RAPID COMMUNICATION

Mutation of the Emx-1 Homeobox Gene Disrupts the Corpus Callosum

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Expression of the Emx-1 homeobox gene is largely restricted to the developing and mature cerebral cortex. To study its function, two lines of mice were generated using gene targeting methods that have a deletion that includes the N-terminal coding region of Emx-1. Mice homozygous for the deletion were viable and fertile and exhibited no obvious behavioral defects. However, 100% of homozygous mice lack most or all of their corpus callosum, the principal fiber tract that connects the left and right cerebral hemispheres. Heterozygotes show partial penetrance for the corpus callosum abnormality. The histology and various molecular properties of the cerebral cortex appear normal in the mutant mice.

INTRODUCTION

The cerebral cortex is the seat of higher neurologic functions. An analysis of its development is now possible using genetic methods due to the identification of candidate regulatory genes (Bulfone et al., 1995) and the availability of gene targeting methods. Several homeobox genes related to Drosophila gap and segment identity genes have been characterized that are expressed in the developing cerebral cortex, including members of the empty spiracle (Emx) and orthodenticle (Otx) families (Simeone et al., 1992a,b). Among these genes, only Emx-1 is primarily expressed in the cerebral cortex, where it is expressed in both proliferating and postmitotic cells (Simeone et al., 1992b; Qiu, Shimamura, and Rubenstein, unpublished). Thus, Emx-1 is a candidate regulator of proliferation, migration, and differentiation. To study its role in cortical development, we made a mouse strain lacking a functional Emx-1 gene.

MATERIALS AND METHODS

Isolation of Emx-1 cDNA and Genomic Clones and Generation of Targeting Vector and Mutant ES Cells

An Emx-1 cDNA was isolated by screening an E11.5 mouse head library (Qiu, unpublished) with a probe homologous to the Drosophila empty spiracle homebox. The cDNA was sequenced on both strands using oligonucleotide primers and the USB sequenase kit. The cDNA was used to isolate genomic DNA encoding Emx-1 from 129-strain mice (from Anton Berns, Amsterdam). The targeting construct was made using pBS KS+ as the cloning vector. PCR generates a 273-bp fragment from exon 1; the primers were 5'-ATGGTGCCACCGGCGGAGT-3' and 5'-GGGTTGCAGCGAGGAGGAGC-3', and the conditions were 1 cycle 94°C 2 min; 35 cycles 94°C 1 min, 63°C 1 min, 72°C 1 min; 1 cycle 72°C 6 min.

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A. *Emx-1* genomic organization and targeting strategy

![Diagram of Emx-1 genomic organization and targeting strategy]

**FIG. 1.** (A) Partial map of the *Emx-1* genomic organization; the location of exons 1 and 2 (boxes), and the position of BamHI (B), EcoRI (E), NotI (N), and XhoI (X) sites are indicated. The positions of probes (P1 and P2) used for Southern analysis are shown. In the middle is shown the *Emx-1* targeting vector, indicating the 2.2-kb deletion that includes exon 1 (between XhoI and NotI), and the insertion of the PGK neo cassette, oriented in parallel to the direction of *Emx-1* transcription. The bottom tier shows the structure of the mutated *Emx-1* locus. (B) Southern analysis of F2 progeny using Probe 1 (P1) and an EcoRI digest; the wild-type fragment is 10.5 kb, and the mutant fragment is 6 kb. (C) Northern analysis of wild-type, heterozygote, and homozygote mutant E14.5 embryos, using probe 2 (P2).

B. Southern analysis of the *Emx-1* F2 Progeny

![Southern blot analysis]

C. Absence of *Emx-1* expression in the E14.5 homozygous embryos

![Northern blot analysis]

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Northern Analysis and in Situ RNA Hybridization

Northern analysis was performed using standard methods (Sambrook et al., 1989). In situ hybridization was performed as described by Qiu et al. (1995). The Otx-1 and Id-2 probes were obtained from Antonio Simeone and Marc Israel, respectively.

Histological Analysis

Postnatal animals were sacrificed and perfusion fixed using 4% paraformaldehyde in PBS. The brains were then removed and fixed in 4% paraformaldehyde in PBS for 12 hr and processed for either cryostat or paraffin sectioning. Sections were Nissl-stained to study the general histology of the brain. DiI was used to study the trajectory of the corpus callosum.

RESULTS AND DISCUSSION

We generated a mutation in Emx-1 that resulted in a 2.2-kb deletion between Notl and Xhol sites that removed exon 1 and part of intron 1 (Fig. 1A); this removed the N-terminus of the EMX-1 protein (140 out of 257 amino acids; Qiu and Rubenstein, unpublished). As a result of this mutation, a PGKneo gene cassette was inserted into the deleted region. Two independent ES clones harboring the mutation were identified using Southern analysis, and both of these clones were used to generate mice chimeric of the mutation. Mice chimeric for cell lines 30 and 44 were made, and both transmitted the mutant allele via their germ line. These heterozygous mice were inbred to generate mice homozygous for the mutant allele (Fig. 1B). The homozygous mutant mice were viable, fertile, and without any obvious morphological or behavioral phenotype. We demonstrated that the homozygous mutant mice lacked Emx-1 transcripts when probed with a DNA fragment from the deleted region (Fig. 1C).

The phenotype of the cerebral cortex was analyzed in Nissl-stained tissue sections of paraformaldehyde-fixed paraffin-embedded Postnatal Day 60 mutant mice brains (Fig. 2). We found that 100% of homozygous mutant mice (13 samples analyzed) lacked most or all of the corpus callosum (a small number of fibers crossed the dorsal midline in the region of the hippocampal commissure; this may be the dorsal commissure of the fornix which is difficult to distinguish from the CC). These findings were confirmed by labeling the subcortical white matter by placing DiI crystals in the cerebral cortex (Figs. 2G, 2H). None of the wild-type mice (0/15) had an abnormal corpus callosum, whereas 5/15 heterozygotes had this phenotype. We observed abundant white matter collecting adjacent to the commissural plate, and occasional ectopic fibers migrating in the ipsilateral cortex (Probst bundle; see Ozaki and Wahlsten, 1993). Thus, it appears as though commissural fibers formed, but were not able to cross the commissural plate. At this point it is not clear whether the primary abnormality is in the commissural fibers or in the commissural plate.

The histological appearance of the neocortex, limbic cortex (hippocampus, entorhinal, cingulate, insular), and paleocortex (olfactory bulb and piriform cortex) in the mutant mice were indistinguishable from wild-type littermates. Molecular properties of the mutant cerebral cortex were analyzed by immunohistochemistry and in situ RNA hybridization (data not shown). The expression of the glutamate GlnR2-3 receptor in wild-type and mutant adult somatosensory cortex was indistinguishable, showing that the pyramidal projection neurons in layers 2, 3, and 5 were present. The expression of Otx-1, Tbr-1, and Id-2 RNA in the cerebral cortex of wild-type and mutant P6 animals was indistinguishable. The spatial distribution of Emx-1 expression was unaltered in the mutant, as judged by using a probe from exon 2 (data not shown). This demonstrates that Emx-1 protein is not required for maintenance of Emx-1 expression.

The CC abnormality can be seen at low penetrance in several inbred mouse strains including 129/J and Balb/c. In these mice, absence of the CC is found in about 20–30% of adults (Ozaki and Wahlsten, 1993). This is of concern because the Emx-1 mutation was made in ES cells derived from 129/J strain mice. However, the penetrance of the CC phenotype is 100% in Emx-1+/−, and we have not detected the CC abnormality in any (0/15) Emx-1+/− litter mates; this strongly suggests that the CC phenotype is due to the Emx-1 deletion and not due to the genetic background of the mice. Because some of the +/− also show a defective CC (5/15), this phenotype may be Emx-1 dosage sensitive. The fact that we have not observed any CC abnormalities in the Emx-1+/− animals may be due to the fact that we have outbred the Emx-1+/− mice with C57BL/6 mice, which do not have the CC abnormality.

FIG. 2. Nissl-stained cryostat coronal sections of wild-type (A, C, E; from different levels of the same brain) and homozygote mutant (B, D, F; from different levels of the same brain) 10-week-old litter mates. While the mutant animals lack most of the corpus callosum (CC) (B and D), a small interhemispheric commissure, which might be a residual CC, can be seen in panel F, overlaying the hippocampal commissure (hcc). A bundle of fibers (Probst bundle, pb), that may include fibers which would otherwise contribute to the CC, are seen in B, D, and F. D shows that the anterior commissure (ac) is present in the mutant. DiI-labeled wild-type (G) and homozygous mutant (H) coronal sections, just rostral to the locations shown in E and F, show that some fibers cross the midline in the mutant. The DiI crystal was applied to the parietal cortex. The dotted line marks the dorsal midline of the cerebral cortex. Other abbreviations: cx, neocortex; sp, septum; st, striatum. The magnification bar in D is 500 μm.
There are multiple etiologies associated with agenesis or small CC, including at least 70 different human syndromes (Norman et al., 1995). In Balb/c and 129/J mice, Ozaki and Wahlsten (1992, 1993) have provided evidence that an abnormality in the “substrates of axon guidance at the midsagittal plane” are the primary defects that predispose to the CC defect. Earlier work in Balb/cCF mice reported that failure to form the CC has been associated with a defective transient glial structure (glial sling) that traverses the dorsal midline prior to the formation of the corpus callosum (Silver and Ogawa, 1983). The molecular nature of these defects is not known. As Emx-1 is expressed in most of the cells in the developing neocortex (Simeone et al., 1992; unpublished data), including those in layers 2 and 3 that are known to contribute processes to the CC, Emx-1 may regulate the expression of cell surface or other molecules that are required to form the CC. Alternatively, Emx-1 may regulate morphogenesis of the telencephalon that secondarily affects formation of the CC.

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