that from E8.5 the signal becomes progressively stronger in the anterior dorsal neuroectodermal regions of the embryo. By E9.5 an anterior boundary delimiting the expression domain appears, overlapping that of *Emx1*, as well as a posterior one, located within the roof of the presumptive diencephalon. At this stage, *Emx2* mRNA is also detected in the ventral roof of the presumptive diencephalon as well as in the olfactory placodes. From E10.0 and during the formation of the cerebral cortex the neuroepithelium becomes the only area of expression of *Emx2*, whereas most postmitotic neurons of the TF and CP lack these transcripts, suggesting a potential role for the gene in neuroblasts proliferation. At E12.5, cerebral cortex essentially coincides with the ventricular zone: here the *Emx2* mRNA signal is uniformly distributed, and shows a gradient along the A/P axis, being stronger in the posterior dorsal telencephalon and gradually decreasing in intensity for the anterior boundary. At this stage the gene is still expressed in ventral thalamus, olfactory bulbs, as well as in the developing olfactory epithelium.

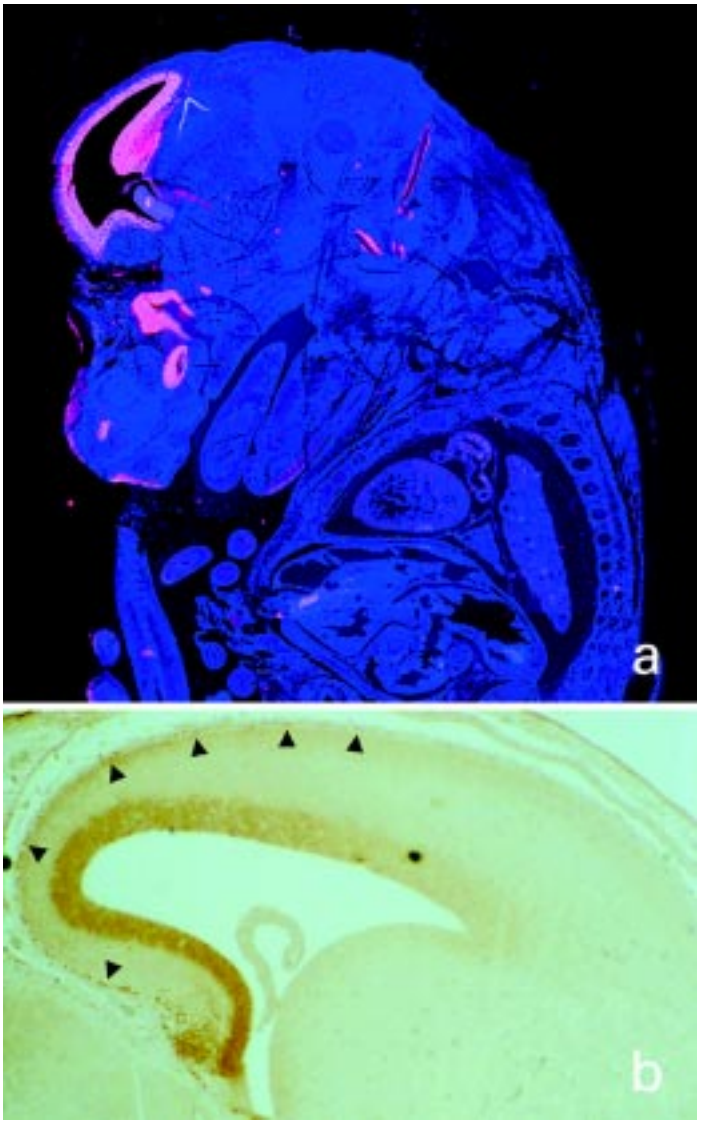

Fig. 1. (a) Distribution of Emx2 mRNA (pink signal) in a sagittal section of a 15.0 day of gestation (E15) mouse embryo using *in situ* analysis. The gene is expressed in the forebrain, olfactory bulbs, inner ear, digits primordia, kidneys and gonads. **(b)** Immunohistochemical analysis of a frontal section of an E14.5 mouse embryonic forebrain using an EMX2 polyclonal antibody (brown staining). Black arrows show the presence of the protein in Cajal-Retzius cells. The signal is very strong in the ventricular zone of the developing cerebral cortex. The staining also shows a gradient of distribution of the protein along the medio-lateral axis.

Between E13.5 and E17.0, the expression domain of *Emx2* is still confined to the proliferative layer of the cortex and from E14.5 onwards the gradient of expression becomes more pronounced, progressively decreasing in intensity in anterior and ventrolateral regions. The presence of *Emx2* in the neuroepithelium of the cerebral cortex makes the gene an excellent marker for this important layer, where neuroblasts divide, giving rise to all neurons subsequently present in the mature cerebral cortex. *Emx2* expres-

sion in the VZ of midgestation mouse embryos follows an antero-posterior gradient of intensity, higher in posterior cortical regions than in anterior ones (Simeone *et al*., 1992b; Gulisano *et al*., 1996). This antero-posterior gradient of expression of the transcript seems to follow the gradient in the maturation of neuroblasts during corticogenesis, the anterior regions having earlier mature cells than the posterior ones, supporting the hypothesis of a role for *Emx2* in neuroblasts proliferation.

By the end of gestation *Emx2* also marks specific cell layers in hippocampus and dentate gyrus. At P2 the number of cells expressing *Emx2* in cerebral cortex becomes irrelevant, as the thickness of the VZ is very limited compared to that of the TF and CP. BrdU pulse-labeling experiments confirmed the *in situ* results, showing that *Emx2* transcript is present in the proliferative layer of the cortex and absent from most postmitotic neurons (Gulisano *et al*, 1996).

We have performed immunohistochemistry experiments in order to directly localize the EMX2 protein and we were able to show that from E9.0 the pattern of distribution of the protein parallels that of the corresponding mRNA (Mallamaci *et al*, 1998). EMX2 has in fact been detected in the anterior CNS, in the olfactory epithelium and in the uro-genital system. In particular, recent data from our laboratory indicate that the distribution of the EMX2 protein also follows a clear antero-posterior and medio-lateral gradient. Interestingly, cortical neurogenesis follows the opposite gradient, with a rostro-lateral maximum and a caudal-medial minimum (Bayer and Altman, 1991). EMX2 might therefore play a role either as an inhibitor of cell proliferation or as a positive regulator of cell differentiation. *Emx2* graded expression, both at the mRNA and protein (Mallamaci *et al*., 1998) levels, suggests a contribution of the gene to cortical polarity and cell identity in the VZ (O'Leary, 1994). Being *Emx2* a regulatory gene coding for a transcription factor, it represents an ideal candidate for generating and/or maintaining a position-dependent signal within the developing cortex.

The high resolution of immunohistochemistry techniques allowed us to look in detail into the cerebral cortex. To our surprise, not only could the protein be detected in the proliferative layer, but interestingly also in the nuclei of Cajal-Retzius (CR) cells, a transient cell population consisting in the first born neurons in neocortex and forming the most superficial cell layer just underneath the pial membrane. They are thought to be responsible for guiding radial migration of neurons from the VZ to their final destination across the CP (Ogawa *et al*., 1995). This finding suggests a potential role for *Emx2* in radial neuronal migration through the CP during corticogenesis. In this respect, analysis of the spontaneous mouse *reeler* phenotype has suggested that REELIN, the gene product of the *Reln* gene, is one of the molecules acting in this process (Ogawa *et al*., 1995; D'Arcangelo *et al*, 1995; Hirotsune *et al*., 1995). REELIN is a large protein secreted by the Cajal-Retzius cells and thought to interact with a target protein present in the matrix or on cell surface. The *reeler* mouse mutant displays an autosomal recessive phenotype manifesting itself by the abnormal layering of the cortical plate. Newborn neurons seem in fact to be unable to bypass pre-existing populations of older neurons, thus originating an inverted "outside-in" pattern. This phenotype has been also observed in *scrambler* mice (Sweet *et al*, 1996), affected by an autosomal recessive mutation in the *scm* gene. Immunocytochemical studies suggest that *scm* may act downstream of *Reln*, in a common signaling pathway controlling neuronal migration during development (Gonzalez *et al*., 1997). Taken together, these results indicate that not only might *Emx2* control the proliferation of cortical neuroblasts, but also regulate their subsequent migration process both directly and indirectly, as it is known that these cells finally settle in the mature cortex according to their birth date (McConnell, 1995).

The analysis of *Emx2*$^{-/-}$ knockout mice has confirmed the expression data obtained for *Emx2* at the mRNA and protein levels (Pellegrini *et al*., 1996; Yoshida *et al*., 1997; Miyamoto *et al*., 1997). Analysis of null embryo brains has indicated major structural changes in the architecture of various regions, including the cerebral cortex. Homozygous null mice show reduced olfactory bulbs, altered patterning of hippocampus and the total absence of dentate gyrus (Pellegrini *et al*., 1996; Yoshida *et al*., 1997). They die within a few hours after birth, owing to the lack of development of the uro-genital system (Miyamoto *et al*., 1997). As the hippocampus and the dentate gyrus are known to be required for declarative memory in humans, a conditional knockout approach could be very informative for studying the processes of learning and memory as well as the structure of cerebral cortex in adult knockout mice.

In order to better understand the function of *Emx2*, we performed a more detailed analysis of the homozygous null mice (Mallamaci *et al*., 1999). Interestingly, we found that in these embryos Cajal-Retzius cells are absent from the first layer of the CP. As a consequence, the settling of radial glia is impaired and neurons display abnormal *reeler*-like migration patterns. We also performed BrdU pulse-labeling experiments between E12.0 and E15.0, i.e. when cortical neurons start migrating to the CP. The data obtained show that at E19.0 the normal layering of the CP is affected in null mutant mice, resulting in a *reeler*-like phenotype, again indicating an involvement of the gene in neuronal migration processes. We thus investigated whether *Emx2* does belong to the same pathway as *Reln* in guiding radial migration during corticogenesis and found that EMX2 distribution in E15 *Reln*$^{-/-}$ mouse embryo cerebral cortex is normal compared to the wild-type (unpublished data). We can therefore conclude that *Emx2* transcription is not dependent on *Reln* transcripts in the control of neuronal migration.

The intriguing hypothesis that *Emx2* might play a role in cortical migration led us to analyze phenotypes related to cortical dysgenesis and particularly to cell proliferation and migration disorders, such as the human schizencephaly, lissencephaly and double cortex syndromes. Schizencephalies are rare human developmental defects of the cerebral cortex (Barkovich and Norman, 1988; Wolpert and Barnes, 1992; Granata *et al*., 1996). These congenital brain malformations are characterized by full-thickness clefts within the cerebral hemispheres. Affected patients display different motor and mental problems, according to the severity and extent of the brain malformation. They are frequently affected by epilepsy. A genetic analysis of 18 schizencephalic patients has shown that 13 of them carry mutations in EMX2 in the heterozygous condition (Faiella *et al*., 1997). For at least some cases a correlation can be made between the molecular defect and the severity of the disease. In fact severe mutations (i.e. frameshifts or splicing mutations) are associated with severe bilateral forms of schizencephaly, whereas molecularly milder or leaky mutations are associated with lighter manifestations of the disorder. This

finding supports the hypothesis that EMX2 is required for the correct formation of the human cerebral cortex. Conversely, we have been so far unable to find an involvement of EMX2 in other types of congenital cortical malformations.

The presence of *Emx1* mRNA can be detected from E9.5 in the cortical region of mouse forebrain. As development continues, the gene is expressed in most of the neurons forming the cortex, either proliferating, migrating, differentiating, or fully differentiated and organized in the mature cerebral cortex (Gulisano *et al*., 1996). *Emx1* is also highly expressed in a subset of subplate neurons. These are thought to take part in early functional circuitry: they receive synaptic inputs from waiting thalamic afferents and make axonal projections to the CP (Allendoerfer and Shatz, 1994). These observations suggest a possible involvement of *Emx1* in some of these crucial events as well as in defining specific cellular identities in the cerebral cortex.

Interestingly, analysis of the distribution of EMX1 protein, which was expected to overlap with that of the corresponding mRNA, revealed some new localizations (Briata *et al*., 1996). Most of the EMX1 signal can be detected in the nuclei of cells of the developing telencephalon, including the presumptive cerebral cortex, olfactory bulbs and hippocampus. Just like the transcript, the protein is present in virtually all cortical neurons, in varying amounts, during proliferation, migration, differentiation and maturation. EMX1 is first detectable in the dorsal telencephalon at E9.5, as the corresponding mRNA, where it persists until E11.5. The signal is particularly strong in the VZ between E10.5 and E17.5. At birth and shortly after it becomes more intense in the forming layers V and VI as well as in the subplate. As previously discussed for the transcript, it is very hard to ascribe a precise function to EMX1 protein in the developing telencephalon. We might speculate about a role in conferring cellular identity to neurons of the cortex, since the protein is expressed throughout corticogenesis.

Totally unexpected was the presence of EMX1 along the entire length of the developing and post-natal olfactory nerve. The protein can in fact be detected from the olfactory epithelium of the developing nose to the terminals touching the olfactory bulb. This localization makes it very difficult to define a role for a transcription factor along axons out of the nucleus. It is though conceivable that EMX1 may assist olfactory sensory neurons in finding their way to specific glomeruli.

These data have been confirmed studying *Emx1*$^{-/-}$ mice, which are viable and display slight defects restricted to the forebrain (Yoshida *et al*., 1997). The CP and the white matter are thinner while the cortical subplate is hardly visible. Hippocampus is generally smaller and the number of fibers of the anterior commissure is unchanged but their guidance is aberrant. It is likely that *Emx1* and *Emx2* genes play interchangeable roles during cerebral cortex formation. *Emx2/Emx1* double mutant analysis should be of great help in this investigation.

In conclusion, it is increasingly clear that *Emx* and *Otx* genes represent powerful tools for the study of the developing brain and in particular of the cerebral cortex during neurogenesis and differentiation. Any alteration occurring in these processes is thought to be responsible for severe developmental defects and pathologies of the nervous system. It is therefore likely that mutations occurring in one of the four genes or in some of their targets, affecting the germ or the somatic line, may underlie a number of brain defects (Guerrini *et al*, 1996).

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