Chapter 21
Reelin and Lissencephaly

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Contents

1   Introduction

The development of the human cerebral cortex is a dynamic process that can be divided into partially overlapping stages occurring during several gestational weeks (Barkovich *et al*., 2005). Migration of postmitotic neurons from the ventricular zone to form the cortical plate comprises one of the most critical stages in brain development. When migration is complete, the cortex is a six-layered structure, with each layer comprising different types of neurons that form discrete connections within the CNS and perform distinct functions (O'Rourke *et al*., 1992). When neurons reach their destination, they stop migrating and order themselves into specific "architectonic" patterns in brain development (Fig. 21.1A). Understanding this complex process has progressed based on studies of human malformations and mouse models with deficient neuronal migration, particularly the malformation known as lissencephaly (LIS).

The term *lissencephaly*, derived from the Greek words *lissos* meaning smooth and *enkephalos* meaning brain, is a neuronal migration disorder characterized by absent (agyria) or decreased (pachygyria) convolutions, producing a smooth cere-

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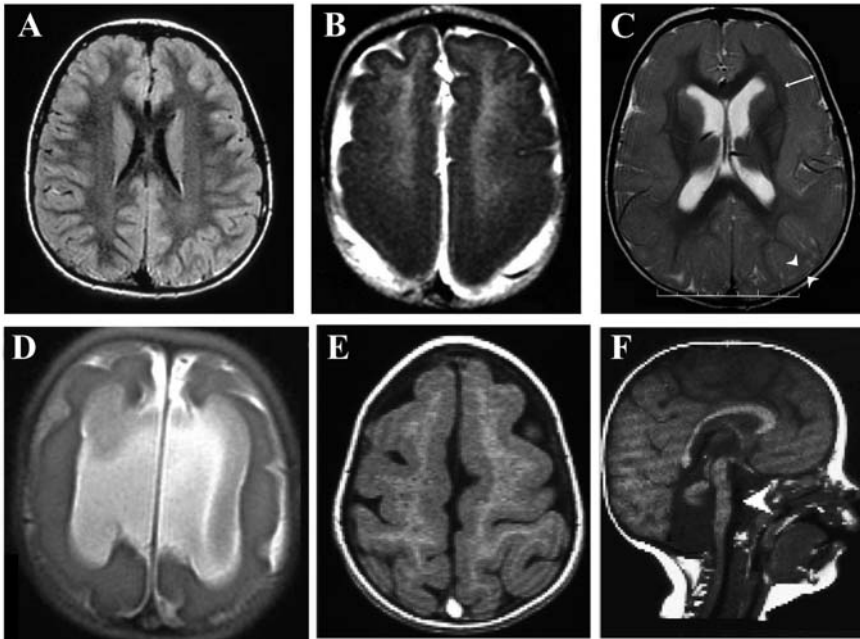
Fig. 21.1 (**A**) Brain MRI scan; axial section of a normal brain. (**B**) Axial section: the cortex in the posterior brain is completely smooth, in the frontal lobes the gyral pattern is simplified and the cortex is thickened. This 4-year-old boy has infantile spasms and a deletion involving the *LIS1* gene. (**C**) Brain MRI scan; axial section: lissencephaly in a girl with *DCX* mutation. There is a typical anterior > posterior malformation pattern, cortical thickness is around 2 cm in the frontal lobes (single arrow) and around 4 mm in the posterior brain (doublearrowheads). (**D**) Axial section: 1-year-old boy with X-linked lissencephaly, with corpus callosum agenesis and ambiguous genitalia due to mutation of the *ARX* gene. Note absence of the corpus callosum with ventriculomegaly and lissencephaly. (**E**, **F**) Brain MRI scans of two patients from a family with LCH type b and a mutation in the *RELN* gene. (**E**) Axial section: the cortex is thickened and the gyral pattern is simplified. (**F**) Sagittal section: the cerebellum is severely reduced in size, with hypoplasia of the inferior vermis and of the hemispheres. The pons (arrowhead) is reduced in size. [Reprinted by permission from Macmillan Publishers Ltd. (Hong *et al.*, *Nature Genet*. 2000; 26:93–96), copyright (2000)]

bral surface (Barkovich *et al.*, 2005). The cytoarchitecture consists of four primitive layers including an outer marginal layer, which contains Cajal-Retzius neurons (layer I), a superficial cellular layer, which contains numerous large and disorganized pyramidal neurons (layer II) corresponding to the true cortex, a variable cell-sparse layer (layer III), and a deep cellular layer composed of medium and small neurons, which extends more than half the width of the mantle (layer IV) (Kato and Dobyns, 2003). The white matter, which is severely reduced in volume, occasionally contains individual neurons or collection of neurons forming heterotopia.

Mechanisms by which cell migration into the cortical plate stops at the appropriate location have been elucidated through the characterization of the *Reeler* mutant mouse (Caviness and Sidman, 1973). In this animal model, the cortical pattern is

opposite with respect to the normal inside-to-outside development of the cerebral cortex. This observation suggests *Reln* to be required for the normal inside-to-outside positioning of cells as they migrate from the ventricular zone; a first component of a signaling pathway guiding cells to the correct location in the cerebral cortex.

2   Lissencephaly Categories

Several different LIS types have been recognized. The most common type, known as classical (or type 1) LIS, features a very thick cortex (10–20 mm rather than the normal 4 mm) and no other major brain malformations. This type of LIS is caused by mutations of the *LIS1* gene (Reiner *et al.*, 1993) and of the *DCX* (or *XLIS*) gene (des Portes *et al.,* 1998; Gleeson *et al.,* 1998). *LIS1* mutations result in more severe LIS in the posterior brain regions (posterior>anterior gradient) (Fig. 21.1B), whereas *DCX* mutations result in more severe LIS in the anterior brain regions (anterior>posterior gradient) (Fig. 21.1C) (Pilz *et al.*, 1998; Dobyns *et al.*, 1999). The interaction of both DCX and LIS1 with microtubules may explain the striking similarities between the lissencephalic phenotypes produced by mutations in these two genes.

Classical LIS is rare with a prevalence of 11.7 per million births. All patients have early developmental delay, early diffuse hypotonia, later spastic quadriplegia and opisthotonus, and eventual severe or profound mental retardation. Rarely, patients with pachygyria may have moderate mental and motor impairment. Some children with LIS have lived more than 20 years, although anecdotal experience suggests that the life span is less than 10 years in most patients. Seizures occur in over 90% of children, with onset before 6 months in about 75%. About 80% of children have infantile spasms, although EEG does not show typical hypsarrhythmia. Later, most children have mixed seizure disorders. As most clinical and neurophysiological studies on children with LIS were conducted before genetic distinction between *DCX* and *LIS1* was made, it is unknown whether these two forms have distinctive electroclinical patterns.

The *LIS1* and *DCX* genes do not account for all known cases of classical LIS, and additional LIS syndromes have been described (Walsh, 1999). Miller-Dieker syndrome (MDS) is caused by a contiguous gene deletion. Classical LIS is accompanied by distinct dysmorphic facial features, including prominent forehead, flattened ear helices, short nose, and anteverted nares. Deletions of 17p13.3, including the *LIS1* gene, are found in almost 100% of patients (Dobyns *et al.*, 1993). Deletion of two additional genes, *CRK* and *14-3-3e*, telomeric to *LIS1,* may contribute to the most severe LIS grade and dysmorphic features observed in MDS. X-linked LIS with corpus callosum agenesis and ambiguous genitalia (XLAG) features LIS with posterior-to-anterior gradient and only moderate increase of the cortical thickness (6–7 mm), absent corpus callosum, and ambiguous genitalia with micropenis and cryptorchidism (Bonneau *et al.*, 2002; Kato *et al.*, 2004) (Fig. 21.1D). Mutations of the X-linked *ARX* gene were identified in individuals with XLAG and in some female relatives (Kitamura *et al.*, 2002). The mutations of the *ARX* gene in XLAG patients are predominantly premature terminations.

3   Lissencephaly with Cerebellar Hypoplasia (LCH)

Malformations in the LIS spectrum can be associated with significant cerebellar underdevelopment and have recently been referred to as lissencephaly with cerebellar hypoplasia (LCH).

Six different subtypes of LCH have been described in patients with LCH with heterogeneous clinical presentations (Ross *et al.*, 2001). Phenotypic features included small head circumference, cortical malformation ranging from agyria to simplification of the gyral pattern, and from near-normal cortical thickness to marked thickening of the cortical gray matter. Cerebellar manifestations range from midline hypoplasia to diffuse volume reduction and disturbed foliation. In the LCHb subgroup the cerebral cortex is pachygyric with mild cortical thickening (4–10 mm). An anterior predominant gradient with fewer, broader gyri in the frontal cortex has been reported. Despite the moderate thickening of cerebral cortex, the hippocampal formation could not be clearly identified. The presumptive hippocampus was straightened and suggested to have marked disorganization of the CA regions and dentate gyrus with marked reduction in the anterior–posterior extent of the parahippocampal cortex. The entire cerebellum was severely hypoplastic with absent folia.

An autosomal recessive form of LCH type b associated with severe abnormalities of the cerebellum, hippocampus, and brainstem was described in two consanguineous pedigrees (Hong *et al.*, 2000). In these patients, the cortex was thickened, and the gyral pattern was simplified (Fig. 21.1E). Both of these abnormalities were more severe frontally and temporally, so that the thickness and gyral pattern of the occipital and parietal cortex were relatively normal. The hippocampus appeared flattened, lacking definable upper and lower blades. The subcortical white matter was decreased in amount but consistently normal in its signal characteristics. The corpus callosum was thin over its entire rostrocaudal extent. The lateral ventricles were enlarged, and the cerebellum was severely reduced in size, with hypoplasia of the inferior vermis and cerebellar hemispheres, devoid of any detectable folds or normal architecture (Fig. 21.1F). The pons was reduced in size in superior–inferior and anteroposterior extent. Affected individuals presented dysmorphic facial features, including bitemporal hollowing, sloping of forehead, widely set eyes, and prominent nasal bridge. Affected children in one family had congenital lymphedema, hypotonia, severe developmental delay, and generalized seizures that were controlled by drugs. Severe hypotonia, developmental delay, and seizures were also reported in the other pedigree. Affected children in both families carried mutations in the *RELN* gene (7q22.1), leading to disrupted splicing of *RELN* cDNA. Western blotting revealed low or undetectable amounts of RELN protein in the serum.

4   Reelin *(RELN)* and Lissencephaly

RELN encodes a large (388 kDa) extracellular matrix protein that acts on migrating cortical neurons by binding to the very-low-density lipoprotein receptor (VLDLR), the apolipoprotein E receptor 2, α3β1 integrin, and cadherin-related receptors

(CNRs) (D'Arcangelo *et al.*, 1995). Mutations of the mouse homologues of *RELN* cause brain defects in mice that resemble LCH (Lambert de Rouvroit and Goffinet, 1998). In mice, *Reln* mutations cause cerebellar hypoplasia, abnormal cortical neuronal migration in the cerebrum, and abnormal axonal connectivity (Lambert de Rouvroit and Goffinet, 1998; Gonzalez *et al.*, 1997). Neurons in affected mice fail to reach their correct location in the developing brain, disrupting the organization of the cerebellar and cerebral cortices and other laminated regions. In this animal model, the cortical layering appears inverted (Caviness, 1976; Ogawa *et al.*, 1995). Thus, Reln is thought to control cell–cell interactions critical for cell positioning in the brain.

RELN mutation analysis is indicated in patients with LCH and an autosomal recessive pattern of inheritance. However, no clear indication on the range of severity of the malformation can be clearly defined at present, due to the paucity of reported cases with proven *RELN* gene defect.

As *RELN* is encoded by 65 exons, covering more than 400 kb of genomic DNA and 12 kb of coding cDNA (DeSilva *et al.*, 1997) the genetic test should be performed in *RELN* cDNA using RT-PCR amplification of RNA from the patients. Rare patients with autosomal recessive LCH, severe epilepsy, mental retardation, and a chromosomal rearrangement causing the disruption of *RELN,* with absence of encoded protein, have been identified (Zaki *et al.*, 2007; Chang *et al.*, 2007). For this reason, cytogenetic analysis, including high-resolution karyotype, FISH, or array-CGH analysis, should be performed as a complement or alternative to *RELN* mutation analysis in patients with LCH.

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Chapter 22
The Role of Reelin in Etiology and Treatment of Psychiatric Disorders

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Contents

1   Introduction

There are many brain proteins that participate in the early growth and development of the mammalian central nervous system. Reelin is a glycoprotein that helps guide brain development in an orderly fashion. Changes in the level of this protein or its receptors or downstream proteins may cause abnormal corticogenesis and alter

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synaptic plasticity. These changes have also been observed in a number of neuropsychiatric disorders. We will discuss more about this protein and its possible involvement in various neuropsychiatric disorders.

2   Structure of Reelin

The Reelin gene (Reln) is localized to chromosome 7 in man (DeSilva *et al*., 1997), and its protein product has a relative molecular mass of 388 kDa (Ogawa *et al*., 1995; D'Arcangelo *et al*., 1995). On SDS-PAGE, Reelin appears as several protein bands, ranging from 410 to 330, 180 kDa, and several smaller fragments (Smalheiser *et al*., 2000; Fatemi *et al*., 2002; Lugli *et al*., 2003; Ignatova *et al*., 2004). Reelin is a secreted extracellular matrix protein containing 3461 amino acids (DeBergeyck *et al*., 1998). Reelin contains a signal peptide followed by an N-terminal sequence and a hinge region upstream from eight Reelin repeats of 350–390 amino acids (DeBergeyck *et al*., 1998). Each Reelin repeat is composed of two subrepeats separated by an EGF motif (DeBergeyck *et al*., 1998). The Reelin protein ends with a highly basic C-terminus composed of 33 amino acids (DeBergeyck *et al*., 1998). An epitope known as the CR-50 is localized near the N-terminus (D'Arcangelo *et al*., 1997) and is composed of amino acids 230–346 of Reelin glycoprotein (Utsunomiya-Tate *et al*., 2000). This epitope is essential for Reelin–Reelin electrostatic interactions that produce a soluble string-like homopolymer, composed of up to 40 or more regularly-repeated monomers, which form *in vivo* (Utsunomiya-Tate *et al*., 2000). Mutated Reelin, which lacks a CR-50 epitope, fails to form homopolymers, and is, thereby, unable to transduce the Reelin signa1 (Utsunomiya-Tate *et al*., 2000). Reelin binds several proteins as likely receptors including apolipoprotein E receptor 2 (ApoER2), very-low-density liproprotein receptor (VLDLR), and α3β1 integrin protein (D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999; Dulabon *et al*., 2000). Reelin binding to ApoER2 and VLDLR receptors induces clustering of the latter receptors, causing dimerization/oligomerization of the adapter protein, disabled-1 (Dab1), on the cytosolic aspect of the plasma membrane (Strasser *et al*., 2004) with eventual tyrosine phosphorylation of Dab1 adapter protein (Cooper and Howell, 1999), facilitating the transduction of signaling pathway from the Reelin-producing cells [GABAergic neurons (Pesold *et al*., 1990) or Cajal-Retzius cells of layer I (Fatemi *et al*., 1999)] to downstream receptor sites on cortical pyramidal cells (Rodriguez *et al*., 2000). *In vivo*, Reelin is processed by cleavage at two locations, i.e., between repeats 2 and 3 and repeats 6 and 7 (de Rouvroit *et al*., 1999), resulting in three final fragments (Jossin *et al*., 2004). The central Reelin fragment is composed of repeats 3–6 and is necessary and sufficient for receptor binding to ApoER2 and VLDLR proteins, causing Dab1 phosphorylation in neuronal cultures (Jossin *et al*., 2004) and able to rescue the reeler phenotype in embryonic brain cultures. Furthermore, Reelin also activates serine-threonine kinases (p35/Cdk5) and Src-tyrosine kinase family (Fyn-kinase), also leading to phosphorylation of Dab1 (Keshvara *et al*., 2001; Beffert *et al*., 2002; Arnaud *et al*., 2003 a,b). Phosphorylated Dab1 can become the substrate for various

kinases, leading to a number of important events, such as synaptic and dendritic spine plasticity (Rodriguez *et al.*, 2000), neurotransmission (Keshvara *et al.*, 2001; Beffert *et al.*, 2002; Arnaud *et al.*, 2003a,b), and inhibition of the level of glycogen synthase-kinase 3β (GSK3β), leading to modulation of pathways of cell survival and growth (Beffert *et al.*, 2002) (Fig. 22.1). Additionally, phosphorylated Dab1 is a substrate for
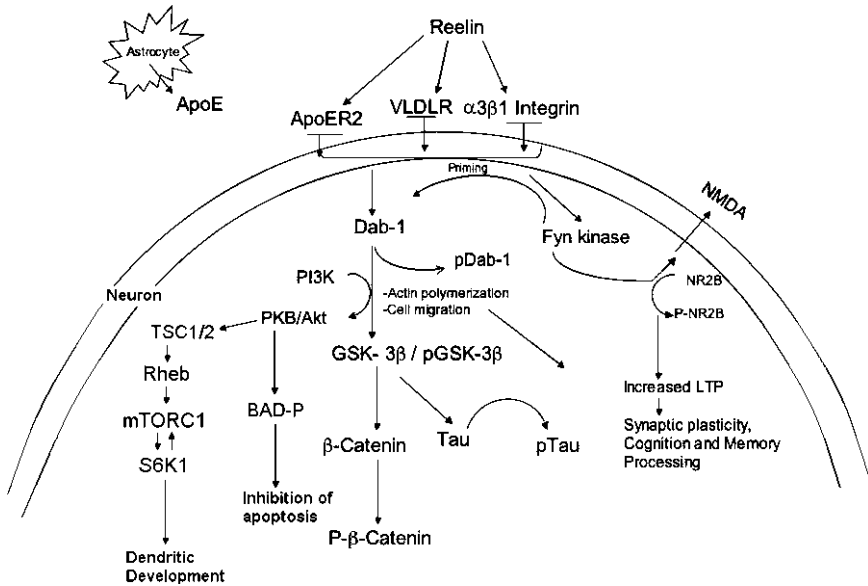
Fig. 22.1 The Reelin signaling system and cognition. Extracellular Reelin glycoprotein is secreted by Cajal-Retzius cells and certain cortical and hippocampal GABAergic cells and cerebellar granule cells. Reelin can bind its receptors ApoER2, VLDLR, and α3β1 integrin directly, initiating the signaling system in the effector cells, i.e., cortical pyramidal cells. Reelin induction of the cascade leads to clustering of the receptors causing dimerization/oligomerization of Dab1 protein and activation of Src-tyrosine kinase family/Fyn-kinase leading to tyrosine phosphorylation of Dab1 protein in a positive-feedback loop. Interaction between Dab1, N-WASP, and ARP 2/3 complex, causes formation of microspikes or filopodia which are important in processes of cell migration and synaptic plasticity. Finally, phosphorylation of a subpopulation of Dab1 molecules causes degradation of Dab1 via ubiquitination, resulting in termination of Reelin signaling cascade. Downstream effector proteins involved in Reelin signaling path include phosphatidylinositol- 3-kinase (PI3K) and protein kinase B (PKB/Akt), which further impact on three other important molecules, glycogen synthase kinase (GSK3β), β-catenin, and tau. Activation of Akt causes phosphorylation of BAD at serine 136 which leads to inhibition of apoptosis. Activation of PI3K and Akt following Dab1 phosphorylation leads to activation of mTor-S6K1 pathway which results in dendritic development. The latter proteins can modulate pathways, affecting cell proliferation, apoptosis, and neurodegeneration, respectively. Finally, Reelin has a direct effect on enhancement of long-term potentiation (LTP), via direct involvement of its receptors VLDLR and ApoER2. Alternately, tyrosine phosphorylation of NR2B subunit of NMDA receptor by Fyn kinase is essential for induction of LTP and modulation of synaptic plasticity, potentially converging on Reelin's role in cognition and memory processing (Fatemi, 2005; Jossin and Goffinet, 2007; Ohkubo *et al.*, 2007). [Modified from Fatemi, S. H. (2005). Reelin glycoprotein in autism and schizophrenia. *Int. Rev. Neurobiol.* 71:179–187]

polyubiquitination-dependent degradation leading to degradation of a subpopulation of Dab1 molecules, via the proteosome pathway (Arnaud *et al.*, 2003a). Dab1 degradation may be an important factor in fine-tuning the Reelin signal and response to it in the CNS (Arnaud *et al.*, 2003a).

Recent work by Suetsugu and co-workers (2004) explains the mechanisms through which Reelin stimulation of Dab1 affects migration of cells. Following induction of Reelin signaling system, Dab1 activates N-WASP [a neuronal type of Wiskott-Aldrich syndrome protein capable of inducing long actin microspikes (Miki *et al.*, 1998)] and stimulates actin polymerization through the Arp 2/3 complex [actin-related proteins 2 and 3, which are essential for initiation of actin assembly (Welch *et al.*, 1997)], causing formation of microspikes or filopodia. Phosphorylation of Dab1 upon Reelin stimulation and via Fyn-Src kinase mediation, causes ubiquitination of Dab1 in a Cbl-dependent manner [Casitas B lymphoma protein, a ubiquitin ligase (Arnaud *et al.*, 2003a,b; Duan *et al.*, 2004)], leading to inhibition of filopodium induction (Figure 22.1) and eventual arrest in cell migration. This mechanism may also underlie abnormal cell migration during brain development observed in the reeler mouse (Ogawa *et al.*, 1995; Arnaud *et al.*, 2003a,b) (vide infra).

Very recently, Jossin and Goffinet (2007) showed that Reelin activates the mTor (mammalian target of rapamycin)-S6K1 (S6 kinase 1) pathway following phosphorylation of Dab1 and activation of PI3K and Akt (PKB, protein kinase B). Moreover, it was proposed that PI3K helps in radial migration of cortical neurons through the intermediate zone independent of Reelin and Akt (Jossin and Goffinet, 2007). In an additional study, Ohkubo *et al.* (2007) showed that Reelin binding of ApoE receptor and activation of the PI3K/Akt pathway causes phosphorylation of Bcl2/Bcl-x associated death promoter (BAD) which helps to protect cells from apoptosis and promotes survival of mature neurons in the brain (Ohkubo *et al.*, 2007).

3   Reelin Mutant Mice

Mutation of the gene for Reelin, as seen in homozygous reeler mutant mice (Goffinet, 1979, 1984), leads to development of ataxia and a reeling gait in the affected mice. Additionally, absence of the Reln gene during embryogenesis leads to development of a brain with multiple histologic defects including a reversal of the normal layering of the brain (Falconer, 1951; Goffinet, 1984, 1992), abnormal positioning of the neurons, and aberrant orientation of cell bodies and nerve fibers (Falconer, 1951; Goffinet, 1984, 1992). The reeler cerebellum is hypoplastic (Magdaleno *et al.*, 2002) and the Purkinje cell number is reduced (Hadj-Sahraoui *et al.*, 1996). Mutations involving ApoER2, VLDLR, and α3β1 integrin receptors result in defective cortical lamination and abnormal neuronal migration (Trommsdorff *et al.*, 1999; Dulabon *et al.*, 2000). Additionally, mice that lack either Reelin or both VLDLR and ApoER2 receptors, exhibit hyperphosphorylation of the tau protein, resulting in dysregulation of neuronal

microtubule function (Hiesberger *et al.*, 1999). Several other reeler-like phenotypes have also been described which produce various neurologic phenotypes similar to the reeler homozygous mutant (for a detailed discussion see Fatemi, 2001). More interestingly, several experimental paradigms and haploinsufficiency in Reln gene in mice also cause decreases in Reelin production with resultant cortical and behavioral abnormalities (Fatemi *et al.*, 1999; Tueting *et al.*, 1999; Fatemi, 2001; Janusonis *et al.*, 2004). In the heterozygous reeler mutation, there is a 50% reduction in Reelin protein and mRNA, decrease in dendritic spine density in frontal cortex, neuropil hypoplasticity, decreased GAD67 expression, and decreased GABA turnover (Carboni *et al.*, 2004). Additionally, the heterozygous reeler mutant mice exhibit decreased prepulse inhibition (Tueting *et al.*, 1999, 2005), a phenomenon observed in schizophrenia and autism (McAlonan *et al.*, 2002; Meincke *et al.*, 2004). Prenatal human influenza viral infection in midterm pregnant mice leads to abnormal corticogenesis (Fatemi *et al.*, 1999), decrease in brain Reelin protein content (Fatemi *et al.*, 1999), and reduced prepulse inhibition (Shi *et al.*, 2003). Finally, exposure of rat pups to 5-methoxytryptamine leads to reductions in brain and blood Reelin levels and abnormal corticogenesis (Janusonis *et al.*, 2004).

4 Reelin's Presence in All Vertebrates

Reelin protein is present in all vertebrates and conserved through evolution (Tissir and Goffinet, 2003). Additionally, the wide distribution of Reelin in the adult lamprey brain is consistent with the existence of different roles for this protein not related to CNS development in the vertebrates (Perez-Costas *et al.*, 2004). For example, Reelin expression in brains of male European starlings was widely distributed in the forebrain including in areas incorporating new neurons in adulthood such as in and around the song control nucleus (Absil *et al.*, 2003). Reelin expression is highly sensitive to testosterone, decreasing markedly in response to exogenous administration of this hormone (Absil *et al.*, 2003). Thus, here, Reelin expression in the brain varies seasonally and could therefore provide a signal that could modulate the seasonal effects in the incorporation of new neurons in the song control system (Absil *et al.*, 2003). In mammals, including rodents, Reelin production begins as early as day 9.5 in the embryonic mouse brain (Ogawa *et al.*, 1995; Ikeda and Terashima, 1997). The cells synthesizing Reelin are Cajal-Retzius cells which act as pathfinding neurons that help in early laminar organization of the cortex (Ogawa *et al.*, 1995). In the adult mammalian brain, Reelin is localized to layer I cortical Cajal-Retzius cells, cortical GABAergic interneurons in layers II–IV (Impagnatiello *et al.*, 1998), cerebellar granule cells (Lacor *et al.*, 2000), and hippocampal interneurons (Fatemi *et al.*, 2000). Presence of Reelin-positive cells in the adult hippocampus indicates that Reelin function is not restricted to the embryonic period but may continue throughout adult life (Abraham and Meyer, 2003).

A recent report demonstrates coexpression of Reelin and Dab1 in Cajal-Retzius cells during cortical development, and in cortical pyramidal cells in the adult CNS (Deguchi *et al*., 2003).

It is now clearly established that Reelin protein serves a dual purpose in mammalian brain: Embryologically, it guides neurons and radial glial cells to their correct positions in the developing brain (Forster *et al*., 2002; Luque *et al*., 2003). After the fetal phase of brain development, levels of Reelin begin to decrease, reaching a plateau by late childhood and remaining constant thereafter in mice (M. Araghi-Niknam and S.H. Fatemi, unpublished data). Moreover, Reelin is largely replaced by Reelin-expressing GABAergic interneurons that are dispersed throughout the mammalian neocortex (Impagnatiello *et al*., 1998) and hippocampus (Fatemi *et al*., 2000; Abraham and Meyer, 2003). Levels of the Reelin receptors ApoER2, VLDLR, and $\alpha 3\beta 1$ integrin and the adapter protein Dab1, which are all essential to the Reelin signaling system, remain expressed in adult brain (Abraham and Meyer, 2003).

5   Reelin and Its Receptors

Previous work by Rodriguez *et al*. (2000) showed an association between Reelin and its receptor $\alpha 3\beta 1$ integrin with synaptic structures, raising the possibility of a potential role in neurotransmission. Recent reports by J. Herz's laboratory (Weeber *et al*., 2002; Herz and Chen, 2006) show that Reelin has a direct effect on enhancement of long-term potentiation (LTP) in hippocampus which is abolished when hippocampus slice cultures are used from VLDLR and ApoER2 knockout mice lacking the receptors for Reelin. These investigators further report that Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning (Weeber *et al*., 2002). Moreover, mice that lack the Reelin receptors ApoER2 or VLDLR have pronounced defects in memory formation and LTP (Weeber *et al*., 2002). More recent work by Beffert *et al*. (2005, 2006) has further demonstrated the importance of ApoER2 on LTP. An amino acid sequence encoded by an exon on the intracellular domain of ApoER2 is required for Reelin-induced tyrosine phosphorylation of NMDA receptor subunits (Beffert *et al*., 2005). Mice lacking this sequence performed poorly in learning and memory tasks (Beffert *et al*., 2005). Beffert *et al*. (2006) further demonstrated that mice that have ApoER2 lacking a sequence required for interaction with Dab1 similarly have abnormalities in LTP and behavior which are different still from knockout mice (Beffert *et al*., 2006).

A recent report by Barr *et al*. (2007) showed the importance of VLDLR and ApoER2 in regulating sensorimotor gating in mice. VLDLR knockout mice revealed deficits in a crossmodal PPI task involving the presentation of acoustic and tactile stimuli while ApoER2 heterozygous and knockout mice showed significant increased crossmodal PPI (Barr *et al.,* 2007).

6   Reelin's Role in Psychiatric Disorders

Several studies now implicate the pathological involvement of Reelin gene or its protein product in six neuropsychiatric disorders, namely, schizophrenia (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Guidotti *et al*., 2000; Costa *et al*., 2003a; Eastwood *et al*., 2003; Eastwood and Harrison, 2003; Abdolmaleky *et al*., 2004; Knable *et al*., 2004; Fatemi *et al*., 2005a; Wedenoja *et al*., 2007), autism (Persico *et al*., 2001; Zhang *et al*., 2002; Fatemi *et al*., 2002, 2004, 2005b; Lugli *et al*., 2003), bipolar disorder (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Guidotti *et al*., 2000; Knable *et al*., 2004), major depression (Fatemi *et al*., 2001; Knable *et al*., 2004), lissencephaly (Hong *et al*., 2000; Miyata *et al*., 2003), and Alzheimer's disease (Saez-Valero *et al*., 2003; Botella-Lopez *et al.,* 2006).

7   Reelin in Schizophrenia, Bipolar Disorder, and Major Depression

Neuroanatomical studies have shown Reelin abnormalities throughout the brain in patients with schizophrenia, bipolar disorder, and major depression. Reelin expression has consistently been shown to be decreased in all three disorders. Impagnatiello *et al*. (1998) used Northern and Western blotting and immunocytochemistry to show reductions in Reelin mRNA and protein in cerebellar, hippocampal, and frontal cortices of patients with schizophrenia and psychotic bipolar disorder. These authors suggested that Reelin might be a vulnerability factor in development of psychosis (Impagnatiello *et al*., 1998). Later, Guidotti *et al*. (2000) confirmed and extended these observations in postmortem frontal cortex of additional subjects with schizophrenia and psychotic bipolar disorder. Reduction in Reelin was associated with a concurrent decrease in glutamic acid decarboxylase 67-kDa (GAD67) protein in the same postmortem brains (Guidotti *et al*., 2000). A later immunocytochemical report (Fatemi *et al*., 2000) showed significant reductions in Reelin immunoreactivity in the hippocampus of patients with schizophrenia, nonpsychotic bipolar disorder, and major depression, suggesting that Reelin deficiency may not be limited to subjects with psychosis alone (Fatemi *et al*., 2000). Knable *et al*. (2004) analyzed molecular abnormalities of the hippocampus in severe psychiatric illness and reconfirmed that GABAergic marker Reelin was decreased in schizophrenia, bipolar disorder, and depression, attesting to reported GABAergic dysfunction in all three disorders. Fatemi *et al*. (2005a) subsequently demonstrated significant reductions in Reelin, as well as GAD65 and 67-kDa proteins, in cerebella of subjects with schizophrenia, bipolar disorder, and major depression, providing further evidence that GABAergic dysfunction is apparent in these disorders (Fatemi *et al*., 2005a). The cerebellar decreases in GAD65 and 67-kDa proteins have been replicated in brains of subjects with schizophrenia by the laboratory of N. Perrone-Bizzozero (Bullock *et al*., 2006), asserting the biological importance of both enzymes in the pathology of psychiatric disorders. Eastwood *et al*. (2003), also showed a trend for reduction in Reelin mRNA in cerebella of subjects with schizophrenia. Interestingly, these reductions in Reelin

mRNA correlated negatively with expression of semaphorin 3A mRNA (Eastwood *et al*., 2003). The authors suggested that these findings were consistent with an early neurodevelopmental origin for schizophrenia, and that the reciprocal changes in Reelin and semaphorin 3A may be indicative of a mechanism that affects the balance between inhibitory and trophic factors regulating synaptogenesis (Eastwood *et al*., 2003). Eastwood and Harrison extended their work to superior temporal cortex and discovered reductions in Reelin mRNA in interstitial white matter neurons (cells representing the adult remnants of the cortical subplate) in brains of subjects with schizophrenia, supporting the contention that the origins of schizophrenia may be neurodevelopmental (Eastwood and Harrison, 2003).

Recent evidence indicates that hypermethylation of the Reelin gene promoter may be responsible for decreased expression of Reelin in brains of subjects with schizophrenia (Costa *et al*., 2003a; Abdolmaleky *et al*., 2004). Costa and co-workers have posited the opinion that alterations in chromatin remodeling related to a selective upregulation of DNA-5-cytosine methyltransferase (DNMT) expression in GABAergic neurons of schizophrenic prefrontal cortex may induce a hypermethylation of Reelin and GAD67 promoter CpG islands, which subsequently downregulate their expression (Costa *et al*., 2003a). These authors suggest that targeting this deficit with inhibitors of histone deacetylases (HDAC) may reduce the DNMT upregulation via covalent modification of nucleosomal histone tails, potentially upregulating Reelin expression in schizophrenic brain (Costa *et al*., 2003a,b; Abdolmaleky *et al*., 2004). Indeed, Veldic *et al*. (2004) have recently shown that mRNA for DNA-methyltransferase 1, which catalyzes the methylation of promoter CpG islands, is increased in cortical GABAergic interneurons but not in pyramidal neurons of schizophrenic brains. More recently, Dong *et al*. (2007) demonstrated in mice that following treatment with L-methionine, which increases RELN and GAD67 promoter cytosine-5-hypermethylation, treatment with HDACs valproate and MS-275 dramatically accelerated RELN and GAD67 promoter demethylation (Dong *et al*., 2007). This suggests the possibility that RELN reactivation may take place due to antipsychotic-induced demethylation (Dong *et al*., 2007).

Despite these biochemical findings, two recent reports failed to find any association between Reln gene polymorphisms and schizophrenia (Akahane *et al*., 2002; Chen *et al*., 2002). Akahane *et al*. examined the polymorphic CGG repeat in the 5´ untranslated region of the Reln gene in 150 schizophrenic and 150 controls matched for age, sex, and ethnicity and found no evidence for any significant association of schizophrenia with polymorphisms for Reln or VLDLR genes (Akahane *et al*., 2002). By the same token, Chen *et al*. studied a single nucleotide polymorphism at the 5′ promoter region of the human Reln gene in 279 Han Chinese schizophrenic patients and 255 controls and could not demonstrate any significant associations in the Reln gene polymorphisms and schizophrenia (Chen *et al*., 2002).

The lack of conclusive results associating RELN, among other genes, with schizophrenia has led to the study of quantifiable traits and endophenotypes (Gottesman and Gould, 2003). A study by Wedenoja *et al*., while demonstrating a lack of association between RELN and a clinical diagnosis of schizophrenia in a Finnish population sample, found that RELN was associated with a number of cognitive traits known to be affected in schizophrenia (Wedenoja *et al*., 2007). Specifically, they found associations between a RELN intragenic microsatellite

marker (RELNSAT6) and attention and working memory, verbal learning and memory, and executive function (Wedenoja *et al.,* 2007). These findings are consistent with RELN's role in synaptic plasticity (Costa *et al.*, 2002; Fatemi, 2005), which is crucial for cognitive abilities (Garlick, 2002).

8   Reelin in Autism

8.1   Brain Abnormalities

In a series of postmortem studies, Fatemi *et al.* (2001, 2004, 2005b) demonstrated reductions in Reelin protein in several brain sites in autism. Brain levels of Reelin 410 kDa were reduced significantly in frontal (Area 9) and cerebellar areas and nonsignificantly in parietal (Area 40) cortex of autistic subjects versus controls. There was also a trend for reduction in Reelin 410 kDa in autistic children, indicating that the reduced Reelin levels were present from childhood (Fatemi *et al.*, 2004). Brain levels of Reelin 330 kDa were reduced significantly in Area 9 and nonsignificantly in Area 40 and cerebellum (Fatemi *et al.*, 2005b). Brain levels of Reelin 180 kDa were reduced significantly in cerebellum and Area 9 and nonsignificantly in Area 40 (Fatemi *et al.*, 2001, 2005b). These results are significant as pathologic findings of the brain are prevalent throughout the brain of subjects with autism including the frontal, parietal, and cerebellar cortices (Palmen *et al.*, 2004).

8.2   Blood Abnormalities

Smalheiser *et al.* (2000) identified three Reelin bands in rat mouse and human blood but these bands were absent in blood from homozygous reeler (*rl/rl*) mice while heterozygous reeler (*rl/+*) mice expressed half as much as wildtype (Smalheiser *et al.*, 2000). Measurement of blood Reelin levels in subjects with autism showed reductions in 410- and 330-kDa species in the subjects with autism versus matched controls (Fatemi *et al.*, 2002). Janusonis *et al.* (2004) found that brain and blood levels of Reelin were decreased in newborn pups (postnatal day 0) born to mice that had been treated with 5-methoxytryptamine. This result suggests that disruption of the serotonergic system, which is known to be altered in autism (Chugani, 2002), produces altered Reelin expression (Janusonis *et al.*, 2004). Finally, Lugli *et al.* (2003) confirmed Fatemi's work, showing significant reductions in 330-kDa plasma protein in a selected group of autistic subjects.

8.3   Genetic Polymorphisms

These biochemical data are bolstered by two association studies showing significant linkage between Reln gene polymorphisms and autism (Persico *et al.*, 2001;

Zhang *et al*., 2002). Recently, Persico *et al*. (2001) described a significant association between autism and Reln gene variants using case–control and family-based designs. They showed a significant association between autistic disorder and the length of a polymorphic GGC repeat located immediately 5' of the Reln gene ATG initiation codon. A further link to autism was also established for specific haplotypes defined by single-base substitutions located in a splice junction of exon 6 and within the coding sequence of exon 50 (Persico *et al*., 2001). These investigators also showed preferential transmission of "long" triplet repeat alleles (i.e., >11 repeats) to autistic patients and correlated this phenomenon with decreases in blood Reelin 330-kDa levels in the autistic offspring (Lugli *et al*., 2003). These authors concluded that transmission of "long" alleles from either parent significantly enhanced the overall probability of a child being affected by autism (Persico *et al*., 2001; Lugli *et al*., 2003).

In a subsequent report, Zhang *et al*. (2002) did not observe any evidence for expansion or instability of transmission of GGC repeats in the autistic subjects but were able to confirm, using a family-based association test, that larger alleles were transmitted higher than expected in the affected children indirectly supporting the work of Persico *et al*. (2001). In contrast, four reports failed to detect any genetic linkage between Reln gene polymorphisms and autism (Krebs *et al*., 2002; Bonora *et al*., 2003; Devlin *et al*., 2004; Li *et al*., 2004). Krebs *et al*. (2002) performed a transmission disequilibrium test analysis of the 5' UTR polymorphism in 167 families, including 218 affected subjects, and could not show any association between this GGC polymorphism of the Reln gene and autism in a population of mixed European descent. Bonora *et al*. (2003), using a positional candidate gene approach, found novel missense variants in the Reln gene with low frequency but could not support a major role for Reln in autism in IMGSAC and German singleton families. Devlin *et al*. (2004) used a large independent family-based sample from the NIH Collaborative Programs of Excellence in Autism (CPEA) Network and could not find any significant association between Reln gene alleles and autism. Finally, Li *et al*. (2004) also could not find any evidence for an association between WNT2 and Reln polymorphisms and autism. However, these authors (Li *et al*., 2004) felt that "association studies of DNA variations are often ineffective in addressing functional alteration of gene products at the level of gene expression" and suggested additional biochemical studies of brain and blood products to further assess the involvement of the Reln gene in autism. Persico *et al*. have demonstrated using *in vitro* studies that the long triplet GGC repeats blunted Reln gene expression (Persico *et al*., 2006). The authors suggest that this may account for the decreased Reelin expression in subjects with autism (Persico *et al.,* 2006). Finally, Serajee *et al*., in a study of 34 Reln single nucleotide polymorphisms (SNP) in a sample of Caucasian families, found an association of autism with a C/T SNP in intron 59 of the Reln gene (Serajee *et al*., 2005).

Despite the controversial nature of genetic association studies, Rakic and co-workers (Janusonis *et al*., 2004) have developed a potential animal model for autism which links prenatal serotonergic abnormalities to reduced brain and blood Reelin levels and abnormal brain development, indicating the relevance of

biochemical/neuroanatomic studies pertaining to the Reelin signaling system in autism.

Other behavioral and biochemical data also show that reductions in levels of Reelin in brain or blood, following postnatal hypoxia (Curristin *et al*., 2002), prenatal viral infection in midgestation (Fatemi *et al*., 1999; Shi *et al*., 2003), immunological challenge with Poly I:C (a viral mimic) (Meyer *et al*., 2007) and in heterozygous reeler mutants (Tueting *et al*., 1999) cause abnormalities in behavior such as decrease in pre-pulse inhibition (PPI), increase in anxiety, and decrease in memory formation. Additionally, mutations in the RELN gene have been associated with significant learning disability, hypoplastic cerebellum, ataxia, and cognitive decline in man and mouse (Goffinet, 1992).

9   Reelin in Lissencephaly

Reelin mutations have also been discovered in a variant of lissencephaly, whereby the affected individuals have very low or undetectable levels of Reelin in their sera (Hong *et al*., 2000; Chang *et al*., 2007). Both Chang *et al*. (2007) and Hong *et al*. (2000) reported that the affected children exhibited congenital lymphedema and hypotonia with brain showing moderate lissencephaly and profound cerebellar hypoplasia. Miyata *et al*. (2003), in a study of a 7-day-old neonate born at 38 gestational weeks with lissencephaly with cerebellar hypoplasia, found altered Reelin expression. Assadi *et al*. (2003) developed compound mutant mice, with disruptions in the Reln gene and PAFAH1B1 (encoding LIS1), which exhibited a higher incidence of hydrocephalus and enhanced cortical and hippocampal layering defects, implicating involvement of both genes in normal brain development.

10   Reelin in Alzheimer's Disease

Finally, Saez-Valero *et al*. (2003) measured Reelin 180-kDa levels in CSF of 13 healthy controls, 14 fronto-temporal dementia, and 20 Alzheimer's disease patients. They reported significant increases in CSF 180-kDa Reelin species in both dementias versus controls, suggesting the involvement of Reelin in neurodegenerative disorders (Saez-Valero *et al*., 2003). More recently, Botella-Lopez *et al*. (2006) found a significant increase in CSF 180-kDa Reelin, and a significant increase in 180-kDa Reelin and total Reelin in frontal cortex in subjects with Alzheimer's disease (Botella-Lopez *et al.,* 2006). Additionally, they found an increase in Reelin/GAPDH mRNA in frontal cortex (Botella-Lopez *et al.,* 2006). In contrast, Ignatova *et al*. (2004) measured CSF Reelin in adults and children and found no correlation with age or neurologic disease (Alzheimer's dementia, multiple sclerosis). However, the latter investigators used a scoring technique which was semiquantitative and had a smaller *N* for each patient population (Ignatova *et al*., 2004).

11   Effects of Psychotropic Medications on Reelin Expression in Rat Brain

Our laboratory has investigated whether chronic administration of psychotropic medications (clozapine, fluoxetine, haloperidol, lithium, olanzapine, and valproic acid) used in the treatment of psychiatric disorders (schizophrenia, major depression, bipolar disorder, etc.) alters mRNA and protein levels for Reelin in rat frontal cortex (FC). FC of drug-treated rats (21 days of intraperitoneal injections) versus saline-treated controls were subjected to SDS-PAGE and Western blotting. Additionally, rat FC mRNAs were also subjected to qRT-PCR. Levels of Reelin were significantly altered in several drug-treated rat FC groups versus controls. These data suggest that changes are due to the psychotropic medications, and that the changes in Reelin expression may help explain the efficacy of these drugs.

11.1   Results

We measured protein levels for Reelin using SDS-PAGE and Western blotting and mRNAs by qRT-PCR for each of the six drug-treatment groups. Reelin molecules appeared on SDS-PAGE as multimeric bands ranging from ~410 to ~330 to ~180 kDa. All values were normalized against β-actin. There were no significant differences in levels of β-actin in the drug-treated brains versus controls.
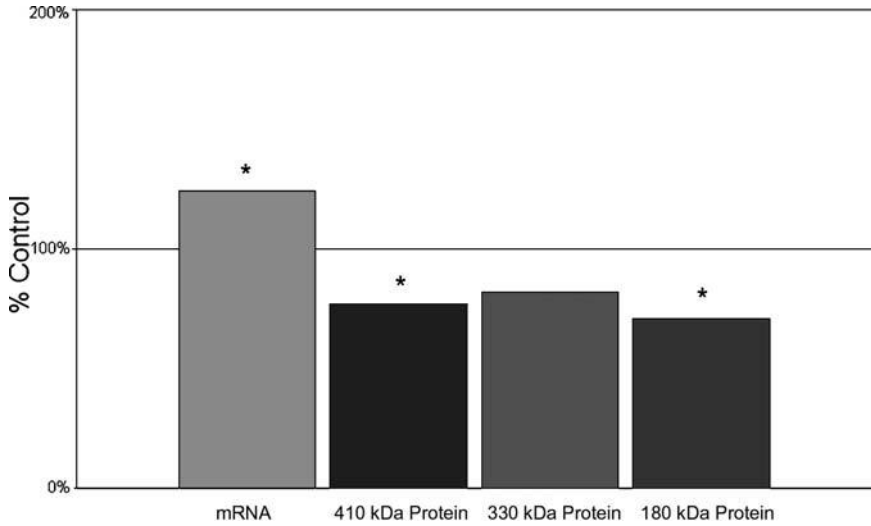
11.1.1   Clozapine

In clozapine-treated rat FC, Reelin protein showed significant downregulation of the 410- and 180-kDa isoforms ($p = 0.0024$ and 0.0099, respectively) while the 330-kDa isoform showed a nonsignificant downregulation. Reln mRNA was significantly upregulated ($p = 0.0006$) versus controls (Fig. 22.2).

11.1.2   Fluoxetine

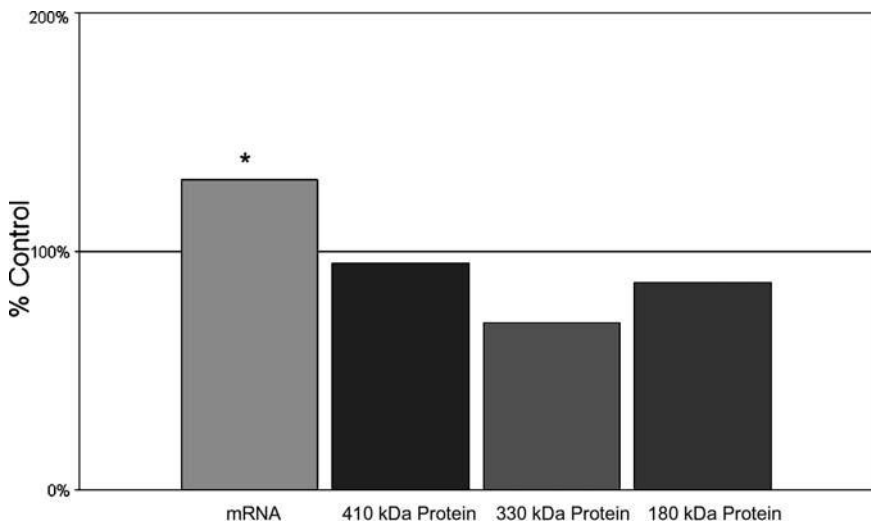Fluoxetine-treated rat FC showed nonsignificant downregulation of all three isoforms of Reelin protein. Reln mRNA was significantly upregulated ($p = 0.0003$) versus controls (Fig. 22.3).

11.1.3   Haloperidol

Reelin protein showed nonsignificant downregulation of the 410- and 330-kDa isoforms while the 180-kDa isoform was significantly downregulated ($p = 0.044$) in

Fig. 22.2 The impact of clozapine on rat brain levels of Reelin. In clozapine-treated rat FC, Reelin protein showed significant downregulation of the 410- and 180-kDa isoforms while Reln mRNA was significantly upregulated versus controls (*See Color Plates*)

Fig. 22.3 The impact of fluoxetine on rat brain levels of Reelin. Reln mRNA was significantly upregulated in fluoxetine-treated rat FC versus controls (*See Color Plates*)

haloperidol-treated rat FC. Reln mRNA level was significantly downregulated in haloperidol versus control rat FC ($p \leq 0.0001$) (Fig. 22.4).

11.1.4 Lithium

Reelin showed nonsignificant downregulation of the 410- and 330-kDa isoforms in lithium-treated FC, while the 180-kDa isoform was significantly downregulated ($p = 0.0066$). Reln mRNA was significantly upregulated ($p = 0.0047$) in lithium versus control rat FC (Fig. 22.5).
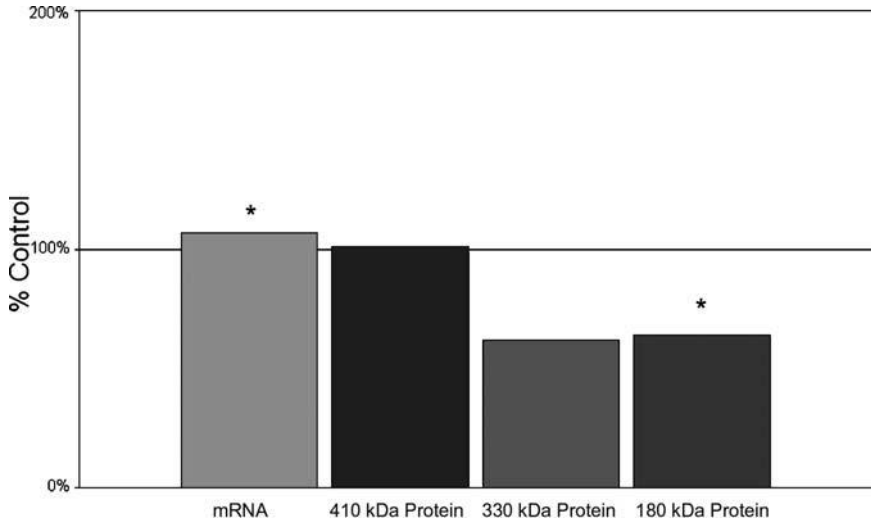
11.1.5 Olanzapine

Olanzapine-treated rat FC showed significant upregulation of the 410- and 180-kDa isoforms of Reelin ($p = 0.0033$ and 0.0001, respectively) proteins (Fatemi *et al.*, 2006), while the 330-kDa protein isoform was nonsignificantly upregulated. Reln mRNA was significantly upregulated ($p = 0.0259$) (Fig. 22.6).

11.1.6 Valproic Acid

The 410- and 330-kDa isoforms of Reelin showed nonsignificant upregulation, while the 180-kDa isoform showed nonsignificant downregulation in VPA-treated
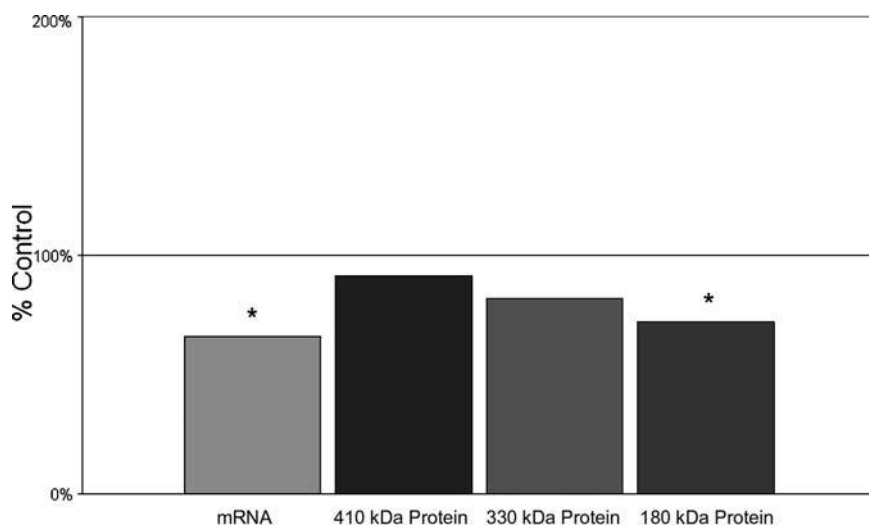
Fig. 22.4 The impact of haloperidol on rat brain levels of Reelin. Reelin protein showed the 180-kDa isoform was significantly downregulated as was Reln mRNA level in haloperidol versus control rat FC (*See Color Plates*)

Fig. 22.5 The impact of lithium on rat brain levels of Reelin. The 180-kDa isoform of Reelin was significantly downregulated following chronic treatment with lithium. In contrast, Reln mRNA was significantly upregulated in lithium versus control rat FC (*See Color Plates*)
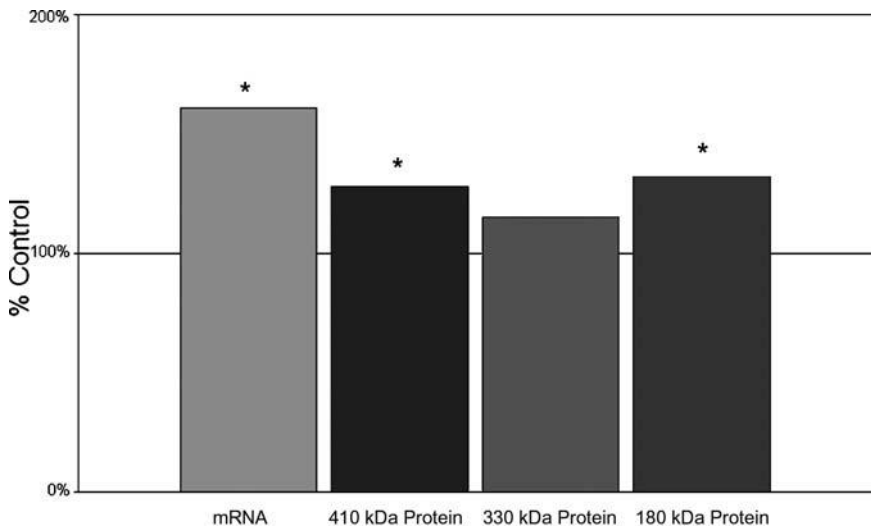

Fig. 22.6 The impact of olanzapine on rat brain levels of Reelin. Olanzapine-treated rat FC showed significant upregulation of the 410- and 180-kDa isoforms of Reelin. Reln mRNA was also significantly upregulated (*See Color Plates*)

versus control rat FC. Reln mRNA was significantly downregulated ($p < 0.0001$) (Fig. 22.7).

11.1.7   Summary

Table 22.1 summarizes the changes in Reelin mRNA and protein expression in rat FC as a result of chronic treatment with psychotropic medications. Importantly, all of the drugs tested altered Reln mRNA with each increasing Reln mRNA except for haloperidol and valproic acid. In contrast, only treatment with olanzapine led to an increase in Reelin protein for both the 410- and 180-kDa isoforms, while clozapine, haloperidol, and lithium led to significant downregulation. Additionally, only haloperidol caused significant downregulation in both mRNA and protein levels for Reelin. Taken together, these results suggest that Reelin is a target of commonly
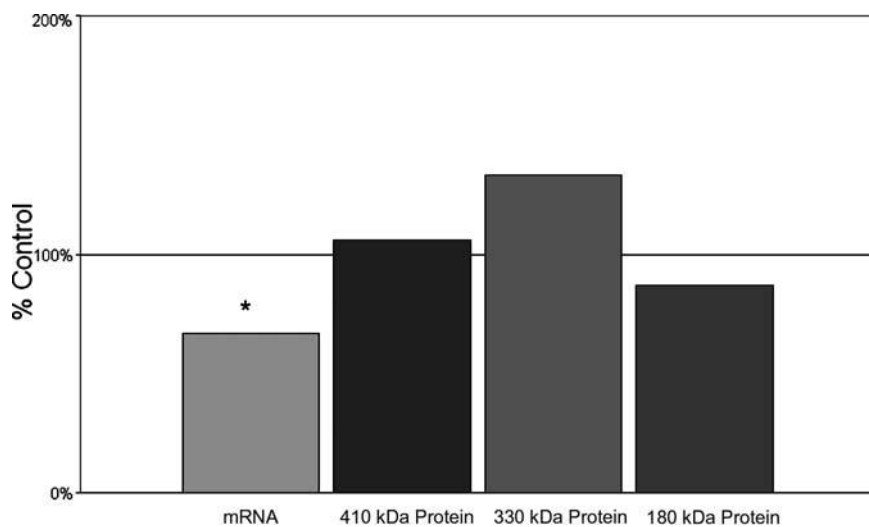
Fig. 22.7 The impact of valproic acid on rat brain levels of Reelin. Reln mRNA was significantly downregulated in rat FC as a result of treatment with VPA (*See Color Plates*)

Table 22.1 qRT-PCR (mRNA) and Western blotting (protein) results on drug-treated versus control rat FC

		Clozapine	Fluoxetine	Haloperidol	Lithium	Olanzapine	VPA
Reelin	Protein	↓*	nc	↓*	↓*	↑*	nc
	mRNA	↑*	↑*	↓*	↑*	↑*	↓*

* $p < 0.05$; nc, no change.

used psychotropic drugs and altered expression of Reelin may partly explain their efficacies.

12 Conclusions

Altered Reelin expression is associated with a number of psychiatric disorders. Reelin expression is increased in cerebrospinal fluid (Saez-Valero *et al*., 2003) and in frontal cortex (Botella-Lopez *et al*., 2006) in subjects with Alzheimer's disease. More commonly, however, Reelin expression is decreased in various psychiatric disorders. Reelin deficiency has been observed in schizophrenia, bipolar disorder, major depression, autism, and lissencephaly. In brains from subjects with schizophrenia, reduced Reelin expression has been observed in cerebellum (Impagnatiello *et al*., 1998; Eastwood *et al*., 2003; Fatemi *et al*., 2005a), frontal cortex (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000), hippocampus (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000), and superior temporal cortex (Eastwood and Harrison, 2003). Decreased Reelin expression in bipolar disorder has been observed in hippocampus (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Knable *et al*., 2004), cerebellum (Impagnatiello *et al*., 1998; Fatemi *et al*., 2005a), and frontal cortex (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000). Subjects with major depression display reduced Reelin in cerebellum (Fatemi *et al*., 2005a) and hippocampus (Knable *et al*., 2004; Fatemi *et al*., 2005a). In autism, decreased Reelin was observed in frontal cortex and cerebellum (Fatemi *et al*., 2005). Decreased Reelin has also been observed in blood (Persico *et al*., 2001; Fatemi *et al*., 2002) with Persico *et al*. (2001) correlating this decrease in blood Reelin with the preferential transmission of "long" triplet repeat alleles of Reln (Lugli *et al*., 2003). Reduced Reelin expression has been observed in brain (Miyata *et al*., 2003) and blood (Hong *et al*., 2000) in lissencephaly.

Various mechanisms may be operational in these neuropsychiatric disorders where Reelin production may be affected selectively by various mutations or selective hypermethylation of the Reelin gene promoter (Costa *et al*., 2003a; Abdolmaleky *et al*., 2004), causing either profound (schizophrenia, autism, lissencephaly) or moderate (bipolar disorder, major depression) cognitive deficits (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Guidotti *et al*., 2000; Hong *et al*., 2000) associated with their respective Reelin levels. The overall picture emerging from these reports suggests that Reelin deficiency may be associated not only with vulnerability to developing psychosis, but also to the development of cognitive dysfunction as clinical symptoms often observed in various neuropsychiatric disorders, such as bipolar disorder (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000), major depression (Fatemi *et al*., 2000), autism (Fatemi *et al*., 2002, 2005a), and lissencephaly (Hong *et al*., 2000). This hypothesis is also supported by animal studies (Rodriguez *et al*., 2000) linking Reelin–integrin interactions with synaptic plasticity. Association of ApoER2 and LDL receptor family with Reelin protein may also link certain neurodegenerative disorders, such as Alzheimer's dementia (Bothwell and Giniger, 2000;

Helbecque and Amouyel, 2000) with dysregulation of the Reelin signaling system.

In summary, Reelin glycoprotein acts as a protease both during embryogenesis and in the adult brain. Absence of Reelin during development leads to abnormal corticogenesis, Purkinje cell loss, and ataxia. Reductions in levels of Reelin during adult life may cause cognitive deficits, as seen in autism, schizophrenia, bipolar disorder, and lissencephaly. Moreover, Reelin is involved in a signaling pathway that underlies memory formation, LTP, and synaptic plasticity. Thus, Reelin's role in early growth and development of the central nervous system makes it an important candidate gene in investigating psychiatric disorders which are associated with gross morphological changes in brain and/or cognitive deficits. Reelin may also have other undefined roles in health and disease because of its presence in diverse areas of the body. Future biochemical, genetic, and neuroanatomic studies will surely expand our knowledge about this important protein and determine its involvement in various neurodevelopmental disorders.

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Chapter 23
Reelin Downregulation as a Prospective Treatment Target for GABAergic Dysfunction in Schizophrenia

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1   Reelin is a Protein Synthesized Almost Exclusively in GABAergic Neurons During Embryonic and Adult Life

The proper functioning of the mammalian cortex depends on the formation of neuronal networks, including principal projection neurons and interneurons that use glutamate and GABA as transmitters, respectively. In the adult brain, cortical interneurons have been implicated in the regulation of the synaptogenesis and neuronal wiring operative in cortical network formation. These neurons are aspiny, express local projecting axons, and their staining with the Golgi method reveals a soma volume smaller than most cortical neurons. They store and synthesize the neurotransmitter GABA and also frequently synthesize and secrete reelin. In embryonic cortex, reelin is synthesized and secreted by the Cajal-Retzius cells, guides neuronal migration and positioning of pyramidal neurons (D'Arcangelo *et al*., 1995). However, postnatally during CNS development and maturation, this protein is synthesized and secreted from GABAergic interneurons and harmonizes the functional plastic interaction of neuronal axons, dendrites, and their spines (Costa *et al*., 2001; Niu *et al*., 2004). Reelin secreted in the extracellular matrix contributes to the modulation of neuronal excitability, firing frequencies, and the morphological properties of the telencephalic neuronal networks regulating their coordinated activity (Liu *et al*., 2001; Costa *et al*., 2001; Weeber *et al*., 2002; Qiu *et al*., 2007).

Recent studies suggest that during conscious states, reelin binding to cortical dendritic spines participates in the modulation of synaptic plasticity and memory processes by: (a) consolidating long-term potentiation (LTP) expression in hippocampal slices, (b) harmonizing protein synthesis locally in dendrites and their spines, and (c) regulating maturation and number of dendritic spines.

Several lines of evidence show that in the brain of schizophrenia (SZ) patients, reelin and GAD67 expressions are severely decreased (Akbarian *et al*., 1995; Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Guidotti *et al*., 2000, 2005; Benes and Beretta, 2001; Costa *et al*., 2001; Eastwood and Harrison, 2003; Veldic *et al*.,

2004, 2005, 2007; Woo *et al.*, 2004; Lewis *et al.*, 2005; Ruzicka *et al.*, 2007). Hence, if cognitive function were to be related to cortical dendritic spine density and maturation and should LTP consolidation be modulated by reelin release, it is possible to entertain the hypothesis that in the presence of a reduced amount of reelin release, as may occur in SZ, dendritic spine maturation may be delayed and distorted, resulting in a decrease in the number of spines associated with this cognitive deficit.

The hypothesis, that in SZ patients these cognitive deficits may be related to a disruption of the inhibitory GABAergic synaptic strength in specific corticolimbic circuits due to an insufficient expression of reelin, is addressed in this chapter.

2   Corticolimbic GABAergic Neurons Secrete Reelin, Which is Important in Modulating CNS Neuronal Plasticity

In the mammalian neocortex and hippocampus, reelin is synthesized almost exclusively by GABAergic neurons (Alcantara *et al.*, 1998; Pesold *et al.*, 1998, 1999; Rodriguez *et al.*, 2002), which secrete this protein in the proximity of dendritic spines (Rodriguez *et al.*, 2000; Costa *et al.*, 2001). In the human cortex, reelin mRNA is expressed in GABAergic neurons of every cortical region or layer studied (Table 23.1); however, the percentage of GAD-positive neurons expressing reelin differs in different layers. For example, approximately 100% of GAD65/67-positive neurons express reelin mRNA in the upper cortical layers, whereas in layers V and VI of different cortical areas, only 50 to 30% of the GAD65/67-positive neurons express reelin mRNA (Table 23.1). The neuronal expression of reelin studied with light microscopic immunoreactivity in rodent, human, and nonhuman primate neocortices reveals that reelin-like immunopositive neurons are not present in all cortical layers but in several areas are confined to layers I and II. In addition, in rodents

Table 23.1 Reelin and glutamic acid decarboxylase 65 (GAD65) expression in Brodmann's area 9 GABAergic neurons of nonpsychiatric subjects (NPS), schizophrenia (SZP), and bipolar disorder patients (BDP)

		Layer I	Layer II	Layer III–IV	Layer V	Layer VI
NPS	Reelin	25 ± 0.85	38 ± 1.6	25 ± 0.78	16 ± 0.22	10.8 ± 0.30
	GAD65	26 ± 0.64	42 ± 0.89	35 ± 1.10	21 ± 0.25	21 ± 0.30
SZP	Reelin	16 ± 0.62*	26 ± 1.69*	19 ± 0.76*	14 ± 0.24	9.7 ± 0.30
	GAD65	27 ± 0.65	44 ± 1.23	36 ± 1.0	23 ± 0.30	23 ± 0.22
BDP	Reelin	18 ± 1.7*	26 ± 1.7*	21 ± 0.99	15 ± 0.25	10 ± 0.37
	GAD65	26 ± 0.77	44 ± 1.1	35 ± 0.86	21 ± 0.33	21 ± 0.37

Counts of reelin and GAD65 mRNA-positive neurons in six layers of BA9 in NPS ($n = 27$), SZP ($n = 20$), and BDP ($n = 14$). Differences were calculated by ANOVA and p values were compared by Bonferroni t-test. *Denotes statistically significant differences ($p \leq 0.013$) when SZP or BDP are compared to NPS. Specimens were obtained from Harvard Brain Tissue Resource Center (Belmont, MA).

and primates, a broad band of diffuse extracellular reelin-like immunoreactivity is detectable in cortical layers I, II, and III (Pesold *et al*., 1998; Guidotti *et al*., 2000; Rodriguez *et al*., 2002). These findings suggest that the reelin storage capacity in cortical GABAergic interneurons may vary and that probably, similar to cerebellar granule cells, reelin may be secreted from cortical GABAergic interneurons into the extracellular space by a "constitutive mechanism" (Lacor *et al*., 2000).

One can consider that reelin may be continuously secreted into extracellular spaces and may undergo rapid metabolic processing by the action of extracellular peptidases. This alternative is supported by the observation that cerebrospinal fluid (CSF) contains a significant amount of reelin processing products (Ignatova *et al*., 2004). The presence of reelin in the extracellular space of upper cortical layers expressing a high density of pyramidal neuron apical dendrites may have functional significance and likely suggests a putative role for extracellular reelin in the maturation of newly formed dendritic spines. In fact, reelin secreted into the extracellular space adheres to dendritic spine postsynaptic densities (Rodriguez *et al*., 2000; Costa *et al*., 2001), and the number of dendritic spines is reduced in the PFC of SZ patients (Glantz and Lewis, 2000; Rosoklija *et al*., 2000), as well as in the frontal cortex and hippocampus of the heterozygous reeler mouse (HRM) (Liu *et al*., 2001).

3   Dendritic Postsynaptic Densities and Spines Are Principal Targets of Extracellular Matrix Reelin

We investigated the possible contribution of extracellular reelin to synaptic plasticity in HRM. This mouse model expresses only 50% of the reelin expressed by the wild-type mouse (WTM) (Tueting *et al*., 1999; Liu *et al*., 2001). The HRM exhibits: (a) increased density of cortical neuronal packing, (b) a decreased cortical thickness due to neuropil hypoplasia, (c) a marked decrease of dendritic spine density on basal and apical dendritic branches of FC pyramidal neurons, and (d) a decrease in dendritic spines expressed on the basal dendritic branches of CA1 pyramidal neurons of the hippocampus (Liu *et al*., 2001).

To establish whether a defect in GAD67 expression, similar to that observed in SZ, is also operative in neuropil hypoplasia, we studied the heterozygous GAD67 mouse. This mouse expresses about 50% of GAD67 mRNA levels and a similar decrease in GABA biosynthesis in the FC (Liu *et al*., 2001; Carboni *et al*., 2004). At the same time, it expresses normal amounts of reelin and fails to show neuropil hypoplasia or a dendritic spine expression downregulation. These findings, coupled with the immunoelectron-microscopic observation that reelin colocalizes with integrin receptors expressed by dendritic postsynaptic densities (Rodriguez *et al*., 2000; Liu *et al*., 2001; Dong *et al*., 2003), suggest that reelin may be a regulatory factor operative in the expression density of cortical dendritic spines. This plastic function is of particular interest, because the brain neurohistochemical phenotypic traits and behavioral deficits exhibited by HRM are similar to those found in post-mortem brains of psychotic patients.

4 Electrophysiological and Behavioral Action of Reelin

In electrophysiological experiments, the addition of reelin enhances LTP induced by tetanus or high-frequency electrical stimulation in mouse hippocampus CA1 region (Weeber *et al*., 2002). In recent studies, reelin was shown (via ApoE2 and VLDL receptors) to potentiate the NMDA receptor-mediated current intensity in CA1 pyramidal neurons as a result of tyrosine phosphorylation of
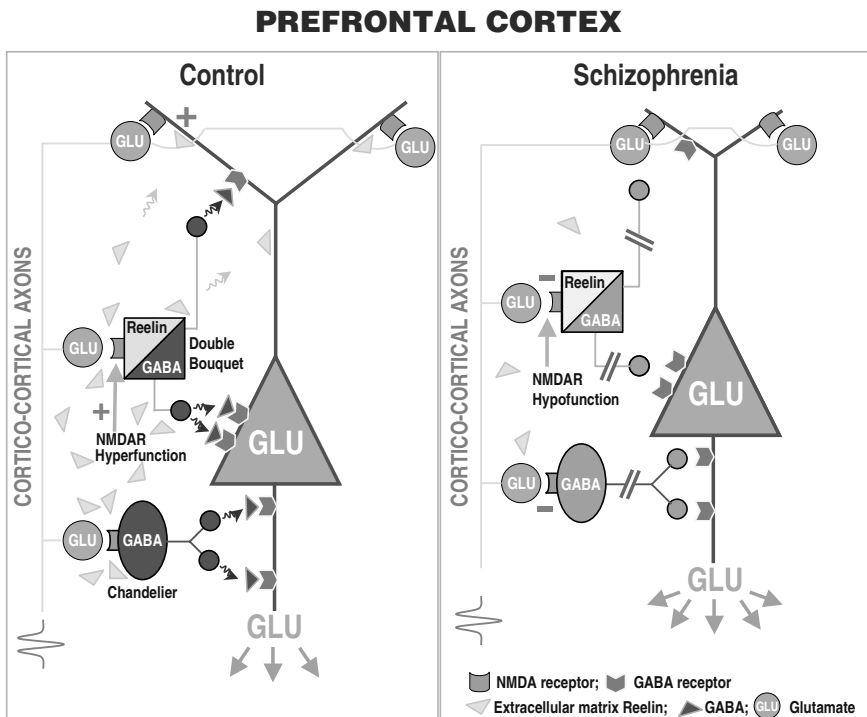

PREFRONTAL CORTEX

Fig. 23.1 (Control) Reelin expressed in double bouquet or horizontal cells in the upper prefrontal cortex layers is secreted by a constitutive mechanism in the extracellular matrix space and: (a) binds to the apical dendritic branches of pyramidal neurons inducing spine formation by facilitating dendritic resident mRNA translation or (b) binds to dendrites or cell bodies of GABAergic interneurons (double bouquet or chandelier cells), facilitating the action of glutamate at NMDA receptors located on GABAergic interneurons and thereby increasing the release of GABA on apical dendrites, cell bodies, and axon initial segments of pyramidal neurons.

(Schizophrenia) Reelin and GAD67 expression and reelin and GABA release are downregulated. The reelin deficit causes: (a) decreased dendritic spine density on the apical dendrites of pyramidal neurons and (b) hypofunction of NMDA receptors located on double bouquet or chandelier cells, eliciting a further decrease of GABA released on the apical dendrites, cell bodies, or axon initial segments of pyramidal neurons. The deficit of GABAergic neurotransmission results in an increased output of glutamate from the axon terminal of pyramidal neurons (*See Color Plates*)

NMDA receptor subunits NR2A and NR2B (Beffert *et al*., 2005; Chen *et al*., 2005). Therefore, reduced expression of reelin in the HRM may result in decreased function of NMDA receptors located on dendrites or cell bodies of GABAergic interneurons innervated by glutamatergic nerve terminals (Fig. 23.1). In addition, the network-driven spontaneous inhibitory postsynaptic currents recorded in CA1 pyramidal neurons of HRM appear to be reduced (Qiu *et al*., 2006). Thus, the GABA-releasing mechanisms may be impaired in HRM due to: (a) a decreased excitatory drive on GABAergic interneuron function and (b) the reduction of GAD67 expression (Liu *et al*., 2001; Carboni *et al*., 2004). Since reelin is localized in the proximity of dendritic postsynaptic densities in cortical and hippocampal regions (Costa *et al*., 2001; Pappas *et al*., 2001), it seems plausible to infer that reelin, by influencing the function of NMDA receptors located on GABAergic interneurons, may selectively facilitate the release of GABA, providing a perisynaptic modulatory milieu for excitatory or inhibitory synapse formation and spine maturation.

Behavioral studies of HRM reveal that reelin haploinsufficiency does not result in a novel specific behavioral pattern but rather causes specific modifications characterized by: (a) a deficit of olfactory discrimination learning (Larson *et al*., 2003), (b) deficits in associative hippocampal learning in contextual fear conditioning tests (Qiu *et al*., 2006), and (c) enhanced susceptibility to the cognitive impairments induced by dizocilpine detected in the eight-arm radial maze (Carboni *et al*., 2004).

The enhanced dizocilpine susceptibility in HRM does not appear to be due to differences in pharmacokinetic characteristics because the levels of dizocilpine in brain cortices of HRM and WTM were virtually equal. We also failed to detect differences between HRM and WTM in glutamate brain content and in the rate of [^{13}C]glucose incorporation into glutamate brain pools. In contrast, we found that the conversion index of glutamate into GABA (an indirect estimation of GABA turnover) is decreased in the cortex, hippocampus, and striatum of HRM compared to WTM (Carboni *et al*., 2004). Qiu *et al*. (2006) reported that downregulation of telencephalic GABAergic transmission may explain the increased susceptibility of HRM to the amnestic action of dizocilpine. Results from our and other laboratories are consistent with the hypothesis that the increased susceptibility of HRM to the amnestic, locomotor, and stereotypic behaviors elicited by dizocilpine may depend on a downregulation of telencephalic GABAergic inhibitory tone. This decrease determines an increase of the intermittent population firing of pyramidal neurons which facilitates increased thalamocortical, corticothalamic, corticocortical, corticostriatal, and corticomesolimbic excitatory transmission. In addition, our findings also point out that in the HRM model, similar to SZ patients, there is a decrease of telencephalic dendritic spine density (Fig. 23.2). Presumably, this decrease in spine density expression is elicited by a combination of GABAergic hypofunction and the downregulation of reelin secretion from GABAergic neurons where reelin is selectively synthesized.
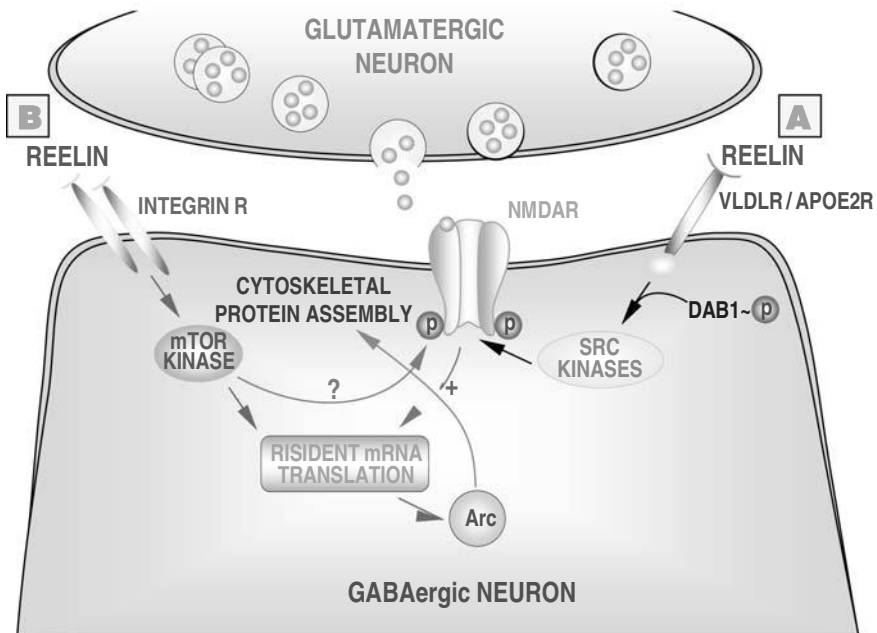
Fig. 23.2 Putative role of reelin in synaptic plasticity. Reelin is depicted binding to a dendritic postsynaptic density of a cortical GABAergic interneuron. Either (A) to VLDL or ApoE2 receptors (VLDLR or APOE2R) or (B) to integrin receptors (INTEGRINR). (A) Reelin modulates NMDA receptor (NMDAR) activity through SRC kinase-mediated tyrosine phosphorylation of the NMDAR intracellular sites (Weeber *et al.*, 2002; Herz and Chen, 2006). (B) Reelin modulates Arc expression and cytoskeletal protein assembly through activation of mTOR kinase (Dong *et al.*, 2003) (*See Color Plates*)

## 5 Reelin Signaling Pathways and the Translation of Dendritic mRNAs

In adult CNS, the molecular mechanisms whereby extracellular matrix reelin, by adhering to cortical dendritic postsynaptic densities, modulates their plasticity, for instance by either changing the density of glutamatergic receptor expression at synapses, facilitating AMPA receptor subunit insertion (Qiu *et al.*, 2007), or increasing the number of dendritic spines (Costa *et al.*, 2001), are not completely understood.

Recent studies suggest that reelin secreted in the extracellular matrix by GABAergic neurons acts as an important indirect modulator of synaptic plasticity in the adult mammalian brain. In fact, reelin binding at synaptic ApoE2 and VLDL and/or integrin receptors (Weeber *et al.*, 2002; Dong *et al.*, 2003) could stabilize

dendritic postsynaptic density expression by providing a molecular scaffold for the assembly of cytoskeletal proteins that facilitate dendritic resident mRNA transport and translation. This could provide a local increase of rapidly inducible protein synthesis (Fig. 23.3) that contributes to LTP consolidation, dendritic spine formation, and ultimately to memory trace formation.

Arc (activity-regulated cytoskeletal protein) is a rapidly inducible cytoskeletal protein whose biosynthesis is encoded by dendrite resident mRNAs located in apical dendrites in spatial proximity to dendritic spines (Steward and Schuman, 2001). This protein is known to be involved in increasing spine formation following LTP consolidation. At dendritic spine postsynaptic sites, Arc mRNA translation is rapidly induced following NMDA receptor stimulation (Steward and Schuman, 2001; Yin *et al.*, 2002). Once Arc biosynthesis is increased, this protein may bind to actin and other cytoskeletal proteins and thus may participate in synaptic remodeling, stabilizing the level of synaptic strength. Arc was found to be decreased in the brains of reeler mice (Lacor *et al.*, 2001, *Soc. Neurosci. Abstr.*27:1759), where the abundance of filopodia-like dendritic spines could be considered an index of spine maturation delay or deficit.

To investigate whether reelin modulates Arc expression by activating its translation directly at dendrites, we (Dong *et al.*, 2003) studied the effects of recombinant reelin on Arc biosynthesis in a synaptoneurosome (SNS) preparation from mouse neocortex. In SNS preparations in which reelin was washed out by a mild Triton X-100 treatment, the application of full-length recombinant mouse reelin results in the displacement of [^{125}I]echistatin binding to integrin receptors with a K_i of 22 pM. On the other hand, echistatin (50–100 nM) completely antagonizes and abates reelin binding to the SNS. The addition of reelin to reelin-free SNS enhances the
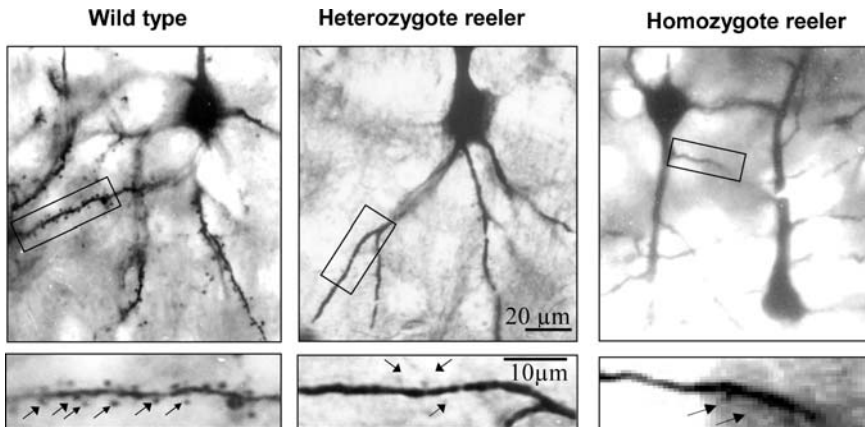
Fig. 23.3 The reeler mouse shows that reelin regulates pyramidal neuron dendritic spine expression. Photomicrographs showing Golgi-impregnated basilar dendritic spines of layer III frontal cortex pyramidal neurons of wild-type (left), heterozygous reeler (middle), and homozygous reeler (right) mice. Note the almost complete absence of spines on dendrites obtained from the heterozygous and homozygous reeler mice. In the homozygous reeler mouse, the laminar structure is disrupted and the pyramidal neuron orientation is altered. (Modified from Liu *et al.*, 2001)

incorporation of [^{35}S]methionine into Arc in a concentration-dependent manner. We also detected that this incorporation is virtually abolished by 50 to 100 nM of rapamycin, a blocker of the mammalian target of rapamycin kinase (mTOR). These data suggest that, in addition to activating Src kinases via ApoE2 and VLDL receptors (Herz and Chen, 2006), reelin may bind to integrin receptors activating mTOR kinases, thereby facilitating dendritic spine resident mRNA translation of Arc and other dendritic mRNAs (Fig. 23.2). Upon reelin addition and activation of mTOR kinase, Arc and other dendritic spine-resident mRNAs very likely acquire a polyadenylation tail that allows them to associate with locally resident polyribosomes to initiate their translation (Richter and Lorenz, 2002).

These findings raise the possibility that reelin binding to integrin receptors at specific spine synapses (i.e., between GABAergic axon terminals and dendritic spines of glutamatergic pyramidal neurons) is a pivotal event which promotes highly selective synaptic modifications during the LTP plasticity associated with memory trace formation.

6   Reelin Deficiency Plays an Important Role in the Pathophysiology of Schizophrenia

Currently, there is compelling evidence that a GABAergic deficit occurs in cortico-limbic regions of patients with either SZ or bipolar disorder; this defect may involve a marked (approximately 50%) decreased expression of GAD67 (one of the two brain isoenzymes that synthesize GABA) (Akbarian *et al.*, 1995; Benes and Beretta, 2001; Woo *et al.*, 2004; Guidotti *et al.*, 2005; Lewis *et al.*, 2005) and reelin (Impagnatiello *et al.*, 1998; Fatemi *et al.*, 2000; Guidotti *et al.*, 2000; Eastwood and Harrison, 2003; Veldic *et al.*, 2004, 2005, 2007; Ruzicka *et al.*, 2007) (Table 23.1). As mentioned, this large-molecular-weight protein, which is synthesized and secreted into the extracellular matrix from GABAergic interneurons and which adheres to dendrites of pyramidal neurons or to somata of GABAergic interneurons, appears to modulate the plasticity of dendritic postsynaptic densities via an activation of a protein kinase that phosphorylates the intracellular consensi of NMDA receptor subunits, thereby increasing their affinity for glutamate (Fig. 23.2) (for reviews see Costa *et al.*, 2001, and Herz and Chen, 2006).

Recent clinical observations and postmortem brain studies suggest that the NMDA receptor density on the somata of cortical or hippocampal GABAergic interneurons is decreased in SZ patients (Krystal *et al.*, 1999; Woo *et al.*, 2004). The decrease of NMDA receptors in GABAergic neurons, together with the decrease of reelin present in the extracellular space, may further reduce the glutamate-mediated release of GABA and the consequent decrease of inhibitory input at postsynaptic sites located on apical dendrites, somata, or initial axon segments of pyramidal neurons in SZ patients (Fig. 23.1).

In this scenario, the downregulation of reelin expression is probably responsible for decreases in dendritic spine density, neuropil hypoplasia, and desynchroniza-

tion associated with the aberrant cortical intermittent population firing that underlie cognitive dysfunctions in SZ (Selemon and Goldman-Rakic, 1999; Glantz and Lewis, 2000; Rosoklija *et al.*, 2000; Black *et al.*, 2004; Spencer *et al.*, 2004).

7   Molecular Basis for Reelin Downregulation in SZ Psychosis

Central to the pursuit of new approaches in SZ treatment is the identification of a molecular basis for the pathophysiological processes that underlie cognitive dysfunction.

The data suggest that in SZ, a defect of reelin secreted from GABAergic neurons may represent a potential pathophysiological target for treatment. Hence, an understanding of whether alterations of genetic or epigenetic mechanisms are responsible for reelin downregulation may offer reelin biosynthesis inhibition as a novel and promising target for antipsychotic drug development.

7.1   Analysis of the Reelin Promoter in Genomic DNA

We mapped the exon/intron structure of the human reelin gene to various BAC sequences present in the human database. The exon/intron structure of *Reln* is remarkably conserved phylogenetically from mouse (Royaux *et al.*, 1997) to human (Chen *et al.*, 2002). The human reelin gene (RELN) maps to chromosome 7q22 (DeSilva *et al.*, 1997) and spans several BAC clones which have been sequenced. We subcloned a 4.2-kb *Eco*RI fragment that contains the entire first exon, 255 bp of the first intron, and some 3.7 kb of 5' flanking DNA. Various unique restriction sites in this clone were used to subclone portions of the region upstream of the ATG start codon into the luciferase reporter construct, pGL-3 basic.

Sequences surrounding the transcriptional start site and first exon form a CpG island, much like that reported for the murine gene (Royaux *et al.*, 1997). Numerous transcription factor search programs identified multiple sequence motifs for different DNA-binding proteins, including CREB, multiple Sp1, and Pax6 sites. We have performed transient transfections and have been able to show that there is an upstream enhancer which contains recognition sites for Tbr1, Sp1, and Pax6. The Sp1 site appears to be critical to the retinoic acid induction of the gene in NT2 cells (Chen *et al.*, 2007).

7.2   Analysis of the 5' UTR

During our initial assessment of potential polymorphic regions in reelin cDNA, we investigated the CGG repeat that is within the 5' untranslated region. This

repeat is unusual because of its proximity to the initiation codon and its potential for formation of hairpin structures that may affect transcription/translation. By far, the most common alleles present in the population studied contained 8 and 10 repeats. Of particular interest is that there is no correlation between any of the repeat-specific alleles and disease phenotype. This is similar to a report by another group who found comparable results (Huang and Chen, 2006). Using transient transfection assays, it has been shown that the amounts of reelin-derived reporter activity negatively correlate with the length of the polymorphic repeat (Persico *et al.*, 2006). This suggests that longer repeats may prove detrimental to stable mRNA expression.

7.3 *Dnmt1 Overexpression and Reelin Promoter Hypermethylation in Telencephalic GABAergic Neurons of SZ Patients*

In spite of the persistent downregulation of reelin mRNA and protein in schizophrenia, evidence of this gene linkage to this disorder is weak. That is, certain reelin alleles are indicated as risk factors only when considered in combination with one of several additional genes that have been implicated (Hall *et al.*, 2007). Very probably, the key factor associated with reelin downregulation is the overexpression of Dnmt1 in telencephalic GABAergic neurons of SZ patients (Veldic *et al.*, 2005, 2007; Ruzicka *et al.*, 2007). Dnmt1 is one member of the DNA methyltransferases, also including Dnmt3a, 3b, and 3L, which methylates genomic DNA in neurons and other cell types (Goll and Bestor, 2005). We have recently reported that in the human cortex, Dnmt1 is expressed at much higher levels than Dnmt3a or 3b in GABAergic interneurons, but its expression is virtually absent in glutamatergic pyramidal neurons. Moreover, Dnmt1 expression is clearly upregulated in cortical GABAergic neurons of layers I and II and in basal ganglion GABAergic medium spiny neurons of SZ brains (Veldic *et al.*, 2005, 2007; Ruzicka *et al.*, 2007). This increase in Dnmt1 correlates with a reproducible decrease in reelin and GAD67 mRNA expression, probably due to promoter hypermethylation (Fig. 23.4).

Consistent with the increased expression of Dnmt1 and the corresponding decrease in reelin and GAD67 expression in cortical GABAergic neurons of SZ patients, we (Grayson *et al.*, 2005) and others (Abdolmaleky *et al.*, 2005) have shown that in SZ patients, portions of the reelin promoter are hypermethylated. We propose that the reduced expression of reelin and also that of GAD67 mRNAs results in a subsequent decrease in interneuron inhibitory tone, which described in the context of SZ appears to be linked to a disruption of pyramidal neuron firing rates (Guidotti *et al.*, 2005; Levenson and Sweatt, 2005; Lewis *et al.*, 2005).

Two reports in the literature show that the reelin promoter is hypermethylated in patients with SZ (Abdolmaleky *et al.*, 2005; Grayson *et al.*, 2005). In the first, the
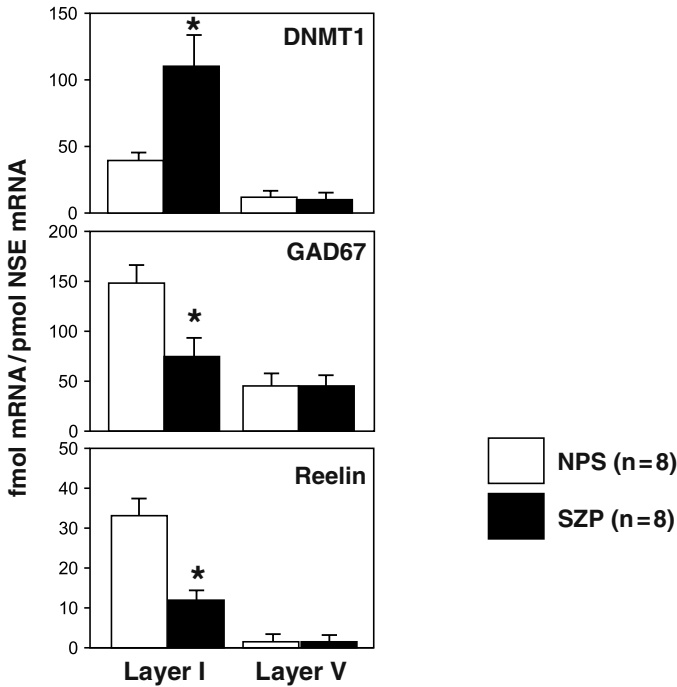
Fig. 23.4 Dnmt1 mRNA is overexpressed and GAD67 and reelin mRNAs are downregulated in prefrontal cortex layer I but not in layer V GABAergic interneurons of SZ patients. Dnmt1, GAD67, reelin, and NSE (neuron-specific enolase) mRNAs were extracted from tissue sections microdissected from layers I and V of Brodmann's area 9 slices and were quantified with nested competitive RT-PCR with internal standards. Data represent the mean ± SE of eight subjects per group. Asterisks denote $p < 0.05$ when SZ patients are compared to nonpsychiatric (NPS) subjects. (From Ruzicka *et al*., 2007)

authors used Brodmann's areas 9 and 10 from the Harvard Brain Tissue Resource Center to extract genomic DNA. Bisulfite analysis and methylation-specific PCR were used to evaluate the extent of methylation. The authors report an increased amount of methylation in 73% of SZ patients compared with 24% in the control group. The more heavily methylated region was located close to the putative CREB binding site positioned around −420 to −398 relative to the RNA start site (Abdolmaleky *et al*., 2005).

In a second report of reelin promoter hypermethylation, we (Grayson *et al*., 2005) examined two different brain cohorts: the Stanley Foundation Neuropathology Consortium and the Harvard Brain Tissue Resource Center. Genomic DNA from occipital cortices was obtained from the first source, while DNA from the PFC was used in the second collection. Bisulfite analysis of genomic DNA followed by nested PCR amplification and sequencing of individual clones was used to identify methylated bases. Interestingly, the analysis of these two patient collections also

showed differences in the methylation patterns among SZ subjects and nonpsychiatric subjects. Genomic DNA from SZ brain was more heavily methylated at two positions, −139 and −134, relative to the RNA start site (Fig. 23.5). While the background methylation patterns were different in both brain collections, the results appeared consistent in these two groups. The two more heavily methylated sites reside within a Pax6 binding site that has recently been shown to be relevant for regulating reelin expression in neural progenitor cells (NT2 cells; Chen *et al.*, 2002, 2007).

In addition to the finding that these sites were hypermethylated in SZ patients, it was also established that double-stranded oligos containing the methylated bases
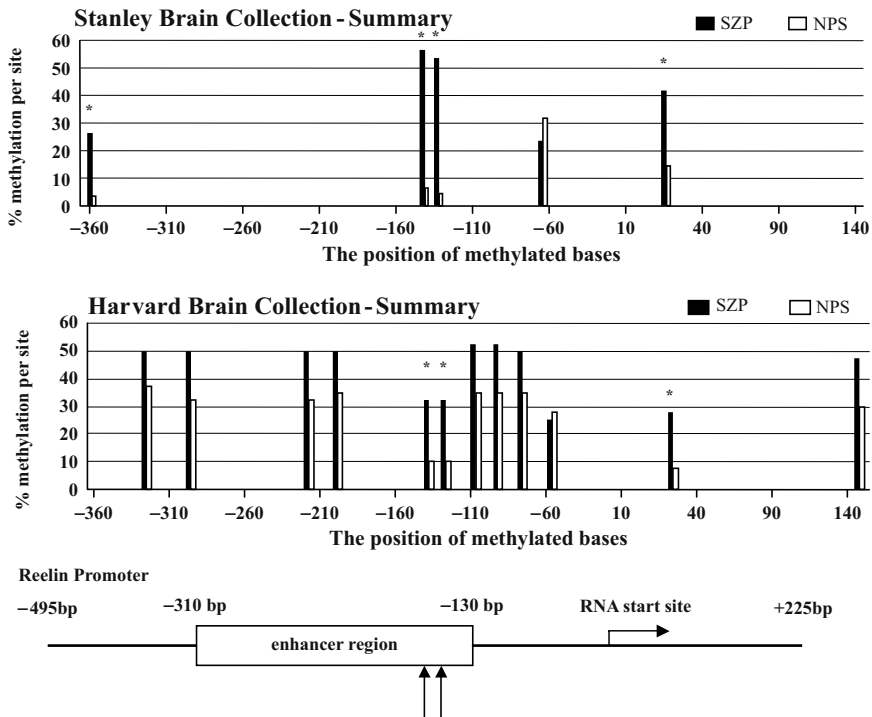

Fig. 23.5 Reelin promoter methylation profile. Genomic DNA was isolated from either occipital cortices (upper) or prefrontal cortices (lower) for bisulfite analysis. (Upper) Summary of data obtained from the Stanley Foundation patients; (lower) data obtained from the Harvard Brain Collection. Following bisulfite conversion, individual DNA strands were amplified, subcloned, and sequenced. The bars show locations of methylated bases. The methylation profiles were different in the two brain collections, which may be due to the brain regions available for this study. A linear representation of the human reelin promoter is shown at the bottom of the figure. Asterisks represent bases consistently methylated in both collections. [Originally published in Grayson *et al.* (2005) and reprinted with permission from the National Academy of Sciences]

bound proteins in nuclear extracts with higher affinity than the nonmethylated oligos. That is, the proteins present in extracts from precursors that do not express the gene showed ~1.7-fold higher affinity for the methylated site compared to the nonmethylated site. This suggests that methyl domain-binding proteins are likely present in NT2 cell nuclear extracts, and that these proteins bind to the methylated bases with a higher affinity. This was confirmed using gel shift competition assays which showed that binding of nuclear proteins was higher at methylated than nonmethylated sites (Grayson *et al*., 2005).

7.4 Point Mutations

To characterize the function of these two sites, we altered each site independently and together in a manner such that only one (m −141, m −136) or both (m −136/−141) base pairs were altered. The remainder of the −514 promoter was left intact and the constructs were transiently introduced into NT2 cells (Fig. 23.6). The data indicate that the −141 bp mutation had little effect on promoter activity, while the m −136 mutant was only half as active as the parent construct, suggesting that this single base pair substitution within the Pax6 binding site is sufficient to disrupt promoter transcription. Although it remains plausible that methylation of this base acts to inactivate the putative Pax6 binding site, it seems more likely that methyl CpG binding proteins, such as MeCP2, bind to the site to repress activity (Grayson *et al*., 2005). While these experiments are interesting, they do not clarify how Dnmt1 or
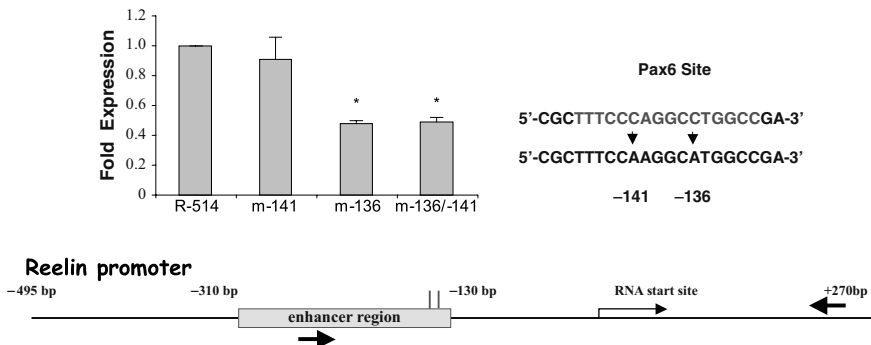
Fig. 23.6 Reelin promoter point mutations. We designed site-directed mutants within the Pax6 binding site that had previously been shown to be more heavily methylated in patients with SZ (Grayson *et al*., 2005). These corresponded to the double (−141/−136), and single promoter mutants (m −141) and (m −136). These minimal mutants were introduced into NT2 cells using transient transfection assays and reporter activity was measured 36 hr later. NT2 cells transfected with the single mutant (m −136) and double mutant construct (m −136/−141) exhibited 50% of the activity of the −514 promoter. *p, 0.05 expressed as a percent of the SV40 promoter and compared with the reelin −514 promoter for statistical purposes (one-way ANOVA followed by Fisher LSD Method) (*See Color Plates*)

other Dnmts act to induce promoter hypermethylation at subsets of promoters operative in the regulation of GABAergic neurons. Answers to these questions will provide clues to the study of mechanisms that may underlie the etiology of SZ and will provide us with additional targets for drug interventions.

8 Reelin Deficiency in an Epigenetic Reeler Mouse Model Relevant to SZ

8.1 Heuristic Value of the Epigenetic Model

The significance of epigenetic downregulation of reelin and GAD67 expression as pathological entities in SZ morbidity would be strengthened by confirming in animal models that reminiscent of SZ, there is a cause–effect relationship between reelin and GAD67 promoter hypermethylation, reelin and GAD67 expression downregulation, and onset of reelin and behavioral dysfunctions. An epigenetic animal model of SZ could be useful not only to clarify the complex mechanisms of gene expression regulation but also to study their pharmacology to reverse the expression of epigenetic mechanisms operative in SZ. Although the HRM model and several other animal models relevant to SZ have been proposed and studied (for reviews, see Gray, 1998; Lipska and Weinberger, 2000; Moser *et al*., 2000; Costa *et al*., 2001; Kilts, 2001; Marcotte *et al*., 2001; Andres, 2002; Murcia *et al*., 2005; Tueting *et al*., 2006), they are all "traditional models" that have been built on genetic but not epigenetic profiling. A new epigenetic model was proposed by Tremolizzo *et al*. (2002), who administered large doses of L-methionine to mice for 15 days to induce reelin and GAD67 promoter CpG-island promoter hypermethylation of genes, such as RELN and GAD67. The rationale for this epigenetic mouse model of SZ is based on several reports of the exacerbation of psychotic symptoms elicited by a 2-week treatment of SZ patients with high daily doses (20–40 g) of L-methionine (Wyatt *et al*., 1971).

8.2 Protracted MET Treatment Induces Reelin and GAD67 Promoter Hypermethylation

The epigenetic mouse model established by administering large dose regimens of L-methionine (MET, 0.25–1 g/kg twice a day for 3 to 15 days) results in: (a) increased FC content of the methyl donor SAM, (b) reelin promoter hypermethylation, and (c) downregulation of the expression of reelin and GAD67 genes by an extent (~50%) that is comparable to the reelin and GAD67 expression deficit measured in the PFC of SZ patients (Guidotti *et al*., 2000; Tremolizzo *et al*., 2002).

The time required for MET treatment to induce a maximum hypermethylation of reelin and GAD67 promoters has been studied using bisulfite DNA promoter sequencing and MSP (methylation-specific PCR) (Tremolizzo *et al.*, 2002, 2005; Dong *et al.*, 2005, 2007). The CpG dinucleotide hypermethylation of RELN and GAD67, but not GAD65 or NSE promoters, increases in a time-dependent manner and reaches its maximum levels within 6–7 days of MET treatment.

Based on bisulfite DNA sequencing data, the analysis of single CpG dinucleotide methylation in the reelin promoter region of mice (from −340 and +160 bp) indicates that after MET treatment, hypermethylation is primarily restricted to CpGs in a region just upstream of the transcriptional start site (Fig. 23.7).

The mechanisms by which MET induces reelin promoter hypermethylation may depend on an increase of brain SAM content that alters high-order chromatin remodeling in GABAergic neurons by: (a) inducing nucleosomal histone (H) tail hypermethylation (i.e., MET—5.2 mmol/kg s.c., twice daily for 15 consecutive days—more than doubled the FC content of dimethyl lysine(K9-H3); and (b) recruiting multifunctional repressor complexes comprising histone methyl transferases (HMTs), histone deacetylases (HDACs), and Dnmts (Burgers *et al.*, 2002; Jenuwein, 2002; Johnstone, 2002; Dong *et al.*, 2005). Further, the recruitment of Dnmt1 methylates reelin and GAD67 promoter CpG dinucleotides.
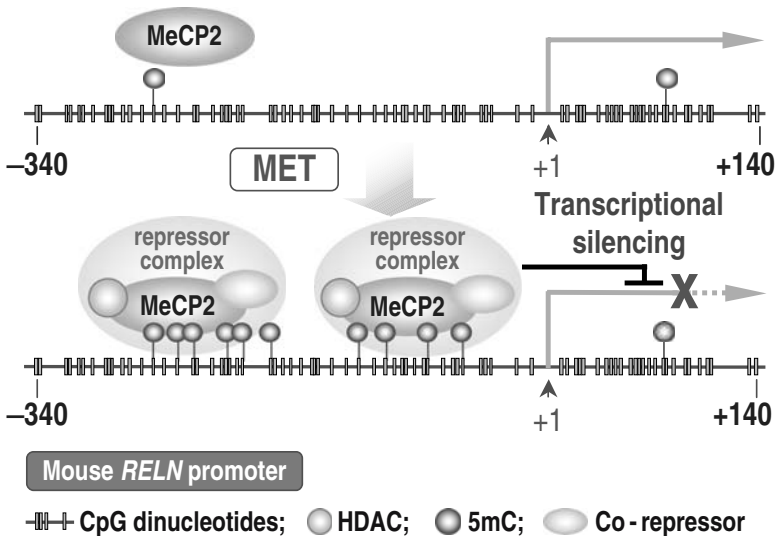
Fig. 23.7 Proposed mechanisms by which mouse *RELN* promoter hypermethylation and recruitment of chromatin remodeling complexes (MeCP2, HDACs, and co-repressors) regulate reelin gene expression. The mouse reelin (*RELN*) promoter region depicted here follows that reported by Tremolizzo *et al.* (2002) and includes the repressor protein complex. Vertical bars represent CpG dinucleotides present in this region. Pink dots denote 5mC present in the sequence. Note the increase of 5mC in MET (methionine)-treated mice. MeCP2 recruits co-repressor complexes including HDACs and induces a state of gene repression (*See Color Plates*)

We have also demonstrated that hypermethylated reelin and GAD67 promoters recruit methyl CpG binding proteins (i.e., MECP2 protein) (Dong *et al*., 2005), which likely contribute to the transition from a transcriptionally active chromatin (euchromatin) to a transcriptionally repressed chromatin (heterochromatin). The proposed mechanism is shown in Fig. 23.7.

8.3   MET-Induced Epigenetic Mouse Model of SZ to Evaluate Prospective Drugs Capable of Increasing Reelin and GAD67 Expression by Inducing DNA-Demethylation

Mice treated with 0.75 g/kg of MET for 15 days, in addition to a decrease of FC reelin and GAD67 that mimics the decrease of reelin and GAD67 observed in the PFC of SZ patients in its intensity, appear to mimic specific phenotypic aspects of SZ. These include a decreased number of spines in apical dendrites of cortical layer II and III pyramidal neurons, and behavioral deficits such as prepulse inhibition of startle (PPI), social interaction, and cognitive abnormalities (Tremolizzo *et al*., 2005). Thus, we have inferred that the MET-induced epigenetic mouse model can be used to study whether drugs that reduce MET-induced behavioral abnormalities related to SZ also reduce reelin and GAD67 promoter hypermethylation and reelin and GAD67 mRNA/protein downregulation.

A logical strategy for the treatment of the reelin/GABAergic dysfunction in SZ would be to normalize the SZ-related increase of Dnmt1 expression in corticolimbic GABAergic neurons by reducing the hypermethylation of reelin and GAD67 promoters with the use of inhibitors of Dnmt1 catalytic activity. However, the most potent Dnmt1 inhibitors (5-aza-cytidine, zebularine) available today fail to readily cross the blood–brain barrier when administered systemically, and are only active in the S-phase of the cell cycle (Brueckner and Lyko, 2004). Hence, a new approach to the treatment of SZ may result from a search for drugs that display direct Dnmt1 catalysis inhibitory activity in nondividing differentiated neurons or drugs that inhibit reelin and GAD67 promoter hypermethylation indirectly by inducing DNA-demethylase activity.

In the epigenetic field, a prevailing concept about the steady-state levels of DNA methylation has been that DNA methylation in somatic cells is almost exclusively maintained by the activities of Dnmts. However, accumulating evidence suggests that the induction of active DNA-demethylases may play an important role in regulating the level of methylated cytosines at various functional stages of differentiation in mammalian cells (Szyf, 2005).

There is also evidence that active demethylation of specific genes is not restricted to differentiating cells but may also take place in somatic postdifferentiated cells, including terminally differentiated neurons. For example, reelin and GAD67 promoters are hypermethylated in the cortex and hippocampus of MET-treated mice, but these promoters can undergo rapid demethylation by the administration of the HDAC inhibitors VPA or MS-275 (Dong *et al*., 2007), presumably via an induction

of DNA-demethylases. Since VPA is gaining clinical importance as a coadjuvant of antipsychotic efficacy in SZ therapy, the benefits of a combination of VPA and atypical antipsychotics in SZ treatment prompted us to study whether epigenetic mechanisms are also included in these treatments with antipsychotics. In preliminary studies, we observed that clozapine (2.5–10 mg/kg) and sulpiride (2.5–10 mg/kg), but not haloperidol (0.5–2.0 mg/kg), exhibited a dose-related increase in the cortical and hippocampal content of acetylated histone-3 (Ac-H3), in 2 hours. Clozapine and sulpiride injected into MET-pretreated mice in doses that increase brain Ac-H3 induce a rapid demethylation of hypermethylated reelin and GAD67 promoters. Furthermore, when clozapine or sulpiride at relatively low doses (i.e., 1.25 mg/kg) was given together with threshold HDAC inhibitor doses of VPA (i.e., 0.75 g/kg; Tremolizzo *et al*., 2002), the two atypical antipsychotics dramatically accelerated the demethylation of the hypermethylated reelin and GAD67 promoters. In contrast, haloperidol, a typical antipsychotic, was ineffective.

We are currently studying other typical and atypical antipsychotics. So far, the results suggest that: (a) atypical antipsychotics may enhance DNA demethylase activity, (b) direct and indirect activators of nuclear DNA-demethylase may have a beneficial action in relieving psychotic symptoms via DNA demethylation of promoters regulating the expression of genes such as reelin and GAD67 downregulated by hypermethylation in SZ, and (c) the coadministration of antipsychotics with HDAC inhibitors may increase their potency and reduce their side effects during SZ treatment.

Hence, a pharmacological strategy with great potential to normalize the reduced amount of reelin, GAD67, or other protein expression in cortical GABAergic neurons of SZ or BP patients is to use drugs that by inhibiting HDACs can reduce the pathology of hypermethylation of reelin and GAD67 promoters via an induction of DNA-demethylases.

The possible success of such a strategy is supported by a report that the short-chain fatty acid VPA (used as an adjunctive with antipsychotics in the medication of SZ morbidity) and the benzamides MS-275 or sulpiride given to animals in doses that increase acetylation of brain chromatin histones (Tremolizzo *et al*., 2002, 2005; Simonini *et al*., 2006), induce reelin and GAD67 promoter demethylation and thereby antagonize MET-induced reelin and GAD67 expression downregulation (Tremolizzo *et al*., 2002, 2005; Weaver *et al*., 2006; Dong *et al*., 2007).

9   Conclusions

Evidence is accumulating that the hypermethylation of reelin and GAD67 promoters is part of the etiopathogenetic process that leads to their transcriptional inactivation and to the GABAergic dysfunction and cognitive deficits found in SZ. This evidence encourages the development of new treatment procedures that can reverse the epigenetically induced reelin and GAD67 transcriptional inactivation by acting on the dynamic interplay of chromatin remodeling processes, including DNA promoter methylation, DNA promoter demethylation, and covalent histone modifications.

Recent studies suggest that DNA methylation and demethylation of genes encoding for reelin and GAD67 and perhaps of other genes are dynamic processes ongoing in the adult brain (Costa *et al*., 2001; Guidotti *et al*., 2005; Dong *et al*., 2007; Miller and Sweatt, 2007). In SZ, the downregulation of reelin and GAD67 expression elicited by hypermethylation of reelin and GAD67 promoters points to a role for epigenetic mechanisms in memory formation.

Presently, one may speculate that a future SZ medication to be considered for normalizing reelin and GAD67 downregulation in the treatment protocols of SZ patients might be the use of Dnmt1 activity antagonists (i.e., procainamide) (Brueckner and Lyko, 2004). These antagonists may be administered with VPA or with its more active HDAC inhibitor analogues in an attempt to induce the activity of neuronal DNA-demethylase.

In fact, so far, the more promising drugs to target epigenetic disorders of cortical GABAergic neurons in SZ are the HDAC inhibitors which have the ability to induce DNA demethylation (Dong *et al*., 2007). Two of these drugs, namely, VPA and sulpiride, are already known to be beneficial in SZ when administered in combination with atypical antipsychotics in patients resistant to antipsychotic monotherapy (Wassef *et al*., 2003; Munro *et al*., 2004).

The analogies of sulpiride action with that of VPA on HDAC activity, which modify reelin and GAD67 expression, suggest that in the treatment of SZ symptomatology, their adjuvant action with atypical antipsychotics may be mediated via an epigenetic modification of GABAergic tone.

To use the GABAergic neurotransmitter system as a target for SZ treatment is now becoming almost mandatory (Guidotti *et al*., 2005); it does not make any sense, and it is not even justified to insist on the continuation of monotherapy with dopamine antagonists, which are not as potent as VPA or HDAC inhibitors combined.

Our data on reelin and GAD67 gene expression regulation point out numerous directions for future research on a therapy targeted to epigenetic mechanisms, including the actions of typical and atypical antipsychotics on the catalytic activities of Dnmts and DNA-demethylases.

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Chapter 24
Epigenetic Modulation of Reelin Function in Schizophrenia and Bipolar Disorder

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1   Introduction

Studies from several laboratories have provided convincing data to support the notion that altered DNA methylation in response to varying physiological and environmental conditions may play a critical role in the fine-tuning of gene expression. However, the establishment of abnormal gene promoter DNA methylation patterns resulting from environmental insults or dysfunctional genes of the DNA methylation machinery may destabilize the normal epigenetic modification of genes. This may affect the equilibrium in the differential gene expression patterns in the normal differentiated cells and tilt the balance toward the disease phenotype. The individuals with genetic susceptibility to specific diseases are likely to be more prone to abnormal DNA methylation. Thus, it is highly likely that the lack of a direct relationship between genotype and phenotype in major psychiatric disorders and the variability in the manifestation of diseases in individuals with identical genetic makeup could be derived from the changes in the DNA methylation patterns.

Recent studies have reported that hypermethylation of DNA at different sites of the promoter region of reelin (*RELN)* with corresponding alterations in gene expression is associated with schizophrenia (SCZ) pathogenesis. It appears that hypermethylation of proximal cytosines of the promoter may recruit inhibitory proteins (i.e., MeCP and MBD2), while the methylation of the distal sites may inhibit binding of the stimulatory factors (SP-1 and CRE), impacting the expression of *RELN*, a hypoexpressed gene in SCZ and mood disorders. In addition to the effects of micro- and macroenvironment during early or later life, dysfunction of dopaminergic and serotonergic systems may also be responsible for the aberrant DNA methylation pattern of *RELN* promoter in SCZ and bipolar disorder (BD).

The findings obtained from the *RELN* studies provide compelling reasons for future validation of these initial observations, as well as for the extension of these studies to other genes with significant roles that are still to be uncovered. In the long term, this line of research will likely play a significant role in helping to develop strategies for early diagnosis, prevention, and therapy of SCZ, BD, and other mental diseases. Importantly, these studies strongly suggest that, in addition to genetic analyses, epigenetic analyses of the candidate genes are necessary before one can formulate a comprehensive picture of the molecular basis of the pathogenesis of complex diseases.

About 10 years ago, after nearly three decades of epigenetic research in medicine, the concept of epigenetics, defined by Waddington in 1940 (reviewed by Morange, 2002), was introduced to the field of psychiatry, and it was followed by extensive discussions and research to establish its potential roles in the pathogenesis of mental disorders (Tasman *et al.*, 1997; Petronis, 2000; Singh *et al.*, 2003; Abdolmaleky *et al.*, 2004a). Based on the current views, epigenetics refers to modifications in gene expression that are controlled by heritable, but potentially reversible, changes in DNA methylation and/or chromatin structure, RNA editing, and RNA interference, which are not accompanied by any change in DNA sequences (Bird, 2002; Jiang *et al.*, 2004; Lavorgna *et al.*, 2004). Although all of the cells of an organism have the

same genetic makeup, each tissue and even individual cell may elicit specified functions in multicellular organisms. Throughout cell differentiation, as well as evolution, epigenetic modifications of the DNA structure could provide the cells with unique identity and function in each cellular network and environmental condition, however in a dynamic manner (Russo *et al.,* 1996; Bird, 2002). Although epigenetics marking is established and developed, due to interactions with other cells and the environment, the cell-specific epigenetic profiles, which govern cell differentiation during embryogenesis, are retained in the genomic memory to be transferred to the next generation of cells (Monk, 1995; Russo *et al.,* 1996; Bird, 2002).

DNA methylation is one of the best-known epigenetic mechanisms for encoding the micro-/macroenvironmental exposures, as well as the inherited epigenetic properties to the developmental memory (Monk, 1995; Russo *et al.*, 1996; Bird, 2002). Methylation of DNA is mediated by methyltransferase enzymes (Bestor, 2000; Kim *et al.*, 2002) by catalyzing the addition of a methyl group (CH_3) to the cytosines which are followed by guanine (CpG). While S-adenosyl methionine is the major methyl donor, folic acid and B12 are involved in remethylation/recruitment of the demethylated S-adenosyl methionine (Fenech, 2001). It has been shown that manipulation of any of these contributors/players can change DNA methylation patterns of genes and influence their expression levels (Bertino *et al.*, 1996; Cooney *et al.*, 2002; Dong *et al.*, 2005; Waterland *et al.*, 2006). Furthermore, it is also well documented that the manner of nurturing in early life could modulate DNA methylation pattern and gene expression levels in later life (Weaver *et al.*, 2004). Therefore, environmental insults on DNA methylation pattern could impact the epigenetic memory leading to the disease phenotype, particularly in individuals with genetic susceptibility to specific diseases. However, flexibility and dynamics of DNA methylation could also allow adaptive fine-tuning of the gene expression in variable environmental conditions or in individuals with dysfunctional polymorphisms (Abdolmaleky *et al.*, 2006).

The emergence of this new branch of science, as a new paradigm to the field of molecular genetics, has promoted a worldwide interest in examining the potential roles of epigenetics in mental illnesses. Accordingly, several studies have directly addressed the epigenetic alterations of specific genes in psychiatric disorders as summarized in the next section.

1.1 A Summary of Epigenetic Aberrations in Major Psychiatric Diseases

Epigenetic modification of DNA, resulting in differential disease phenotypes, has been well documented in other complex diseases, such as cancer. The first observations in neuroscience came from studies of fragile X and Rett syndrome (reviewed by Robertson and Wolffe, 2000), and the brain laterality of DRD2 promoter DNA methylation (Popendikyte *et al.*, 1999). In fragile X, DNA hypermethylation of the

expanded CGG repeats in the *FMR1* gene, and its association with the severity of the disease was reported (Weinhausel and Haas, 2001). In Rett syndrome, a mutation in *MECP2* influences the interplay of MECP2 protein with methylated DNA (reviewed by Akbarian, 2003; Akbarian *et al*., 2006) that normally inhibits the binding of transcription factors (Kaludov and Wolffe, 2000). Following these observations, activity-dependent DNA methylation of BDNF promoter at CRE binding sites was reported (Martinowich *et al.,* 2003) as one of the most important observations of the current decade in the field of neuroscience that was followed by elegant studies addressing the effects of early life experiments on steroid receptor promoter DNA methylation status in the hippocampus (Weaver *et al*., 2004). Furthermore, the established epigenetic modifications of the DNA structure during early life periods were manipulated in adult animals, causing change in the corresponding phenotype (Weaver *et al*., 2005).

In addition, several investigators reported the following methylation changes in various genes and diseases: (1) DNA hypermethylation of *RELN* promoter (Abdolmaleky *et al*., 2005; Grayson *et al*., 2005) and *SOX10* (Iwamoto *et al*., 2005) in SCZ; (2) hyperexpression of DNMT1 (Veldic *et al*., 2004, 2007; Ruzicka *et al*., 2007) and increase in S-adenosyl methionine content (Guidotti *et al*., 2007) in cortical interneurons of patients with SCZ and psychotic BD; (3) hypermethylation of genomic DNA for alpha synuclein (Bonsch *et al*., 2005), as well as HERP gene promoters in alcoholism (Bleich *et al*., 2006); and (4) hypomethylation of *MB-COMT* promoter in SCZ and BD (Abdolmaleky *et al*., 2006).

These observations have now provided a convincing rationale in accepting the emerging science of epigenomics and methylomics, as complementary to the study of genomics, in uncovering the dilemmas of pathogeneses in major mental diseases. As 30% of human genes expressed in the brain (Davies and Morris, 1997) and ~50% of all genes harbor CG-rich promoters (Costello and Plass, 2001), approximately 5000 genes are legitimate candidates for methylation analyses in the field of neuroscience. Since methylation pattern of each gene could vary in each region of the brain, epigenetic profiling of the gene methylome, particularly targeted to the regulatory elements, could be an expanding field of research in the next decade and may serve a bridge to the next revolution in psychiatry.

1.2   Epigenetic Modulation of RELN Functions

RELN is mainly expressed by GABAergic interneurons of the brain and encodes a large extracellular matrix protein that acts on apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR). *RELN* is involved in neuronal migration, cellular positioning, axonal branching, synaptogenesis, and memory formation throughout development of the brain and in later life (Pesold *et al*., 1999; Fatemi *et al*., 2000; Costa *et al*., 2001, 2006; Beffert *et al*., 2005). Binding of RELN to lipoprotein receptors (LPR) activates a tyrosine kinase (TK)-dependent cascade leading to Dab1 phosphorylation (Fig. 24.1) and activation of Src-family
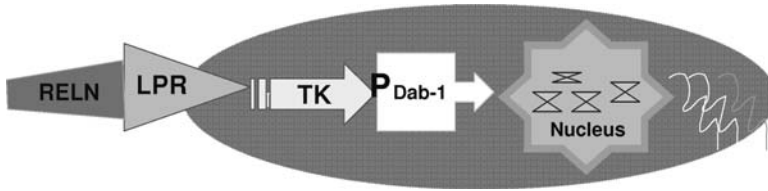
Fig. 24.1 Binding of RELN to lipoprotein receptors (LPR) activates a tyrosine kinase (TK)-dependent cascade leading to Dab1 phosphorylation and expression of several genes that lead to long-lasting structural changes (*See Color Plates*)

kinases and Akt that coordinately orchestrate the expression of several genes that lead to long-lasting structural changes (Pesold *et al.*, 1999; Homayouni *et al.*, 2003; Beffert *et al.*, 2005, 2006). RELN also causes an increase in *DRD2* expression (Ballmaier *et al.*, 2002) and modulates NMDA activity mediated by Dab1 (Chen *et al.*, 2005) and ApoER2 (Beffert *et al.*, 2005, 2006). ApoER2 also forms a functional complex with NMDA receptors and facilitates memory formation in adulthood (Beffert *et al.*, 2005). It has been reported that NMDA receptors influence acetylation of histone H3 in hippocampus (Levenson *et al.*, 2004), which is likely to be tied to DNA methylation changes. NMDA signaling is also known to participate in the regulation of several neurotransmitters, including dopamine and norepinephrine, through intracellular mechanisms involving CREB (Martucci *et al.*, 2003; Marrone *et al.*, 2006). Thus, any malfunction of the *RELN* gene could affect glutamatergic and dopaminergic pathway functions as well. Consistent with this notion, reeler mice, which are haploinsufficient for *RELN* expression, showed a decrease in dopamine transporter (DAT1) and DRD2, an increase in DRD3 levels, and a failure of dopaminergic neuron migration (Ballmaier *et al.*, 2002; Nishikawa *et al.*, 2003).

Currently, there are no polymorphisms for *RELN* that are known to be associated with SCZ or BD. Although the long allele of polymorphic GGC repeat in the 5' untranslated region of *RELN* is correlated with reduced gene expression compared to the common 8- and 10-repeat alleles (Persico *et al.*, 2006), this polymorphism has not yet been positively linked to SCZ (Akahane *et al.*, 2002; Huang and Chen, 2006). However, it is interesting to note that while animal studies showed that chronic use of antipsychotic drugs, such as olanzapine, could increase *RELN* expression and protein level (Fatemi *et al.*, 2006), individuals carrying the 10-repeat allele may have a better response to antipsychotic treatment (Goldberger *et al.*, 2005).

Human postmortem studies showed that *RELN* expression is reduced in the brains of patients diagnosed with SCZ, BD, depression, and autism (Impagnatiello *et al.*, 1998; Guidotti *et al.*, 2000; Fatemi *et al.*, 2000, 2005a, 2005b; Eastwood and Harrison, 2003; Ruzicka *et al.*, 2007; Veldic *et al.*, 2007). These observations, supported by methionine-induced exacerbation of psychotic symptoms in SCZ and BD, led to the conclusion that hypoactivity of *RELN* may be due to hypermethylation of

the gene's promoter (Chen *et al*., 2002). Animal studies also showed that L-methionine, a methyl donor, is linked to a decrease in *RELN* mRNA, which was associated with an increase in the degree of *RELN* promoter methylation (Tremolizzo *et al*., 2002), while valproate could prevent methionine-induced *RELN* promoter hypermethylation (Chen *et al*., 2002; Tremolizzo *et al*., 2002). Other, more recent studies have also found that hypoexpression of *RELN* was associated with the hyperexpression of *DNMT1*, and a corresponding increase in S-adenosyl methionine content in postmortem brains of patients with SCZ and BD (Guidotti *et al*., 2007).

RELN harbors one of the most CpG rich promoters in the human genome with 72 candidate cytosines for methylation and several regulatory elements (e.g., CRE, SP1, and the consensus GC box) in 450 base pairs upstream of the first exon (Fig. 24. 2). Most of these binding elements harbor **CG** islands in their sequence, such as CRE (TGA**CG**TCA), SP1 (GGG**CG**G), and consensus GC box (GGGG**CG**GG**CG**CC), that affect cAMP-induced activities (Park-Sarge and Sarge, 1995).

Human studies have revealed that various human tissues, such as liver, stomach, and breast, express *RELN* mRNA (Abdolmaleky *et al*., 2005) indicating that *RELN* has a pleiotropic role in human development. Further studies on some of these tissues show an inverse correlation between *RELN* promoter methylation and expression levels (Fig. 24.3), providing direct support that *RELN* promoter methylation modulates gene expression. Our analyses of several cancer cell lines have also shown that, unlike normal tissues, *RELN* promoter is extensively hypermethylated and underexpressed in some types of cancers. For example, the MCF7 cell line contains a totally methylated *RELN* promoter (Fig. 24.3) and, as a result, the gene expression is almost 15 times less than in normal breast tissue (Abdolmaleky, Zhou, and Thiagalingam, unpublished). These observations indicate that *RELN* has diverse roles in human development and diseases that are yet to be uncovered.

Fig. 24.2 A view of *RELN* promoter sequence. *RELN* harbors a CG-rich promoter with 72 candidate cytosine (C) sites for methylation and several regulatory binding sites located in 450 base pairs upstream of the coding region. A CRE binding site is underlined in the first line and several SP1 binding sites (GGGCGG) and a consensus GC box are underlined in other locations. The boldface **C**s that are followed by G are candidates for methylation, while other Cs or unmethylated Cs will be converted to T during bisulfite treatment (*See Color Plates*)
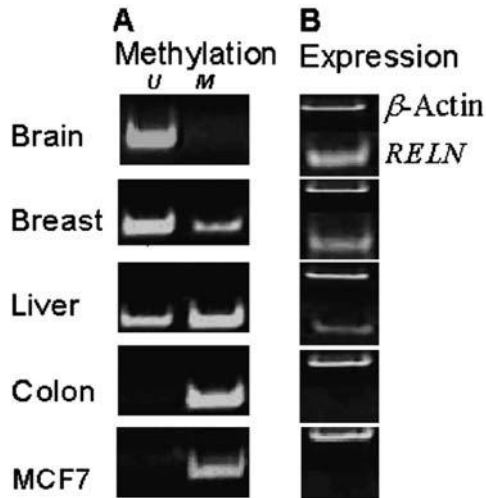
Fig. 24.3 *RELN* expression as a function of promoter methylation. *RELN* methylation and expression analyses in different human tissues showed a direct correlation between methylation of CRE and SP1 binding sites and expression levels and the brain, with the least methylation and greatestt expression levels compared to the other tissues. *U* and *M* in panel A refer to unmethylated and methylated PCR products, respectively. The upper and lower bands in panel B show β-actin and *RELN* expression levels, respectively

Examination of postmortem brains of patients with SCZ versus control subjects, analyzed independently by two groups using completely different sets of samples, although provided from the same brain banks [Harvard Brain Tissue Resource Center (HBTRC) and the Stanley Medical Research Institute (SMRI)], revealed that the *RELN* promoter DNA is hypermethylated in SCZ with a concomitant decrease in the transcript levels (Abdolmaleky *et al*., 2005; Grayson *et al*., 2005). Here, details of the existing data and methodology for the analyses of the methylome of *RELN* gene promoter are outlined to promote the worldwide search for this and other epigenetically altered candidate genes in psychiatric diseases.

2 Methods for the Analysis of DNA Methylation Status

2.1 Bisulfite Sequencing and Methylation-Specific PCR (MSP)

Bisulfite-treated genomic DNA sequencing and MSP have been successfully used to map the differentially methylated CpG islands in the promoter regions of the *DRD2*, *RELN, COMT*, and other genes in psychiatric disorders (e.g., Popendikyte *et al*., 1999; Abdolmaleky *et al*., 2005, 2006; Grayson *et al*., 2005; Murphy *et al*.,

2005). To accomplish this goal, initially, the methylation status of candidate CpG islands could be evaluated using bisulfite sequencing to assess the overall methylation pattern of the target CpG islands. Then, MSP analyses could be used to screen and determine the frequency of DNA methylation (methylated product) of the selected group of specific CpGs. In brief, test genomic DNA is chemically modified by sodium bisulfite to convert the unmethylated cytosines to uracils, while methylated cytosines remain unaffected. Then, primers are synthesized to amplify the target fragment for sequencing (Frommer *et al*., 1992). To perform MSP, primers are synthesized to selectively amplify methylated and unmethylated DNA in separate PCR reactions (Herman *et al*., 1996). PCR reactions are resolved on nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. The bisulfite-modified placental DNA and *in vitro* methylated placental DNA are used as negative and positive controls, respectively, for methylation.

2.2   SYBR Green-Based Quantitative MSP (qMSP) and Quantitative Multiplex MSP (QM-MSP)

Although the standard MSP is an efficient approach for screening the presence or absence of promoter DNA methylation of genes, an evaluation of the degree of methylation could be quantified by performing the qMSP when both controls and the subjects have exhibited different degrees of methylation with significant physiological endpoints. The detailed methodology for this type of analysis is presented elsewhere (Abdolmaleky *et al*., 2008). In summary, in order to evaluate the differential DNA methylation levels of the candidate genes, we established a real-time PCR-based qMSP and quantitative multiplex MSP (QM-MSP) using SYBR green in our laboratory.

In general, for qMSP or QM-MSP, bisulfite-treated DNA was used as the template, and unmethylated or methylated DNA-specific primers (50–100 nM) were used for PCR amplification in separate reactions (Table 24.1) (Abdolmaleky *et al*., 2008). For relative quantification of the methylated product, the method of $\Delta\Delta C_T$ was used and normalized with the C_T (cycle threshold) for β-actin gene (Fackler *et al*., 2004). An additional step in the PCR cycling (72–77°C) was introduced to eliminate the potential confounding effects of nonspecific product or primer dimer formation (Chan *et al*., 2004).

For QM-MSP, first, the promoter regions of the several candidate genes were amplified in the same reaction using primes (Table 24.1), which correspond to the CG free regions of the gene promoters (Swift-Scanlan *et al*., 2006). Then, 1 μL of the 40-fold diluted PCR product was used (as the template) to amplify the methylated and unmethylated templates, using methylation- or nonmethylation-specific primers in separate reactions. By employing this approach, the generation of nonspecific products that may compromise the reliability of SYBR green-based real-time PCR was efficiently eliminated, in addition to the remedy that prevented dimer formation due to the use of the minimal amount of MSP primers (<10 pg) necessary for the second-round PCR (Abdolmaleky *et al*., 2008).

Table 24.1 Primers for methylation analyses of RELN (M: methylated; U: unmethylated specific primers)

Genes and primer type	Forward (5'-3')	Reverse (5'-3')	Ann. Tem.°C (fragment size, bp)
β -actin Promoter Amplification	TTGGGAGGGTAGTT TAGTTGTGGT	CAAAACAAAACA CCTTTTACCCTAA	60 (197)
β -actin for qMSP	GGTGGGTTTAGATTT AGGTTGTGTA	CTACCTACTTTTA AAAATAACAATCAC	60 (125)
RELN Promoter Amplification	GTATTTTTTAGGAAA ATAGGGT	CTCCCAAAAT TACTTTAAA	56 (506)
RELN MSP M1	CGGGGTTTTGACGT TTTTC	CGCCCTCACG AACTCGACG	60 (184)
RELN MSP U1	TATTTTGGTTA TTGTTGTGT	CACCCTCACA AACTCAACA	60 (184)
RELN MSP M2	CGGGAGGTGTTTTT TGCGGGGTTTTGAC	CCGAAAAAAC AAAAAAAA ACGCCCG	60 (115)
RELN MSP U2	TGGGAGGTGTTTTTT GTGGGGTTTTGAT	CCCAAAAAAA CAAAAAAAA ACACCCA	60 (115)
RELN MSP M3	GTCGTCGAGTTAG TTCGAGAGGGC	GACCAAACCTAAA AAAACGCCCG	60 (150)
RELN MSP U3	GTTGTTGAGTTAGTT TGAGAGGGT	AACCAAACCTAAA AAAACACCCA	60 (150)
RELN, nested for sequencing	GTTAAAGGGGTTGGTT (or reverse of RELN promoter amplification primer)		57

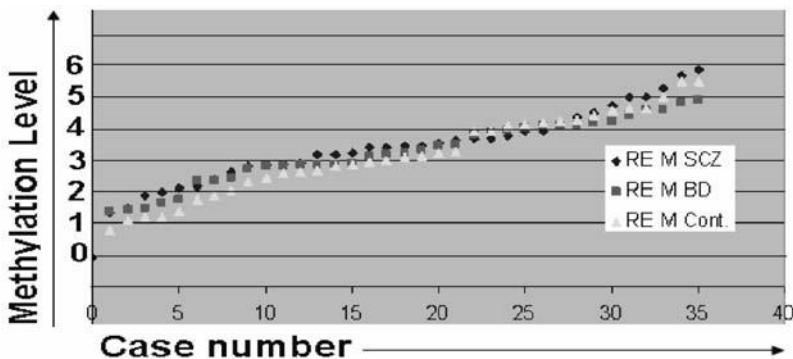# 3 Hypermethylation of *RELN* Promoter Localized to the CRE and SP1 Binding Sites in SCZ and BD

3.1 MSP Analyses for Evaluation of RELN Promoter Methylation Status in SCZ

In a preliminary study, bisulfite sequencing and MSP analysis were employed (Abdolmaleky *et al.*, 2005) to map the promoter methylation status in 10 postmortem brain samples from the frontal lobe Brodmann's area 10 (BA10) of male patients with SCZ and controls with a mean age of 46 (SD = 2.7) donated by HBTRC. These studies using tissue samples from four different locations showed a significantly higher frequency of promoter methylation in SCZ when compared to the control subjects (Abdolmaleky *et al.*, 2005). Subsequent studies using an additional nine brain samples donated by HBTRC (four from SCZ patients and five from the control subjects) with a narrow range of age (mean = 38 and 45, SD = 11 and 5, respectively) showed a similar pattern of promoter methylation and corresponding gene expression changes in BA10.

The same experiments, using a different sample set (105 postmortem brain-derived DNA and RNA samples, including 35 patients with SCZ, 35 BD, and 35 control subjects) from BA46 provided by the SMRI, showed the same trend but at a higher degree and frequency of promoter methylation in both the patients and control subjects (SCZ 83% methylated, versus controls 68%). It is to be noted that the dissected brain tissues from the gray matter (BA10) were used for the isolation of DNA from the HBTRC brain samples, while homogenized brain tissues from the cortical brain regions (BA46), which likely contained more cells from the white matter, were used to extract DNA for the SMRI samples by the provider. As the higher level of methylation in SMRI samples could have arisen from the cells of the white matter (the high sensitivity of MSP enabled it to be detected as a positive methylated signal in both the patients and controls), the level of *RELN* promoter methylation was determined using qMSP, as detailed under methods.

3.2   qMSP Analyses of RELN Promoter Methylation in SCZ

qMSP analysis showed that in SCZ, the level of *RELN* promoter methylation, particularly at the CRE binding site, was significantly increased in comparison to the controls subjects (Fig. 24.4). Furthermore, these analyses revealed that alcohol abuse was associated with a higher degree of *RELN* methylation in SCZ. With the use of qMSP analyses, we also uncovered that the degree of promoter methylation increased with age in both SCZ and the control subjects.

Fig. 24.4 Comparison of DNA methylation levels by qMSP, revealing that the degree of *RELN* methylation in SCZ and BD is almost twice that of the controls. To visualize the differential levels of *RELN* promoter methylation in the patients and controls, the ΔC_T of methylated product for *RELN*, normalized with the C_T of β-actin, was sorted from minimum to maximum. Thus, the increase in the percent of methylation would be exponential. As shown, the base level of *RELN* promoter DNA methylation was greater in SCZ and BD compared to the control subjects (almost twofold). This difference remained nearly the same across the entire samples; however, patients with BD showed a lesser degree of *RELN* methylation in the last part of the curve, where the level of methylation was relatively high (*See Color Plates*)

## 3.3 MSP and qMSP Analyses of the RELN Promoter Methylation in BD

By performing MSP analyses, we found that while 100% (5/5) of manic patients had a severe degree of *RELN* methylation, those patients who were classified as depressed had a significantly lower frequency and intensity of *RELN* promoter methylation in CpGs proximal to the first SP1 binding site. Additionally, from the evaluation of the variables that could be associated with a relatively lower frequency of *RELN* promoter methylation in BD patients, we detected a highly significant association between the use of serotonin-specific reuptake inhibitors (SSRIs) and decrease in the frequency of *RELN* promoter methylation of these CpG sites [38% in SSRI users versus 86% in nonusers ($p=0.004$)]. However, this was not associated with a significant change in gene expression level due to small sample size. Furthermore, qMSP analysis that targeted the CRE binding site revealed that patients with BD who were less than 50 years of age showed a drastically high level of DNA hypermethylation of *RELN* promoter compared to the controls and the other BD patients, although the methylation level generally exhibited a tendency to decrease after age 45 in BD. The level of methylation reached a minimum after age 50 compared to the younger ages (less than 40%). Excluding those patients who were older than 50 (73% of them were under valproate and/or SSRI treatment at the time of death), the methylation level of *RELN* promoter in BD was 50% more than the control subjects. In addition, there was a clear correlation between the degree of *RELN* promoter methylation and the younger age of disease onset in BD.

3.4 Influence of RELN Promoter Methylation Localized to the CRE and SP1 Binding Sites on Gene Expression

Examination of several human tissues provided further validation that methylation of the *RELN* promoter (particularly at the CRE and SP1 binding sites) is associated with reduced gene expression (Abdolmaleky *et al*., 2005) (Fig. 24.3). Similarly, in the normal human brain, the expression of *RELN* in half of the samples who exhibited lower levels of methylation was 1.5 times higher than the other half with higher level of methylation ($p=0.04$), as determined by quantitative real-time PCR (qRT-PCR) and qMSP, respectively. Expression analysis of HBTRC samples using qRT-PCR also showed a significant degree (almost 40%) of hypoexpression of *RELN* in SCZ compared to the control subjects ($p=0.015$). The same analysis on SMRI samples revealed that SCZ and BD patients had a lower expression of *RELN* compared to the controls (almost 20%). However, this difference reached a significant level only in half of the patients with a high level of *RELN* methylation, compared to the entire group of control subjects ($p=0.04$; two-tailed *t*-test). In other words, the decrease in *RELN* expression in SCZ patients with low levels of *RELN* promoter methylation was insignificant compared to the control subjects, but the mean level of expression was decreased by at least 15% in this group compared to the same group of the controls.

3.5 Inverse Correlation Between the Expression of RELN Versus DRD1, DRD2, and MB-COMT

Since our previous studies (Abdolmaleky *et al.*, 2006) revealed that *MB-COMT* promoter hypomethylation was associated with *DRD2* and *RELN* promoter hypermethylation, we analyzed the expression of *DRD2* and *RELN* genes in the same samples. In order to quantify the correlation between *MB-COMT* versus *DRD2 or RELN* expression, we stratified the total samples, controls, and patients into two subgroups (low and high *MB-COMT* expression) as sorted by *MB-COMT* expression levels (Fig. 24.5). The expression of *MB-COMT* was inversely correlated with the expression of *DRD2* or *RELN* in the entire samples ($p = 0.001$ for both *DRD2* and *RELN*, in 52 low- versus 52 high-*MB-COMT*-expressing groups of the SMRI samples; two-tailed t-test). We also examined the trend within the patients and the control groups of the SMRI samples. The expression of *DRD2 and RELN* in 17 SCZ patients with high levels of *MB-COMT* showed significant reduction compared to the same group with low levels of *MB-*
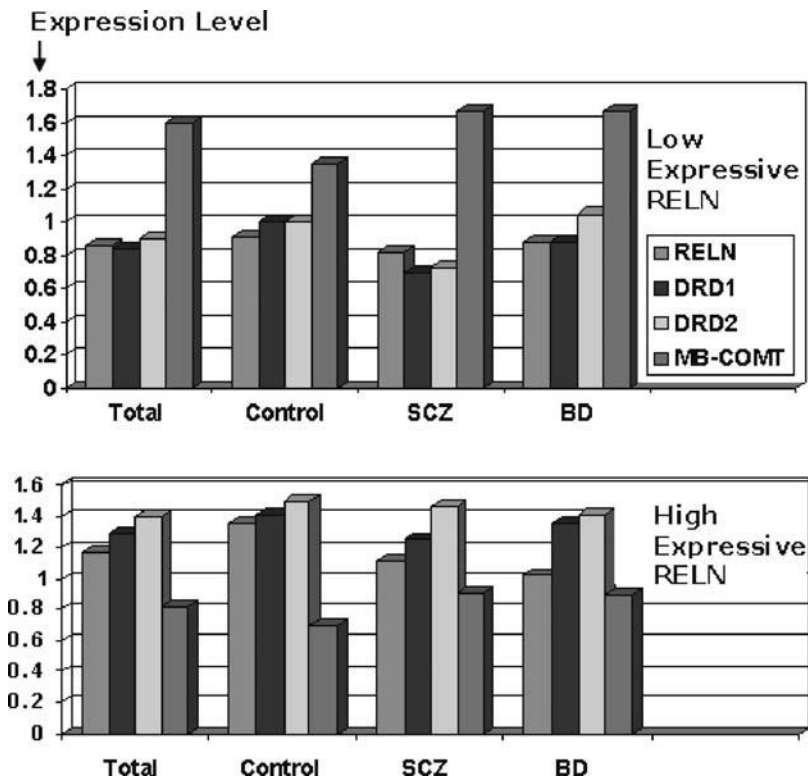
Fig. 24.5 Inverse correlation between the expression of *RELN* and *DRD1*, *DRD2*, and *MB-COMT*. Consistent with the promoter methylation status, expressions of *RELN, DRD1,* and *DRD2* appear to be correlated, but are inversely correlated with the *MB-COMT* expression in both controls and the patients, as well as in total samples. As a result, *RELN* hypoexpression could be associated with hypoactivity of dopaminergic neurotransmission in the frontal lobe (*See Color Plates*)

COMT expression (*p*=0.01 and 0.04, respectively; two-tailed *t*-test). A similar trend for such relationship in the control subjects was also detected (*p*=0.05 and 0.03, respectively). Furthermore, it is noteworthy that the expression of *DRD2* and *RELN,* in high-*MB-COMT*-expressing SCZ and BD patients, was significantly lower than in the control subjects (*p*=0.005 and 0.03, respectively).

Additionally, a direct correlation between the expression of *DRD1* and that of *DRD2* and *RELN* was detected (*p*=0.0000005 and 0.025, respectively, in 50% of the total samples with minimum level of *DRD1* expression versus the other 50% with maximum level of *DRD1* expression). This association remained significant in SCZ and controls, as well as in BD for *DRD2* (*p*=0.0005, 0.003, and 0.05, respectively). Overall, as shown in Fig. 24.5, the expressions of *DRD1, DRD2,* and *RELN* were highly correlated, whereas they were inversely correlated with that of *MB-COMT*. These observations suggest that *MB-COMT* hyperactivity causing a reduction in the synaptic dopamine level and *DRD1* or *DRD2* understimulation are likely to influence *RELN* promoter methylation/hypoexpression contributing to the pathogenesis of SCZ and BD. However, the possibility of a converse correlation remains to be excluded, i.e., *RELN* hypermethylation may lead to *DRD1/2* hypoexpression and/or *MB-COMT* hyperexpression. In support of the latter possibility, SCZ subjects with a low level of *RELN* methylation assessed by qMSP showed almost 95% and 60% higher expression of *DRD1* and *DRD2*, respectively, and 50% lower expression of *MB-COMT* compared to those individuals with a maximum level of *RELN* methylation (half of the samples with low versus high *RELN* methylation as determined by sorting the methylation level, *p*=0.016, 0.03, and 0.005, respectively; two-tailed *t*-test). The same trend for *DRD1*, as well as *DRD2* in the control samples, was also detected (60% less, *p*=0.06). Furthermore, the expression of *MB-COMT* was significantly increased in low versus high *RELN* methylation in SCZ, BD, and the entire samples (*p*=0.005, 0.05, and 0.002, respectively; two-tailed *t*-test). Overall, the observations suggest that there could either be a cause–effect relationship between the expressions of these genes or they are simultaneously influenced by the same unknown factor(s).

3.6 The Effect of the Functional Status of the Dopaminergic System on the Modulation of RELN Promoter Methylation at SP1 and CRE Binding Sites

As has been reported elsewhere (Abdolmaleky *et al.*, 2006), our studies showed a significantly high frequency of *MB-COMT* hypomethylation in SCZ and BD compared to control subjects. Additionally, there was a significant correlation between *MB-COMT* hypomethylation and concurrent hypermethylation of *DRD2* and *RELN* promoters in SCZ (11/35) and BD (12/35) versus control subjects (0/35) (*p*=0.001). The qMSP analysis of the same samples revealed that the level of *RELN* promoter methylation in SCZ and BD with a methylated *DRD2* promoter was greater than that in other SCZ or BD patients (30% and 100%, respectively). In contrast, in controls an inverse relationship was detected (>100% less in individuals with methylated *DRD2*, compared to unmethylated *DRD2*). Furthermore, as shown in Fig. 24.5,

there was also a direct correlation between *RELN, DRD2,* and *DRD1* expression, and an inverse correlation between the expression of these genes and *MB-COMT*.

Altogether, these data along with the significant correlation observed between the presence of valine allele of *COMT* (the overactive allele) and the frequency of *RELN* promoter methylation (Abdolmaleky *et al.,* 2006) suggest that methylation of the *RELN* promoter could be under the influence of brain dopaminergic systems. However, the likelihood of alternative conclusions, such as *RELN* coordinately orchestrating the expression of dopaminergic genes or the existence of a reciprocal interaction, needs to be investigated.

3.7   The Effects of the Brain Serotonergic System
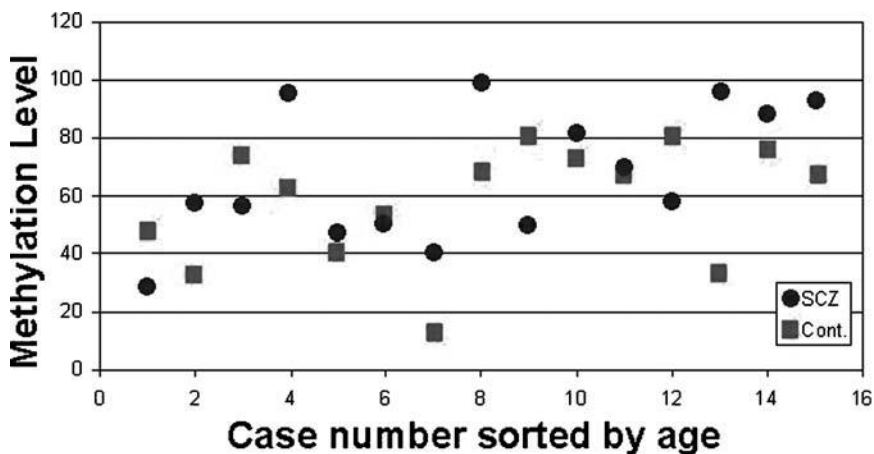###   on RELN Promoter Methylation

As the use of SSRIs was correlated with a significant decrease in *RELN* promoter methylation, we examined the association of the T102C polymorphism of *HTR2A* with the *RELN* promoter methylation level, as determined by qMSP. In SCZ and BD, the degree of *RELN* promoter methylation in individuals with the CC genotype of the T102C polymorphism of *HTR2A* was three and four times higher than the control subjects, respectively. This difference was not drastic in the TC heterozygotes or TT homozygotes. The expression of *RELN* in SCZ and BD with the CC genotype was 30% and 48% lower, respectively, compared to the control subjects with the same genotype. However, the differences were only significant in BD, and these observations indicate that the reported association between the C allele of the T102C polymorphism of *HTR2A* and SCZ, as confirmed by two meta-analyses (Williams *et al*., 1997; Abdolmaleky *et al.,* 2004b), could be related to effects mediated by the CC genotype on *RELN* promoter methylation and expression levels.

4   RELN Promoter Methylation Localized
to −139 to −131 Cytosines

Grayson *et al*. (2005), a group with a decade of research interest in the *RELN* signaling pathway analysis, reported that increased methylation at positions −134 and −139 are critically important in determining the promoter activity of *RELN* through recruitment of MeCP2, MBD2, and/or Dnmt1 to the methylated bases. However, their approach to examining *RELN* promoter methylation status was conducted on a completely different set of brain samples, provided by the same brain banks. They showed that the *RELN* promoter DNA is hypermethylated in SCZ with a concomitant decrease in the transcript levels (Grayson *et al*., 2005). Although this group reported the highest levels of *RELN* promoter methylation of cytosines at positions −139 and −134 compared to the other cytosines in the promoter region, our earlier and recent studies showed low levels of cytosine methylation at these sites. A careful

examination of the samples used by Grayson *et al*. (2005) revealed that discrepancies in the results between the two studies could be primarily due to a large difference in demographics (age, in particular) of our HBTRC samples versus their samples, despite both having been provided by the same brain bank (HBTRC). A comparison of demographic data of their (supplementary Table 2 in Grayson *et al*., 2005) and our samples (Table 1 in Abdolmaleky *et al*., 2005) clearly indicated that almost all of their HBTRC samples were different from our samples. For example, all of our samples were from males and under age 49 (mean age 45.5 and SD=2.7), while 60% of their samples were female and all were over 49 with a mean age of 65 (60% over 65). Furthermore, while 60% of our samples were from the left brain, 30% of their samples were from the left brain. However, there was one case in their HBTRC samples from a male patient with an age of 49. This case exhibited the heaviest methylation level among all of their samples, including the samples from SMRI (see supplementary Table 2 in Grayson *et al*., 2005). Considering that the level of DNA methylation of *RELN* changes with age and that the mean age of their HBTRC samples was 20 years older that ours, it is highly likely that the discrepancy could have arisen from the mean age differences between the two sample sets.

Although a new study on the epigenetic aberration of human *RELN* in SCZ reported an age-related increase in *RELN* methylation only in the control subjects (Tamura *et al*., 2007), our statistical analysis for the effect of age on *RELN* methylation level of the Grayson *et al*. (2005) samples (supplementary Table 2 in Grayson *et al*., 2005) showed that consistent with our data the degree of DNA methylation of *RELN* promoter is significantly increased in both SCZ and the control subjects (Fig. 24.6) (p=0.025 for either SCZ or normal controls; student's t-test). Thus, it would be interesting to perform follow-up studies to uncover the potential impact

Fig. 24.6 Age-dependent increase in *RELN* promoter methylation. The degree of *RELN* promoter methylation (*Y* axis), extracted from Grayson *et al*. (2005, supplementary Table 2), was sorted by age (*X* axis). As shown, the degree of promoter methylation increased by age in both SCZ and the control subjects (*See Color Plates*)

of *RELN* methylation status on the age of disease onset. Our studies so far have established a clear correlation between the degree of *RELN* promoter methylation and the younger age of disease onset in BD.

5   Summary

Studies from our and other laboratories support that altered DNA methylation in response to environmental insults or dysfunctional genes may destabilize the normal epigenetic fine-tuning of genes in psychiatric disorders (Abdolmaleky *et al.*, 2005, 2006; Bonsch *et al.*, 2005; Grayson *et al.*, 2005; Iwamoto *et al.*, 2005; Bleich *et al.*, 2006). Aberrant DNA methylation patterns of *RELN* promoter in SCZ and BD, its association with the dysfunctions of dopaminergic and serotonergic systems and similarity of the promoter DNA methylation status of the patients at young age with the normal controls at old age, and occurrence of an early onset and age-inappropriate *RELN* promoter DNA methylation in the patients, have provided compelling evidence that calls for more investigations in this arena. Thus, further studies will be needed for validation of these initial observations using other sets of brain samples to provide a comprehensive molecular insight for the genesis of SCZ and BD. These studies will have future implications for early diagnosis, prognosis, and management of these diseases, and will provide clues for preventive measures and identification of nodal points for effective therapeutic interventions.

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