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***Cux-1* and *Cux-2* control the development of Reelin expressing cortical interneurons**

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Summary

Homeodomain transcription factors play important roles in the specification and differentiation of neuronal subpopulations. In the cerebral cortex, the expression patterns of *Cux-1* and *Cux-2* in the medial ganglionic eminence (MGE) suggest a role for these transcription factors in the development of interneurons, a heterogeneous neuronal population. In this report, we describe expression of *Cux-1* and *Cux-2* proteins in Reelin-secreting interneurons of the cortical plate, but not in calretinin or parvalbumin subpopulations. The role of *Cux* genes in the development of Reelin positive neurons was studied using *Cux-1* and *Cux-2* knockout mice. These experiments demonstrate that *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation is embryonically lethal. Although this phenotype is highly penetrant, a small proportion of mice develop to birth (P0). Analysis of these animals demonstrate that expression of Reelin is completely absent in layers II–IV of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice, but it is not affected in the cortex of *Cux-1*^{-/-} or *Cux-2*^{-/-} single mutants. No *Cux-1*^{-/-}; *Cux-2*^{-/-} double-mutant were collected after P0. Since, GABA-ergic populations mature at late postnatal stages, this did not allow us to analyze the expression of subclass specific markers and define the affected interneuron subpopulations. Our analysis of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant thus demonstrates essential yet redundant roles for *Cux-1* and *Cux-2* in specifying Reelin expressing cortical interneurons.

Keywords

cerebral cortex; *Cux-1*; *Cux-2*; interneurons

Introduction

Inhibitory GABA-ergic interneurons are a heterogeneous population as defined by their morphology, electrophysiological properties and expression of cellular markers. This diversity reflects distinct neuronal molecular identities and precursor origin. However, little is known about the intrinsic factors that define each interneuron subpopulation and direct the differentiation of their specific precursors.

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Interneurons originate in the ventral telencephalon during development and migrate tangentially to reach the cortical plate. Most interneurons are born at the medial ganglionic eminence (MGE), and not the lateral ganglionic eminence (LGE), but some subpopulations are born at the caudal ganglionic eminence (CGE) (reviewed in (Flames and Marin, 2005)).

Reelin is a large extracellular matrix glycoprotein secreted by several populations of neocortical neurons (Alcantara et al., 1998). Most known is the expression of Reelin by the Cajal-Retzius neuronal population of layer I during embryonic development (D'Arcangelo et al., 1995; D'Arcangelo et al., 1997). At postnatal stages, expression of Reelin gradually disappears from layer I and appears in subsets of neurons distributed across cortical layers II–VI (Alcantara et al., 1998). The majority of these Reelin positive neurons in the cortical plate express markers of GABA-ergic interneurons such as somatostatin, neuropeptide Y or calretinin (Alcantara et al., 1998). Thus, Reelin is expressed by GABA-ergic interneurons but does not mark a specific homogeneous interneuron population.

Homeobox transcription factors are involved in embryonic patterning and cell type specification. The transcription factors *Cux-1* and *Cux-2* are homologous to the *Drosophila* homeobox gene *Cut*. In the adult brain, expression of *Cux-1* and *Cux-2* selectively marks the upper cortical layers (II–IV) of the cerebral cortex, with only a few scattered neurons in the lower layers (V–VI) and the hippocampus expressing *Cux-1* and *Cux-2* (Nieto et al., 2004). During development, *Cux-1* and *Cux-2* genes are early markers of neuronal differentiation and are expressed in neural precursors in the telencephalon (Nieto et al., 2004; Zimmer et al., 2004). In the ventral telencephalon, *Cux-1* is expressed both in the ventricular zone (VZ) and the subventricular zone (SVZ) of the LGE, MGE and CGE (Nieto et al., 2004). In contrast, *Cux-2* marks the SVZ of the MGE and is not expressed in the LGE or the CGE (Nieto et al., 2004; Zimmer et al., 2004). The expression of *Cux* genes in these ventral telencephalon regions thus suggests possible roles in interneuron differentiation. Moreover, the overlapping expression of *Cux-1* and *Cux-2* in the MGE indicates possible redundant functions for *Cux* proteins in neurons originating in this region. A previous report showed that *Cux-1* knockout (ko) mice (*Cux-1*^{-/-}) die shortly after birth and show abnormal growth, but have no phenotype specifically related to the development of the nervous system (Luong et al., 2002). We have generated and analyzed *Cux-2* ko mice (*Cux-2*^{-/-}), which survive normally but present excessive production of upper layer neurons, that packed at abnormally high cellular densities (Cubelos et al., 2007).

Here we report the expression of *Cux-1* and *Cux-2* by a subpopulation of Reelin expressing interneurons (Alcantara et al., 1998) that occur throughout the cortical plate (layers II–VI) of perinatal wild-type (WT) animals. To investigate the roles of *Cux-1* and *Cux-2* in the specification of these neuronal subpopulations we set out to analyze Reelin expression in the brains of *Cux-1*^{-/-} and *Cux-2*^{-/-} single mutant mice as well as in *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant animals. In the course of these experiments we found that *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation is embryonically lethal, suggesting a function for *Cux* genes early in embryonic development. However, although this phenotype is highly penetrant, a small proportion of mice develop to birth. Analysis of the expression of upper and lower cortical layer markers, such as *Brn-1* and *Foxp-1* (Sugitani et al., 2002; Ferland et al., 2003), suggests that the majority of upper and lower pyramidal neurons of the *Cux-1*^{-/-} and *Cux-2*^{-/-} single mutants, and of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice, correctly acquire their early laminar identity. In contrast, the development of cortical interneurons was impaired by the loss of *Cux* function: while Reelin expression in the cortical plate of *Cux-1*^{-/-} or *Cux-2*^{-/-} single mutants was not affected, it was absent from cortical layers II–VI of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice. In conclusion, our data indicate novel and important roles for *Cux* genes in interneuron differentiation.

Methods

Animals

All animal procedures were approved by the Centro Nacional de Biotecnología Animal Care and Use Committee in compliance with National and European Legislation. The generation of *Cux-2* null allele (*Cux-2*^{-/-}) is reported elsewhere (Cubelos et al. 2007). Briefly, a targeting construct was designed to conditionally eliminate exons 22 and 23, which encode the third Cut repeat and part of the homeodomain. Mice carrying the conditional allele (*Cux2loxP*) were mated with mice expressing CRE recombinase under the human beta actin promoter (Tg (ACTB-CRE)2Mrt deleter mice; Jackson Laboratories) to obtain mice giving germline transmission of the floxed null allele. *Cux-2*^{+/-} animals were mated to obtain *Cux-2* homozygous mutant mice (*Cux-2*^{-/-}). *Cux-1*^{-/-} mice have been described previously (Luong et al., 2002), and were obtained from A.J. van Wijnen (Umass. MA. USA). Animals were maintained on a C57BL6: Swiss Webster background. Morning of the day of the appearance of the vaginal plug was defined as embryonic day (E) 0.5.

Antibodies, immunohistochemistry and histology

Mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by cold 4% paraformaldehyde in PBS. The perfused brains were removed and post-fixed in 4% paraformaldehyde at 4 °C. Brains were embedded in parafin and sectioned (5µm) or were cryoprotected in 30% sucrose in PBS and sectioned on a cryostat to produce either 10–20 µm cryosections on Superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA) or 50–100 µm floating cryosections. Sections were blocked for 1 h at room temperature (r.t.) with 5% horse serum in PBST (PBS containing 0.5% Triton-X 100; blocking solution) and then incubated for 1 h at r.t. or overnight at 4 °C with primary antibodies diluted in blocking solution. Fluorescent-tagged secondary antibodies (in PBS, 5% horse serum) were applied for 1 hour at r.t., and sections were counterstained with Hoechst 33342 (Molecular probes, Eugene,OR) and mounted in Aqua-polymount mounting medium (Poly-Labo). Peroxidase/diaminobenzidine immunohistochemical (IHC) staining was performed as described (Cubelos et al., 2005). Briefly, after incubation with primary antibody, sections were incubated with biotinylated donkey anti-rabbit IgG (Sigma) for 1 h. Sections were then washed 3 times in PBS, incubated with streptavidin-biotinylated horseradish peroxidase complex, washed, and incubated with 0.1 mg/ml H₂O₂, 0.5 mg/ml diaminobenzidine in PBS. Sections were mounted in glycerol-gelatin.

The following primary antibodies were used at the dilutions indicated: rabbit polyclonal anti-Cux-1 (clone M222) (1:10) and anti-Brn-1 (1:50) (Santa Cruz Biotechnologies, inc. CA); rabbit polyclonal anti-phospho-histone H3 (pH3) (1:500) (Upstate, Spartanburg, SC); rabbit polyclonal anti-Cux-2 (antibody 356, a gift from Dr. Alex Nepveu of McGill University Health Centre, Canada). Mouse anti-Reelin CR50 (1:50) was kindly provided by M. Ogawa, (RIKEN Brain Science Institute, Wako, Japan) and rabbit anti-Reelin (1:500) and anti-parvalbumin (clone PARV-19) were from Sigma (St. Louis, Missouri). Alexa 488- and 594-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Molecular Probes) were applied at 1:500. Before staining for Cux-1, Cux-2, sections were boiled for 30 min in the antigen retrieval vector solution (Vector, CA). This was followed, in the case of staining for BrdU, CldU, IdU and pH3, by 30 min treatment with 2N HCl.

Confocal microscopy and imaging

Confocal microscopy was performed with a Radiance 2100 (Bio-Rad) Laser Scanning System on a Zeiss Axiovert 200 microscope. For fluorescence excitation, an argon ion laser (488 nm), a Krypton-Neon laser (543 nm) and a red diode (637 nm) were employed. The combination of filters used was as follows: a 560 DCLPXR beam splitter and an HQ 515/30

emission filter for detection of Alexa 488; and a 650 DCLPXR beam splitter with an HQ 590/70 for Alexa 594. Sequential images were taken with LaserSharp v5.0 software (Bio-Rad) and analyzed using LaserPix v.4 image software (Bio-Rad).

Results

Cux-1 and Cux-2 label populations of Reelin expressing neurons in the cortical plate

Cux-1 and *Cux-2* label the majority of neurons of the upper layers of the cerebral cortex and also scattered neurons distributed throughout the deep layers (Nieto et al., 2004). The expression pattern in the deep layers overlaps with the expression of Reelin in the cortical plate (Alcantara et al., 1998). Reelin is secreted by Cajal-Retzius cells in layer I, which are eliminated during postnatal development, and by cortical neurons that appear at late embryonic and early postnatal stages. To observe both populations of Reelin positive cells, we performed double labeling immunofluorescence studies at P0 to determine whether neurons in this region coexpress Reelin and Cux-1 and Cux-2 proteins. This analysis showed that Cux-1, but not Cux-2, is expressed in the Cajal-Retzius neurons of layer I (Fig 1, panels A, B), and that both Cux-1 and Cux-2 proteins are expressed in Reelin expressing neurons (Fig 1, panels C, D) distributed throughout the layers II–VI. These Cux-positive neurons display a bipolar morphology characteristic of some interneuron populations. Cux-1 and Cux-2 immunoreactivity was also detected in scattered cells in the marginal zone of the hippocampus, but neither Cux protein was localized in Reelin positive cells in this region (Fig 1, panels E, F).

Most of the markers for specific interneuron subpopulations are expressed only in the late postnatal and adult cortex in mature neurons. Double labeling experiments with Cux-1 and Cux-2 rabbit polyclonal antisera are confined to the availability of antibodies generated in different species. With the aim to define the subpopulation of interneuron that expresses Cux proteins we performed double staining with two markers of interneuron subpopulations, parvalbumin and calretinin proteins. Neither of these two markers colocalized with Cux-1 or Cux-2 protein (Fig 1, G–J). Thus, we concluded that *Cux-1* and *Cux-2* are expressed in a subset of cortical interneurons.

Cux-1^{-/-}; Cux-2^{-/-} double mutation is lethal during embryonic development

We next set out to analyze the development of Reelin expressing neurons of the dorsal telencephalon in WT, *Cux-1*^{-/-} and *Cux-2*^{-/-} single knockouts and in *Cux-1*^{-/-}; *Cux-2*^{-/-} double ko animals. Mice carrying null mutations of one or both *Cux* genes were generated by crossing *Cux-1* and *Cux-2* heterozygous mice, and progeny were collected at embryonic and postnatal stages for analysis. Table I shows the numbers of animals collected for each genotype and the expected proportion of animals that would be recovered according to a Mendelian transmission of both alleles. As shown in table I, *Cux-1*^{-/-} and *Cux-2*^{-/-} animals were collected at the expected Mendelian ratios at embryonic and postnatal (P0) stages. Only 1.3% of total animals collected were *Cux-1*^{-/-}; *Cux-2*^{-/-}, which represents an 80% reduction from the predicted Mendelian proportion (Table I). The lethality of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation was observed at embryonic stages (E9 to E19) as well as P0. This indicates that *Cux-1*^{-/-}; *Cux-2*^{-/-} embryos do not develop normally and die before E19. A few *Cux-1*^{-/-}; *Cux-2*^{-/-} were collected at P0 but none after the first postnatal day (Table I). A previous report showed that *Cux-1*^{-/-} mice are born normally but that 70% of the animals die within a week (Luong et al., 2002). Accordingly, we observed that *Cux-1* mice survive the first postnatal days with no overt phenotype but die subsequently (not shown). In addition, we detected that most *Cux-1*^{-/-} mice had no milk in their stomachs during the first 8 to 12 hours after birth. In contrast, their wild-type (WT) and *Cux-2*^{-/-} littermates fed promptly after birth, with milk observed in their stomachs within the first

hours (Fig 2, panel A). This feeding problem is overcome after the first postnatal day, and milk was observed as normal in those *Cux-1*^{-/-} animals that survived beyond P1 (not shown). We reason that *Cux-1*^{-/-} animals are less able to compete with their littermates for nursing initially, but are eventually able to feed. None of the animals carrying the *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation had milk in their stomachs, and these animals appeared dehydrated, cyanotic, and in a generally bad state (Fig 2 C). Although we did not identify any *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice among the animals analyzed after P0 (Table I), the number of animals analyzed at P1–P5 is insufficient to allow determination of survival rates at these stages. Nonetheless, the general bad state of the *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice indicates that the very few animals that survive to birth would be unable to survive beyond the first hours.

Pyramidal neurons of the cerebral cortex of *Cux-1*^{-/-}; *Cux-2*^{-/-} animals normally express markers of their appropriate cortical layers

The overlapping expression patterns of *Cux-1* and *Cux-2* in the cerebral cortex suggest redundancy in their functions during the specification of neuronal populations, and predict that these functions should be revealed in the cerebral cortex of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutants. We therefore investigated possible defects in cortical lamination and in the molecular identity of cortical neurons of the *Cux-1*^{-/-}; *Cux-2*^{-/-} mice that escape the lethal phenotype. We analyzed the expression of neuronal markers that are restricted to specific cortical layers. Brn-1 (layers II, III, IV and V) and Foxp-1 (layers III, IV and V) were correctly expressed in WT, *Cux-1*^{-/-}, *Cux-2*^{-/-} P0 animals. Expression of Foxp1 and Brn1 was also observed in *Cux-1*^{-/-}; *Cux-2*^{-/-} upper layers (Fig 3). We have reported increased cellular density of the upper layer of *Cux-2*^{-/-} animals (Cubelos et al., 2007). Foxp1 and Brn1 staining revealed defects in fine lamination also in *Cux-1*^{-/-} and *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutants (Fig 3). Increased cellular density, as estimated by the number of Foxp1 positive cells per area, was observed in the upper layers of *Cux-1*^{-/-} (3521 ± 356 cells/mm²); *Cux-2*^{-/-} (4155 ± 361 cells/mm²) and *Cux-1*^{-/-}; *Cux-2*^{-/-} (3973 ± 250 cells/mm²) mutants compared to WT (2998 ± 135 cells/mm²) (Supplementary Fig 1). Double mutant mice show also reduced thickness of the upper layers (Fig 3). Id-2 (layers II, III, and V) and Tbr-1 (layer VI) were also observed in the expected neuronal subpopulations in all genotypes (data not shown). These results strongly suggest that inside-outside migration of most cortical pyramidal neurons proceeds normally and that the majority of pyramidal neurons acquire their correct molecular identity in *Cux-1*^{-/-}, *Cux-2*^{-/-} and *Cux-1*^{-/-}; *Cux-2*^{-/-} mice.

Reelin expression is absent from neurons of the cortical plate of *Cux-1*^{-/-}; *Cux-2*^{-/-} P0 mice

Since *Cux-1* and *Cux-2* label subpopulations of Reelin expressing neurons, we next analyzed the differentiation of these neurons in the absence of *Cux* genes. In WT animals, Reelin is abundantly expressed in layer I of the cortical plate, where it is secreted by the Cajal-Retzius neurons, which are strongly immunoreactive for this protein (Fig 4A and 5A, panel a). Reelin is also observed in scattered interneurons distributed throughout layers II–III, IV, V and VI, being more abundant in the deep layers (V and VI) (Fig 4A and B, panel a). In *Cux-1*^{-/-} and *Cux-2*^{-/-} single knockout mice, the expression of Reelin is normal both in layer I and in layers II–VI (Fig 4A and B, panels c and d). Reelin is also correctly expressed by Cajal-Retzius neurons of layer I in *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant animals (Fig 4A, panel b and Fig 5A, panel b). But surprisingly Reelin-expressing cells were completely absent from layers II–VI of *Cux-1*^{-/-}; *Cux-2*^{-/-} animals (Fig 4A and B, panel b). This phenotype is observed in all regions of the cerebral cortex and at all rostral, caudal, medial and lateral levels (not shown).

On the other hand, analysis of the hippocampus of single and double *Cux* knockouts showed that Reelin-expression was present in cells of the hippocampal marginal zone and was indistinguishable across all genotypes (Fig 5B, panels a to d). All together, these results indicate that *Cux-1* and *Cux-2* genes are involved in the differentiation and/or proliferation of Reelin expressing interneurons of the cortical plate but do not regulate the expression of Reelin in other neuronal populations such as the Cajal-Retzius and hippocampal cells. These findings support the distinct anatomical and molecular origins of the different interneuron subpopulations and situate *Cux* genes among the players regulating their specific identities.

Discussion

Expression of *Cux-1* and *Cux-2* homeobox in the developing and adult brain suggests possible functions in the development of the nervous system. Such a role is supported by the reported functions of their invertebrate counterpart *Drosophila Cut* in neuronal specification (Boulder-Committe, 1970; Bodmer et al., 1987; Brewster et al., 2001; Grueber et al., 2003). In the ventral telencephalon, the expression of *Cux-1* and *Cux-2* in the MGE suggests a role in interneuron differentiation. However, this possibility has not previously been explored, and the expression of *Cux* genes in GABA-ergic interneurons has not been reported (Nieto et al., 2004; Zimmer et al., 2004; Butt et al., 2005). Here, we provide the first description of the expression of *Cux-1* and *Cux-2* in a subpopulation of cortical interneurons. These interneurons are characterized by their expression of Reelin, and we demonstrate that their development depends on redundant functions of the two *Cux* genes. Unfortunately, most of the specific markers defining GABA-ergic subpopulations appeared only in the late postnatal brain and the lethality of the double mutation did not allow us to further define the specific interneuron population affected.

Our studies demonstrate that *Cux-1* and *Cux-2* double gene deletion is embryonically lethal as a result of embryonic defects manifest at E19 or earlier. This suggests an earlier function of *Cux* genes in other tissues, and underlines their importance and the redundancy of their functions in cell fate specification. The cause of this lethality is currently unknown, and the early expression patterns of *Cux-1* and *Cux-2* from E0 to E8 have not been reported. The reported embryonic expression patterns at subsequent stages do not suggest obvious reasons for the observed embryonic lethality (Quaggin et al., 1996; Vanden Heuvel et al., 1996; Nepveu, 2001; Iulianella et al., 2003; Sharma et al., 2005). Further studies are therefore required to identify the affected tissues and processes in which *Cux* functions are indispensable for the development of a viable embryo.

Our results provide the first demonstration that *Cux-1* and *Cux-2* proteins are expressed in neurons of the cortical plate that also express Reelin at P0. These data indicate that *Cux* transcription factors are expressed in a population of inhibitory interneurons. Previous reports have provided detailed descriptions of the expression of markers of GABA-ergic interneurons in Reelin expressing cells of the cortical plate (Alcantara et al., 1998; Flames and Marin, 2005), whereas Reelin is not secreted by pyramidal neurons of layers II–IV. Hence, this is the first description of the expression of *Cux-1* and *Cux-2* in GABA-ergic interneurons.

Inhibitory interneurons originate in the ventral portion of the developing telencephalon and subsequently migrate tangentially to the dorsal cerebral cortex. Most of the interneurons are thought to be born from precursors in the MGE. However, strong evidence support that calretinin-expressing interneurons are born in the CGE (Flames and Marin, 2005). We show that parvalbumin and calretinin-expressing interneurons do not express *Cux-1* or *Cux-2* proteins. In the ventral portion of the developing telencephalon, *Cux-2* expression is restricted to the SVZ of the MGE. This expression pattern is clearly suggestive of possible

roles in the early specification or proliferation of an interneuronal subpopulation. In contrast, *Cux-1* is broadly expressed in the VZ and SVZ of the MGE, LGE and most of the ventral telencephalon (Nieto et al., 2004). While not precluding similar roles, this pattern does not suggest the same level of specificity. The evidence is suggestive of *Cux* genes functioning specifically in certain MGE derived interneurons, and not in GABA-ergic neurons derived from other regions, although it should be considered the possibility that expression of *Cux* genes shuts off in the postmitotic interneuron. They also support the different anatomical origin of calretinin-expressing interneurons. Having found evidence for *Cux-1* and *Cux-2* expression in Reelin interneurons, we analyzed the expression of Reelin in the telencephalon of *Cux* mutants. We found that expression of Reelin in the telencephalon of *Cux-1*^{-/-} and *Cux-2*^{-/-} single mutant animals is not affected in layer I, layers II–VI, or in the hippocampus. In contrast, Reelin expression is specifically and completely lost in layers II–VI in *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant P0 animals, but not in the hippocampus or the Cajal-Retzius cells of layer I. Thus, loss of either *Cux-1* or *Cux-2* can be compensated for by the corresponding homolog in Reelin expressing interneurons, but these neurons require *Cux* function for their correct development.

Our data thus indicate redundant and essential functions for *Cux-1* and *Cux-2* in the development of an interneuron subpopulation. At present, it is not clear if the absence of Reelin-positive interneurons from the cortical plate in *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant animals is due to a failure of these cells to develop or to later differentiation defects. Failure of these interneurons to develop would indicate early specification problems, proliferation defects or alterations to migration routes. Regarding migration, it is not known whether GABA-ergic interneurons express Reelin in the course of their migration through layer I, and the SVZ, but we did not observe any accumulation of Reelin expressing cells in any regions of the ventral telencephalon. Alternatively, our data could be interpreted as indicating that *Cux-1* and *Cux-2* regulate later aspects of neuronal differentiation and control expression of Reelin. In summary, although it remains to be determined whether *Cux* genes control early or late aspects of neuronal differentiation, the current report provides the first unequivocal demonstration that *Cux-1* and *Cux-2* play an important role in interneuron development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

E	embryonic
Ko	knockout
LGE	lateral ganglionic eminence
MGE	medial ganglionic eminence
P	postnatal
SVZ	subventricular zone

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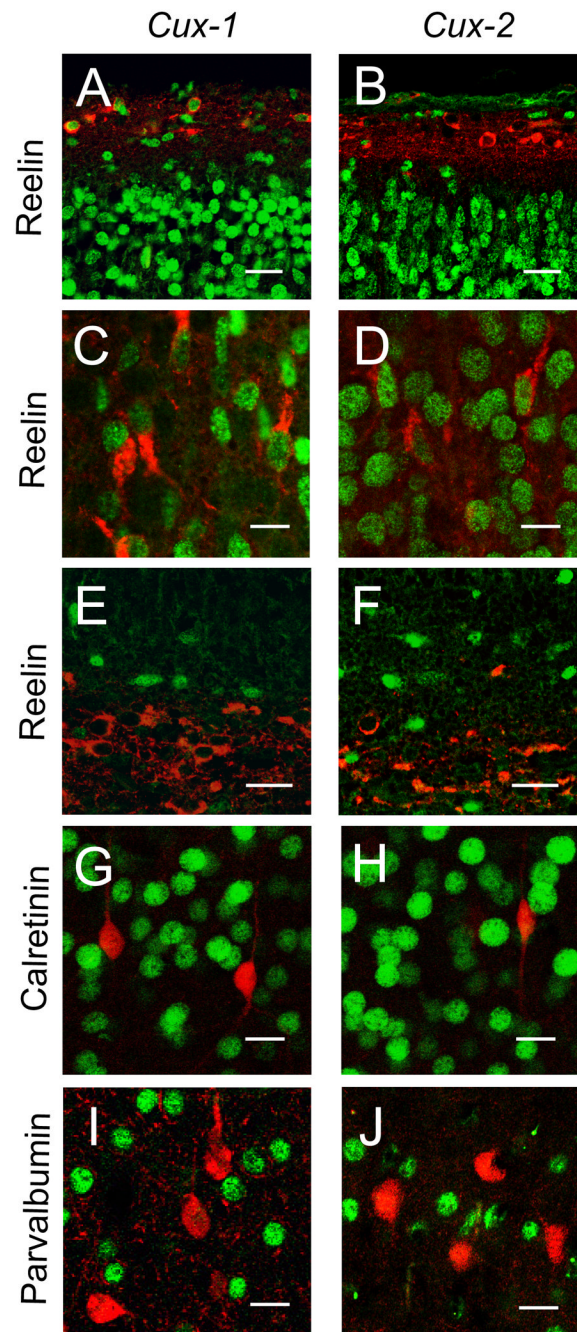


Figure 1. Expression of Cux-1, Cux-2 and Reelin in the mouse cortex

Panels A and B show double-staining of Cux-1 (A), Cux-2 (B) and Reelin in layer I in the dorsal cortex of P0 animals. Scale bar, 25 μ m. Panels C and D show colocalization of Cux-1(C) or Cux-2 (D) and Reelin in neurons of layers II–VI Scale bar, 10 μ m. Panels (E and F) show expression of Cux-1 (E) or Cux-2 (F) and Reelin in the marginal zone of the developing P0 hippocampus. Scale bar 20 μ m. Double-staining of Cux-1 (G, I), Cux-2 (H, J) and calretinin (G, H) or parvalbumin (I, J) in P21 brain. Scale bar, 15 μ m

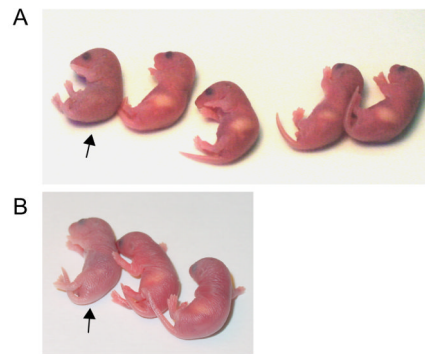


Figure 2. Early embryonic lethality of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation and nursing defects associated to the *Cux-1* phenotype

(A and B) Photographs showing P0 animals of two litters obtained from breeding *Cux-1*^{+/-} and *Cux-2*^{+/-} heterozygous animals. Arrows indicate animals with little or no detectable milk in their stomachs. Subsequent genotyping demonstrated that the animals marked with arrows bear the *Cux-1*^{-/-} *Cux-2*^{+/-} null mutation (A) and the *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation (B).

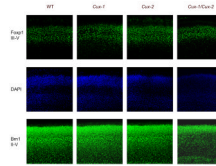


Figure 3. Expression of cortical layer-specific markers in Cux mutant mice

Immunofluorescence staining showing the expression patterns of Foxp-1 and Brn-1 in the cortical plates of P0 WT and *Cux-1*^{-/-}, *Cux-2*^{-/-} and *Cux-1*^{-/-}; *Cux-2*^{-/-} mutant mice. Middle panels show nuclear staining corresponding to sections shown in the upper panels.

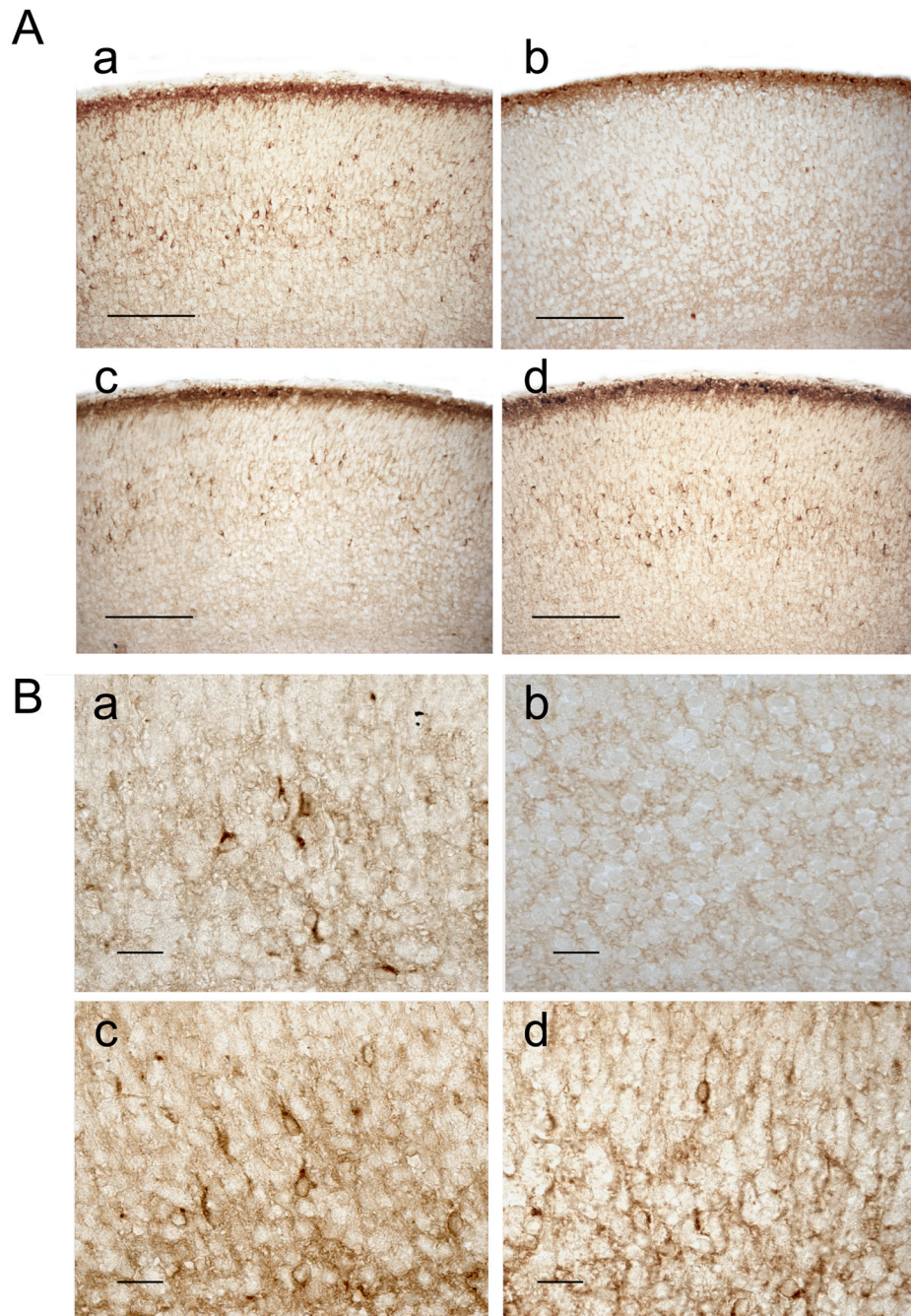


Figure 4. Expression of Reelin in P0 Cux mutant mice

(A and B) Micrographs showing the expression of Reelin in the cortical plate of WT (a), *Cux-1*^{-/-}; *Cux-2*^{-/-} (b), *Cux-1*^{-/-} (c), and *Cux-2*^{-/-} (d) P0 mice. Panel A shows low magnification images of P0 cortical sections. In all genotypes, Reelin immunoreactivity is detected in the marginal zone, where it strongly labels Cajal-Reztius cells. Reelin immunoreactivity is also detected in scattered neurons in layers II–VI in WT (a), *Cux-1*^{-/-} (c) and *Cux-2* (d) animals, but is completely absent in layers II–VI of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice (b). Scale bars = 100 μ m. (B) High power micrographs showing Reelin expressing cells in the cortical plate of WT (a), *Cux-1*^{-/-} (c), and *Cux-2*^{-/-} (d) animals but not in *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutant mice (b). Scale bars = 10 μ m.

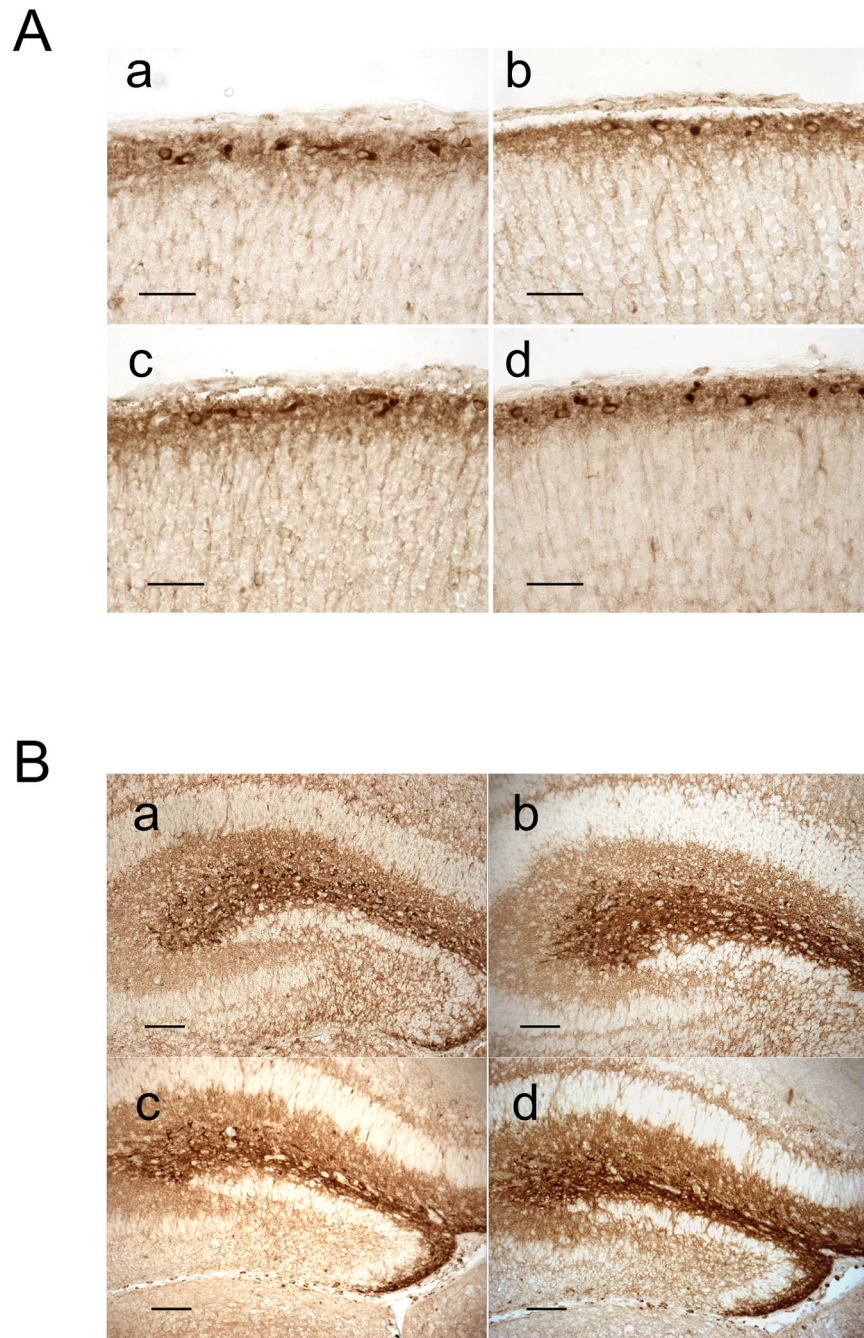


Figure 5. Expression of Reelin in cortical layer I and hippocampus of Cux mutant mice
 (A) Micrographs showing Reelin immunoreactivity in the cortical plate of WT (a), *Cux-1*^{-/-}; *Cux-2*^{-/-} (b), *Cux-1*^{-/-} (c), and *Cux-2*^{-/-} (d) P0 mice. Scale bars = 50 μ m. (B) Micrographs showing Reelin immunoreactivity in the hippocampus of WT (a), *Cux-1*^{-/-}; *Cux-2*^{-/-} (b), *Cux-1*^{-/-} (c), and *Cux-2*^{-/-} (d) P0 brains. Reelin is strongly expressed in the marginal zone of the hippocampus in mice of all genotypes. Scale bars = 100 μ m.

Table 1

Early embryonic lethality of *Cux-1*^{-/-}; *Cux-2*^{-/-} double mutation

	<i>Cux-1</i> ^{+/?} ; <i>Cux2</i> ^{+/?}	<i>Cux-1</i> ^{-/?} ; <i>Cux2</i> ^{+/?}	<i>Cux-2</i> ^{-/?} ; <i>Cux-1</i> ^{+/?}	<i>Cux-1</i> ^{-/?} ; <i>Cux2</i> ^{-/-}	<i>Total</i>
E9	2	3	2	0	7
E11-E13	10	3	4	0	17
E15-E16	25	5	6	1	37
E19	46	13	15	0	74
E9-E19	83	24	27	1*	135
P0	121	49	48	4**	222
P1-P5	11	5	4	0	20
Total	215	78	79	5***	377
Total %	57%	20,7%	21%	1,3%	100%
Expected	56%	18,75%	18,75%	6,25%	100%

Genotypes and numbers of progeny, collected at different embryonic and postnatal stages, from crosses between *Cux-1*^{+/?} and *Cux-2*^{+/?} heterozygotes. Total %: the percentages of each genotype recovered. Expected: the predicted percentages of progeny genotypes according to Mendelian allelic transmission.

* p<0.1,

** p<0.05,

*** p<0.01 as compared to the expected Mendelian distribution using a chi square test.