Chapter 17
Reelin, Liver, and Lymphatics

Brigitte Samama and Nelly Boehm

Contents

1 Introduction

Reelin, as an extracellular glycoprotein involved in neuronal migration and cerebral cortex layering pattern, has received much attention since the discovery of the gene responsible for the disturbed central nervous system development in the reeler mouse, because of its fundamental functions in cerebral development, its modulatory effects on synaptic plasticity in adult rodents, and its potential involvement in psychiatric disorders (for recent reviews and references see: Jossin, 2004; D'Arcangelo, 2005; Fatemi, 2005; Forster *et al*., 2006; Herz and Chen, 2006). Our knowledge about effects that reelin may have on peripheral organs, however, remains very scarce. Reelin mRNA and protein have been detected during development and adulthood in several peripheral organs. In the present chapter, we will focus on the presence of reelin in liver and lymphatics and discuss some hypotheses about the functional significance of this presence.

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2 Localization of Reelin in Peripheral Tissues

2.1 Reelin in Liver Cells

Hirotsune *et al*. (1995) observed by RT-PCR the presence of mRNA in adult mouse liver, while DeSilva *et al*. (1997) detected by Northern hybridization the presence of transcripts in fetal human liver. A cellular localization of hepatic reelin was first provided by Ikeda and Terashima (1997) who observed by *in situ* hybridization very strong mRNA expression in livers of fetal mice from embryonic day 9.5 (E9.5) to E16.5 and adults; these authors concluded that reelin was localized in sinusoid endothelial cells. Reelin protein has also been detected in liver; Smalheiser *et al*. (2000) noted that "in well perfused rat liver, little reelin-like immunoreactivity was observed, except for a zone surrounding the sinusoids." By immunocytochemistry using a reelin antibody specific for the N-terminal domain of the protein, generously provided by Dr. Goffinet (De Bergeyck *et al*., 1998), we observed the presence of reelin-like immunoreactivity in livers of humans (Fig. 17.1A), mice and rats during development (Fig. 17.1B,C), and in adult livers of rats (Fig. 17.1D) (Samama and Boehm, 2005, and unpublished data). Reelin was present in the youngest embryos we studied [i.e., E10.5 in mice, E12.5 in rats, and gestational week 5 (GW5) in humans]. The stained cells lined the sinusoids and appeared as spindle-shaped or stellate cells; their number progressively decreased during development, probably because of the expansion of hepatocyte number; in adult rats, reelin-positive cells were scattered in liver parenchyma (Fig. 17.1D). The cells were present in the space of Disse, between hepatocytes and endothelial cells, and had the same distribution as glial fibrillary acidic protein (GFAP)-positive cells (Buniatian *et al*., 1997), suggesting that these cells were stellate cells (Ito cells) (Fig. 17.1E); they were identified by immunoelectron microscopy in rat adult liver as stellate cells by their localization between sinusoid endothelial cells and hepatocytes and by their lipid content (Fig. 17.1F,G).

2.2 Reelin in Lymphatics

When analyzing reelin immunoreactivity in vessels, we observed a unique staining in endothelial lining of lymphatic capillaries but not of blood capillaries or vessels (Samama and Boehm, 2005, and unpublished data). Staining was present at E13 in rats in the jugular lymphatic sac (Fig. 17.2A) and in scattered mesenchymal cells in the whole body, and at later fetal stages, in many lymphatics. This staining was also present in lymphatics of adult rats and mice as illustrated in Fig. 17.2B–D, where a strong immunostaining in rat ovarian medulla (Fig. 17.2B) or around Peyer's patches in rat gut (Fig. 17.2C,D) can be seen. In human fetuses, we first observed a clear staining at GW7 in clefts we could identify as lymphatic capillaries. These clefts were especially prominent in skin and lungs; in addition, scattered

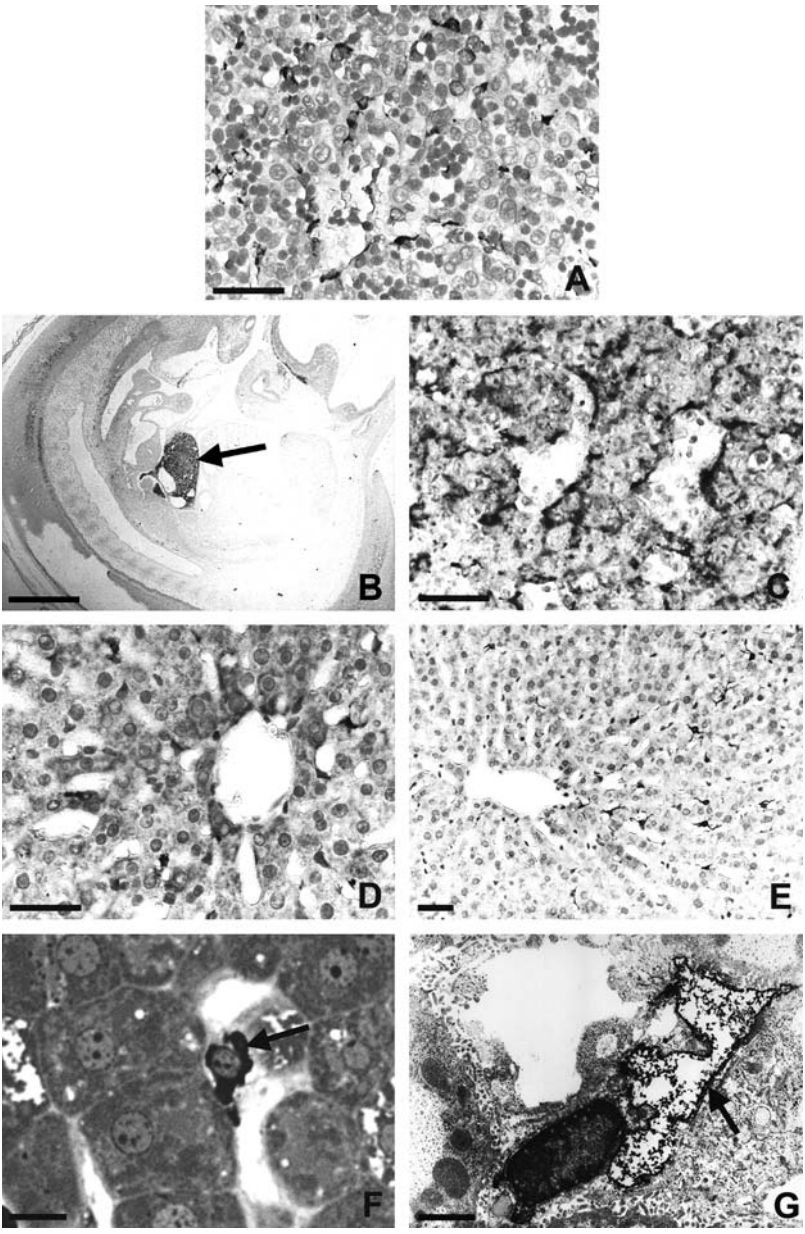

Fig. 17.1 Reelin (**A–D**, **F**, **G**) and GFAP (**E**) expression in human (**A**) and rat (**B–G**) liver. **(A)** Reelin immunostaining in stellate cells of human fetus at GW7. (**B**) Reelin immunostaining in liver of rat fetus at E13 (arrow). **(C)** Reelin immunostaining in stellate cells of rat fetus at E13; **C** is a high magnification of **B**. **(D)** Reelin immunostaining in adult rat stellate cells. **(E)** GFAP immunostaining in adult rat stellate cells. **(F)** Reelin immunostaining in a stellate cell of adult rat observed on a semithin section stained with toluidine blue (arrow). **(G)** Reelin immunostaining in a stellate cell of adult rat: electron microscopic examination; staining is observed in rough endoplasmic reticulum (arrow). Scale bars = 40 μm (**A**, **C–E**), 800 μm (**B**), 10 μm (**F**), and 2 μm (**G**) (*See Color Plates*)

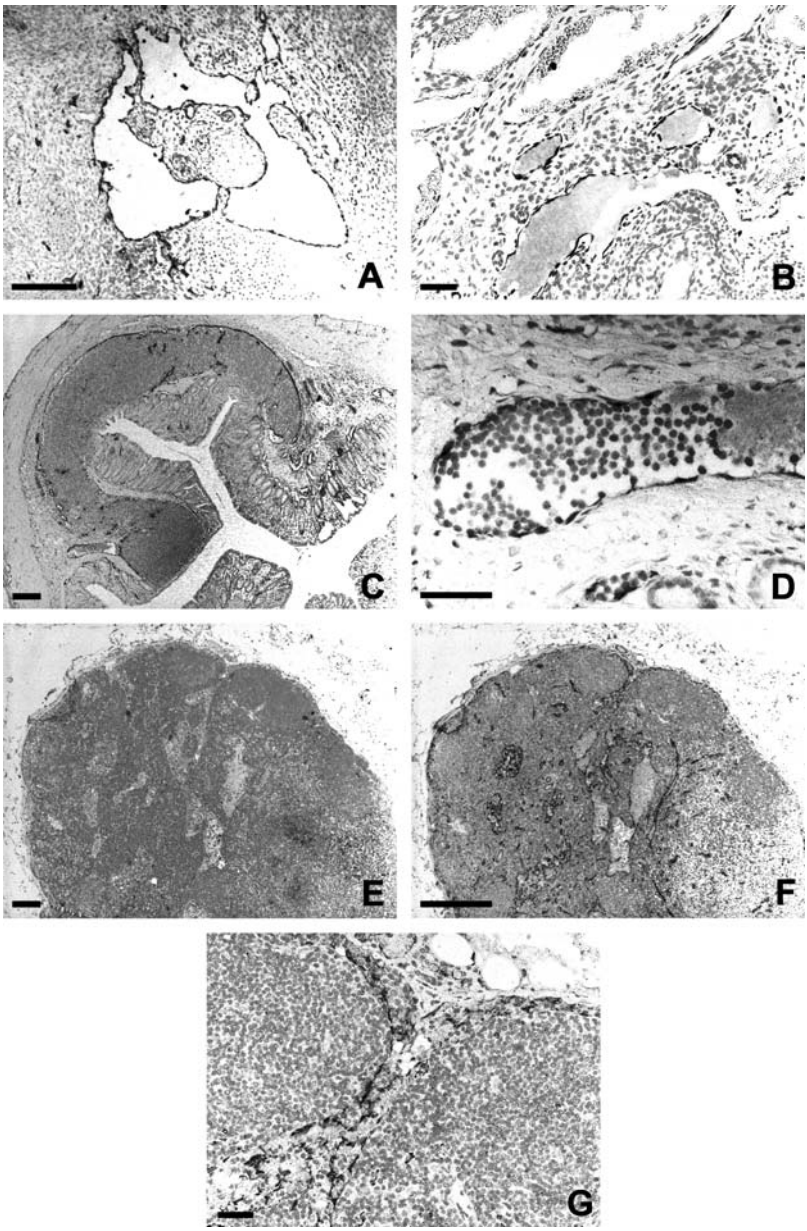

Fig. 17.2 Reelin (**A–E**) and CD31 (**F**, **G**) expression in rat fetus (**A**), adult rat (**B–D**), and adult human (**E–G**). **(A)** Reelin immunostaining in the jugular lymphatic sac of rat fetus at E13. **(B)** Reelin immunostaining in lymphatics of adult rat ovarian medulla. **(C, D)** Reelin immunostaining of lymphatics around Peyer's patches in adult rat gut; **D** is a high magnification of **C**. **(E)**Absence of reelin immunostaining in adult human lymph node. **(F, G)** CD31 immunostaining in adult human lymph node; **G** is a high magnification of **F**. Scale bars = 150 μm (**A**, **C**, **E**) and 40 μm (**B**, **D**, **F, G**) (*See Color Plates*)

elongated cells in mesenchyme were stained (Samama and Boehm, 2005). When comparing this staining with that observed with blood vessel markers or whole vasculature markers, reelin-positive cells were clearly present in lymphatic endothelial cells. No reelin immunoreactivity could be detected in lymph nodes of rats and humans as illustrated in Fig. 17.2E–G.

3 Functional Significance of Liver and Lymphatic Reelin

The presence of reelin-immunoreactive cells in peripheral organs raises two questions: Is the synthesized glycoprotein secreted? What may be its function?

3.1 Is Peripheral Reelin a Secreted Glycoprotein?

In the central nervous system during development, reelin is a glycoprotein of the extracellular matrix secreted by several groups of neurons. In the marginal zone of the cerebral cortex, reelin secreted by Cajal-Retzius cells is involved in the correct layering of cortical neurons in an inside-out manner (Curran and D'Arcangelo, 1998; Tissir and Goffinet, 2003; Soriano and Del Rio, 2005). Reelin immunoreactivity, most probably originating locally and not from the plasma, has also been detected in cerebrospinal fluid of humans as two main fragments, reflecting *in vivo* cleavage at two principal processing sites (Ignatova *et al*., 2004). In peripheral blood, reelin has been detected as full-length protein and as two cleaved fragments in the serum of rats, mice, and humans (Smalheiser *et al*., 2000; Lugli *et al*., 2003). No correlation between modifications of cerebrospinal fluid reelin and plasma reelin has been detected in normal human subjects and Alzheimer's disease patients, suggesting that cerebrospinal fluid and peripheral blood reelin have different origins (Botella-Lopez *et al*., 2006). Reelin, which is detectable in adult rat liver extracts, is also detected in conditioned medium when dissociated liver cells are placed in serum-free medium (Smalheiser *et al*., 2000). Reelin production *in vitro* is modulated by hormonal influences, since dexamethasone greatly inhibits the accumulation of reelin in both cells and conditioned medium (Smalheiser *et al*., 2000). All of these data suggest that peripheral reelin may be secreted and participates in the circulating pool of reelin. However, Roberts *et al*. (2005), in a postmortem study of human brains, observed that while the intracellular localization of reelin was quite similar in adult and fetal cortex, extracellular labeling was absent in adults and present in fetuses. After discussion of technical pitfalls, the authors concluded that reelin in the adult cortex was not a secreted protein. In our experiments, we did not observe reelin immunoreactivity in the extracellular matrix of liver or lymphatics. Further studies would be necessary to ascertain the nature of reelin as a secreted or a strictly intracellular glycoprotein. The last hypothesis raises the question of an intracrine effect of reelin as suggested or demonstrated for some peptides (Re and Cook, 2006).

3.2 Role of Peripheral Reelin?

3.2.1 Role of Reelin in Liver

Stellate cells (also referred to as Ito cells, fat-storing cells, lipocytes) represent a minor cell population of the liver (Geersts, 2004). Indeed, the adult hepatic lobule consists predominantly of anastomosing plates of hepatocytes limiting sinusoids feeding into the central venules. Stellate cells lie in the space of Disse between the endothelial lining and vascular domain of hepatocytes; this space contains scattered collagen fibrils but lacks basal laminae. The cells extend long cytoplasmic processes around sinusoids and their cytoplasm is characterized by the presence of lipid droplets, which have been demonstrated to store the major part of vitamin A of the whole body. They have important functions in adults, such as vitamin A storage and metabolism, production and remodeling of Disse space extracellular matrix, production of growth factors and cytokines, and regulation of sinusoidal lumen (for review, see Senoo, 2004). These cells present two phenotypes: when quiescent, they exhibit the fat-storing phenotype of the normal liver; when activated, they proliferate and display a myofibroblast-like phenotype, characteristic of stellate cells' response to liver injury (Senoo, 2004; Gressner and Weiskirchen, 2006). The origin of stellate cells remains debated. The liver develops from the anterior endoderm as a hepatic bud growing in the septum transversum, where mesoderm-derived cells promote growth of hepatocytes (Le Douarin, 1975). It is believed, although the exact lineage has not yet been demonstrated, that septum transversum mesenchymal cells give rise to stellate cells (Geersts, 2004). Recently, it has been shown that they do not derive from neural crest (Cassiman *et al*., 2006). Morphological aspects of liver stellate cell development have been reviewed by Enzan *et al*. (1997). At E10 in mice and rats and at GW5 in humans, when hepatic cords grow into the mesenchyme of the septum transversum, sinusoids are still present and probable progenitors of stellate cells are trapped in the subendothelial space. At E12–14 in mice and rats and GW6–8 in humans, stellate cells are characterized by one or more lipid droplets; this developmental pattern corresponds to our observation of reelin in these cells. One may then suggest that reelin could play a role as a paracrine factor in liver development. However, Ikeda and Terashima (1997), when comparing reeler and wild-type mice, did not notice any difference in liver morphology; it is not excluded that subtle differences may exist, for example, at the ultrastructural level.

Hepatic stellate cells are believed to be a major source of collagen type I production during hepatic fibrosis. In response to liver injury, quiescent cells undergo rapid activation with loss of vitamin A storage and upregulation of α-smooth muscle actin and desmin, Kobold *et al*. (2002) studied reelin expression during activation of stellate cells *in vitro*. They observed that expression was restricted to those cells, was absent in other liver myofibroblasts, and remained stable, while other activation markers were up- or downregulated. During liver injury *in vivo*, they observed an upregulation of reelin in both hepatocytes and stellate cells mainly in the damaged zones, while in the periportal undamaged zone the number of reelin-positive

cells remained more or less unchanged. However, at present, no role can be attributed to this upregulation, since reeler mice did not show differences over the complete time course of liver injury as compared to heterozygous and wild-type mice (Kobold *et al*., 2002).

3.2.2 Role of Reelin in Lymphatics

Concerning lymphangiogenesis, the developmental origin of lymphatic endothelial cells from deep embryonic veins or mesenchymal lymphangioblasts remains to be clearly determined. At present, a dual origin of lymphendothelial cells is mostly accepted. A first type of cells is located in specific segments of the venous system and mostly present in segments where blood and lymph vessels are fused, permanently or transiently. A second type of lymphendothelial cell derives from scattered mesenchymal cells (Wilting and Becker, 2006, and references therein). Interestingly, we observed reelin immunoreactivity in both lymphatic capillaries and scattered elongated cells, suggesting that reelin may be an early marker of lymphatic endothelial cells. The role lymphatic reelin may play in lymphangiogenesis can only be highly speculative. To our knowledge, there is no report about lymphatic malformations in reeler mice. However, Hong *et al*. (2000) reported an autosomal recessive form of lissencephaly (smooth brain) with severe abnormalities of cerebellum, hippocampus, and brainstem, associated with two mutations in the human gene encoding reelin, resulting in low or undetectable levels of reelin protein in blood. It is noteworthy that some patients showed persistent lymphedema neonatally, resulting in accumulation of chylous ascites fluid in one patient who required peritoneal shunting (Hourihane *et al*., 1993). Lymphedema is caused by insufficient lymph transport, as a result of lymphatic hypoplasia, impaired lymphatic function, or obstruction of lymph flow. That observation points to a possible role of reelin in lymphangiogenesis and lymphatic structure homeostasis.

It is noteworthy that both liver stellate cells and lymphatic endothelial cells are surrounded by a poor extracellular matrix, mostly lacking a continuous basal lamina, although collagen IV is secreted in the space of Disse and in the subendothelial space of lymphatics. Moreover, lymphatic capillaries do not recruit pericytes as do blood capillaries. Although we studied the collecting lymphatic vessels extensively, afferent and efferent lymphatics of lymph nodes were never reelin immunoreactive (unpublished data).

3.3 Reelin Signaling Pathway

Some clues about reelin functions in peripheral organs may come from the localization of the reelin signaling pathway. In the developing cortex, transmission of reelin signal to migrating neurons involves preliminary binding to reelin receptors. Two main types of receptors are known: lipoprotein receptors, i.e., very-low-density

lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) (Tissir and Goffinet, 2003; May *et al.,* 2005) and $\alpha 3\beta 1$ integrin (Dulabon *et al*., 2000). In peripheral organs, VLDLRs are abundant in heart, muscle, adipose tissue, and brain and are barely detectable in liver. They are also present in macrophages and endothelial cells of capillaries and arterioles, and in endothelial cells of coronaries but not in aorta or in veins or venules. In human liver, only sinusoidal lining cells but not hepatocytes express this receptor (review in Takahashi *et al*., 2004, and references therein). ApoER2 transcripts are numerous in postmitotic neurons, testis, and ovary (Kim *et al*., 1996).

In brain, binding of reelin to ApoER2 and VLDLR induces tyrosine phosphorylation of an adapter protein, disabled-1 (Dab1) by Src family kinases (Jossin *et al.,* 2003; Forster *et al.,* 2006; Stolt and Bock, 2006), resulting in nucleation of multiprotein complexes which modulate cytoskeleton dynamics. Dab1 is highly expressed and tyrosine phosphorylated in developing central nervous system and serves as a substrate for Src family kinases. In the central nervous system, reelin-expressing cells are adjacent to Dab1-immunoreactive cells, which are targets for reelin and are disturbed in their migration in the absence of reelin. In human and mouse cerebral cortex, during development, Dab1, VLDLR, and ApoER2 are expressed during neuronal migration in immature neurons of the cortical plate and may thus be responsive to reelin secreted by Cajal-Retzius cells in the marginal zone (Meyer *et al*., 2003; Perez-Garcia *et al*., 2004). Similarly, reelin protein is secreted by neurons adjacent to migrating sympathetic neurons, which, in turn, express Dab1 (Yip *et al*., 2000, 2003, 2004; Kubasak *et al*., 2004). Dab1 is mainly expressed in fetal and adult brain; during development, Howell *et al*. (1997) reported Dab1 expression and phosphorylation in some peripheral nerves in the mouse; in cultured cells, only P19 embryonal carcinoma (EC) cells and hematopoietic cell lines expressed Dab1. Smalheiser *et al*. (2000) noted that Dab1 immunoreactivity was present in the posterior lobe of the rat pituitary gland while reelin was present in the intermediate lobe. In human tooth, reelin is expressed in fully differentiated odontoblasts and the reelin signaling pathway is present in the trigeminal ganglion, suggesting that reelin might be involved in the terminal innervation of the dentin–pulp complex (Maurin *et al*., 2004). Dab1 protein was not detectable in the adult rat liver either by immunocytochemistry or by Western blotting (Smalheiser *et al*., 2000). We did not observe Dab1 immunoreactivity in livers of fetal and adult mouse and rat (unpublished data). Moreover, Dab1 mutants have no obvious peripheral phenotype.

An alternative hypothesis would be that reelin, secreted in the extracellular matrix, blood, or lymph may use a signaling pathway different from that previously described. It has been shown that reeler mice of both sexes had a reduced number of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus and that seminiferous tubules were reduced in number and dilated (Cariboni *et al*., 2005). Reelin expressed along the intracerebral route of these migrating cells has an inhibitory role in guiding these neurons. However, mutant mice lacking reelin receptors or Dab1 have a normal complement of GnRH neurons, showing that the effect of reelin is independent of Dab1.

It is now clear that although several groups observed the presence of reelin in peripheral organs, such as liver and lymphatics and in peripheral blood, the significance of peripheral reelin during development and adulthood needs to be determined.

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Chapter 18
Reelin and Cajal-Retzius Cells

Jean-Marc Mienville

Contents

1 Introduction

Cajal-Retzius (CR) cells comprise a population of neurons found in the marginal layer of the developing cerebral cortex and hippocampus of amniotes. Their name originates from their codiscovery in the 1890s by Santiago Ramón y Cajal, who was using Golgi staining techniques on brain sections from small mammals such as rabbits (Ramón y Cajal, 1891), and by Gustaf Retzius, who referred to the cortical marginal cells he observed in human fetuses as "Cajal's cells" (Retzius, 1893). Despite this common classification, it should be noted that the morphology of CR cells from primates versus small mammals is not homogeneous. Drawings of primate marginal cells provided by Retzius and other authors (Meyer *et al*., 1999) indicate rather complex and variable morphologies, and Retzius even initially considered "his" CR cells as glia (König, 1978). By contrast, CR cells present in the rat marginal zone—or layer I of the more mature cortex—display fairly homogeneous aspects, so that their identification is straightforward based on three morphologic criteria: fusiform or ovoid shape; bipolarity, i.e., presence of one axon and one dendrite; and tangential orientation of the latter (Fig. 18.1). Due to their facilitated access in a widely used species, a large body of data have been collected regarding

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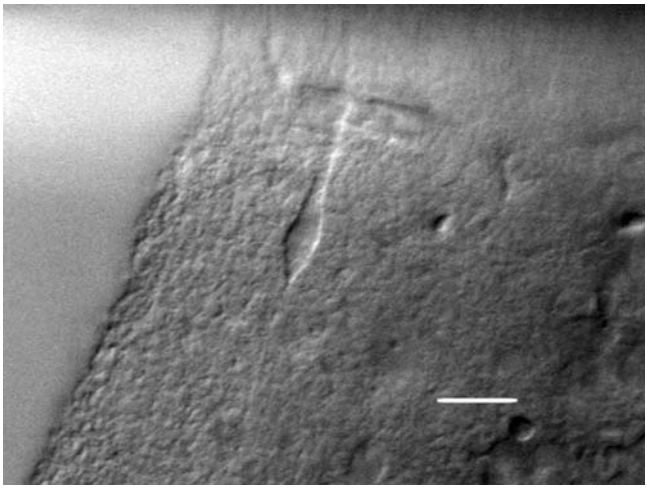

Fig. 18.1 Infrared differential interference contrast image of a Cajal-Retzius cell in a slice of postnatal day 11 rat neocortex. Bar = 20 μm. (Modified from Mienville, 1999, by permission from Oxford Journals)

the physiologic properties of rat CR cells (Mienville, 1999). In 1995, a novel criterion for identifying these neurons emerged with the discovery of reelin, their secreted protein, which is necessary for correct cortical lamination (D'Arcangelo *et al*., 1995; Ogawa *et al*., 1995). While reelin is produced by other cells (see below), certainly the combination of morphologic and immunocytochemical criteria now should allow unambiguous identification of CR cells.

Besides aiding with identification, the presence of reelin in CR cells may allow their provisional classification among those cells permitting neuron placement in a developing multilayered structure. This generalization beyond a role in the neocortex proper is necessary, given the presence of CR cells in lower animals devoid of such a structure (i.e., nonmammals). An ontogenic parallel to this observation is provided by the presence of CR cells in the mammalian hippocampus, where reelin is required for proper laminar organization (Nakajima *et al*., 1997; Fatemi *et al.,* 2000). In the case of the hippocampus, however, the concept of "neuron placement" may be too restrictive, as reelin-producing CR cells also have a role in axonal growth and pathfinding, being required for the layer-specific targeting of developing entorhinal afferents (Del Río *et al*., 1997). In addition, reelin is not the only signal produced by CR cells. For one thing, it is now generally admitted that they are glutamatergic neurons (Hevner *et al*., 2003), though CR cell-induced postsynaptic currents have not been demonstrated yet. As for other substances, Clark *et al*. (1997) found that the products of genes whose mutations are responsible for a lissencephalic phenotype are heavily expressed in CR cells (as well as in the ventricular neuroepithelium). As lissencephaly appears to be caused by a developmental migratory defect, this reinforces the organizational role of CR cells, and suggests that some aspects of this role might be independent of reelin production.

Reciprocally, reelin is not exclusively secreted by CR cells. Reelin mRNA has been detected in many zones of the developing CNS, and in several cell types other than CR cells, including mitral cells of the olfactory bulb, granule cells of the cerebellum, and retinal ganglion cells (Schiffmann *et al*., 1997; Alcántara *et al*., 1998).

2 Life and Death of CR Cells

CR cells are among the first neurons to be produced during cortical development; they can be detected and are found to express reelin as early as embryonic day 10 in rodents (Ogawa *et al*., 1995; Alcántara *et al*., 1998), and at gestational week (GW) 6 in humans (Zecevic *et al*., 1999). Progenitor commitment to CR cell fate is suppressed by the transcription factor Foxg1, which accounts for the time- and space-limited production of these cells (Hanashima *et al*., 2004; Muzio and Mallamaci, 2005; Shen *et al*., 2006). During initial stages of corticogenesis, CR cells, perhaps guided by some autocrine function (Meyer *et al*., 2003; Perez-Garcia *et al*., 2004), or more likely by interactions with the meningeal chemokine CXCL12 (Yamazaki *et al*., 2004; Borrell and Marín, 2006), settle in a phylogenetically and ontogenetically primitive structure called the preplate or primordial plexiform layer (Zecevic *et al*., 1999). The concept that their site of origin may be the subjacent ventricular zone (Zecevic *et al*., 1999; Jiménez *et al*., 2003; Shen *et al*., 2006) coexists with numerous observations of migratory imports from the ganglionic eminence (Lavdas *et al*., 1999; Zecevic and Rakic, 2001; Rakic and Zecevic, 2003) and, in fact, from multiple anatomic sites according to time-, origin-, and destination-dependent patterns (Meyer and Wahle, 1999; Meyer *et al*., 2002a; Rakic and Zecevic, 2003; Takiguchi-Hayashi *et al*., 2004; Muzio and Mallamaci, 2005; Borrell and Marín, 2006; Yoshida *et al*., 2006; Cabrera-Socorro *et al*., 2007; García-Moreno *et al*., 2007; see Hevner *et al*., 2003, and Bielle *et al*., 2005, for a specific focus on mouse CR cells). Upon formation of the cortical plate, CR cells remain in the marginal zone, which is the future layer I of the neocortex.

The survival, function, and subsequent fate of CR cells appear to be determined by a number of extrinsic factors in their environment. For instance, their maintenance during corticogenesis seems to depend on a trophic contribution from meningeal cells, as pharmacologic destruction (Supèr *et al*., 1997) or genetic alteration (Hartmann *et al*., 1999) of the latter leads to a major loss of CR cells. Potential candidate factors for meningeal trophic support of CR cells include CXCL12 (also known as stromal cell-derived factor-1; Stumm *et al*., 2003) and a TGF-β inhibitor (Kim and Pleasure, 2003). Extrinsic monoaminergic afferents may also influence the function and/or fate of CR cells (Naqui *et al*., 1999; Janušonis *et al*., 2004). On the pathologic side, expression of reelin by CR cells is decreased by prenatal infection with viruses such as human influenza H1N1 (Fatemi *et al*., 1999), while exposure to ethanol indirectly disrupts reelin expression in the marginal zone, leading to the formation of brain heterotopias (Mooney *et al*., 2004).

The normal genesis and differentiation of CR cells also depend on the expression of specific genes such as the transcription factor *Tbr-1* (Lambert de Rouvroit and Goffinet, 1998; Hevner *et al*., 2001), the *Drosophila* head gap gene orthologs *Emx1* and *Emx2* (Shinozaki *et al*., 2002; Bishop *et al*., 2003), *LIS-1*, a gene coding for a microtubule-associated protein (Meyer *et al*., 2002b), on the integrity of the heat-shock factor 2 → p35/39 → cyclin-dependent kinase 5 pathway (Chang *et al*., 2006), and on the turning off of the transcription factor COUP-TFI (Studer *et al*., 2005); in turn, CR cell positioning relies on the correct expression of the integrin *β1* gene (Graus-Porta *et al*., 2001), while their migration rate is controlled by the transcription factor Pax6 (Stoykova *et al*., 2003) and by CXCL12 (Borrell and Marín, 2006). As expected, alterations of the above genetic factors lead to abnormalities in cortex development.

Although a matter of occasional debate (Fairén *et al*., 2002), it is generally admitted that CR cells are not only pioneer but also transient neurons. While some CR-like cells may persist in adult brain (see below), a major disappearance of CR cells occurs between postnatal weeks 2 and 3 in rodents, and around GW 27 in humans. A mechanism of "physiologic excitotoxicity" combining high expression of NMDA receptors and low resting potential (Fig. 18.2) has been proposed to account for this disappearance (Mienville and Pesold, 1999). This view is consistent with the increased survival of hippocampal CR cells in reeler due to decreased glutamatergic entorhinal afferentation (Coulin *et al*., 2001), and with the massive loss of depolarized cortical CR cells in presenilin-1 (PS1) knockouts (Kilb *et al*., 2004). Hypotheses evoking programmed cell death also have been proposed. For instance, it has been suggested that p73, a member of the p53 tumor-suppressor family, may be involved in the survival and death of CR cells (Meyer *et al*., 2002a). Brain-derived neurotrophic factor (BDNF) appears to favor survival of late CR cells, but, interestingly, it is also a downregulating factor of reelin production, and it alters the morphology and organization of CR cells, all events suggesting that BDNF induces a change of function in these cells (Ringstedt *et al*., 1998; Alcántara *et al*., 2006). Perhaps relevant to this finding, early human cortical CR cells initially display the same bipolar shape and tangential orientation as those seen in rodents, but subsequently switch (while hippocampal CR cells do not; see Abraham and Meyer, 2003) to the polymorphic aspect originally described by Retzius (Meyer and Goffinet, 1998; Cabrera-Socorro *et al*., 2007). Such phenotypic changes are compatible with the marked transcriptomic changes observed in developing CR cells (Yamazaki *et al*., 2004). Around the same early midgestational period, the subpial granular layer supplies a new wave of reelin-expressing cells that resemble CR cells, including those of the "polymorphic" type (Meyer and Wahle, 1999; Rakic and Zecevic, 2003), and that may persist into adulthood (Meyer and Goffinet, 1998). This additional source of reelin, along with the further differentiation of early born CR cells, has been tentatively linked to the dramatically increasing complexity of the primate neocortex and the need to accommodate a protracted neurogenesis (Meyer and Goffinet, 1998; Meyer and Wahle, 1999; Cabrera-Socorro *et al*., 2007). A similar biphasic scenario has been proposed regarding human hippocampal CR cells (Abraham *et al*., 2004a). It is noteworthy that CR or CR-like

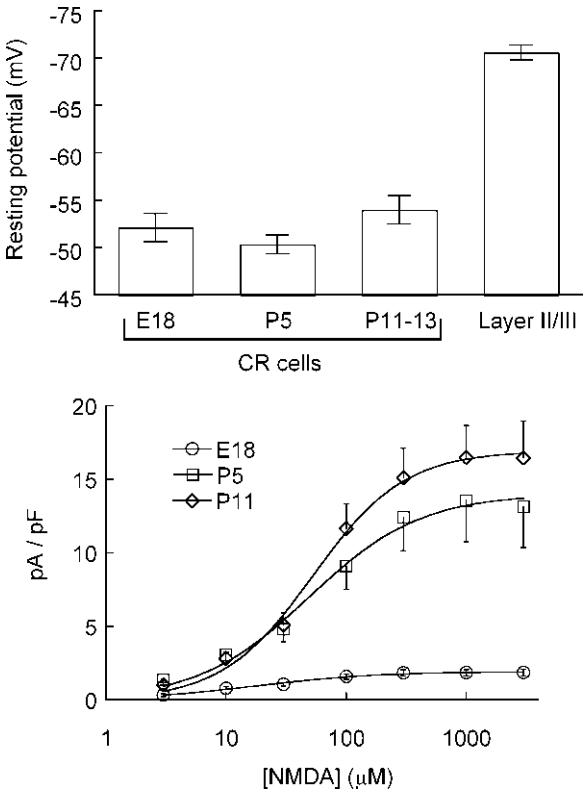

Fig. 18.2 (Upper panel) Low resting potential of rat neocortical Cajal-Retzius cells from embryonic day 18 to postnatal day 13 (mean ± SEM). The mean resting potential of Layer II/III neurons is shown for comparison. (Lower panel) Dose–response curves showing an increase in the density of NMDA receptor-mediated current from embryonic to postnatal stages of development. pA/pF = picoamperes per picofarad. (Modified from Mienville and Pesold, 1999, by permission from the Society of Neuroscience)

cells persist in the adult hippocampus of several mammalian species including mouse (Alcántara *et al*., 1998), rat (Drakew *et al*., 1998), macaque (Martínez-Cerdeño *et al*., 2002), man (Fatemi *et al.,* 2000; Abraham and Meyer, 2003), and pig (Abraham *et al*., 2004b), somehow consistent with the late survival of CR cells in more primitive brains (Blanton and Kriegstein, 1991).

The observation that CR or other types of reelin-secreting cells are present in the adult brain (Pesold *et al*., 1998; Zecevic and Rakic, 2001; Abraham *et al*., 2005) prompts the question of the functional role of reelin in the adult organism. In the cases of the adult entorhinal cortex and postnatal dentate gyrus, owing to the large number of reelin-expressing CR cells therein and the possibility that both structures may be sites of adult neurogenesis, one may hypothesize mere continuity in the same neuron-guiding role throughout life (Riedel *et al*., 2003; Abraham and Meyer,

2003). On a more general level, an exciting perspective is the putative role of reelin in dendritic spine plasticity (Liu *et al*., 2001; Abraham *et al*., 2005; Roberts *et al*., 2005) and long-term potentiation (Weeber *et al*., 2002).

3 Reelin-Independent Functions of CR Cells

It is very likely that the most important role of CR cells is related to their capacity to synthesize and secrete reelin. The discovery of this protein and of its key function in cortical lamination was made possible by the availability of the *reeler* mutant mouse (D'Arcangelo *et al*., 1995; Ogawa *et al*., 1995). In this animal, several laminated brain structures such as cortex, hippocampus, and cerebellum are disorganized, which results in impaired motor function. It is important to note that CR cells are present in reeler (indeed, they even are found in greater number than in wild-type; see Coulin *et al*., 2001), various mutations of their *reeler* gene preventing either synthesis or secretion of reelin, with the deleterious consequences just mentioned.

It has now become evident that reelin signaling is not the only developmental function of CR cells. Though it may seem technically difficult to perform selective ablation of CR cells, especially in early embryos, several such attempts indicate that lack (or strong reduction) of CR cells does not yield phenotypes equivalent to that of reeler. For instance, PS1-deficient mice have a dramatically reduced CR cell number, but display cortical dysplasia reminiscent of lissencephaly rather than *reeler* phenotype (Hartmann *et al*., 1999; but see Hong *et al*., 2000). *Emx1/2* double mutants incur a complete loss of CR cells and have lamination defects much more severe than those of reeler (Shinozaki *et al*., 2002). CR cell-depleted $p73^{-/-}$ knockouts fail to develop a hippocampal fissure whereas the latter appears normally in reeler (Meyer *et al*., 2004). Unexpectedly, in the latter knockouts, lamination of the rostral cortex is not significantly altered despite the ubiquitous loss of CR cells. Similarly, genetic ablation of the cortical hem, a major source of CR cells, does not disrupt lamination of the rostral neocortex (Yoshida *et al*., 2006). Therefore, the role of reelin and CR cells in rostral areas may have to be redefined.

Nongenetic approaches have used pharmacologic ablation of CR cells versus reelin blockade by CR-50 antibody (or *reeler* mutants) to study the trophic effects of CR cells on axonal growth and pathfinding in the hippocampus. It was found that CR cell ablation prevents the ingrowth of entorhinohippocampal afferents, whereas reelin blockade or *reeler* mutation only affects their collateral branching and innervation density (Del Río *et al*., 1997). Reelin-independent effects of CR cells can thus be separated; they appear to include both a permissive action on entorhinal afferent ingrowth, possibly via their own projections to the entorhinal cortex (Ceranik *et al*., 1999; see below), and an inhibitory action on commissural axons via contact inhibition (Borrell *et al*., 1999). Similar experimental strategies applied to cortical areas indicated that CR cell ablation induces a loss of radial glial processes, the "scaffold" system used by neuroblasts to migrate out of the ventricular zone, and thereby arrests migration. By contrast, the radial glia scaffold persists in

reeler, though with a somewhat altered organization (Luque *et al*., 2003), indicating that the lack of reelin is not the main factor for the loss of radial glia (Supèr *et al*., 2000). However, the glial scaffold of the dentate gyrus is severely affected in reeler (Frotscher *et al*., 2003).

The question therefore remains as to the reelin-independent mechanisms used by CR cells to affect developmental processes. As mentioned above, they might secrete soluble signals other than reelin, but our current state of knowledge does not allow expanding on that issue (Derer *et al*., 2001; Yoshida *et al*., 2006). Alternatively, the action of CR cells may be of a more physical nature. For instance, it has been proposed that CR cell projections to the entorhinal cortex may serve as a template or guiding scaffold for outgrowing entorhinal afferents (Ceranik *et al*., 1999). Finally, a promising lead concerns the electrical activity of CR cells. Their participation in network activity of the developing neocortex has been demonstrated both at the single-unit (Soda *et al*., 2003) and multi-unit recording level (Aguiló *et al*., 1999). In the latter case, this activity did not reveal any major difference when recorded from reeler mice, confirming a lack of involvement of reelin, and was blocked by neurotransmitter antagonists, suggesting involvement of synaptic contacts. Indeed, spontaneous (Kilb and Luhmann, 2001) and evoked postsynaptic currents (Radnikow *et al*., 2002) have been demonstrated in CR cells. Altogether, these results suggest that CR cells may function similarly to subplate cells, whose transient synaptic contacts allow establishment of thalamocortical connections (Friauf *et al*., 1990). In that context, CR cells may serve as a temporary interface necessary for implementing hippocampal (Del Río *et al*., 1997) as well as cortical layer I inputs. Their developmentally regulated spontaneous firing (Mienville *et al*., 1999) and possible outputs (Radnikow *et al*., 2002) in turn may be related to the maturation of the apical dendrites of cortical pyramidal cells (Marín-Padilla, 1998; Mienville, 1999).

4 Reelin Secretion by CR Cells

One may wonder whether this input–output activity of CR cells is also related to triggered reelin secretion. This is very unlikely because reelin secretion follows a constitutive mode, as would be expected for an extracellular matrix protein, and is independent of classical stimulus–secretion coupling processes (Lacor *et al*., 2000). Studies of the Orleans *reeler* mutation point to the importance of the C-terminal region of reelin for secretion (de Bergeyck *et al*., 1997), and work on alternatively polyadenylated reelin mRNA has located an important secretory signal to between residues 3328 and 3428 of the protein (Lambert de Rouvroit *et al*., 1999). From a cellular viewpoint, the secretion of reelin from CR cells is postulated to occur along their axon, from specialized smooth cisterns therein (Derer *et al*., 2001). However, the potential problem of CR cell axon early myelination (Ramón y Cajal, 1891; Marín-Padilla, 1998) in regard to this type of mechanism needs to be addressed in future studies. Interestingly, Roberts *et al*. (2005) saw reelin labeling in unmyelinated

axons of adult and fetal human brains. As with other extracellular matrix proteins, secretion is in equilibrium with production (Lacor *et al*., 2000; Derer *et al*., 2001), which still leaves the possibility of a regulation at the transcriptional level. Such a regulation upon CR cells may be operated both upstream, e.g., by DNA topoisomerase IIβ (Lyu and Wang, 2003), and downstream by hormones (Alvarez-Dolado *et al*., 1999), neurotransmitters (Martínez-Galán *et al*., 2001), and in general through the electrical input activity mentioned above.

5 CR Cells, Reelin, and Disease

Concomitant with the above-described advances in the understanding of reelin and CR cell physiology, a growing number of neurologic cases are being linked to defects in the reelin pathway. As many chapters herein are devoted to these cases, we limit our account to cases where CR cells specifically appear to be involved. Curiously, several pathologies display CR cell hyperplasia or persistence in cortex and hippocampus from human fetuses and adults. These pathologies include cyclencephaly (Auroux, 1969), polymicrogyria (Eriksson *et al*., 2001), epilepsy-linked cortical architectural dysplasia (Garbelli *et al*., 2001), and hippocampal sclerosis linked to temporal lobe epilepsy (TLE; Thom *et al*., 2002). Regarding TLE, there is disagreement with another study reporting CR cell hypoplasia possibly due to cytotoxic focal ischemia (Haas *et al*., 2002). Remarkably, CR cell number or morphology appear not to be involved in Alzheimer's disease pathogenesis (Riedel *et al*., 2003; Miettinen *et al*., 2005), although the reelin pathway may be involved independently of CR cells. Thus, it may be speculated that fine tuning of CR cell number and reelin levels, both in the embryo and in the adult, is required for proper brain development and for specific neural functions in the adult. In that respect, though not specifically focused on CR cells, it is important to mention the first discovery of human lissencephaly cases diagnosed as reeler mutations (Hong *et al*., 2000), as it may open the road to more discoveries of neurologic diseases linked to reelin and/or CR cell dysfunction.

6 CR Cells, Reelin, and Evolution

Beyond Cajal's initial (and substantiated) belief that "his special" cells were involved in the brain's ontogenic development, there are now solid reasons to believe that these cells also represent key players in evolutionary drive. Probably the most convincing evidence has emerged recently from a study showing that CR cells of the developing human cortex express *HAR1F*, a novel RNA gene containing a highly evolving "human accelerated region" (Pollard *et al*., 2006). The fact that this type of noncoding gene is probably only involved in the space-time expression of protein-coding genes is not inconsistent with the phylogenic changes (mirrored by ontogenic

changes) observed in CR cells. These changes may be both quantitative, e.g., expressed in CR cell densities and/or degree of reelin expression (Goffinet *et al*., 1999; Bernier *et al*., 1999, 2000; Tissir *et al*., 2003), and qualitative, some peculiar examples being the change of direction of primate CR cell soma and neurites from horizontal to vertical (Retzius, 1893; Zecevic and Rakic, 2001; Cabrera-Socorro *et al*., 2007), or the exclusive expression in human CR cells of the whole downstream part of the reelin pathway (Perez-Garcia *et al*., 2004). Similarly, increased proportions of migratory versus local supply of CR cells may correspond to evolutionary steps from reptile to rodent to man (Bielle *et al*., 2005; Cabrera-Socorro *et al*., 2007). Consistent with their plasticity, CR cells are evolutionarily conserved, as reflected by their ubiquity over all amniotes and even amphibians. To better understand their developmental roles, it would be essential, whenever possible, to distinguish those related to reelin function from unrelated ones. For instance, it appears that reelin is involved in other, perhaps more fundamental processes than cortical lamination (Kikkawa *et al*., 2003; Tissir *et al*., 2003), such as tangential (in addition to radial) migration (Morante-Oria *et al*., 2003). In fact, the reelin gene is expressed in all vertebrates (Goffinet *et al*., 1999), and its sequence is highly conserved, consistent with the idea that lability is geared toward space-time expression rather than molecular structure (Bernier *et al*., 1999). Moreover, this fits a potential regulation by *HAR1F* (Pollard *et al*., 2006). Thus, for instance, a particular coordination between increased spatialization of CR cells and their reelin secretion timing may have provided a framework for the three-dimensional architecture of the mammalian neocortex (Fairén *et al*., 2002; Nishikawa *et al*., 2002; Alcántara *et al*., 2006).

At the base of their most conspicuous reelin-independent role, the electrical activity of CR cells is probably related to transient priming of maturing cortical wiring. Several aspects of this activity are suggestive of a state of persistent immaturity (Mienville, 1999), which is entirely consistent with high labile potential. To summarize, we may suggest that CR cells were recruited as instructing constituents of the archicortex, where they subserve, in the adult, a still undefined role perhaps linked to synaptic plasticity. Their presumptive high labile potential may have promoted them to an active part of neocortical evolution. The reason for which this "special mission" was coselected with their timed degeneration remains to be discovered.

The past 10 years have seen much progress in our understanding of the detailed mechanisms that govern cortical lamination, wherein CR cells play a pivotal role. As such, these cells and their product(s) should continue inspiring investigators, inasmuch as they stand at a unique crossroads between phylogeny, ontogeny, and disease.

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Chapter 19
Reelin and Odontogenesis

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Contents

1 Introduction

Human tooth is made of three different hard tissues: enamel—recovering the crown and corresponding to the most mineralized tissue found in the body; cementum—deposited on the root surface; and dentine—underlying the enamel and cementum and forming the bulk of the tooth. Dentine covers the pulp, which lies in the center

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of the tooth. Pulp, the vital mesenchymal tissue, contains: (1) a specialized layer of cells at its periphery (the odontoblast layer) that is responsible for the dentine matrix synthesis and (2) blood vessels and nerves. During development, tooth pulp acquires a profuse nociceptive and sympathetic innervation from the trigeminal ganglion (TG) and the superior cervical ganglion, respectively.

The main symptom of tooth hypersensitivity, or more precisely, dentine hypersensitivity, is a sharp sudden pain of short duration in response to thermal stimuli, such as intake of cold or hot foods, but may also arise from tactile stimuli, e.g., the use of a toothbrush. Dentine hypersensitivity may arise as a result of loss of enamel by attrition, abrasion, or erosion and/or root surface denudation with exposure of the underlying dentine. Up to 30% of adults experience this situation at some time during their lifetime. And now, an increasing number of adolescents are concerned due to excessive dietary acids, such as citrus juices and carbonated drinks.

The mechanism underlying dentine sensitivity has been widely discussed over the years, and several hypotheses have been proposed to explain this process (Dowell and Addy, 1983). The "hydrodynamic theory," developed in the 1960s, postulates that rapid shifts, in either direction, of the fluids within the dentinal tubules, following a stimulus application, result in activation of a nerve receptor sensitive to pressure, which leads to the transmission of the stimuli in the pulp/inner dentine region of the tooth (Brannstrom, 1962). However, trigeminal sensory nerve endings penetrate no more than 0.1–0.2 mm in coronal dentine tubules. This fact does not fully support the view that intradentinal axons could be directly elicited by stimuli applied to the teeth. Recently, an array of evidence has shown that odontoblasts, rather than nerve fibers, may operate as sensor cells. These neural crest-derived cells play a central role during the formation of dentine. They extend a long cell process in the dentinal tubules and their body is enclosed in a dense network of sensory pulpal axons.

Two kinds of mechanosensitive K^+ channels (KCa and TREK-1) have been identified in human odontoblasts (Allard *et al*., 2000; Magloire *et al*., 2003). Moreover, human odontoblasts *in vitro* produce voltage-gated tetrodotoxin (TTX)-sensitive Na^+ currents in response to depolarization under voltage clamp conditions and are able to generate action potentials (Allard *et al*., 2006). These findings indicate that odontoblasts may be able to convert pain-evoking fluid displacement within dentinal tubules into electrical signals, strengthening their possible role as tooth sensor cells that initiate tooth pain transmission. These results raise the question of how the firing of odontoblasts is transmitted to the neighboring nerve cells. In fact, the way odontoblasts and nerve cells may communicate remains unclear.

Recently, we have shown that reelin, a large extracellular matrix glycoprotein elaborated by odontoblasts, could promote adhesion between nerve endings and cells (Maurin *et al*., 2004). This close association suggested that odontoblasts and nerve endings may directly interact, although no synaptic structures or any junction could be detected between them (Ibuki *et al*., 1996).

This chapter will review the development of dental innervation, the signaling molecules involved in this process, and the putative role of reelin in odontogenesis.

2 Establishment of Tooth Innervation

2.1 The Embryonic Stages of Tooth Development

Several studies of the localization of nerve fibers in human and murine teeth have shown that dental axon growth and patterning take place in a spatiotemporally controlled manner and are tightly linked with advancing tooth morphogenesis (Pearson, 1977; Mohamed and Atkinson, 1983; Tsuzuki and Kitamura, 1991; Fristad *et al*., 1994; Hildebrand *et al*., 1995; Luukko, 1997; Kettunen *et al*., 2005). Tooth development is generally divided into three main stages, defined as initiation, morphogenesis, and cell differentiation. Trigeminal axons are present in the maxillary and mandibular processes before any structural signs of tooth formation are detectable. Then, a plexus of nerve fibers is seen in the mesenchyme, beneath the thickened oral epithelium, during the formation of dental lamina, which is the first histological sign of tooth formation. As the dental epithelium thickens and the underlying mesenchyme undergoes condensation, axon sprouts grow toward the mesenchyme and continue to the epithelium as lingual and buccal branches. During the next stages of morphogenesis, at the cap and early bell stages, local tooth primordial axons form a plexus at the base of the primitive dental papilla and come into contact with the dental follicle. Sensory axons first enter the dental pulp in the late bell stage, at the onset of enamel formation (Fig. 19.1A–D; for review, see Hildebrand *et al*., 1995).

2.2 The Postnatal Stages of Tooth Development

Newly erupted human teeth, with an incomplete root formation, have a sparse innervation. They are less sensitive to electrical or thermal stimulation. A rapid development of sensory pulpal axons arises during root formation after tooth eruption, leading to the formation of the subodontoblastic plexus of Raschkow. The sensory nerve endings originating from the plexus of Raschkow increase in the odontoblastic layer, coiling around the cell bodies and processes of odontoblasts in the dentinal tubule (Fig. 19.1E–H). Dentinal innervation begins at the tips of the pulp horn, and the pioneer axons enter the dentine, just before tooth eruption.

Trigeminal nerve endings are distributed within a gradient, with the greatest innervation at the tip of the pulp horn where the sensitivity is also the greatest. A less dense innervation is observed as distance to the pulp horn tip increases, and root dentine is only slightly innervated. However, a greater number of nerve fibers are observed in human root dentine than in the rodent one. This situation could explain the high sensitivity of exposed human root dentine during thermal stimulation and carious process.

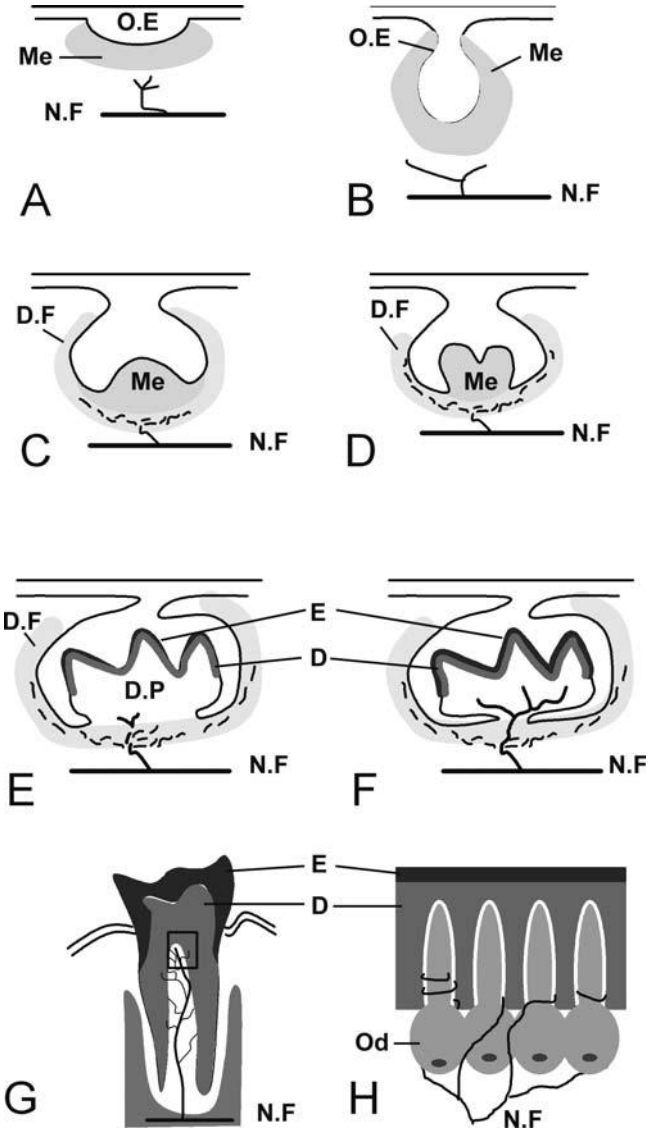

Fig. 19.1 Schematic representation of dental innervation during tooth development from embryonic stages **(A—D)** to postnatal stages **(E—H). (A**) Epithelial thickening stage. A plexus of nerve fibers is observed in the mesenchyme beneath the thickened oral epithelium. (**B**) Bud stage. The oral epithelium thickens and the mesenchyme undergoes a condensation. Axon sprouts grow toward the mesenchyme and continue to the epithelium as lingual and buccal branches. (**C**) Cap stage. Local axons form a plexus at the base of the primitive dental papilla and come into contact with the dental follicle. (**D**) Early bell stage. The number of axons increases in the dental follicle. (**E**) Late bell stage. At the onset of amelogenesis and dentinogenesis, the first sensory axons enter the dental papilla. (**F**) During early root formation, the number of pulpal axons increases. (**G**) During tooth eruption and with the advancing root formation, a rapid development of sensory pulpal axons leads to the formation of the subodontoblastic plexus of Raschkow. (**H**) Enlarged schematic representation of the dentin pulp complex innervation. The sensory nerve endings originating from the plexus of Raschkow coil around the cell bodies and processes of odontoblasts in the dentinal tubules. D, dentin; D.F, dental follicle; D.P, dental papilla; E, enamel; Me, mesenchyme; N.F, nerve fiber; Od, odontoblasts; O.E, oral epithelium (*See Color Plates*)

Anterograde axonal transport labeling methods in animal molars have shown that sensory nerves innervating dentine can extend up to 0.1–0.2 mm into the dentinal tubules at the tip of the crown (more than 50% of the dentinal tubule can be innervated in this area) (Byers *et al*., 2003). They usually penetrate shorter distances in other coronal regions. Some of the nerve fibers appear to form adhesive contact with the odontoblasts inside the dentinal tubules. An extracellular space is retained between these two cells, but the junctions are not of the gap or synaptic form (Ibuki *et al*., 1996). This space—about 20 nm wide—could be used for the delivery of signaling substances in molecular or ionic forms.

In human teeth, several years elapse between eruption and the root apex closure corresponding to a slow neural maturation. Then, by the time occlusion has been established, most of the dentinal innervation at the tip of the cusp has formed. (for review, see Hildebrand *et al*., 1995).

3 Molecular Signaling and Pulp Innervation

As described above, the pulpal nerve development is closely coordinated with tooth development. It seems that growth and establishment of nerve terminals are controlled by local molecular signals. The specificity of dental sensory innervation and the highly plastic formed system imply that signals involved in the regulation of axon growth must be selective in terms of sensory modalities. The regulation of the development of tooth sensory nerve supply at a molecular level is beginning to be elucidated. There is accumulating evidence that the molecules involved in the development of the peripheral nervous system are also involved in dental axon guidance (for a review, see Luukko *et al*., 2005). Among them, neurotrophic factors [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-line-derived neurotrophic factor (GDNF)], axon guidance molecules (semaphorins, netrins, and ephrins), and extracellular matrix proteins (laminin, fibronectin, and tenascin) have been shown to play a role in the establishment of the mesenchymal dental axon pathway (Nosrat *et al*., 1997, 1998; Loes *et al*., 2002; Mitsiadis *et al*., 2003; Lillesaar and Fried, 2004; Fried *et al*., 2005; Kettunen *et al*., 2005; Maurin *et al*., 2005).

NGF, GDNF, and netrin-3, which exert positive effects on axon growth, are specifically expressed in the mesenchymal dental axon pathway when pioneer dental axons are navigating toward the early bud stage tooth germ (Luukko *et al*., 2005). Subsequently, *Ngf* becomes expressed together with *Gdnf* in the dental follicle target field (Luukko *et al*., 1997; Nosrat *et al*., 1998). Interestingly, semaphorin 3a, a secreted chemorepellant, is present in the developing tooth in sites that are devoid of nerve fibers, and its pattern of expression is complementary to the one of NGF and GDNF. Together, these molecules draw a corridor in the jaw mesenchyme for the trigeminal axons toward the tooth target area.

Postnatally, laminin-8 (Lm-411) and tenascin-C are expressed by tooth pulp fibroblasts and can therefore participate in the dental mesenchyme innervation (Sahlberg *et al*., 2001; Fried *et al*., 2005). Laminin-8 has been shown to promote

neurite outgrowth from trigeminal ganglion sensory neurons. Both NGF and BDNF genes are upregulated in the odontoblastic target area of the dental papilla consisting of dentine, predentine, odontoblasts, and the underlying mesenchymal subodontoblastic area before axon ingrowth to the dental papilla (Luukko *et al*., 1997), though they appear not to initiate axon growth to the dental papilla.

NGF, however, seems to be essential for the development of the pulpal innervation. Indeed, treatment of newborn rats with anti-NGF antibodies reduces the amount of sensory nerve fibers in dental papilla (Qian and Naftel, 1996). Moreover, NGF knockout mice are devoid of pulpal innervation (Byers and Närhi, 1999).

During root formation, NGF, GDNF, and BDNF mRNAs are mainly detected in the dental pulp, the odontoblast layer, and the subodontoblast zone. Their pattern of expression correlates with onset of dentine innervation. Moreover, their receptors, TrkA, GFR-a1, and TrkB, respectively, are expressed by trigeminal ganglion neurons (Nosrat *et al*., 1997). We have also shown that semaphorin-7A (Sema 7A) is specifically expressed at this stage by odontoblasts and is closely correlated with the odontoblast/dentine innervation process (Maurin *et al*., 2005).

All of these molecules can then promote the final step of dentinal innervation by guiding pulpal developing axons into this final target field.

4 Pulpal Nerve Fiber Plasticity: Putative Role of Odontoblasts

Neuronal pulpal changes occur throughout the life of the tooth, and remodeling of nerve distribution occurs alongside, with the shift from the primary to the permanent dentition, and with aging and dental injury. During pulpal inflammatory processes (Byers and Närhi, 1999), sensory nerve fibers react to dentine injury by sprouting extensively of their terminal branches in the adjacent surviving pulp. In addition, extent and duration of the response (localized in time and space) depend on the severity and the nature of the injury, as well as the survival of odontoblasts. During reactive dentinogenesis following minor dentine injuries, original odontoblasts are not altered, and the sprouting of sensory nerves is related to the increase of dentinal sensitivity noted in human teeth after cavity drilling (Anderson *et al*., 1967). There is evidence that neurotrophic factors might be involved in this process, and the transient increase in the level of NGF originating from adjacent pulp cells surrounding the injured pulp has been strongly suggested (Byers *et al*., 1992). In contrast, a deep dentin cavity or a small pulp exposure causes destruction of odontoblasts, and the absence or reduction in the sensory innervation in the underlying pulp which is probably related to the laying down of reparative dentine by odontoblast-like cells. Thus, the regeneration of pulpal axons seems to be under the control of factors originating from odontoblasts. Laminin, an adhesive molecule bathing the odontoblast layer and, particularly, the α 1 and 2 subunits (Salmivirta *et al*., 1997), has also been suspected of guiding the regenerating axons (Fried *et al*., 1992). In this context, the role of extracellular matrix molecules, such as tenascin and reelin, elaborated by odontoblasts, in the growth and orientation of nerve endings during tooth injury remains an open question.

5 Expression of Reelin and Its Receptor During Odontogenesis

Recently, reelin was identified in a subtractive cDNA library from human odontoblasts (Buchaille *et al*., 2000). This finding raised the question of the role of this molecule, described first as an axon guidance molecule in the central neural system, during tooth development, and particularly during the terminal innervation of the dentine pulp complex.

5.1 Reelin: A Role in Tooth Morphogenesis?

To date, very few studies have mentioned the reelin expression during tooth development and developing tooth germ. Ikeda and Terashima (1997), analyzing reelin mRNA localization during mouse development, reported some transient or permanent expressions in different peripheral organs, i.e., liver, kidney, testis, ovary. They

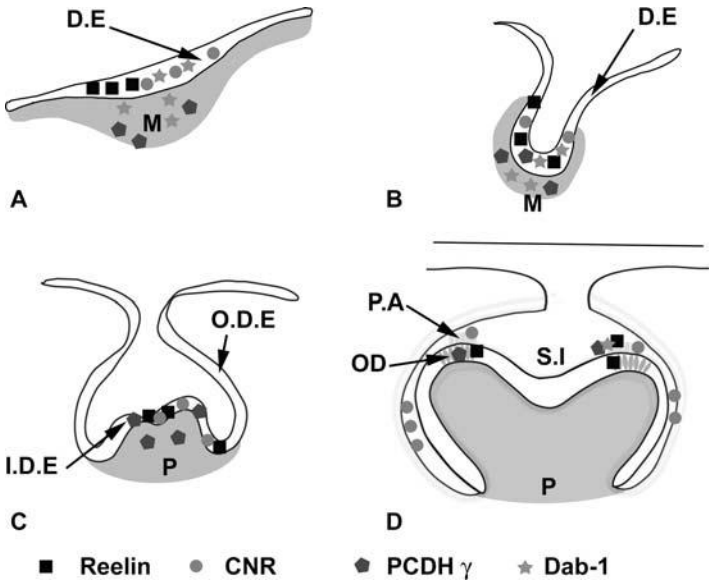

Fig. 19.2 Schematic representation of reelin gene expression and its receptors during successive stages of odontogenesis. Reelin is first detected in the oral epithelium from the initiation stage through the early bell stage. Then, reelin expression shifts in differentiating odontoblasts at the late bell stage. Dab1 is mainly expressed in both oral epithelium and dental mesenchyme during the initiation stages (epithelial thickening and bud stages). CNRs are present in the epithelium through the tooth development whereas PCDH-γ is expressed in both epithelial and mesenchymal compartments. D.E, dental epithelium; I.D.E, inner dental epithelium; M, mesenchyme, OD, odontoblasts; O.D.E, outer dental epithelium; P, dental papilla; P.A, preameloblasts; S.I, stratum intermedium (*See Color Plates*)

observed a positive signal in the tooth at the cap stage. Another study reports the expression patterns of reelin and the new protocadherin families CNRs and PCDH-γ during mouse odontogenesis (Heymann *et al*., 2001).

During the thickening of the oral epithelium (corresponding to the initiation stage), expression of reelin transcripts is restricted to the epithelial layer, whereas reelin receptors —cadherin-related neuronal receptor (CNR) and protocadherin γ (PCDH-γ) —are detected in the mesenchyme underlying the dental epithelium (Heymann *et al*., 2001). Dab1 (disabled-1), the reelin cytoplasmic adapter, first similarly distributed in both epithelium and mesenchyme, decreases as a function of the increasing level of differentiation of ameloblasts and odontoblasts. During this last step of tooth development (bell stage), PCDH-γ is also identified in differentiating ameloblasts (Fig. 19.2). Taken together, these findings show that a correlation could exist between expression of reelin and its receptors during odontogenesis, thus giving putative roles in morphogenesis and differentiation processes.

Given the role of reelin in architectonic brain development, however, its expression in mature human odontoblasts (Maurin *et al*., 2004), and the unique nerve–odontoblast relationship, it is tempting to speculate on a relevant physiological role for reelin in the plasticity of dental pulp axons (adhesion/recognition) in adult teeth.

5.2 Reelin: A Role in Dental Pain Transmission?

In situ hybridization and immunohistochemistry performed on human pulp tissues show a restricted expression of reelin in the odontoblast layer at the gene and protein level (Fig.19.3) (Maurin *et al*., 2004). *In vitro*, using cultured odontoblasts differentiated from pulp cells (Couble *et al*., 2000), reelin is identified as large patches in the microenvironment of the odontoblast membrane. In addition, the coculture of trigeminal axons with odontoblasts (Maurin *et al*., 2004) successfully mimics the *in vivo* situation demonstrating that a single neurite could be associated with an odontoblast cell through a varicosity (bead nerve terminal) (Fig. 19.3). Interestingly, this close relationship is also reelin-dependent. These findings raise the question of a possible expression of reelin receptors by neighboring trigeminal ganglion afferent axons. Recently, we provided evidence that VLDLR, CNR, and Dab1 (assumed as reelin receptors and cytoplasmic adapter) were expressed in rat trigeminal ganglion, suggesting that reelin could constitute a critical functional link between odontoblast cell membrane and nerve terminals (Maurin *et al*., 2004). This close association between cells could be assumed as the earliest step of tooth pain transmission. Indeed, based on the spatial situation of odontoblasts, nerve endings, and fluid movements in dentinal tubules, nociceptive responses may result from an increase in intradentinal pressure, which, in turn, might activate nerve endings. Thus, reelin could have a pivotal role in the extension and branching of pulp axons in target areas as in other tissues, such as the retina, the hippocampus, or the olfactory system (Borrell *et al*., 1999; Rice and Curran, 2000; Teillon *et al*., 2003), thus participating in events underlying the sensory transduction in teeth. A similar role has recently

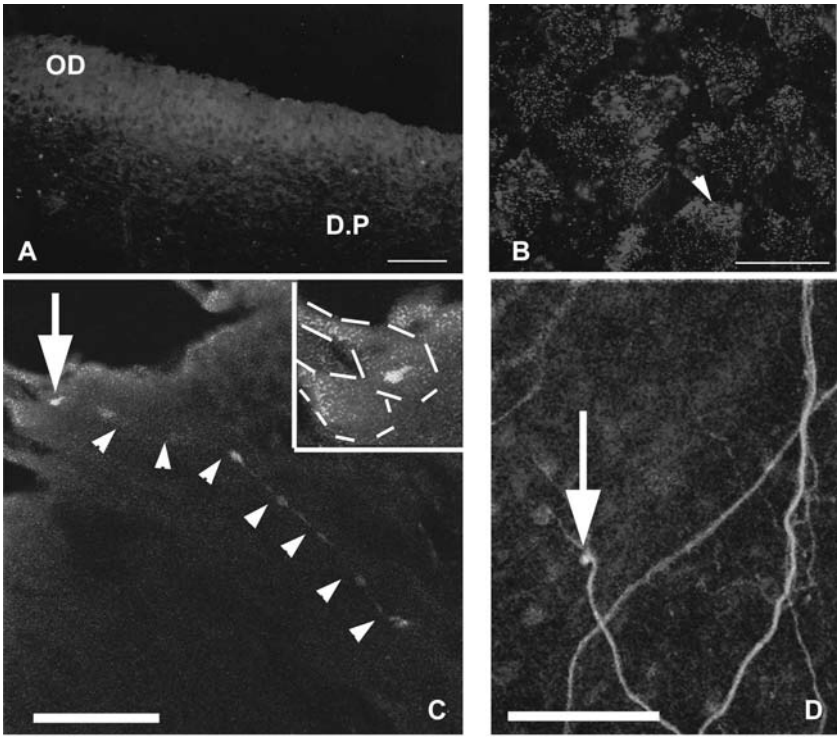

Fig. 19.3 Expression of reelin in human odontoblasts. (**A**) An immunolabeling of reelin performed with anti-reelin antibody 142 shows a signal in the odontoblast layer (OD). No staining is observed in dental pulp cells (D.P) (bar is 100 µm). (**B**) Immunofluorescence labeling with the same antibody, and without permeabilization of the cells, appears as reelin-positive patches localized around the cultured odontoblast cell membrane (arrowhead) (bar is 100 µm). (**C**) A double immunostaining with the monoclonal anti-reelin antibody and a polyclonal anti-neurofilament H on a human dental pulp section was analyzed by confocal microscopy. The nerve fiber course in the pulp can be followed (arrowheads). A yellow patch observed in a nerve varicosity, indicates a colocalization between nerve fiber and reelin close to the odontoblast membrane (arrow and insert) (bar is 20 µm). (**D**) Coculture of human odontoblasts and rat trigeminal ganglion shows the same colocalization (yellow) of reelin (red) and the varicosity (green) in the odontoblast cell layer (bar is 20 µm). [Modified from Maurin *et al*. (2004). Expression and localization of reelin in human odontoblasts. *Matrix Biol*. 23:277–285, with permission from Elsevier] (*See Color Plates*)

been demonstrated using the mutant *reeler* mice showing that reelin signaling is essential for the development of central circuits underlying nociception (Villeda *et al*., 2006). Accordingly, the putative role of reelin in tooth pain transmission is strengthened by the recent evidence for excitable properties of odontoblasts, concentration of mechanosensitive channels (Allard *et al*., 2000; Magloire *et al*., 2003) in the borderline between cell extension, and cell bodies and clustering of key molecules at the site of odontoblast–nerve contact. Finally, taken together, these findings strongly suggest that odontoblasts may operate as sensor cells (Allard *et al*., 2006).

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Chapter 20
Homozygous and Heterozygous Reeler Mouse Mutants

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1 Introduction

Reelin is a large-molecular-weight protein secreted during early embryonic development into the extracellular matrix by Cajal-Retzius cells, a temporary population of GABAergic neurons located in the uppermost layer of the developing cortex (marginal zone). In the adult, reelin is exclusively located in GABAergic interneurons of the upper layers of the cortex, and following secretion into the extracellular matrix, surrounds dendritic spines of pyramidal cells located in every layer (Costa *et al*., 2002). Glutamic acid decarboxylase 67 (GAD67)-positive interneurons of Layer I also exclusively contain DNA methyltransferase 1 (DNMT1) (Ruzicka *et al*., 2007).

The RELN gene, located on human chromosome 7q22, encodes a 3460-amino-acid protein that is 94% identical to the mouse protein. The promoter region of RELN is rich in CpG islands and, therefore, prone to DNA methylation which results in transcription downregulation of reelin (Chen *et al*., 2002). Significantly, GABAergic neurons of postmortem brains of schizophrenia and bipolar disorder patients with psychosis are characterized by both reelin downregulation and DNMT1 upregulation (Veldic *et al*., 2004, 2005). These findings suggest the possibility that reelin promoter methylation by DNMT1 is involved in the dendritic spine hypoplasticity and GABAergic tone downregulation operative in schizophrenia (Costa *et al*., 2001).

2 The *Reeler* Mouse and Neurodevelopmental Disorders

The homozygous *reeler* mouse lacks reelin and has been instrumental in deciphering reelin's role in embryonic neurodevelopment. There are several strains of *reeler*; all have abnormalities in the coding region of the reelin gene resulting in various degrees of reelin expression downregulation, but there are discrete strain differences in loss of reelin function depending on which region of the gene is affected (D'Arcangelo, 2006).

Studies of *reeler* mouse have shown that reelin expression is critical for the normal inside-out layering of the cerebral cortex; earlier born neurons normally stop their radial migration in deeper layers of the cortex, and later born neurons navigate around them into upper layers. In *reeler* mutant mice, cortical layering is reversed (Caviness, 1982). During development, GABAergic neurons from the ganglionic eminence migrate tangentially to the cortex, but this migration does not appear to be directly affected by lack of reelin (Hevner *et al*., 2004). The subsequent radial migration of these interneurons toward an appropriate cortical layer (Yabut *et al*., 2007) occurs in a manner that preserves interneuron/pyramidal neuron laminar position even though cortical layers are inverted (Manent *et al*., 2006; Pla *et al*., 2006). Areas of the brain besides the cortex are abnormal in reelin deficient mouse, among them the hippocampus (Caviness and Sidman, 1973), cerebellum (Goffinet *et al*., 1984), olfactory bulb (Wyss *et al*., 1980), striatum (Marrone *et al*., 2006), and the rostral telencephalic cholinergic system (Sigala *et al*., 2007). The loss of cellular organization in the cerebellum is responsible for the reeling gait after which the

mutant is named. Not surprisingly, given their substantial neurobiological abnormalities, a large number of behavioral dysfunctions in addition to a reeling gait have been recognized in *reeler* mouse (Salinger *et al*., 2003; Laviola *et al*., 2006; Marrone *et al*., 2006).

The homozygous *reeler* mouse has been suggested as a model for understanding abnormal neuronal migration and even for human lissencephaly (D'Arcangelo, 2006). A human equivalent of the null *reeler* mutant apparently does exist, but afflicted individuals are extremely rare (seven individuals have been described to date). They have severe lissencephaly and cerebellar atrophy, probably preventing adult survival (Hong *et al*., 2000; Chang *et al*., 2007). It is possible that the null mutant genotype is more prevalent in human populations, but the fetus is usually not viable.

However, if given special care, *reeler* mice can survive into adulthood and may even be fertile, serving as a practical model for studying reelin function. Human stem cells, injected into the lateral ventricles of *reeler* mice, fail to migrate or display typical differentiation patterns, while stem cells injected into wild-type mice (WT) migrate and differentiate normally (Kim *et al*., 2002). Won *et al*. (2006) have reported that *reeler* mice have a defect in adult neurogenesis, resulting in larger brain infarcts than WT following middle cerebral artery occlusion and larger excitotoxic lesions after intracerebral injection of *N*-methyl-D-aspartate (NMDA). Also, several other mutant or knockout mice that serve as models for severe neurological and psychiatric disorders involve mutations that include components of the reelin signaling pathway, or proteins that are operative in the reelin signaling pathway (recently reviewed by D'Arcangelo, 2006). Appropriate examples are mice with mutations of DAB1 (the adapter protein for reelin), mice heterozygous for Lis1, and mice with mutations of the receptors for reelin [apolipoprotein ER2 receptor (ApoER2) and very-low-density lipoprotein receptor (VLDLR), or integrin $\alpha 3$] (D'Arcangelo, 2006). These mutants may have a phenotype similar to *reeler* and have helped to provide valuable insight into the reelin signaling pathway and the consequences of its disruption. Significantly, reelin is overexpressed in cortex and CSF in several neurodegenerative diseases, including Alzheimer's disease (Botella-Lopez *et al*., 2006). The mechanism is unknown, but Herz and Chen (2006) have proposed a role for reelin lipoprotein receptors in Alzheimer's disease.

In a potentially significant new development with implications for future understanding of reelin function, Pollard *et al*. (2006) have reported that a noncoding RNA gene (HAR1F) is coexpressed with reelin in Cajal-Retzius neurons of human cortex from 7 to 9 gestational weeks which is a critical period for cortical neuron migration. Presumably, a similar situation is operative in mice.

3 The Heterozygous Reeler Mouse (HRM) as a Model for Nondegenerative Psychiatric Disorders

Disorders such as schizophrenia have long puzzled neuroanatomists because of the failure to find evidence of obvious abnormalities or degeneration and cell loss commensurate with the extreme behavioral manifestations. Therefore, interest in the

role of reelin in psychosis escalated when reelin was found to be 50% downregulated in psychotic postmortem brain (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000). Downregulation of reelin in schizophrenia and in bipolar disorder has been replicated in other laboratories and brain cohorts and is considered the most consistent finding in schizophrenia postmortem brain tissue (Torrey *et al*., 2005). Moreover, the decrease in reelin expression has been extended to other similar nondegenerative disorders of neurobiological function, including autism (Fatemi *et al.,* 2001, 2002, 2005), suggesting a link among these psychiatric disorders.

A small number of heterozygous individuals with mutations of the RELN locus have been described in the families of the seven homozygous probands studied to date (Hong *et al.,* 2000; Chang *et al*., 2007). These heterozygous relatives appear to express various psychiatric pathologies but have no specific psychiatric diagnosis in common. It is possible that they could have other gene mutations in addition to the reelin gene mutation, or that the expression of reelin in GABAergic neurons might vary in individuals due to the embryonic environment, epigenetic factors, or to compensatory mechanisms for the reelin deficit. Supporting a possible epigenetic role in reelin and GABAergic tone downregulation is the finding that DNMT1 is upregulated in GABAergic neurons of psychotic postmortem brain (Veldic *et al*., 2004, 2005; Abdolmaleky *et al*., 2005; Ruzicka *et al*., 2007).

While the null mutant *reeler* mouse is not suitable as a stand-alone model of psychosis because of the mutant's extreme structural brain impairment and associated behavioral abnormalities, knowledge about the role of reelin in the neurodevelopment of *reeler* mouse is important for the HRM model. The schizophrenia literature strongly suggests the importance of neurodevelopment in setting the stage for schizophrenia vulnerability (Rehn and Rees, 2005). Environmental insults, such as drugs, malnutrition, hypoxia, and viral infection, are hypothesized to interact with specific stages of embryonic development and with the presence of schizophrenia-related genes, including reelin. A complex model in which nonspecific genetic factors that increase susceptibility to developmental abnormalities interact with specific genetic factors and epigenetic and compensatory events has been proposed to explain the etiology of schizophrenia (Avila *et al*., 2003).

3.1 Importance of Reelin in Neurogenesis and Synaptic Plasticity

The pleiotropic nature of the RELN gene accounts for its essential role in normal neurogenesis and synaptic function in the adult cortex and hippocampus. Following reelin secretion into the extracellular matrix by GABAergic interneurons located in the upper cortical layers, reelin surrounds spines of glutamatergic pyramidal cell dendrites, where there is evidence that it plays a role in modulating pyramidal neuron dendritic spine structures and morphology in excitatory synapses, which include the NMDA receptor implicated in various aspects of memory function (Costa *et al*., 2001; Dong *et al*., 2003). Importantly, dendritic spine density in prefrontal cortical pyramidal neurons is decreased in schizophrenia (Glantz and Lewis, 2000; Hill *et al*., 2006).

In support of reelin's role in synaptic function, Qiu *et al*. (2006; Qiu and Weeber, 2007) reported that HRM exhibits profound impairment in hippocampal CA1 excitatory postsynaptic potentials, paired pulse facilitation ratio, long-term potentiation and depression, as well as a reduction in spontaneous inhibitory postsynaptic currents. Qiu *et al*. (2006) also provide evidence that these synaptic impairments in hippocampal plasticity and functional inhibition at excitatory synapses may underlie behavioral deficits in hippocampal associative function, including the deficits in contextual fear conditioning and prepulse inhibition of startle (PPI) in HRM.

3.2 Reelin Downregulation and GABAergic Tone Deficit in Psychosis

Reelin downregulation in psychosis has been linked to GABAergic tone deficit (Costa *et al*., 2001; Carboni *et al*., 2004). Glutamic acid decarboxylases 65, 67 (GAD65, 67) catalyze the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) from glutamate. Downregulation of the GAD67 isoform is a critical aspect of the HRM model of psychosis (Liu *et al.,* 2001) because, along with reelin, GAD67 mRNA and protein are also downregulated in postmortem cortex and hippocampus of psychotic patients (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000; Akbarian and Huang, 2006). GAD65 appears not to be downregulated in psychotic postmortem brain, but this may depend on diagnosis, area of the brain studied, or other factors (Fatemi *et al*., 2002). Importantly, the localization of the two GAD isoforms may differ in different subpopulations of GABAergic neurons (Volk *et al*., 2000) and in different brain areas. Thus, there is increasing evidence that the neurobiological abnormalities and behavioral dysfunctions observed in schizophrenia are related to an epigenetically induced and coordinated downregulation of GAD67 in specific types of GABAergic interneurons (Grayson *et al*., 2005, 2006; Lewis *et al*., 2005), which is likely to be related to the decrease in reelin and to the increase in DNMT1 expressions in these same neurons (Veldic *et al*., 2004, 2005; Ruzicka *et al*., 2007).

GAD67 downregulation in HRM is only about 30% (Liu *et al*., 2001), i.e., the downregulation of GAD67 expression in HRM is less severe than in psychotic patients where the GAD67 downregulation is approximately 50% (Guidotti *et al*., 2000). Interestingly, GAD67 heterozygous mice do not reveal downregulation of reelin expression (Liu *et al*., 2001; Carboni *et al.,* 2004). Together, these findings suggest that a number of factors, in addition to reelin and GAD67, may be contributing to the onset of psychosis.

3.3 Controversy over the Usefulness of the HRM Model

HRM was an obvious animal model for evaluating the role of a 50% downregulation of reelin in psychosis, and it is now apparent that a complete model of reelin downregulation in psychosis needs to include methylation of the reelin gene pro-

moter in GABAergic neurons (Tremolizzo *et al*., 2002). Unlike the homozygous *reeler,* which shows obvious structural and behavioral impairment, the HRM behavioral and biological phenotype appeared similar to WT at first. Only a closer examination revealed neurobiological and behavioral abnormalities reminiscent of abnormalities found in psychosis. We have recently reviewed the HRM model (Tueting *et al*., 2006), and consequently, will only highlight and update this evidence here.

Neurobiological findings in HRM (previously summarized in Table 1 of Tueting *et al*., 2006, p. 1067) are similar to abnormalities found in postmortem brain of psychotic patients and include a decrease in neuropil, especially in frontal cortex, together with increased pyramidal cell density, an increased number of GABAergic cells in underlying white matter, decreased GABA turnover, and indirect evidence for a dysregulation of glutamate, dopamine, and related neuroendocrine functions. Importantly, the decreased spine density on dendrites of pyramidal cells located in Layer III of frontal cortex has been reported both in HRM (Liu *et al*., 2001) and in psychotic patients (Glantz and Lewis, 2000; Hill *et al*., 2006). Since publication of our review (Tueting *et al*., 2006), a report on rostral regions of the basal forebrain and medial cortex indicates significant redistribution of cholinergic neuron innervation in HRM (Sigala *et al*., 2007). There is also a recent report on Purkinje cell loss in male HRM occurring in an earlier age (Biamonte *et al*., 2006) than originally reported (Hadj-Sahraoui *et al*., 1996). Another new report indicates a decrease in the density of μ-opioid receptors in the midbrain of HRM (Ognibene *et al*., 2007a).

However, it is the HRM behavior, not the substantial neurobiological findings summarized briefly above, that has sparked a controversy over the usefulness of the HRM as a psychosis model. A complete absence of behavioral deficits in HRM on standard behavioral test batteries has been vigorously defended (Salinger *et al*., 2003; Podhorna and Didriksen, 2004). Tests that were negative in the Salinger *et al*. (2003) and Podhorna and Didriksen (2004) studies included assessments of sensory function, social behavior, anxiety level, spatial working memory, fear conditioning, and PPI. The absence of a behavioral deficit in HRM, despite extreme neurobiological deficit, is explained by the ability of the developing nervous system to compensate for the consequences of the decrease in reelin (Salinger *et al*., 2003).

On the other hand, the evidence suggesting behavioral abnormalities in HRM compared to WT is substantial and has been recently reviewed (see Table 2 in Tueting *et al*., 2006, p. 1069) and includes deficits in PPI, social interaction and social recognition, contextual fear conditioning, olfactory discrimination learning, and radial arm maze performance (especially following MK801 administration). In addition, there are new reports of behavioral differences between HRM and WT that have been published since our review. These findings include HRM deficits in executive function (Brigman *et al*., 2006) and in learning (Krueger *et al*., 2006), as well as differences between the genotypes in anxiety, risk assessment, motor impulsivity, morphine-induced analgesia (Ognibene *et al*., 2007a), and in subsonic

vocalization and locomotor response during an amphetamine challenge (Laviola *et al*., 2006). Despite the mounting evidence for the existence of subtle behavioral differences between the two genotypes, the usefulness of the HRM model continues to be discounted (e.g., Patterson, 2006).

There are several possible explanations for failure to find behavioral deficits in HRM in certain circumstances, many of which we have reviewed previously (Tueting *et al*., 2006). Here we will expose this work to further scrutiny and revisit the controversy focusing on prepulse inhibition of startle (PPI).

3.4 PPI Deficit in HRM

The fact that the PPI deficit observed in psychotic patients and their close relatives who fail to express a psychiatric disorder is especially intriguing, because PPI is considered to be an endophenotype marker for psychosis that can be studied comparatively in humans and animals. Another advantage of PPI is that much is known about the underlying brain circuitry, anatomy, pharmacology, and genetics with respect to psychosis (Koch, 1999; Geyer *et al*., 2001; Swerdlow *et al*., 2001; Hauser *et al*., 2005).

PPI deficit in HRM was originally reported by Tueting *et al*. (1999) and recently replicated by Qiu *et al*. (2006). However, both Salinger *et al*. (2003) and Podhorna and Didriksen (2004) failed to find a significant PPI deficit in HRM compared to WT. Failure to find a deficit in HRM for an accepted endophenotype for psychosis vulnerability is surprising and requires explanation. The issues that need further scrutiny appear to be failure to measure reelin and variation in breeding procedures, as well as variation in specific parameters of the behavioral experiments and in the social environment, which we will explore in greater depth here.

Methods. Mice used in our experiments to be described were progeny of WT (paternal) and HRM (maternal) pairings in our colony of B6C3F, Edinburgh reelin mutation, originally obtained from Jackson Laboratories. The colony has been maintained continuously in our temperature- and light-controlled vivarium for 20+ generations. After weaning at 21 days, mice are normally housed five of the same sex in a plastic cage with random assignment of genotypes to a cage. Mice were genotyped (Tueting *et al*., 1999) and behaviorally tested (l0:00 and 16:00 hr) with the investigator blind to genotype. They were 4–7 months old, older than in previous studies, and perhaps more vulnerable to environmental adverse events and sensitive to epigenetic influences. Importantly, quantitative reelin mRNA levels were measured using RT-PCR including internal standards. Our results expand on our earlier finding of a PPI deficit in HRM using a broader range of prepulse intervals and suggest the possibility that the extent of reelin and GABAergic deficiencies in neural circuits underlying PPI can be compromised by factors, such as experiment duration and social isolation, to such an extent that normal adaptive neural responses underlying PPI may be altered.

3.4.1 PPI Deficit in HRM Is Present over an Extended Range of Silent Interval Prepulse–Startle Delays

That variation in experimental parameters can affect PPI is well documented. Prepulse intensity and duration, startle intensity and duration, prepulse interval, the interstimulus interval, stimulus modality, the nature and number of the different trial types used, and the noise and lighting background all affect PPI. The 100-ms silent prepulse interval is the most common interval used in human and animal PPI evaluation relevant to translational research in psychopharmacology (Geyer *et al*., 2001). In the current study, we used a wider range of prepulse intervals. The SR-Lab Startle Response System (San Diego Instruments) was programmed so that a trial consisted of a 115-dB startle pulse 30 ms in duration (startle trial) or of the same startle pulse preceded by an 85-dB prepulse 20 ms in duration (prepulse trial). The *Unfilled Interval* program consisted of a random sequence of startle pulse [only] trials and five types of prepulse trials in which the silent interval between prepulse and startle pulse was varied from 40 to 420 ms. The *Filled Interval* program was the same as the *Unfilled Interval* program, except that the prepulse remained on during the prepulse interval up to 20 ms before presentation of the startle pulse. Ratios reflecting the amount of prepulse-induced inhibition of the startle reflex were calculated by subtracting the mean peak amplitude for prepulse trials from the mean peak amplitude for startle pulse [only] trials and dividing by mean peak amplitude for startle pulse trials to normalize, and finally multiplying by 100 to calculate percent inhibition.

In Fig. 20.1A, HRM is compared to WT for the two conditions. When the prepulse was kept on during the prepulse interval (*Filled Interval*), HRM and WT showed equally high levels of prepulse inhibition at all prepulse intervals, and PPI was not systematically related to length of interval. For the *Unfilled Interval* condition, HRM show decreased inhibition compared to WT, and the extent of inhibition decreased as the prepulse interval increased for both genotypes. These findings confirm and extend our earlier finding of PPI deficit in HRM which was based on the 100-ms prepulse silent interval and the same startle intensity (Tueting *et al*., 1999).

3.4.2 The PPI Deficit in HRM Is Greater in Later Trials of the Session

PPI is generally considered to be a stable and reliable measurement between and within sessions when the same parameters are used. The means shown in Fig. 20.1A were calculated on the basis of all 72 trials in the session. The session of 72 trials was designed in three equivalent 24-trial blocks so that changes in PPI during the session could be detected. For this analysis, the data shown in Fig. 20.1A were collapsed across all five prepulse intervals. Fig. 20.1B shows that the PPI deficit in HRM was significantly larger in later trials of the session for the *unfilled* condition. For the *filled* interval condition, PPI decreased during the session equally for WT and HRM. Startle response amplitude for startle [only] trials decreased (habituated)

during the session. However, neither startle response amplitude nor startle habituation differed as a function of genotype (WT versus HRM) or condition (*filled* versus *unfilled* interval). Thus, it is possible that the PPI deficits in HRM compared to WT would fail to be significant in short sessions.

What could explain the differential PPI changes in HRM and WT over the session for the *Unfilled Interval* condition? Since startle response habituation and PPI for the *Filled Interval* condition were similar for HRM and WT over trials, the increased PPI deficit in HRM later in the session for the *Unfilled Interval* condition is probably not related to muscle fatigue, but rather may be related to a functional failure in interconnected neural networks of frontal cortex, hippocampus, amygdala, and nucleus accumbens that underlie inhibition of the startle reflex (Swerdlow *et al*., 2001). The differential PPI changes in HRM and WT over the

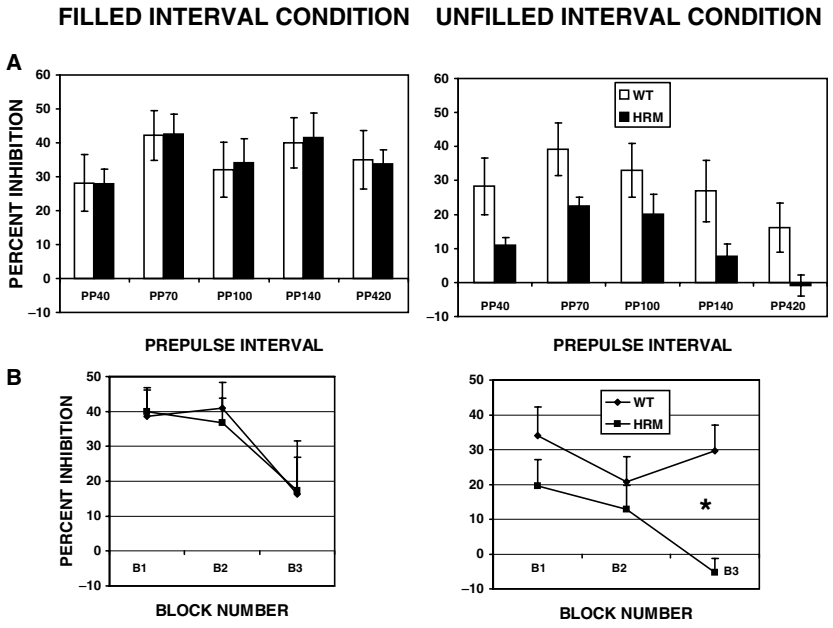

Fig. 20.1 (**A**) HRM show a PPI deficit when the interval between prepulse and startle pulse is silent (*unfilled* interval condition). No deficit is present for the *filled* interval condition not requiring a memory trace of the prepulse. The data were collapsed across intervals and a two-way repeated measures ANOVA performed on the mean PPI difference between the *filled* and *unfilled* conditions. There was a significant effect of genotype (p=0.042) and prepulse interval (p<0.001) [8 male WT, 6 male HRM group-housed mice; 72 trials]. (**B**) PPI in the same experiment was collapsed across the five prepulse intervals and the means shown separately for each of the three consecutive equivalent blocks of 24 trials each. There was no significant difference in PPI between WT and HRM for the *filled* condition for any trial block. An interaction between Genotype and Block was significant for the *unfilled* condition (F=3.550, p=0.045), and post-hoc comparisons (Neuman Keuls) revealed a significant difference between WT and HRM for Block 3. Two-way repeated measures ANOVA (1 factor repetition)

session could be associated with GABAergic tone downregulation and excitatory synapse deficits in HRM with consequent failure of glutamatergic, dopaminergic, acetylcholinergic, or other components of PPI neural circuitry to express the normal compensation for the loss of inhibition.

3.4.3 GABAergic Positive Allosteric Modulators (Agonists) Correct PPI Deficit in HRM in Later Trials of the Session

We initially reported that young male HRM show anxiety on the elevated plus-maze (Tueting *et al*., 1999), which could be interpreted to reflect a downregulation of GABAergic tone in HRM. To test whether the PPI deficit in HRM is related to GABAergic downregulation, we studied whether imidazenil, a specific positive allosteric modulator of the action of GABA at $GABA_A$ receptors (Costa *et al*., 2002), is able to correct the PPI deficit operative in the later trials. Fig. 20.2 shows a replication of the PPI deficit in later trials of the session in a sample of female HRM, confirming the finding in males (Fig. 20.1B). (The deficit occurs earlier than in the male study, but the PPI paradigm was also different.) The deficit in the later trials was corrected by subcutaneous injection of as little as 0.3 and 0.6 mg/kg of imidazenil, injected 20 min before the PPI session. Neither mean startle response nor startle habituation was affected by genotype or by imidazenil.

Increased PPI deficit in HRM in later trials of the session could be related to a nonspecific action (stress?) included in the PPI testing procedure, the effects of which accumulate over the session duration in the presence of a downregulation of GABAergic tone, and a related failure of other neuronal systems due to a decrease of synaptic efficacy (Carboni *et al*., 2004). These systems normally can compensate for some loss of inhibition. This interpretation is supported by the imidazenil-mediated correction of the PPI deficit in HRM in the later trials of the session, since this drug is a positive allosteric modulator of the action of GABA at specific $GABA_A$ receptors, including $\alpha5$ subunits, and is devoid of action on receptors containing $\alpha1$ subunits (Guidotti *et al*., 2005). Interesting in this regard is the fact that the $\alpha5$ subunit of the $GABA_A$ receptor in mutant mice has been related to PPI deficit (Hauser *et al*., 2005). In addition, there is a recent report of decreased social interaction in HRM that can be normalized by imidazenil treatment, a finding associated with an upregulation of the $\alpha5$ subunit of the $GABA_A$ receptor by the benzodiazepine (Doueiri *et al*., 2006).

3.5 PPI in WT is More Sensitive than the HRM Genotype to the Effects of Social Isolation

The two controversial reports that concluded that WT and HRM do not differ in PPI involved a combined sample of group-housed and socially isolated mice (Salinger *et al*., 2003) or socially isolated mice only (Podhorna and Didriksen, 2004). Fig. 20.3

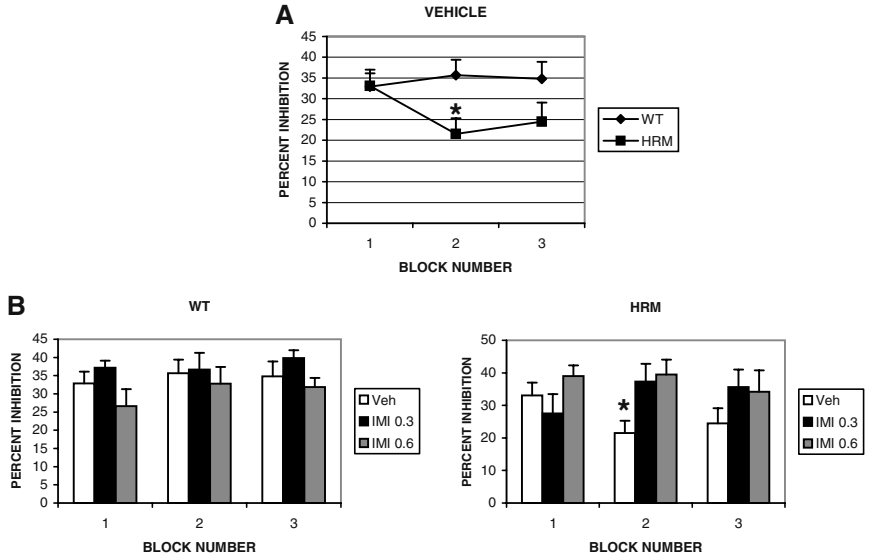

Fig. 20.2 (**A**) There is a significant difference between HRM and WT in Block 2 but not in Block 1. (**B**) Imidazenil (0.3 and 0.6 mg/kg, s.c.) corrects the deficit in PPI present in HRM for the vehicle condition in later trial blocks of the session. Five female WT, five female HRM, 5 months old. (An *unfilled* interval trial sequence consisting of startle only trials was randomly presented with 85- and 80-dB prepulse–startle trials with the prepulse–startle delay constant at 100 ms) *$p < 0.05$

shows that PPI was significantly decreased in HRM compared to WT when mice were group housed, replicating in older mice our earlier findings obtained with young mice (Tueting *et al*., 1999). After 2 weeks of isolation, however, WT and HRM no longer differed in PPI, due to the fact that percent inhibition (averaged across prepulse intervals) declined for WT following isolation (32 ± 4% in group housed but 15 ± 6% following 2 weeks of isolation, $t = 2.689$, $p = 0.031$) and failed to change in HRM or even increased slightly (16.4 ± 3.7% in group housed compared to 16.9 ± 4.2% in isolated).

A decrease in PPI in WT following isolation is consistent with other reports showing that PPI decreases in isolated compared to socially housed rodents (Powell and Geyer, 2002; Sakaue *et al*., 2003). There were no significant differences in mean startle amplitude over blocks as a function of isolation or genotype. Thus, it appears that social isolation in adulthood can obscure the PPI deficit in HRM compared to WT, which could explain the negative findings published by Salinger *et al*. (2003) and Podhorna and Didriksen (2004).

Following 4 weeks of isolation, the reelin expression was measured in the frontal cortex as previously described (Tueting *et al*., 1999; Tremolizzo *et al*., 2002), and its expression was 59% lower in HRM than in WT. In addition, reelin expression varied considerably among individuals both within the WT (80–250 pmole/0.5 µg RNA) and within the HRM group (40–100 pmole/0.5 µg mRNA). Presumably, this

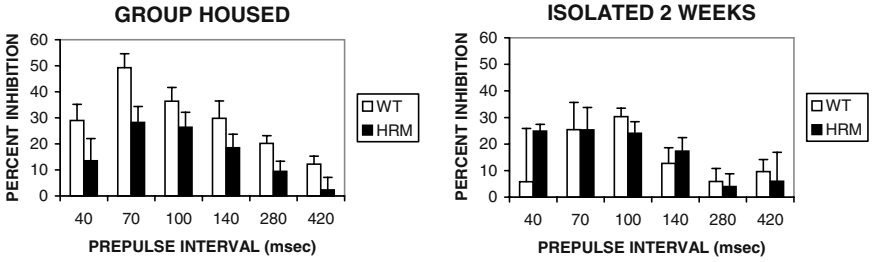

Fig. 20.3 Group-housed male HRM show a deficit in PPI (F=7.509, p=0.017, two-way repeated measures ANOVA with interval as the repeated measure). After 2 weeks of isolation, the difference between WT and HRM was no longer present due to a significant decrease in PPI in WT (t_7=3.071, p=0.018) and no decrease in PPI in HRM. Eight male WT, six male HRM. (Interval trial types collapsed for statistical analysis)

Table 20.1 DNMT1 expression (ratio of DNMT1 to NSE mRNA) is increased in frontal cortex of mice isolated for 3 weeks

	Mean	S.D.
Group housed	1.477	0.289
Socially isolated	2.556*	0.190

* t_7 = 4.321, p =0.003.

increased variance among individual mice is due to epigenetic variance since the coding gene dosage is 100% for WT and 50% for HRM. Moreover, there was a positive correlation between reelin mRNA and PPI when PPI was measured post-isolation and close to sacrifice in both genotypes (WT= +0.25, HRM = +0.854, $p<0.05$). This observation confirms an association between higher levels of reelin and greater prepulse inhibition (implicit in the group housed WT vs HRM difference in PPI) even in mice with the same genetic code for reelin.

The effect of social isolation on HRM and WT was assessed by Salinger *et al.* (2003), but their conclusion that social isolation was without effect was based on higher order statistical interactions that were complex, as both male and female mice and the *reeler* genotype were included in the sample together with WT and HRM. There are known to be substantial sex differences in neurobiological and behavioral responses to social isolation (Pinna *et al.*, 2003, 2004).

We have recently noted a significant increase in DNMT1 in the frontal cortex of isolated Swiss male mice as shown in Table 20.1. An isolation-induced increase in DNMT1 in WT is consistent with methylation of the reelin promoter and could be related to our failure to find a difference in PPI between WT and HRM following isolation. A next step would be to compare DNMT1 expression in HRM and WT in group-housed versus socially isolated mice.

The differential effects of social isolation in HRM and WT mice and the increase in DNMT expression in WT following isolation are findings that are consistent with

a complex interaction between genetic and epigenetic factors influencing reelin expression. Such a complex interaction has recently been reported by Laviola *et al*. (2006) in a study involving a comparison between the locomotor activity of *reeler*, WT, and HRM following amphetamine challenge and by Ognibene *et al*. (2007b) in a study of the consequences of maternal separation on infant *reeler*, WT, and HRM.

4 Discussion and Conclusions

4.1 Interaction of Genetic and Epigenetic Influences on Reelin Downregulation

Studies of HRM have increased our understanding of the neurobiological and behavioral consequences of reelin downregulation. However, a new version of a model to study reelin downregulation in psychosis must also consider the extent of methylation of the RELN and GAD67 promoters, in addition to the genetic coding of these genes (Grayson *et al*., 2005, 2006). In human postmortem psychotic brain, there is evidence that downregulation of reelin is related to hypermethylation of the reelin promoter, as DNMT1 mRNA is overexpressed in cortical GABAergic neurons in which reelin and GAD67 are downregulated (Veldic *et al*., 2004, 2005; Grayson *et al*., 2006; Ruzicka *et al*., 2007). Grayson *et al*. (2005) isolated, bisulfite treatment amplified, and sequenced genomic DNA from the cortices of schizophrenia patients and nonpsychiatric subjects and found increased methylation within the CpG islands of the reelin promoter at positions −134 and −139 (base pairs). These two positions overlap with functionally defined *cis*-acting elements which bind repressor factors which leads to a compromised RELN promoter function. A coordinated hypermethylation of the RELN and GAD67 promoters is likely to be operative (Kundakovic *et al*., 2007).

Additional evidence for epigenetic influences on reelin and GABAergic downregulation is provided by the fact that methionine exacerbates psychotic symptoms and, when given to WT mice, leads to downregulation of reelin and GAD67 (Tremolizzo *et al*., 2002) and to an increased recruitment of methyl-binding domain proteins expressed by RELN and GAD67 promoters (Dong *et al*., 2005). Moreover, HRM and methionine treated WT mice share similar behavioral consequences, presumably due to GABAergic downregulation, including similar PPI deficits and deficits in social interaction and recognition (Tremolizzo *et al*., 2002, 2005).

Now that it appears that DNMT1 expression is greater in socially isolated than group-housed WT mice, the obvious next step is to compare HRM and WT, with respect to epigenetic changes induced by isolation. These future studies might involve measurement of the expression of DNMT1, reelin, GAD67, and methyl-binding proteins in group-housed and socially isolated HRM and WT. Ideally, such future studies would incorporate measurements of the extent of promoter methylation.

4.2 Evaluation of Age and Sex in Future Studies of Reelin Downregulation in Psychosis

Since, in human psychosis vulnerability, age and sex are known to be important variables, these two variables also need to be considered in an animal model to evaluate their role in reelin downregulation in psychosis. Purkinje cell loss has been reported in male, but not female HRM, and is influenced by estrogen (Biamonte *et al*., 2006) and age (Hadj-Sahraoui *et al*., 1996). In addition, reelin deficiency in HRM exacerbates neuronal death resulting from ischemic injury (Won *et al*., 2006), which often increases with age. Our unpublished data suggest that PPI from 1 to 6 months of age is significantly positively correlated with age in WT mice, but not in HRM. Thus, interpreting the relationship between reelin downregulation and behavior will require systematic age-related studies of behavior in both sexes, along with quantitative measurements of reelin and GAD67 expression in the two different genotypes.

4.3 The Need to Account for Compensatory Mechanisms for Reelin and GABAergic Downregulation

The fact that multiple transmitter systems (e.g., dopaminergic, serotonergic, cholinergic) and endocrine function are altered both in psychotic patients (e.g., Lewis and Lieberman, 2000) and in HRM (Tueting *et al*., 2006), suggests a need to explore the mechanisms and the time course of possible compensatory events in those systems that are invoked to balance the reduction of reelin and GABAergic function. Comparing HRM and WT treated with methionine versus saline is a possible strategy that could be explored in greater depth along with direct *ad hoc* measurements of probable indicators of compensations that are operative in other neurochemical pathways. In our studies of deficits in HRM and methionine-treated WT mice (Tremolizzo *et al*., 2002), we detected similar deficits in PPI and in social behavior whether reelin was reduced by genetic code or by methionine, which is interesting, given that compensatory and epigenetic events for compromised reelin and GABA functions have been presumably occurring since conception in HRM but only for a short time in methionine-treated mice.

4.4 Experimental Design Issues for the Future

In evaluating the HRM model for schizophrenia, it is important to consider that conclusions are based on difference scores between WT and HRM. Our failure to find a PPI deficit in HRM following isolation could be explained by the downregulation of RELN and GAD67 genes, and that of other genes mediated by methylation that could differ in extent or in consequence in WT and HRM. Thus, laboratory

differences could reflect sample differences in WT only, in HRM only, or in both WT and HRM. This issue becomes crucial when one hypothesizes that the difference score reflects reelin downregulation as a consequence of genetic coding plus epigenetic and compensatory factors.

Given that epigenetic events may not have the same consequences in all subjects because of baseline differences in the current state of the epigenome, the exploration of within-subject treatment strategies may be useful. Within-subject studies are more common in the human psychosis literature, due to the advantage of having each subject serve as their own control, as patients vary considerably from one to another even within the same diagnostic group. Different experimental controls and potential confounds apply in within-study designs than in between-subject designs. For example, ideally our study of pre- and post-social isolation could be replicated using a separate control group of mice not subjected to isolation.

In conclusion, there is mounting evidence that HRM and WT mice differ in behavior and underlying neurobiology in ways consistent with continued use of HRM as a model for psychosis and related disorders. In addition, the interaction between DNA coding sequences and epigenetic modification in GABAergic neurons that underlie reelin and GAD67 downregulation can be best understood by combining DNA coding and epigenetic profiling (Petronis, 2004; Schumacher *et al*., 2006). Further study of HRM is warranted because reference points for the genetic code are needed in order to interpret the contribution of epigenetic or compensatory influences at each neurodevelopmental stage (Tueting *et al*., 2006).

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