

Reelin immunoreactivity in the adult neocortex: A comparative study in rodents, carnivores, and non-human primates

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ABSTRACT: Recent evidence indicates that, in addition to playing a crucial role in early cortical development, intercellular signaling mediated by the protein Reelin may be widely active in the adult neocortex. The extent of Reelin distribution and its functional role in the adult are not clear yet. Here, we have examined Reelin immunoreactivity in the neocortex of an adult rodent (rat, *Rattus norvegicus*), a carnivore (ferret, *Mustela putorius*), and a primate (macaque monkeys *Macaca nemestrina*, *Macaca mulatta*) at the optic microscope level. Our data show that the neocortex of all three species contains several morphologically distinct populations of interneurons whose perikaryon and proximal dendritic processes are heavily immunoreactive for Reelin. The laminar distribution of these cells is species-specific. In addition, discrete reelin-immunoreactive pericellular structures are present in virtually all neocortical neurons of macaques. © 2002 Elsevier Science Inc.

KEY WORDS: Neocortex, Layer I, Cajal-Retzius cells, Interneuron, Extracellular matrix.

INTRODUCTION

Reelin was identified in 1995 [2] as the protein product of the gene mutated in *reeler*, a mice strain characterized by widespread disruption of neuronal positioning in neocortex and other brain regions (see [1] for review). Reelin is a high molecular weight secretable glycoprotein synthesized by particular neuron types [9], and other body tissues [12]. In the embryonic cortex, Reelin is secreted by neurons positioned mainly in the marginal zone, and may act as a signaling molecule crucial for the neural–glial interactions that stabilize radially migrating neuroblasts at their correct position (see [4], for a recent review). The low-density lipoprotein receptor 2, β 1-integrins [13], and possibly cadherin-like proteins [11] may act as Reelin receptors.

Recent studies in rodents and primates, including humans, have shown that Reelin mRNA and protein remain widely present in the neocortex throughout life [6–8,10], although the precise distribution and functional role of Reelin in the adult neocortex is less understood. There is evidence suggesting that Reelin may play roles in dendritic spine plasticity [10] or in neurotransmitter synthesis regulation [5,8]. Immunocytochemical studies revealed that some γ -aminobutyric acid (GABA)-ergic interneuron types contain large amounts of intracytoplasmic Reelin in rats [8]. Similar observations were made in monkeys [9] and humans [5]; interest-

ingly, the laminar distribution of these cells appears to differ markedly between species. Rodent studies suggest that secreted Reelin may be located mainly in the specialized extracellular matrix domains known as perineuronal nets [8]. Primate studies indicate that, in addition, some pyramidal cortical neurons may contain some Reelin [10], although this aspect has not been investigated further. No data are available in the literature regarding Reelin distribution in adult carnivores.

To gain insight into the specific neocortical circuits and neuronal populations that may be under the direct influence of Reelin in the adult, we have conducted a comparative analysis of Reelin-immunoreactive (ReIn-ir) neuron types in the adult cerebral neocortices of placental mammals belonging to three separate phyla: rodents (rat, *Rattus norvegicus*), carnivores (ferret, *Mustela putorius*) and primates (macaque monkeys, *Macaca nemestrina*). We focused on the morphological features, staining patterns, and laminar position of the various ReIn-ir neuron types.

MATERIALS AND METHODS

Brain tissue from two adult rats (male and female, aged 6 months), three ferrets (females, aged 2–3 years), and two Macaca nemestrina (adult males) was used for the present study. All procedures involving live animals were carried out in accordance to European Community's Council Directive 86/609/EEC guidelines. Animals were overdosed with sodium pentobarbital (80 mg/kg, intraperitoneal), and subsequently perfused through the heart with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were then removed from the skull. Rat and ferret brains were postfixed for 24 h in the same solution. Before sectioning, tissue was cryoprotected by soaking in 30% sucrose in PB for 24–48 h at 4°C. Serial coronal 40- μ m-thick sections were subsequently obtained on a freezing microtome. Monkey brain sections were temporarily stored in a 20% glycerol PB solution at -80°C before immunostaining, while ferret and rat tissue was processed shortly after sacrifice. Sections were pretreated with a 1% hydrogen peroxide + 10% methanol phosphate-buffered saline (PBS) solution for 20', rinsed, and then blocked with 10% horse serum + 3% bovine serum albumin (Boehringer Mannheim) + 0.5% Triton X-100 in PBS. As an additional pretreatment in some experiments, sections were microwaved (700 W) in citrate buffer (pH 6.0) for 2 min. Sections were then incubated for 48 h at room temperature in mouse monoclonal

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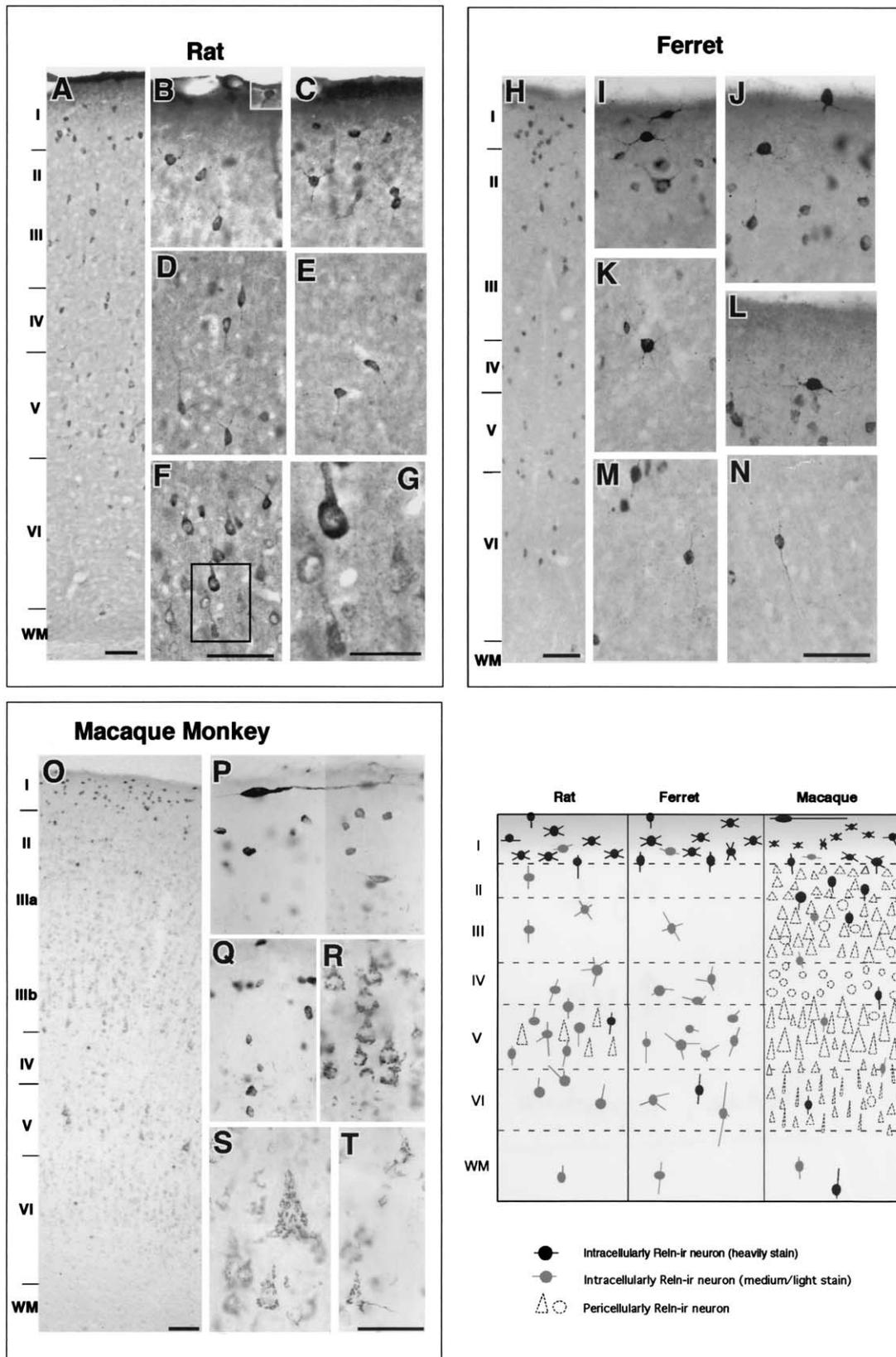


FIG. 1. Reelin immunoreactivity (ReIn-ir) in the temporal neocortex of the rat. (A–G, upper left panel) Suprasylvian gyrus of the ferret (H–N, upper left panel), and middle parietal gyrus of the macaque monkey (O–T, lower right panel). The cortical pial surface is always to the top. Scale bar: 100 μm for all photomicrographs, except for (G), where scale bar is 50 μm . The diagram in the lower right panel summarizes our observations in the three species. (A) Low-power view of a section through all cortical layers of the rat's temporal cortex. Note the prevalence of darkly stained neurons in layer I and V, and the neuropil labeling in layer I. (B–C) High-power view of ReIn-ir neurons in layer I. Note their multipolar dendritic arrangement; in (B), note the "inverted" neuron in the inset with enhanced brightness. (D–F) Multipolar and fusiform neurons of layers I–II (D), III–IV (E). (F) Interneurons and pyramidal cells labeled in layer V. (G) Perisomatic ReIn-ir in some pyramidal neurons of layer V. The image in (G) corresponds to

IgG 142 (1:400), directed against the amino terminal tiers of Reelin [3], a generous gift of Dr. A.M. Goffinet (Namur, Belgium). A biotinylated horse anti-mouse IgG (Pierce, 1:200) was used as secondary antibody. Immunoreagents were diluted in PBS containing 3% NHS and 0.1% Triton X-100. Slices were subsequently incubated in avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) in PBS, and developed with 0.01% H₂O₂ + 0.04% 3,3'-diaminobenzidine tetrahydrochloride in acetate buffer. In some experiments, we enhanced the opacity of the reaction product by including 2.5% nickel sulfate in the developing medium. Multiple rinses in PBS were intercalated between each of the above steps. The specificity of the monoclonal IgG 142 is well characterized [3]. In addition, we included primary antibody-free controls in each experiment, and in preliminary experiments, we also tested and optimized the concentration of primary antibody. Selected sections were counterstained with cresyl violet for cytoarchitectonic identification. Slices were finally mounted on gelatin-coated glass slides, air-dried, dehydrated, cleared in xylene, and coverslipped with DePeX. Sections were examined and photographed at 40–1000× under bright and dark-field illumination using a Zeiss Axiophot microscope. Serial sections covering all the neocortex of rats and ferrets were examined. In macaques, we immunostained and analyzed whole coronal sections selected at various anteroposterior levels of the cerebral hemisphere which included samples from a variety of prefrontal, frontal, parietal, medial limbic, temporal, and occipital areas.

RESULTS

In the three species investigated (Fig. 1) the neocortex displays widespread ReIn-ir. For description purposes, ReIn-ir structures can be grouped into three general categories: (a) intracytoplasmically labeled cells with relatively high levels of staining which typically display interneuron morphology; (b) fainter deposits of labeling that wrap around non-labeled, preferentially pyramidal cells; and (c) continuous neuropil staining without discrete structure.

Intracytoplasmically immunoreactive neurons are in general heavily stained. At low magnification, they are the most salient feature of the labeling (Figs. 1A,H,O). At higher magnification, ReIn-ir typically fills the somatic and proximal dendritic cytoplasm of these neurons, but leaves their nucleus conspicuously free of labeling. In the dendrites labeling becomes fainter and discontinuous distally, but second or third-order dendritic branches are often stained (Figs. 1C–F, I–N, and P–Q). In addition, some neurons have a sizable segment of the axons labeled by the immunoprecipitate (Fig. 1P). Experiments without nickel enhancing revealed that, among the intracytoplasmically labeled cells, some stain more intensely than others; this suggests that there may be substantial differences in Reelin content among these cells. In all three species, the majority of heavily ReIn-ir neurons is located in layer I and superficial part of layer II. In deeper layers, paler intracellularly labeled interneurons are numerous, particularly in layer V.

Morphological features of the intracellularly ReIn-ir cells are layer-specific. In layer I, most cell somata are relatively small, and

their dendrites display bitufted or multipolar arrangements (Figs. 1B–D,I–J,P). In rats and ferrets, some of these cells have their soma bulging into the pial surface and dendrites extending downwards (Fig. 1B inset, J). In macaques, large horizontal neurons with a single long, poorly branched dendrite are occasionally found just beneath the pia (Fig. 1P). Neurons in layers II–V have small and stellate or rounded bodies (Figs. 1D–F,K–M, and Q). Fusiform neurons are also present in these layers, but are particularly prevalent in layer VI and in the subcortical white matter (Figs. 1G,N).

The second category of labeling involves clump-like or filiform immunoprecipitate deposits that appear to “wrap around” the somata and dendritic shafts of cells that are otherwise non-labeled (Figs. 1F–G,P–T). Nissl counterstain revealed that the majority of the cells wrapped by these perisomatic deposits are pyramidal neurons. It should be emphasized that, at least in monkeys, virtually all cortical neurons display this type of ReIn-ir labeling around their somata. Interestingly, these structures are absent from layer I cells. In our material, this type of labeling was markedly less evident in rats and ferrets. Neuropil staining without discrete cellular elements is evident in layer I, particularly immediately beneath the pial surface.

Although the patterns described above are on the whole consistent for all the neocortical fields in the three species investigated, it is also true that we observed minor variations in the staining intensity, number, and/or layer distribution of the labeled cells across the various cortical areas. In general, these fluctuations were most evident in the perisomatic labeling aggregates, and largely reflected the underlying cytoarchitectonic differences among areas.

DISCUSSION

Our findings confirm previous reports that Reelin is present in high amounts within numerous interneuronal populations of the neocortex of adult rats and primates, and reveal that this is also the case in carnivores. In addition, our observations indicate that pericellular Reelin deposits in adult primates are substantially more ubiquitous than previously noted.

Our observations regarding cell types and laminar distribution of ReIn-ir interneurons in the rat and monkey neocortex are in agreement with previous studies [7–8,10]. In layer V of rats and monkeys only a faint labeling was noted [10], that probably corresponds to the one that we include in the “pericellular” category. The morphological features of the intracellularly labeled neurons observed in our material are compatible with the notion that certain types of GABAergic interneurons may be the only neocortical cells that synthesize Reelin [10]. These cells are present in all cortical layers, although a majority of them have their somata located in layer I.

Perisomatic ReIn-ir in monkeys is substantially more extensive than previously reported. This observation suggests that, at least in monkeys, Reelin-mediated signaling may influence the function of virtually all pyramidal cells. The nature of this perisomatic labeling is unclear, but given that Reelin is synthesized in interneurons, and that pyramidal cells do not express Reelin mRNA [7], it is

the inset in (F). (H) Low-power view of a section of the suprasylvian cortex of a ferret. (I–J) High-power views of intracellularly labeled horizontal and multipolar neurons in layers I and II. (K) Multipolar interneuron of layer II. (M–N) Fusiform/bitufted interneurons in layer V (M) and VI (N). Note the absence of labeled pyramidal cells. (O) Low-power view of a cortical column of macaque parietal cortex immunoreacted for reelin. (P–Q) High-power views of layer I and the superficial part of layer II. Note in (P) the horizontal neurons with a large horizontal dendrite extending to the right, and a thin axon to the right. Note in (P) the paler horizontal neuron near the border of layer II and, in (Q), a multipolar interneuron in layer II. (R–T) Perisomatic ReIn-ir labeling in layer IIIb (R), layer V (S), and layer VI (T). The same type of labeling can also be observed in the lower part of the images in (P) and (Q). Note the clump-like or filiform aspect of the immunoprecipitate that abruptly fades beyond the initial segment of the dendritic shafts.

reasonable to speculate that perisomatic Reelin is anterogradely transported to the terminals of interneuron axons that ramify around the somata of other cells [10]. In any case, it follows from our observations that intercellular signaling mediated by Reelin in the neocortex of adult mammals may be remarkably widespread, involving many neuron types and cortical circuits.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. André Goffinet for the generous gift of the reelin monoclonal antibody, Dr. Carmen Cavada for the excellent macaque tissue samples (grant PB 99/0064), and Dr. Alfonso Fairén for discussion. Support: FIS (98/0286), DGES (PB 97/015). V.M. is the recipient of a predoctoral fellowship BEFI 99/9160.

REFERENCES

1. Caviness, V. S.; Crandall, J. E.; Edwards, M. A. The reeler malformation. Implications for neocortical histogenesis. In: Peters, A.; Jones, E. G., eds. *Cerebral cortex*, vol. 7. New York: Plenum Press; 1988: 59–89.
2. D'Arcangelo, G.; Miao, G. G.; Chen, S. C.; Soares, H. D.; Morgan, J. I.; Curran, T. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* 374:719–723; 1995.
3. De Bergeyck, V.; Naerhuyzen, B.; Goffinet, A. M.; Lambert de Rouvroit, C. A panel of monoclonal antibodies against reelin, the extracellular matrix protein defective in reeler mutant mice. *J. Neurosci. Methods* 82:17–24; 1998.
4. Gleeson, J. G.; Walsh, C. A. Neuronal migration disorders: From genetic diseases to developmental mechanisms. *Trends Neurosci.* 23: 352–359; 2000.
5. Guidotti, A.; Auta, J.; Davis, J. M.; Gerevini, V. D.; Dwivedi, Y.; Grayson, D. R.; Impagnatiello, F.; Pandey, G.; Pesold, C.; Sharma, R.; Uzunov, D.; Costa, E. Decrease in reelin and glutamic acid decarboxylase 67 (GAD67) expression in schizophrenia and bipolar disorder: A postmortem brain study. *Arch. Gen. Psychiatry* 57:1061–1069; 2000.
6. Impagnatiello, F.; Guidotti, A. R.; Pesold, C.; Dwivedi, Y.; Caruncho, H.; Pisu, M. G.; Uzunov, D. P.; Smalheiser, N. R.; Davis, J. M.; Pandey, G. N.; Pappas, G. D.; Tueting, P.; Sharma, R. P.; Costa, E. A decrease of reelin expression as a putative vulnerability factor in schizophrenia. *Proc. Natl. Acad. Sci. USA* 95:15718–15723; 1998.
7. Pesold, C.; Pisu, M. G.; Impagnatiello, F.; Uzunov, D. P.; Caruncho, H. J. Simultaneous detection of glutamic acid decarboxylase and reelin mRNA in adult rat neurons using in situ hybridization and immunofluorescence. *Brain Res. Prot.* 3:155–160; 1998.
8. Pesold, C.; Liu, W. S.; Guidotti, A.; Costa, E.; Caruncho, H. J. Cortical bitufted, horizontal, and Martinotti cells preferentially express and secrete reelin into perineuronal nets, nonsynaptically modulating gene expression. *Proc. Natl. Acad. Sci. USA* 96:3217–222; 1999.
9. Rice, D. S.; Sheldon, M.; D'Arcangelo, G.; Nakajima, K.; Goldowitz, D.; Curran, T. Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain. *Development* 125:3719–3729; 1998.
10. Rodriguez, M. A.; Pesold, C.; Liu, W. S.; Kriho, V.; Guidotti, A.; Pappas, G. D.; Costa, E. Colocalization of integrin receptors and reelin in dendritic spine postsynaptic densities of adult nonhuman primate cortex. *Proc. Natl. Acad. Sci. USA* 97:3550–3555; 2000.
11. Senzaki, K.; Ogawa, M.; Yagi, T. Proteins of the CNR family are multiple receptors for Reelin. *Cell* 99:635–647; 1999.
12. Smalheiser, N. R.; Costa, E.; Guidotti, A.; Impagnatiello, F.; Auta, J.; Lacor, P.; Kriho, V.; Pappas, G. D. Expression of reelin in adult mammalian blood, liver, pituitary pars intermedia, and adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* 97:1281–1286; 2000.
13. Trommsdorff, M.; Gotthardt, M.; Hiesberger, T.; Shelton, J.; Stockinger, W.; Nimpf, J.; Hammer, R. E.; Richardson, J. A.; Herz, J. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell*. 97:689–701; 1999.