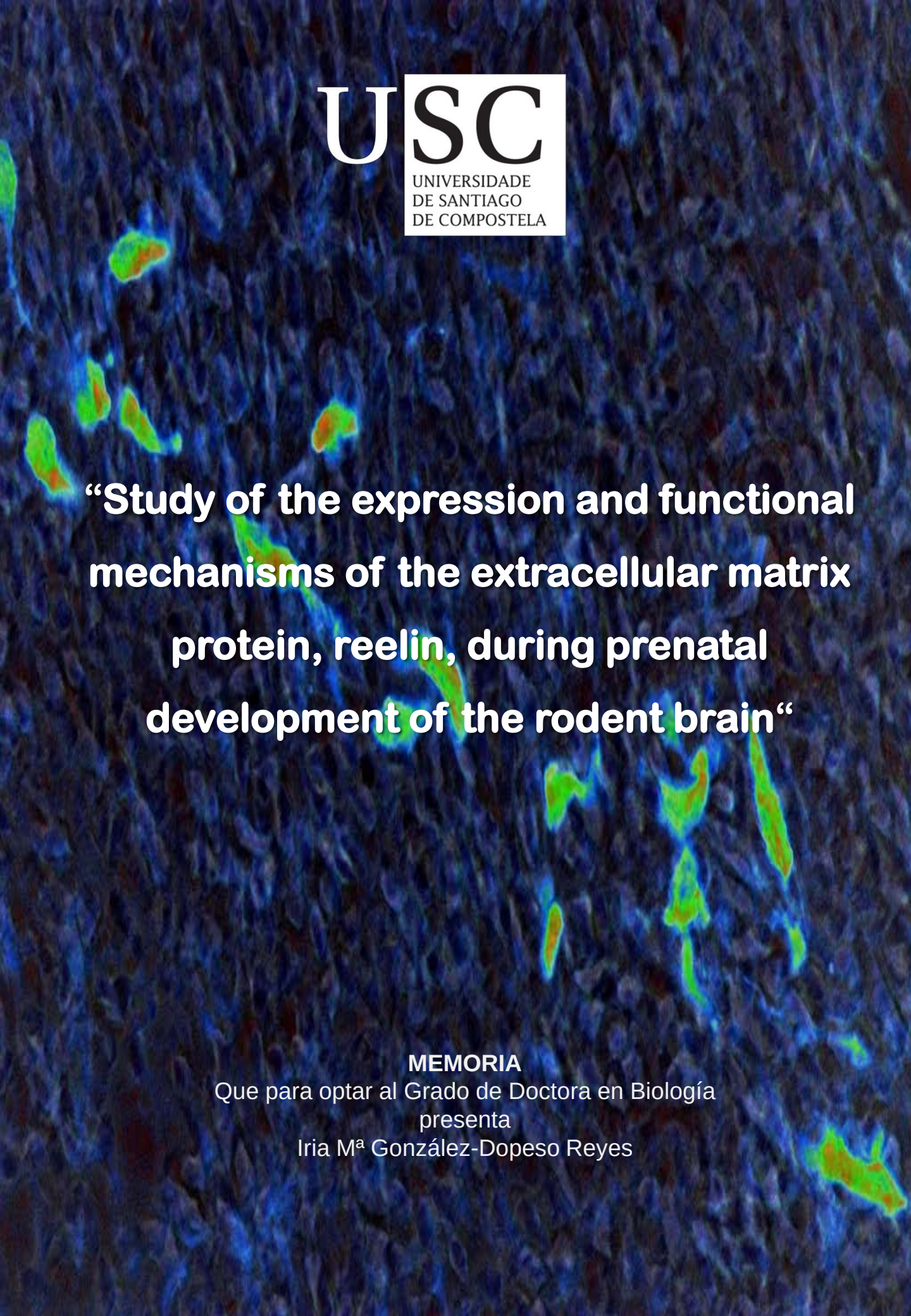

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

“Study of the expression and functional mechanisms of the extracellular matrix protein, reelin, during prenatal development of the rodent brain“

MEMORIA

Que para optar al Grado de Doctora en Biología

presenta

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE BIOLOXÍA CELULAR E ECOLOXÍA

HÉCTOR J. CARUNCHO MICHINEL PROFESOR TITULAR DEL ÁREA DE BIOLOGÍA CELULAR Y MIGUEL A. RODRÍGUEZ DÍAZ, PROFESOR CONTRATADO DOCTOR DEL ÁREA DE BIOLOGÍA CELULAR DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA,

CERTIFICAN:

Que la presente memoria titulada "**Study of the expression and functional mechanisms of the extracellular matrix protein, reelin, during prenatal development of the rodent brain**", que para optar al Grado de Doctor en Ciencias Biológicas presenta Dña. IRIA Mª GONZÁLEZ-DOPESO REYES, ha sido realizada bajo nuestra dirección. Y considerando que constituye trabajo de TESIS, autorizamos su presentación al Consejo de Departamento correspondiente.

Y para que así conste, expedimos el presente certificado en Santiago de Compostela, 13 de octubre de 2009.

Los directores de Tesis:

Fdo: Héctor J. Caruncho Michinel

Fdo: Miguel A. Rodríguez Díaz

La Doctoranda

Fdo: Iria Mª González-Dopeso Reyes

Esta Tesis Doctoral forma parte de los Proyectos de Investigación: “Papel de la vía de señalización de la reelina en la patogénesis de la esquizofrenia” Financiado por la Fundació La Marató de TV3; “Reelina, agrupamientos de proteínas y dominios de membrana en esquizofrenia” (SAF-2004-00951); “Reelina e agrupamento de proteínas de membrana en depresión maior” (PGIDT06PXIB200166PR); “Correlaciones de alteraciones en plasticidad neural en esquizofrenia, trastorno bipolar y depresión mayor” (SAF-2007-62766).

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Tampoco puedo olvidarme de aquellos que pelearon porque no me quedase en el camino tiempo atrás y gracias a los que he podido llegar hasta aquí. Y sobre todo a mis padres porque me enseñaron a levantarme cada vez que me caí, "a forzar mi corazón, mi nervio y mi fuerza para que sirvan aún después de que se hayan ido" y así he terminado una etapa más del camino.

Y a toda esa gente que ha pasado por mi vida y que la ha enriquecido, a tod@s

MUCHAS GRACIAS

“La Ciencia comercia con el saber, obtenido mediante la duda. Al tratar de impartir saber a todos los hombres sobre todas las cosas, aspira a hacer que todos los hombres duden. [...] ¿Para quién estáis trabajando? Yo sostengo que el único objetivo de la Ciencia es aliviar las fatigas de la existencia humana. Si los científicos, intimidados por los poderosos egoístas, se contentan con acumular Ciencia por la Ciencia misma, se la mutilará, y vuestras nuevas máquinas significarán sólo nuevos sufrimientos.”

Vida de Galileo. Bertolt Brecht

“Itaca te brindó tan hermoso viaje.
Sin ella no habrías emprendido el camino.
Pero no tiene ya nada que darte.

Aunque la halles pobre, Itaca no te ha engañado.
Así, sabio como te has vuelto, con tanta experiencia,
entenderás ya qué significan las Itacas”.

Itaca. C.P. Kavafis

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General Introduction

Brain development is a complex process that requires the coordinated genesis, migration and maturation of all its constituents. Our understanding of the mechanisms involved in neuronal signalling and development, as well as the function of the major sensory and motor systems of the brain, has benefited from studies of human malformations and mouse mutants. Genetic studies have identified the genes disrupted in many mutants, allowing a molecular interpretation of the phenotypes, but the elucidation of the underlying causes of many neurological and psychiatric disorders has proved more difficult. Neurological and psychiatric disorders are among the most common and most serious health problems in developed societies. For example, schizophrenia has a high vital risk in the general population of about 1%. The etiology of this illness probably involves many genes acting together with environmental factors, which increase the difficulty of understanding the neurobiology of this disorder and developing new effective treatments. It becomes even more difficult because patients show extreme behavioural manifestations but there are no evidences of gross neuroanatomical abnormalities or degeneration and lost of cells.

In 1998 was reported the decreased expression of reelin protein and mRNA (about 50%) in post-mortem brains of schizophrenic patients (Impagnatiello et al., 1998). After that seminal finding, other reports also found significant reductions of reelin levels in post-mortem brains of patients with schizophrenia, bipolar disorder, major depression (Fatemi et al., 2000; Guidotti et al., 2000; Fatemi et al., 2001b; Torrey et al., 2005), and also in autism (Fatemi et al., 2001a; 2002; 2005). These findings support the so-called neurodevelopmental hypothesis of schizophrenia and a possible role for reelin in the pathogenesis of this disorder. Neurodevelopment comprises genetic, epigenetic, and environmental events associated with cell birth, cell differentiation, neural migration, programmed cell death, synaptogenesis and the formation of neuronal circuits, as well as axon pruning and myelination. The developmental hypothesis of schizophrenia suggests that several genetic, epigenetic and/or environmental factors associated with early developmental stages can interfere with these early events and have a negative influence in the formation of the neuronal circuits, cell organization and/or in critical signal transduction pathways (reviewed by Rapoport et al., 2005). Reelin is critical for normal cortex development at early developmental stages, it plays a role in the establishment of the inside-out pattern of the developing cortex, it is involved in the control of radial migration of neuroblasts in neocortex during embryogenesis, and it plays a role in the regulation of synaptogenesis and dendrite maturation (reviewed by Huang and D'Arcangelo, 2008). Changes in the expression of this protein, and/or in the downstream signal transduction pathway results in abnormal corticogenesis and synaptic plasticity. The study of the *reeler* mutant (with null expression of reelin) has clearly helped to have a better knowledge of corticogenesis.

The null *reelin* mutations in humans result in lissencephaly, but there is no evidence of mutations in the *reelin* gene in schizophrenia (Huang and Chen, 2006). The high deficit in reelin

(50% of both protein and mRNA) found in several brain areas of schizophrenic patients suggested that this deficit could be caused by a deficiency in gene regulation, and/or epigenetic control of the *reelin* gene (reviewed by Abdolmaleky and Smith, 2008). The heterozygous *reeler* mouse is a *reelin* haploinsufficient animal, expressing about 50% less reelin, and exhibiting some neuroanatomical and behavioural abnormalities that resemble those described in schizophrenia. Therefore, this animal could be an acceptable model to study the role of reelin in different brain areas, as well as the importance of reelin as a vulnerability factor in schizophrenia.

The study of well-defined animal models for the different mental illnesses is important for the development of new treatments and prevention. Previous to the development of animal models we should gain a better understanding of the physiological roles played by the molecules that are implicated in neurodevelopment and plasticity. Reelin plays an important role in embryogenesis and is also highly expressed during adult life. There are numerous evidences that relate reelin with psychiatric disorders; and in fact the heterozygous *reeler mouse* was proposed as an animal model for schizophrenia. However it is still necessary to understand in more detail the developmental reelin expression pattern and the functional roles of reelin.

1. *The reeler* mutant mouse

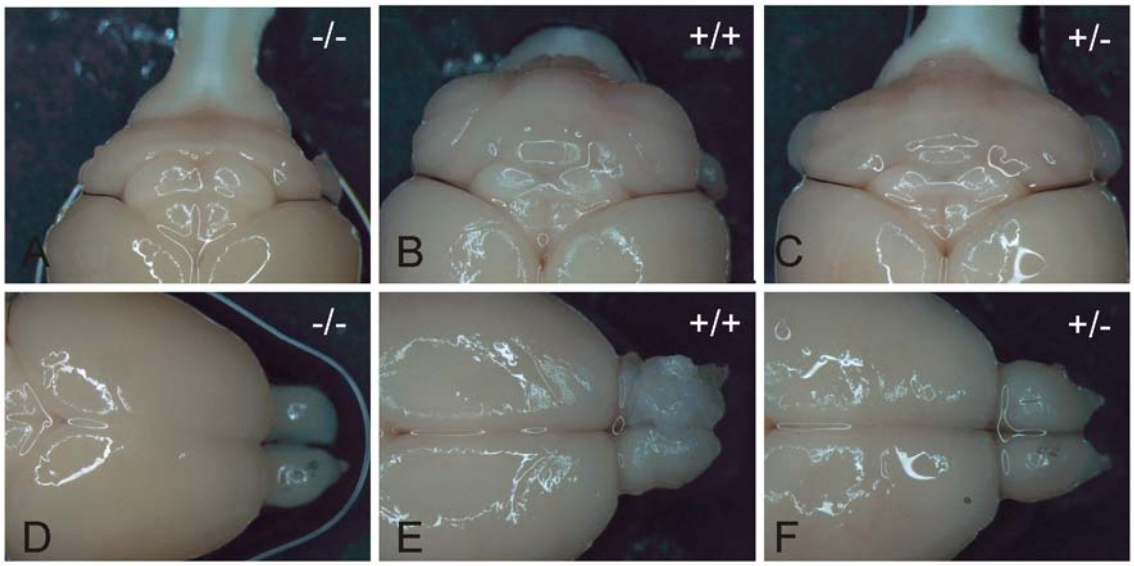

Figure 1. Photographs of reeler, wild type and heterozygous mice brains. A-C Cerebellum of *reeler* mouse (A), wild type mouse (B), and *heterozygous* mouse (C). There are not differences in size between the heterozygous and wild type mice cerebellum, while that the reeler mouse cerebellum is much smaller than the others. D-F Cerebral hemispheres and olfactory bulb of *reeler* mouse (D), wild type mouse (E), and heterozygous mouse (F). There are no apparent differences in cortical size, but the olfactory bulb of the *reeler* mouse is smaller than in heterozygous and wild type.

The *reeler* mutation was described by Falconer in 1951 as a spontaneous mutation in a mildly inbred stock. *Reeler* homozygotes show limited mobility, an important ataxia and falling over onto their sides when walking, as well as a reducing viability and fertility. The name *reeler* comes from the distinct "reeling" gait that is caused by hypoplasia of the mouse cerebellum, in which the normal cerebellar folia are missing (Falconer, 1951). This phenotype is caused by an autosomal recessive mutation (revised by Rakic and Caviness, 1995; Lambert de Rouvroit and Goffinet, 1998; Tueting et al., 2008). Following the discovery of the reeler mutant mouse numerous anatomical brain abnormalities affecting laminated and non-laminated brain structures have been described in this mutants (revised by Goffinet, 1984; Lambert de Rouvroit and Goffinet, 1998; Katsuyama and Terashima, 2009).

The *reeler* mutation affects the ability of postmitotic neurons to assume correct positions in the developing brain (Falconer, 1951; Caviness and Sidman, 1973; Goffinet, 1980; revised by Goffinet et al., 1984; Lambert de Rouvroit and Goffinet, 1998; Meyer, 2007). The most striking anatomical abnormality in *reeler* mice is the lack of foliation and a marked reduction in the cerebellum size (Fig. 1, 2), the external granular layer is also reduced in size and the Purkinje cell layer is absent. The cerebral cortex exhibits an apparent inverted orientation of the cortical layers (Fig. 2), the preplate does not split and the cortical plate develops ectopically underneath preplate neurons, and radial glia is also affected. In the hippocampus of *reeler* mice the pyramidal neurons are less densely packed, and the dentate gyrus is not compact (Fig. 2). There are defects in the radial glia scaffold and the pyramidal layer is disrupted. (reviewed by Curran and D'Arcangelo, 1998; Lambert de Rouvroit y Goffinet, 1998; Förster et al., 2006; Katsuyama and Terashima, 2009). Structural abnormalities have also been reported in non-laminar areas of brain like the thalamus, mesencephalon, brainstem and spinal cord (Molnar et al., 1998; Yip et al., 1998; Nishikawa et al., 2003; Yamamoto et al., 2003; Yip et al., 2003a; Yip et al., 2003b; Li et al., 2005; Baba et al., 2007; Yip et al., 2009).

The heterozygous *reeler* mouse (HRM) shows a decrease in reelin mRNA and protein levels about 40% to 60% of the brain reelin content of wild-type mice. Although HRM do not exhibit important structural abnormalities such as those observed in the homozygous *reeler* mice, they present some brain abnormalities due to the reelin deficit and have a significant decrease in the number of cells expressing reelin in several brain areas (Tueting et al., 1999; Liu et al., 2001). HRM show several neuroanatomical abnormalities: decreased cortical neuropil and increased cortical cell packing density, which correlate with a reduction in cortical thickness (Liu et al., 2001), reduction in dendritic spine density of pyramidal cells in frontal cortex and hippocampus (Liu et al., 2001), reduction in dendritic branching in hippocampus (Niu et al., 2004), abnormal dendrite development of cortical interneurons (Yabut et al., 2006), increased number of NADPH-Diaphorase positive cells in cortical white matter (Tueting et al., 1999), decreased number of

cerebellar Purkinje cells in male (Hadj-Sharaoui et al., 1996; Biamonte et al., 2009), and reduced stem cell migration in hippocampus and olfactory bulb (Kim et al., 2002).

Examination of the HRM brain also reveals neurochemical alterations: downregulation of GABAergic function, an important decrease in glutamic acid decarboxylase 67 (GAD67) mRNA and protein in prefrontal cortex (Liu et al., 2001; Pappas et al., 2001) and alteration of glutamatergic and dopaminergic function (Isosaka et al., 2006; Ballmaier et al., 2002). HRM became of interest as a possible animal model for psychosis (Tueting et al., 1999; Brigman et al., 2006) because it was determined that the anatomical and neurochemical alterations observed in the HRM, such as down-regulation of reelin and GAD67 in prefrontal cortex and hippocampus, are similar in magnitude to that observed in psychotic post-mortem brains (Carboni et al., 2004; Costa et al., 2001; Liu et al., 2001; Impagnatiello et al., 1998; Guidotti et al., 2000). HRM mice are also impaired in some behavioural test (reviewed in Costa et al., 2002; Podhorna and Didriksen, 2004; Tueting et al., 2006).

Using antibodies against the N-terminal region, reelin protein was detected in the brain of Orleans mutant mice but it could not be detected with antibodies directed against its C-terminal part (revised by Lambert de Rouvroit and Goffinet, 1998). In this mice reelin is produced by Cajal-Retzius cells but not secreted, and accumulates in the endoplasmic reticulum (de Bergeyck et al., 1997; Derer et al., 2001). These results indicated that the C-terminal region of reelin is essential for its secretion and suggested that the Orleans *reeler* phenotype is due to defective reelin secretion rather than to secretion of an inactive protein. Posterior *in vitro* studies showed that most of the C-terminus is not essential for reelin secretion, although it is important for secretion efficiency (Nakano et al., 2007). In the Orleans mutant, reelin is unable to go through the secretory pathway and becomes accumulated in the endoplasmic reticulum because the protein is truncated in the middle repeats of the reelin molecule (Nakano et al., 2007).

Other mouse mutations with a *reeler*-like phenotype appeared in different laboratories in the 90s, like *scrambler* and *yotari* mice (Sweet et al., 1996; Sheldon et al., 1997; Yoneshima et al., 1997). The phenotype of *scrambler* and *yotari* is very similar to that of *reeler*, showing unstable gait and tremors. The cerebellum of homozygous *yotari* is hypoplastic and has no foliation. A molecular and a granular cell layer can be identified, but Purkinje cells are scattered throughout both the granular layer and white matter. Reelin immunoreactivity is present in the *scrambler* and *yotari* cortex showing a normal expression pattern, but the laminar structure of the cerebral cortex is disrupted (González et al., 1997; Yoneshima et al., 1997). Molecular genetic studies revealed that *scrambler* and *yotari* arose from independent mutations in the disabled-1 (Dab1) gene (Sheldon et al., 1997; Ware et al., 1997). Mice deficient in Cdk5, p35, or both p35 and p39 also display some hallmarks of disturbed cortical development, including cortical layer inversion, and hippocampal and cerebellar alterations (Ohshima et al.,1999; Chae et al., 1997; Gilmore et

al., 1998; Kwon and Tsai, 1998; Ko et al., 2001). In the neocortex, *Cdk5*-/- and *p35*-/- mice exhibit normal neuronal migration up to the point of layer VI formation, after which subsequent waves of migrating neurons appear incapable of surpassing the layer of earlier-born neurons (Ohshima et al., 1999; Chae et al., 1997; Gilmore et al., 1998).

Other mutants presenting a *reeler*-like phenotype affect the Very-low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2), and both receptors can bind Dab1 on their cytoplasmic side (Howell et al., 1999a). VLDLR and ApoER2 are expressed in cortical and cerebellar layers close to layers that express reelin (Pérez-García et al., 2004). VLDLR mutant has an important disruption in the cerebellum, poor development of the anterior lobules and large number of ectopic Purkinje cells, while the cerebral cortex show just little alterations. On the other hand, ApoE2 receptor mutant shows defects in the patterning of the cerebral cortex and hippocampus while the defects in the cerebellum are less pronounced. Double knockout mice, for both receptors show a phenotype indistinguishable from the *reeler* mouse (Trommsdorff et al., 1999).

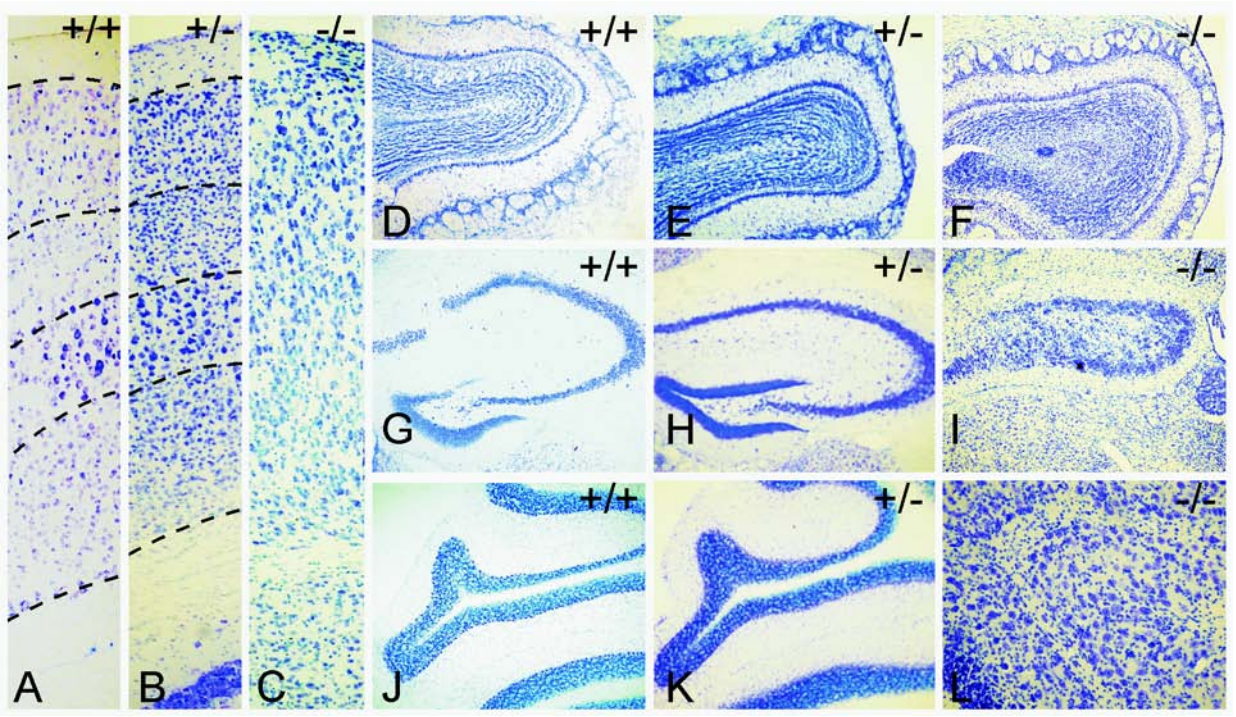

Figure 2. Photographs of reeler, heterozygous, and wild type mice brains. A-C Cortex of mice showing the differences in the layer organization between wild type and heterozygous with *reeler*. Wild type (A),heterozygous mouse (B), and *reeler* mouse (C). **D-F** Olfactory bulb shows not apparent differences between wild type, heterozygous and *reeler* in the layer organization. Wild type mouse (D), heterozygous mouse (E), and *reeler* mouse (F). **G-I** Hippocampus of wild type (G), heterozygous (H) and *reeler* mice (I), showing differences in the layer organization. **J-L** Cerebellum of wild type (J), heterozygous (H) and *reeler* mice (I).

The study of these mutants is of great interest to understand cortical development mechanisms and reelin signalling pathway.

2. Structure of the reelin protein and gene.

Reelin gen was cloned in Tom Curran's laboratory (D'Arcangelo et al., 1995). The original *reeler* allele was found to correspond to a large genomic deletion of about 150 Kb (Bar et al., 1995; D'Arcangelo et al., 1995).

Reelin is a glycoprotein of the extracellular matrix of 3,461 amino acids and 450 KDa (D'Arcangelo et al., 1995; 1997). The N-terminus site comprises a signal peptide and a region of similarity with F-Spondin (about 250 amino acids), a protein that directs neural crest cell migration and neurite outgrowth (Klar et al., 1992; Burstyn-Cohen et al., 1999; Debby-Brafman et al., 1999). After the first 500 amino acids, the main body of reelin consists of a series of eight internal repeat sequences of 350-390 amino acids. Each repeat contains two related sub-domains A and B, separated by a stretch of 30 amino acids with conserved cystein residues related to epidermal growth factor-like motifs. These motifs present a high grade of conservation with sites for glycosylation, xylosylation and mirystylation. Cystein residues are related to these modifications in other extra cellular proteins such as tenascin C, tenascin A, restrictine and the integrin family of receptors. The C-terminus contains a short stretch of 33 basic amino acids, positively charged (D'Arcangelo et al., 1997; de Bergeyck et al., 1997) (Fig. 3).

Several reelin isoforms are present in the brain, liver and supernatant of primary neural cultures (Ikeda and Terashima, 1997; Smalheiser et al., 2000; Lambert de Rouvroit et al., 1999a; D'Arcangelo et al., 1999); these isoforms arise via cleavage of full-length reelin into smaller proteins. Reelin is cleaved *in vivo*, in the extracellular environment, at two sites: N-terminus site located after domain 2, and C-terminus site located between domains 6 and 7. By using antibodies against N-terminal, C-terminal or central fragments, five main reelin isoforms have been detected in cell cultures or tissue lysates (D'Arcangelo et al., 1999; Lambert de Rouvroit et al., 1999a). The activity of proteases generates fragments of 180 KDa (N-terminus to domain 2), 190 KDa (central, domains 3 to 6), 80 KDa (C-terminus, domains 7 and 8), and two intermediate fragments of 370 KDa (N-terminus to domain 6) and 270 KDa (domains 6 to 8) (Lambert De Rouvroit et al., 1999a; Jossin et al., 2004, 2007). This processing did not occur in Reln-Orl mutant mice in which reelin is not secreted, and it was prevented in explant cultures of normal mice by brefeldin treatment, suggesting that it takes place extracellularly or in a postendoplasmic reticulum compartment (D'Arcangelo et al; 1997). *In vitro* experiments suggest that reelin is processed by a metalloprotease in the extracellular matrix in the N-terminus site, but the protease that acts in the C-terminus remains unknown (Lambert de Rouvroit et al., 1999b).

Reelin function is dependent of the central fragment (190 Kb), which binds to ApoE2 and VLDLR receptors triggering Dab1 tyrosine phosphorylation in neuronal cultures, as well as rescue the *reeler* trait in embryonic brain slices, while N-terminal and C-terminal fragments appear to be inactive (Jossin et al., 2004; 2007). The N-terminus mediates the formation of stable homodimers composed of full-length protein, which appear to stimulate reelin signalling pathway more efficiently than the central fragment, (Kubo et al., 2002). The central cleavage fragment can diffuse farther into the developing cortical plate than the full-length homodimers (Jossin et al., 2007).

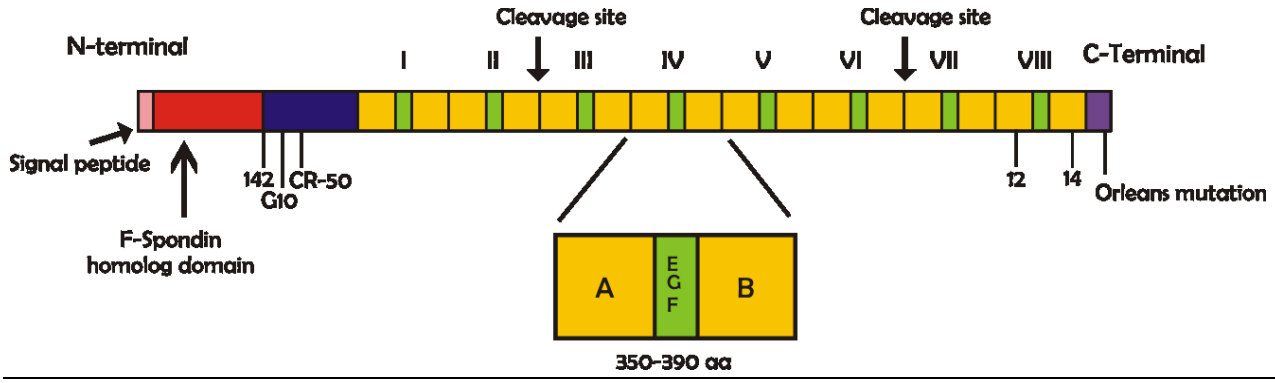

Figure 3. Scheme of the protein reelin. This protein has a signal peptide in the N-terminus, followed by a region with similarity to F-Spondin, aminoacids 28-190. A unique region, specific of reelin, aminoacids 191-500, contains the epitopes recognized by antibodies 142, G-10 and CR-50. After this region there are eight repeats of about 350 aminoacids, each repeat contains an epidermal growth factor motif (EGF) flanked by two subrepeats, A and B, which show weak similarity to each other. Last repeated region contains the epitopes recognized by antibodies 12 and 14. The C-terminus have a region with 33 aminoacids, that is rich in basic residues. Taken and modified from de Bergeyck et al., (1998).

The C-terminus of reelin protein is not necessary for its secretion *in vitro*, but is important for an efficient induction of the proteins implicated in the reelin pathway. The C-terminus seems to interact with an unknown co-receptor in the plasma membrane. Positive charges situated in the C-terminus could contribute to bind the protein to the plasma membranes, while the central fragment is related with the signalling capacity (Jossin et al., 2007; Nakano et al., 2007).

The *reelin* gene in mouse is very large, between 400 and 450 Kb, due to the presence of large introns; that could be involved in the regulation of transcription rate time, or contain enhancer or silencer elements involved in the regulation of gene expression (Royaux et al., 1997). The *reelin* gene is composed of 65 exons, 51 of which encode eight reelin repeats. The presence of eight large introns in reelin repeats suggests that the gene evolved by duplication. Comparison of 8 intron positions in mouse and human reelin genes reveals a highly conserved genomic structure, suggesting a similar structure of the whole gene in both species.

The *reelin* gene is conserved in many vertebrate species, including humans. The predicted mouse and human proteins are similar in size (388 kDa) and the amino acid and nucleotide

sequences are 94.2% and 87.2% identical, respectively (D'Arcangelo et al., 1995; DeSilva et al., 1997) The *reelin* gene is located in chromosome 4 in mouse, chromosome 5 in rat and in chromosome 7 (7q21-22) in man (revised by Lambert De Rouvroit and Goffinet, 1998; Meyer, 2008). Reelin mRNA is also present in chicken, zebrafish, crocodile, lizard and turtle (Bernier et al., 2000; Bernier et al., 1999; Goffinet et al., 1999; Costagli et al., 2002; Tissir et al., 2003). Reelin would be present in all vertebrates, because the protein is present from Amphioxus to human (Pérez-García, 2001; 2004; Pérez-Costas et al., 2002, 2004; Abrahám et al., 2004; Martínez-Cerdeño et al., 2002; Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al., 2003; Ramos-Moreno et al., 2006).

There are several antibodies raised against N-terminal, central and C-terminal regions of reelin (de Bergeyck et al., 1998; Jossin et al., 2007). CR-50 was the first antibody raised against reelin, which labels Cajal-Retzius cells and cerebellar granular cells in normal mice but not in *reeler* mice (Ogawa et al., 1995; Miyata et al., 1997). CR-50 recognized an epitope situated in the specific region of the reelin protein, between aminoacids 230 and 346 (D'Arcangelo et al., 1997) and perturb the action of reelin *in vivo* and *in vitro* (Miyata et al., 1997; Nakajima et al., 1997). There are also available two specific antibodies (142 antibody and G-10 antibody) that recognize an epitope located in the region downstream from the F-Spondin similarity region, and upstream from the reelin repeats (de Bergeyck et al., 1998): the 142 antibody was used to perform studies of reelin in human pathology, and the G-10 antibody recognizes an epitope situated between aminoacids 189 and 245 in rodents.

3. Receptors and signal transduction pathways of reelin

The reelin signalling pathway has been partially elucidated through the study of different mutant mice strains that exhibit a *reeler*-like phenotype.

In 1991 was described a new spontaneous mutant mouse named *scrambled* (Sweet et al., 1996) with a *reeler*-like phenotype. In 1996 scrambled phenotype was associated with an autosomal recessive mutation in the Dab1 gene. Other induced mutant mice, which exhibited a *reeler*-like phenotype (*yotari*) also have a mutation in chromosome 4, in the *disabled-1* (DAB) gene (Howell et al., 1997a, b; Sheldon et al., 1997). Dab1 is a cytoplasmatic protein that contains a PI/PTB domain (protein interaction/Phosphotyrosine binding domain) similar to Shc adaptor protein and on the C-terminus a series of five tyrosine residues that are potential target sites for the Src protein family, implying that Dab1 acts as a cytoplasmatic adapter protein with enzymatic activity, as well as a scaffold for the assembly of a multiprotein signalling complex (Howell et al., 1999a, b; Sheldon et al., 1997; Ware et al., 1997; Howell et al., 2000).

Dab1 is mostly expressed by neurons of the cortex including the ventricular zone (Rice et al., 1998; Luque et al., 2003), hippocampus, cerebellum, and by radial glia precursors in the

ventricular and subventricular zones during neurodevelopment (Sheldon et al., 1997; Rice et al., 1998; Bar et al., 2003; Luque et al., 2003). *Scrambled* and *yotari* mutants have a *reeler*-like phenotype but reelin expression is not affected (Goldowitz et al., 1997). Dab1 levels in *reeler* mice are higher than in the wild type, but they exhibit a normal Dab1 pattern in all brain areas during neurodevelopment (Rice et al., 1998). All these findings together suggest that reelin and Dab1 are involved in the same signalling pathway related with positioning of neurons in the central nervous system (CNS) during development (Goldowitz et al., 1997; González et al., 1997; Sheldon et al., 1997; Yoneshima et al., 1997; Rice et al., 1998 Howell et al., 1999a, b). Both proteins reelin and Dab1 are also present in the adult brain. Dab1 is present in cortical and hippocampal pyramidal cells and reelin in many GABAergic neurons. In the cerebellum reelin was expressed in glutamatergic granule cells, these cells are the primary synaptic input of Purkinje cells, which expressed high levels of Dab1 (reviewed by Rice and Curran, 2001).

The double mutant for reelin and Dab1 evidenced that both proteins act in the same pathway (Howell et al., 1999a). *In vivo* and *in vitro* experiments showed that reelin signalling stimulates tyrosine phosphorylation of Dab1, and reduce the Dab1 levels during development (Rice et al., 1998; Howell et al., 1999a). Animals with mutations in the tyrosine phosphorylate sites of Dab1 exhibit a *reeler*-like phenotype (Howell et al., 2000), although recent studies indicated that Dab1 phosphorylation alone is not sufficient to rescue *reeler* phenotype (Jossin et al., 2004).

Once Dab1 was identified as a molecule that acts downstream of reelin, it was used as a "tool" to find the receptors related with the reelin pathway. The PI/PTB domain of Dab1 interacts with the cytoplasmatic transmembrane domain of APP, LRP and LDL receptor families (Howell et al., 1999b; Trommsdorff et al., 1999). In 1999 Trommsdorff and colleagues using gene-targeting techniques, generated knockouts for two proteins of the low-density-lipoprotein receptors family (LDLR): apolipoprotein E receptor type 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR), and also a double knockout for both proteins. In absence of both receptors, (double knockout) mice exhibited a *reeler*-like phenotype characterized by layer inversion in the cortex and a hypoplasia in the cerebellum, although showed a normal mDab1 distribution pattern protein levels are increased (Trommsdorff et al., 1999). Single knockout mice for ApoER2 showed alterations in cortex and hippocampus but not in the cerebellum, while single knockout for VLDLR showed alterations in the cerebellum but not in cortical structures, which suggests that VLDLR and ApoER2, were in the reelin pathway and both were necessary to the correct positioning of neurons in laminar structures during neurodevelopment. However the differences in the single receptor mutants revealed that VLDLR seems more important for the cerebellum development and ApoER2 for cortical lamination (Trommsdorff et al., 1999; Benhayon et al., 2003). Cortical development of single mutants showed some differences: ApoER2 mutants did not show abnormalities in the lower layers of the cortex, but their superficial layers were severely

altered; in VLDLR single mutants numerous neurons invaded the marginal zone and a separate layer I could not be discerned. Further studies demonstrated that VLDLR and ApoER2 have different functions during cortical development, thus binding of reelin to VLDLR seems to act as a stop signal for early generated neurons while, ApoER2 could be involved in the control of the migration of late generated neurons destined to the superficial layers (Hack et al., 2007).

Further analysis evidenced that VLDLR and ApoER2 act as receptors in migrating neurons, inducing the phosphorylation of Dab1 in the tyrosine residues adjacent to the PTB domain, through a kinase cascade (Hiesberger et al., 1999; D'Arcangelo et al., 1999). *In vitro* experiments also demonstrated that ApoE2 and CR-50 are able to inhibit the Dab1 phosphorylation induced by reelin (D'Arcangelo et al., 1999; Howell et al., 2000). CR-50 inhibits reelin action by blocking the N-terminal region, so the first hypothesis was that the receptor-binding domain was in the N-terminal fragment but *in vitro* experiments showed that the N-terminal portion was not able to bind to the receptors itself (Hiesberger et al., 1999). Reelin binds to VLDLR and ApoER2 by the central fragment, while the N-terminal region mediates the formation of homodimers; the presence of homodimers might be more efficient than the central fragment alone to induce efficient tyrosine phosphorylation of Dab1 (Utsunomiya-Tate et al., 2000; Kubo et al., 2002; Jossin et al., 2004; 2007).

Reelin interacts with two or more lipoprotein receptors simultaneously and seems to induce homo- or heterodimerization or clustering of VLDLR and ApoER2 (Andersen et al., 2003; Strasser et al., 2004). Using *in vitro* techniques it was shown that VLDLR and ApoER2 were in distinct subdomains of the plasma membrane (ApoER2 is situated in lipid rafts while VLDLR is excluded from these domains) and also demonstrated that the signalling activity of reelin receptors was not related to a localization of receptors in lipid rafts and/or caveolae (Mayer et al., 2006). In the other side, different results support the idea of a co-receptor involved in the reelin signalling through VLDLR and ApoER2, but remains to be demonstrated (Jossin et al., 2004).

Parallel to the discovery of VLDLR and ApoER2 as reelin receptors in the developing brain, was described the presence of integrin receptors in the developing cortical neurons and their association with radial glial cells, as other possible receptors for reelin (Anton et al., 1999). Integrins are a family of cell surface adhesion receptors, that modulate specific cell-cell and cell-matrix adhesion, promoting adhesion between neurons, radial glia and the extracellular matrix, and acting as structural links between the extracellular matrix and the cytoskeleton. These receptors are composed of noncovalenty associated alpha and beta subunits that form heterodimeric transmembrane receptor complex (revised by Clegg et al., 2003). During cortical development, integrin receptor subunits show a differential spatial and temporal expression. The alpha subunits play a determinant role in ligand specificity and physiological response of the

individual integrin receptor (revised by Schmid and Anton, 2003).

Beta 1 subunit is widely expressed in the cerebral cortex and alpha subunits dimerize with it. Knockout mice for the alpha 3 subunit lack the laminar organization of the cortex, while the cortical plate in alpha 3 mutant splits normally, the subsequent cell migration to the cortical plate is disrupted, showing that alpha 3 subunit of integrins modulates neuron-glia recognition, and glial-guided migration during cortical development (Schmid et al., 2004). In absence of alpha 3 integrins, neuroblasts adhere to other neurons rather than to their glial guides (Anton et al., 1999; Dulabon et al., 2000; Schmid et al., 2004). However, alpha 3 mutant mice did not phenocopy the *reeler* phenotype: although the cortical plate did not show a laminar organization, Dab1 levels were not increased (Dulabon et al., 2000; Schmid et al., 2004).

Using recombinant reelin, it was demonstrated that alpha 3 beta 1 integrin associates with the N-terminal region of reelin, while VLDLR and ApoER2 bind the central fragment of reelin, so different regions of reelin seem to bind with different receptors (Schmid et al., 2005). Reelin can induce alpha 3/ beta 1 integrin clustering, as has been demonstrated for ApoER2 and VLDLR receptor, as well as endocytosis (Strasser et al 2004). Other studies evidenced that integrins are not an essential component of the reelin pathway during cortical development in mice, and reelin signalling mediated by VLDLR and ApoER2 is independent of beta 1 integrin (Belvindrah et al., 2007). Actually there is still a controversy about the functional link between reelin and integrin during development.

Integrins are also important in the adult nervous system, since they appear implicated in synaptic plasticity involved in learning and memory (reviewed by Clegg et al., 2003). In adult mammalian cortex and hippocampus reelin is synthesized by GABAergic interneurons and secreted in the extracellular matrix surrounding dendrites and dendritic spines (Pappas et al., 2001; Dong et al., 2003). In the cortex, reelin colocalizes with the integrin α3 subunit at the postsynaptic density of the dendritic spines (Dulabon et al., 2000; Rodríguez et al., 2000). *In vitro* experiments evidenced that interaction of reelin with integrin receptors, could stabilize the dendritic spine postsynaptic density and induce protein translation in synaptosomes (Dong et al., 2003).

The protocadherin cadherin-related neuronal receptor-1 (CNR1) is widely expressed in the cortical plate and in synaptic junctions. *In vitro* experiments proposed that CNR1 could form a reelin-receptor complex in the plasma membrane with VLDLR and ApoER2 (Senzaki et al., 1999), although posterior results did not demonstrated detectable interactions between CNR1 and reelin (Jossin et al., 2004).

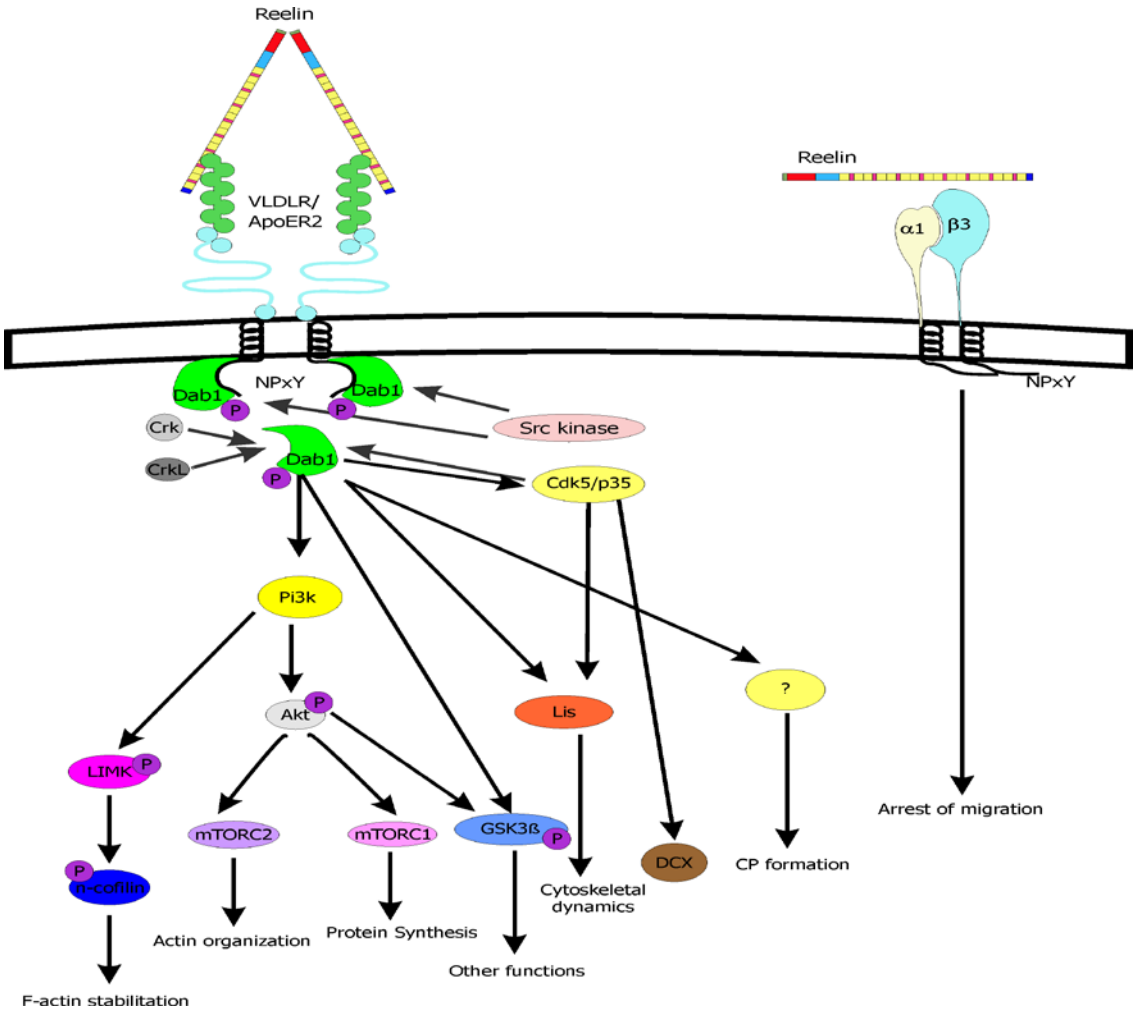

Figure 4. Scheme of the reelin signalling pathway. Schematic view of the possible effects of reelin on different proteins and different downstream pathways. Scheme is based in Jossin and Goffinet, (2007); Chai et al., (2009).

The signalling pathway downstream the reelin receptors, has been partially elucidated (Fig. 4); while reelin and its receptors are anatomically and temporally restricted in the brain, the downstream molecules may comprise some general signalling proteins. *In vitro* and *in vivo* experiments implicated the SFK family of non-receptor protein kinases (Src, Fyn, Yes, Lck and Lyn) in reelin signalling pathway (Arnaud et al., 2003; Bock and Herz, 2003; Kuo et al., 2005). SFK protein kinases are widely expressed in the brain and are abundant in neurons; they have been implicated in proliferation, differentiation and motility during development of the CNS. Proteins of this family share a common mechanism of activation and many of them can phosphorylate the same substrate proteins, being partly redundant members of the same signalling pathways (reviewed by Kalia et al., 2004). *In vitro* experiments, using inhibitors of SFK-family, reduce Dab1 tyrosine phosphorylation and inhibit preplate splitting and formation of a normal cortical plate in cortical slice cultures (Arnaud et al., 2003; Bock and Herz, 2003; Jossin et al., 2003), while mice with inactivated Fyn, Src or Yes do not shown a *reeler*-like phenotype

(Arnaud et al., 2003). Studies of Src and Fyn mutants have provided additional evidences about the role of those proteins in the reelin signalling pathway. Although single mutants for Src or Fyn do not show reeler-like phenotype, double mutants for those proteins show reduced Dab1 tyrosine phosphorylation and degradation in response to reelin, showing a phenotype that is similar to *Dab1* or *reeler* mutants, although less severe (Arnaud et al., 2003; Bock and Herz, 2003; Kuo et al., 2005). Reelin binds to receptor clusters activating Fyn and Src, which in turn phosphorylate Dab1 in specific tyrosine residues (Arnaud et al., 2003; Bock and Herz, 2003; Ballif et al., 2004; Howell et al., 1999a; Keshvara et al., 2001).

Tyrosine phosphorylated Dab1 is a short life molecule that could interact with several SH2 and SH3 containing multi-adaptor proteins, which regulate cell motility and adhesion through interactions with multiple binding proteins, acting in different pathways that contribute in the control of neuronal migration by changes in cytoskeletal dynamics that determine cell motility and morphology (reviewed by Stolt and Bock, 2006), but most of the events are incompletely characterized and not integrated in a coherent scheme. *In vitro* studies showed that phosphorylated Dab1 recruits the p85 subunit of phosphatidylinositol 3 kinase (PI3K), resulting in the phosphorylation of the serine threonine kinase Akt (Beffert et al., 2002; Bock et al., 2003). During cortical development, normal activity of PI3K and Akt is required during the preplate splitting and cortical plate layering (Jossin and Goffinet, 2007). Reelin signal activates several partners of the PI3K/Akt signalling pathway and different targets downstream from Akt mediate the effects of reelin during development or in adult brains. Phosphorylation of Akt could follow two different pathways, inhibiting glycogen synthase kinase 3ß (Bock et al., 2003) or activating mTor kinase (Jossin and Goffinet 2007). Glycogen synthase 3ß activity have been related with the activation of Tau microtubule-associated protein, which could explain the observed increase in Tau phosphorylation in reelin-deficient mice (Hiesberger et al., 1999; Beffert et al 2002; Ohkubo et al., 2003), but this pathway has not been demonstrated *in vivo.* Glycogen synthase 3ß mutant mice display normal brain organization with no indication of neuronal migration defects, because of the probable redundancy and embryonic lethality (Brachmann et al., 2005; Dummler et al., 2006). Reelin may activate, through Akt phosphorylation, the mTORC1-S6K1 pathway and mTORC2 complex. The mTORC2 complex could modulate cytoskeletal dynamics through Rho and Rac, and mTORC1-S6K1 pathway stimulates protein synthesis (Jossin and Goffinet, 2007).

Reelin, SFK family and PI3K are also involved in another signalling pathway related with cofilin, which is related with the stabilization of the actin cytoskeleton of neuronal processes (Chai et al., 2009). Cofilin is an actin-depolymerizing protein that binds to actin filaments and promotes their disassembly. PI3K phosphorylates LIM-kinase1, which in turn phosphorylates cofilin; as a consequence actin dynamics and process elongation are inhibited (reviewed by Frotscher et al., 2009). Crk, Crk-like and Nck are also implicated in the reelin-signalling pathway

(Pramatarova et al., 2003; Ballif et al., 2004; Chen et al 2004; Matsuka et al., 2008). Crk family proteins are expressed ubiquitously and they have been proposed to participate in a variety of biological processes, including several signalling pathways. Laminar brain structures in Crk KO mice exhibit a very similar phenotype to *reeler* mice, while Crk-L mutants just show some similarities to the *reeler* phenotype in the spinal cord but not in the neocortex (Park and Curran, 2008). Studies of Crk KO mice suggest that glycogen synthase 3ß and the IP3K-Akt pathways both depend on the interaction of Crk/CrkL with Dab1 in reelin signalling (Park and Curran, 2008; Feng and Cooper, 2008). However, it is not clear which are the signalling molecules downstream of Crk family proteins and how these signals are integrated with other molecules involved in the reelin pathway.

Other molecules that interact with Dab1 and have the potential to modify the microtubule cytoskeleton are Lis1 and Cdk5. Lis1 is encoded by Pafah1b1 and is mutated in a human lissencephaly syndrome (Reiner et al., 1993). Reelin pathway and the Pafah1b complex interact genetically and biochemically, the regulatory subunit of Pafah1b interacts with phosphoDab1 (Assadi et al., 2003). Lis1 also can bind the microtubule motor cytoplasmic dynein and regulates cytoskeletal dynamics (reviewed by Olson and Walsh 2008; Cooper et al., 2008). Cdk5 is a homologue of the CDK protein family of serine/threonine kinases, is mainly active in postmitotic cells. Its activity is regulated by a neuron-specific regulatory subunit p35 or its isoform p39. Cdk5 modulates the actin cytoskeleton dynamics through phosphorylation of different proteins as doublecortin, Nudel or Collapsin. Studies using different mutants suggest that reelin and Cdk5 function together in a parallel manner during brain developing (reviewed by Ohshima, 2008). But there are some possible molecular mechanisms underlying the relation between reelin and Cdk5, one of them is via the phosphorylation of Dab1 by Cdk5, but the functional significance of this pathway remains to be elucidated. Reelin and Cdk5 may share downstream targets, which could explain the synergistic function of these two pathways (reviewed by Ohshima, 2008). Recently, Notch was proposed as a molecule that could control the morphology and migration of postmitotic neurons, through reelin and Dab1 signalling, although the interaction between reelin and Notch should be further studied (Hashimoto-Torii et al., 2008; Keilani and Sugaya, 2008).

4. Comparative distribution of reelin expression in vertebrates

Reelin is present in the developing and adult CNS and is widely expressed in the brain, showing a highly conserved expression pattern in non-mammalian and mammalian vertebrates.

The presence of reelin was first described during brain development (Ogawa et al., 1995; Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcántara et al., 1998), and several studies in rodents have shown that reelin signal is usually higher during development than in the adult.

Studies using mRNA probes in mouse showed that reelin is firstly expressed at embryonic day 10 (E-10), with the ongoing development there is an overall increase in the expression of reelin in many telencephalic and diencephalic regions (Schiffmann et al., 1997; Alcántara et al., 1998). Maximum levels occur between embryonic day 14 (E-14) and postnatal day 5 (P5).

In the developing telencephalon reelin is located in cells of the cortical marginal zone, they are arranged in a continuous band that covered the entire telencephalic vesicles, including hippocampal region, entorhinal cortex, piriform area and septal region; this pattern remains similar between E-11 and newborn pups (Ogawa et al., 1995; Schiffmann et al., 1997; Alcántara et al., 1998).

The olfactory bulb shows high levels of reelin expression in mitral cells during development and the first postnatal week. After birth, reelin expression levels decrease in mitral cells whereas reelin-positive cells appear in the innermost part of the glomerular layer (Ogawa et al., 1995; Schiffmann et al., 1997; Alcántara et al., 1998).

One of the most studied reelin-positive cells is the Cajal-Retzius cell type, which is located in the marginal zone of the cortex and expresses high amounts of reelin. Cajal-Retzius cells are immunostained for reelin at E-11, and reelin expression levels in these cells remain high during brain development and decrease during postnatal stages (D'Arcangelo et al., 1995; Ogawa et al., 1995; Schiffmann et al., 1997; Alcántara et al., 1998; Meyer and Goffinet, 1999). In the neocortex, reelin-positive neurons are observed in the cortical plate at E-18; there is a progressive decrease in the levels of reelin expression, although reelin-positive cells are still observed in the adult neocortex.

At E-16 the hippocampus shows a layered pattern. reelin is expressed by Cajal-retzius cells in the outer marginal layer (prospective stratum lacunosum-moleculare), and there is a second reelin-positive cell population in the inner zone (prospective stratum radiatum) and in the subplate (prospective stratum oriens). The number of reelin-positive cells in the stratum radiatum and oriens increases at perinatal stages, and some reelin-positive cells are also obseverd in the dentate hilar region of newborn pups. Double labelling studies during hippocampal development showed that reelin is highly expressed in glutamate descarboxylase (GAD) negative cells (Schiffmann et al., 1997; Alcántara et al., 1998).

Reelin is also present in subcortical areas during development: variable levels of reelin expression are found in the prospective fields of the medial and lateral septum, bed nucleus and stria terminalis, as well as in the olfactory tubercle and amygdaloid regions. The ganglionic eminences, the prospective basal ganglia, also show reelin-positive in the caudate-putamen but not in the globus pallidus (Schiffmann et al., 1997; Alcántara et al., 1998).

The diencephalon also shows reelin-positive cells from E-12 onwards. The epithalamus

shows moderate levels of reelin in the habenula and the paraventricular nucleus. While the dorsal thalamus is devoid of reelin, the ventral thalamus shows reelin-positive cells in the zona limitans intrathalamica, reticular nucleus, zona incerta and ventral lateral geniculate nucleus. Reelin expression decreases in the diencephalon after P5 (Schiffmann et al., 1997; Alcántara et al., 1998).

The distribution of reelin-positive cells in mesencephalon and rombencephalon has received less attention, only in the cerebellum and spinal cord there have been performed some detailed studies. The superficial tiers of the tectum, and some areas of the tegmentum show reelin-positive cells (Schiffmann et al., 1997; Alcántara et al., 1998). During cerebellar development, reelin is secreted by granule cells located in the external granular layer, and also a weak labelling appeared associated with neuroepithelial cells in the germinal region adjacent to the fourth ventricle (Schiffmann et al., 1997). Reelin mRNA is also weakly expressed in deep cerebellar nuclei, as well as in the ventral field of the pons and medullary reticular formation (Ogawa et al 1995; Schiffmann et al., 1997). There are discrepancies regarding reelin expression in the inferior olivary complex and facial nerve nuclei, while immunostochemical studies using CR-50 describe immunoreactive neurons in these areas (Ogawa et al., 1995), *in situ hybridization* studies evidenced the lack of reelin in the inferior olivary complex and facial nerve nuclei, although other study detected mRNA in the inferior olivary complex (Ikeda and Terashima, 1997; Schiffmann et al., 1997).

A robust expression of reelin is detected in the entire mouse spinal cord from early embryonic stages, (Schiffmann et al., 1997; Kubasak et al., 2004). Reelin expressing cells are distributed along the dorsal and medial borders of the preganglionic neuronal column, as well as in the superficial dorsal horn and intermediate gray. At early developmental stages, several immunoreactive axons and a small number of reelin-positive cells are detected in the ventral commissure. There is also a group of immunoreactive cells in the ventral ventricular zone. Interestingly there are some reelin-positive cells associated with the roof plate in rat but not in mouse (Kubasak et al., 2004).

Studies of reelin expression in other vertebrates during development also showed the wide expression of reelin in different brain structures (Bernier et al., 1999; Goffinet et al., 1999; Bernier et al., 2000; Costagli et al., 2002; Pérez-Costas et al 2002; Tissir et al., 2003; Candal et al 2005; Cabrera-Socorro et al., 2007). In fact reelin is also observed in species showing a laminar-type brain like the sea lampreys (Pérez-Costas et al., 2002). The most significant differences in reelin expression among different species are observed in the telencephalon: the lamprey, zebrafish and turtle brains do not show reelin-positive cells in the marginal zone, which is a characteristic of the mammalian brain (Bernier et al., 1999; Costagli et al., 2002; Pérez-Costas et al., 2002), while in the chick, lizard and crocodile the cortex contains subpial reelin-positive cells, suggesting that these cells could be homologous to mammalian Cajal-Retzius cells (Bernier et al., 2000; Goffinet

et al., 1999; Tissir et al., 2003; Cabrera-Socorro et al., 2007). The lizard cortex differs from those of turtles, crocodiles, birds and mammals in the presence of reelin-positive cells in the subplate (Tissir et al., 2003). Different studies reveal an evolutionarily conserved pattern of reelin expression in other areas of the brain. Thus, reelin is also present in the olfactory bulb, septal nuclei, amygdala, striatum, habenula, lateral geniculate nucleus, reticular nucleus, hypothalamus, some layers of the optic tectum, the external granular layer of the cerebellum, the reticular formation and spinal cord (Bernier et al., 1999; Goffinet et al., 1999; Bernier et al., 2000; Candal et al., 2002; Costagli et al., 2002; Pérez-Costas et al., 2002; Tissir et al., 2003).

In adult vertebrates, studies of reelin distribution have mostly focused on the cerebral cortex (Miyata et al., 1997; Pesold et al., 1998; Impagnatiello et al., 1998; Rodríguez et al., 2000; Zecevic and Rakic 2001; Pérez-García et al., 2001; Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al., 2002; 2003; Costagli et al., 2002; Pérez-Costas et al., 2004; Abrahám et al., 2005; Ramos-Moreno et al., 2006). Different reports confirm that reelin mRNA and protein are present in high amounts within different GABAergic interneurons of the neocortex in mammals (Pesold et al., 1998; Rice et al., 1998; Pesold et al., 1999; Fatemi et al., 2000; Guidotti et al., 2000; Martínez-Cerdeño and Clascá, 2002), while Dab1 is expressed by pyramidal neurons (Rice et al., 1998). Reelin-positive neurons are particularly located in cortical layer I, but they are also common in layers II-VI, although the laminar distribution of these reelin expressing cells appears to differ between species. Rodents and carnivores show a few superficial and rounded small reelin-positive cells in layer I, as humans do after the first year of life (Pérez-García et al., 2001; Martínez-Cerdeño et al., 2003; Fatemi et al., 2000; reviewed by Meyer, 2008). Other vertebrates like the odontocetes show a high number of reelin-positive cells in the depth of cortical sulci and folds (Pérez-García et al., 2001).

The presence of reelin-positive cells in deeper layers of the neocortex has been studied in rodents, where they are scattered throughout all cortical layers (Alcántara et al., 1998; Ramos-Moreno et al., 2006), and about 3/4 of them show colocalization with GABA. The majority of reelin-positive GABAergic interneurons in the cortex also coexpress other markers such as calbindin, calretinin, neuropeptide Y or somatostatin (Alcántara et al., 1998; Pesold et al., 1999). Some pyramidal cells in layer V of the rodent cortex also express reelin, but protein levels are very low (Pesold et al., 1999; Ramos-Moreno et al., 2006). Non-human primates also show reelin-positive GABAergic interneurons located throughout all cortical layers, with the highest number in layer II (Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al 2002; 2003; Rodríguez et al., 2002), while in humans reelin positive cells below the layer II are more scarce (Pérez-García et al., 2001). It is under discussion the expression of reelin by pyramidal cells in non-human primates and humans, some authors have found reelin almost exclusively in GABAergic interneurons (Fatemi et al., 2000; Pérez-García et al., 2001; Rodríguez et al., 2002), while other

authors also indicate reelin expression in some pyramidal cells and dendritic spines (Rodríguez et al., 2000; Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al 2002, 2003L; Roberts et al., 2005; revised by Meyer, 2008).

The presence of reelin was also reported in the adult telencepahlon of lampreys, teleost fishes, amphibians and reptiles (Pérez-García et al., 2001; Costagli et al., 2002; Pérez-Costas et al., 2004).

Other brain regions show reelin in a variety of species: mouse, rat, ferret, zebrafish and lamprey, revealing a conserved basic pattern of reelin protein localization in homologous neuronal populations, axonal tracts and neuropil areas (Alcántara et al., 1998; Costagli et al., 2002; Martínez-Cerdeño et al., 2003; Pérez-Costas et al., 2004; Ramos-Moreno et al., 2006). In the telencephalon of adult lampreys, teleost fishes and rodents, reelin is distributed in the olfactory bulb, and also in the subpallium; in the diencephalon, the most prominent reelin immunolabelling is associated with the hypothalamus. In addition, reelin is observed in the ventral thalamus and pretectum of teleosts and rodents. In the mesencephalon, lampreys did not show reelin immunoreactivity while its presence was detected in the zebrafish and rodents. In caudal brain areas, reelin is present in cells of the reticular formation, medullar motor nuclei in the spinal cord (Costagli et al., 2002; Pérez-Costas et al., 2004; Ramos-Moreno et al., 2006).

Using immunohistochemical techniques it has been shown that reelin synthesising neurons contain reelin immunoreactivity in the endoplasmatic reticulum and Golgi complex (Pappas et al., 2001; Martínez-Cerdeño et al., 2002; Martínez-Cerdeño and Clascá, 2002), and also in axons that transport it to be secreted (Pesold et al., 1998; Derer et al 2001; Martínez-Cerdeño et al., 2002; Martínez-Cerdeño and Clascá, 2002; Pappas et al., 2003), as well as in the extracellular matrix of synaptic neuropils like cortical layer I, stratum lacunosum-moleculare of hippocampus, piriform cortex layer Ia, or the molecular layer of the cerebellar cortex (Miyata et al., 1997; Pesold et al., 1998; Rodríguez et al., 2000; Pérez-García et al., 2001; Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al., 2002; Deguchi et al., 2003; Roberts et al., 2005).

5. Reelin functional roles

The observation that the formation of cortical layers is disrupted in reeler and reeler-like mutants, demonstrated that reelin and its signalling pathway are crucial during development of laminated structures, and suggests that one of the main functions of reelin is the control of radial migration and the formation of cellular layers during prenatal brain development (Caviness and Sidman, 1973; reviewed by Huang and D'Arcangelo, 2008).

In order to better knowledge the reelin function during brain development we will first summarize in a few paragraphs the main stages of neurodevelopment at the cellular and molecular levels:

The vertebrate brain consists of an enormous number of neurons and glial cells, and presents a high complexity of fibres connections and neural networks. The CNS develops from a small number of highly plastic cells that proliferate, acquire regional identities and produce different cell types, neurons and glia. These cells have been defined as neural stem cells. To understand all the complexity of the brain development, it could be divided in three major phases, although these are not independent and are overlapped:

- Cell proliferation and generation of specific neuronal types.
- Migration of postmitotic cells to their final positions.
- Guidance of axons to their targets, dendrite extension and formation of synapses.

Layer formation is a distinct feature of all laminated structures including the cerebral cortex, hippocampus and cerebellum (reviewed by Sanes et al., 2006a; 2006b).

The mammalian neocortex consists of six different layers (I-VI), and each layer has neurons with distinct functional and morphological identities. Two major neuronal cell classes are found in the cortex: projection neurons (excitatory) and interneurons (mostly inhibitory), both neuronal classes types can be divided in several subtypes. The formation of individual neuronal layers involves migration of neurons in radial and tangential directions to their final destination. These layers are generated in an inside-out pattern, with early born neurons located in the deep layers and late born cells in the superficial layers (reviewed by Huang, 2009).

Early neural tube is formed by a neuroepithelium, consisting in a single type of neuroepithelial cells in various stages of the mitotic cycle. These cells are attached to both the ventricular and meningeal surfaces of the neural tube. Initially, neuroepithelial cells divide symmetrically to increase the pool of stem cells, and the neuroepithelium is a proliferative compartment and the thickness of the neural tube increases (Fig. 5A). This period is followed by a period of asymmetrical cell divisions that generate a stem cell that remains in the ventricular zone and a daughter cell that migrates radially outward the ventricle (revised by Nieuwenhuys, 1998a; 1998b; Sanes et al., 2006b) (Fig 5B). Some cells withdraw from the cell cycle to develop as the first cortical neuroblasts. These neurons migrate through the intermediate zone to form a distinct layer just beneath the pial surface, the preplate (Fig. 5B). The preplate consists in two distinct cell types: Cajal-Retzius cells in a more superficial marginal zone and subplate neurons located in deeper zones. Cajal-Retzius cells were described as the earliest born neurons that migrated from the ventricular zone to the marginal zone. Actually there is an overall consensus that Cajal-Retzius cells are an heterogeneous group of cells derived from different birth places, that by subpial tangential migration invade the marginal zone of the neocortex (Meyer and Goffinet, 1998; Meyer and Whale, 1999; Meyer et al., 2002; Hevner et al., 2003; Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006; Meyer, 2007). Cajal-Retzius cells are

related with different signalling capacities, and are characterized by the presence of high levels of reelin (D'Arcangelo et al., 1997; Schiffman et al., 1997; Alcántara et al., 1998; Deguchi et al., 2003). In mice, only reelin and VLDLR are present in the Cajal-Retzius cells, but human Cajal-Retzius express more components of the reelin pathway (VLDLR, ApoER2 and Dab1) suggesting that reelin might have an autocrine and/or paracrine function, and could play a role controlling the position of Cajal-Retzius cells (Pérez-García et al., 2004).

Most of the cortical projection neurons are generated in the ventricular zone of the pallium, and new postmitotic neurons migrate radially from the ventricular zone towards the pial surface splitting the preplate into marginal zone, where the Cajal-Retzius cells are situated, and the intermediate zone where the subplate neurons are located. Between the marginal and the intermediate zones is located the cortical plate, where the new postmitotic neurons accumulate. Each new cohort of migrating projection neurons bypass the older ones in their way toward the marginal zone, generating the typical six cellular layers of the mammalian cortex in an inside-out pattern (revised by Nieuwenhuys, 1998a; 1998b; Sanes et al., 2006b) (Fig. 5C).

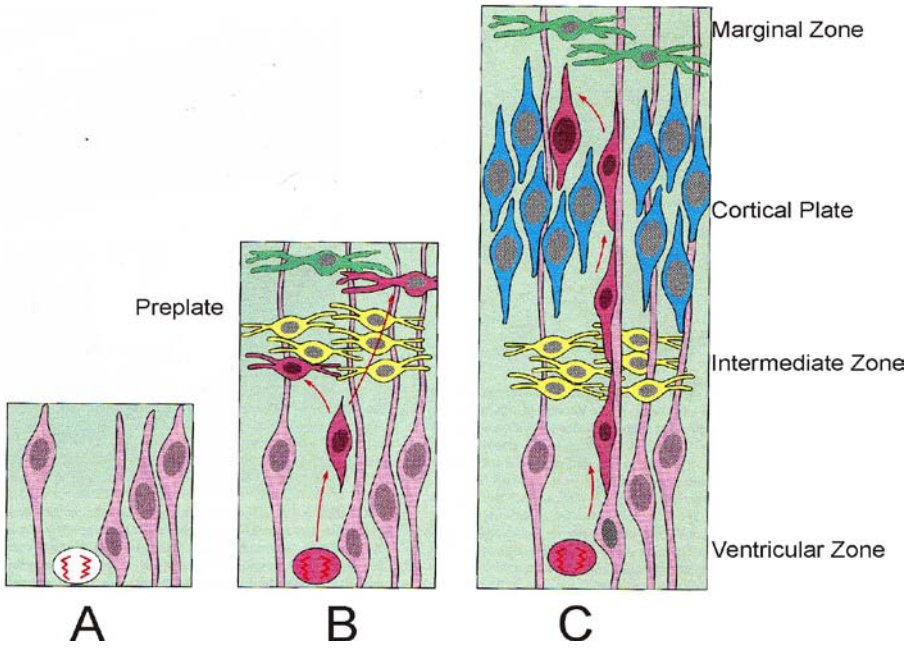

Figure 5. Scheme of the three phases of histogenesis in cerebral cortex. In the first phase, (A) the wall of the cerebral cortex is made up of the progenitor cells (ventricular zone). In a second phase (B) early born neurons exit the cell cycle (red) and accumulate in the preplate. In the third phase (C), newly generated neurons (red) migrate along radial glia fibres and form the cortical plate (blue cells) (Sanes et al, 2006b).

There are two major modes of radial migration used by cortical neurons to move their cell bodies: somal translocation, and glia-guided locomotion. Somal translocation seems to be the predominant mode used by neurons at early corticogenesis; during late development glia-guided locomotion becomes more common, and neurons just switch to somal translocation when their

leading process reaches the pial surface (reviewed by Huang, 2009). To guide the newly generated cortical neurons there is a scaffold of radial glia, with the migrating neurons wrapped around the radial glial process. During the last decade different studies using time-lapse imaging and retrovirus to label small numbers of cortical progenitor cells in slices cultures showed that, contrary to the previous theories, the radial glia and the progenitor cells are not generated separately. Noctor (2002) found that the radial glia themselves were the neuronal progenitors in the cortex. Radial glial cells function during CNS development as both neural progenitors (Malatesta et al., 2000, 2003; Miyata et al., 2001; Noctor et al., 2002) and as a scaffold upon which nascent neurons migrate (reviewed by Nieuwenhuys, 1998a).

Cells situated in the upper cortical plate express VLDLR, ApoER2 and Dab1 (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999a; Trommsdorf et al., 1999; Pérez-García et al., 2004). A current model suggests that a soluble form of reelin produced by Cajal-Retzius cells is secreted into the extracellular matrix where it diffuses towards the developing cortical plate (reviewed by D'Arcangelo, 2005; Jossin et al., 2007) (Fig. 6). Reelin promotes the extension of the neuroblast leading edge and radial migration activating Dab1 pathways through the binding with ApoER2 (Howell et al., 1999; Frotscher et al., 2009; Hack et al., 2007). Once these cells reach the top of the cortex, where reelin levels are higher than in the cortical plate or there is an immobilized form of reelin, cells stop (Anton et al., 1999; Dulabon et al., 2000; Beffert et al., 2002; Hack et al., 2007). It is under discussion which is the receptor involved in the reelin role as a stop signal. *In vitro* and *in vivo* experiments showed that reelin had an effect upon migrating neurons, retarding neuronal migration and inducing neuronal detachment from radial guides through alpha 3 beta 1 integrins (Anton et al., 1999; Dulabon et al., 2000), while other studies suggest that integrin beta 1 is not required for radial glia-guided migration (Belvindrah et al., 2007). VLDLR also seems to mediate a stop signal from reelin, phosphorylating Dab1, which in turn leads to the detachment of the migrating neuron from the radial glia (Hack et al., 2007). So, reelin could act both as a stop signal (Anton et al., 1999; Dulabon et al., 2000) and as a chemoattractant signal (reviewed by Gilmore and Herrup, 2000), the different signals could be mediated by different reelin receptors or by differential recruitment of Dab1-interacting proteins that interact with the actin and microtubule cytoskeleton (see the section on Receptors and the signal pathway of reelin).

Some of the molecules related with the reelin pathway, such as ApoE2 and VLDL receptors and Dab1 are also located in radial glia during corticogenesis (Förster et al., 2002; Luque et al., 2003). Actually, another hypothesis about the role of reelin during cortical development postulates that neural stem cells receive a functional reelin signalling, activating in the ventricular zone the transduction pathway first through the radial glia and thereafter through the newborn radial glia process (Luque et al., 2003; Frotscher et al., 2009). According to

this model, reelin could act as a signal for perikaryon translocation, stimulating and directing radial glia fibre growth (Förster et al., 2002; Luque et al., 2003). Other recent studies suggest that reelin could be related with the control of the cell fate because absence of reelin might preclude a premature differentiation of radial cells into astrocytes (Förster et al., 2002; Frotscher et al., 2003; Zhao et al., 2004; 2007; Hashimoto-Torii et al., 2008; Keilani and Sugaya, 2008; Sibbe et al., 2009).

In the past decades numerous studies revealed that there are cortical neuronal subpopulations generated in non-cortical areas of the developing brain. In rodents, the main sources of cortical interneurons are the medial and caudal ganglionic eminences. Subpopulations of GABAergic neurons are generated in the ganglionic eminences and migrate from at different stages of development to enter the cortical plate following a tangential migratory pathway. After reaching the cortex, interneurons adopt radial migration to invade cortical layers, occupying cortical layers in an inside-out pattern according to their birthdates (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; 2001; Anderson et al., 2001; Valcanis et al., 2003; Xu et al., 2004; reviewed by Wonders and Anderson, 2006).

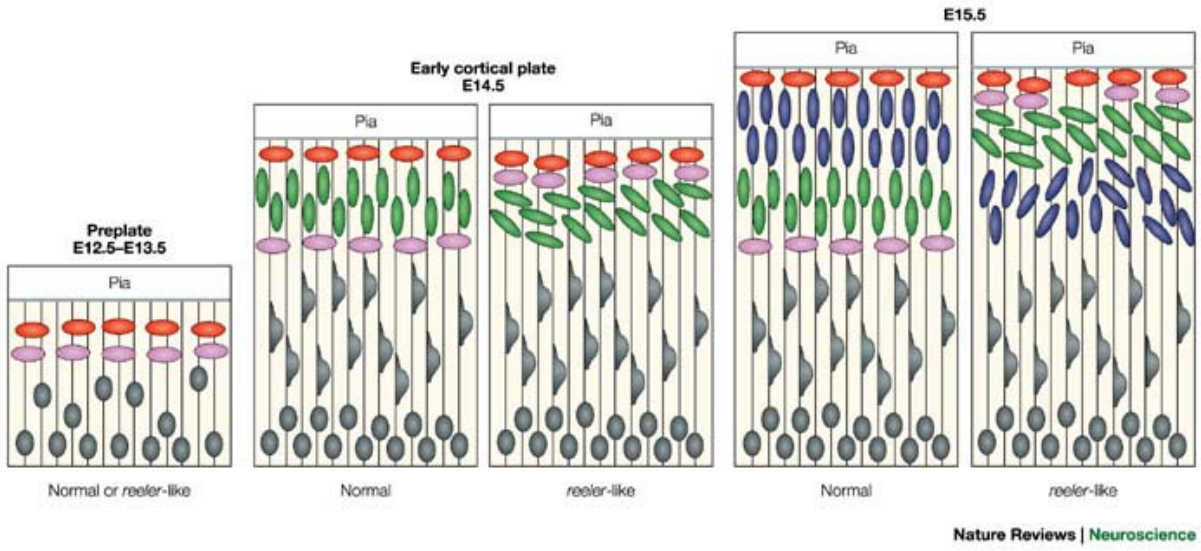

Figure 6. Cortical development in a wild type mouse and a reeler mouse. The reeler cortical plate is populated with cells in an oblique orientation and does not split the preplate into two components at E15.5, a second cohort of cells (blue) migrates through the normal cortical plate and settles superficially, forming an inside to outside gradient. In reeler mutants, the second cohort settles in the deep tiers of the cortical plate, forming an outside to inside gradient. Tissir and Goffinet, (2003).

As reelin is expressed by GABAergic interneurons, some reports studied the possible roles of reelin in regulating the migration of GABAergic neuroblasts. While most reports appear to indicate that reelin does not play a role in regulating migration of GABAergic neuroblasts (López-Bendito et al., 2004; Pla et al., 2006), a recent study by Hammond et al (2006) indicates

that late-born cortical interneurons could rely on reelin signalling for their correct positioning.

Reelin acts using similar mechanisms in other laminated regions of the brain, such as the hippocampus and cerebellum, where principal neurons are born near the ventricle and migrate away from it by using radial glia in the hippocampus and Bergmann fibres in the cerebellum (reviewed by Förster et al., 2006; Larouche et al., 2008).

A last step in the development of the brain circuits is the guidance of axons to their targets and the formation of synapses. Axons make a journey to connect to their targets, growing axons being able to recognize several molecules on the surfaces of other axons and cells, and use these signals as cues to reach their particular destination. Synaptogenesis is the result of a complex series of events that include the acquisition of synaptic competence and the apposition of presynaptic and postsynaptic anatomical structures (Sanes et al., 2006c). Several studies performed in the hippocampus have found that reelin is not involved in axon guidance, since the general topography of hippocampal connections is preserved in *reeler* mice (Borrell et al., 1999; Wu et al., 2008). However, reelin could modulate axonal targeting, adhesive fasciculation, as well as influence axonal branching; and synaptogenesis it might be related with the formation or stabilization of dendritic spines in the postnatal hippocampus (Borrell et al., 2007; Niu et al., 2008). ApoER2, VLDLR and Dab1 are also crucial during dendritic growth and synaptogenesis, probably through the PI3K/Akt/mTOR pathway (Jossin and Goffinet, 2007). Synaptic maturation is a dynamic process that continues long after synaptogenesis and enables synapses to stabilize their presynaptic and postsynaptic properties. *In vitro* studies showed that reelin facilitates the developmental maturation of hippocampal glutamatergic function in the postnatal mice: reelin controls changes in the subunit composition and number of NMDA receptors during postnatal maturation of glutamatergic central synapses, involving receptors of the integrin family (Sinagra et al., 2005; Groc et al., 2007; Qiu and Weeber, 2007). Reelin could also be implicated during cortical development in the maturation of apical dendrites of pyramidal neurons of layers II/III (Chameau et al., 2009).

During adult life, neurogenesis and neuronal migration continue in some brain regions, such as the hippocampus and the olfactory bulb (reviewed by Gould, 2007). The most apparent migration in rodent adult brain is the rostral migratory stream, in which neuronal precursors are generated in the ventricular zone and migrate tangentially to the olfactory bulb, using a characteristic mode of translocation in a homotypic chain-like organization. Once the precursor cells arrive to the olfactory bulb, they change their behaviour from a tangential migration to individual radial migration before their differentiation. These new generated cells will differentiate into GABAergic interneurons of the granular and periglomerular layers (reviewed by Lledo et al., 2008). The olfactory bulb shows a severe reduction in size and the number of newly generated neurons is also decreased in the reeler mice (reviewed by Lambert de Rouvroit

and Goffinet, 1998; Kim et al., 2002). In the adult olfactory bulb reelin is not a directional cue or a stop signal for neuronal precursors migrating in the rostral migratory stream, but it seems that reelin induces detachment of these cells from their migratory substrate (Hack et al., 2002). Reelin expression is also persistent in the adult dentate gyrus, where it maintains the normal integration of newborn neurons in the neonatal and adult mammalian hippocampal formation, regulating the migration of adult dentate granule cells progenitors (Fournier et al., 2009a, b; Lussier et al., 2009). Reelin could have a role leading the detachment of chain-migrating dentate gyrus neuroblasts, as it occurs in the olfactory bulb (Gong et al., 2007).

Because reelin is related with the synaptic machinery, different studies were focused on the role of reelin in the postnatal and adult brain, as regards synaptic plasticity and memory (reviewed by Herz and Chen, 2005). *In vitro* and *in vivo* experiments demonstrated that reelin promotes long-term potentiation through the activity of ApoE2 and VLDL receptors (Weeber et al., 2002; Beffert et al., 2006). Reelin, secreted by GABAergic neurons, could be necessary to maintain the subunit composition of NMDA receptors as well as to activate SFK family proteins regulating the activity of the NMDA receptor, controlling calcium entry and thus regulating synaptic plasticity (Chen et al., 2005; Beffert et al., 2006; Campo et al., 2009). Dendritic spine formation and maintenance could also be related with reelin function in hippocampus (Niu et al., 2008). For this role, reelin could provide a molecular scaffold for the assembly of cytoskeletal proteins that facilitate dendrites resident mRNA translations, increasing protein synthesis required for memory process (Dong et al., 2003).

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Aims of this Thesis

During brain development there are crucial events that determine the correct position of different populations of neurons and the establishment of neuronal connections and networks. Numerous molecules are involved in regulating these processes, and it is of interest to know in detail their patterns of expression and functional roles. Reelin is one of these molecules, having been demonstrated to play essential roles in regulating neural migration, synaptogenesis and synaptic plasticity. In addition, reelin has been shown to be downregulated in several psychotic disorders increasing the interest of the studies on this protein.

Several reports have shown that a decrease in reelin expression during development could give rise to some brain abnormalities that resemble those observed in schizophrenia brains, where reelin is also downregulated. Although some general works reporting reelin expression during brain development have already been published, we believe that it is of interest to focus on more detail in the developmental pattern of reelin expression in some brain areas that have been shown to be important in schizophrenia symptomatology, such as the cerebral cortex, striatum, piriform cortex, and thalamus. In addition, we present a detailed study of reelin prenatal expression in midbrain and hindbrain that were not reported in detail in other previous works on reelin distribution.

Therefore the present work has 5 main aims:

1) To investigate reelin expression in the developing cortex: To this aim we study reelin expression at different developmental stages and the coexpression of reelin with several neurochemical markers. The results are presented in Chapter 1 entitled "Reelin and Tbr1 colocalization in a subpopulation of neuroblasts in the cortical subplate/intermediate zone".
2) To study reelin expression in striatal patches (striosomes), as well as the colocalization of reelin with other striatal markers. The results are reported in Chapter 2 entitled "Correlation of the temporal expression of reelin, DARPP-32 and tyrosine hydroxylase during prenatal development of striatal compartments in the rat brain".
3) To study reelin expression in the piriform cortex in relation to the expression of other neurochamical markers. The results are presented in Chapter 3 entitled "Reelin expression during development of the rat piriform cortex".
4) To investigate reelin expression in thalamic nuclei. The results are reported in Chapter 4 entitled "Reelin, Tbr1 and Pax6 expression in the ventral thalamus during rat prenatal development and newborn pups".
5) To provide a detailed description of reelin expression in the developing midbrain and hindbrain. The results are reported in Chapter 5 entitled "Reelin expression in the midbrain and hindbrain during rat prenatal development and in newborn pups".

Chapter 1

Reelin and Tbr1 colocalization in a subpopulation of neuroblasts in the cortical subplate/intermediate zone

1. INTRODUCTION

Reelin is an extracellular matrix protein involved in neuronal positioning, synaptogenesis and neural plasticity. During cortical development reelin is mainly expressed by Cajal-Retzius cells and secreted into the extracellular matrix (D'Arcangelo et al. 1995; Ogawa et al. 1995). In the adult cortex reelin is expressed by GABAergic interneurons and also by a few scattered interstitial cells in the cortical white matter (Alcántara et al; 1998; Pesold et al. 1998; Pesold et al. 1999; Eastwood and Harrison, 2003; Ramos-Moreno et al., 2006).

Reelin containing Cajal-Retzius cells are mostly, but not exclusively, originated in the cortical hem and undergo tangential migration through the marginal zone where they will be finally located (Meyer et al., 2001; Bielle et al., 2005).

In the rodent adult cortex, the vast majority of neurons that express reelin are cortical GABAergic interneurons belonging to specific cortical GABAergic subtypes, being mostly GABAergic neurons that express either calbindin, calretinin, somatostatin or NPY, but not parvalbumin (Alcántara et al., 1998; Pesold et al., 1998, 1999). The main sources of cortical interneurons are the ganglionic eminences, from where subpopulations of GABAergic neurons generate and migrate at different stages of development to enter the cortical plate following a tangential migratory pathway (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Anderson et al., 2001; Xu et al., 2004; Wonders and Anderson, 2006).

Since reelin is expressed by cortical GABAergic interneurons, there have been several reports studying the putative role of reelin in regulating the migration of GABAergic neuroblasts. While most reports appear to indicate that reelin does not play a role in regulating migration of GABAergic neuroblasts (Lopez-Bendito et al., 2004; Pla et al., 2006), a recent study by Hammond et al (2006) indicates that late-born cortical interneurons could rely on reelin signaling for their correct positioning.

There is an additional population of reelin expressing cells in the adult cortex, constituted by some white matter interstitial cells that probably represent a population of survivors subplate neurons (Eastwood and Harrison, 2003).

In a previous report, we showed that a few reelin containing neuroblasts could be seen in the intermediate zone during the third week of rat embryonic development (Caruncho et al., 2004). The presence of reln-ir cells in the intermediate zone suggests different alternatives: they could represent some tangentially migrating GABAergic neuroblasts, or cells of pallial origin such as Cajal-Retzius cells, or a population of subplate cells. Therefore the aim of the present study is the characterization of this population of reelin expressing cells by using immunohistochemical techniques and BrdU birthdating experiments.

2. MATERIAL AND METHODS

Animals

Adult males and pregnant female Spragle-Dawley rats were used in this study. All procedures for handling and euthanasia are in accordance with the European Commission guidelines (86/609/CEE) and were approved by the bioethics committee of the University of Santiago de Compostela. Pregnant rats were deeply anaesthetized, and euthanized by cervical dislocation. For calculation of gestation and embryonic development stages, the day when the vaginal plug appears it was day 0 and the pups were born on E-21 (P0). The embryos were removed by caesarean surgery at different developmental stages corresponding with embryonic day 13, 14, 15, 17, and 19 (E-13, E-14, E-15, E-17, and E-19). Newborn pups (P0) and P10 were also used. For birthdating experiments using BrdU, pregnant female rats were injected with a single dose of BrdU (100 mg/kg I.P.) at E-13.

The animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer 0.1M (PB). Some E-15 embryos and P0 were also perfused transcardially with 4% paraformaldehyde 2% glutaraldehyde in 0.1 M PB for GABA immunohistochemistry. The brains removed from the skull were cryoprotected in a sucrose solution (30% sucrose in PB) and sectioned at 20 or 40μm thickness in a cryostat. For long-term storage, the tissue sections were kept at -80ºC.

Antibodies

The following primary antibodies were used in single and double immunohistochemistry procedures: 1) Mouse anti-reelin (reln) monoclonal antibody G-10, 1:1000 or 1:500 in fluorescent immunolabelling (two different sources: a generous gift of Dr. A. Goffinet, University of Louvain, Belgium and a commercial antibody (Chemicon, code MAB 5364). For details on the characterization of this antibody see De Bergeyck et al. (1998). 2) Rabbit anti-Calbindin-D28k polyclonal antibody (CB) diluted 1:10000 (Swant, code CB-38a). 3) Rabbit anti-Calretinin polyclonal antibody (CR) diluted 1:1000 (Swant, code 7699/4). 4) Rabbit anti-Doublecortin antibody (DCX) diluted 1:500 (Cell Signalling, code 4604). 4) Rabbit anti GABA diluted 1:500 (Affinitty, code GA1159). 5) Rabbit anti-Tbr1 1:500 (Chemicon, code AB9616). 6) Mouse anti P73 1:100 (Neomarkers, code MS-762). 7) Rabbit anti-Pax6 1:500 (Chemicon, code AB5409). 8) Rabbit anti-BrdU 1:250 (Affinity BioReagents, code PA1-28373).

In the present study, we have used the following secondary antibodies: Biotinylated goat anti-mouse and goat anti-rabbit antibodies (Dako product numbers, Z0420 and E0432, respectively). Alexa Fluor 488 coupled goat anti-rabbit and Alexa Fluor 546 coupled goat anti-mouse antibodies (Molecular Probes product numbers A11008 and A11003 respectively).

Immunohistochemistry

For BrdU detection, sections were immersed previously to immunolabelling in 2N HCl for 30 minutes at 37ºC, and washed in a solution of sodium borate (0.1M, pH 8.5) during 10 minutes, followed by Tris buffer saline (TBS) (pH 7.4) for another ten minutes. For single immunohistochemistry, sections were incubated overnight at room temperature with the appropriate primary antibodies diluted in a solution of 15% normal goat serum (NGS) and 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in TBS.

After rinsing with TBS sections were sequentially incubated with the appropriate secondary antibodies for one hour, diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100. After the secondary antibody the sections were incubated with ABC complex (Vector Laboratories), developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine (DAB Sigma), and finally dehydrated and coverslipped.

For double immunofluorescence, sections were incubated overnight at room temperature with mouse anti-reelin combine with the following antibodies: anti-GABA, anti-DCX, anti-CB, anti-CR, anti-Pax6 and anti-Tbr1. In all cases, the antibodies were prepared in a solution containing 15% NGS, 1% BSA and 0.2% Triton X-100 diluted in TBS. After rinsing with TBS, sections were incubated with appropriate fluorescent secondary antibodies diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100 for one hour. Some sections were counterstained with bisbenzimide 0,4%. Finally, sections were rinsed in distilled water, air-dried, and coverslipped using Mowiol (Calbiochem).

For all antibodies, a series of control sections was stained in which the primary antibody was omitted, and not staining was seen.

In situ Hybridization

In situ hybridization (ISH) for reelin was performed using a riboprobe labelled with digoxigenin-d-UTP (Roche) by *in vitro* transcription of a cDNA fragment encoding mouse reelin (D'Arcangelo et al., 1995) with T3 polymerase (Roche). Sections were permeabilized 2 minutes with proteinase K (5 μg/ml), fixed 10 minutes in 4% paraformaldehyde, and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridized at room temperature for 3 hours in a solution containing 50% deionised formamide, 10% dextran sulphate, 1X Denhardt's solution, 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 2X SSC, 250 μg/ml yeast t-RNA and 250 μg/ml denatured salmon sperm DNA. Labelled anti-sense cDNA was added to the pre-hybridization solution (500 ng/ml), and the hybridization was performed at 60ºC overnight. Sections were then rinsed sequentially in 1X SSC (10 minutes at 65ºC), 1.5X SSC (10 minutes at 65ºC), and 2X SSC (40 minutes at 37ºC), digested with RNase A (30 minutes at 37ºC) and rinsed in 0.2X SSC (1 hour at 55ºC). After that, sections were rinsed in maleic acid buffer containing 0.5% Tween 20 (15 minutes), incubated with a blocking solution containing MABT (100 mM maleic

acid, 150 mM NaCl and Tween 20) and normal sheep serum (3 hours at room temperature), and finally incubated overnight with an alkaline phosphatase-conjugated antibody to digoxigenin (1:2000, Roche). The reaction was developed with BM purple (Roche), and coverslipped with Mowiol.

Photography

Sections were observed using an Olympus Bx51 microscope. Photomicrographs were taken with an Olympus DP-71 color digital camera. Sections labeled for double immunofluorescence were observed using a Leica DMRE microscope. Confocal images were acquired with a Leica TCS-SP2 laser confocal microscope. The images were converted to a gray scale (for black and white figures), and adjusted for brightness and contrast by using Corel Photo-Paint 13. Picture set up was achieved with Corel-Draw 13. The full resolution was maintained until the micrographs were cropped and assembled, at which were adjusted to a resolution of 300 dpi.

3. RESULTS

3.1. Reelin is expressed by a subpopulation of neuroblasts in the subplate/intermediate zone during cortical development

In the present study, we have characterized a population of reelin immunoreactive (reln-ir) neuroblasts, which is apparent at the beginning of the third week of embryonic development in the cortical intermediate zone.

At E-13, cortical reln-ir cells (probably Cajal-Retzius cells) are observed just underneath the pial surfaces, but there is no evidence of reln-ir cells in the intermediate zone (Fig. 1A). E-14 is the first developmental stage at which some reln-ir cells appear in the intermediate zone with their long axis parallel to the pial surface (Fig. 1B).

The number of reln-ir cells in the intermediate zone clearly increases by E-15. In cortical oblique sections that include the retrobulbar region, ganglionic eminences, thalamus and cortical hem is possible to discern a row of reln-ir neuroblasts in the intermediate zone of the caudal-lateral cortex (see Fig. 1C-D and serial sections in Fig. 1E-J). At this developmental stage, reelin staining appears mostly as a heavy intracytoplasmic labelling. In fact, the degree of intracytoplasmic staining of these cells appears to be similar to that observed in reln-ir Cajal-Retzius cells located in the cortical marginal zone (Fig. 2A). These results are confirmed by in situ hybridization experiments showing that at E-15 there are heavily labelled reelin mRNA expressing neurons in the cortical intermediate zone (arrows in Fig. 2B). The staining level of reelin mRNA in cells in the intermediate zone is comparable to that observed in Cajal-Retzius cells in the cortical marginal zone (Fig 2B). Reln-ir neuroblasts in the intermediate zone show fusiform shape with processes that run parallel to the pial surface. Most of them present a thick process extended

towards the lateral cortex (Fig. 2C, D, E), although some show the main process towards the medial cortex (Fig. 2F). In addition, there are a few multipolar reln-ir cells near the pallium-subpallium limit (Fig. 2G).

At E-17 a few reln-ir cells are seen in the upper intermediate zone of the lateral cortex, near the subplate as well as in the lower intermediate zone (Fig. 3A-C). At embryonic day 19, some reln-ir cells are observed in the subplate region. These cells have a fusiform shape and keep their long axis parallel to the pial surface (Fig. 3D).

The subplate zone of the newborn pups shows a higher number of reln-ir cells than in previous stages (Fig. 3F, 3G), the cortical plate also shows an increase in the number of reln-ir cells (Fig. 3F). At postnatal day 10, the subplate continues showing reln-ir cells that are accumulated mostly in the dorsal cortex area (Fig. 3H-I). Interestingly, most of these cells show a multipolar shape instead of the fusiform shape that was observed in previous stages. In the adult cortex layer VIb there was a general absence of reln-ir cells, with the exception of some reln-ir cells in cingulate cortex layer VIb (Fig. 3J-K).

3.2. Reln-ir cells observed in the intermediate zone at E-15 originate before E-13, these cells are Tbr1, DCX and calretinin positive but do not express Pax6, P73, GABA or calbindin.

To perform birthdating studies we injected BrdU at E-13, previously to the appearance of reln-ir cells in the cortical intermediate zone. At E-15 reln-ir cells in the intermediate zone failed to show any BrdU labelling, indicating that these cells are originated before E-13 (Fig. 4A-C).

To characterize the subpopulation of reln-ir cells in the intermediate zone, we have used several neurochemical markers for cortical neuroblasts (antibodies against Pax6, Tbr1, DCX, CR, CB, GABA, and P73) in brains from E15 embryos, the stage at which those cells show the highest degree of cytoplasmatic reln-ir.

Double labellings of reln and Pax6 showed that Pax6 immunoreactivity is restricted to the ventricular zone while reln-ir cells are situated in the intermediate zone. There was no colocalization of reelin and Pax6 (Fig. 4D).

Tbr1 is a transcription factor expressed in cells distributed along the marginal zone, preplate and intermediate zone, but not in the ventricular zone (Fig. 4E). Reln-ir cells in the intermediate zone showed a clear colocalization with Tbr1, with the cell nucleus Tbr1-ir and the cytoplasm reln-ir (Fig. 4E-H). In the intermediate zone every neuron immunoreactive for reelin was also DCX positive, representing a small percentage of DCX immunoreactive cells (Fig. 4I-L)

P73 immunolabelling is restricted to the marginal zone of the developmental cortex but there are no P73-ir cells in the intermediate zone at E-15 (Fig. 5A), suggesting that P73 is not expressed in reln-ir cells in this zone.

Double immunolabelling for reelin and GABA demonstrates that reln-ir and GABA-ir neuroblasts are observed in the same region in the intermediate zone, but those markers do not colocalize within the same cells (Fig. 5C-D).

Labelling of calcium-binding proteins shows two different expression patterns, while all reln-ir cells are also positive for calretinin (Fig. 5E-G); there was no colocalization with calbindin (Fig. 5B).

To study the evolution of reln-ir neurons in the subplate/intermediate zone, we also ascertained if these colocalization patterns described at E-15 are also observed in reelin positive cells at later prenatal developmental stages. Colocalization studies performed on E-17 showed that in the upper intermediate zone there are scattered reln-ir cells that are also stained with Tbr1 (Fig. 6A-D), DCX (Fig. 6E-H), and calretinin (Fig. 6I-L). At E-19 reln-ir cells in the subplate remain coexpressing Tbr1 (Fig. 7A-C), calretinin (Fig. 7D-F), and DCX (although DCX levels are already lower) (Fig. 7G-I).

3.3. Neurochemical characterization of subplate reln-ir cells in postnatal stages

In newborn pups, reln-ir subplate cells coexpress Tbr1 (Fig. 7J-L), calretinin (Fig. 7M-O), and a faint stain for DCX (Fig. 7P-R), but do not express GABA (Fig. 7S-V). Reln-ir cells are also observed in different layers of the cortex; where they colocalize with DCX, and some of those reln-ir cells also show colocalization with calretinin in the upper layers of the developmental cortex. However, colocalization of reelin with the markers (DCX, calretinin and Tbr1) only appears in the subplate and also in layer I.

At P10, reln-ir cells in the subplate accumulate mostly in the dorsal cortex and show a clear colocalization with Tbr1 (Fig. 8A-D). DCX immunolabelling decreases and is not observed in reln-ir cells of the subplate (Fig. 8E-H). Calretinin immunostaining decreased in the subplate, most reln-ir cells in the subplate showed colocalization with calretinin but not all of them (Fig. 8I-L).

A few reln-ir cells were observed in layer VIB of the anterior cingulate cortex, but not in other cortical regions. These cells also express Tbr1 (Fig. 8M-P).

4. DISCUSSION

The present work evidences a population of reln-ir cells that starts to be seen in the IZ of the lateral cortex in oblique sections at E-14 rat embryos. Many of these cells have the typical appearance of neuroblasts undergoing tangential migration. None of these cells appears to cross the cortical-striatal border and we have no clear evidence of any of these cells reaching the piriform cortex area, although it cannot be rejected.

BrdU birthdating studies indicate that reln-ir cells in the subplate-intermediate zone are born before E-13. We selected to inject BrdU at E-13 because at that stage there was no evidence of reln-ir cells in the subplate-intermediate zone. These data appears to point to an identification of these cells as a pioneer cell population within the cortex. In fact the cells of the cortical preplate (Cajal-Retzius cells that will remain in the marginal zone and those that will form the subplate) are predominantly originated before E-13 (Bayer and Altman, 2004).

To help discerning the nature of these reln-ir cells population we also used different markers that allow us to demonstrate that during developmental stages these cells are also labelled for Tbr1, DCX and calretinin but do not express Pax6, P73, GABA.

The localization of some of these cells in the IZ close to the cortical-striatal border at E14-15 and the presence of some cells with the main process towards the lateral border could mean that this cell population pertains the lateral cortical stream towards ventral telencephalic regions (i.e. the piriform cortex and amygdala). However, we could not follow these cells towards the piriform cortex area but they are also negative for Pax6 (a marker of cells of pallial origin within the lateral cortical stream), which argues that the reln-ir/Tbr1-ir cell populations in the IZ will not be part of the lateral cortical stream because this includes two main population of cells, one Pax6 positive, which is not the case, and other that is Dlx2 positive (indicating subpallial origin), and therefore not labelled for Tbr1 (Bulfone et al., 1995; Carney et al., 2006; Hevner et al., 2006). In any case we should not rule out that some of these cells could reach the piriform cortex and perhaps represent part of the reln-ir cell population originated at the pallium-subpallium border.

The location of these cells in the IZ of the developing cortex in rat embryos argues that they could represent a population of cortical reln-ir cells. During cortical development reelin expression is observed mostly in Cajal-Retzius cells in the marginal zone, while in the adult cortex reelin is preferently expressed by different subtypes of GABAergic interneurons and also by a few interstitial white matter cells. Therefore one should consider that the presence of reln-ir cells in the cortical SP/IZ of E-15 rat embryos could represent one of these populations.

Cajal-Retzius cells have been identified mostly by the presence of reelin and calretinin (Alcántara et al., 1998; Hevner et at, 2001; Ogawa et al., 1995; Meyer and Goffinet, 1998). Therefore it could be logic to think that reln-ir and calretinin-ir cells describe in the present study it could represent a small subpopulation of Cajal-Retzius cells. However, there are several findings that make this very unlikely: First, we describe the novel population of reelin migrating neuroblasts in the intermediate zone at E14-17, while Cajal-Retzius cells are clearly identified in the marginal zone, covering up the whole developing cortex, already at E-13 (the first stage that we have studied, although are already visualized at E-12). Most Cajal-Retzius cells are stained by protein p73 (a protein that belongs to the family of tumor-supressing protein p53), which in fact it has been proposed as a more accurate marker for Cajal-Retzius cells than reelin (Meyer et al.,

1999; Meyer et al., 2002). In E-15 rat embryos P73 labelling is restricted to cells in the marginal zone and not in the IZ, where the row of reln-ir cells is found. Therefore these findings appear to indicate that the population of reln-ir migrating neuroblasts found in the intermediate zone at E-15 is not a subpopulation of Cajal-Retzius cells, although it should not be completely discarded.

The second option to be considered was that these cells could represent tangentially migrating GABAergic neuroblasts originated in the ganglionic eminences. Several subtypes of cortical GABAergic interneurons express reelin in the adult brain, and due to the role of reelin in regulating neural migration it could be thought that reelin could be already expressed in migrating GABAergic neuroblasts and could play a role in this migration. However, most reports indicate that reelin does not play an important role regulating tangential migration of GABAergic neuroblasts, (Pla et al., 2006) although some studies suggest that some specific subpopulation of GABAergic neuroblasts could rely on reelin as a molecule regulating their tangential migration (Hammond et al., 2006). Since we have identified a very specific subpopulation of reln-ir cells in the cortical IZ, it could be possible that these cells were identified as GABAergic neuroblasts. However, there was not colocalization of reelin and GABA in this cell population, but these cells were also stained for Tbr1 (a marker for glutamatergic neurons of pallial origin). Therefore we can discard that these cells represent a subpopulation of migrating GABAergic neuroblasts.

The third possibility to be considered is that they could represent a subpopulation of subplate neurons, some of those could survive in the adult brains as interstitial white matter cells. Subplate neurons have different origins: glutamatergic neurons originated in the cortical neuroepithelium that express markers as Tbr1 and interneurons which are produced from progenitors in the ganglionic eminences and express transcription factors as Dlx (Hevner et al., 2001; 2002; 2006). Some reln-ir cells are seen just below the cortical white matter, which appear thereafter to accumulate in the subplate. Double-labellings of reelin and Tbr1 or calretinin (which labels many subplate cells) confirm that with prenatal development progression, cells coexpreessing reelin and Tbr1 are not observed anymore in the intermediate zone but accumulate in the subplate. When they reach the subplate lose the labelling for DCX and switch from a fusiform to a multipolar shape, which probably indicates that at this time they stop their migration and differentiate. In the adult brain there is no clear evidence of reln-ir cells that coexpress Tbr1 in the cortex with the exception of a few weakly stained cells in layer VIB of the cingulate cortex.

Interestingly, reln-ir cells in the white matter did not show labelling with Tbr1, indicating that they represent a different population from that we have described in the present study. This collection of data appears to indicate that we are describing a novel population of reelin expressing cells with a pallial origin, which migrate tangentially through the intermediate zone

and reach the subplate. This cell population is not observed in the adult brain suggesting that they stop expressing reelin or they cannot visualized because they have degenerated by apoptosis.

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6.FIGURES

Figure 1: Reelin expression in rat telencephalon at E-13, E-14 and E-15. (A) Coronal section immunostained with anti- G-10 antibody, showing reln-ir Cajal-Retzius cells in the marginal zone of the cortex at E-13, while no immunopositive cells are observed in the intermediate zone of the cortex and ganglionic eminences. Inset: High magnification of Cajal-Retzius reln-ir cells in the marginal zone. (B) Oblique section at E-14. Note the presence of reln-ir cells in the lateral intermediate zone (arrows), as well as in the marginal zone. (C) Schematic drawing of a rat brain at E-15, showing the orientation and the level of the oblique sections in which is possible to visualize the row of reln-ir cells in the intermediate zone. (D) Oblique section and schematic drawing of rat brain coronal section at E-15 showing the distribution of reln-ir cells (grey dots) in the marginal zone, intermediate zone, cortical hem and ganglionic eminences. (E-J) Serial oblique sections at E-15: is observed in Cajal-Retzius cells in the marginal zone and in a row of cells in the intermediate zone. Abbreviations: PP, preplate; GE, ganglionic eminences; V, ventricle; MZ, marginal zone; IZ, intermediate zone, CH, cortical hem; CP, cortical plate; VZ, ventricular zone. Scale bars: A: 50 μm, high magnification 25 μm; B: 200 μm; D: 2 mm; E-J: 100 μm.

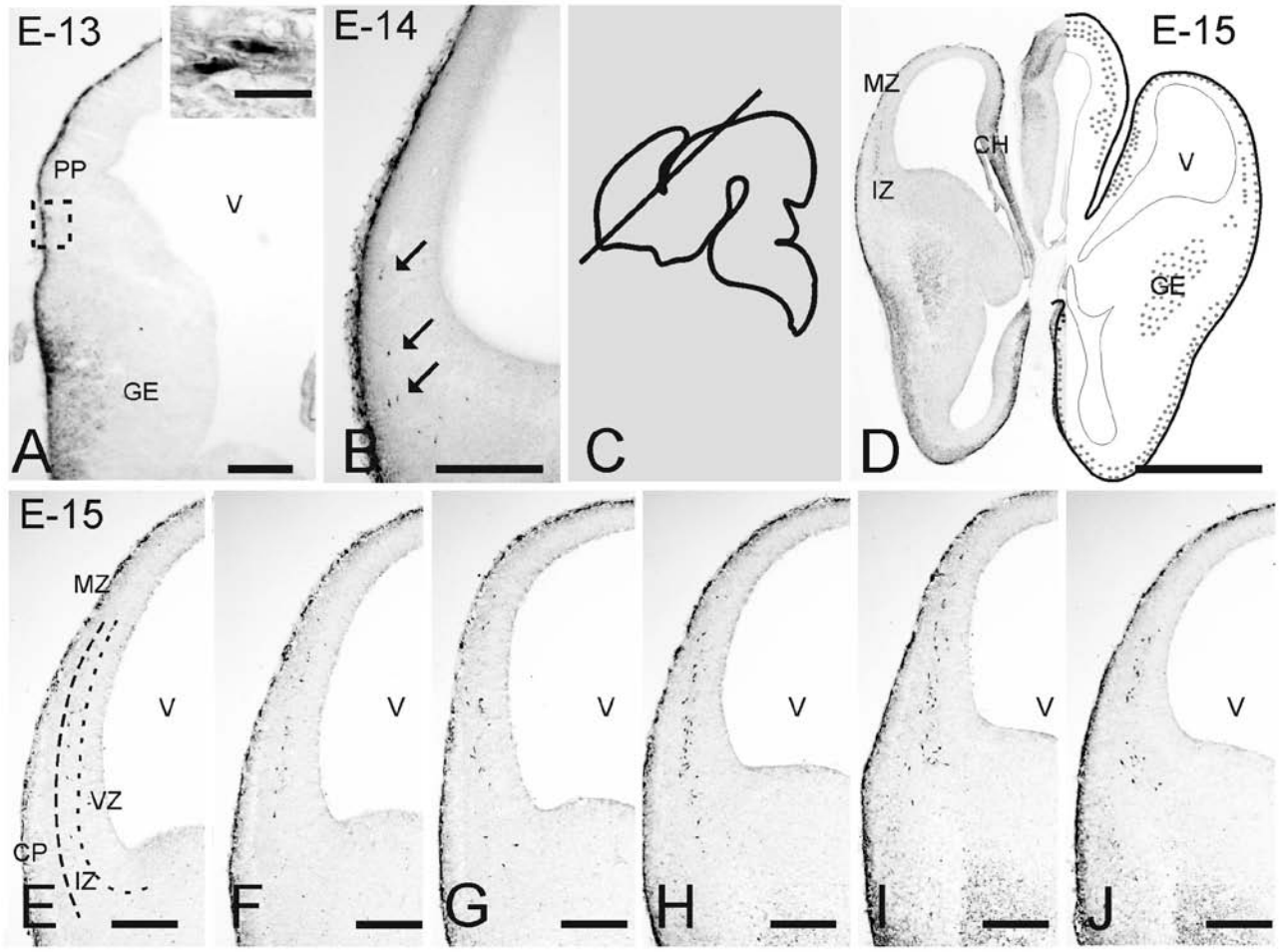

Figure 1

Figure 2: Reelin expression in the intermediate zone at E-15. (A) Nomarski microscopy image of the lateral cortex from an oblique section. Reln-ir is observed in the subpial zone Cajal-Retzius cells, and in a row of elongated cells in the intermediate zone. (B) In situ hybridization photomicrograph showing cells the intermediate zone (arrows) and in the marginal zone. (C-G) High magnification of reln-ir cells in the IZ: Most of them show the shape of elongated neuroblasts (C-F) while a few cells located near the pallium/subpallium border have a multipolar shape (G). Abbreviations: CP, cortical plate; IZ, intermediate zone, VZ, ventricular zone; V, ventricle. Scale bars: A: 500 μm; B: 200 μm; C-G: 20 μm.

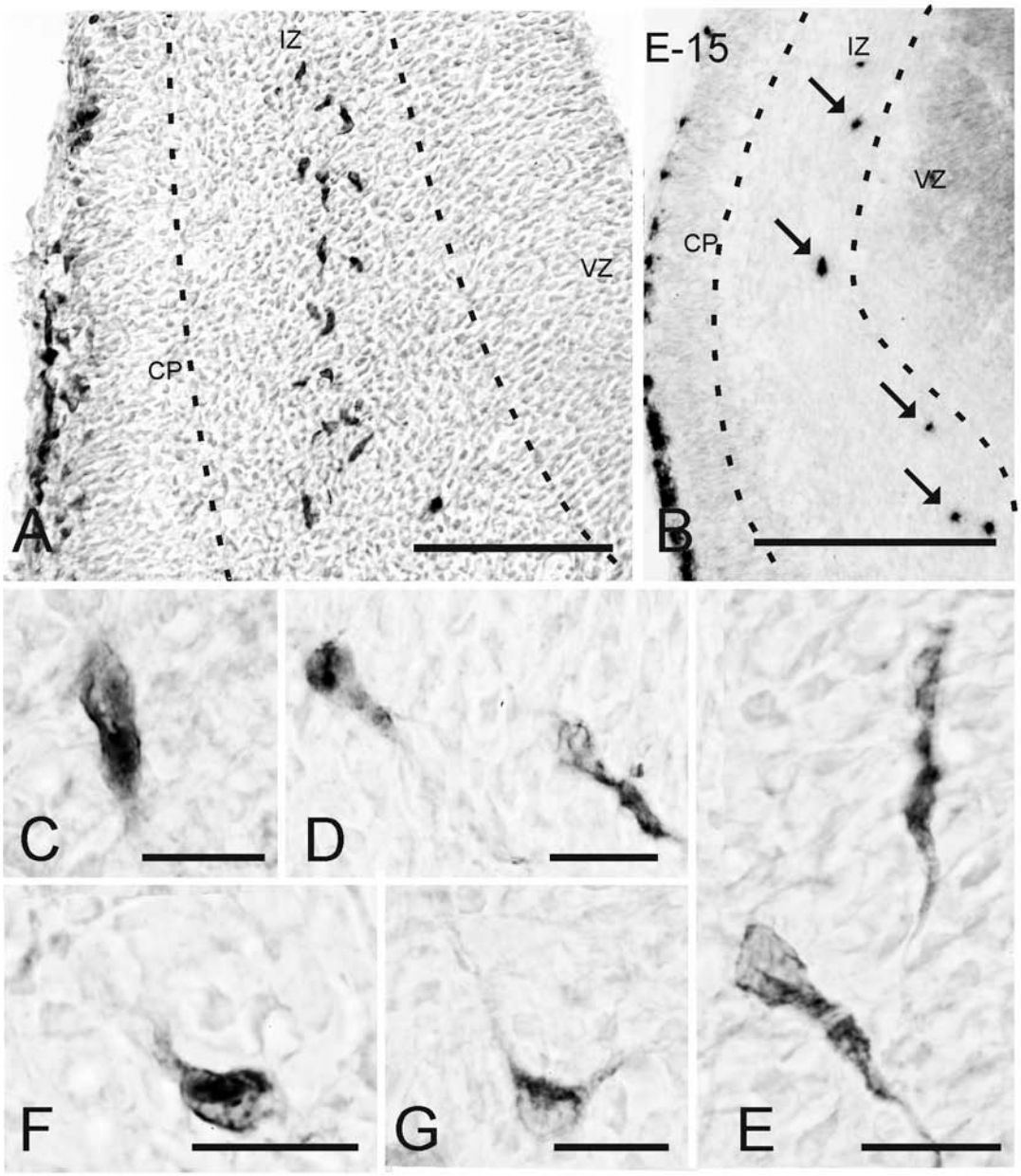

Figure 2

Figure 3: Reelin expression during E-17, E-19, newborn pups, postnatal stage 10 and adult cortex. (A-C) Coronal sections of the developing cortex at E-17, (A) mRNA containing cells (arrows) are situated in the intermediate zone, as well as in the marginal zone. (B) Coronal section at E-17 showing a reln-ir cell (arrow), in the subplate/intermediate zone and the heavy labelling observed in the marginal zone. (C) High magnification of the fusiform reln-ir cell in the subplate/intermediate zone in B. Note the high level of intracytoplasmatic labelling in the cell body and main process. (D, E). Images of reln-ir fusiform cells located in the cortical subplate at E-19. (F-G) Expression of reelin in newborn pups. (F) Low magnification micrograph showing the presence of reln-ir cells in the subplate (arrows) and cortical plate (arrowheads). The dotted line indicates the putative limit between cortical plate and subplate. (G) High magnification of a reln-ir fusiform cell in the subplate. (H-I) Reelin expression in postnatal day 10 cortex: (H) Reln-ir cells in the subplate (arrows) and in the cortical plate (arrowheads) at P10. (I) High magnification of a multipolar reln-ir cell. (J, K) Reln-ir in adult rat cortex: (J) Low magnification of anterior cingulate cortex, showing reln-ir cells in the cortical plate (arrowheads) and a few scattered reln-ir cells in the layer VIb (arrows). (K) High magnification of a reln-ir cell in the layer VIb. Note the decrease in labelling intensity in comparison to previous stages. Abbreviations: MZ, marginal zone; CP, cortical plate; SP/IZ, subplate/intermediate zone; VZ, ventricular zone; WM, white matter. Scale bars: A: 200 μm; B: 100 μm; C-E: 10 μm; F: 200 μm; G: 20 μm; H: 200 μm; I: 20 μm; J: 200 μm; K: 20 μm.

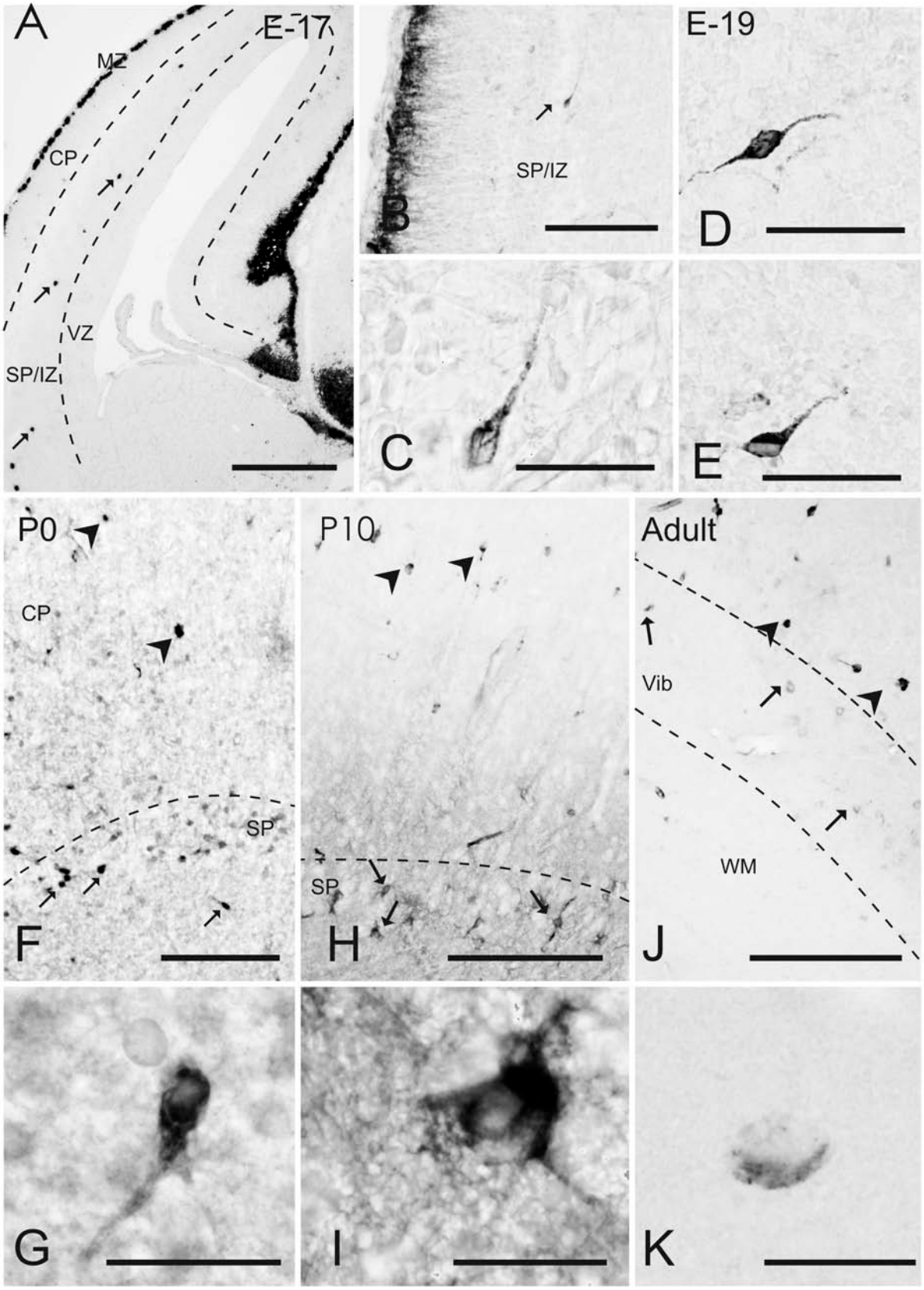
A
E-17
MZ
CP
VZ
SP/IZ
B
SP/IZ
C
D
E-19
E
P0
CP
SP
F
P10
SP
H
Adult
Vib
WM
J
G
I
K

Figure 3

Figure 4: Double labelling of reelin and BrdU, Pax6, Tbr1 and DCX at E-15. (A-C) Double labelling of reelin and BrdU, in animals injected with BrdU at E-13 and sacrificed at E-15. Note the absence of BrdU labelling in reln-ir cells. (D) Reln-ir fusiform cells (red) are observed in the intermediate zone while Pax6 immunoreactivity (green) is only present in the ventricular zone. (E) Low magnification micrograph showing reln-ir (red) and Tbr1-ir (green). (F-H) High magnification of the are area enclosed by a square in B. (F) Reelin immunoreactive cells in the intermediate zone (G) Tbr1 immunoreactivity in the nucleus of intermediate zone cells (H) Overlay image showing Tbr1 in the nucleus of reln-ir cells. (I) Double labelling of (red) and DCX (green), nuclei are stained with bisbenzimide (blue). (J-L) Coexpression of reln-ir (red) and DCX (green), nuclei are stained with bisbenzimide (blue). Abbreviations: CP, cortical plate; IZ, intermediate zone, VZ, ventricular zone. Scale bars: A-C: 25 μm; D-E: 200 μm; F-H: 25 μm; I: 200 μm; J-L 10 μm.

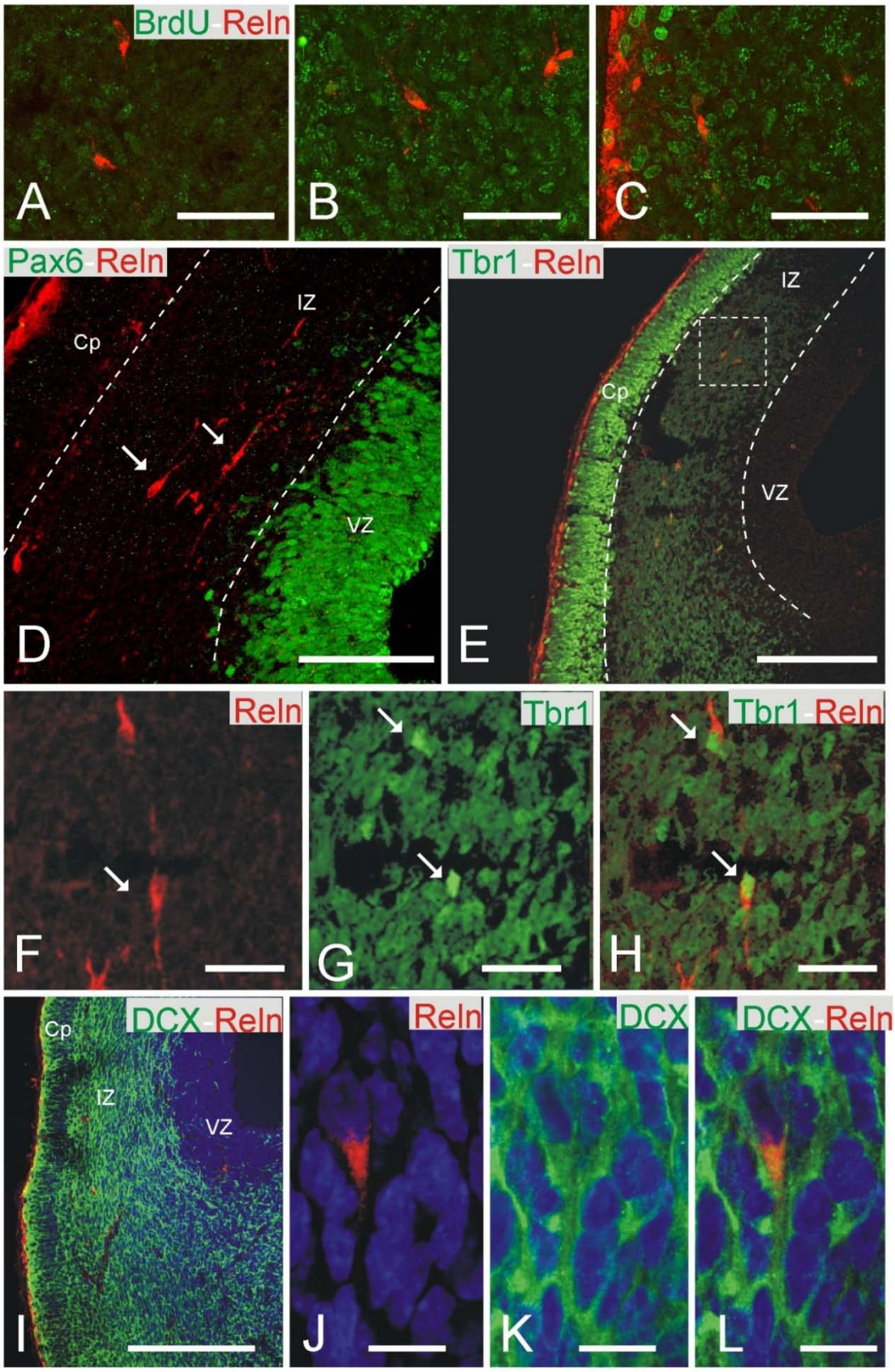
BrdU Reln
A
B
C
Pax6 Reln
Cp
IZ
VZ
D
Tbr1 Reln
IZ
Cp
VZ
E
Reln
F
Tbr1
G
Tbr1 Reln
H
DCX Reln
Cp
IZ
VZ
I
Reln
J
DCX
K
DCX Reln
L

Figure 4

Figure 5: Colocalization of reelin and calbindin, GABA and calretinin at E-15. (A) P73 immunoreactivity at E-15 is observed in the marginal zone, but not in the intermediate and ventricular zones. (B) Calbindin-ir cells (green) are observed in the marginal zone and cortical plate but not colocalization with reelin is observed (red). (C) Low magnification micrograph of reln (red) and GABA (green) in the intermediate zone. (D) High magnification of area enclosed in a square in C showing lack of colocalization of reln (red) and GABA (green) in the intermediate zone. (E) Reelin (red) and calretinin (green) immunoreactivity at E-15. Calretinin-ir cells are observed in the marginal zone and intermediate zone where colocalization with reelin is observe. Calretinin-ir is also observed in the cortical plate and subplate but did not show colocalization with reelin. (F) Reln-ir fusiform cells that show colocalization with calretinin. (G) Calretinin immunoreactive cells situated in the intermediate zone also show mRNA. Abbreviations: MZ, marginal zone, CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; SP, subplate. Scale bars: A: 200 μm; B, C and E: 100μm; D and F: 10 μm; G: 25 μm

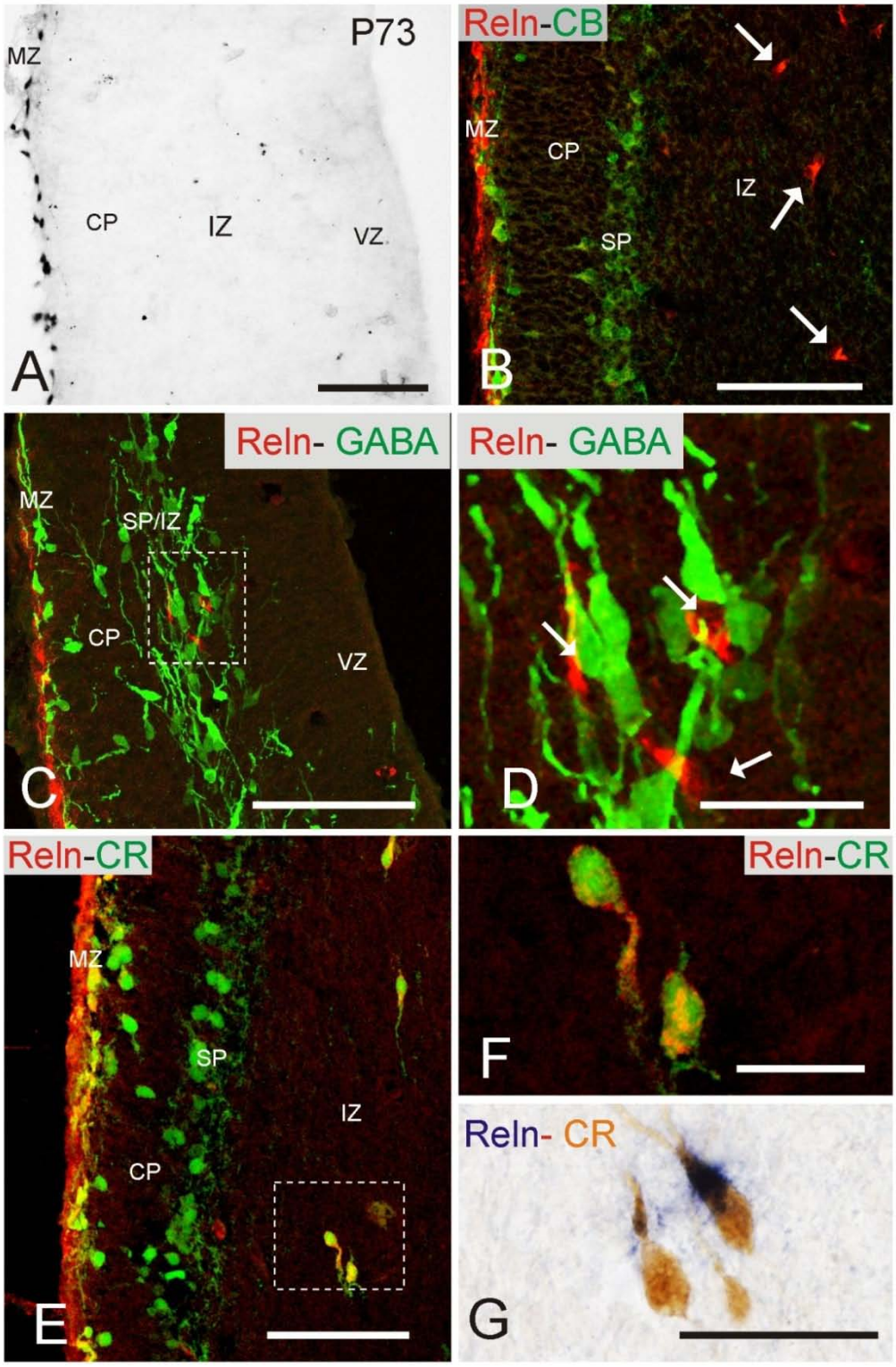
P73
MZ
CP
IZ
VZ
A
Reln-CB
MZ
CP
IZ
SP
B
Reln- GABA
MZ
SP/IZ
CP
VZ
C
Reln- GABA
D
Reln-CR
MZ
SP
IZ
CP
E
Reln-CR
F
Reln- CR
G

Figure 5

Figure 6: Colocalization of reelin and Tbr1, DCX and calretinin in the intermediate zone of E-17. (A) Double labelling of reelin (red) with Tbr1 (green). (B-D) High magnification of the area enclosed by a square in A. (B) Reln-ir cells. (C) Tbr1-ir cells. (D) Overlay image showing colocalization of reln and Tbr1. (E) Double labelling of reelin (red) and DCX (green). Nuclei stained with bisbenzimide (F-H) High magnification of the region enclosed by a square in E. (F) Reln-ir fusiform cell. (G) DCX-ir cells. (H) Overlay image showing the presence of DCX (green) in a reln-ir cell. (I) Low magnification micrograph showing reln-ir (red) and calretinin-ir (green). (J-L) High magnification of area enclosed by a square in I. (J) Reln-ir fusiform cells. (K) Calretinin-ir cell. (L) Overlay image showing a cell where calretinin and colocalize cell. Abbreviations: CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Scale bars: A and I: 100 μm; B, C, D, F, G, H, J, K and L: 10 μm; E: 200 μm.

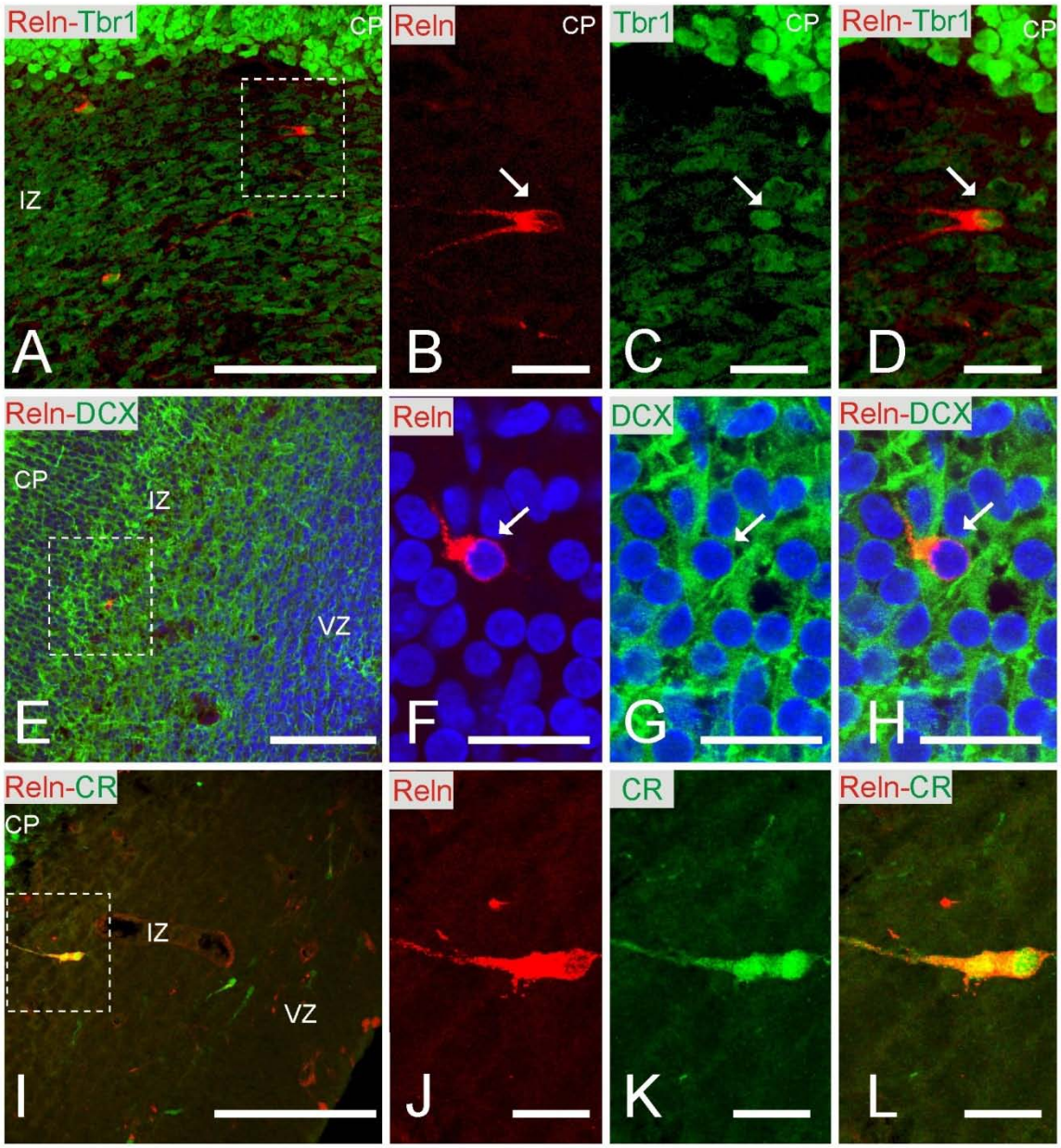
Reln-Tbr1
CP
IZ
A
Reln
CP
B
Tbr1
CP
C
Reln-Tbr1
CP
D
Reln-DCX
CP
IZ
VZ
E
Reln
F
DCX
G
Reln-DCX
H
Reln-CR
CP
IZ
VZ
I
Reln
J
CR
K
Reln-CR
L

Figure 6

Figure 7: Confocal images from coronal sections at E-19 (A-I) and newborn pups (J-V). Colocalization of reelin (red) with Tbr1, calretinin or DCX (green) in the subplate at E-19 (A-I) and newborn pups (J-R). Nuclei are stained with bisbenzidine (blue). (S-V) Double labelling of reelin (red) and GABA (green) in the subplate of P0: Note the lack of colocalization of the two markers. Arrows indicate reln-ir and their colocalization with other markers. Scale bars: 50 μm.

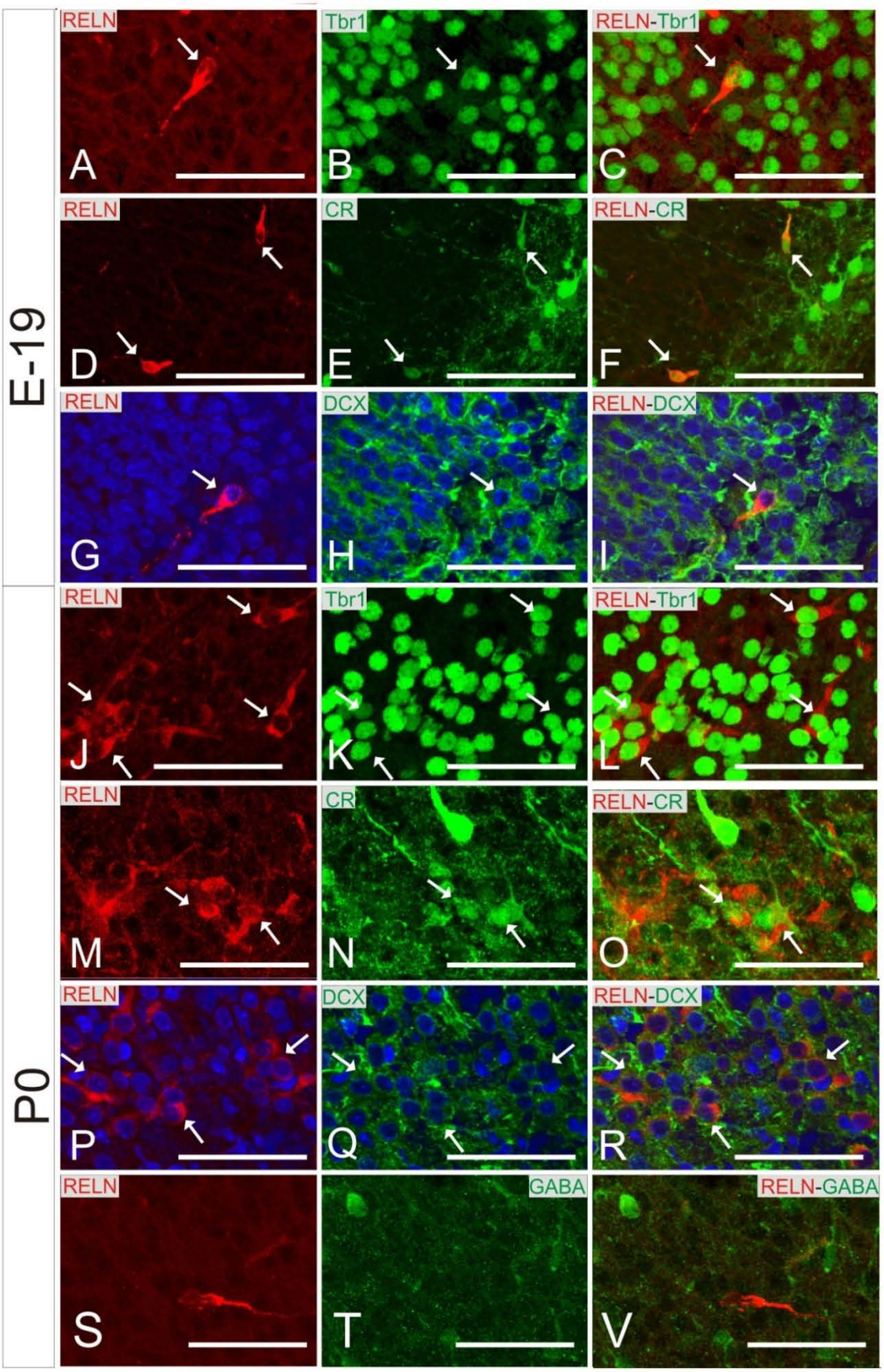
E-19
P0
RELN
Tbr1
RELN-Tbr1
A
B
C
RELN
CR
RELN-CR
D
E
F
RELN
DCX
RELN-DCX
G
H
I
RELN
Tbr1
RELN-Tbr1
J
K
L
RELN
CR
RELN-CR
M
N
O
RELN
DCX
RELN-DCX
P
Q
R
RELN
GABA
RELN-GABA
S
T
V

Figure 7

Figure 8: Confocal images from coronal sections of postnatal stages (A-L) and adults (M-P). Colocalization of reelin (red) with Tbr1, Calretinin or DCX (green) in the subplate at postnatal stages (A-L) and adult (M-P). Note the colocalization of reelin and Tbr1 (A-D), but positive cells in the subplate do not show colocalization with DCX at P10 (E-H). A few reln-ir cells colocalize with calretinin (I-L). In adult only a few reln-ir cells show colocalization with Tbr1 (M-P). Arrows indicate reln-ir and their colocalization with other markers. Scale bars: A, I, M: 250 μm; E: 100 μm; B-D, F-H, J-L, N-P: 20μm.

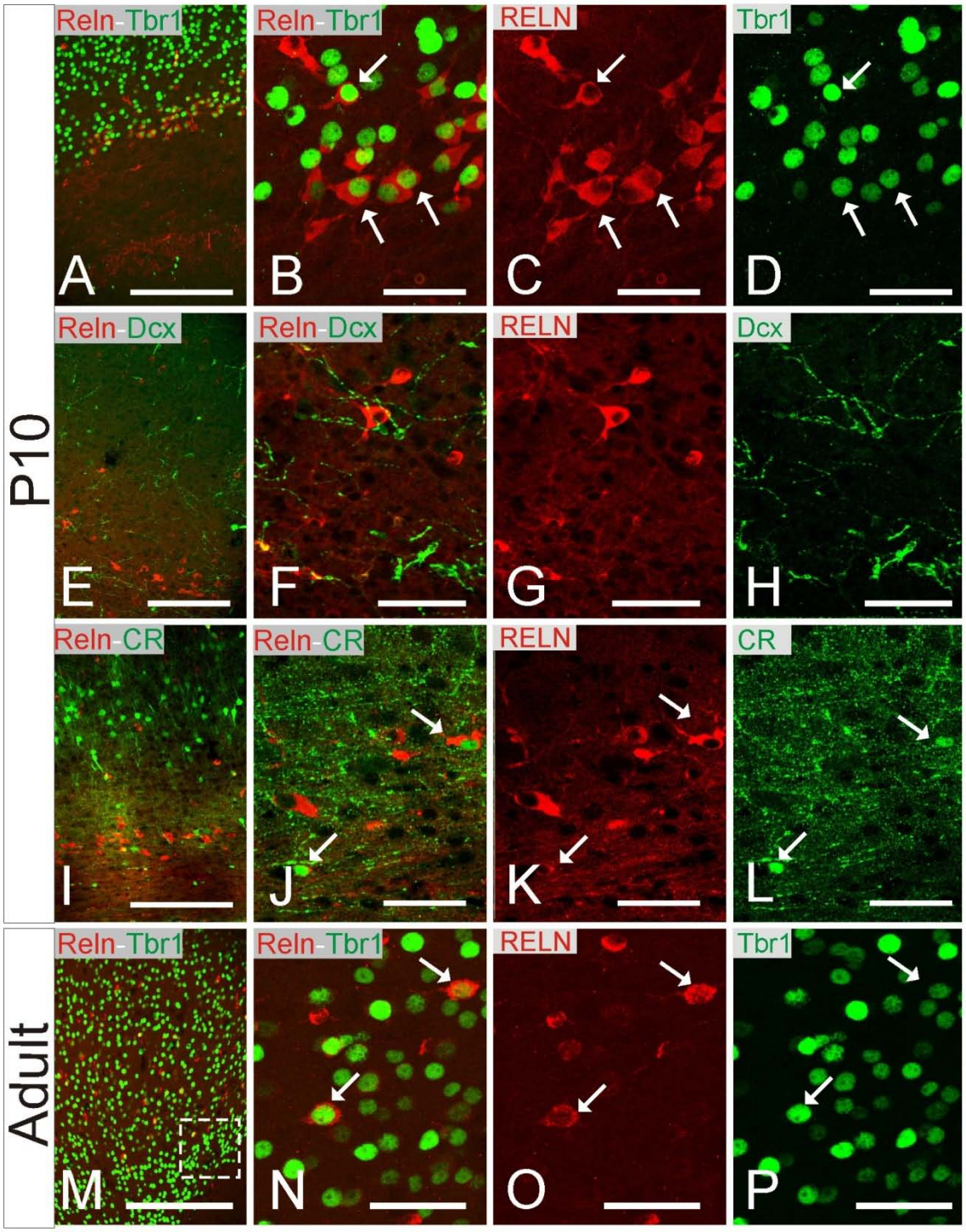
P10
Adult
Reln-Tbr1
Reln-Tbr1
RELN
Tbr1
A
B
C
D
Reln-Dcx
Reln-Dcx
RELN
Dcx
E
F
G
H
Reln-CR
Reln-CR
RELN
CR
I
J
K
L
Reln-Tbr1
Reln-Tbr1
RELN
Tbr1
M
N
O
P

Figure 8

Chapter 2

Correlation between the temporal expression of reelin, DARPP-32 and tyrosine hydroxylase during prenatal development of striatal compartments in the rat brain

1. INTRODUCTION

The striatum is the largest afferent component of the basal ganglia circuit, and plays a central role in the coordination of movement, emotions, and cognition. The ganglionic eminences are the primary source of striatal neurons: striatal projection neurons are generated in the ventricular zone of the lateral ganglionic eminence whereas the interneurons arise from the ventricular zone of the medial ganglionic eminence (Olsson et al., 1995, 1998; Marin et al., 2000; Wichterle et al., 2001; Stenman et al., 2003). Neuroblasts from the ventricular zone of the ganglionic eminences migrate into the mantle zone, where they undergo terminal differentiation into striatal neurons, although other telencephalic regions like the piriform preplate may also give rise to a subpopulation of striatal neurons (Hamasaki et al., 2003, 2004).

During prenatal stages of development there are many evidences of neuroanatomical striatal compartmentalization, as indicated by the patchy expression of different neurochemical markers such as dopamine and cyclic adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32) and tyrosine hydroxylase (TH) (Foster et al., 1987, 1988; Labandeira-García et al., 1991; see also Joel and Weiner, 2000).

Neurons located in the striosomes (patches) or outside the patches (matrix) are generated at different times during striatal development: In rats, striosome neurons first appear on embryonic day 13, and form the striatal primordium. Matrix cells are generated in the subventricular zone of the lateral ganglionic eminence after embryonic day 16, and then move into the striatal primordium separating the striosome neurons in patches (reviewed by Hamasaki et al, 2003).

During postnatal development some neuronal markers such as DARPP-32 and TH both become widely expressed in striosomes and matrix, while the level of expression of other markers like the glial-cell line derived neurotrophic factor GDNF) [which is also expressed in striosomes during development], decreases and becomes almost undetectable with immunohistochemical techniques, appearing as very weak diffuse immunolabelling in the adult rat striatum (López-Martín et al., 1999). It is known that prenatal striatal expression of DARPP-32 occurs before the arrival of TH fibres in the striatum (Foster et al., 1987, 1988; Labandeira-García et al., 1991). In a previous study we also have shown that GDNF expression precedes that of TH-immunopositive fibres, and it was suggested that GDNF released by developing striatal cells may attract dopaminergic afferents towards the patchy compartments (López-Martín et al., 1999). However, much remains to be discovered about the molecular mechanisms that direct striatal compartmentalization during development (Jain et al., 2001).

Reelin is an extracellular matrix protein that plays important roles during development of the nervous, as it is involved in regulating neuronal positioning and synaptogenesis, and synaptic plasticity in the adult brain (for recent reviews see D'Arcangelo, 2006; Herz and Chen, 2006;

Meyer, 2007; Levenson et al., 2008). Reelin is expressed in striatum during prenatal development (Alcántara et al., 1998), exhibits a patchy distribution during postnatal striatal development, and decreases in the adult striatum, suggesting that it may be mostly operative in regulating striatal compartmentalization (Nishikawa et al., 1999). Interestingly, *reeler* mice [that lack of functional reelin protein] appear to have a conventional distribution of different markers in the striatal compartments during the first postnatal week (Nishikawa et al., 2003). In addition, *reeler* or reelin haploinsufficient heterozygous mice show different anatomical, neurochemical and functional alterations in the striatum or other basal ganglia structures, such a decreased dopaminergic activity in the basal ganglia (Ballmaier et al., 2002; Nishikawa et al., 2003; Matsuzaki et al., 2007), defects in the striatal cholinergic system (Sigala et al., 2007) and reduced density of striatal parvalmunin-positive neurons (Ammasari-Teule et al., 2009). In addition, electrophysiological and behavioural techniques have revealed abnormal striatal plasticity in adult *reeler* mice (Marrone et al., 2006).

In order to gain some insight into the possible roles of reelin in the development of striatal compartments, we undertook a detailed study of the expression of reelin in the developing striatum and its correlation with the expression of two well known striatal markers (tyrosine hydroxylase and DARPP-32). The results show that reelin is expressed in striosome neurons prior to the arrival of TH afferent fibres and that complete colocalization of reelin and DARPP-32 does not occur during prenatal striatal development.

2. MATERIAL AND METHODS

Animals

Adult pregnant female Sprague-Dawley rats were used in the study. All procedures for handling and euthanasia are in accordance with the European Commission guidelines (86/609/EEC) and were approved by the bioethics committee at the University of Santiago de Compostela. Pregnant rats were anesthetized and rat fetuses at E-13, E-15, E-17, E-19 (number of embryonic days after formation of the vaginal plug) were extracted under cesarea. Newborn pups (P0) were also used.

The animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer 0.1M (PB). The brains were removed from the skull, cryoprotected in a sucrose solution (30% sucrose in PB), and sectioned at 20μm thickness in a cryostat.

Antibodies

The following primary antibodies were used in single and double immunohistochemistry procedures: 1) Mouse anti-reelin, G-10 anti-reelin monoclonal antibody from two different sources: a generous gift from Dr. A. Goffinet, University of Louvain, Belgium and a commercial

antibody (Chemicon MAB 5364) diluted 1:1000 (for details on the characterization of this antibody see De Bergeyck et al., 1998); 2) Rabbit anti-Tyrosine Hydroxylase (Chemicom AB152) diluted 1:500; 3) Rabbit anti-DARPP-32 (Cell Signalling Technology 2306) diluted 1:500.

In addition, we used the following secondary antibodies: biotinylated goat anti-mouse and goat anti-rabbit antibodies (Dako product numbers, Z0420 and E0432, respectively); Alexa Fluor 488 coupled goat anti-rabbit, and Alexa Fluor 546 coupled goat anti-mouse antibodies (Molecular Probes product numbers A11008 and A11003 respectively).

Immunohistochemistry

Sections were incubated overnight at room temperature with the appropriate primary antibodies diluted in a solution of 15% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.2% Triton X-100 in Tris-buffer saline (TBS), pH 7.4.

For single immunohistochemistry, sections were rinsed with TBS and incubated for one hour with the appropriate secondary antibodies diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100. The sections were incubated with ABC complex (Vector Laboratories), the reaction was revealed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine (DAB Sigma-Aldrich), and finally dehydrated and coverslipped.

For double immunofluorescence, the sections were rinsing with TBS, them incubated with the appropriate fluorescent secondary antibodies diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100 for one hour. Finally, sections were rinsed in distilled water, air-dried, and coverslipped with Mowiol (Calbiochem).

For all antibodies, a series of control sections was stained in which the primary antibody was omitted, and no staining was seen.

Double *In situ* Hybridization-immunolabelling

In situ hybridization for reelin was performed using a riboprobe labelled with digoxigenin-d-UTP (Roche) by *in vitro* transcription of a cDNA fragment encoding mouse reelin (D'Arcangelo et al., 1995) with T3 polymerase (Roche). Sections were permeabilized 2 minutes with proteinase K (5 μg/ml), fixed for 10 minutes in 4% paraformaldehyde, and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridized at room temperature for 3 hours in a solution containing 50% deionised formamide, 10% dextran sulphate, 1X Denhardt's solution, 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 2X SSC, 250 μg/ml yeast t-RNA and 250 μg/ml denatured salmon sperm DNA. Labelled anti-sense cDNA was added to the pre-hybridization solution (500 ng/ml), and the hybridization was performed at 60ºC overnight. Sections were then rinsed sequentially in 1X SSC (10 minutes at 65ºC), 1.5X SSC (10 minutes at 65ºC), and 2X SSC (40 minutes at 37ºC), digested with RNase A (30 minutes at 37ºC) and rinsed in 0.2X SSC (1 hour at 55ºC). The sections were then rinsed in maleic acid buffer containing 0.5% Tween 20 (15

minutes), incubated with a blocking solution containing MABT (100 mM maleic acid, 150 mM NaCl and Tween 20) and normal sheep serum (3 hours at room temperature), and finally incubated overnight with an alkaline phosphatase-conjugated antibody anti-digoxigenin (1:1000, Roche). The reaction was developed with BM purple (Roche).

After *in situ* hybridization was developed, sections were fixed 10 minutes in 4% paraformaldehyde. Thereafter, the sections were rinsed in citrate buffer pH 6.0 (for antigen retrieval), for 30 minutes at 95ºC. Sections were incubated overnight at room temperature with rabbit anti-TH, or anti-DARPP-32 diluted in a solution of 15% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in TBS. Thereafter, sections were rinsed with TBS, then sequencially incubated with biotinylated goat anti-rabbit or biotinylated goat anti-mouse (1h), and ABC complex (Vector Laboratories), developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine, and finally coverslipped with Mowiol.

Photography

Sections were observed in an Olympus Bx51 microscope. Photomicrographs were taken with an Olympus DP-71 colour digital camera. Sections labelled for double immunofluorescence were observed in a Leica DMRE microscope. Confocal images were acquired with a Leica TCS-SP2 laser confocal microscope. The images were converted to a gray scale (for black and white figures), and adjusted for brightness and contrast by using Corel Photo-Paint 13. Picture set up was achieved with Corel-Draw 13. The full resolution was maintained until the micrographs were cropped and assembled, at which were adjusted to a resolution of 300 dpi.

3. RESULTS

3.1. Reelin expression during development of the ganglionic eminences and striatal mantle

At E-13, the first stage studied, only a few very weakly stained reelin immunoreactive (reln-ir) cells were visualized in the developing striatal mantle, while many reln-ir cells were observed in the cortical marginal zone and piriform cortex (Fig. 1A).

However, already at E-15 there was widespread expression of reln-ir cells in the striatal mantle (differentiating striatal region) of both the medial and lateral ganglionic eminences but not in the proliferation zone (ventricular and subventricular zone) (Fig. 1B). In the rostral striatum reln-ir cells accumulated mostly in ventrolateral areas (Fig1B), while in more caudal regions reln-ir cells were preferentially observed in the medial striatal mantle just below the subventricular zone (Fig. 1 B-C). Interestingly, in some coronal-oblique sections and in sagittal sections it was possible to observe a row of reln-ir cells connecting the olfactory tubercle and the striatal mantle (Fig. 1C-D). At high magnification reln-ir cells displayed the typical elongated (fusiform)

appearance of neuroblasts and intense cytoplasmic labelling (Fig. 1E). In fact, the intensity of cytoplasmic immunolabelling decreased at later developmental stages, whereas there was an increase in the diffuse labelling (possibly extracellular) surrounding reelin positive cells (see Fig. 1B, 1E and 1G).

At E-17 clusters of reln-ir cells were observed at the striatal border and in small patches, which were more clearly observed by *in situ* hybridization (Fig. 1F) than by immunohistochemistry (Fig. 1G).

At E-19, the labelling of reln-ir cells in the striatal border and patches was more defined than at previous stages, although the intensity of reelin labelling decreases relative to E-17 (Fig. 1H). Finally, reelin expression in the striosomes became even more clearly defined at P0 (Fig. 1I).

3.2. Correlation berween reelin and TH expression during prenatal striatal development

To analyze the relationship between expression of reelin and TH during striatal development we carried out a double labelling experiment, with *in situ* hybridization for reelin, and immunolabelling for TH.

Since striatal reelin expression was almost nonexistent at E-13, the first stage studied was E-15. In the rostral striatum reelin expression was observed in ventral areas of the striatal mantle while there were very few fibres positive for TH (Fig. 2A). In the central striatum, reelin continued to predominate in the ventral region whereas fine TH positive fibres were observed in most of the striatal mantle (Fig. 2B-C), and in the caudal striatum TH expression was more evident than reelin expression (Fig. 2D).

At E-17, reelin and TH showed stronger labelling (Fig. 2E). In the rostral striatum, reelin positive cells were mainly observed in the striatal border, whereas TH expression was observed throughout the striatal mantle and the subjacent white matter (Fig. 2E). In the central striatum, reelin expression was mainly observed in the striatal border and TH expression was distributed throughout the striatal mantle (Fig. 2F-G). In the caudal striatum, reelin labelling was observed in the striatal border and in some striosomes located close to the border, whereas TH labeling was mainly observed in medial areas (Fig. 2H).

At E-19 the general pattern of spatial colocalization of both markers resembles E-17, but there was greater coexpression of reelin and TH in the same areas, mainly in the central striatum (Fig. 2I-L).

At P0 both reelin and TH labelling tended to be located at the striatal borders and in the striosomes in the rostral and central striatum (Fig. 2M-O). In the caudal region of the striatum reln-ir was clearly present in the striosomes, whereas TH was more widely expressed, particularly in the ventral striatum (Fig. 2P).

3.3. Correlation of reelin and DARPP-32 expression during prenatal striatal development

To analyze the possible spatial and cellular colocalization of reelin and DARPP-32, we used double immunofluorescent labelling, as well as double labelling with *in situ* hybridization for reelin and immunohistochemistry for DARP32 in P0.

At E-15 reelin expression was more abundant in the rostral striatum than in the caudal striatum, whereas DARPP-32 immunoreactivity was greater in the caudal than in the rostral striatum. Scarce cells showing both markers (Reelin and DARPP-32) were observed in the rostral striatum, although in the central and caudal regions of striatum there was an increase in the amount of cells where both substances colocalize (Fig. 3A-C).

The same pattern of expression was observed at E-17, but the number of cells displaying cellular colocalization of reelin and DARPP-32 [mainly observed in the central and caudal striatum] was somewhat higher than at E-15 (Fig. 3D-F).

At E-19, the number of DARPP-32 positive cells increased and most displayed colocalization with reelin, not only in the caudal and central striatum, but also in the rostral striatum (Fig. 3G-I).

In newborn pups (P0), both reelin and DARPP-32 were observed in striosomes and at the striatal border, and there was cellular colocalization of both markers in all rostro-caudal areas throughout the striatum, although a few reln-ir cells were immunonegative for DARPP-32 (Fig. 3J-L). At this stage colocalization of reelin mRNA and DARPP-32 immunolabelling was also observed at the border of the striatum (Fig 3E) and striosomes (Fig 3M-N).

4. DISCUSSION

The main findings of the present study are that striatal reelin expression follows a caudal-rostral gradient during prenatal development and that this is the opposite of the patterns of expression of TH and DARPP-32. In addition, we show that TH expression is widespread throughout the striatum and becomes accumulated in striosomes towards the end of prenatal development, once reln-ir cells are already clustered in striosomes. Another interesting finding is the almost absolute lack of spatial and cellular colocalization of reelin and DARPP-32 in the first stages studied (E-15), while in newborn pups striosome neurons express both markers. Reelin immunohistochemistry also indicates strong intracytoplasmic immunostaining by E-15, whereas at E-17 there was a reduction in the intracytoplasmic labelling and concomitant diffuse (i.e. extracellular) labelling; this correlates with the clustering of reln-ir cells in striosomes. Finally, at E-15 there was evidence of a continuous row of reln-ir cells between the olfactory tubercle and the striatum.

The vast majority of striatal neurons originate in the ganglionic eminences (reviewed by Hamasaki et al., 2003), but it has also been shown that a transient population of cells migrating from the piriform preplate are also incorporated in the developing striatum where they may regulate the connectivity of intrastriatal axon bundles, and thereafter suffer cell death by apoptosis (Hamasaki et al., 2004). However, the latter authors also report that these cells fail to express reelin, and therefore appear to represent a different population than the reln-ir cells that we have observed as a continuous row from the olfactory tubercle to the striatum at E-15. Interestingly, the retrobulbar area, including the olfactory tubercle, is considered one of the sources of pioneer reln-ir telencephalic neurons (Cajal-Retzius cells), which will migrate tangentially towards the cortical marginal zone (Meyer et al., 1998). Our observations suggest that a subpopulation of reln-ir cells originated in the retrobulbar compartment may enter the striatum and represent the population (or at least part of it) of reln-ir cells observed in the striatum at E-15. Therefore, during prenatal development the striatum comprises a population of projection neurons that originate in the lateral ganglionic eminence, interneurons that arise from the medial ganglionic eminence, a population of transient neurons migrating from the piriform preplate, and reln-ir cells that appear to originate from the retrobulbar compartment.

E-15 reln-ir striatal cells display a high degree of intracytoplasmic labelling, but already at E-17 the intensity of this labelling decreased, and there was a concomitant increase in diffuse (i.e. extracellular) labelling surrounding reln-ir cells. This may indicate the release of reelin to the extracellular matrix. Interestingly, the change in the reln-ir expression pattern (from intracytoplasmic to diffuse) shows a temporal correlation with the first evidence of reln-ir cells clustering in striosomes (Fig. 1F). It is therefore tempting to speculate that reelin release may regulate the aggregation of striosome cells in patches, perhaps by promoting their adhesiveness (Krushel et al., 1995). However, well-defined striosome compartments are also found in reeler mice (with null expression of reelin) (Nishikawa et al., 2003), which indicates that while reelin may play a role in the formation of striatal compartments, it is not essential for their formation.

We observed an increase in the number of cells expressing reelin and DARPP-32 throughout development. Such colocalization is more evident in late embryonic stages and at P0, almost all DARPP-32 positive cells contain reelin. These observations may indicate that reln-ir cells increase DARPP-32 expression in late stages of prenatal development. However, we cannot discount the possibility that some reln-ir cells may suffer apoptotic cell death after the aggregation of reln-ir cells in striosomes and that secondary striosome DARPP-32-ir cells will transiently express reelin during the first weeks of postnatal development. Clearly there is a need for further studies to clarify what occurs.

TH and DARPP-32 temporal pattern expression are different as the onset of DARPP-32 expression starts prior to the arrival of TH-ir fibers in the striatum (Foster et al., 1987, 1988;

Labandeira-García et al., 1991), and therefore show important differences with respect to reelin expression. TH-ir is more abundant in the caudal than in the rostral striatum at E-15, while reelin displays the opposite expression pattern. In addition, striatal TH-ir is more widely distributed and clustering of TH-ir fibers in striosomes occurs after striosomal aggregation of reln-ir cells. This temporal pattern of expression indicates that reelin may play a role in the synaptogenesis of dopaminergic fibers on striosome cells, in the same way that reelin has been shown to regulate synaptogenesis in the hippocampus (Borrell et al., 1999). In fact, although reeler mice develop well-differentiated striatal compartments, they display alterations in the position of dopaminergic nigral cells and dopaminergic striatal innervation (Nishikawa et al., 2003). Interestingly, in reelin haploinsufficient heterozygous reeler mice, the mesolimbic dopamine pathway, but not the nigrostriatal pathway appears to be affected (Ballmaier et al., 2002).

Low levels of reelin protein were detected in thepostnatal striatum of different vertebrate species adults (Martínez-Cerdeño et al., 2002; Martínez-Cerdeño et al., 2003; Ramos-Moreno et al., 2006), Reelin mRNA expression can be detected by RT-PCR and a considerable downregulation of reelin mRNA expression has been found in the striatal caudate nucleus in schizophrenia (Impagnatiello et al., 1998). Therefore, taking into account the pattern of reelin expression in the developing striatum and the neuroanatomical, neurochemical and physiological alterations observed in the heterozygous *reeler* mice [which display similar levels of reelin to those observed in schizophrenia], it would be of interest to analyze whether or not any of the striatal alterations found in heterozygous *reeler* mice are replicated in schizophrenia brains.

In conclusion, the present indicates a possible role for reelin in the fine organization of the striosomes and in the correct development of synaptic circuits in the striatum.

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6. FIGURES

Figure 1. Reelin expression during striatal development. (A) At E-13 there is almost no reelin immunoreactivy in the striatal mantle. (B-D) Ventral telencephalon at E-15. (B) Coronal-oblique section of the rostral striatum at E-15 showing numerous reln-ir neurons in the striatal mantle but not in the ventricular or subventricular zones. The lateral border of the striatum is indicated by arrowheads. (C) Coronal-oblique section of caudal striatum showing a row of reln-ir cells from the marginal zone of the olfactory tubercle to the striatal mantle (arrows). (D) Saggital section at E-15 showing a row of reln-ir cells between the olfactory tubercle and the striatum (arrows). (E) High magnification of reln-ir cells in the striatal mantle at E-15 showing a high level of intracytoplasmic labelling. (F-G) Coronal sections of striatum at E-17. (F) Reelin mRNA expression (*in situ* hybridization) showing the clustering of reelin positive cells in the border of the striatal mantle at E-17. (G) Reln-ir at E-17. There is an increase of diffuse labelling in the striatum that possibly reflects extracellular reelin. (H) Reln-ir at E-19. Note the accumulation of reln-ir cells at the striatal border (arrows) and patches (arrowheads). (I) In newborn pups reln-ir cells are more clearly accumulated in the striatal border (arrows) and striosome patches (arrowheads). The border between the striatal mantle and the subventricular zone is indicated by dotted lines. Abbreviations: ac, Anterior commisure; Cx, Cerebral Cortex; DB, Diagonal band of Broca; ic, Internal capsule; LGE, Lateral ganglionic eminence; MGE, Medial ganglionic eminence; Pir, Piriform cortex; St, Striatal Mantle; Tu, Olfactory tubercle; V, Ventricle; VZ, Ventricular zone. Calibration bars: A-D: 250 μm; E: 10 μm ; F-I: 250 μm.

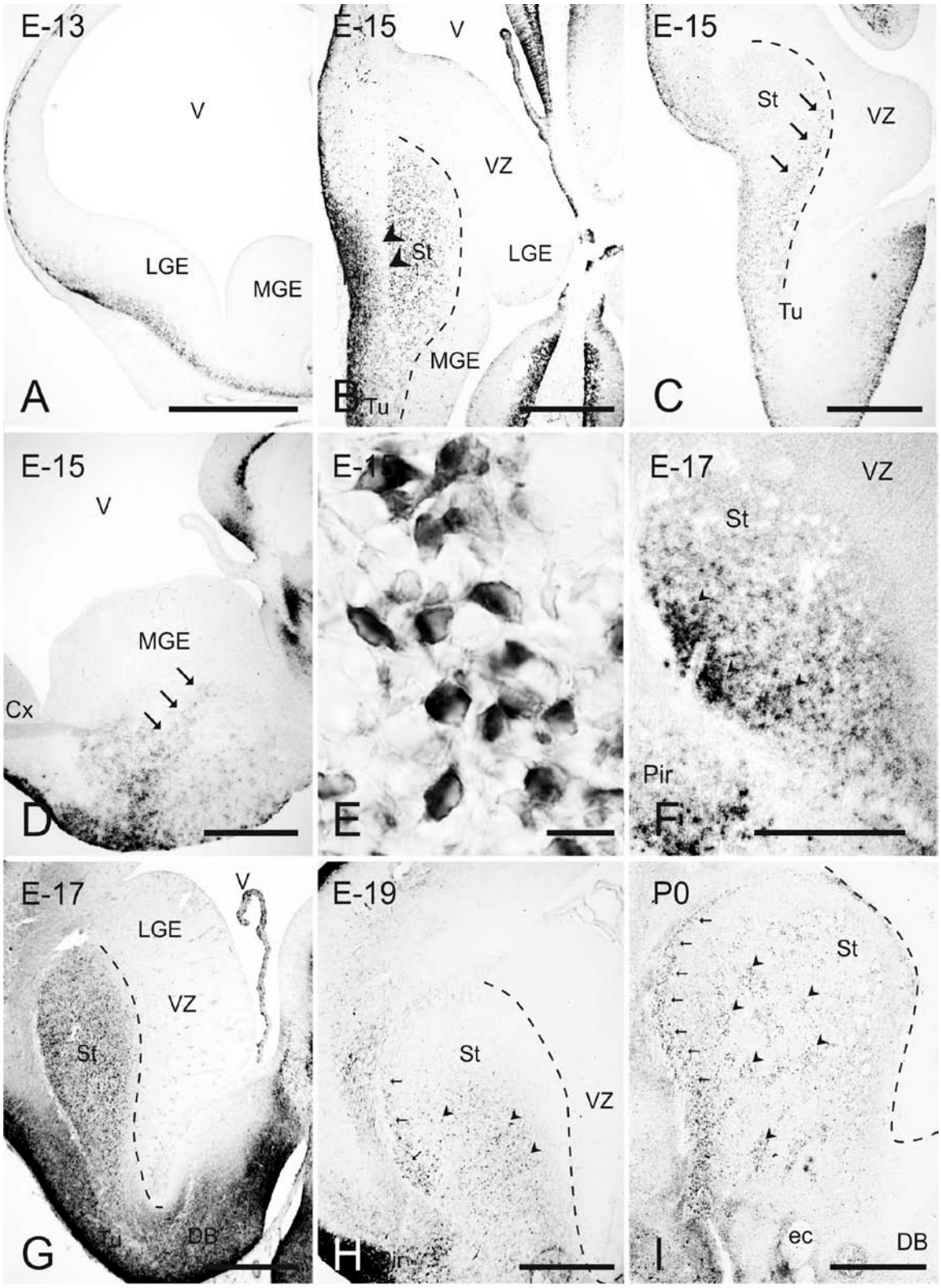
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Figure 1

Figure 2. Reelin mRNA (blue) and tyrosine hydroxylase (brown) expression in the developing striatum at E-15 (A-D), at E-17 (E-H), at E-19 (I-L) and P0 (M-P). Left panels (A, E, I, M) show images from coronal sections of the rostral striatum; central panels (B-C, F-G, J-K, N-O) show a general view and a detail of coronal sections in the central striatum; right micrographs (D, H, L, P) show the caudal striatum. At all stages studied reelin expression is more abundant in the rostral and central striatum than in caudal regions. The opposite expression pattern is shown by TH. Reelin positive cells and patches are indicated with arrowheads, and the border between the striatal mantle and the subventricular zone with dotted lines. Calibration bars: A-D: 250 μm. High magnification A-D 50 μm.

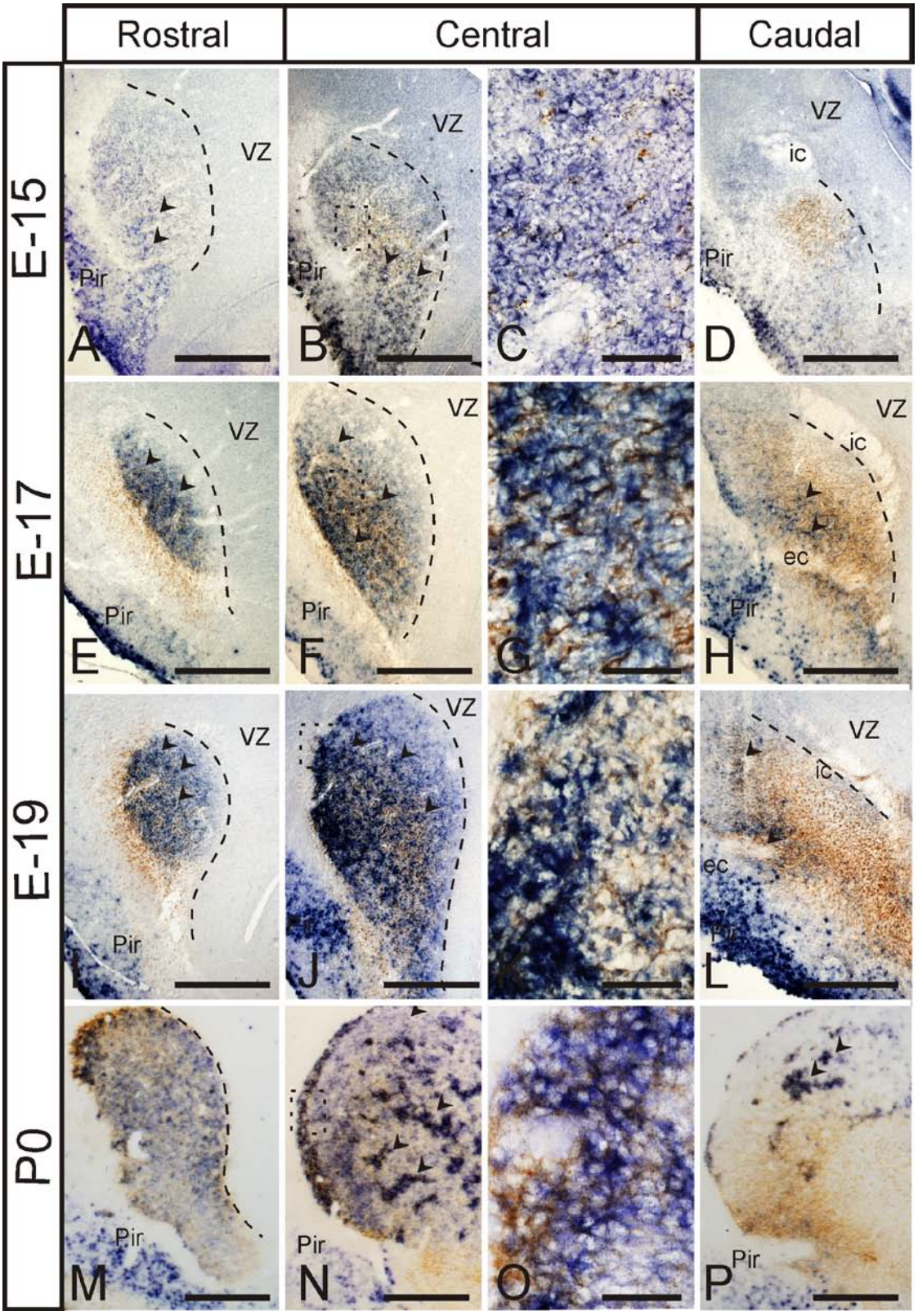
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Figure 2

Figure 3. Reelin and DARPP-32 expression during striatal development. (A-C) Overlay images of the double immunolabelling of reelin (red) and DARPP-32 (green) at E-15. Reln-ir cells are more abundant in rostral than caudal regions. A few reln-ir cells are also positive for DARPP-32 mainly in the central and caudal portions of the striatal mantle. (D-I) Overlay images of the double immunolabelling of reelin (red) and DARPP-32 (green) at E-17 (D-F) and E-19 (G-I). Note the increase in the amount of DARPP-32 immunoreactive cells and of cells expressing both substances throughout striatum. (J-L) Overlay images of the double immunolabelling of reelin (red) and DARPP-32 (green) at P0. Note that cellular colocalization of reelin and DARPP-32 (yellow in the overlay pictures) is observed more clearly in newborn pups. (M-N) Reelin mRNA (blue) and DARPP-32 immunoreactivity (brown) at P0. M corresponds to the striatal border and N is a high magnification of a striosome. Note the colocalization of both markers in the striatal border and patches. Calibration bars: A-E: 25 μm.

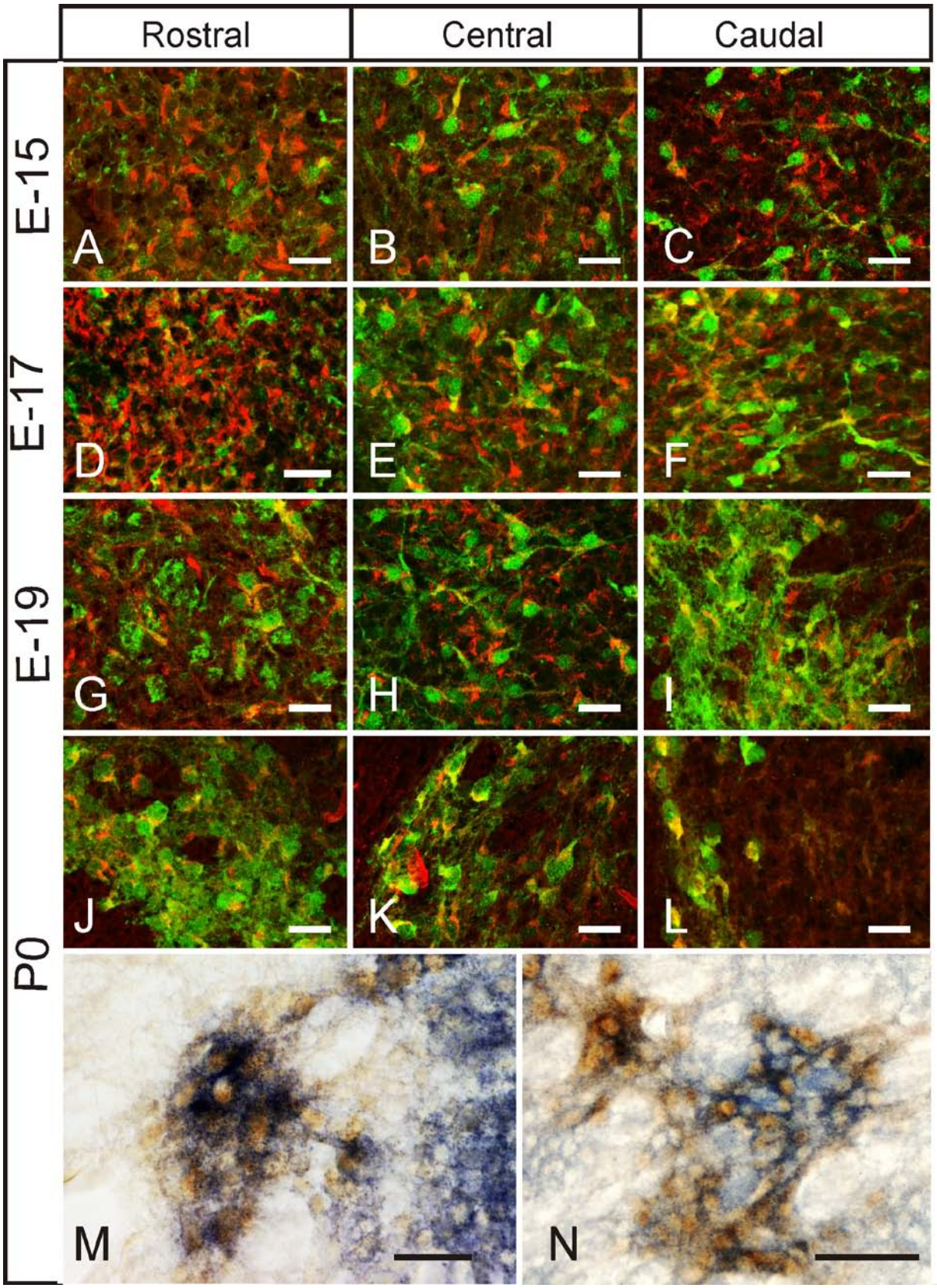

Figure 3

Chapter 3

Reelin expression during development of the piriform cortex

1. INTRODUCTION

Reelin, a large extracellular matrix protein involved in regulating neuronal migration, synaptogenesis and neural plasticity, is expressed in the rodent olfactory system throughout life. In fact, the olfactory bulb is one of the brain regions that show the highest levels of Reelin during development and in the adult brain (see Alcántara et al., 1998; Pappas et al., 2003, Ramos-Moreno et al., 2006).

In the olfactory bulb, reelin is mainly expressed by mitral/tufted cells, but some periglomerular and granule cells also appear to contain some reelin immunoreactivity (reln-ir) (Alcántara et al., 1998, Bar et al., 2000, Hack et al., 2002, Pappas et al., 2003, Okuyama-Yamamoto et al., 2005, Ramos-Moreno et al., 2006). Although in some brain areas (i.e. neocortex, hippocampus or cerebellum) reelin is primarily expressed by regional interneurons (Alcántara et al., 1998; Pesold et al., 1998, 1999, Ramos-Moreno et al., 2006) in the olfactory bulb reelin is mainly expressed by projection neurons (mitral/tufted). However, olfaction is the only sensory system that reaches the cortex without a thalamic or brainstem relay, and mitral cells do not act only as projection neurons (sending their axons to the olfactory cortex through the lateral olfactory tract), because they are also involved in dendro-dendritic reciprocal synapses in the olfactory bulb (reviewed by Neville and Haberly, 2004).

Surprisingly, even though it has been shown that the olfactory bulb of *reeler* mice (reln -/-) show smaller size than of wild-type mice (reln +/+), there are no apparent alterations in neuronal migration and layer formation such as those present in most other laminated brain regions of the *reeler* mouse (reviewed by Lambert de Rouvroit and Goffinet, 1998; Katsuyama and Terashima, 2009). However, detailed ultrastructural studies have shown a wide array of morphological alterations in the olfactory bulb of *reeler* mice and also in the haploinsufficient heterozygous *reeler* mouse (reln +/-) (Pappas et al., 2003).

Reelin is also expressed in other olfactory brain structures such as the olfactory cortex (see Alcántara et al., 1998; Teillon et al., 2003, Ramos-Moreno et al., 2006), but to our knowledge there are no detailed studies of reelin expression in the rodent piriform cortex (anterior olfactory cortex), a three layered structure that receives afferents from the olfactory bulb mostly in layer IA (reviewed by Haberly, 2001).

The piriform cortex is involved in learning of olfactory discrimination and association (Nevile and Haberley, 2004). The expression of reelin in this region during development and adult life could be important for some of these roles, as it has been shown that reelin regulates the formation and stabilization of synapses, synaptic strength, and synaptic plasticity (Chen et al., 2005; Beffert et al., 2006; Jossin and Goffinet, 2007; Niu et al., 2008).

Interestingly, different studies have shown that reelin is involved in modulating long-term potentation and learning in some brain areas (Weeber at al., 2002; Beffert et al., 2006),

enhancing NMDA receptor activity or maintaining the composition of NMDA receptors (Chen et al., 2005; Campo et al., 2009).

Taking into account the deficits in olfactory learning observed in heterozygous *reeler* mice (Larson et al., 2003) as well as the presence of alterations in olfactory perception in schizophrenia (Hudry et al., 2002), a disorder that presents a significant downregulation of reelin mRNA and protein expression (Impagnatiello et al., 1998; Guidotti et al., 2000; Costa et al., 2001) a detailed study of reelin expression during development of the rodent piriform cortex would be of interest.

In the present study single and double immunolabellings were used to determine the cellular distribution of reelin expression during development of the rat piriform cortex.

2. MATERIAL AND METHODS

Animals

Sprague-Dawley rats were used in the study, and all experiments are in accordance with the European Commission guidelines (86/609/EEC) and were approved by the bioethics committee at the University of Santiago de Compostela.. Pregnant rats were anesthetized and the fetuses were extracted under caesarean at E-13, E-15, E-17, E-19 (number of embryonic days after formation of the vaginal plug). In addition, newborn, two-days, one-week and two-weeks old pups, as well as adult rats were used. The animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer 0.1M (PB). The brains were removed from the animals and then cryoprotected in a sucrose solution (30% sucrose in PB) and 20-40 μm thick sections were obtained in a cryostat. For long-term storage, the tissue sections were kept at -80ºC.

Antibodies

In the present study we have used the following primary antibodies: 1) Mouse anti-reelin, G-10 anti-reelin monoclonal antibody from two different sources: a generous gift of Dr. A. Goffinet, University of Louvain, Belgium and a commercial antibody (Chemicon, code MAB 5364) diluted 1:1000 (for details on the characterization of this antibody see De Bergeyck et al., 1998). 2) Rabbit anti-Tbr1 1:500 (Chemicon, code AB9616). 3) Sheep anti-GAD 65/67 using a sheep polyclonal antibody 1:75.000 (a generous gift of Dr Mugnaini, Northwestern University, Chicago). 4) Rabbit anti-Glial Fibrillary Acidic Protein 1:500 (GFAP) (DAKO, code Z0334). 5) Rabbit anti-NeuN 1:1000 (Chemicon, code MAB377). 6) Rabbit anti-vesicular glutamate transporter 2 (VGLUT2) 1:2000 (Synaptic Systems, code 135 403). 7) Rabbit anti-synaptophysin 1:1000 (LabVision, code RB-9044). 8) Rabbit anti-tyrosine hydroxilase (TH) 1:1000 (Chemicon, code AB152). 9) Rabbit anti-serotonin (5-HT) 1:10.000 (INCSTAR, code 20080).

We also used the following secondary antibodies: Biotinylated goat anti-mouse and goat anti-rabbit antibodies (Dako product numbers, Z0420 and E0432, respectively). Alexa Fluor 488 coupled goat anti-rabbit, Alexa Fluor 546 and 488 coupled goat anti-mouse, Alexa Fluor 546 coupled donkey anti sheep antibodies (Molecular Probes product numbers A11008, A11003, A11001 and A21098 respectively).

Immunohistochemistry

For single immunohistochemistry, sections were incubated overnight at room temperature with the appropriate primary antibodies diluted in a solution of 15% normal goat serum (NGS) or normal donkey serum (NDS) and 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in Tris Buffer Saline (TBS).

After rinsing with TBS sections were incubated for one hour with the appropriate secondary antibodies diluted in a solution containing 10% NGS or NDS, 1% BSA and 0.2% Triton X-100. Afterwards the sections were incubated with ABC complex (Vector Laboratories), developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine (DAB Sigma-Aldrich), and finally dehydrated and coverslipped.

Double-labelling of reelin and Tbr1, synaptophysin, (GFAP), VGLUT2 and GAD 65/67 in brain sections from newborn pups and/or adult rats was performed by simultaneous overnight incubation of the brain sections with the aforementioned antibodies diluted in a solution of 15% normal goat serum (NGS) and normal donkey serum (NDS) and 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in TBS. After rinsing with TBS, sections were incubated with the appropriate fluorescent secondary antibodies diluted in a solution containing 10% NGS and NDS, 1% BSA and 0.2% Triton X-100 for one hour. Finally, sections were rinsed in distilled water, air-dried, and coverslipped using Mowiol (Calbiochem, 475904).

In situ Hybridization

In situ hybridization for reelin was performed using a riboprobe labelled with digoxigenin-d-UTP (Roche, 11277073910) by in vitro transcription of a cDNA fragment encoding mouse reelin (D'Arcangelo et al., 1995) using T3 polymerase (Roche). Sections were permeabilized 2 minutes with proteinase K (5 µg/ml), fixed 10 minutes in 4% paraformaldehyde, and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridized at room temperature for 3 hours in a solution containing 50% deionised formamide, 10% dextran sulphate, 1X Denhardt's solution, 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 2X SSC, 250 µg/ml yeast t-RNA and 250 µg/ml denatured salmon sperm DNA. Labelled anti-sense cDNA was added to the pre-hybridization solution (500 ng/ml), and the hybridization was performed at 60ºC overnight. Sections were then rinsed sequentially in 1X SSC (10 minutes at 65ºC), 1.5X SSC (10 minutes at 65ºC), and 2X SSC (40 minutes at 37ºC), digested with RNase A (30 minutes at 37ºC) and rinsed in 0.2X SSC (1 hour at 55ºC). After that, sections were rinsed in maleic acid buffer containing 0.5%

Tween 20 (15 minutes), incubated with a blocking solution containing MABT (100 mM maleic acid, 150 mM NaCl and Tween 20) and normal sheep serum (3 hours at room temperature), and finally incubated overnight with an alkaline phosphatase-conjugated antibody anti-digoxigenin (1:1000, Roche). The reaction was developed with BM purple (Roche).

After *in situ* hybridization was developed, sections were fixed 10 minutes in paraformaldehyde 4% and coverslipped with Mowiol.

Photography

Sections were observed using an Olympus Bx51 microscope. Photomicrographs were taken with an Olympus DP-71 colour digital camera. Sections labelled for double immunofluorescence were observed using a Leica DMRE microscope. Confocal images were acquired with a Leica TCS-SP2 laser confocal microscope. The images were converted to a gray scale (for black and white figures), and adjusted for brightness and contrast by using Corel Photo-Paint 13. Picture set up was done by using Corel-Draw 13.

3. RESULTS

3.1 Prenatal expression of reelin in the rat piriform cortex

At early stages of development reelin is mostly observed in the marginal zone of the prospective piriform cortex. At E-13, the first stage studied, a diffuse reelin immunostaining together with a few reln-ir neurons were observed in the anterior piriform cortex region (Fig. 1A). In situ hybridization (ISH) for reelin also shows some reelin-positive cells in the piriform cortex marginal zone but no diffuse immunostaining (Fig. 1B). Diffuse reelin immunostaining was also discerned throughout the piriform cortex region at E-15 (Fig. 1C). Furthermore, at this stage of embryonic development two groups of reln-ir elongated neuroblasts were apparent in the area of the piriform cortex: the first one, a group of reln-ir neuroblasts was located in a wide pathway below the LOT (Fig. 1C), and the second group were reln-ir cells observed within the LOT (Fig. 1C inset). Similar observations are found with ISH (Fig. 1D).

The piriform cortex of E-17 showed an intense reelin immunostaining in the LOT (Fig. 1E). Below the LOT, layer I piriform cortex also showed a diffuse reln-ir albeit less intense than that in the LOT. In addition layer I and layer III contained some reln-ir cells (Fig. 1E). At this stage, the differences between immunolabelling and mRNA probes were more significant: Reelin mRNA staining was absent in the LOT (Fig. 1F), although, there were reelin mRNA-positive cells in layers I and III (Fig. 1F).

The appearance of reelin expression in the piriform cortex at E-19 resembles that observed at E17 both with immunolabelling (Fig. 1G) and ISH (Fig. 1H).

To better understand the topographical relation between reelin expression and developmental markers in the piriform cortex, we performed a double labelling of reelin and Tbr1. As expected both labelling are expressed in different compartments. At E-13 and E-15 Tbr1 immunoreactive cells (Tbr1-ir) are located in the marginal zone and cortical plate, a high amount of reelin is present in the neuropil in the same region but both substances did not show colocalization (Fig. 2A-F). At E-17 and E-19 Tbr1 and reelin expression increase, reelin immunoreactivity is observe in the LOT and some cells in layers I and III , while Tbr1- ir labels many cells that start to accumulate in layer II (Fig. 2G-L)

3.2 Reelin expression during postnatal development in the rat piriform cortex

In newborn pups (P-0), there was a high degree of reelin immunostaining in the LOT and in some cells of layers I and III (Fig. 3A), while the LOT was devoid of reelin mRNA labelling (Fig. 3C). Interestingly, reln-ir in the LOT in newborn pups was primarily observed as some intensely stained round spots of about 1 μm in diameter, that appear to punctuate parallel lines running through the LOT from the marginal zone to layer I (Fig. 3B).

To better understand the nature of the intensely stained reln-ir spots in the newborn LOT, we performed double immunolabellings with different neurochemical markers: As during prenatal development there was a different topographical distribution for reelin and Tbr1 labellings (Fig. 4A-C). Labelling of glial fibrilary acid protein (GFAP) also failed to show any relation to reln-ir in the LOT (Fig. 4D). The immunostaining of presynaptic terminals with an antibody for synaptophysin showed a punctuate labelling more evident in layer I than in the LOT, where its appearance was similar to the punctuate labelling in parallel lines evidenced by reelin immunoreactivity (Fig. 4E). However no colocalization of both markers is observed; synaptophysin immunoreactive terminals were more abundant in the LOT regions closer to layer I, while reln-ir spots were more prominent in the LOT regions away from layer I (Fig. 4E). Labelling of GABAergic terminals with GAD or glutamatergic terminals with VGLUT2 also fails to show any colocalization with reln-ir spots (Figs. 4F and 4G, respectively).

At postnatal day 2 a high degree of reelin expression persists in the LOT and layer I (Fig. 5A), and there is still evidence of reln-ir spots in the LOT (Fig. 5B). At postnatal day 7 there was an intense diffuse reelin immunolabelling in both the LOT and layer I, while reln-ir spots in the LOT are difficult to discern (Fig. 5C). At postnatal day 15 there is a decrease in the level of reln-ir diffuse staining in the LOT and layer I, and no evidence of reln-ir spots in the LOT (Fig. 5D). In addition, reln-ir cells are observed in layer IA and layer III (Fig. 5D).

3.3 Reelin immunoreactivity in the adult rat olfactory cortex

Figure 6 illustrates the distribution of reelin immunoreactivity in the adult piriform cortex. To better understand the areas of reelin labelling, we provide an image of NeuN

immunostaining with the identification of the several piriform cortical layers (Fig. 6A), as well as a double labelling of reelin and Tbr1 (Fig. 6E). Reln-ir is observed primarily in layers IA and III, while the LOT is devoid of labelling (Fig. 6B). In layer IA there is an intense diffuse immunostaining, as well as several reln-ir cells preferentially located in the border with the LOT (Fig. 6C). In layer III there are some heavily stained reln-ir cells, while other cells show a much lower degree of intracytoplasmic staining (Fig. 6D).

As reelin has been show to play a role in synaptic plasticity in the adult brain, we have compared reelin immunoreactivity distribution with some markers of different neurotransmitter systems, which it was evidenced as glutamatergic, GABAergic, dopaminergic and serotonergic innervations in the piriform cortex. All of these markers showed different degrees of immunostaining in layer IA where there was a clear accumulation of diffuse reelin immunolabelling (Fig. 7A-D).

4. DISCUSSION

The present findings suggest that reelin might play a role in regulating the synaptogenesis of LOT axons during piriform cortex development, and thereafter may be involved in the regulation of synaptic plasticity in layer I of the piriform cortex, where reelin could be secreted by olfactory bulb mitral cell axons and also by terminals of piriform cortex GABAergic interneurons that impinge onto pyramidal neurons dendrites.

During prenatal development reelin immunolabelling in the piriform cortex is particularly evident in the LOT as a heavy diffuse immunolabelling, and also in cells in the developing layers I and III. However, reelin mRNA labelling is evident in some cells in layers I and III (in concordance with reelin immunostaining) but there is no evidence of reelin mRNA expression in the LOT (present results). The presence of a heavy diffuse reelin immunolabelling is not restricted to the LOT (or to layer IA in later developmental stages), such labelling has also seen in the hippocampus stratum lacunosum-moleculare where it has been proposed it represents reelin secreted into the neuropil extracellular matrix (Pesold et al., 1998). Accordingly, the diffuse immunolabelling observed in the LOT during development could represent secreted reelin protein and therefore not be stained by *in situ* hybridization.

Tbr1 is expressed by virtually all post-mitotic glutamatergic neurons in several cortical areas and hippocampus (reviewed by Hevner et al., 2006). As expected, double labellings of reelin and Tbr1, a marker of glutamatergic cells failed to show cellular colocalization in the developing piriform cortex, indicating that the heavily stained reln-ir cells of layers I and III represent some kind of interneurons (possibly GABAergic) as it happens with reln-ir cells in neocortex and hippocampus (Alcántara et al., 1998; Pesold et al., 1998, 1999).

In newborn pups, the pattern of reelin immunostaining in the piriform cortex is different than in prenatal development, being the heavily stained reln-ir round spots in the LOT the main

difference. At this stage, the LOT is mainly composed of incoming axons from olfactory bulb mitral cells that would synapse with apical dendrites of olfactory cortex pyramidal neurons, and entorhinal cortical neurons (among others) in more caudal regions. Olfactory bulb mitral cells express reelin, and reln-ir products have been observed inside mitral cell axons (Pappas et al., 2003). One explanation for the presence of these reln-ir spots in the LOT of newborn pups may be that they represent the accumulation of reelin in some areas of mitral cell axons or axon endings that will establish synapses with pyramidal cell dendrites, which implies that reelin may play a role in olfactory cortex synaptogenesis, as it has been suggested in different brain areas (Derer et al., 2001., Martínez-Cerdeño and Clascá, 2002; Martínez-Cerdeño et al., 2002, 2003; Ramos-Moreno et al., 2006; Roberts et al., 2005). This may be a similar role to that played by reelin in fibres of the perforant pathway that reach the dentate girus and have their origin in the entorhinal cortex (Del Rio et al., 1997; Borrell et al., 1999).

Doublelabelling experiments in newborn pups demonstrated not only a lack of colocalization of reln-ir and glial fibrilary acid proteins (as could be expected), but also a lack of colocalization with presynaptic markers as synaptophysin, GAD and VGLUT2 (present results). Both reln-ir in the LOT and the immunolabelling of presynaptic markers show a punctuate appearance with heavily stained round spots. However, while synaptophysin and VGLUT2 positive spots are mostly observed in layer I and in the regions of LOT closer to layer I (GAD terminals are not observed in the LOT); reln-ir spots are observed in the LOT areas more away from layer I. A possible explanation could be that reln-ir spots represent an accumulation of reelin by mitral cell axons and it is secreted in areas where glutamatergic synapses will be developed and established, but clearly further experimentation would be needed to demonstrate this possibility.

During the first two weeks of postnatal development the main changes in the distribution of Reelin in the anterior olfactory cortex were the disappearance of reln-ir round spots present in the LOT of newborn pups, the decrease of reelin immunoreactivity in the LOT, and the increase in diffuse reln-ir in layer IA which reached highest levels in the adult brain. As olfactory bulb mitral cells continue to exhibit high levels of reelin expression in the adult brain (Pappas et al., 2003). In addition, it has been shown that reelin can be transported and secreted in long axonal pathways such as the lateral olfactory tract or the perforant pathway in the adult macaque and ferret adult brain (Martínez-Cerdeño et al., 2002, 2003; see also Pesold et al., 1998). These findings indicate the possibility that even if reelin is present in the adult LOT the presence of myelin may in part prevent the clear visualization of reelin immunolabelling by light microscopy.

The reelin pattern distribution in the adult anterior olfactory cortex closely resembles the pattern present in the mature hippocampus (also part of the palleocortex) where intense diffuse immunostaining is observed in the stratum lacunosum-moleculare and where many GABAergic

interneurons are reln-ir (Pesold et al., 1998). Moreover, as was shown by Martínez-Cerdeño et al. (2003) in the ferret piriform cortex, the intense diffuse immunolabelling observed in layer IA matches the map of mitral axonal endings in the olfactory cortex, suggesting that a high amount of reelin is secreted in this region. It is also important to note that a recent electron microscopy study by Roberts et al. (2005) has shown that reelin immunoreactivity is present inside axons in postmortem human brain samples. Finally, the presence of reelin in some subpopulations of GABAergic interneurons resembled that observed in the neocortex and hippocampus (Pesold et al., 1998, 1999)

The functional roles of reelin in the adult brain appear to be related to synaptic plasticity events (reviewed by Herz and Chen, 2005). In fact, reelin has been shown to be involved in LTP formation in hippocampus, and this action is mediated by its interaction with VLDL and ApoE2 receptors (Weeber et al., 2002; Beffert et al., 2006). Another possible action of reelin is promote protein synthesis in dendritic spines, a process in which reelin may act through integrin receptors (Rodríguez et al., 2000; Dong et al., 2003). Freeze-fracturing analysis of synaptosomal membranes indicate that incubation with recombinant Reelin may induce the clustering of membrane proteins in postsynaptic or presynaptic membranes (Dong et al., 2003), and other studies have shown that receptor clustering is involved in reelin signalling (Strasser et al., 2004). All of these findings suggest that reelin may be a pleiotropic molecule with several molecular functional roles which in the adult brain may be related to synaptic plasticity.

The expression of reelin in olfactory regions that are essential in olfactory learning and memory indicates that Reelin may be important in the regulating of olfactory learning. Taking into account that olfactory deficits and decrease in reelin levels have been reported in schizophrenia patients (Impagnatiello et al., 1998; Moberg et al., 1999; Fatemi et al., 2000; Guidotti et al., 2000; Hudry et al., 2002; Fatemi, 2001; Moberg and Turetsky, 2003; Eastwood and Harrison, 2003) as well as in the heterozygous *reeler* mouse (postulated as an animal model for this disease) (Tueting et al., 1999; Liu et al., 2001; Pappas et al., 2001; Larson et al. 2003). Detailed knowledge of reelin distribution in the normal olfactory system may provide an important insight into the pathophysiology of the olfactory system in schizophrenia.

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6. FIGURES

Figure 1: Reelin expression in coronal sections of the piriform cortex during prenatal development. Reelin immunolabelling is shown in the left panel (A, C, E, G), and reelin mRNA expression in the right panel (B, D, F, H). Note the presence of a heavy reelin immunolabelling in the developing LOT while there is no reelin mRNA labelling in this area. Scale bars: A-H 500 μm; inset: 10 μm.

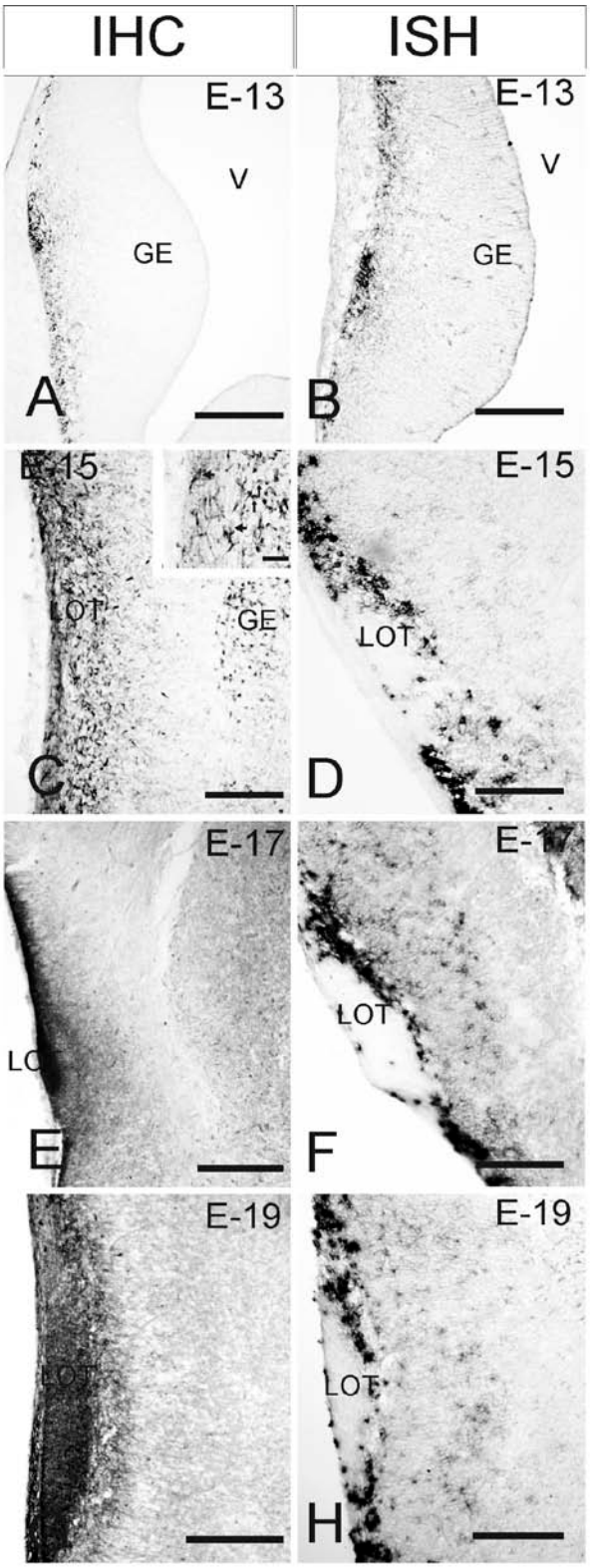
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Figure 1

Figure 2: Reelin (green) and Tbr1 (red) expression during prenatal development (A-L). Note the lack of spatial colocalization between the two markers (A-C) Piriform cortex at E-13 showing reln-ir (green) and Tbr1-ir (red) cells. (D-F) Piriform cortex at E-15, marginal zone shows reln-ir cells (green) and immunorrecativity in the extracellular matrix while Tbr1-ir cells (red) are located in the marginal zone and cortical plate. (G-L) Reelin imunorreactivity (green) at E-17 (G-I) and E-19 (J-L) is mainly located in the LOT and layer I of the piriform cortex, where there is a lack of colocalization of reln and Tbr1 (red). Scale bars: A-L 50 μm.

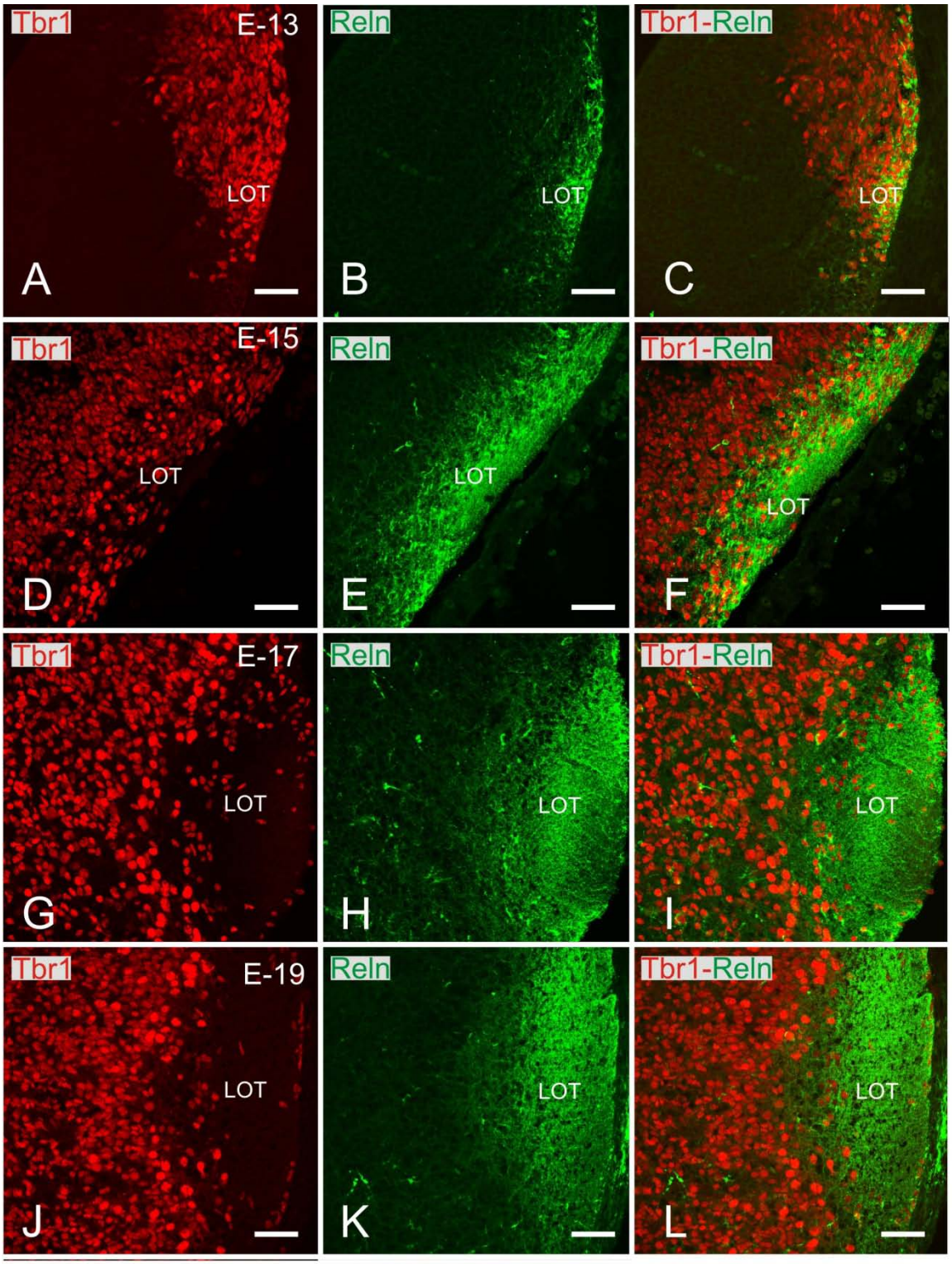
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Figure 2

Figure 3: Reelin expression during postnatal development. (A) Low magnification image showing a general view of reelin immunostaining in piriform cortex of newborn pups. There is diffuse reelin immunoreactivity in the LOT and layer IA (arrow), as well as some reln-ir cells in layers IA and III (arrows). (B) High magnification micrograph showing a detail of the LOT reln-ir round spots (arrowheads). (C) Reelin mRNA expression in the priform cortex of P0. Reelin mRNA positive cells are located mainly in layer I and III, hwile there is no evidence of Reelin mRNA positive spots in the LOT. Scale bars: A) 250 μm, B) 10 μm, C) 500 μm.

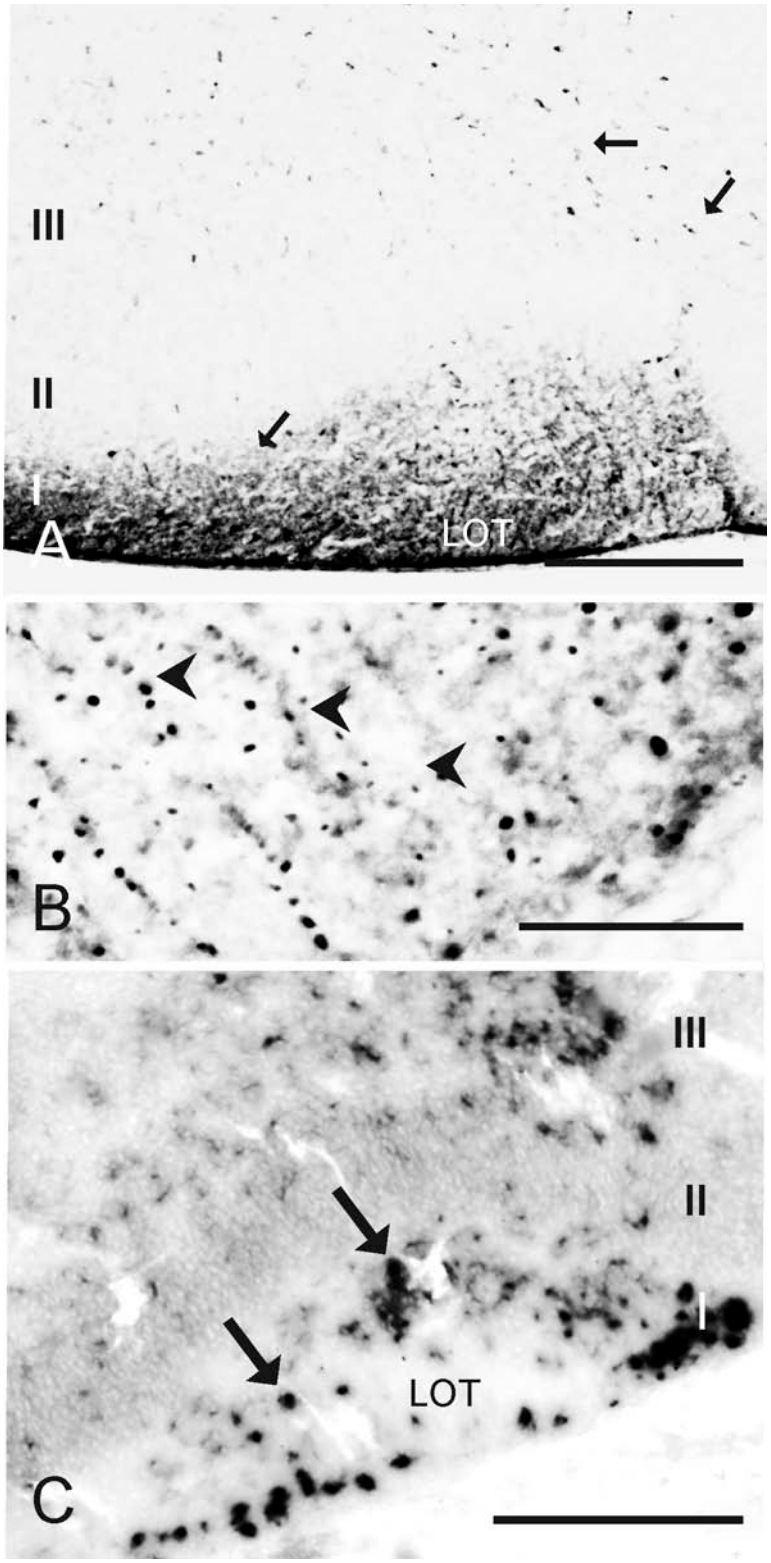

Figure 3

Figure 4: Confocal microscopy images from coronal sections of the piriform cortex at P0. (A-C) Double labelling of reelin (green) and Tbr1 (red) at P0, showing the lack of colocalization between both markers in piriform cortex. (D) Detail of reln-ir round spots (green) and GFAP immunoreactivity (red). (E) Double labelling of reelin (green) and synaptophysin (red) in the LOT. F) Detail of the LOT with reln-ir (green) and GABAergic synaptic terminals, GAD-ir (red). (G) Labelling of reelin (green) and VGLUT2 (red) in the LOT and layer I. Note that there is no colocalization of reelin with any of the other markers used. Scale bars A-C) 500 μm, D-G) 50 μm.

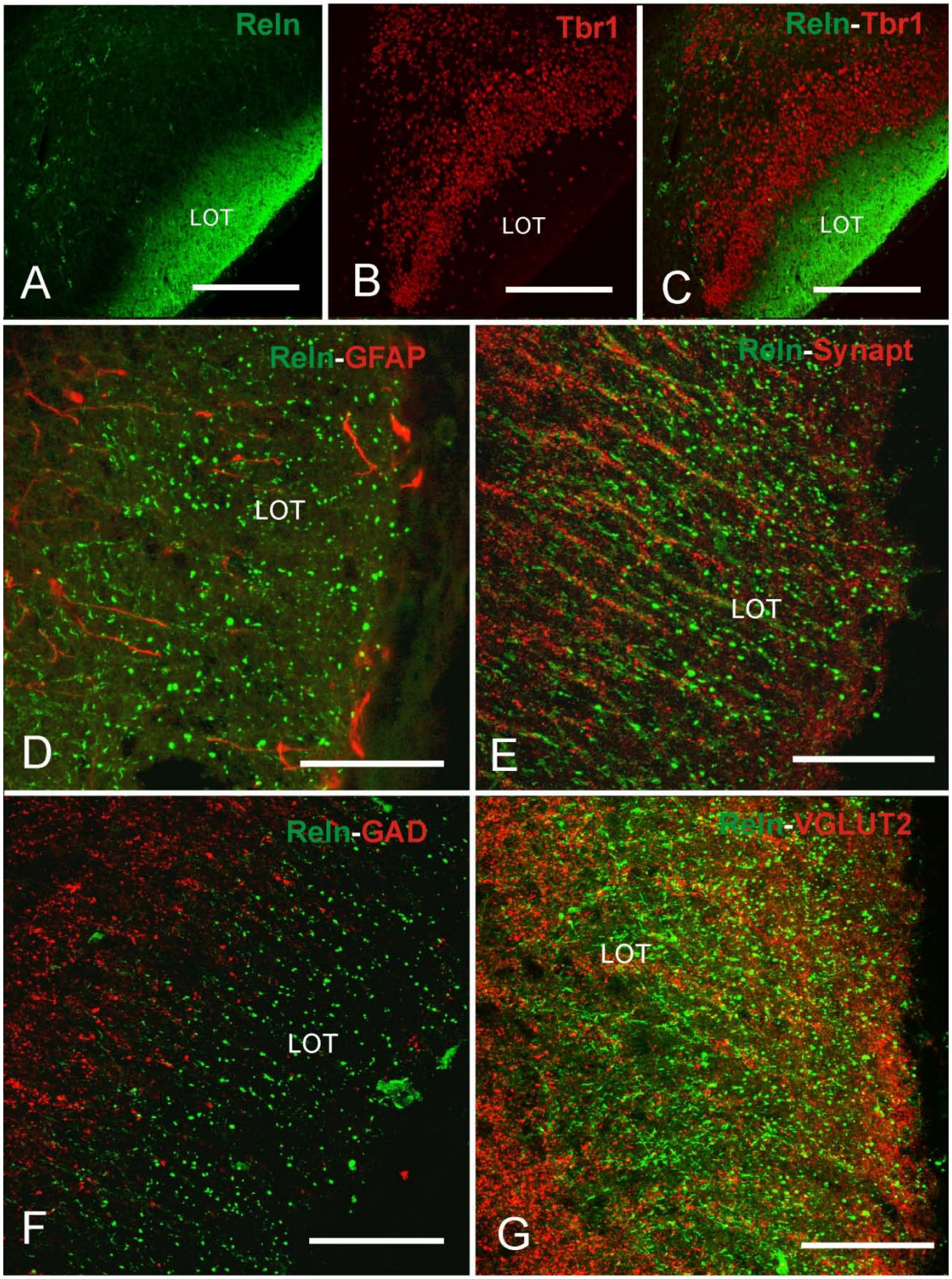
Reln
LOT
A
Tbr1
LOT
B
Reln-Tbr1
LOT
C
Reln-GFAP
LOT
D
Reln-Synapt
LOT
E
Reln-GAD
LOT
F
Reln-VGLUT2
LOT
G

Figure 4

Figure 5: Reelin expression in coronal sections of the piriform cortex at postnatal stages between P2 and P15. (A) The piriform cortex at P2 presents an intense diffuse labelling in the LOT, and layer I. (B) High magnification image of the LOT at P2 showing reln-ir round spots (arrowheads) and reln-ir diffuse labelling. (C) Labelling of reln-ir at P7 showing an increase in diffuse labelling in the LOT and layer IA. (D) Reln-ir at P15 showing an intense diffuse reln-ir labelling in layer IA as well as some reln-ir cells (arrows). Note that the LOT at P7 and P15 is basically devoid of the reln-ir round spots observed in previous stages. Scale bars: A and D) 100 μm, B) 10 μm, and C) 250 μm.

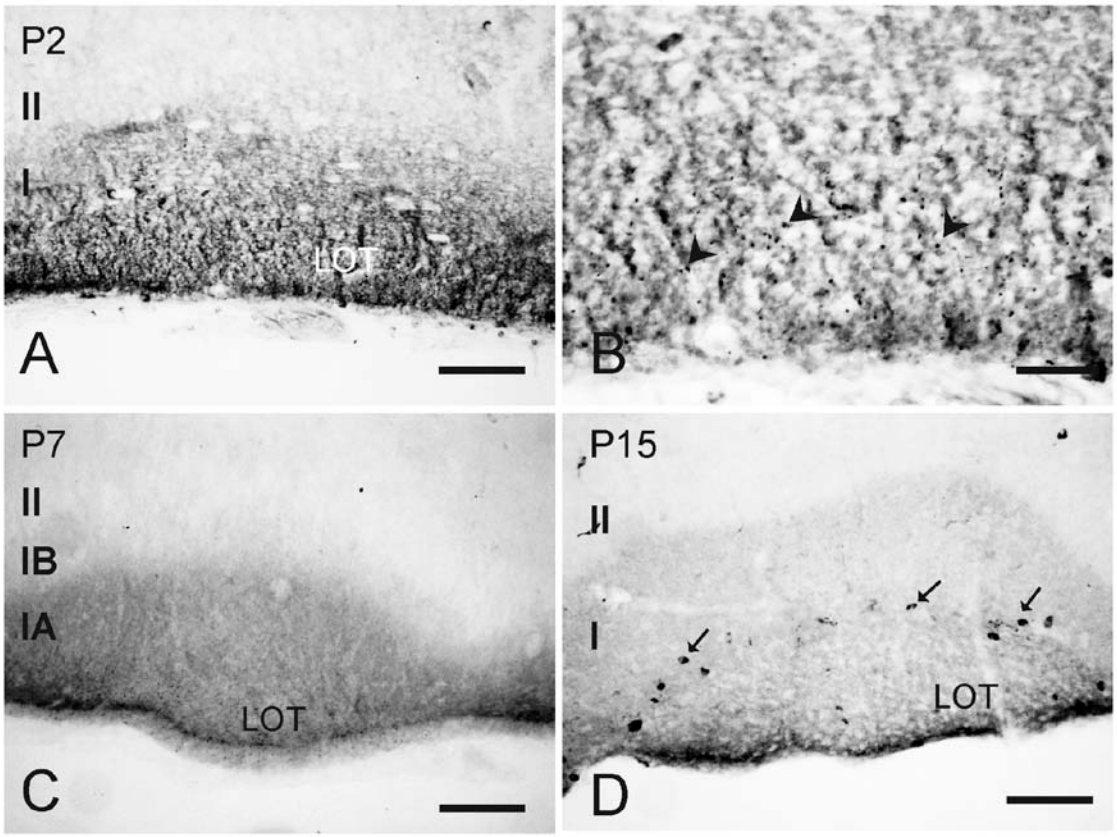

Figure 5

Figure 6: Reelin expression in coronal sections of the adult rat piriform cortex. (A) NeuN immunostaining showing the distribution of neuronal cell bodies in the different layers of the piriform cortex. (B) Reelin immunostaining reveals the presence of diffuse immunolabelling in layer IA as well as the presence of some reln-ir cells in layers IA, and III (arrows). (C) Detailed image of the LOT and layer IA showing some reln-ir cells (arrows). Note also the intense diffuse reelin immunostaining present in layer IA while the LOT is devoid of this kind of staining. (D) High magnification of image B showing reln-ir cells in layer III of the piriform cortex. (E) Double labelling of reelin (green) and Tbr1 (red) in the adult piriform cortex. Scale bars: A-B) 250 μm, C-D) 10 μm, E) 100 μm.

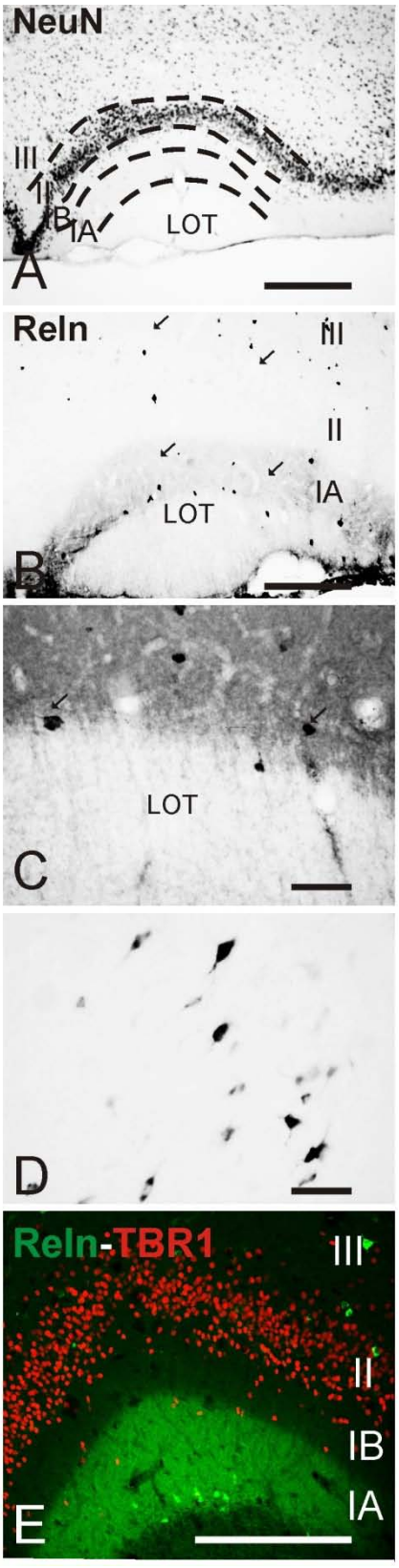
NeuN
III
II
IB
IA
LOT
A
Reln
III
II
IA
LOT
B
LOT
C
D
Reln-TBR1
III
II
IB
IA
E

Figure 6

Figure 7: Immunolabelling of neurotransmitter systems markers in the adult rat piriform cortex. (A) VGLUT2 immunoreactivity is present as diffuse labelling throughout all layers. (B) There is GAD immunoreactivity in all layers of piriform cortex. In layer IA the GAD-ir terminals are distributed uniformly. (C) There are a high amount of TH-ir fibres in layer IA and dopaminergic innervation is lower in layers II and III. (D) There are numerous 5HT-ir fibres in all layers of the piriform cortex. Scale bars A-D) 250 μm.

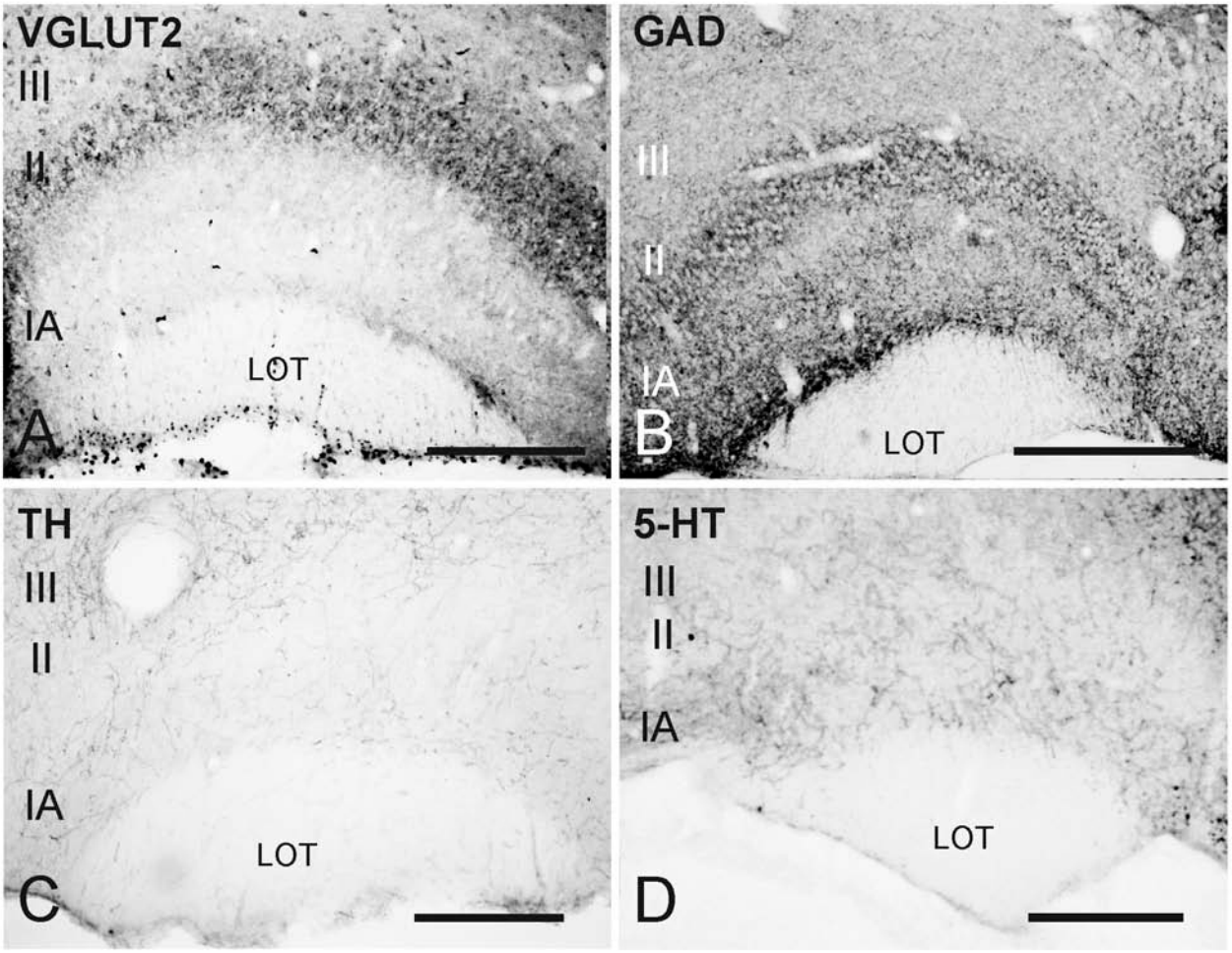
VGLUT2
III
II
IA
LOT
A
GAD
III
II
IA
LOT
B
TH
III
II
IA
LOT
C
5-HT
III
II
IA
LOT
D

Figure 7

Chapter 4

Reelin, Tbr1 and Pax6 expression in the ventral thalamus during rat prenatal development and in newborn pups

1. INTRODUCTION

Reelin is an extracellular matrix protein that plays a role in neuronal migration and positioning during development of the central nervous system (CNS) (reviewed by Huang and D'Arcangelo, 2008). Besides this role in early development, reelin is also expressed in the adult brain, where it has been related with synaptic plasticity by enhancing the induction and maintenance of long-term potentiation (Weeber et al., 2002; Beffert et al., 2006), and with dendrite and dendritic spine development (Dong et al., 2003; Niu et al., 2004; 2008; Beffert et al., 2006; Campo et al., 2009). During ontogenesis of the CNS, reelin mRNA is expressed at embryonic day 10 in mouse (E-10), and the protein is detected at E-13 in several discrete brain regions (Schiffmann et al., 1997; Alcántara et al., 1998). The reelin expression pattern was mostly studied in laminar structures of the mouse brain, where important alterations have been described in the *reeler* mutant [cerebral cortex, hippocampus and cerebellar cortex]. However, no detailed studies were done in subcortical, diencephalic, mesencephalic and rhombencephalic areas, although important alterations were also described in some of these areas in the *reeler* mouse (reviewed by Lambert de Rouvroit and Goffinet, 1998; Katsuyama et al., 2009).

The thalamus is a major relay structure in the diencephalon, each of the thalamic nuclei exhibits a unique pattern of gene expression and connectivity. In mammals, a vast majority of thalamic nuclei send axons to unique sets of distinct neocortical areas; principal sensory nuclei establish topographic and area-specific thalamocortical projections to primary sensory areas in the neocortex (reviewed by Mólnar et al, 2006). In the classic model of thalamus development, neuroblasts are generated in the ventricular area from where these differentiating neuroblasts migrate to the marginal zone (reviewed by Nieuwenhuys, 1998), but new studies in rodents have revealed the presence of neuroblasts migrating tangentially in the thalamus during early development (Frassoni et al., 1998; Ortino et al., 2003). Despite the functional importance of the thalamus, the molecular mechanisms that control the specification of thalamic nuclei and its thalamocortical connections are not well understood. Reelin is present in the thalamus of rodents from early embryonic stages (Ikeda and Terashima, 1997; Schiffman et al., 1997; Alcántara et al., 1998), and persists in some nuclei of the ventral and dorsal thalamus during the adult life (Ramos-Moreno et al., 2006). In other brain areas, reelin is involved in the control of the neuronal migration, but it is not clear what role plays during development of the thalamus.

The knowledge of the expression of transcription factors during development has provided new tools to study the morphological development of the brain. The use of transcription factors enabled tracing the fates of progenitor cells expressing a particular gene and the limits between specific brain regions. Numerous studies have revealed lineage relationships between each of these progenitor domains and distinct classes of postmitotic neurons (reviewed by Puelles and Rubenstein, 2003). In the present study we have used a combination of different transcription

factors that are expressed differentially in the embryonic diencephalon to study the compartmental expression of Reelin in different progenitor populations in the diencephalon: 1) Sonic Hedgehog (shh) is expressed in the diencephalic basal plate (i.e. ventral to the thalamus) and in the zona limitans intrathalamica (Shimamura et al., 1995; revised by Puelles and Rubenstein, 2003). 2) Pax6 has been detected during rodent development in ventral telencephalon as well as in the ventricular zone of eminentia thalami; mature derivatives of this area include the zona incerta (Stoykova et al., 1996; Puelles et al., 2000). 3) Tbr1 expression was described during development in the mantle zone of the eminentia thalami. Some of the adult territories originated from this area are the bed nucleus of the stria medullaris, and the posterior part of the bed nucleus of the stria terminalis (Puelles et al., 2000). We have also used antibodies against calretinin and calbindin as markers of some early generated neuronal populations (Frassoni et al., 1998); both proteins show specific expression patterns during development in ventral and dorsal thalamus (Puelles et al., 1992; Frassoni et al., 1998).

The aim of the present study was to examine in detail the spatiotemporal expression of reelin in the developing rat diencephalon and its relationship with the expression of some transcription factors (shh, Pax6, Tbr1), two neuronal markers (calbindin and calretinin) of early generated neurons and the proliferating cell nuclear antigen (PCNA), a general proliferation marker.

2. MATERIAL AND METHODS

Animals

Pregnant female Spragle-Dawley rats were used in this study. All the procedures for handling and euthanasia are in accordance with the European Commission guidelines (86/609/CEE) and were approved by the ethics committee of the University of Santiago de Compostela. Pregnant rats were deeply anaesthetized, and euthanized by cervical dislocation. For calculation of gestation and embryonic development stages, the day when the vaginal plug appears was day 0 and pups were born on E-21 (P0). The embryos were removed by caesarean surgery at different developmental stages corresponding with embryonic day 13, 14, 15, 17, and 19 (E-13, E-14, E-15, E-17, and E-19).

The animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer 0.1M (PB). Some E-15 embryos and P0 were also perfused transcardially with 4% paraformaldehyde. The brains removed from the skull were cryoprotected in a sucrose solution (30% sucrose in PB) and sectioned at 20 μm thickness in a cryostat. For long-term storage, the tissue sections were kept at -80ºC.

Antibodies

The following primary antibodies were used in single and double immunohistochemistry procedures: 1) Mouse anti-reelin (reln) monoclonal antibody G-10 (Chemicon, code MAB 5364; dilution 1:1,000 or 1:500 in immunofluorescent labelling). For details on the characterization of this antibody see de Bergeyck et al. (1998). 2) Rabbit anti-Calbindin-D28k polyclonal antibody (CB) diluted 1:10,000 (Swant, code CB-38a). 3) Rabbit anti-Calretinin polyclonal antibody (CR) diluted 1:1,000 (Swant, code 7699/4). 4) Rabbit anti-Tbr1 1:500 (Chemicon, code AB9616). 5) Rabbit anti-Pax6 1:500 (Chemicon, code AB5409). 6) Rabbit anti-Proliferating cell nuclear antigen (PCNA) 1:500 (Sigma-Aldrich, code P8825). 7) Rabbit anti-sonic hedgehog (shh) 1:100 (Santa Cruz, code sc-9024).

In the present study, we used the following secondary antibodies: Biotinylated goat anti-mouse and goat anti-rabbit antibodies (Dako, product numbers, Z0420 and E0432, respectively). Alexa Fluor 488 coupled goat anti-rabbit and Alexa Fluor 546 coupled goat anti-mouse antibodies (Molecular Probes, product numbers A11008 and A11003, respectively).

Immunohistochemistry

For single immunohistochemistry, sections were incubated overnight at room temperature with the appropriate primary antibodies diluted in a solution of 15% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in TBS.

After rinsing with TBS sections were sequentially incubated with the appropriate secondary antibodies for one hour, diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100. After rinsing in TBS, the sections were incubated with ABC complex (Vector Laboratories), rinsed, developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine (DAB Sigma), and finally dehydrated and coverslipped.

For double immunofluorescence, sections were incubated overnight at room temperature with one of the cocktails of reelin and the following antibodies: anti-CR, anti-Pax6 and anti-Tbr1. In all cases, the antibodies were prepared in a solution containing 15% NGS, 1% BSA and 0.2% Triton X-100 diluted in TBS. After rinsing with TBS, sections were incubated with appropriate fluorescent secondary antibodies diluted in a solution containing 10% NGS, 1% BSA and 0.2% Triton X-100 for one hour. Finally, sections were rinsed in distilled water, air-dried, and coverslipped using Mowiol (Calbiochem).

In situ Hybridization

In situ hybridization (ISH) for Reelin was performed using a riboprobe labelled with digoxigenin-d-UTP (Roche) by in vitro transcription of a cDNA fragment encoding mouse Reelin (D'Arcangelo et al., 1995) using T3 polymerase (Roche). Sections were permeabilized 2 minutes

with proteinase K (5 μg/ml), fixed 10 minutes in 4% paraformaldehyde, and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridized at 60ºC for 3 hours in a solution containing 50% deionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 2X SSC, 250 μg/ml yeast t-RNA and 250 μg/ml denatured salmon sperm DNA. Labelled anti-sense cDNA was added to the pre-hybridization solution (500 ng/ml), and the hybridization was performed at 60ºC overnight. Sections were then rinsed sequentially in 1X SSC (10 minutes at 65ºC), 1.5X SSC (10 minutes at 65ºC), and 2X SSC (40 minutes at 37ºC), digested with RNase A (30 minutes at 37ºC) and rinsed in 0.2X SSC (1 hour at 55ºC). After that, sections were rinsed in maleic acid buffer containing 0.5% Tween 20 (15 minutes), incubated with a blocking solution containing MABT (100 mM maleic acid, 150 mM NaCl and Tween 20) and normal sheep serum (3 hours at room temperature), and finally incubated overnight with an alkaline phosphatase-conjugated antibody to digoxigenin (1:2000, Roche). The reaction was developed with BM purple (Roche).

After the *in situ* hybridization, sections were fixed 10 minutes in paraformaldehyde 4%. Thereafter, sections were rinsed in citrate buffer (pH 6.0) 30 minutes at 95ºC for antigen retrieval. Sections were incubated overnight at room temperature with rabbit anti-CR, anti-PCNA, anti-CB or anti-Pax6, diluted in a solution of 15% normal goat serum (NGS) and 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in TBS. After rinsing with TBS the sections were sequentially incubated with biotinylated goat anti-rabbit or biotinylated goat anti-mouse for one hour, followed by incubation with ABC complex (Vector Laboratories), developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine, and finally coverslipped with Mowiol.

Photography

Sections were observed using an Olympus Bx51 microscope. Photomicrographs were taken with an Olympus DP-71 colour digital camera. Sections labelled for double immunofluorescence were observed using a Leica DMRE microscope. Confocal images were acquired with a Leica TCS-SP2 laser confocal microscope. The images were converted to a gray scale (for black and white figures), and adjusted for brightness and contrast by using Corel Photo-Paint 13. Picture set up was done by using Corel-Draw 13.

3. RESULTS

The present study reports the expression of reelin mRNA and protein in the developing rat diencephalon from E-13 to P0. Rat diencephalic structures were identified by using the Atlas of prenatal rat brain development (Altman and Bayer, 1995). In our descriptions we will follow the terminology proposed by Puelles et al. (2000), because they have used some of the

transcription factors used in the present study. Also, the orientation of sections through the diencephalon used in the different embryos was approximately parallel to the coronal axis of the brain, horizontal sections defined by these authors.

Reelin expression (protein and mRNA) was detected in all the embryonic stages studied as well as in newborn pups. Reelin immunoreactive (reln-ir) cells were fusiform and showed one or several immunoreactive processes; Reelin was also observed as a diffuse (i.e. extracellular) immunolabelling around cells in some brain areas, which was observed from E-15 rats and increased during later embryonic stages.

At E-13 the portion of the diencephalic vesicle was almost exclusively composed by neuroepithelium, although a zone of differentiating cells was already present. At this stage the ventricular zones did not show reelin immunoreactivity, some scattered reln-ir cells were detected in the habenula in the mantle zone (Fig. 1A). In this area the intensity Reelin mRNA was higher than the protein immunoreactivity (Fig. 1A, 1E). Double immunolabelling with shh showed the presence of reln-ir cells in the lateral portion of the zona limitans intrathalamica (Fig. 1K-P), but also a lack of colocalization of both substances in this area (Fig. 1P). Reln-ir cells were observed in the eminentia thalami and the ventral thalamus ["prethalamus" of Puelles and Rubenstein (2003)] (Fig. 1B-D) showing codistribution with Tbr1 (Fig. 2A-D) and Pax6, but not a clear colocalization (Fig. 2E-H). In addition, dorsal thalamus ["thalamus" of Puelles and Rubenstein (2003)] showed some scattered reln-ir cells (Fig. 1B-D, F-H; Fig 2E-P).

In caudal regions of the ventral thalamus (near the zona limitans) some reln-ir cells were also calretinin immunoreactive (cr-ir) (Fig. 2I-L), while the rostral part of the dorsal thalamus showed cells expressing reelin but not calretinin. Reln-ir cells situated in this area showed a radial orientation, as well as some of them displayed perpendicularly from the radial migratory stream (Fig. 2I-L). Basal regions of ventral and dorsal thalamus (towards the basal plate) showed some reln-ir cells also immunostained for calretinin (Fig. 2M-P).

At E-15 the overall pattern of Reelin expression resembled the E-13 pattern. The lateral habenula showed reelin mRNA and reln-ir cells (Fig. 3A-B, D-E); the intensity of the reelin immunoreactivity in this area is more prominent than the expression of the mRNA. Reln-ir positive cells were scattered in the lateral dorsal thalamus (Fig. 3A, C), although the expression of the reelin mRNA is weak and does not show colocalization with calbindin; calbindin immunoreactive cells are located in the subventricular area but not in lateral areas of the dorsal thalamus (Fig. 3D-F).

In order to show the actual localization of cells as regards the interprosomeric borders, we used double labelling with shh and reelin. This procedure showed a lack of colocalization of shh and reelin at this stage in the zona limitans intrathalamica, as also described at E-13 (Fig. 3G-I).

Shh shows clearly that the reelin-ir populations are present at both sides of the zona limitans, i.e. there are reelin-expressing populations in both the caudal prethalamus and rostral thalamus. In addition, the zona limitans appeared as a PCNA-negative discontinuity where reelin mRNA was not present (Fig. 3J-L).

In the eminentia thalami and ventral thalamus there was a robust expression of reelin immunoreactivity and mRNA. The lateral area of the eminentia thalami showed reln-ir cells that also were cr-ir, but not all the reln-ir cells co-express calretinin (Fig. 4A-B). The eminentia thalami was identified as the region containing Tbr1-ir cells, following Puelles et al., (2000). In the dorsal portion of the eminentia thalami, although Tbr1 and reelin were located in the same area there was not a clear colocalization of both markers (Fig. 4C, G), while in the basal areas of the eminentia thalami were observed some reln-ir cells that were also immunoreactive to Tbr1 (Fig. 4K, O); this area could correspond to the bed nucleus of the stria medullaris. There was a lack of colocalization between reelin and Pax6 in the eminentia thalami, since Pax6 immunoreactive cells (Pax6-ir) were present in the ventricular area, while reln-ir cells were located in the mantle area (Fig. 4D, H). In the ventral thalamus reelin was strongly expressed in the region adjacent to the zona limitans intrathalamica, and the positive population extended from the ventricle to lateral areas of the ventral thalamus (Fig. 4E, I, M). Some of reln-ir cells were also positive for Pax6 (Fig. 4H, L, P); this area could correspond with the zona incerta. In the lateral area was present another intense reln-ir cell population; this zone could correspond to the primordium of the ventral lateral geniculate nucleus, which express high levels of reelin at later stages (Fig. 4I). Some reln-ir cells in the ventral portion of the lateral geniculate nucleus also showed immunoreactivity to calretinin and Pax6 (Fig. 4F, J, L, N, P).

During later stages of development and newborn pups the pattern of reelin distribution resembled that described at E-15. Accordingly, only those structures showing changes with respect to previous stages will be highlighted.

At E-17, reelin continued being expressed in the lateral habenula and at E-19 was also present in the medial habenula. In newborn pups reelin mRNA was highly expressed in the medial and lateral habenula but the reelin mRNA expression was more prominent in the medial than in the lateral habenula (Fig. 5I).

The dorsal thalamus at E-17 andE-19 showed a few reln-ir positive fibres and some scattered reln-ir cells in lateral areas, but reelin mRNA was not observed in the same area. At P0 only a few scattered cells showed reelin immunoreactivity in the dorsal thalamus (data not showed).

As in earlier stages, at E-17, E-19 and P0 the lateral area of the eminentia thalami showed reln-ir cells that also were cr-ir, but not all the reln-ir cells co-express calretinin (Fig.5 A-B, E, I, M).

Also as in earlier stages, the eminentia thalami showed Tbr1-ir and reln-ir cells at E-17, E-19 and P0 (Fig. 5C). The major change in this region was in the ventral area of the eminentia thalami, the bed nucleus of the stria medullaris, where there were some cells that expressed clearly both markers, reelin and Tbr1 (Fig 5G, K, O).

The area corresponding to the zona limitans intrathalamica at E-17 was devoid of shh immunoreactivity (data not showed), although reelin expression remained near the ventricle in this area. There were two reelin positive populations flanking the region of the zona limitans intrathalamica. However, the number of reelin positive cells decreased at this level compared with the previous embryonic stages (Fig. 5E). The Reln-ir cells located in the rostral dorsal thalamus, at E-17 and E-19 showed a similar pattern to that observed at E-15: reln-ir cells situated in the region adjacent to the zona limitans intrathalamica in the ventral thalamus continued expressing both markers, reelin and Pax6, while reln-ir cells adjacent to the zona limitans intrathalamica located in the dorsal thalamus did not showed Pax6 immunoreactivity (Fig 5D, H). This expression patter was also observed at P0, where some of reln-ir cells located in the ventral thalamus showed immunoreactivity to Pax6 (Fig 5L, P). In the ventral thalamus we also observed reln-ir cells coexpressing calretinin (Fig 5F, J, and N).

The lateral portions of the ventral thalamus at E-17, E-19 and P0 showed a robust reelin labelling (Fig 5A, E, H-I, M-N, P). A few scattered cells showed colocalization with calretinin and Pax6 in the dorsal portion (Fig 5N, P), while the ventral portion only showed colocalization with Pax6 (Fig 5H, P).

We have also found mRNA expressing cells that colocalize with calretinin in the paraventricular nuclei of the dorsal thalamus at P0 (data not show).

4. DISCUSSION

In this study we performed an immunohistochemical analysis of reelin in parallel with reelin mRNA analysis. The same regions that showed immunostaining with the antibody against reelin were also positive with the reelin mRNA probe, with the exception of the reln-ir population in the dorsal thalamus. The onset of reelin mRNA expression observed in the present work is in agreement with results of previous studies in mouse where reelin expression appears by E-11.5-12 (Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcántara et al., 1998). We also provide new evidence of reelin protein expression in early developmental stages and its correlate with the pattern that was described for reelin mRNA.

Previous studies have described the presence of reelin mRNA or protein in discrete diencephalic structures of different vertebrates (Schiffmann et al., 1997; Alcántara et al., 1998; Bernier et al., 1999; Goffinet et al., 1999; Bernier et al., 2000; Costagli et al., 2002; Pérez-Costas et

al 2002; Tissir et al., 2003), revealing a conserved basic pattern of reelin expression in the diencephalon. We have also used different transcription factors (shh, Pax6 and Tbr1), calretinin, calbindin and PCNA whose expression patterns in the developing thalamus have been characterized in detail for mouse (Puelles et al., 1992; Shimamura et al., 1995; Stoykova et al., 1996; Frassoni et al., 1998; Martínez and Puelles, 2000; Puelles et al., 2000; reviewed by Puelles and Rubenstein, 2003), in order to investigate in detail the patterns of reelin expression.

Reelin is expressed by cells located in the eminentia thalami. To identify this area we used reelin in combination with the transcription factors Tbr1 and Pax6. In mouse, the eminentia thalami was distinguished by strong Tbr1 expression and the presence of Pax6-positive cells only in the ventricular zone (Stoykova et al., 1996; Puelles et al., 2000). Reln-ir cells in this area showed a codistribution with Tbr1 from early developmental stages to newborn pups, being conspicuous the colocalization of both markers in later stages. Previous studies defined that mature derivatives of the eminentia thalami include the bed nucleus of the stria medullaris (Puelles et al., 2000). Here, the study of colocalization of reelin and Tbr1 in the developing diencephalon clearly showed that reelin is present in the bed nucleus of the stria medullaris, which was not described in previous rodent studies (Schiffmann et al., 1997; Alcántara et al., 1998). Some disagreements with previous anatomical interpretations appear to be related with the use in the present study of Tbr1, which allow us a more precise description of the reelin expression pattern in this complex region.

The zona limitans intrathalamica is a transverse boundary between the dorsal thalamus [thalamus in Puelles and Rubenstein, 2003] and the ventral thalamus [prethalamus in Puelles and Rubenstein, 2003]. This zone has been described in the neural tube of different vertebrates (Martínez and Puelles, 2000; Echevarría et al., 2001; Vieira et al., 2005; 2006; Vue et al., 2007; 2009). The neuroepithelial cells that originate the zona limitans intrathalamica exhibit clonal restriction and a low proliferation rate, and shh expression is considered a marker for the zona limitans (Martínez and Puelles, 2000; Echevarría et al., 2001; Vieira et al., 2005; reviewed by Puelles and Rubenstein, 2003). Our results demonstrate that the reln-ir cells located in the caudal regions of the ventral thalamus and rostral regions of the dorsal thalamus did not show colocalization with shh, as well as that this PCNA immunonegative area is devoid of reelin whereas reelin positive cell populations flanked both sides of this low proliferative zone. Presumably, there are two reelin positive populations flanking the zona limitans intrathalamica that should not be considered part of the zona limitans due to the lack of shh expression by both populations. By contrast, previous studies in different vertebrates described reelin expression in the zona limitans intrathalamica (Alcántara et al., 1998; Costagli et al., 2003). Our results indicates the presence of two populations flanking the zona limitans intrathalamica of early embryos, but the individuality of these two populations is very difficult to define without using specific makers of the zona limitans intrathalamica such as shh or Pax6 .

At E-13 there are reln-ir cells in lateral areas of the flanking regions of the zona limitans intrathalamica. We also observed fusiform reln-ir cells, oriented radially or perpendicularly to the zona limitans, some of them also expressing calretinin. This finding indicates that some of the early generated diencephalic neurons also express reelin. The orientation of some cells in the zona limitans allows to hypothesize that these reelin positive cells located in lateral areas of the ventral and dorsal thalamus could correspond with cell population migrating from the ventral to the dorsal thalamus, which also occurs with GABAergic cells. Previous studies of the diencephalon during development showed that calretinin is expressed by migrating neurons (Frassoni et al., 1998); some of them follow a tangential migration route instead of running through the predominant radial route, and some neurons detach from the cluster of cells migrating along the zona limitans intrathalamica and ventral thalamus and move towards to the dorsal thalamus (Frassoni et al., 2000; Ortino et al., 2003). Using Pax6 as a marker of the ventral thalamus (Stoykova et al., 1996), we showed at E-13 the presence of a reln-ir population located in caudal regions of the ventral thalamus, showing codistribution with Pax6 but not a clear colocalization, and a reln-ir population located in a Pax6 negative zone that corresponds with rostral regions of the dorsal thalamus. It has been demonstrated that cr-ir cells in the boundary region between dorsal and ventral thalamus and flanking the zona limitans intrathalamica also express GABA (Ortino et al., 2003; Invernardi et al., 2007).

At E-15 and later developmental stages, reelin was strongly expressed in cells of the region adjacent to the zona limitans intrathalamica, extending in the ventral thalamus from the ventricle to lateral areas and showing codistribution with Pax6. Pax6 is expressed in a restricted domain, which allowed differentiating derivatives of the ventral thalamus from those of the dorsal thalamus. Pax6 positive cells are located in the zona incerta and the ventral part of the dorsal geniculate body (Stoykova et al., 1996). Our results show that although the overall reelin expression during the development of the diencephalon in vertebrates shows a conserved pattern, there are some discrepancies between the results of previous studies in vertebrates and our study as regards the presence of reelin positive cells in the reticular nucleus (Schiffmann et al., 1997; Alcántara et al., 1998; Bernier et al., 1999; Goffinet et al., 1999; Bernier et al., 2000; Costagli et al., 2002; Tissir et al., 2003). We cannot confirm the presence of reelin during development in the reticular nucleus because we did not use any specific marker for this nucleus, but with double immunolabelling experiments using reelin and Pax6 we can assess the presence of reelin in precursor neuroblasts and in the neurons of the zona incerta as well as in the ventral region of the lateral geniculate body. Our results also demonstrate that in the geniculate body two reelin positive cells populations can be differentiated, one in the ventral region that colocalizes with Pax6 and another located in dorsal regions that does not show colocalization with Pax6. This suggests that early on in development of diencephalic structures reelin is expressed in dorsal and

ventral lateral geniculate nucleus primordia, as it was described for these parts in adult rat (Ramos-Moreno et al., 2006), but not during mouse prenatal development (Alcántara el al., 1998).

Previous studies in mouse did not show the presence of the reelin mRNA in the lateral portions of the dorsal thalamus (Schiffmann et al., 1997; Alcántara et al., 1998), which is similar to our results with in situ hybridization, but using the antibody against reelin we observed some scattered reln-ir cells and fibres in this dorsal thalamus area throughout development. In later developmental stages the number of reln-ir cells and fibres decrease. This discrepancy between immunohistochemical and in situ hybridization data could be related with the concentration of protein and mRNA. It is possible that levels of mRNA in the cells and fibres in this area were too low to be detected by the in situ hybridization, but protein levels were enough to be revealed using immunohistochemistry. A different possibility is that exogenous reelin from other neurons was internalized in cells of the thalamus after binding to lipoprotein receptors, as it was proposed by Ramos-Moreno et al. (2006).

Previous studies have shown that reelin is implicated during development in neuronal migration, as a signal involved in the correct positioning of neuroblasts in laminated structures (reviewed by Huang and D'Arcangelo, 2008) and non-laminated structures. In the hindbrain reelin appears to act as a repulsive molecule during migration of sympathetic preganglionic neurons (Yip et al., 2009). In the present study we have found reelin-expressing populations in close proximity to the zona limitans intrathalamica, a major interprosomeric boundary involved in the organization of the dorsal and ventral thalamus. This association with the zona limitans suggests that reelin could be acting as a guidance molecule for migration of some neuroblast populations as was described in other brain areas (Yip et al., 2009), and it could be a cofactor that allows diencephalic neuroblasts respond to their normal cues during migration (Magdaleno et al., 2002). Further investigations are necessary to elucidate whether reelin is a signal involved in the correct positioning of neuroblasts in the various thalamic nuclei during diencephalic development.

The thalamus is a major brain relay structure receiving fibres from different brain areas (cortex, retina, mesencephalon, rhombencephalon and spinal cord, and also from other diencephalic nuclei), which project to different thalamic nuclei. The lateral geniculate body of mammals is an important relay nucleus in the visual system, the dorsal part of the geniculate nucleus receivies major afferent projection from ganglionic cells of the retina and projects to the primary visual cortex. Our results show a robust and specific reelin expression in the lateral geniculate body in prenatal stages, which is in agreement with previous studies that showed reelin-positive cells in rodent embryos and adults (Alcántara et al., 1998; Ramos-Moreno et al., 2006). During development growing axons are guided to their final targets where they will

establish synapses in specific patterns; growth cones have the ability to sense the environment, and different types of molecules and receptors guide the growing direction. Reelin does not appear to exert any attractive or repulsive effects on growth cones or leading processes in the telencephalon (Jossin and Goffinet, 2001; Teillon et al., 2003), although the interaction of reelin with growing retinal axons has not been investigated yet.

The optic axons grow along the diencephalon, and at E-17 (after establishment of robust reelin expression in the lateral geniculate body) the optic fibres invade the lateral dorsal geniculate nucleus (Bunt et al., 1983). After reaching their targets, the optic fibres must first establish connections with neurons in a first stage, and subsequently refine these connections in order to establish the mature retinotopic map (reviewed by Sanes et al., 2006). In the lateral geniculate body reelin could be implicated in the modulation of axonal targeting, axonal fasciculation in the optic tract and axonal branching in the nucleus as well as, in the formation and stabilization of synapses, as it was proposed in previous studies in the hippocampus (Borrell et al., 1999, 2007; Niu et al., 2008). As far as we are aware, in rat ganglion cells or in the growing optic fibres expression of Dab1 or reelin receptors has not been investigated during eye development. Studies in the adult rodent retina have not detected Dab1 in retinal ganglion cells, however is highly expressed in a subtype of amacrine cell (Rice et al., 2000). Curiosly, the presence of cells expressing Dab1 has been reported in the ganglion cell layer of the developing chick retina (Katyal et al., 2004). Accordingly, it cannot be ruled out that reelin expressed in the lateral geniculate body would acts as signal via Dab1 signalling pathway to direct or facilitate the establishment of the connections between the optic tract and the lateral geniculate body. This possibility might explain the notable difference observed in reelin expression pattern between this thalamic nucleus and other thalamic regions during development.

Overall, the present study showed that: a) Reelin mRNA and protein showed the same expression pattern in the rat diencephalon during development, with the exception of the lateral portion of the dorsal thalamus. b) Reelin positive cells are present in the rat dorsal thalamus. c) Two reelin positive cell populations are flanking the zona limitans intrathalamica during development, but they do not show colocalization with shh, a marker of this zone. d) Reelin is present in a migrating calretinin-immunoreactive population of the diencephalon at early embryonic stages.

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6. FIGURES

Figure 1. Rostral-caudal oblique sections showing reelin and shh expression in the diencephalon at E-13. (A-D) Reelin immunoreactive cells in the habenula (asterisk) (A-B), eminentia thalami (A-B), ventral and dorsal thalamus (B-D) and the lateral portion of the zona limitans intrathalamica (B-D). (E-H) Reelin mRNA expression in habenula (E), eminentia thalami (E), ventral and dorsal thalamus (F-H) and the lateral portion of the zona limitans intrathalamica (F-H). (K-P) Double immunolabelling for reelin (red) and shh (green). Reln-ir cells are situated in the lateral portion of the developing diencephalon (arrows), showing a codistribution with shh immunoreactivity, but there is a lack of colocalization of both substances in the same cells. Abbreviations: EMT, eminentia thalami; Hb, habenula; VT, ventral thalamus; DT, dorsal thalamus; zli, zona limitans intrathalamica; V, ventricle. Calibration bars: A-O: 500 μm; P: 50 μm.

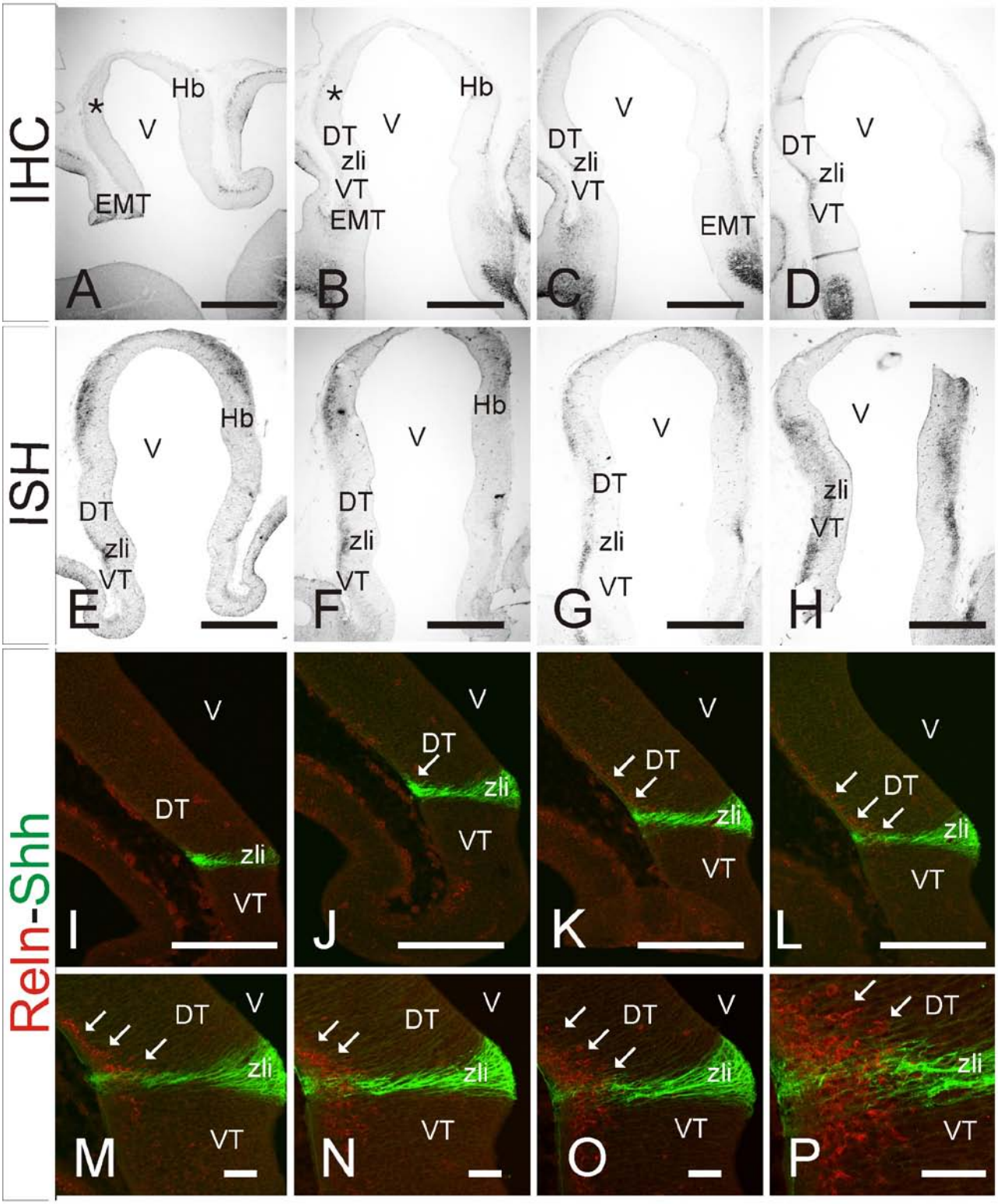

Figure 1

Figure 2. Oblique sections from rostral to caudal portions of the diencephalon at E-13 showing reelin and Tbr1 (A-D), Pax6 (E-H) and CR (I-P) immunopositive cells. (A-D) Expression of reelin (red) and Tbr1 (green) in the eminentia thalamica, although do not show a clear colocalization of both markers (B-D). (E-H) The expression of Pax6 immunoreactivity in the ventral thalamus indicates that reelin is present in the ventral thalamus showing a codistribution with Pax6. Note that reelin is also expressed in a Pax6 immunonegative area in the dorsal thalamus. (I-P) Calretinin is expressed by reln-ir cells in the caudal and ventral regions of the ventral thalamus (I-L), and in ventral regions of the dorsal thalamus (M-P). Abbreviations: EMT, eminentia thalami; VT, ventral thalamus; DT, dorsal thalamus; zli, zona limitans intrathalamica; V, ventricle. Calibration bars: A, E, F, G: 500 μm; B-D: 50 μm ; H: 50 μm; I, K, M, O: 500 μm ; J, L, N, P: 50 μm

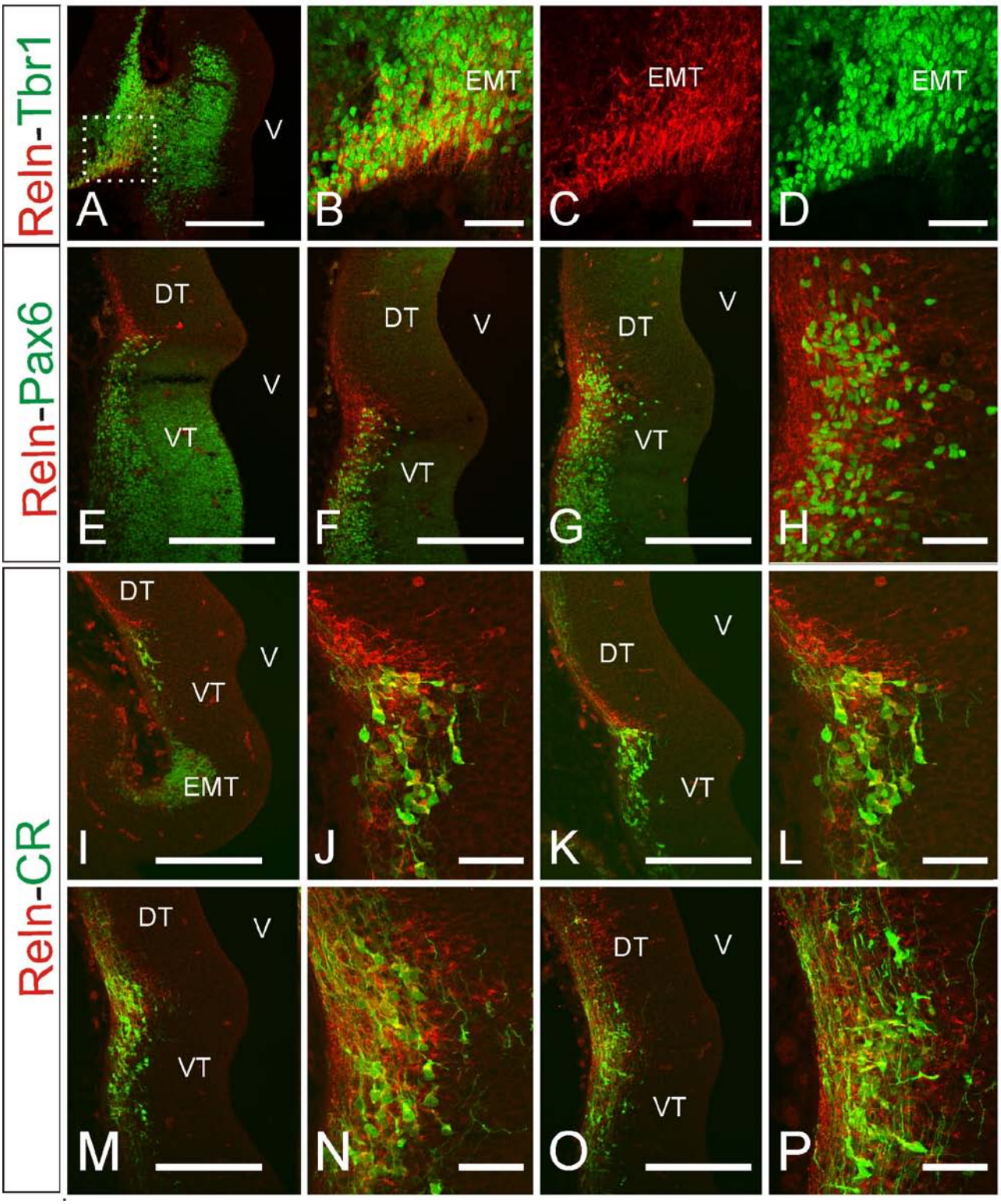

Figure 2

Figure 3. Oblique sections from rostral to caudal portions of the diencephalon at E-15. (A-C) Distribution of reelin immunoreactivity in the diencephalon, showing the presence of the protein in the habenula (A-B), dorsal thalamus (D-F) and ventral thalamus. (D-F) Expression of reelin mRNA (blue) and calbindin protein (brown), showing the lack of colocalization of reelin and calbindin in the dorsal thalamus. Reelin mRNA is present in the eminentia thalami (D), habenula (D) and ventral thalamus (D-F). (G-I) Double immunolabelling for reelin (red) and shh (green). Shh immunoreactivity is present in the area of the zona limitans intrathalamica near the ventricle, while reln-ir cells are situated on both sides of the zona limitans in the lateral portion of the developing thalamus. Note the reln-negative area between both reln-ir populations. (J-L) Reelin mRNA expression (blue) and PCNA immunoreactivity (brown). Rostral diencephalic regions show the eminentia thalamica (J) and more caudal regions of the thalamus show a PCNA and reelin negative region in the zona limitans intrathalamica (K-L). Abbreviations: EMT, eminentia thalami; DT, dorsal thalamus; PT, pretectum; V, ventricle; VT, ventral thalamus; zli, zona limitans intrathalamica. Calibration bars: A-D: 500 μm; E-F: 100 μm ; G-I: 500 μm; J-L: 500 μm.

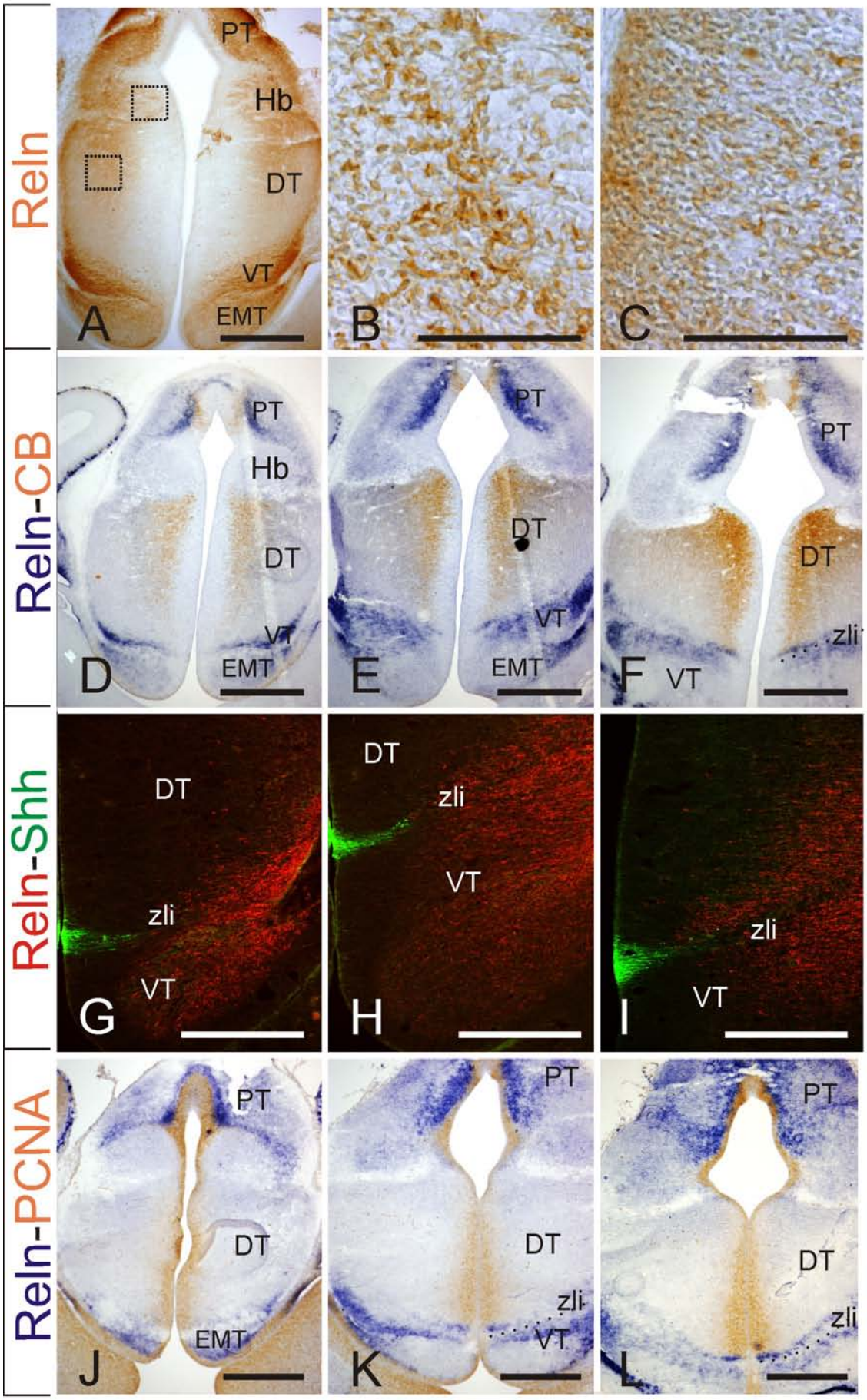
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Figure 3

Figure 4. Rostral-Caudal oblique sections showing the expression of reelin (blue or red), calretinin (brown or green), Tbr1 (green) and Pax6 (green) at E-15. (A-D) Eminentia thalamica showing the presence of reelin mRNA and protein in the mantle but not in the ventricular zone. Some reln-ir cells located in lateral portions of the eminentia thalamica show also colocalization with calretinin (B), Tbr1 is also present in the eminentia thalamica but there is not a clear colocalization between both markers(C). Reln-ir cells are situated in the mantle zone while Pax6-ir cells are in the ventricular zone (D). (E-J) Ventral thalamus showing the presence of reelin and positive cells and cr-ir cells (E, I). Double immunolabelling for reelin, calretinin, Tbr1 and Pax6 in the ventral thalamus showing the presence of cells where reelin and calretinin colocalize (F, J), while this region is devoid of Tbr1 immunoreactive cells (G, K) and some reln-ir cells are also immnureactive to Pax6 (H, L). (M-P) Double immunolabellings shows colocalization of reelin and calretinin in the lateral portion of the zona limitans (N), while cells where reelin and Pax6 colocalize are observed only in the ventral thalamus (P), and reelin and Tbr1 are observed in the ventral region of the eminentia thalami (O). Abbreviations: EMT, eminentia thalami; DT, dorsal thalamus; Hb, habenula; V, ventricle; VT, ventral thalamus; zli, zona limitans intrathalamica. Calibration bars: A, E, I, M: 500 μm; B-D: 50 μm ; F: 25 μm; G-L: 50 μm ; N-P: 50 μm.

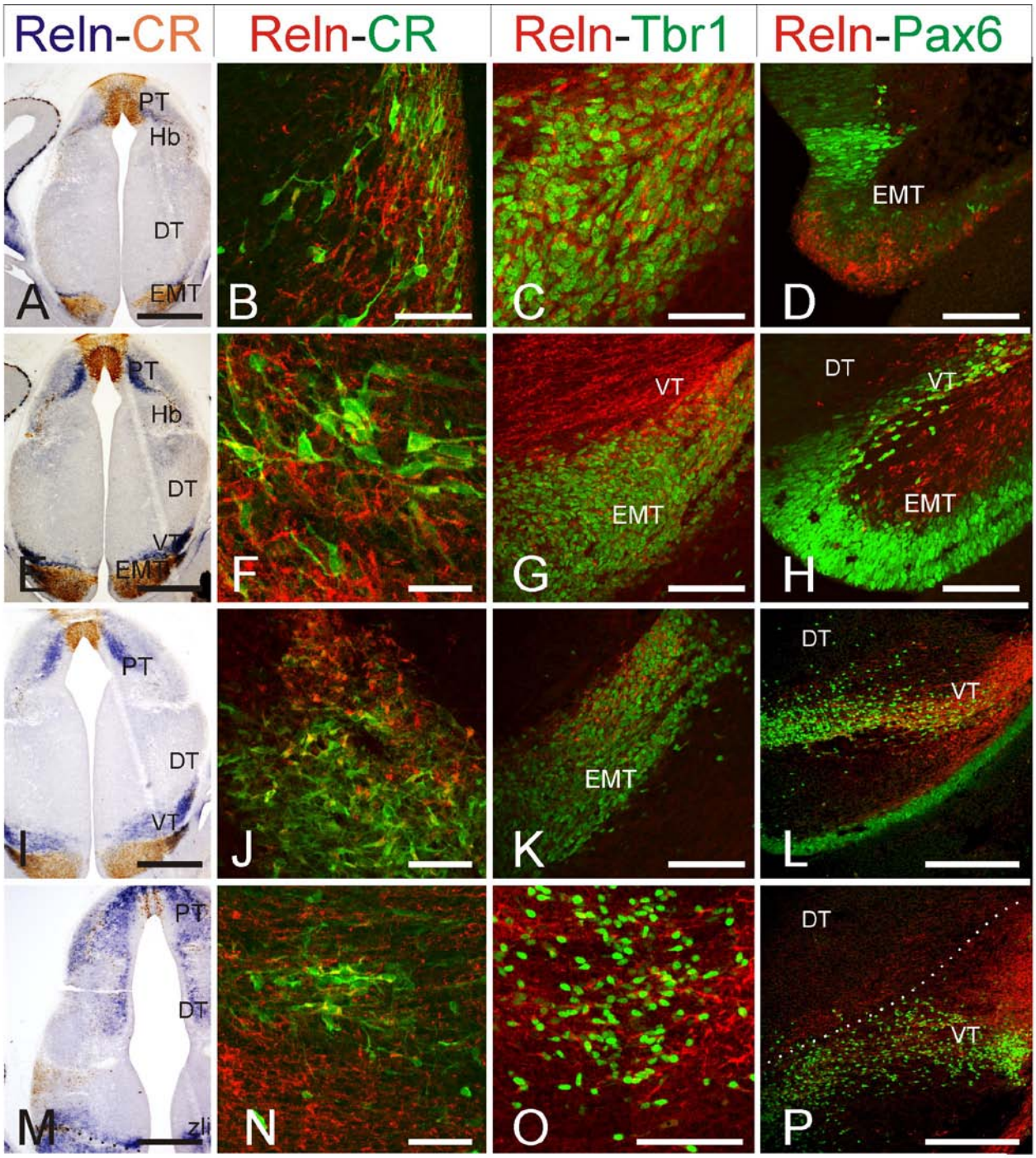
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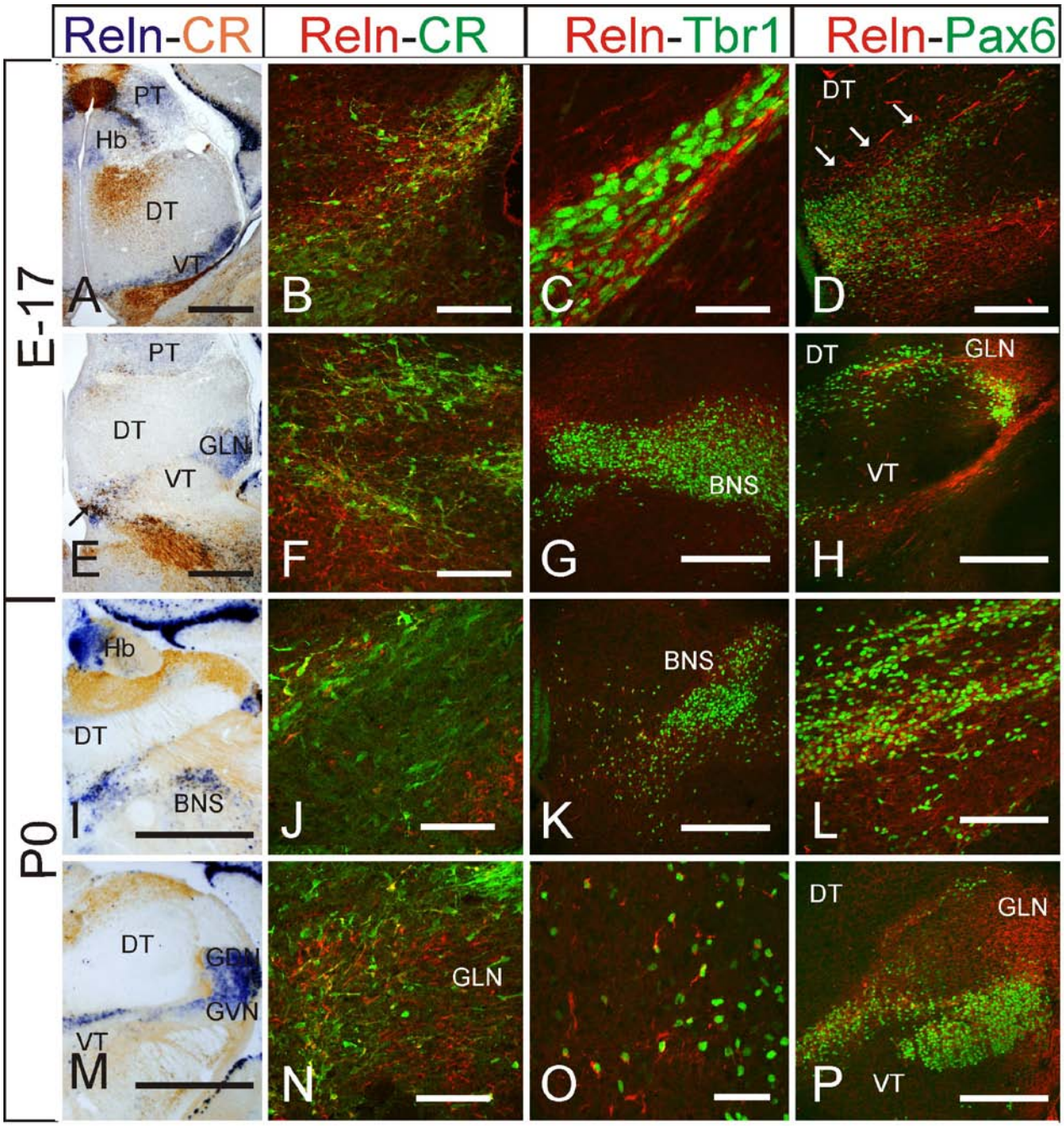
Figure 4

Figure 5. Oblique sections from rostral to caudal portions of the diencephalon at E-17 (A-H) and P0 (I-P) showing reelin (blue or red), calretinin (brown or green), Tbr1 (green) and Pax6 (green) expression. (A-H) Reelin expression at E-17. Eminentia thalami showing reln-ir cells that also were cr-ir in the lateral region (A-B), while Tbr1 and reelin do not show a clear colocalization(C). (D-F) Zona limitans intrathalamica showing the presence of two flanking reelin positive populations, one in the dorsal thalamus devoid of Pax6 and other in the ventral thalamus; some of the reln-ir cells in the ventral thalamus also express Pax6 (D) or calretinin immunoreactivity (F). The bed nucleus of the stria medullaris showing some reln-ir cells also Tbr1 immunoreactives (G). Lateral portions of the ventral thalamus showing a robust reelin immunoreactive population in the lateral geniculate body. Some reln-ir cells in the ventral portion are also Pax6 immunoreactive (H). (I-P) Reelin expression at P0. Although reelin continue being expressed in the lateral habenula, the medial habenula shows higher labelling intensity (I). Ventral thalamus showing the presence or some reln-ir cells also cr-ir (J) and Pax6-ir (L). The bed nucleus of the stria medullaris shows some cells that are reln-ir and Tbr1-ir (K, O). In the lateral geniculate nucleus we observe an important reln-ir populations, some of the reln-ir cells in the dorsal portion are also cr-ir, while in the ventral portion we observe a group of reln-ir cells that area also Pax6-ir. Abbreviations: BNS, bed nucleus of the stria medullaris; DT, dorsal thalamus; EMT, eminentia thalami; GDN, geniculate dorsal nucleus; GLN, geniculate lateral nucleus; GVN, geniculate ventricular nucleus; Hb, habenula; PT, pretectum; V, ventricle; VT, ventral thalamus; zli, zona limitans intrathalamica. Calibration bars: A, E, I, M: 500 μm; B-C: 100 μm ; D: 50 μm; F: 100μm; G-H: 50 μm ; J: 100 μm; K-L: 50μm; N: 100 μm ; O-P: 50 μm.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Chapter 5

Reelin expression in the midbrain and hindbrain during rat prenatal development and in newborn pups

1. INTRODUCTION

Reelin, a large extracellular matrix glycoprotein (D´Arcangelo, 1995, 1997) is expressed in different regions of the central nervous system during brain development in rodents (Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcántara et al., 1998); avians (Bernier et al., 2000); reptiles (Bernier et al., 1999; Goffinet et al., 1999; Tissir et al., 2003), and fishes (Costagli et al., 2002; Pérez-Costas, 2002; Candal et al., 2005). Using in situ hybridization techniques, Ikeda and Terashima, (1997) detected reelin expression in discrete brain areas in early embryonic stages of the mouse; more detailed studies have shown that reelin is highly expressed in several forebrain areas. However, the abundance of reelin in the prosencephalon contrasts with low and/or undetected reelin expression in the midbrain and brainstem (Schiffmann et al., 1997; Alcántara et al., 1998). The distribution of reelin immunoreactive cells in the spinal cords of mice and rats during embryonic development was found to be similar but not identical (Kubasak et al., 2004).

Mutation of the gene that codifies reelin produces a disruption in the correct lamination pattern of the cortex, cerebellum, hippocampus and inferior olive in the reeler mutant mouse (D´Arcangelo, 1995, 1997). Other studies have also shown cytoarchitectonic alterations in several areas of the midbrain (superior colliculus, mesencephalic trigeminal nucleus and substantia nigra), hindbrain (pontine nuclei, dorsal cochlear nucleus, inferior olivary nucleus, ambiguous, facial and motor trigeminal motoneurons) and spinal cord (reviewed by Katsuyama and Terashima, 2009). However, some of these areas that are disorganized in the *reeler* mouse did not display reelin expression in the normal mouse during embryonic development (Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcántara et al., 1998).

During early development of the central nervous system, reelin is expressed by the Cajal-Retzius cells in the marginal zone of the cortex, as well as in the marginal cells of the hippocampus, and plays a key role in neuronal migration and correct positioning of neurons in these laminated forebrain areas (Förster et al., 2006; Herz and Chen, 2006; Frotscher et al., 2009). It has recently been shown that reelin triggers a signalling cascade necessary for the cytoskeletal reorganization that occurs during neuronal migration (Chai et al., 2009). On the other hand, several studies have shown that reelin is also associated with the correct positioning of positive gonadotropin-releasing hormone hypothalamic neurons (Cariboni et al., 2005), inferior olive neurons (Ohshima et al., 2002), hindbrain efferent neurons (Rossel et al., 2005), cerebellar cells (Miyata et al., 1996; Zhao et al., 2004), precerebellar nuclei (Bloch-Gallego et al., 2005; Tanaka et al., 2007) and sympathetic preganglionic neurons (reviewed by Yip et al., 2009) in the *reeler* mutant mouse. Reelin is also thought to be involved in the development and synaptogenesis of hippocampal connections during development (Borrell et al., 2007; Wu et al., 2008). In the postnatal brain, reelin has been associated with the changes from tangential migration to radial migration in the olfactory bulb (Hack et al., 2002), detachment of postnatal

subventricular cells (Simó et al., 2007), and more recently with the dendritic maturation of cortical pyramidal neurons (Chameau et al., 2009) and hippocampal neurons (MacLaurin et al., 2007; Niu et al., 2008).

Ramos-Moreno et al., (2006) mapped reelin distribution in the adult rat by use of immunocytochemical techniques, and observed a more widespread distribution than previously reported in the adult mouse brain, including numerous nuclei in the pretectum, mesencephalon, pons and medulla oblongata. In addition to the numerous populations of reelin positive cells found in the adult rat brain, the protein has also been observed in several neuropil areas (Ramos-Moreno et al., 2006), as also described in the ferret (Martínez-Cerdeño et al., 2003) and non-human primate brain (Martínez-Cerdeño et al., 2002). Electron microscopy studies have demonstrated that reelin accumulates in the extracellular matrix around dendrites and spines of cortical pyramidal neurons in non-human primates and rodents (Rodríguez et al., 2000).

We therefore considered it of interest to carry out a more detailed study of prenatal reelin expression in rat midbrain and hindbrain at various stages of development, by using in situ hybridization and immunocytochemical techniques.

2. MATERIAL AND METHODS

Animals

Adult pregnant female Spragle-Dawley rats were used in the study. All the procedures for handling and killing the animals used in the study were in accordance with the European Commission guidelines (86/609/CEE). The day that the vaginal plug was found was counted as embryonic day 0 (E0) and the day of birth as postnatal day 0 (P0). Pregnant rats were deeply anesthetized and killed, by cervical dislocation. Embryos at different stages of development (E-13, E-15, E-17 and E-19) and also newborn pups (P0) were used.

E-13-E-19 embryos and postnatal rats (P0) were anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (PFA). The brains were removed from the animals, then cryoprotected in 30% sucrose solution. Serial coronal sections (20 μm thick sections) were cut with a cryostat. For long term storage the tissue sections were maintained at -80ºC.

Immunohistochemistry

For the immunocytochemical procedure, sections were incubated with a mouse anti-reelin monoclonal antibody G-10 1:1000 (Chemicon, code MAB 5364), overnight at room temperature, in a solution containing Tris-buffered saline (TBS; pH 7.4), 15% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.2% Triton X-100. The sections were washed several times in TBS then incubated in biotinylated goat anti mouse immunoglobulin (1:500; Dako) for one hour

in a solution containing 5% NGS, 1% BSA and 0.2% Triton X-100. The sections were then rinsed several times in TBS, and incubated in an avidin-biotin complex (Vector). The sections were again rinsed several times, and the reaction was developed with 0.03% hydrogen peroxide and 0.05% 3-3'-diaminobenzidine (DAB). The sections were dehydrated through graded alcohols, cleared in xylene and coverslipped. In control sections in which the primary antibody was omitted, no immunolabelling was observed.

In situ hybridization

Sections from E-13-E-19 and newborn pups (P0) were hybridized with riboprobes labelled with digoxigenin-11-d-UTP (Roche). The in vitro transcription of a cDNA fragment encoding mouse reelin (D'Arcangelo et al., 1995) was carried out with T3 polymerase (Roche). Tissue sections were permeabilized with proteinase K (5 μg/ml), fixed for 10 minutes in 4% PFA (10) and blocked in 0.2% glycine for 5 minutes. Thereafter, slides were incubated for 3 hours at room temperature with hybridization buffer (50% formamide deionizade, 10% dextran sulphate, 1X Denhardt´s solution, 5 mM EDTA, 0.1% Tween 20, 0.1% CHAPS, 0.1 mg/ml heparin, 2X SSC, 250 μg/ml yeast t-RNA and 250 μg/ml denatured salmon sperm DNA). Sections were then hybridized overnight at 60ºC with riboprobe (500 ng/ml) diluted in the hybridization buffer. The sections were then rinsed at 65ºC in 1X SSC and in 1.5X SSC for 10 minutes each, and for 40 minutes in 2X SSC at 37ºC. Sections were then treated for 30 minutes with RNase A (20 μg/ml) at 37ºC and washed in 0.2X SSC for 1 hour at 55ºC. The sections were then rinsed in maleic acid buffer containing 0.5% Tween20, and blocked for 2 hours with a blocking solution containing MABT and 20% normal sheep serum. The slides were then incubated overnight at room temperature with anti-DIG antibody conjugated with the alkaline phosphatase (1:1000, Roche). The slides were rinsed several times in buffer and alkaline phosphatase activity was detected with BM purple (Roche). The sections were finally mounted with Mowiol (Calbiochem).

Photomicrography and histological terminology

Photomicrographs were taken with an Olympus AX-70 photomicroscope equipped with a colour digital camera (Olympus DP-71). The photographs were adjusted for brightness and contrast by use of Corel Photo-Paint 13 software. Photomontage and lettering were done with Corel Draw 13.

For the study and determination of labelled structures in the midbrain and hindbrain, the nomenclature used in the Atlas of Prenatal rat brain development (Altman and Bayer, 1995) and the Atlas of the developing rat nervous system (Paxinos et al., 1994) was followed; for the description of the labelled structures the nomenclature of Paxinos et al., (1994) was used.

3. RESULTS

Reelin expression was detected in all the embryonic stages studied and in newborn pups. Reelin mRNA expression was also detected in all midbrain and hindbrain areas where reelin protein was found. In general reelin immunoreactive cells were fusiform, with one or several immunoreactive processes, reelin immunoreactivity occurred in the cytoplasm, and labelling was absent from the cell nucleus. Reelin was also observed as extracellular diffuse immunolabelling around the cells in some brain areas, in E-15 rats.

Reelin was found in several areas of the pretectum, mesencephalon, cerebellum, pons and medulla at initial stages of prenatal brain development (E-13), and was also detected in the germinal trigone (upper and lower rhombic lip), as well as in regions of the roof plate.

In E-15 rats, reelin was detected in the same regions as in the previous embryonic stage, and was also observed in other areas of the pons, cerebellum, and medulla; reelin immunoreactivity also appeared both as cytoplasmic labelling and extracellular diffuse immunolabelling around the cells in some brain regions in these embryos. From E-17 until birth, the pattern of reelin transcripts remained the same, and there was a clear increase in the number of reelin immunoreactive neurons.

3.1. Pretectum and mesencephalon

In E-13 rats, the pretectum displayed reelin immunoreactive (reln-ir) cells dispersed throughout the marginal and subventricular zone; numerous immunoreactive fibres were also observed in the marginal zone (Fig. 1A-C). From E-15 to E-17, although the reelin expression pattern remained the same in the pretectum, an increase in immunoreactivity (cells and fibres) was observed at this level (Fig. 1D-G). Scattered reelin immunoreactive cells were observed in the posterior pretectal nucleus and olivary pretectal nucleus at E-19 and neonates, and strong reelin expression was observed in the dorsal region of the central gray from E-19 until birth (Fig. 1H-K).

From E-13 to E-15, a high density of reelin positive cells and fibres were observed in the ventral and lateral tegmentum; a group of reelin immunoreactive cells was observed in the subventricular area of the lateral tegmentum, and based on their location were identified as red nucleus (Fig. 2A-H). Although at E-17 and E-19, reelin continued to be expressed in the ventral and lateral tegmentum, at these stages reelin immunoreactivity was mostly observed as diffuse immunolabelling around the cells in the lateral tegmentum and substantia nigra (Fig. 2I-M). At P0, cells and fibres containing reelin were visualized in the ventral tegmental area and substantia nigra (Fig. 2N). At E-13, a low level of reelin expression was observed in the lateral portions of the central gray, and this increased gradually during prenatal brain development (Fig. 2H).

At E-13, numerous weakly immunoreactive cells were present in the lemniscus nuclei and pontine nucleus (Fig. 3A-C). At E-15-P0, around the motor trigeminal nucleus, which was not

positive for reelin; numerous reelin expressing neurons were observed in the lateral lemniscus and pontine nuclei (Fig. 3D-I).

Few immunoreactive cells were present in the marginal zone of superior colliculus and inferior colliculus at E-13 (Fig. 3J). At E-15-P0, strong reelin expression was detected in the subventricular and marginal zone of the superior and inferior colliculus, and although the number of cells containing reelin decreased in the subventricular zone, numerous reln-ir fibres were present at this level; in addition, diffuse reelin immunolabelling was visualized around the cells in the marginal zone of both superior and inferior colliculus (Fig. 3K-L; 4A-B). Reelin expression was observed from E-13 to E-17 in regions of the roof plate; reelin was absent in this region in E-19 rats and in neonates (Fig. 4C-E).

3.2. Hindbrain

At E-13, a band formed by a few immunoreactive cells with very faint labelling was distributed throughout the nuclear transitory zone of the cerebellum (Fig. 4F, G); numerous immunoreactive fibres were observed dorsal to this band. From E-13 until birth reelin expression was also observed in the medial ventricular zone of the cerebellum, an area close to the roof plate (Fig. 4H, J). From E-15 to E-17, strong reelin expression was present in the external granular layer and nuclear transitory zone; scattered immunoreactive cells were observed throughout the cortical transitory zone, and a few immunoreactive cells were visualized beneath the ventricular zone (Fig. 4L-M). At E-19-P0, reelin expression increased in the cerebellum; a band of diffuse reelin immunolabelling was observed in the external granular layer, and numerous reelin positive cells were observed throughout the internal granular layer (Fig. 4N, O; Fig. 5A).

Generally, the ventricular zones of the brainstem were immunonegative for reelin, with the exception of the above-mentioned regions adjacent to the roof plate, and the germinal trigone (upper and lower rhombic lip) where strong reelin expression was observed from the rostral portions to caudal levels at E-13 (Fig.5B-D). From E-15 to E-17 strong expression was observed in the upper and lower rhombic lip; reelin expression extends from the upper rhombic lip along the subpial stream to the nuclear transitory zone of cerebellum (Fig. 4I, K-L). In the lower rhombic lip, reelin expression extended from the lateral portion of the lower rhombic lip along the auditory and vestibular neuroepithelium, as well as throughout the region of the anterior precerebellar extramural migratory stream (Fig. 5E-H); reelin expression was maintained in the rhombic lip at E-19 and in neonates, although to a lesser degree (Fig. 5I).

From E-15 to early postnatal stages, numerous reelin immunoreactive cells were visualized ventral to the auditory neuroepithelium in the dorsal cochlear nucleus (Fig. 5F, G, I).

At E-13, weak reelin expression was observed in the spinal trigeminal nucleus; in later embryonic stages and neonates, there was an increase in the number of reelin immunoreactive cells in the sensory trigeminal nucleus associated with the descendent trigeminal root (Fig. 5J, K).

Also in E-13 rats, scattered weakly stained reelin positive cells were observed in the intermediate and lateral reticular area of the medulla oblongata; from E15 until birth, an increase in the amount of reelin immunoreactivity was observed in the intermediate and lateral reticular area, as well as in the reticular zone that lies just dorsal to the inferior olive (Fig. 5L, M; 6A-D, G).

At E-15 a few weakly immunoreactive neurons were observed in several raphe nuclei (dorsal raphe nucleus, median raphe nucleus, magnus raphe nucleus, obscurus raphe nucleus) (Fig. 6E); Reelin expression was higher in later embryonic stages and neonates at this level.

At E-19, numerous reelin immunoreactive neurons were observed in the medial accessory olive, and faint immunolabelling was first observed in the principal olive of neonates (Fig. 6F, H, I).

4. DISCUSSION

In the present study we investigated the pattern of reelin gene expression in the rat midbrain and hindbrain from the second week of prenatal brain development until birth. We found that reelin is expressed in several midbrain and hindbrain nuclei, although it was not found in these areas in previous studies of the mouse brain. Results of the analysis of the spatiotemporal pattern of reelin expression suggest that reelin may be involved in many aspects of midbrain and hindbrain development. The findings also demonstrate that knowledge of reelin function in central nervous system development remains incomplete.

4.1. Comparison of the observed pattern of reelin expression during prenatal development with previous observations in vertebrates

We detected the presence of reelin in the pretectum, mesencephalon, cerebellum and medulla oblongata from E-13 onwards. The onset of reelin expression observed in the present study is consistent with previous findings in the mouse, in which reelin expression appears by E-11,5-12 (Ikeda and Terashima, 1997; Schiffmann et al., 1997; Alcántara et al., 1998), and in the rat in which it appears by E-13 in the spinal cord (Kubasak et al., 2004). Ikeda and Terashima (1997) detected reelin expression in discrete brain regions (mesencephalic tectum, tegmentum, pons, cerebellum and medulla) in early mouse embryos (E-11, E-12), as in the present study, although the latter authors did not provide details of the different populations of reelin positive cells present in these brain areas. Shiffmann et al., (1997) observed weak reelin expression in putative reticular neurons and tegmentum, moderate reelin expression in the tectum, and strong expression associated with the external granular layer of the cerebellum from early mouse embryonic stages (E-13) until postnatal stages. In the present study, we detected reelin in several areas of the pretectum, mesencephalon and hindbrain (red nucleus, ventral tegmental area,

substantia nigra, lateral lemniscus, pontine reticular nuclei, raphe nuclei, sensory trigeminal nucleus, intermediate and lateral reticular area, and inferior olive, and external and internal granular layer of the cerebellum). We also observed strong reelin expression in the upper and lower rhombic lip, as well as in regions of the roof plate, which indicates that reelin is more widely expressed in the midbrain and hindbrain of the rat brain than previously reported in the mouse. As in the present study, reelin was observed in the dorsal cochlear nucleus of late mouse embryos and new born pups (Takaoka et al., 2005); however, this is the first study in which reelin expression has been detected in the regions of the auditory and vestibular neuroepithelium (the auditory neuroepithelium is the source of the neurons that form the cochlear nucleus). A comparative study of developmental patterns of reelin expression in the spinal cord of mouse and rat revealed that the distribution of reelin expressing cells is similar in both species, but not identical (Kubasak et al., 2004).

Numerous mesencephalon and rhombencephalon nuclei are cytoarchitectonically disrupted in the reeler mutant mouse, and visual corticotectal and retinotectal fibres course normally in the superficial layers in the superior colliculus (Baba et al., 2007); cytoarchitectonic alterations are also observed in the dorsal cochlear nucleus where layer I is thinner than in the wild type and the granule cells are ectopically distributed (Takaoka et al., 2005). In addition, the inferior olivary complex exhibits abnormal folding (Lambert de Rouvroit and Goffinet, 1998); dopaminergic neurons of the substantia nigra do not migrate correctly in the reeler (Nishikawa et al., 2003) and several precerebellar nuclei are also cytoarchitectually disorganized (Tanaka et al., 2007). Most of these areas displayed reelin expression from E-13 in the rat brain (present results), which supports the important role of this protein in the correct architectonic pattern of forebrain, even in the midbrain and hindbrain. Although all these findings together provide evidence of interspecific differences in reelin expression pattern within rodents, such differences may be attributable to methodological differences. On the other hand, the results demonstrate the importance of comparative studies of different species within the same vertebrates group, which would be useful for further investigations of reelin function in the development of the central nervous system.

Previous studies in the lizard (Goffinet et al., 1999), chick (Bernier et al., 2000) and zebrafish (Costagli et al., 2002) development have demonstrated the presence of reelin in the ventral and lateral tegmentum, as in the rat brain (present results). Strong reelin expression was observed in the superior colliculus, which is consistent with the results in avians (Bernier et al., 2000), reptiles (Bernier et al., 1999; Goffinet et al., 1999) and fishes (Candal et al., 2005; Pérez-Costas et al., 2002). In the present study, we detected reelin in the inferior colliculus from E-15 to birth. This area is an obligatory relay centre for most ascending auditory tracts. Reelin has also been observed in the cochleovestibular system of avians (Bernier et al., 2000) and reptiles

(Bernier et al., 1999), as well as in the reticular formation of crocodile (Tissir et al., 2003) and chick (Bernier et al., 2000). Strong reelin expression has been observed in the cerebellum of reptiles (Goffinet et al., 1999; Bernier et al., 1999; Tissir et al., 2003) and avians (Bernier et al., 2000), as in the rat brain. The cerebellum receives afferents from many sources in the brainstem known as precerebellar nuclei, some of which, such as the pontine nucleus, reticular nucleus, trigeminal nucleus, and inferior olivary nucleus display reelin expression during brainstem rat development (present results). Furthermore, the red nucleus, which is also associated with cerebellar function, also expresses reelin. Together these findings indicate that reelin expression is highly conserved in the cerebellum, visual and auditory system during brain development in vertebrates.

The pattern of reelin mRNA expression exhibited by E-15 embryos was maintained in later embryos and newborn pups, however, we observed an increase in the number of reelin immunoreactive cells and also found that reelin accumulates throughout development in the extracellular matrix of some of the areas that exhibit reelin expression. Although reelin was not detected in the extracellular matrix in previous studies in the mouse (Ikeda and Terashima, 1997; Schiffmann et al., 1997), faint or dense reelin immunolabelling was detected in the neuropil of numerous areas of the adult rat brain, including inferior and superior colliculus, substantia nigra, cerebellum (Ramos-Moreno et al., 2006); in the present work a diffuse reelin immunolabelling was also observed from the second week of prenatal development in the inferior and superior colliculus, and from the third week in the substantia nigra and cerebellum, indicating that this type of labelling is not restricted to the adult brain; the extracellular matrix of these areas also showed similar labelling in non-human primates (Martinez-Cerdeño et al., 2002) and ferret (Martínez-Cerdeño et al., 2003).

4.2. Reelin expression is associated with proliferative regions and regions of the roof plate during prenatal brain development in rat

During the development of the central nervous system, long distances separate the final destination of migratory neurons from their birthplace. The rhombic lip is an embryonic proliferative neuroepithelium in the alar plate and forms the wall of the fourth ventricle, and along the long axis of the hindbrain is divided into the rostral rhombic lip and caudal rhombic lip (upper and lower rhombic lip, respectively) (Altman and Bayer, 1997).

Although in a previous study in mouse, reelin was not detected in the neuroepithelium adjacent to the fourth ventricle (Ikeda and Terashima, 1997), Schiffmann et al., (1997) described weak labelling in neuroepithelial cells of the germinal zone adjacent to the fourth ventricle at E-13-E-15, which was not detected in later embryos. More recently, Fink et al., (2006) described reelin expression in the upper rhombic lip of E-13,5 mice showing the same expression pattern that we have observed in the upper rhombic lip in the rat during prenatal development. However,

we also observed strong reelin expression in the lower rhombic lip; such expression appears in E-13 and persists until later embryonic stages and neonates. Reelin has been observed in the rhombic lip at stage 19 and stage 21 in the crocodile, equivalent to stages E-12 and E-14, respectively, in the mouse, (Tissir et al., 2003). Expression of the basic-helix-loop transcription factor mouse atonal homolog 1 (Math1, Atoh1) has revealed a migratory stream from the upper rhombic lip via which neurons migrate to the isthmus, pons, deep cerebellar nuclei and external granule layer, and three migratory streams from the lower rhombic lip (the cochlear extramural stream that generates neurons of the cochlear nuclei; the anterior precerebellar extramural migratory stream that is the route used by the neurons destined to the reticulotegmental and pontine nuclei, and the posterior extramural precerebellar stream that gives rise to neurons of the lateral reticular and cuneate nucleus) (Wang et al., 2005). In the present study we described reelin expression in the auditory and pontine neuroepithelium, the origins of neurons that migrate through the cochlear extramural migratory stream and anterior precerebellar extramural migratory stream, respectively. We also observed reelin expression in the cochlear nucleus and pontine nucleus, which indicates that reelin may be involved in the development of precerebellar nuclei and auditory nuclei.

In addition, we detected reelin expression in regions of the roof plate in the mesencephalon and cerebellum during the second and third week of prenatal brain development; similar labelling was also observed in the roof plate of the spinal cord in rat embryos (E-13), but was not observed at the same level in the mouse spinal cord (Kubasak et al., 2004). The roof plate is an organizational centre that produces molecules that control several aspects of the dorsal central nervous system development, and which has been best described in the developing spinal cord where it controls specification, proliferation, differentiation and axon guidance of neurons, although signals from the roof plate are also essential for dorsal hindbrain patterning (for review see Chizhikov and Millen, 2005). Further studies are necessary to elucidate the role of reelin at this level, although its presence in regions of the roof plate suggest that it may be involved in the correct patterning of dorsal structures in the mesencephalon and rhombencephalon.

4.3. Putative role of reelin in the midbrain and hindbrain

Numerous studies have provided evidence of the important role of reelin in neuronal migration. Reelin is an important signal in the correct migration of developing neuronal populations in laminated forebrain structures (Förster et al., 2006; Herz and Chen, 2006). In the olfactory bulb the presence of reelin produces the detachment of neuronal precursors and allows them to migrate individually, although its absence leads to accumulation of neuronal precursors in clusters (Hack et al., 2002). In the hindbrain, radial migration toward the pial surface is controlled by reelin, and it has been suggested that reelin is involved in changes in the direction of

migration (Rossel et al., 2005). Reelin appears to act as a repulsive molecule during migration of sympathetic preganglionic neurons (Yip et al., 2009). Several studies have confirmed that neurons migrating through the cochlear migratory stream (Takaoka et al., 2005) and anterior extramural migratory stream (Tanaka et al., 2007) are ectopically distributed in the reeler mutant mouse. In the present study we detected reelin expression in the auditory and vestibular neuroepithelium, as well as in the anterior extramural migratory stream, which suggests that reelin may be directly involved in the migratory behaviour of the neurons that use these migratory streams. Moreover, we detected reelin expression in the germinal trigone from E-13. Numerous cell specification factors and molecules involved in guidance are expressed in the rhombic lip (Wingate, 2001) and some, such as netrin 1, act as a repulsive cue for postmitotic migrating neurons from the lower rhombic lip, whereas postmitotic neurons from the upper rhombic lip do not respond to netrin 1 (Alcántara et al., 2000). Moreover, we detected reelin expression in regions of the roof plate during early embryonic stages. The roof plate expresses inhibitory molecules (keratan sulfate) that form a barrier at the roof plate and prevent the entry of axons into the region (Snow et al., 1990). Further investigations are necessary to elucidate whether reelin provides support and orientation for neuronal migration and/or forms boundaries between permissive and non-permissive areas in the rhombic lip and roof plate.

In addition to its role in neuronal migration, reelin is also thought to be involved in axonal branching and synaptogenesis (Förster et al., 2006; Herz and Chen, 2006; Borrell et al., 2007; Wu et al., 2008) and in the maturation of dendrites in the cortex and hippocampus (MacLaurin et al., 2007; Niu et al., 2008; Chameau et al., 2009). Several studies have shown that reelin is very abundant in dendrite spine-rich neuropil (Martínez-Cerdeño et al., 2000, 2003; Ramos-Moreno et al., 2006), and electron microscopically studies have shown that the reelin present in the extracellular matrix is adhered to dendritic shafts and/or postsynaptic densities (Rodríguez et al., 2000). During rat prenatal development we have detected a diffuse reelin immunolabelling in the neuropil of lateral tegmentum, substantia nigra, superior and inferior colliculus and cerebellum (present results), suggesting that reelin could be implicated in synaptogenesis process. In the brain of primitive vertebrates without laminated cortex, where most of the neurons remain in a paraventricular position and only a limited amount of neurons migrate away, reelin is mainly expressed in fibres, which suggests that its first evolutionary role may be in synaptogenesis (Pérez-Costas et al., 2002). It is therefore not surprising that reelin is present in numerous neuronal nuclei and not only in layered areas.

Together these findings suggest that reelin may be directly involved in the correct neuronal migration of both laminated and non-laminated brainstem nuclei, and may also play an important role in the organization and/or remodeling of specific synaptic connections in the cerebellum, precerebellar system, and auditory and visual system in the rat brainstem.

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6. FIGURES

Figure1. Reelin expression in the developing pretectum. Coronal sections processed by in situ hybridization (C, E, G, I, K) and immunocytochemical techniques (A, B, D, F, H, J). (A) Coronal section from E-13 showing reelin immunoreactivity in the marginal zone of the pretectum (arrows). (B) High magnification of the previous image, showing reelin immunoreactive cells in the marginal zone of the pretectum (arrowheads). (C) Coronal section from E-13 showing reelin mRNA expression in the pretectum (arrows). (D-G) Photomicrographs of transverse sections of E-15 (D, E) and E-17 (F, G) embryos showing reelin immunoreactive cells in the pretectum (D, F) and reelin mRNA expression (arrows) (E, G) in the pretectum. (H) Coronal section from E-19 showing reelin immunoreactive cells in the anterior pretectal nucleus (arrows). (I) Photomicrograph of a coronal section showing reelin mRNA expression in the central gray (arrows) at E19. (J-K) Images from a newborn pup showing reelin immunoreactive cells (arrowheads in J) and reelin mRNA expression (arrows in K) in the anterior pretectal nucleus. Abbreviations: D3V, dorsal third ventricle. Calibrations bars: A, C, E: 200μm; G, I-K: 100 μm; B, D, F, H: 50μm.

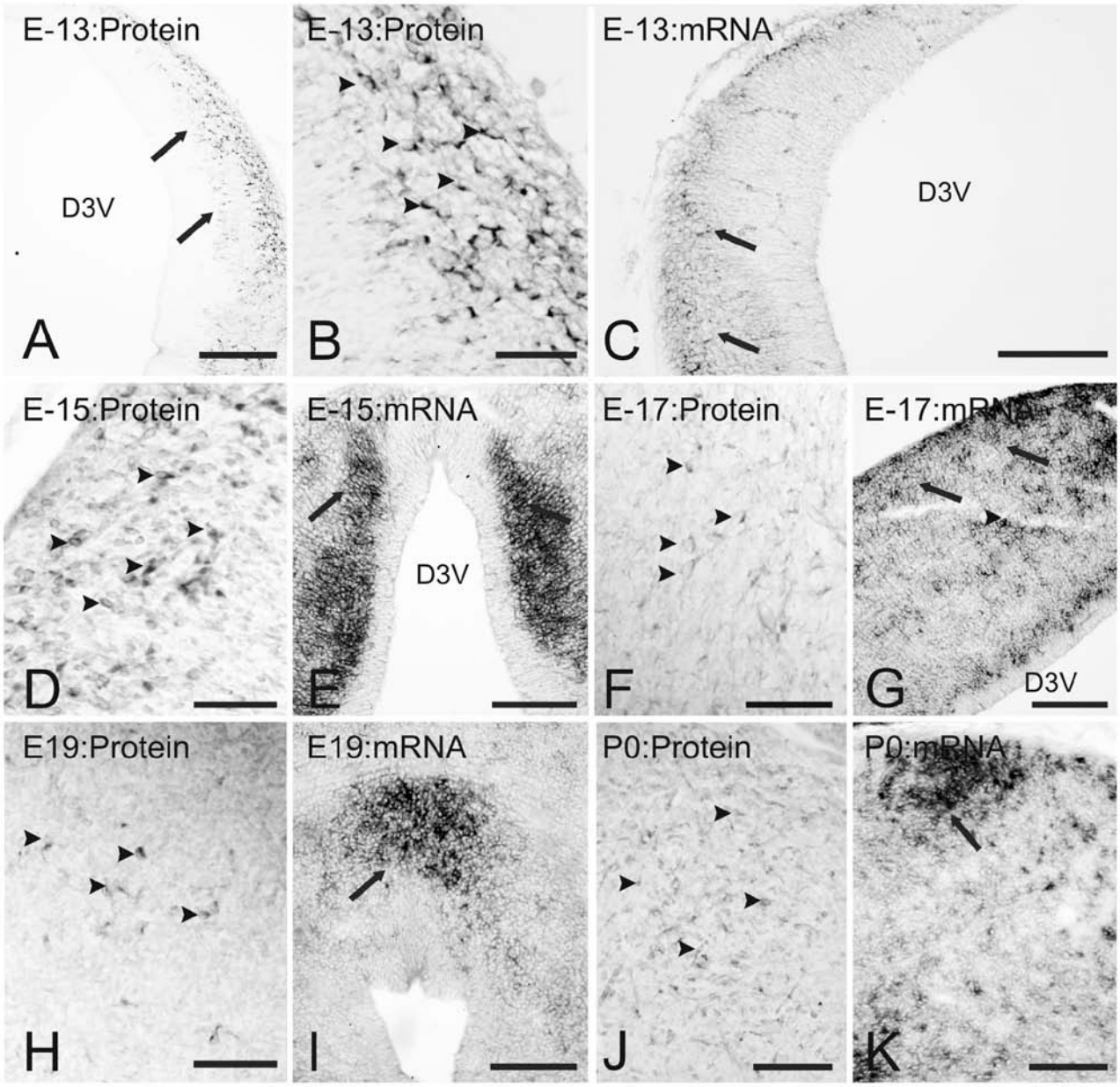
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Figure 1

Figure 2. Reelin expression in the developing rostral mesencephalon. Coronal sections processed by in situ hybridization (E, F, H, I, M) and immunocytochemical techniques (A-D, G, J, K, L). (A-D) Panoramic (A) and higher magnification (B-D) photomicrographs from a coronal section of E-13 showing reelin immunoreactivity (cells and fibres) in the ventral tegmentum (star in A, image B), red nucleus (arrowhead in A, image C) and lateral tegmentum (arrow in A, image D). (E-F) reelin mRNA expression in the red nucleus (arrow in E) and ventral tegmentum (arrowheads in F) at E-13. (G) At E15, reelin expresses in the ventral tegmental area (star), red nucleus (arrow) and lateral tegmentum (arrowhead). (H) reelin mRNA expression in the ventral tegmental area (star) and periaqueductal gray (arrowhead) at E-15. (I) Photomigraph from E-17 showing reelin mRNA expression in the red nucleus (arrow) and ventral tegmental area (star). (J-K) Higher magnification of reelin immunoreactive cells (arrowheads) in the red nucleus (J) and ventral tegmental area (K) at E17. (L-M) Images from E-19 showing diffuse reelin immunolabelling (arrows in L) and reelin mRNA expression (arrows in M) in the substantia nigra. (N) High magnification of reln-ir cells (arrowheads) in the ventral tegmental area at birth. Abbreviations: Aq, aqueductus of Sylvius. Calibration bars: A: 500 μm; E, F: 200 μm; H, I 100 μm; B-D, J-N 50 μm.

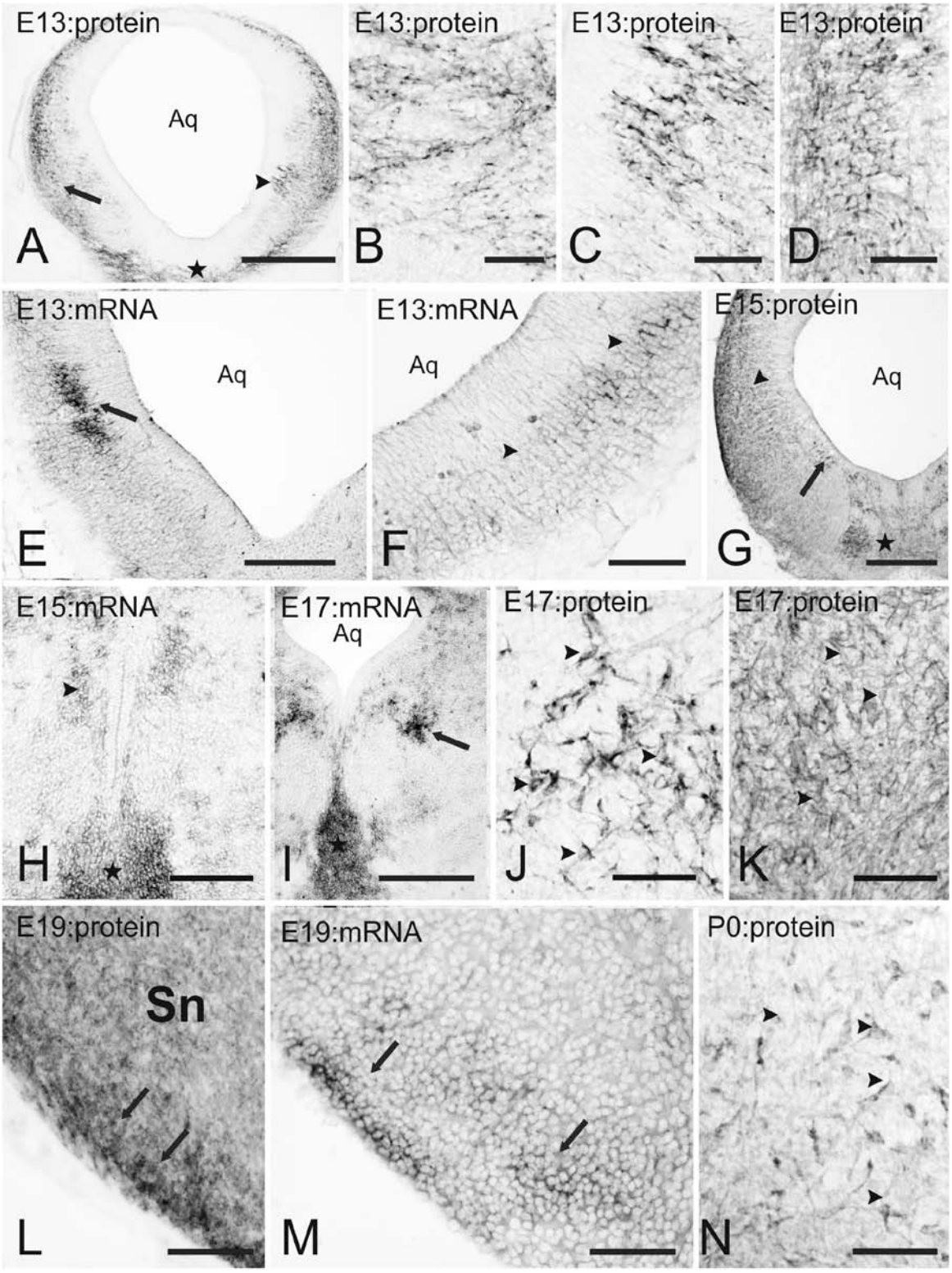
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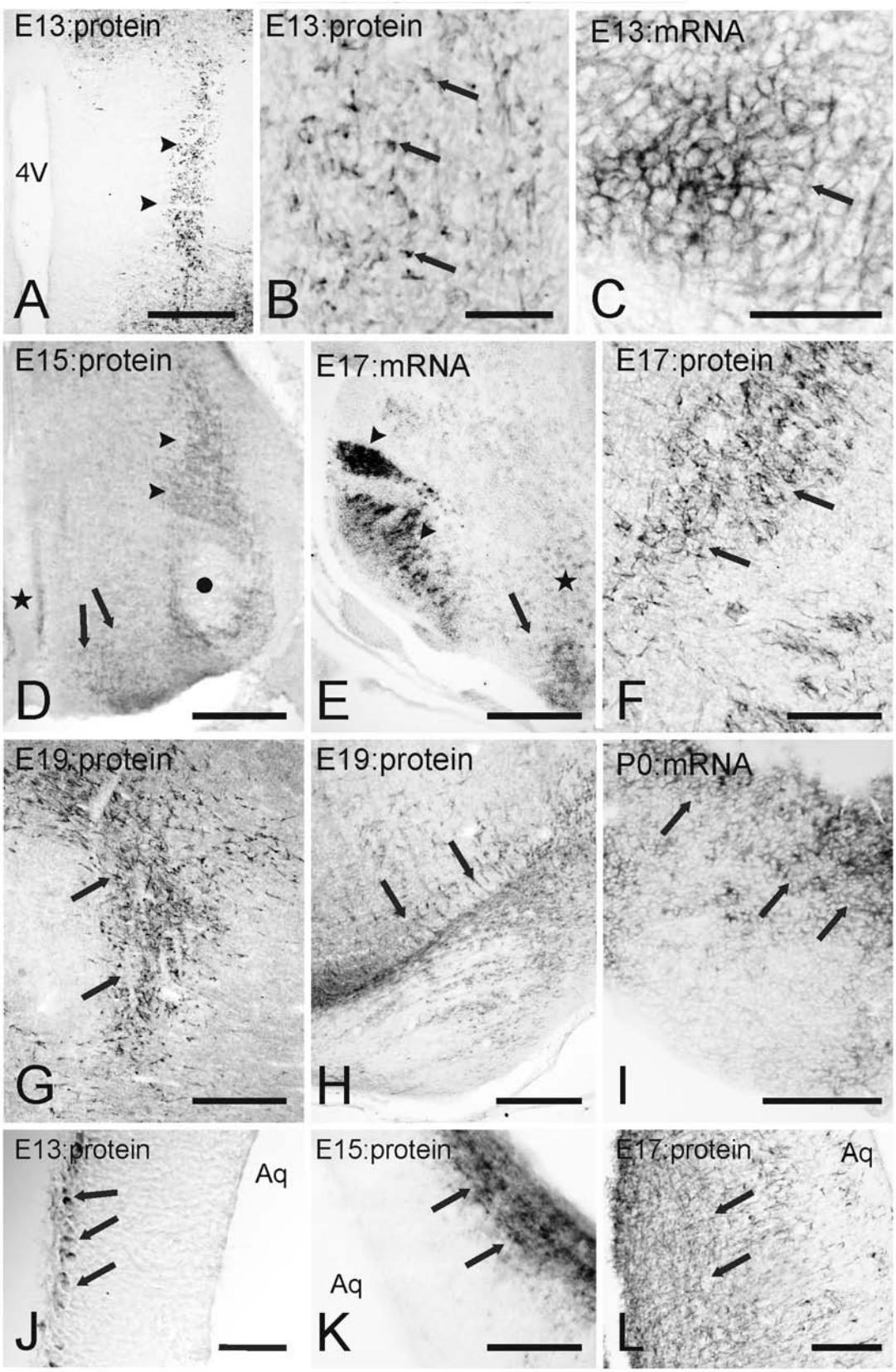
Figure 2

Figure 3. Reelin expression in the developing caudal mesencephalon. Coronal sections processed by in situ hybridization (C, E, F) and immunocytochemical techniques (A, B, D, G, H, J-L). (A) Photomicrograph from E-13 embryo showing reelin immunoreactivity in the pontine nuclei (arrowheads). (B-C) Higher magnification showing reelin immunoreactive cells (arrows in B) and reelin mRNA expression (arrow in C) in the pontine nucleus at E-13. (D) Photomicrograph at the level of motor trigeminal nucleus (circle) from E-15 embryos, which is not positive for reelin (circle). Note the presence of reelin immunoreactivity in the lateral lemniscus (arrowheads), pontine reticular nucleus (arrows) and median raphe nucleus (star). (E) Image from E-17 embryos at the level of caudal mesencephalon showing strong reelin mRNA expression in the pontine reticular nucleus (arrows), median raphe nucleus (star) and lateral lemniscus (arrowheads). (F) High magnification of reelin immunoreactive cells in the lateral lemniscus (arrows) at E-17. (G-H) Images from E19 showing the high density of reelin immunoreactive cells in the lateral lemniscus (arrows in G) and pontine reticular nucleus (arrows in H). (I) Reelin mRNA expression (arrows) in the pontine region at P0. (J-L) High magnification of superior colliculus at E-13 (J), E-15 (K) and E-17 (L). Note the scarce reelin immunoreactive cells observed at E-13 (arrows), and the increase in the reelin immunoreactivity (cells and diffuse immunolabelling) (arrows) in the marginal zone of the superior colliculus at E-15 and E-17. Abbreviations: 4V, fourth ventricle; Aq, aqueductus of Sylvius. Calibration bars: G, H, I: 200 μm; C, E, F, K, L: 100 μm; A, B, D, J: 50 μm.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Aq
K
E17:protein
Aq
L

Figure 3

Figure 4. Reelin expression in the developing rostral hindbrain. Coronal sections processed by in situ hybridization (A, B, D, G, K, L) and immunocytochemical techniques (C, E, F, H, I, J, M, N, O). (A-B) High magnification showing reelin mRNA expression in the superior colliculus (A) and inferior colliculus (B) at P0. (C-E) Images from E-17 showing reelin immunoreactivity (arrowheads in C and E) and reelin mRNA expression (arrowheads in D) in regions of the mesencephalic roof plate. (F-G) Reelin immunoreactive cells (arrows in F) and reelin mRNA expression (arrows in G) in the nuclear transitory zone of the cerebellum at E-13. (H) Image at the caudal cerebellum showing reelin immunoreactivity (arrowheads) in the ventricular zone at E-13. (I) Image from E-15 showing intense reelin immunoreactivity in the external granular layer, scattered reelin immunoreactive cells in the nuclear transitory zone (arrowheads). Note the presence of reelin immunoreactivity in the upper rhombic lip (star) and along the subpial stream from the upper rhombic lip (arrow). (J) Image from E-15 at the central portions of the cerebellum showing diffuse reelin immunolabelling (arrows) in the pial surface of the nuclear transitory zone and scarce reelin immunoreactive cells (star) in the same area. Note the reelin immunoreactivity in regions of the mesencephalic and cerebellar roof plate regions (arrowheads). (K) Reelin mRNA expression in the external granular layer, in the subpial stream from the upper rhombic lip to the nuclear transitory zone (arrow) and in the auditory and vestibular neuroepithelium (arrowheads) at E-15. (L) Photomicrograph from E-17 showing intense reelin expression in the external granular layer, subpial stream (arrow) and upper rhombic lip (arrowhead). Note also the reelin expression in the internal granular layer (circle) and vestibular neuroepithelium (star). (M) High magnification showing numerous reelin positive cells and fibres in the external granular layer at E-17. (N-O) Images from E-19 at lateral (N) and medial (O) level of the cerebellum showing large number of reelin positive cells in the external granular layer (arrows) and numerous scattered reelin immunoreactive cells in the internal granular layer (circle). Note the reelin diffuse immunolabelling present in the external granular layer. Abbreviations: Aq, aqueductus of Sylvius; 4V, fourth ventricle; LR4V, lateral recess of the fourth ventricle. Calibration bars: L: 1000 μm; I, J, N, O: 200 μm; A-C, K: 100 μm; D, E, F-H, M: 50 μm.

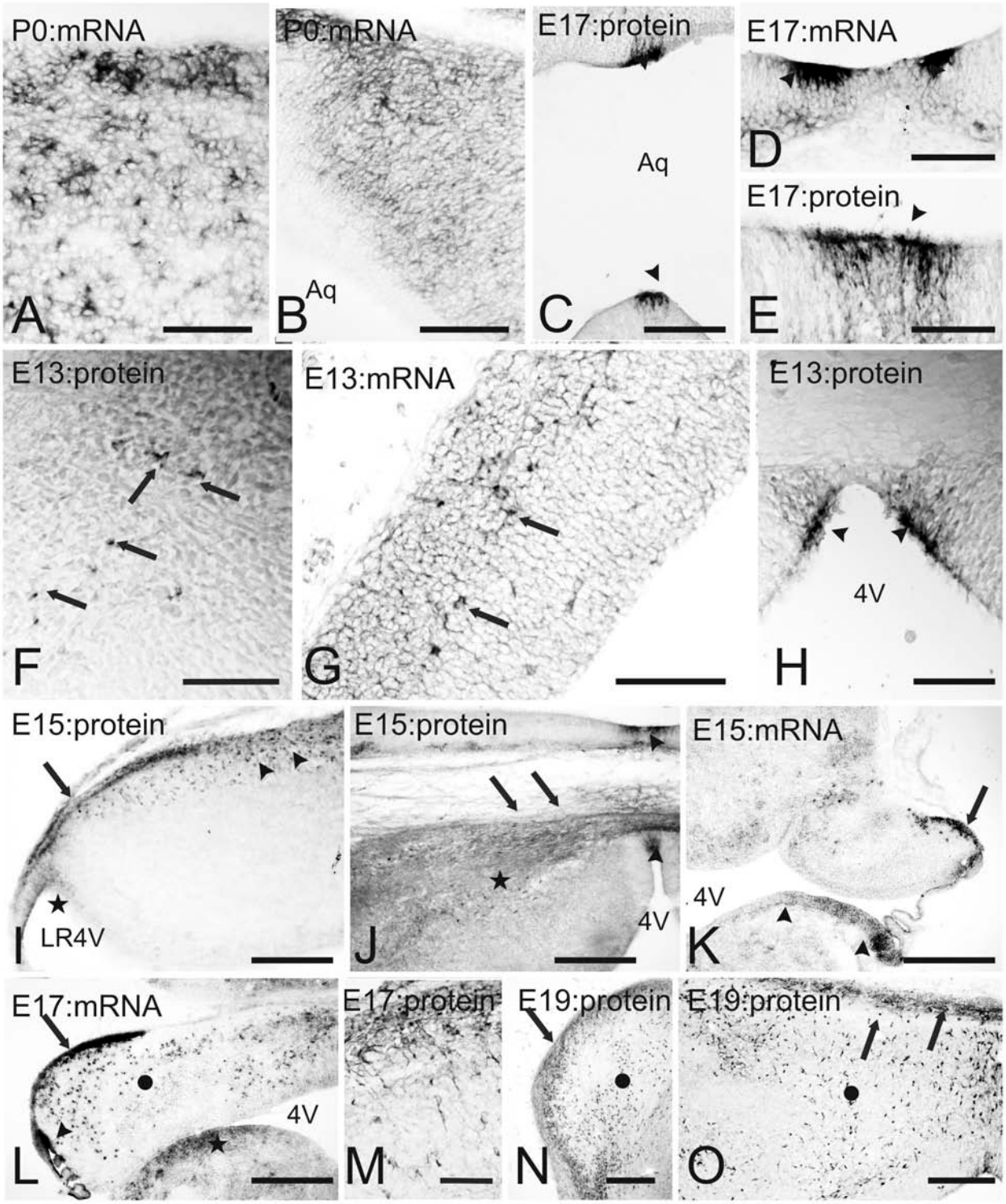
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Figure 4

Figure 5. Reelin expression in the developing hindbrain. Coronal sections processed by in situ hybridization (A, D, G, H, J) and immunocytochemical techniques (B, C, E, F, I, K, L, M). (A) High reelin expression in the external granular layer (arrowheads) and internal granular layer (arrow) of cerebellum at birth. (B) Reelin immunoreactivity (arrowheads) in the germinal trigone at E-13. (C-D) High magnification showing reelin immunoreactivity (arrowhead in C) and reelin mRNA expression (arrowhead in D) in the lower rhombic lip at E-13. (E) Photomicrograph showing reelin immunoreactivity (arrowheads) in the auditory and vestibular neuroepithelium at E15. (F-G) Photomicrographs showing reelin immunoreactivity (F) and reelin mRNA expression (G) in the lower rhombic lip (arrowheads) and cochlear nucleus (arrows) at E-17. (H) Low magnification showing reelin mRNA expression in the ventromedial and ventrolateral part of the medulla (star), in the neuroepithelium of the lower rhombic lip (arrow) at E-17. Note the presence of reelin immunoreactivity in the anterior precerebellar extramural migratory stream (arrowhead). (I) Image from a coronal section showing reelin immunoreactivity in the auditory (arrow) and vestibular (arrowheads) neuroepithelium at E-19, as well as numerous reln-ir cells in the cochlear nucleus (circle). (J) reelin expression (arrowhead) in the sensory trigeminal nucleus at E-13. (K) reelin immunoreactive cells (arrows) in the sensory trigeminal nucleus at E-15. (L) Image from a coronal section showing reelin immunoreactive fibres (arrowheads) in the ventrolateral reticular area of medulla at E-15. M) Image from a coronal section in the medulla of the E-15 showing weakly reelin immunoreactive cells located in ventromedial portions. Abbreviations: LR4V, lateral recess of the fourth ventricle. Calibration bars: H: 1000 μm; A, E, I: 200 μm; B, F, G, K: 100 μm; C, D, J, L, M 50 μm.

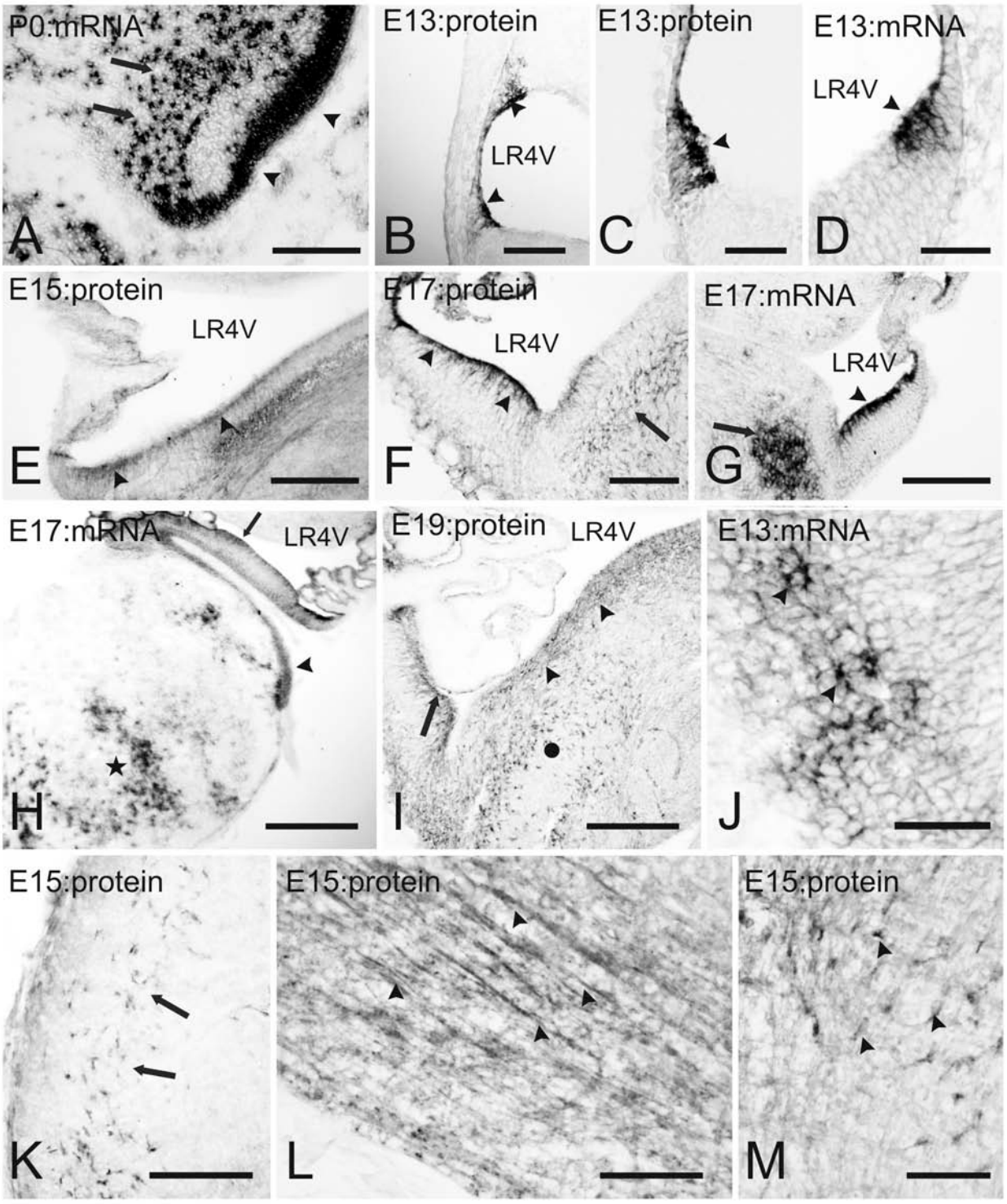
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Figure 5

Figure 6. Reelin expression in the developing caudal medulla. Coronal sections processed by in situ hybridization (A, H) and immunocytochemical techniques (B-F, G, I). (A-B) Photomicrographs from coronal sections of E-17 showing reelin mRNA expression (A) and reelin immunoreactivity (B) in the intermediate reticular zone (arrowhead), lateral reticular zone (arrow) and gigantocellular reticular nucleus (star) in the caudal medulla. (C-D) Details at high magnification of image B, showing reelin immunoreactive cells in the gigantocellular reticular nucleus (C), and lateral reticular zone (D). (E) High magnification of a coronal section in the medulla of E-17 showing reelin immunoreactive cells in the raphe magnus nucleus at E-17. (F) Image from E-19 at the level of the inferior olive, showing numerous reelin immunoreactive neurons in the medial accessory olive (arrowheads), and gigantocellular reticular nucleus and raphe obscurus nucleus (arrows). (G) Reln-ir cells in the intermediate (arrowhead) and lateral reticular area (arrow) at E-19. (H-I) Images from neonates showing reelin mRNA expression (H) and reelin protein (I) in the inferior olive (arrowheads). Note the presence of reelin immunoreactive cells in the gigantocellular reticular nucleus (arrows). Calibration bars: A: 1000 μm; B: 500 μm; F, G, H: 200 μm; C, D, I: 100 μm.

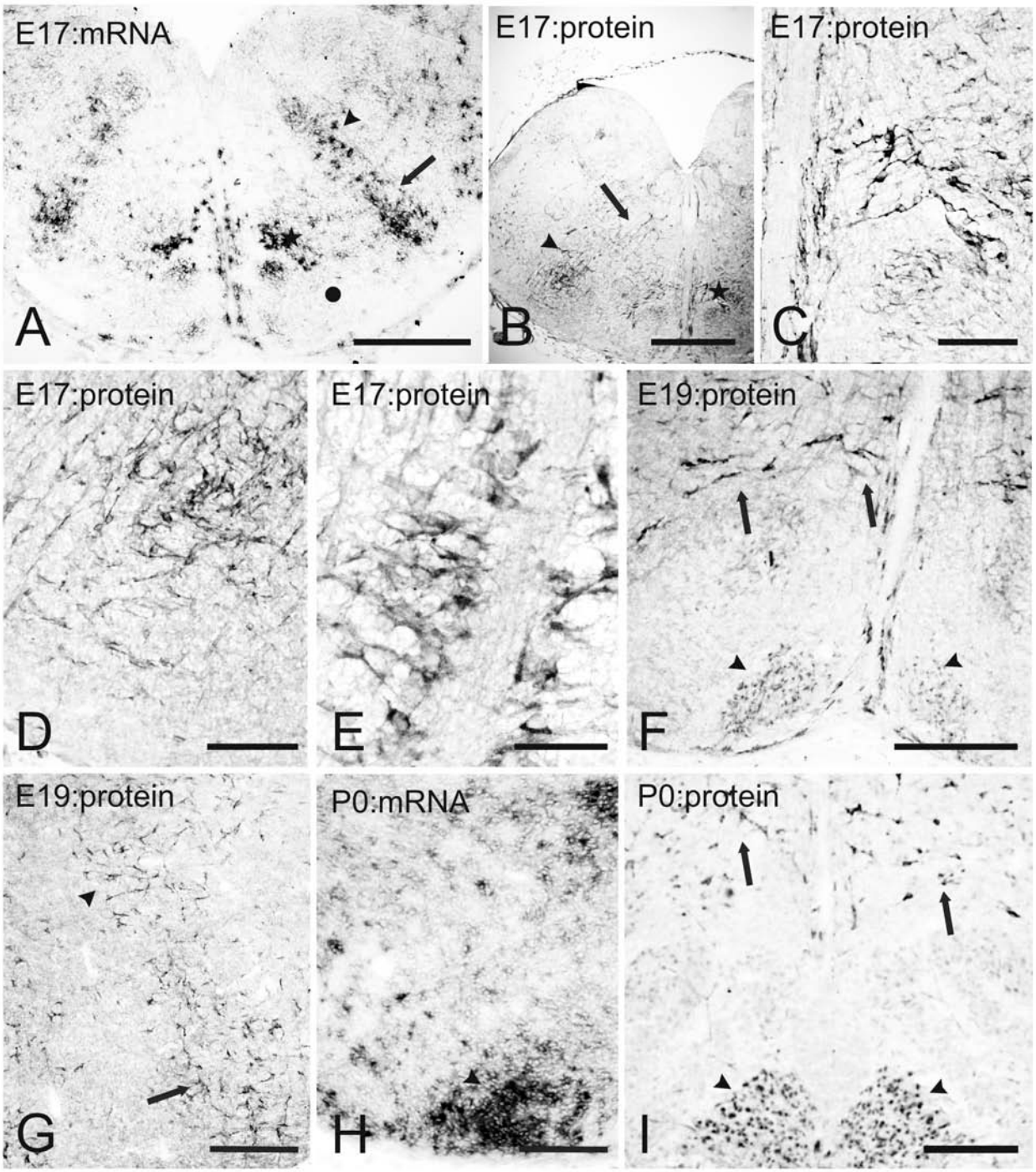
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Figure 6

Conclusions

The results obtained in the present Thesis lead us to the following conclusions:

1. There is a subpopulation of reelin expressing cells that is observed in the cortical intermediate zone in the second prenatal week in rodents, and in the third week is incorporated into the subplate. These cells also express Tbr-1 (a marker of glutamatergic neurons), as well as calretinin and doublecortin, but not P73, Pax6 or GABA.
2. Although these cells express some markers characteristic of Cajal-Retzius cells, their distribution in the intermediate zone/subplate indicates that is not a typical subpopulation of Cajal-Retzius cells. We suggest that it represents a novel population of subplate cells that will probably degenerate during postnatal development.
3. During the second prenatal week, reelin expression in the striatum follows a caudal-rostral pattern that is opposite to that observed for tyrosine hydroxylase and DARPP-32. These reelin-positive neurons might originate in the retrobulbar area and show a strong intracytoplasmatic reelin labelling at E-15, while at E-17 there is a decrease in the levels of intracytoplasmatic reelin labelling and an apparent increase in extracellular labelling, indicating the possible secretion of reelin into the extracellular matrix.
4. Reelin and DARP-32 coexpression in striatal neurons is not observed during the second prenatal week of striatal development. However, in newborn pups almost all DARPP-32 immunoreactive cells contained reelin. Double labelling of reelin and TH indicates that TH-positive fibres become accumulated in striosomes towards the end of prenatal development, while striosomal reelin expression takes place at an earlier stage, suggesting that reelin could play a role in regulating the synaptic impinging of dopaminergic fibres onto striosome cells.
5. During prenatal development reelin protein is observed in the lateral olfactory tract (LOT) as a heavy diffuse immunolabelling although as expected reelin mRNA is not detected at this level. During the first postnatal week the LOT shows heavily stained reln-ir round spots and a diffuse immunolabelling is also observed in layer IA of the piriform cortex. These findings suggest that reelin may accumulate in mitral axons and be secreted into the extracellular matrix, where it may play a role in synaptogenesis of LOT axons during piriform development.
6. In later stages of postnatal development and in adult brain, there is an increase in reelin diffuse immunolabelling in layer IA of the piriform cortex, although reelin immunoreactive round spots in the LOT are not observed, perhaps due to lack of penetration of the antibody at this stage related to the increased myelination of the LOT, suggesting that reelin could be transported and secreted by axons of the mitral cells into the layer IA

where it could be related with plasticity events.

7. Double immunolabelling of reelin and shh at E-13 and E-15 indicate that reelin is expressed by cells flanking the zona limitans intrathalamica in the dorsal and ventral thalamus, but not by neurons in the zona limitans intrathalamica.

8. At the end of the second week of prenatal development reelin and calretinin coexpression is observed in cells located in lateral portions of the ventral and dorsal thalamus, suggesting that these could represent an early-generated thalamic population that migrate tangentially across the zona limitans intrathalamica and move towards the dorsal thalamus.

9. From the second week of prenatal development until birth, reelin protein and mRNA is observed in the pretectum, the red nucleus, ventral and lateral tegmental areas, substantia nigra, lateral lemniscus, cochlear nucleus, several raphe nuclei, inferior and superior colliculli, external and internal granular layer of the cerebellum, precerebellar nuclei (pontine reticular nuclei, trigeminal nucleus, and inferior olive), intermediate and lateral reticular areas, and upper and lower rhombic lip, which evidences that reelin is widely expressed in the midbrain and hindbrain during rat prenatal development, suggesting that reelin could play an important role in the cytoarchitectonic pattern of midbrain and hindbrain.

10. A reelin diffuse immunolabelling is observed in the neuropil of the superior and inferior colliculli at the end of the second week of prenatal development, while at the end of the third week is also expressed in lateral tegmentum, substantia nigra and cerebellum; suggesting that reelin may play an important role in the organization and/or remodelling of specific synaptic connections in the cerebellum, auditory and visual system in the rat brainstem.

11. From the second week of prenatal development until birth, reelin expression is observed in proliferative regions such as the upper and lower rhombic lips (i.e. the auditory and pontine neuroepithelia), and is also present in the anterior precerebellar extramural migratory stream, suggesting that reelin may be implicated in the development of the precerebellar and auditory nuclei.

12. During the second and third week of prenatal development we have observed reelin expression in regions of the roof plate in both the mesencephalon and cerebellum; the roof plate is an important organizational centre that control several aspects of the dorsal brain patterning, which suggests that reelin could be involved in the correct patterning of dorsal structures in the mesencephalon and rhombencephalon.

Resumen

Introducción General

El desarrollo del sistema nervioso central (SNC) es un proceso complejo durante el cual tienen lugar procesos de neurogénesis, migración, maduración de neuronas y establecimiento de las conexiones nerviosas. En la actualidad existe un gran número de enfermedades neurológicas y mentales de las que no sabemos sus causas, aunque constituyen uno de los problemas de salud más comunes en los países desarrollados. En 1998 se publicó un estudio en el que se mostraba una reducción de aproximadamente el 50% en los niveles de proteína y de ARN mensajero de reelina en muestras de cerebros de pacientes esquizofrénicos. Estudios posteriores mostraron que muestras de cerebros provenientes de pacientes con otras patologías mentales también presentaban alteraciones en los niveles de esta proteína.

Actualmente no hay evidencias de que una mutación en el gen que codifica para la reelina esté involucrada en el desarrollo de la esquizofrenia, pero la reducción tanto en la expresión de la proteína como del ARN mensajero sugieren que este déficit podría estar relacionado con una menor expresión de este gen, ya sea por un defecto en la regulación de la expresión o debido a factores epigenéticos. El ratón heterocigoto *reeler* presenta algunas alteraciones neuroanatómicas y de comportamiento semejantes a las observadas en pacientes que padecen esquizofrenia. Por ello este ratón podría ser un buen modelo animal para llevar a cabo estudios sobre la esquizofrenia, lo que implicaría tener un amplio conocimiento del patrón de expresión de la reelina durante el desarrollo y de los papeles que desempeña.

La mutación *reeler* fue descrita por primera vez en 1951, este fenotipo está causado por una mutación recesiva, y afecta a la capacidad de algunos neuroblastos postmitóticos para llegar a su posición correcta durante el desarrollo del cerebro. Los defectos neuroanatómicos más destacables en el cerebro de los ratones *reeler* son: 1) reducción del tamaño del cerebelo, la capa granular externa es de menor tamaño que en los ratones salvajes y además no se observa una capa de células de Purkinje. 2) la corteza cerebral muestra un patrón invertido, ya que la placa cortical se desarrolla ectópicamente por debajo de la preplaca. 3) La capa de neuronas piramidales del hipocampo así como el giro dentado presenta alteraciones citoarquitectónicas. Aunque los defectos más llamativos se han observado en áreas laminadas del encéfalo, también se han descrito alteraciones en áreas no laminadas como tálamo, mesencéfalo, o médula espinal.

El ratón heterocigoto *reeler* expresa un 50% de ARNm de reelina y de proteína en relación al ratón salvaje. Aunque no presenta las mismas alteraciones que las observadas en el ratón *reeler*, se ha descrito una reducción en el número de células que expresan reelina, una reducción en el tamaño del neuropilo, así como un aumento en el empaquetamiento de las células de la corteza cerebral. Por otra parte, se ha observado una reducción en la densidad de espinas dendríticas en las células piramidales de la corteza prefrontal e hipocampo, así como un

descenso en el número de ramificaciones de las dendritas en el hipocampo.

Además de las alteraciones citoarquitectónicas que acabamos de mencionar también se han observado diversas alteraciones neuroquímicas como una reducción en los niveles de ARNm del enzima descarboxilasa del ácido glutámico o alteraciones en el sistema glutamatérgico y dopaminérgico. Estas alteraciones se correlacionan con las observadas en muestras de cerebros provenientes de pacientes esquizofrénicos, por los que este ratón podría ser un buen modelo para el estudio de la esquizofrenia.

Otros ratones que presentan alteraciones o fenotipos similares al ratón *reeler* y que han aportado información sobre la reelina y su cascada de señales también han sido objeto de estudio, entre ellos están: los ratones de la cepa Orleans, las cepas de ratones *Scrambled* y *Yotari,* ratones con mutaciones en los genes que codifican para el receptor de proteínas de baja densidad o el receptor de la proteína ApoE2.

La reelina es una glicoproteína de la matriz extracelular de 3.461 aminoácidos que pesa 450 KDa. En el extremo amino-terminal posee un péptido señal seguido de una región similar a la F-Spondina y de una región específica de esta proteína. A continuación hay ocho secuencias repetidas de 300-350 aminoácidos. Cada repetición tiene dos subdominios, A y B, separados por una secuencia altamente conservada de 30 aminoácidos. Por último el extremo carboxi-terminal presenta una secuencia de 33 aminoácidos cargados positivamente. La reelina se procesa *in vivo,* sufre proteolísis. La actividad de las proteasas genera fragmentos de 370, 270,190, 180 y 80 KDa. Las funciones de la reelina dependen de su fragmento central de 190 KDa, a través de él se une a los receptores de proteínas de baja densidad y de ApoE2, mientras que el extremo amino-terminal interviene en la formación de homodímeros estables. El extremo carboxi-terminal parece que interactúa con un co-receptor situado en la membrana plasmática, y su carga positiva permite una mejor interacción de la proteína con la membrana plasmática.

El gen de la reelina es un gen de gran tamaño, entre 400 y 450 Kb. Este gen está altamente conservado en vertebrados, la secuencia de aminoácidos de la proteína de ratón y de humano presenta un 94,2% de similitud y un 87,2% de similitud en la secuencia nucelotídica. El ARN mensajero de esta proteína está presente en numerosos vertebrados a lo largo de la escala evolutiva.

Mediante el estudio de diversos mutantes que muestran un fenotipo similar al *reeler* se ha estudiado parcialmente la cascada de señales de la reelina. La presencia de reelina en el encéfalo de los mutantes *scrambled* y *yotari* que presentan un fenotipo similar al *reeler*, permitió la identificación de la proteína Dab1 como integrante de la cascada de señales de respuesta a la reelina. Esta proteína citoplasmática contiene un dominio PI/PTB en el extremo amino-terminal similar al de la proteína adaptadora Shc y en el extremo carboxi-terminal tiene una serie de

residuos de tirosina que constituyen un sitio de unión para las proteínas de la familia Src. Dab1 puede actuar como una molécula adaptadora en la cascada de tirosina quinasas que responde a la señal de reelina.

El extremo PI/PTB de la Dab1 puede interaccionar con el extremo citoplasmático de proteínas de la familia de receptores de proteínas de baja densidad o el receptor de la proteína ApoE2, la activación de estos receptores fosforila la Dab1 a través de una cascada de quinasas. La reelina se une a estos receptores a través de su fragmento central, mientras que el extremo N-terminal es importante en la formación de homodímeros que hacen que la proteína sea más eficiente para inducir la fosforilación de la Dab1.

Las integrinas, son una clase de receptores transmembrana que intervienen en la migración celular. Experimentos *in vitro* han demostrado que las integrinas se unen a la región amino-terminal de la reelina; la reelina induce a la dimerización de estos receptores como ocurre con los receptores de proteínas de baja densidad o el receptor de la proteína ApoE2. La vía de señalización de reelina a través de la integrina alfa 3 beta 1 es independiente de la vía de señalización de los receptores de proteínas de baja densidad o el receptor de la proteína ApoE2. Colocalización de estos receptores con la reelina se ha observado en la matriz extracelular rodeando a las espinas dendríticas, y además se ha detectado la presencia de integrinas en las densidades postsinápticas lo que sugiere que la reelina podría intervenir en los procesos de estabilización de las espinas dendríticas.

La cascada de señales que se activa en respuesta a la reelina sólo está parcialmente estudiada, debido a que las proteínas que parecen implicadas en dicha cascada son proteínas que intervienen en más cascadas de señales lo que hace más complejo su estudio. En la actualidad se piensa que las proteínas de la familia proteína quinasas no receptores Fyn y Src forman parte de esta cascada de señales fosforilando Dab1. Una vez fosforilada puede activar diferentes proteínas:

- Dab1 recluta a la subunidad p85 de la fosfatidilnositol 3 quinasa, la cual fosforila Akt. Una vez fosforilada Akt puede seguir dos vías de señalización: inhibiendo la glicógeno sintetasa 3ß, o activando mTor. De esta manera la reelina podría estar modulando a través de estas proteínas el citoesqueleto o la síntesis proteica y por tanto la motilidad celular.
- La fosfatidilinositol 3 quinasa puede a su vez actuar a través de otra cascada de señales relacionada con la cofilina. Esta proteína está relacionada con la estabilización del citoesqueleto de actina.
- Otras moléculas que parecen estar implicadas en la vía de señalización de reelina son Lis1 y Cdk5. Lis1 puede unirse a la dineina y de esta manera regular el citoesqueleto.

Cdk5 también modula el citoesqueleto de actina a través de la fosforilación de doblecortina, Nudel o colapsina.

Recientemente Notch ha sido propuesta como molécula implicada en la vía de señalización de reelina, pero son necesarios nuevos estudios para confirmarlo.

La reelina está presente en el SNC de vertebrados a lo largo de la escala evolutiva, desde la lamprea a humanos. Aunque la presencia de esta proteína en el sistema nervioso es mayor durante el desarrollo, también se ha observado reelina en el SNC de animales adultos.

En la actualidad existen diversos trabajos sobre la expresión de reelina a lo largo del desarrollo de diferentes vertebrados. Los primeros estudios se llevaron a cabo en ratón y han demostrado la presencia de reelina en el telencéfalo, diencéfalo, mesencéfalo y rombencéfalo durante el desarrollo del SNC.

En el telencéfalo está principalmente en la zona marginal y en las células mitrales del bulbo olfatorio, en el hipocampo y en áreas subcorticales. Las células de Cajal-Retzius situadas en la zona marginal de la corteza expresan reelina desde estadios tempranos y a medida que avanza el desarrollo disminuye la expresión de esta proteína. En el diencéfalo de roedores se ha encontrado reelina en el epitálamo y tálamo ventral, y su expresión disminuye después del nacimiento. Aunque el patrón de expresión de reelina se ha estudiado en el mesencéfalo y en el rombencéfalo, este ha sido objeto de un número menor de estudios y se ha analizado en menor profundidad; aún así la reelina se ha detectado en el techo, tegmento, cerebelo, algunos núcleos precerebelares, formación reticular y regiones ventrales del puente. En la médula espinal la reelina está presente desde estadios tempranos de desarrollo.

En adultos los estudios se han centrado principalmente en la corteza cerebral, en donde diversas subpoblaciones de interneuronas GABAérgicas expresan reelina, mientras que Dab1 se expresa en las neuronas piramidales. Las neuronas reelina inmunorreactivas están presentes en las diferentes capas de la corteza, aunque el patrón varía entre especies. Actualmente está en discusión la presencia de reelina en neuronas piramidales de la corteza cerebral, algunos autores solo han encontrado reelina en interneuronas GABAérgicas, mientras que otros autores han encontrado reelina en las dendritas y espinas dendríticas de las neuronas piramidales.

Durante el desarrollo y en etapas adultas en otros vertebrados se han descrito poblaciones de células reelina inmunorreactivas, demostrando que hay un patrón de expresión conservado a lo largo de la escala evolutiva. La reelina está presente en el bulbo olfatorio, paleo, subpaleo, tálamo ventral, hipotálamo, pretecho, y en regiones caudales del encéfalo.

La neocorteza de mamíferos consiste en seis capas, donde se localizan dos tipos principales de neuronas: las neuronas de proyección y las interneuronas, cada una de ellos se pueden dividir en subtipos. La corteza se desarrolla siguiendo un patrón de dentro a fuera, las neuronas más

jóvenes se sitúan en las capas más superficiales mientras las neuronas que se originan primero se sitúan en las capas más profundas. Durante el desarrollo de la corteza las neuronas migran radial y tangencialmente hasta llegar a sus destinos.

El tubo neural está formado por un neuroepitelio, en el cual en los primeros estadios del desarrollo sus células se dividen simétricamente de forma que se incrementa el número de células y el grosor del neuroepitelio. A continuación las células se dividen asimétricamente, de forma que una célula permanece en la zona ventricular mientras que la otra célula hija migra radialmente para situarse en la preplaca. La preplaca consiste en dos tipos celulares: células de Cajal-Retzius y células de la subplaca.

Las células de Cajal-Retzius son las neuronas que nacen más temprano, y constituyen un grupo heterogéneo de células que nacen en diferentes lugares y que migran invadiendo la capa marginal de la neocorteza. Una de las características de las células de Cajal-Retzius es la expresión de reelina.

La mayoría de las neuronas de proyección se generan en la zona ventricular, migran radialmente hacia la superficie pial dividiendo la preplaca en una zona marginal en la que se sitúan las células de Cajal-Retzius, zona intermedia y subplaca. Entre la zona marginal y la zona intermedia se localiza la placa cortical donde se acumulan las neuronas postmitóticas, se organizan siguiendo el patrón de dentro a fuera, formando la estructura de seis capas típica de la neocorteza. La traslocación somal parece que es el principal modo de migración durante las primeras etapas de la corticogenesis, mientras que en las etapas tardías la migración mediante la guía de la glia radial es la principal.

Las células de las capas altas de la placa cortical expresan Dab1, y los receptores de las proteínas de baja densidad y ApoE2. Con todos estos datos se ha propuesto un modelo de actuación de la reelina en el que se propone que las células de Cajal-Retzius producen reelina que es secretada a la matriz extracelular en donde difunde a la placa cortical. La reelina promueve la extensión de los procesos celulares, y la célula avanza hacia la superficie pial, en donde la concentración de reelina es mayor y las células se paran para ocupar su destino final. Por tanto la reelina en el desarrollo de la neocorteza podría actuar como señal de atracción y como señal de parada, esto dependería del receptor que se activase en cada caso.

Algunas de las moléculas implicadas en la vía de transducción de la reelina están presentes en la glia radial, por lo que se ha propuesto otro papel para esta proteína durante el desarrollo, donde la reelina podría actuar sobre las células madre de la zona ventricular, actuando sobre la diferenciación de las células madre en células de la glia. Otra posibilidad es que la reelina actúe como señal para la traslocación del soma de las células de la glia para que se forme el andamio de células de la glia de la neocorteza en desarrollo.

Las interneuronas a diferencia de las neuronas de proyección se generan principalmente en áreas no corticales. En roedores la principal fuente de interneuronas GABAérgicas son las eminencias ganglionares, en donde se originan siguiendo un patrón espacio temporal, y migran tangencialmente hacia la corteza, donde para situarse en las diferentes capas utilizan la migración radial siguiendo el mismo patrón de dentro a fuera que las neuronas de proyección. Aunque diferentes subpoblaciones de interneuronas GABAérgicas continúan expresando reelina en la corteza cerebral del adulto, no está claro qué papel desempeña esta proteína durante el desarrollo sobre las interneuronas. Al igual que en la corteza, la reelina parece actuar de una manera similar en otras áreas laminadas como el hipocampo o el cerebelo.

Diversos datos indican que la reelina no actúa como molécula señal en la guía axónica, pero sí parece que podría modular el crecimiento, fasciculación y la ramificación de los axones. Además parece que la reelina está involucrada en procesos de sinaptogénesis, mediante la formación o la estabilización de las espinas dendríticas. Mediante estudios *in vitro* se ha visto que en el hipocampo de roedores la reelina actúa en la estabilización de las sinapsis glutamatérgicas durante el desarrollo postnatal. La reelina también se ha relacionado con la maduración de las dendritas apicales de las neuronas piramidales.

En algunas áreas del encéfalo como el hipocampo y bulbo olfatorio se sigue produciendo neurogénesis y migración neuronal en individuos adultos. La corriente migratoria rostral está formada por células que se generan en la zona ventricular y migran tangencialmente hacia el bulbo olfatorio. Una vez que llegan al bulbo olfatorio, como ocurre en la corteza, cambian de conducta migratoria, y utilizan la migración radial para alcanzar su destino final. En la corriente migratoria rostral la reelina no actúa como molécula señal (señal de parada o de atracción), pero sí parece que la reelina actúa induciendo la separación de los neuroblastos de la corriente migratoria rostral. En el giro dentado también persiste la expresión de reelina durante la vida adulta de los roedores, la reelina actuaría igual que en el bulbo olfatorio, en este caso en la integración de las nuevas neuronas en la formación hippocampal.

En el encéfalo adulto la reelina está involucrada en procesos de plasticidad sináptica, estudios *in vitro* e *in vivo* han demostrado que la reelina promueve la potenciación a largo plazo a través de la activación de los receptores de proteínas de baja densidad y ApoE2. Así mismo a través de las integrinas la reelina podría actuar en el mantenimiento de las espinas dendríticas.

Justificación y Objetivos

Las posiciones que alcanzan las diferentes poblaciones neuronales así como las conexiones entre ellas son procesos cruciales que tienen lugar durante el desarrollo del SNC. La reelina es una de las proteínas que interviene en estos eventos, se ha demostrado que juega un papel importante en la regulación de la migración, sinaptogénesis y plasticidad sináptica durante el desarrollo. Esta proteína es de interés debido a que se ha observado un descenso en la expresión de esta proteína en muestras que procedían de pacientes que padecían esquizofrenia. Diferentes trabajos han demostrado que un descenso en la expresión de reelina durante el desarrollo puede ocasionar alteraciones citoarquitectónicas y neuroquímicas que recuerdan a las observadas en pacientes esquizofrénicos.

Aunque existen algunos trabajos describiendo la expresión de reelina durante el desarrollo, creemos que es necesario un estudio en detalle de la expresión de reelina en diversas áreas cerebrales que se ha visto que están alteradas o intervienen en la sintomatología de la esquizofrenia, como son la corteza cerebral, el estriado, la corteza piriforme y el tálamo. Además presentamos los resultados de un estudio detallado de la expresión de reelina en el mesencéfalo y rombencéfalo, áreas del encéfalo que han sido objeto de un menor estudio.

Los objetivos de la presente Tesis son:

1) Estudiar la expresión de reelina durante el desarrollo de la corteza cerebral: para realizar este objetivo hemos estudiado la expresión de reelina durante diferentes estadios de desarrollo y la colocalización con varios marcadores neuroquímicos. El resultado de este estudio se presenta en el capítulo 1 con el título "Una población de neuroblastos de la subplaca/zona intermedia presenta colocalización de reelina y Tbr1".
2) Estudiar la expresión de reelina en los parches estriatales y la colocalización con otros marcadores estriatales. Los resultados se presentan en el capítulo 2 titulado: "Correlación temporal de la expresión de reelina, DARPP-32 y tirosina hidroxilasa durante el desarrollo prenatal de los compartimentos estriatales de rata".
3) Estudiar la expresión de reelina en la corteza piriforme y su relación con otros marcadores neuroquímicos. Los resultados se presentan en el capítulo 3 con el título: "Expresión de reelina durante el desarrollo de la corteza piriforme de rata".
4) Estudiar la expresión de la reelina en los núcleos talámicos durante el desarrollo embrionario. Estos resultados se describen en el capítulo 4 titulado: "Estudio de la expresión de reelina, Tbr1 y Pax6 durante el desarrollo prenatal del tálamo ventral de rata".

5) Estudio detallado de la expresión de reelina durante el desarrollo del mesencéfalo y rombencéfalo de rata. Estos resultados se presentan en el capítulo 5 con el título: "Estudio de la expresión de reelina en cerebro medio y posterior en embriones y neonatos de rata".

Material y Métodos

Para la realización del presente trabajo hemos utilizado ratas Spagle-Dowley. Todos los procedimientos se realizaron de acuerdo con las normativa europea (86/609/EEC) y fueron aprobados por la comisión de Bioética de la Universidad de Santiago de Compostela. Las ratas fueron anestesiadas y se extrajeron los fetos de 13, 15, 17 y 19 días (E-13, E-15, E-17, E-19), además se utilizaron ratas recién nacidas (P0), así como postnatales de 2, 7 y 14 días (P2, P7 y P14). Los animales fueron anestesiados y perfundidos utilizando paraformaldehído al 4%. Posteriormente se crioprotegieron utilizando una solución de sacarosa al 30% y se cortaron en secciones de 20 µm en un criostato.

Inmunohistoquímica simple y doble

Las secciones se incubaron toda la noche en una solución que contenía: anticuerpo primario (ver tabla 1), 15% de suero normal de cabra (SNC) y 0,2% de Triton X-100 diluido en tampón Tris salino (TTS) a pH 7.4 que contenía seroalbúmina bovina al 1%. En las inmunohistoquímicas dobles se combinó anticuerpo anti-reelina con anticuerpos anti-GABA, anti-DCX, anti-calbindina, anti-calretinina, anti-Pax6, anti-Tbr1, anti-DARPP-32, anti-sinaptofisina, anti-GFAP, anti-GAD, anti-shh, anti-BrdU y anti-VGLUT2 a las diluciones que se indican en la tabla 1.

Después de lavar las secciones en TTS se incubaron en una solución que contenía: anticuerpo secundario apropiado (ver tabla 2), 10% de SNC y 0,2% de Triton X-100 diluido en TTS con seroalbúmina bovina al 1%. Las secciones que se procesaron para doble inmunohistoquímica fueron incubadas en la misma solución pero se utilizaron mezclas de anticuerpos secundarios acoplados a un fluoróforo (ver tabla 2).

Las secciones se lavaron TTS y posteriormente se incubaron con el complejo ABC en el caso de las secciones que se procesaron para una inmunohistoquímica simple. El revelado de la reacción se llevó a cabo utilizando una solución de 0.6 mg/ml de 3,3´-tetrahidrocloruro de diaminobenzidina (DAB) en TTS, con 0´003% de H_2O_2. Finalmente las secciones se deshidrataron en una batería de alcoholes con una gradación creciente, se aclararon en Xilol y se montaron con Eukitt. Las secciones que se procesaron para doble inmunohistoquímica se lavaron en agua y se dejaron secar, después se montaron utilizando Mowiol.

Tabla 1

Anticuerpo 1º	Tipo de anticuerpo	Casa Comercial	Dilución utilizada
Reelina	Monoclona de ratón	Chemicon, MAB5364	1:500
Reelina	Monoclona de ratón	Dr. A. Goffinet	1:1000
DCX	Policlonal de conejo	CellSignalling, 4604	1:500
DARP32	Policlonal de conejo	CellSignalling, 2306	1:500
Tbr1	Policlonal de conejo	Chemicon AB9616	1:500
Calretinina	Policlonal de conejo	Swant, 7699/4	1:1000
Calbindina	Policlonal de conejo	Swant, CB-38a	1:10000
GABA	Policlonal de conejo	Affinitty, GA1159	1:500
Pax6	Policlonal de conejo	Chemicon AB5409	1:500
PCNA	Monoclona de ratón	Sigma-Aldrich P8825	1:200
P73	Monoclona de ratón	Neomarkers MS-762	1:200
VGLUT2	Policlonal de conejo	Synaptic System 135403	1:5000
TH	Policlonal de conejo	Chemicom, AB152	1:1000
GAD	Policlonal de oveja	Dr. Mugnaini	1:60.000
Serotonina	Policlonal de conejo	INCSTAR 20080	1:15.000
BrdU	Policlonal de oveja	Affinity Bioreagents PA1-28373	1:500
Synaptophisyn	Policlonal de conejo	Labvison RB-9044	1:1000
Shh	Policlonal de conejo	Santa Cruz, sc-9024	1:100
GFAP	Policlonal de conejo	Dako Z0334	1:500

Para testar la especificidad de cada uno de los anticuerpos utilizados hemos realizado controles negativos que consistieron en la omisión del anticuerpo primario; y ninguna estructura immunorreactiva fue observada en estos controles.

Hibridación *in situ*

La hibridación *in situ* se llevó a cabo utilizando una sonda marcada con digoxigenina, que se generó mediante transcripción *in vitro* de un fragmento de cDNA del gen de la reelina mediante la enzima T3. Las secciones se permeabilizaron mediante distintos tratamientos. La

prehibridación se llevó a cabo a temperatura ambiente durante 3 horas en una solución de pre-hibridación que contenía: 50% formamida desionizada, 10% Dextran sulfato, 1X solución Denharts, 5nM EDTA, 0,1% Tween 20, 0,1% CHAPS, 0,1mg/ml heparina, 2X SSC, 250 µg/ml t-RNA de levadura, 250 µg/ml ADN de esperma de salmón. A continuación se añadió a la solución de pre-hibridación la sonda (500 ng/ml) y se incubó durante 16 horas a 60°C. Las secciones se lavaron secuencialmente en SSC 1X (65°C), SSC 1,5X (65°C) y SSC 2X (37°C), en una solución de ARNasa a 37°C y por último en SSC 0,2X (55°C). A continuación las secciones se lavaron en una solución de ácido maleico que contenía Tween 20 al 0,5% y se incubaron en una solución de bloqueo y finalmente se incubaron con el anticuerpo anti-digoxigenina toda la noche a 4°C. El revelado de la reacción se llevó a cabo con BMP.

En aquellos casos en los que se llevó a cabo una inmunohistoquímica combinada con la hibridación in situ las secciones se postfijaron en paraformaldehído al 4% y a continuación se lavaron en TTS. Se llevó a cabo una recuperación antigénica utilizando tampón citrato a pH 6,0 a 95°C durante 30 minutos. A continuación se sigue el protocolo detallado en el apartado de inmunohistoquímica.

Tabla 2

Anticuerpo 2º	Acoplado a:	Casa Comercial	Dilución
Cabra anti-conejo	Biotina	Dako, Z0420	1:500
Cabra anti-conejo	Alexa 488	Molecular Probes A11008	1:200
Cabra anti-ratón	Biotina	Dako E0432	1:500
Cabra anti-ratón	Alexa 546	Molecular Probes A11003	1:200
Cabra anti-ratón	Alexa 488	Molecular Probes A11001	1:200
Burro anti-oveja	Biotina	Vector Laboratories BA6000	1:200
Burro anti-oveja	Alexa- 546	Molecular Probes A21098	1:200

Fotografía

Las fotografías fueron sacadas utilizando un microscopio Olympus BX-51acoplado a una cámara Olympus DP-71 o con un microscopio confocal Leica-SP2. Las imágenes se convirtieron a escala de grises utilizando el Corel Photo-Paint 13. Las figuras se montaron utilizando Corel-Draw 13.

Capítulo 1: Una población de neuroblastos de la subplaca/zona intermedia presenta colocalización de reelina y Tbr1

Durante el desarrollo del SNC de roedores, la reelina se expresa principalmente en las células de Cajal-Retzius de la corteza cerebral, mientras que en adultos está presente en determinadas subpoblaciones de interneuronas GABAérgicas de la corteza y en algunas células situadas en la sustancia blanca. Las poblaciones celulares telencefálicas que expresan reelina tienen diferentes orígenes, así las células de Cajal-Retzius parecen originarse principalmente en el límite coroideo telencefálico, mientras que las interneuronas GABAérgicas lo hacen en las eminencias ganglionares, desde estas regiones migran tangencialmente hacía su posición final en la corteza cerebral. La población de células que expresan reelina localizada en la sustancia blanca, podrían ser células de la subplaca que han sobrevivido y se mantienen en la corteza cerebral adulta.

Nuestros resultados muestran una población de células que expresan reelina y que aparece entre los estadios E-14 y E-15 en la zona intermedia de la corteza en desarrollo. Utilizando secciones oblicuas del encéfalo de embriones de rata hemos observado que en los embriones de 15 días se puede observar una fila de células que son inmunorreactivas a reelina y que presentan abundante ARNm de reelina. En este estadio la inmunorreactividad es principalmente intracitoplasmática y comparable en intensidad a la mostrada por las células de Cajal-Retzius situadas en la capa marginal de la corteza. Las células presentan una morfología fusiforme con uno varios procesos que se sitúan principalmente paralelos a la superficie pial.

En estadios posteriores de desarrollo hemos observado células que expresan reelina primero en las regiones más superficiales de la zona intermedia (en E-17 y E-19) y posteriormente en la subplaca (E-19 y P0). Además se puede observar un cambio en la morfología celular, mientras en los estadios iniciales en los que se observa esta población, las células reelina inmunorreactivas presentan una morfología fusiforme, en los neonatos estas células presentan una morfología multipolar.

Para caracterizar neuroquímicamente esta población hemos utilizado diversos marcadores y hemos observado que estas células expresan Tbr1, DCX y calretinina, pero no expresan Pax6, P73, GABA ni calbindina. Algunas células reelina inmunopositivas que se sitúan en la subplaca continúan expresando Tbr1 y calretinina en estas últimas etapas de desarrollo sin embargo ya no expresan DCX. En el adulto el número de células reelina inmunorreactivas en la subplaca es menor y solo podemos encontrar algunas células en la capa VIb de la corteza cingulada que además expresan Tbr1.

Esta población de células inmunorreactivas a reelina que hemos estudiado parece tener un origen palial, ya que expresan Tbr1; creemos que son células que migran a través de la zona

intermedia de la corteza en desarrollo ya que expresan DCX y posteriormente se sitúan en la subplaca. Aunque esta población celular expresa los mismos marcadores que las células de Cajal-Retzius creemos que no son una subpoblación de las mismas debido a que no presentan P73 y porque están situadas en la zona intermedia/subplaca en un estadio en el que las células de Cajal-Retzius ya están situadas en la capa marginal. Otra posibilidad es que sean una subpoblación de interneuronas GABAérgicas que migran desde las eminencias ganglionares hacia la corteza cerebral, pero la ausencia de GABA nos hace pensar que no forman parte de esa población. Estos datos parecen indicar que podría tratarse de una subpoblación de células de las subplaca, que tiene un origen palial, pero que solo se observa durante el desarrollo, ya sea porque durante la etapa adulta no expresan reelina o porque han degenerado.

Capítulo 2: Correlación temporal de la expresión de reelina, DARPP-32 y tirosina hidroxilasa durante el desarrollo prenatal de los compartimentos estriatales de rata

El estriado es uno de los componentes del circuito de los ganglios de la base, interviene en la coordinación de movimientos, emociones y en procesos de cognición. Las eminencias ganglionares son el principal origen de las neuronas estriatales. Las neuronas que forman parte de los parches del estriado se originan en rata en el día embrionario 13, mientras que las neuronas que formarán parte de la matriz estriatal lo hacen el día 16. En el estriado se pueden diferenciar dos compartimentos: los parches y la matriz estriatal. Durante el desarrollo se puede observar ya una compartimentalización neuroanatómica utilizando marcadores como DARPP-32 o TH. La expresión de DARPP-32 precede a la llegada de las fibras TH procedentes de la sustancia negra, pero se desconoce el mecanismo molecular que controla la compartimentalización estriatal durante el desarrollo.

La reelina, que interviene en procesos de migración neuronal, sinaptogénesis y plasticidad neuronal, está presente en el estriado durante su desarrollo. Durante el desarrollo postnatal esta proteína se localiza en los parches estriatales, y sus niveles disminuyen en la etapa adulta hasta ser casi indetectables. Aunque el ratón mutante *reeler* no presenta alteraciones en la expresión de los marcadores de los parches del estriado, si se han observado algunas alteraciones neuroquímicas y funcionales en el estriado que podrían estar relacionadas con el déficit de la proteína. En este capítulo analizamos la correlación temporal de la reelina, el DARPP-32 y la TH durante el desarrollo del estriado de rata.

Nuestros resultados muestran que la reelina se puede observar en el manto del estriado desde el día de desarrollo embrionario 15 y que comienza a acumularse en el borde del estriado y en los parches estriatales a partir del día embrionario 17. La expresión de reelina en los parches aumenta en los estadios tardíos de desarrollo, E-19 y P0. Las células reelina inmunorreactivas son

más numerosas en el estriado rostral que en el caudal en E-15 y E-17, mientras que la inmunorreactividad de la tirosina hidroxilasa y DARPP-32 es más abundante en las porciones caudales del estriado que en las rostrales a esta edad. Sin embargo, en el siguiente estadio estudiado se produce un cambio en este patrón y la inmunorreactividad de las tres sustancias, reelina, tirosina hidroxilasa y DARPP-32 presentan un patrón muy similar, incluida la colocalización de la reelina y DARPP-32 en las mismas células.

Estos resultados nos sugieren que la reelina podría desempeñar un papel en la organización de las conexiones dopaminérgicas (marcadas con la tirosina hidroxilasa) en las células que son inmunorreactivas a DARPP-32 y que se acumulan en los parches estriatales. La falta de reelina podría estar relacionada con defectos en el establecimiento de estas conexiones dopaminérgicas y por tanto ser la responsable de algunos de los defectos de la función estriatal observados en los ratones *reeler*.

Capítulo 3: Expresión de reelina durante el desarrollo de la corteza piriforme de rata

La reelina es una proteína de la matriz extracelular que está implicada en la regulación de la migración neuronal, la sinaptogénesis y la plasticidad neural, esta proteína está presente en el sistema olfatorio de roedores durante el desarrollo embrionario y en individuos adultos. En el bulbo olfatorio las células que expresan reelina son principalmente las células mitrales y las células en doble penacho, la presencia de reelina en estas poblaciones celulares, que son neuronas de proyección, representa una gran diferencia en relación con el resto de células que expresan reelina en otras áreas cerebrales que son interneuronas. Aunque la reelina está presente en el bulbo olfatorio de roedores durante toda su vida, el mutante *reeler* no presenta alteraciones neuroanatómicas remarcables, sólo un menor tamaño con respecto al bulbo olfatorio del ratón salvaje. Sin embargo, los estudios ultraestructurales realizados si muestran alteraciones morfológicas. Además la reelina está presente en otras áreas del encéfalo relacionadas con el sistema olfatorio como es la corteza piriforme.

La corteza piriforme está involucrada en el aprendizaje de la asociación y la discriminación de olores. La presencia de reelina durante el desarrollo y en la vida adulta de roedores podría estar relacionada con estos procesos ya que esta proteína está implicada en la formación y estabilización de sinapsis, así como en la modulación de la potenciación a largo plazo y el aprendizaje en otras áreas cerebrales.

El primer estadio en el que encontramos reelina en la corteza piriforme es E-13 aunque a E-15 seguimos observando un patrón muy similar, la corteza piriforme presenta unas pocas células inmunorreactivas a reelina y un marcaje difuso. Estos resultados se confirman mediante hibridación in situ.

En estadios más tardíos de desarrollo, E-17 y E-19, se observa una fuerte inmunorreactividad a reelina en el tracto olfatorio lateral, la capa I presenta inmunorreactividad difusa a reelina y algunas células reelina inmunorreactivas, que también están presentes en la capa III. Mediante hibridación in situ se ha confirmado la presencia de reelina en células de la capa I y III, pero el ARNm no está presente en el tracto olfatorio lateral. Técnicas imunohistoquímicas dobles utilizando anticuerpos contra reelina y Tbr1 muestran que la reelina no está presente en las células Tbr1 inmunorreactivas de la capa II.

Los neonatos presentan un patrón de expresión de reelina similar al descrito en E-19. El tracto olfatorio presenta una serie de puntos inmunorreactivos a reelina de aproximadamente 1 μm de diámetro. Mediante la utilización de doble inmunofluorescencia hemos observado que la distribución topográfica de la reelina y Tbr1 es diferente, además no hay colocalización entre GFAP y reelina en el tracto olfatorio lateral. Además la reelina no colocaliza con sinaptofisina, GAD o VGLUT2.

A medida que avanza el desarrollo postnatal, la inmunorreactividad difusa en la capa I, así como las células inmunorreactivas a reelina en la capa I y III se mantienen mientras que los puntos inmunorreactivos que observábamos en el tracto olfatorio lateral ya no se observan.

En la corteza piriforme del adulto observamos células inmunorreactivas a reelina en la capa IA y en la capa III, así como una importante inmunorreactividad difusa en la capa IA. La capa IA además presenta inervación GABAérgica, glutamatérgica, dopaminérgica y serotoninérgica.

Estos resultados sugieren que la reelina podría estar actuando en procesos de sinaptogénesis de los axones del tracto olfatorio lateral durante el desarrollo, y en procesos de plasticidad sináptica en la capa IA posteriormente.

Capítulo 4: Estudio de la expresión de reelina, Tbr1 y Pax6 durante el desarrollo prenatal del tálamo ventral de rata

El tálamo constituye la principal puerta de entrada de señales al telencéfalo, está atravesado por numerosas fibras aferentes y eferentes, además cada núcleo talámico exhibe un patrón característico de expresión génica y de conectividad. En los modelos clásicos las neuronas de los núcleos talámicos se generan en la zona ventricular desde donde migran a la zona marginal, pero estudios recientes han demostrado la presencia de neuroblastos que migran tangencialmente durante el desarrollo del diencéfalo. La reelina está presente en el diencéfalo de roedores durante el desarrollo y en adultos, pero no se conoce cuál es el papel que esta proteína podría estar desempeñando.

En el presente trabajo hemos utilizado factores de transcripción: Tbr1, Pax6 y shh, proteínas de unión a calcio: calretinina y calbindina y marcadores de proliferación celular: PCNA,

para estudiar en detalle la expresión espacio temporal de la reelina durante el desarrollo del diencéfalo de rata.

Nuestros resultados muestran que a partir de E-13 se observa reelina (proteína y ARNm) en diversas regiones diencefálicas, y mediante doble inmunohistoquímica hemos identificado las regiones en las que se localizan células inmunorreactivas a reelina.

Utilizando Tbr1 y Pax6 hemos identificado células reelina inmunorreactivas en la eminencia talámica desde E-13 hasta P0, en base a esto hemos identificado estos territorios como parte del núcleo del lecho de la estría terminal. Aunque se observa una clara codistribución entre reelina y Tbr1 no podemos afirmar hasta P0 que hay algunas células que expresan ambos marcadores.

Utilizando shh, PCNA y Pax6 hemos identificado la zona limitans intratalámica. Los dobles marcajes con shh y reelina muestran que estas dos sustancias no colocalizan. Existe una zona claramente inmunorreactivas a shh que no lo es para reelina; flanqueando la zona limitans intratalámica encontramos dos poblaciones reelina inmunorreactivas, una en el tálamo dorsal y otra en el tálamo ventral. Las células reelina inmunorreactivas situadas en el tálamo ventral codistribuyen con Pax6, y algunas expresan ambas sustancias desde E-13. Utilizando anticuerpos contra la PCNA también hemos podido identificar la zona limitans intratalámica, la cual no presenta inmunorreactividad a PCNA ni a reelina.

En E-13 y E-15 hemos observado en las porciones laterales del diencéfalo una población de células inmunorreactivas a reelina, que además expresan calretinina. Son células fusiformes orientadas radial y perpendicularmente al ventrículo, y que pueden observarse en el tálamo ventral y en el tálamo dorsal. Podría tratarse de células originadas en estadios tempranos en el tálamo ventral y que migran tangencialmente al tálamo dorsal, coincidiendo con algunas poblaciones GABAérgicas descritas en diferentes trabajos.

A partir de E-15 podemos diferenciar en las porciones laterales del diencéfalo el núcleo geniculado lateral, que presenta numerosas células inmunorreactivas a reelina. Utilizando Pax6 podemos diferenciar una porción ventral, en la que encontramos células que coexpresan reelina y Pax6 y una porción dorsal con células inmunorreactivas a reelina, pero no a Pax6. Además la utilización de Pax6 nos permite describir la presencia de reelina en la zona incierta, aunque no podemos descartar también su presencia en el núcleo reticular talámico.

En el tálamo dorsal a partir de E-15 hemos encontrado algunas células inmunorreactivas a reelina, aunque no hemos observado la presencia de ARNm, esto podría ser debido a que la cantidad de ARNm presente en esta área es muy baja y no podemos detectarla o a que las células reelina inmunorreactivas han internalizado la reelina producida por otras células.

Estos resultados sugieren que la reelina podría estar actuando en el diencéfalo en la guía

axónica no como molécula señal, pero si como molécula necesaria para que los axones respondan correctamente a las señales de guía axónica. Además la presencia en el núcleo geniculado lateral sugiere que esta proteína puede estar implicada en procesos de sinaptogénesis o de refinamiento de conexiones procedentes de la retina o de otras áreas talámicas.

Capítulo 5: Estudio de la expresión de reelina en cerebro medio y posterior en embriones y neonatos de rata

Durante el desarrollo embrionario la reelina se expresa principalmente en áreas laminadas del encéfalo en donde se ha relacionado con el posicionamiento de las neuronas; y además se ha descrito su presencia en varios núcleos cerebrales. Aunque numerosos estudios han analizado el patrón de expresión de esta proteína en el encéfalo de vertebrados, la mayor parte de ellos se han centrado en el cerebro anterior y no en las regiones medias y caudales del encéfalo. En el ratón *reeler* además de las alteraciones citoarquitectónicas descritas en la corteza cerebral y en el cerebelo, numerosos núcleos del mesencéfalo y el rombencéfalo muestran también importantes alteraciones, por lo que consideramos de interés realizar un estudio detallado de la expresión de reelina en el cerebro medio y posterior durante el desarrollo de rata.

En el presente trabajo hemos estudiado la expresión de reelina tanto con inmunorreactividad como con la presencia del ARN mensajero en todos los estadios estudiados, desde E-13 a P0, además el patrón de distribución de la proteína coincide con el patrón de distribución del ARN mensajero. A partir de E-15 observamos inmunorreactividad en el citoplasma celular y en algunas zonas de la matriz extracelular. En el estadio E-13 hemos observado la presencia de inmunorreactividad a reelina en el pretecho, el tegmento, el cerebelo, puente y bulbo raquídeo. El número de células que expresan reelina aumenta en E-15 en el pretecho, colículo superior e inferior, el núcleo rojo, la sustancia negra, el área tegmental lateral y ventral, capa granular externa del cerebelo, núcleos del rafe, núcleos precerebelares (núcleos basales del puente, núcleo trigémino, oliva inferior) núcleo coclear, zona reticular, labio rómbico (superior e inferior) y en los territorios de la placa del techo del mesencéfalo y del cerebelo. Además en este estadio se puede observar inmunorreactividad difusa en los colículos superior... Este patrón de expresión observado a E-15 se mantiene hasta el P0, con la única diferencia de que a partir de E-17 se observa inmunorreactividad difusa, además de las zonas ya descritas, en el tegmento lateral, la sustancia negra y el cerebelo.

Hemos observado que la reelina está presente en áreas proliferativas, en el labio rómbico superior e inferior (neuroepitelio vestibular y auditivo) y en alguna de las corrientes migratorias que parten de estas áreas proliferativas como es la corriente migratoria subpial de la zona nuclear transitoria del cerebelo y en la corriente extramural anterior precerebelar; también está presente en los núcleos en los que se sitúan las neuronas que se originan en estas áreas como el

núcleo coclear y núcleos pontinos. Estos datos sugieren que la reelina podría estar implicada en el desarrollo de los núcleos pontinos y coclear.

Regiones de la placa del techo en regiones mesencefálicas y cerebelares también presentan inmunorreactividad a la reelina. Durante el desarrollo, esta zona actúa como centro de organización, controlando la proliferación, especificación y diferenciación de los neuroblastos así como controlando la guía axónica. Estos resultados sugieren que la reelina durante el desarrollo del cerebro medio y posterior de rata podría desempeñar un papel en la proliferación neuronal y en el control y en el correcto posicionamiento de las neuronas en la zona dorsal.

Por tanto, durante el desarrollo del encéfalo de rata, la reelina podría estar implicada en el control de la migración de áreas laminadas y no laminadas del encéfalo posterior, así como en la organización o en la remodelación de las conexiones sinápticas en el cerebelo, el sistema precerebelar y en los sistemas visual y auditivo en regiones caudales del encéfalo.

Conclusiones

Tras el análisis de los resultados del presente trabajo hemos obtenido las siguientes conclusiones:

1. Hay una población de células inmunorreactivas a reelina en la zona intermedia de la corteza en desarrollo que puede ser observada en la segunda semana de desarrollo prenatal en roedores, y que durante la tercera semana se incorpora a la subplaca. Estas células además expresan Tbr1 (marcador de neuronas glutamatérgicas), calretinina y DCX, pero no P73, Pax6 o GABA.
2. Aunque estas células expresan marcadores de las células de Cajal-Retzius, su distribución en la zona intermedia/subplaca indica que no es una subpoblación típica de las células de Cajal-Retzius. Estos resultados sugieren que se trata de una nueva población de células de la subplaca que posiblemente degeneran durante el desarrollo postnatal.
3. Durante la segunda semana de desarrollo prenatal, la expresión de reelina en el estriado sigue un gradiente rostro-caudal que es contario al observado con tirosina hidroxilasa y DARPP-32. Estas neuronas que expresan reelina podrían originarse en la región retrobulbar, muestran una intensa inmunorreactividad en el citoplasma a E-15, mientras que a E-17 los niveles de la inmunorreactividad intracitoplasmática descienden, indicando una posible secreción de la reelina a la matriz extracelular.
4. La colocalización de reelina y DARPP-32 en el estriado no se observa durante la segunda semana de desarrollo prenatal. Sin embargo, en neonatos la mayoría de las células inmunorreactivas a DARPP-32 contienen reelina. Los dobles inmunomarcajes de reelina y TH indican que las fibras inmunorreactivas a TH se acumulan en los estriosomas hacia el final del desarrollo prenatal, mientras que la expresión de reelina por parte de las células

de los estriosomas es anterior, esto sugiere que la reelina podría desempeñar un papel en la regulación del desarrollo de sinapsis de las fibras dopaminérgicas en los estriosomas.

5. Durante el desarrollo prenatal la reelina se observa en el tracto olfatorio lateral (TOL) en forma de intenso marcaje difuso, aunque el ARN mensajero que codifica para la reelina no se ha detectado en esta zona. Durante la primera semana postnatal el TOL presente unos intensos puntos inmunorreactivos a reelina, mientras que en la capa IA de la corteza piriforme se observa inmunorreactividad difusa a reelina. Estos resultados sugieren que la reelina podría acumularse en los axones de las células mitrales y ser secretada a la matriz extracelular, donde podría desempeñar un papel en los procesos de sinaptogénesis de los axones del TOL durante el desarrollo de la corteza piriforme.
6. Durante estadios más tardíos de desarrollo postnatal y en el encéfalo adulto se produce un incremento de la inmunorreactividad difusa en la capa IA de la corteza piriforme de rata. Sin embargo, los puntos inmunorreactivos a reelina ya no se observan en el TOL, quizás debido a la penetración del anticuerpo, sugiriendo que la reelina podría ser trasportada y secretada por los axones de las células mitrales en la capa IA, donde podría intervenir en procesos de plasticidad sináptica.
7. Experimentos de doble inmunorreactividad de reelina y shh indican que la reelina se expresa en células que flanquean la zona limitans intratalámica, tanto en el tálamo dorsal como en el ventral, pero no la expresan las células de la zona limitans intratalámica.
8. Al final de la segunda semana de desarrollo prenatal, se observa en la porción lateral del tálamo dorsal y ventral una población de células que inmunorreactivas a reelina y a calretinina, sugiriendo que podría tratarse de una población que se genera temprano en el tálamo ventral y que migra tangencial hacia el tálamo dorsal cruzando la zona limitans intratalámica.
9. Desde la segunda semana de desarrollo prenatal hasta el nacimiento, observamos la presencia de reelina, proteína y ARNm, en el pretecho, el núcleo rojo, el área lateral y ventral del tegmento, la sustancia negra, el lemnisco lateral, el núcleo coclear, diversos núcleos del rafe, colículo inferior y superior, capa granular externa del cerebelo, núcleo precerebelares (núcleos reticulares pontinos, núcleo trigémino y oliva inferior), áreas reticulares laterales e intermedias y labio rómbico superior e inferior, lo que demuestra una amplia expresión de esta proteína en el cerebro medio y caudal durante el desarrollo prenatal del encéfalo de rata y que sugiere que la reelina podría desempeñar un papel en el establecimiento del patrón citoarquitectónico del cerebro medio y caudal.
10. Al final de la segunda semana de desarrollo prenatal se observa un marcaje difuso en el

neuropilo del colículo inferior y superior, mientras que a partir de la tercera semana de desarrollo prenatal este marcaje difuso se observa también en el tegmento lateral, la sustancia negra y el cerebelo, lo que sugiere que la reelina podría desempeñar un papel en la organización y/o remodelación de conexiones sinápticas específicas en el cerebelo y en los sistemas auditivo y visual del tronco encefálico.

11. Desde la segunda semana de desarrollo prenatal hemos observado la expresión de reelina en regiones proliferativas como el labio rómbico superior e inferior (neuroepitelio pontino y vestibular), así como en la corriente precerebelar extramural anterior, sugiriendo que la reelina podría estar implicada en el desarrollo de los núcleos precerebelares y auditivos.

12. Durante la segunda y tercera semana de desarrollo embrionario hemos detectado la expresión de reelina en regiones de la placa del techo en el mesencéfalo y rombencéfalo; la placa del techo es un importante centro organizador que controla diversos aspectos del desarrollo del patrón dorsal del encéfalo, lo que sugiere que la reelina podría estar implicada en el control del establecimiento del patrón dorsal del mesencéfalo y rombencéfalo.