Chapter 13
Protein Kinases and Signaling Pathways that Are Activated by Reelin

Jonathan A. Cooper, Nathaniel S. Allen, and Libing Feng

Contents

1 Introduction 193
2 Core Components of the Reelin Signaling Pathway 195
2.1 The Reelin Receptors 195
2.2 Src-Family Kinases 196
2.3 Dab1 197
3 Possible Downstream Signaling Pathways 200
3.1 Kinase Pathways 200
3.2 Dab1-Binding Proteins 202
3.3 Adhesion Molecules 204
3.4 Other Genes Involved in Neuron Migrations: Lis1 and Cdk5 205
3.5 Ubiquitin–Proteasome System 207
3.6 Phosphatases 208
4 Summary and Conclusions 209
References 209

1 Introduction

Defects in the cortex, hippocampus, inferior olive, and cerebellum of Reeler mutant mice were first detected many decades ago (Caviness and Rakic, 1978; Rice and Curran, 2001). Recently, a plethora of other developmental and adult phenotypes have been detected in mutant mice, including misplacement of olfactory

J. A. Cooper
Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, Seattle, WA 98109
e-mail: jcooper@fhcrc.org

N. S. Allen
Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, Seattle, WA 98109

L. Feng
Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, Seattle, WA 98109

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interneurons (Hack *et al*., 2002), facial motor neurons (FMNs) (Ohshima *et al*., 2002; Rossel *et al*., 2005), sympathetic preganglionic neurons (SPNs) (Yip *et al*., 2000), and gonadotropin-releasing hormone (GnRH) neurons (Cariboni *et al*., 2005), reduced dendrite outgrowth in the hippocampus (Niu *et al*., 2004), and defective long-term potentiation (LTP) and memory (Weeber *et al*., 2002). In some genetic backgrounds, the Reeler mutation also causes neurodegeneration and early death, but these phenotypes are not detected in other backgrounds and are likely to be indirect (Brich *et al*., 2003; Goffinet, 1990).How Reelin, the Reeler gene product, creates these different phenotypes is still incompletely understood.

The discovery that Reelin is secreted (D'Arcangelo *et al*., 1995; Ogawa *et al*., 1995) and acts nonautonomously, as shown by chimera experiments (Terashima *et al*., 1986; Yoshiki and Kusakabe, 1998), led to the idea it might be a positional signal. The discoveries of other mutations that recapitulate the Reeler phenotype (Sweet *et al*., 1996; Trommsdorff *et al*., 1999; Yoneshima *et al*., 1997) but act (more or less) cell autonomously (Hammond *et al*., 2001; Yang *et al*., 2002), led to the identification of proteins that are required in cells receiving the Reelin signal. Biochemical experiments in cultured neurons and brain slices, backed up by *in vivo* observations, provide a reasonable model (described in Section 2) for how these components fit together to form a Reelin-activated signaling pathway (reviewed by Gupta *et al*., 2002; Jossin, 2004; Lambert de Rouvroit and Goffinet, 2001; Rice and Curran, 2001). This pathway seems to be needed for almost all of the known Reelin functions—LTP and dendrite outgrowth, as well as positioning of most classes of neurons. However, there are three outstanding questions:

- When and where does Reelin act during development of the cortex and cerebellum? Both migrating neurons and the radial glia are affected by Reelin signaling (Dulabon *et al*., 2000; Forster *et al*., 2002; Hartfuss *et al*., 2003; Hunter-Schaedle, 1997; Olson *et al*., 2006; Pinto-Lord *et al*., 1982; Tabata and Nakajima, 2002; Zhao *et al*., 2004), but it is not clear whether abnormal lamination in the Reeler mutants results from defects in the neurons, radial glia, or both.
- At the cellular level, what is the effect of Reelin? Is it a change in radial glia differentiation or branching or attachment at the pial surface? Or does Reelin regulate the leading edge, nucleokinesis, or cell–cell adhesion of migrating neurons? Or all of the above?
- What other genes and proteins link the known intracellular signaling components to the Reelin-regulated cellular responses that result in a normal brain structure?

The purpose of this chapter is to briefly review the core components and signaling mechanism of the Reelin pathway, and then to present evidence on possible downstream components. Issues related to the first two questions, the timing and site of Reelin action and the possible changes in cell biology, are left for other chapters.

2 Core Components of the Reelin Signaling Pathway

The central core of the Reelin response is shown in Fig. 13.1 This pathway operates in all known Reelin-induced events, including neuron positioning, dendrite outgrowth, and LTP, with the conspicuous exception of the positioning of GnRH neurons (Cariboni *et al*., 2005). In the core pathway, Reelin binding to either or both of the Reelin receptors, VLDLR and ApoER2, induces receptor clustering, which is translated, on the inside of the membrane, into the mutual activation of the tyrosine kinases Src and Fyn and the phosphorylation on tyrosine of the adapter protein Dab1. Evidence supporting this core pathway has come from a wealth of experiments, described below, that have made use of mouse mutants, cultured neuronal progenitor cells, differentiating neurons, brain slices, and reconstituted systems.

2.1 The Reelin Receptors

VLDLR and ApoER2 are two transmembrane apolipoprotein E (ApoE) receptors that are expressed in the developing CNS. ApoER2 is expressed at higher levels in the cortex and hippocampus, and VLDLR at higher levels in the developing cerebellum (Trommsdorff *et al*., 1999), but it appears that, in most respects, their roles are highly overlapping and essentially redundant. An important exception, a nonredundant role of a specific splice form of ApoER2 in Reelin-regulated LTP, will be discussed briefly in Section 3.1.1.

Trommsdorff *et al*. (1999) first showed that *apoer2-/-* mice have subtle developmental defects in the cortex and hippocampus, while *vldlr-/-* have a defective cerebellum, but the double mutant develops the Reeler phenotype. The ectodomains of ApoER2 and VLDLR bind to recombinant Reelin—specifically the central four repeats (R3–7) (Jossin *et al*., 2004)—with similar affinity (D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999). Reelin may bind to other cell surface proteins, but functional

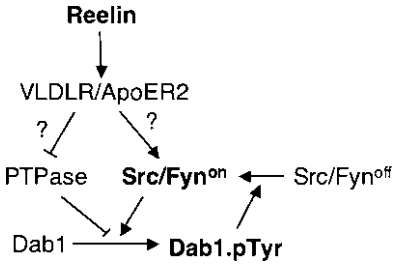

Fig. 13.1 Core components of the Reelin signaling pathway. This figure shows proteins and causality relationships demonstrated by study of biochemical events and phenotypes of mutant mice and neurons

blockade or knockout of VLDLR and ApoER2 is sufficient to block Reelin-induced activation of Dab1 (Benhayon *et al*., 2003; D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999), and expression of either receptor on NIH3T3 cells allows coexpressed Dab1 to be tyrosine phosphorylated in response to added Reelin (Mayer *et al*., 2006). Furthermore, antibody-induced cross-linking of either VLDLR or ApoER2 on cultured neurons activates Dab1 (Jossin *et al*., 2004; Strasser *et al*., 2004). The cytoplasmic tails of VLDLR and ApoER2 contain canonical endocytosis motifs found in all of the LDLR family, FXNPXY, and these motifs bind to the Dab1 adapter protein (Homayouni *et al*., 1999; Howell *et al*., 1999b). A mutant of ApoER2, in which the NFDNPVY stretch was replaced with an EIGNPVY signal, does not bind Dab1 and shows classic Reeler-like defects in the cortex, hippocampus, cerebellum, and LTP (Beffert *et al*., 2006). These results demonstrate that VLDLR and ApoER2 are necessary for Reelin-dependent brain development *in vivo* and are necessary and sufficient for Reelin-dependent Dab1 phosphorylation in neurons.

2.2 Src-Family Kinases

Src and Fyn are two Src-family kinases (SFKs), which contain an N-terminal myristoylation site for membrane association, SH3 and SH2 domains for intramolecular regulation and for protein–protein interactions when activated, a tyrosine kinase domain, and a C-terminal inhibitory phosphorylation site (Sicheri and Kuriyan, 1997). SFKs undergo conformation switching between an inactive, C-terminally phosphorylated state and a fully activated state in which the C-terminus is dephosphorylated and the activation loop of the kinase domain is phosphorylated. Both Src and Fyn are highly expressed in the developing CNS. *Src-/-* mice have no obvious brain defects (Soriano *et al*., 1991). *Fyn-/-* mice have an undulating instead of straight layer of pyramidal neurons in the CA3 region of the hippocampus (Grant *et al*., 1992), which does not resemble the Reeler phenotype. Also, *fyn-/-* layer V projection neurons have misaligned dendritic arbors (Sasaki *et al*., 2002). Interestingly, this is exacerbated in semaphorin 3a (*sema3a-/-*) mutants, and projection neurons of all layers are misoriented in Reeler mutants. Late-generated neurons of *fyn-/-* cortex undermigrate (Yuasa *et al*., 2004), resembling Reeler. However, when both Src and Fyn are mutants, a more obvious Reeler phenotype results, with inefficient preplate splitting, inverted cortical plate, and undermigration of Purkinje cells (Kuo *et al*., 2005). Unfortunately, it is not known whether a complete Reeler phenotype results, because double mutant pups die perinatally.

Neurons from *src-/- fyn-/-* embryos have greatly reduced tyrosine phosphorylation of Dab1 when stimulated with Reelin (Kuo *et al*., 2005). Moreover, pharmacological inhibition of SFKs impairs Dab1 phosphorylation in neurons (Arnaud *et al*., 2003b; Bock and Herz, 2003). Application of SFK inhibitors to cortical plate cultures prevents preplate splitting *in vitro* (Jossin *et al*., 2003b). Reelin stimulates SFK activity when added to neurons (Arnaud *et al*., 2003b; Bock and Herz, 2003). This activation is rather slight, suggesting that a small proportion of the total kinase

population is activated at any one time. The activation depends on both Reelin and Dab1, suggesting that Dab1 is needed for SFK activation, as well as vice versa (Ballif *et al*., 2003). There is thus a mutual interdependence (positive feedback loop), linking SFKs and Dab1 as dual outcomes of Reelin receptor clustering on the surface.

As is the case for VLDLR and ApoER2, Src and Fyn do not have completely overlapping roles. Overall, the absence of Fyn has a bigger effect than absence of Src, and Src only plays a detectable role when Fyn is absent (Arnaud *et al*., 2003b; Bock and Herz, 2003). However, Src and Fyn may play different roles in different types of neurons, and, given that Fyn is more closely associated with lipid rafts than Src, they may contribute differently to Reelin signaling in different membrane microdomains.

2.3 Dab1

Dab1 is a cytoplasmic protein that lacks a catalytic domain and appears to act as an adapter, to create signaling complexes via protein–protein interactions (Howell *et al*., 1997a). Dab1 gene deletion, or reduced expression due to retrotransposon insertion, causes the Reeler phenotype (Howell *et al*., 1997b; Sheldon *et al*., 1997; Ware *et al*., 1997). At the protein level, Dab1 is activated by tyrosine phosphorylation in Reelin-stimulated cultures, and the stoichiometry of Dab1 phosphorylation is decreased in embryonic brains of Reeler mutants in which Reelin is missing (Howell *et al*., 1999a). Dab1 tyrosine phosphorylation is also completely, or nearly completely, abolished in *vldlr-/- apoer2-/-* and in *src-/- fyn-/-* neurons and brains (Benhayon *et al*., 2003; Kuo *et al*., 2005) or by SFK inhibitors *in vitro* (Arnaud *et al*., 2003b; Bock and Herz, 2003). Dab1 is an *in vitro* substrate for Src and Fyn, binds to Src and Fyn SH2 domains after it is tyrosine phosphorylated, and activates Src and Fyn when coexpressed (Bock and Herz, 2003; Howell *et al*., 1997a), although very little if any Src or Fyn forms a stable complex with Dab1 in Reelin-stimulated neurons (Bock *et al*., 2003). Finally, a mutant form of Dab1, Dab1 5F, in which the potential phosphorylation sites are mutated to phenylalanine, is not phosphorylated *in vivo* and cannot support normal brain development (Howell *et al*., 2000). Dab1 5F homozygous animals exhibit all the phenotypes of Reeler mutants that have been studied, proving that Dab1 phosphorylation is required for its Reelin-induced functions.

Dab1 contains an N-terminal PTB-related domain which can bind to PIP_2 (Howell *et al*., 1999b; Stolt *et al*., 2003; Yun *et al*., 2003). Indeed, even though much of the Dab1 in neurons seems to be cytoplasmic, a fraction partitions to the membrane. The Dab1 PTB domain has a second binding site for the FXNPXY signal found in the cytoplasmic tails of the Reelin receptors VLDLR and ApoER2 and in proteins related to amyloid precursor protein (APP) (Homayouni *et al*., 1999; Howell *et al*., 1999b). Dab1 binds independently and noncooperatively to PIP_2 and NPXY (Howell *et al*., 1999b; Stolt *et al*., 2003, 2004). PIP_2 binding promotes basal (Reelin-independent) Dab1 tyrosine phosphorylation (Huang *et al*., 2005), but both

binding sites are needed for Reelin-stimulated Dab1 tyrosine phosphorylation in neurons (Stolt *et al*., 2005; Xu *et al*., 2005). Even though Dab1 activation is driven by ApoER2/VLDLR clustering (Strasser *et al*., 2004), Dab1 binding to the receptors in neurons is barely detectable by coimmunoprecipitation (Bock *et al*., 2003), and it is not known whether Dab1 binding to receptors increases if they are clustered. However, small regions of Dab1 immunoreactivity are transiently detected adjacent to sites of immunoreactive Reelin on the surface of Reelin-stimulated neurons (Morimura *et al*., 2005), suggesting mutual coclustering. Why this makes clustered Dab1 a better substrate for SFKs [or a worse substrate for phosphotyrosine phosphatases (PTPs)] remains unclear. Conformational changes may be involved, or an as yet unidentified tyrosine kinase may initiate the process.

One question raised by the PIP_2-binding activity of the Dab1 PTB domain is whether signaling occurs preferentially from lipid-ordered membrane microdomains (lipid rafts) which are enriched for cholesterol and PIP_2. ApoER2 partitions to lipid rafts (Riddell *et al*., 2001), as does Fyn and some Src (Mukherjee *et al*., 2003). Cholesterol depletion of neurons reduces Reelin-induced Dab1 tyrosine phosphorylation *in vitro* (Bock *et al*., 2003). However, VLDLR is excluded from lipid rafts (Mayer *et al*., 2006) but can activate Dab1 in the absence of ApoER2, leaving open the role of lipid rafts.

Potential tyrosine phosphorylation sites in Dab1 were mapped by mutation to four tyrosine residues (a, Y185; b, Y198; c, Y220; d, Y232) contained in four repeated exons. These residues come in pairs (a and b; c and d) of very similar local sequence (Fig. 13.2). Cotransfected Src causes phosphorylation of Dab1 at multiple sites, and mutants lacking various combinations of sites show progressive loss of phosphotyrosine (Howell *et al*., 2000). Src can phosphorylate all four sites, as detected by specific phosphopeptide antibodies (Keshvara *et al*., 2001) and, for sites b and c, by mass spectrometry (Howell *et al*., 2000). In neurons, Reelin stimulates phosphorylation of sites b and c, detected using phosphopeptide antibodies (Keshvara *et al*., 2001), and site d, detected using phosphopeptide mapping (Ballif *et al*., 2004) and mass spectrometry (B. Ballif, personal communication). It is not known whether site a is phosphorylated in response to Reelin. There has been one attempt to functionally test the importance of individual phosphorylation sites for Dab1 function *in vivo*: Sanada *et al*. (2004) used *in utero* microinjection to express wild-type or point mutant Dab1 proteins in *dab1-/-* brain and followed the location of the altered neurons using green fluorescent protein. They found that wild-type Dab1 rescued the ability of neurons to enter the cortical plate and to detach from their sister radial glia fiber. Surprisingly, mutation of the b site had no effect on rescue, while mutation of sites c or d abolished rescue. Reciprocally, expression of wild-type Dab1 or b mutant Dab1 had no deleterious effects on neurons in a normal brain, while the c and d mutants acted as dominant negatives. These results suggest essential roles for the c and d sites, and a nonessential (or redundant) role for the b site.

Dab1 is alternatively spliced (Howell *et al*., 1997a). One form (555^*) is relatively highly expressed in the VZ compared to the canonical form (555, or p80) (Jossin *et al*., 2003a). Knockin mutants demonstrate that p80 can fulfill all of the

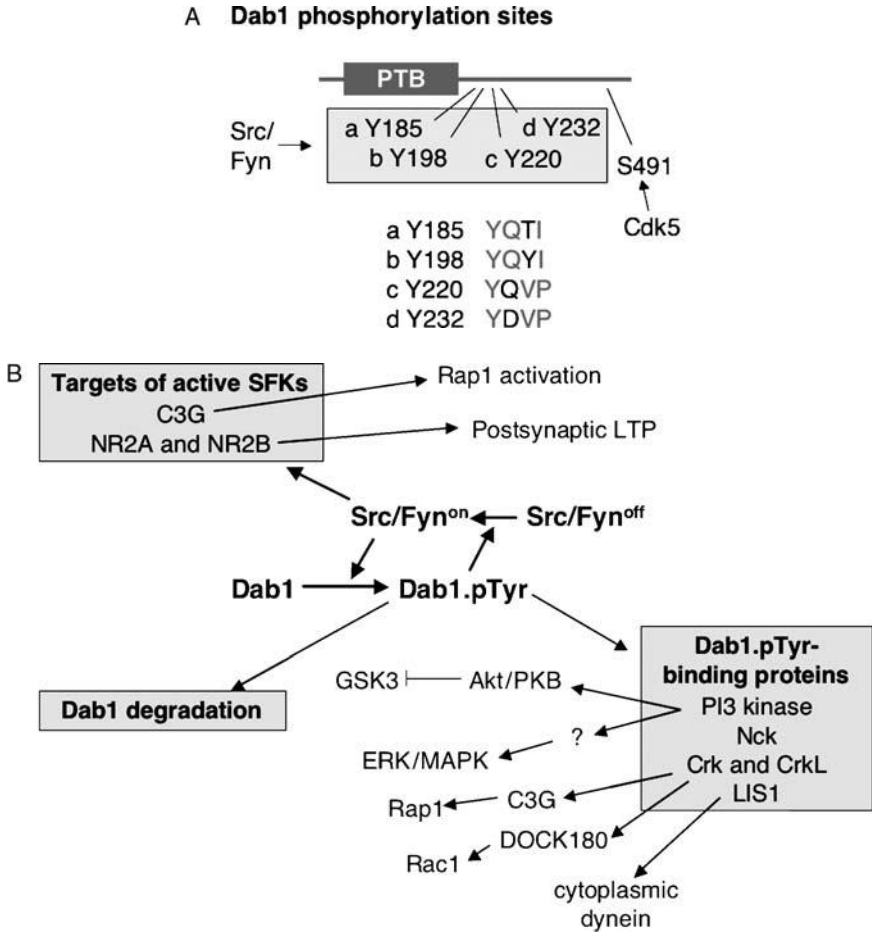

Fig. 13.2 Events downstream of Dab1 phosphorylation and SFK activation. (**A**) Phosphorylation sites in Dab1 that are phosphorylated by SFKs and Cdk5. (**B**) Events that may be important in Reelin signaling are shown separated into two categories: those triggered by active SFKs and those dependent directly on Dab1 phosphorylation (*See Color Plates*)

functions of Dab1 (Howell *et al*., 2000). Another splice form, p45, which lacks most sequence C-terminal to the PTB domain and tyrosine phosphorylation sites, is expressed at very low level in normal brain but at increased level in *cdk5-/-* and *reelin-/-* brain (Ohshima *et al*., 2007). p45 can substitute when p80 is absent, but does so inefficiently, such that a single copy of the p45 allele causes a phenotype if p80 is absent (Herrick and Cooper, 2002). Other splice forms, which lack two or more tyrosine phosphorylation sites, were detected in cDNA libraries and may correspond to small proteins detected with certain Dab1 antisera in embryonic brain (Howell *et al*., 1997a). However, in chicken retina, expression of a short

(two site) form of Dab1 is detected in early, proliferating, retinal cells and the long (four site) form of Dab1 in later, differentiating cells (Katyal and Godbout, 2004). Forced expression of the long form induces differentiation in cultured retinal cells (Katyal and Godbout, 2004). Thus, regulated splicing of Dab1 may permit different signaling events.

3 Possible Downstream Signaling Pathways

It is not known how Dab1 phosphorylation leads to downstream signaling. However, there are two obvious mechanisms, either or both of which may be important. First, Dab1 tyrosine phosphorylation is needed to activate SFKs (Fig. 13.2B) (Ballif *et al*., 2003; Bock and Herz, 2003). Other proteins phosphorylated by the active SFKs may transmit downstream responses. Second, phosphorylated Dab1 may directly regulate other proteins by binding to them. Phosphorylation of proteins such as Dab1 allows them to bind to other proteins that contain SH2, PTB, or (rarely) C2 domains (Sondermann and Kuriyan, 2005). Phosphorylated Dab1 could influence the activity of bound proteins by inducing a conformation change, holding them near membranes, or bringing two bound proteins into proximity so that one acts on the other. In the following sections, we review several ways in which active SFKs or phosphorylated Dab1 may regulate cellular machinery downstream of Reelin signaling.

3.1 Kinase Pathways

3.1.1 SFK Activation and Possible Other Substrates: C3G and NMDAR

Once SFKs are stimulated by Reelin in the presence of phosphorylated Dab1, they may phosphorylate other cell proteins to relay the signal. However, simple examination of Western blots probed with antibodies to phosphotyrosine shows that tyrosine phosphorylation events elicited by Reelin do not occur wholesale, and phosphorylation of many proteins does not change detectably. Indeed, apart from Dab1 itself and the activation loop tyrosine residues of Src and Fyn, few other tyrosine phosphorylation events have been detected.

When the adapter proteins Crk and Crk-like (CrkL) were found to bind to phosphorylated Dab1 (see Section 3.2.2), Ballif *et al*. (2004) investigated whether Reelin might regulate another Crk/CrkL-binding protein, C3G, also known as Rapgef1. Indeed, when immunoprecipitated from Reelin-treated neurons, C3G contains increased levels of phosphotyrosine, while phosphorylation of another Crk-binding protein, p130Cas, did not change (Ballif *et al*., 2004). C3G tyrosine phosphorylation was also increased in developing normal embryo brain compared with Dab1 5F mutant brain (Ballif *et al*., 2004). This suggests that C3G

tyrosine phosphorylation is increased by Reelin, dependent on Dab1 tyrosine phosphorylation. One possible downstream consequence of this phosphorylation is increased activity of C3G on its substrate, Rap1. C3G is a guanine nucleotide exchange factor, or GEF, for the small GTPase Rap1 (Tanaka *et al*., 1994). Human C3G activity was previously reported to be stimulated by phosphorylation on a specific tyrosine residue (de Jong *et al*., 1998; Ichiba *et al*., 1999). This tyrosine residue is conserved in mouse C3G but its local environment is altered, and it is not known if this same tyrosine is phosphorylated in response to Reelin. However, slight but significant increases in Rap1 activity were detected in Reelin-stimulated neurons (Ballif *et al*., 2004). Whether this occurs *in vivo* is not clear, but Rap1 has been implicated in regulating the actin cytoskeleton via another small GTPase, Rac1, and in regulating adhesion (Ohba *et al*., 2001). The importance of C3G or Rap1 in the Reeler phenotype has not been assessed.

Another protein whose phosphorylation is regulated by Reelin is the NMDA receptor. This is important for the effect of Reelin on LTP (Beffert *et al*., 2005; Chen *et al*., 2005). The same phosphorylation may or may not occur during Reelin-regulated neuron migrations, but it is unlikely to be important for neuron positioning because it depends on a specific splice form of one of the Reelin receptors, ApoER2, and this splice form (exon 19+) is not needed for normal cortical lamination or cerebellar development (Beffert *et al*., 2005). Reelin treatment of hippocampal slices sensitizes neurons to glutamate, and this sensitization requires the Reelin receptors, as well as Dab1 and SFK activity (Beffert *et al*., 2005; Chen *et al*., 2005). Reelin stimulates the phosphorylation of the NR2A and NR2B subunits of the NMDA receptor (Chen *et al*., 2005), which are known to regulate channel function (Yu *et al*., 1997). Both splice forms of ApoER2 bind to NMDA receptors, but only the exon 19+ form of ApoER2 binds to PSD95 (Beffert *et al*., 2005; Gotthardt *et al*., 2000), which also binds to NMDA receptors and promotes their phosphorylation by SFKs (Tezuka *et al*., 1999) and localizes to postsynaptic density (Beffert *et al*., 2005). These results provide strong evidence that Reelin regulates tyrosine phosphorylation of NR2B via Dab1 and SFKs, and thus regulates LTP. However, the results also prove that this signaling is not needed during Reelin-regulated neuron positioning events.

3.1.2 PI3 Kinase, Akt, and GSK3

Reelin stimulates the protein kinase Akt (also called PKB), leading to phosphorylation and inhibition of another kinase, GSK3, in cultured neurons (Ballif *et al*., 2003; Beffert *et al*., 2002). Beffert *et al*. (2002) also detected a decrease in tau phosphorylation, consistent with GSK3 inhibition. Consistent with this pathway operating *in vivo*, mutation of Reelin causes an increased level and activity of GSK3 and tau phosphorylation (Ohkubo *et al*., 2003). This effect may be related to the observed changes in dendrite morphology in Reeler mutant brain and to the postnatal accumulation of phosphorylated tau and associated neurotoxicity (Brich *et al*., 2003; Hiesberger *et al*., 1999). In other cell types, Akt is activated by 3´-phosphoinositides generated through the activity of PI3 kinase (Vanhaesebroeck and Alessi,

2000). A role for PI3 kinase in Reelin activation of Akt was inferred from the effects of various PI3 kinase inhibitors on phosphorylation of Akt and GSK3 but not Dab1 (Beffert *et al*., 2002; Bock *et al*., 2003), and small amounts of PI3 kinase were subsequently found to coimmunoprecipitate with Dab1 from Reelin-stimulated neurons (Bock *et al*., 2003). Whether this binding is involved in or required for PI3 kinase activation remains unknown. An important role for PI3 kinase in cortical plate formation has been inferred from slice culture experiments, in which PI3 kinase inhibitors prevent normal cortical plate formation (Bock *et al*., 2003), but the relevance *in vivo*, or the roles of Akt and GSK3, are unclear.

3.1.3 MAP Kinase Pathways

Recently, Simo *et al*. (2007) detected an increase in MAP kinase (ERK) activity in Reelin-stimulated cortical neuron cultures. The effect was specific in that two other MAP kinases, p38 and JNK, were not activated, it required SFKs, and was not detected in *dab1*-/- neurons. The level of activity was less than that induced by a growth factor (BDNF) and may have been overlooked by other investigators (Ballif *et al*., 2003). The mechanism of activation is unclear—in other cell types, ERK is activated via the small GTPase Ras and the upstream kinase Raf1, but in Reelin-stimulated neurons, Ras activity does not appear to increase and, unexpectedly, ERK activation is prevented by PI3 kinase inhibitors (Simo *et al*., 2007). While Ras-independent ERK activation has been detected in other systems, a role for PI3 kinase independent of Ras and upstream of ERK is unusual but not unprecedented (Schmidt *et al*., 2004; Takeda *et al*., 1999; York *et al*., 1998). ERK activation was also detected by immunofluorescence in chain-migrating subventricular zone neurons stimulated with Reelin, and exposure of these cultures to an ERK inhibitor prevented Reelin-induced scattering of the chain-migrating cells (Simo *et al*., 2007). Moreover, Reelin was found to induce expression of an early response gene, Egr1, and this induction was inhibited when ERK activation was blocked (Simo *et al*., 2007). Therefore, ERK activation is involved in at least some aspects of the Reelin response.

3.2 Dab1-Binding Proteins

Some Dab1-binding proteins, including the Reelin receptors (Section 2.1), PI3 kinase (Section 3.1.2), and Lis1 (Section 3.4.1), are discussed in other sections. Here we discuss proteins that bind the PTB domain and phosphotyrosines of Dab1.

3.2.1 Nck

Various methods have been used to identify proteins that might bind to Dab1 after it is phosphorylated. Pramatarova *et al*. (2003) used the yeast two-hybrid system,

in the presence of Src, and identified Nckβ (also called Nck2 or Grb4) as a phosphorylation-dependent Dab1-binding partner. In tissue culture, Nckβ but not Nckα (Nck1) could bind to phosphorylated Dab1. Both proteins contain three SH3 domains and one SH2 domain. SH2 domains bind to specific tyrosine-phosphorylated sequences, and SH3 domains to proline-rich peptides (Pawson, 1995). Even though the SH2 domains of Nckα and Nckβ bind comparable sequences *in vitro* (Frese *et al*., 2006), there are reports that they have different activities in cells (Chen *et al*., 2000). To test whether Dab1 and Nckβ might affect nonneuronal cells, they were coexpressed in fibroblasts and developing fruitfly eye. In both assays, the effects of combined Dab1 and Nck were worse than with either protein on its own (Pramatarova *et al*., 2003).

To test whether Nckβ might be involved in Reelin signaling in neurons, Pramatarova *et al*. (2003) showed that Nckβ is expressed in Reelin-responsive Purkinje cells and cortical neurons, and that Nckβ relocalizes with Dab1 to growing neurites when early differentiating neurons are exposed to Reelin. Unfortunately, complexes between phosphorylated Dab1 and Nckβ were not detected in Reelin-stimulated neurons or developing brain, and it is possible that the Reelin-induced relocalization of Nckβ and Dab1 is indirect.

Nck family proteins are involved in axon pathfinding in fruit flies and mice (Cowan and Henkemeyer, 2001; Garrity *et al*., 1996) and in regulating actin dynamics in mouse fibroblasts and kidney cells (Bladt *et al*., 2003; Jones *et al*., 2006), by binding to transmembrane proteins that contain phosphorylated pYDXV sequences (Frese *et al*., 2006; Jones *et al*., 2006). No site in Dab1 is a perfect candidate to bind Nck, but sites c and d are implicated (Pramatarova *et al*., 2003). Nck SH3 domains bind a variety of proteins, of which N-WASP and PAK1 are good candidates to regulate the actin cytoskeleton downstream of Nck *in vivo* (Eden *et al*., 2002; Li *et al*., 2001; O'Sullivan *et al*., 1999; Rohatgi *et al*., 2001). However, it remains unclear whether Nck is directly regulated by Dab1 during Reelin signaling *in vivo*.

3.2.2 Crk and CrkL

The closely related Crk and CrkL adapter proteins were identified as binding to Dab1 by use of various affinity purification methods (Ballif *et al*., 2004; Chen *et al*., 2004; Huang *et al*., 2004). Importantly, Crk and CrkL form a complex with Dab1 following Reelin stimulation of neurons and in embryonic brain. Indeed, a large fraction of the tyrosine phosphorylated Dab1 can be immunoprecipitated from neurons with antibodies against Crk and CrkL (Ballif *et al*., 2004). Crk and CrkL both contain a single SH2 domain and one or two SH3 domains (Feller, 2001). The SH2 domain of Crk binds to sequences containing pY(D/k/n)(H/f/r)P (Songyang *et al*., 1993), in good agreement with the sequences of Dab1 sites c (pYQVP) and d (pYDVP). Indeed, mutation of either or both sites c and d abolishes binding of phosphorylated Dab1 to the Crk or CrkL SH2 domain *in vitro* (Ballif *et al*., 2004; Huang *et al*., 2004). Crk/CrkL SH3 domains bind a variety of

proteins, including the guanine nucleotide exchange factors DOCK180 (also called DOCK1) and C3G (Hasegawa *et al*., 1996; Tanaka *et al*., 1994). Evidence that Reelin stimulates C3G tyrosine phosphorylation and activates its substrate, Rap1, was reported in Section 3.1.1. Evidence that DOCK180 is regulated by Reelin in neurons is lacking, but abnormal fruit-fly eye development caused by ectopic murine Dab1 expression is ameliorated by mutation of the fruit-fly DOCK180 homologue, suggesting that Dab1 can activate endogenous DOCK180 under some conditions.

The importance of Crk/CrkL for Reelin signaling *in vivo* awaits genetic testing. Mutation of the *crkl* gene reportedly causes either no phenotype (Hemmeryckx *et al*., 2002) or perinatal death (Guris *et al*., 2001; Hemmeryckx *et al*., 2002), depending on mouse strain background. A Reeler-like phenotype has not been reported. Crk is more highly expressed than CrkL in the developing cortex. Knockout of the *crk* gene was recently reported to cause late gestation or perinatal lethality (Park *et al*., 2006), so that conditional mutations in the *crk* or *crkl* or both genes will likely be needed to assess their importance in brain development.

3.2.3 Proteins that Bind to the PTB Domain

In addition to the Reelin receptors VLDLR and ApoER2, several proteins have been described that bind to the Dab1 PTB domain, at least *in vitro* or in tissue culture. These proteins include APP (Homayouni *et al*., 1999; Howell *et al*., 1999b), Dab2IP (Homayouni *et al*., 2003), and N-WASP (Suetsugu *et al*., 2004). However, even if these interactions occur in neurons, it seems unlikely that binding would be affected by Dab1 tyrosine phosphorylation state, which is essential for downstream signaling (Howell *et al*., 2000). Therefore, these proteins are more likely to be involved in events upstream of Dab1 activation rather than downstream. For example, they could act as endogenous competitors of the Reelin receptor–Dab1 interaction, and thus increase the threshold level of Reelin needed to activate Dab1.

3.3 Adhesion Molecules

One effect of the Reeler mutation is increased interaction between neurons and radial glia *in vivo* (Pinto-Lord *et al*., 1982) and abnormal reaggregation of neurons in culture (DeLong and Sidman, 1970; Ogawa *et al*., 1995). This suggests changes in adhesive properties of neurons. Anton *et al*. (1999) noted that antibodies to integrin $\alpha 3\beta 1$ inhibited migration *in vitro*, and that integrin $\alpha 3$-/- cortex showed abnormal layering. Indeed, $\alpha 3$-/- neurons migrate abnormally in culture systems (Schmid *et al*., 2004). A surprisingly direct link between integrin $\alpha 3\beta 1$ and the Reelin pathway came with the report that Reelin, $\alpha 3\beta 1$, and Dab1 could

all be coprecipitated from neurons (Dulabon *et al*., 2000), suggesting that α3β1 is a Reelin receptor. Dab1 binds to integrin tails *in vitro* (Calderwood *et al*., 2003). However, α3β1 was not needed for Reelin-induced Dab1 tyrosine phosphorylation (Dulabon *et al*., 2000), and α3β1 binds to the nonessential N-terminus of Reelin (Jossin *et al*., 2004; Schmid *et al*., 2005), suggesting that α3β1 may be a nonessential coreceptor. More recently, Sanada *et al*. (2004) showed that α3β1 levels are elevated in *dab1-/-* brain relative to normal brain, and α3β1 levels were locally increased in marked clones of cells in which Dab1 was mutant. RNAi-mediated knockdown of α3 rescued positioning defects caused by inhibiting Dab1 function. These results led to a model where α3β1 may be downregulated following receipt of a Reelin signal, permitting detachment of migrating neurons from radial glia.

These models were questioned when it was found that although targeted knockout of the *β1* gene caused brain abnormalities, the phenotype did not resemble Reeler and was likely due to a requirement for β1 in the basement membrane (Graus-Porta *et al*., 2001) and defects in radial glia (Forster *et al*., 2002). However, gene deletion and RNAi knockdown may cause different effects if integrin subunits can switch between different partners with distinct specificities.

3.4 Other Genes Involved in Neuron Migrations: Lis1 and Cdk5

3.4.1 Lis1

The gene encoding Lis1 (Pafah1b1) is mutated in a human lissencephaly syndrome (Reiner *et al*., 1993). Lis1 biochemistry and biology is complex: it is a component of at least two biochemical complexes, one that catalyzes breakdown of platelet-activating factor, an intracellular second messenger that is present in brain cells, and another that interacts with and regulates cytoplasmic dynein/dynactin complexes (Feng and Walsh, 2001; Reiner, 2000). Mutations of other components of the second complex also cause neuron migration defects—typically slower migration and uncoupling of the centrosome from the nucleus of the migrating cell (Shu *et al*., 2004). In addition, mutation of Lis1 and Lis1-binding components can affect neurogenesis, possibly by affecting mitotic spindle function (Feng and Walsh, 2004; Sheen *et al*., 2006). The cellular phenotypes are not obviously related to those caused by mutation of Reelin pathway components (Gupta *et al*., 2002).

Nevertheless, Assadi *et al*. (2003) tested for genetic and biochemical interactions between the Reelin pathway and Lis1. They made use of *lis1* and *dab1* mutants to test for genetic interactions. The most conspicuous phenotype was hydrocephalus, possibly due to partial blockage of the aqueduct of Sylvius, which implies abnormalities in the ependymal cells and is probably not related directly to changes in Reelin signaling. However, they also detected subtle changes in

layering of neurons in the cortex and hippocampus. Lis1+/– has a partial dominant phenotype, with some disorganization of the hippocampus. The hippocampus was more disrupted in *lis1*+/– *dab1*+/–, although many of these mice had severe hydrocephalus, complicating the interpretation. There was also some disorganization of deep layer cortical plate neurons in the double heterozygotes. It is possible that some of these effects are secondary to a change in neural progenitors in the ventricular zone, since these are the cells that give rise later to the ependyma. If so, this would be the first evidence for a role for Dab1 in the progenitors. Next, they tested for a direct interaction between Dab1 and Lis1 proteins. In tissue culture cells, Lis1 and Dab1 can coimmunoprecipitate, depending on tyrosine phosphorylation of Dab1 at sites b and c and on residues in Lis1 that are mutated in human lissencephaly patients. Strikingly, Dab1 and Lis1 also coimmunoprecipitate from embryonic brain extracts, dependent on Reelin. These results provide strong evidence that Reelin may signal via an inducible Dab1–Lis1 complex to regulate neuron migrations.

3.4.2 Cdk5

The serine/threonine protein kinase, Cdk5, and its activators p35 and p39, have essential functions in neuron migrations in the cortex, hippocampus, and cerebellum, as shown by the phenotypes of *cdk5*-/- (Chae *et al*., 1997; Ohshima *et al*., 1996) or double mutant *p35*-/- *p39*-/- (Ko *et al*., 2001) mice. However, the *cdk5*-/- phenotype differs from the Reeler phenotype in several important respects. First, in the *cdk5*-/- or *p35*-/- cortex, preplate splitting does occur (Chae *et al*., 1997; Ohshima *et al*., 1996), although the splitting is abnormal (Rakic *et al*., 2006). Second, cortical plate layers are intermixed, rather than inverted as in Reeler (Gupta *et al*., 2002; Kwon and Tsai, 1998). Nevertheless, there are signs of interactions between the Reelin pathway and Cdk5 at both the genetic and biochemical level.

Several investigators have studied the phenotypes of mice with compound mutations in Cdk5 or p35 and Reelin, Dab1, ApoER2, or VLDLR. Cerebellar and facial motor neuron development is more severely affected in *dab1*-/- *p35*-/- than in either *dab1*-/- or *p35*-/- (Ohshima *et al*., 2001, 2002). The fact that p35 plays a role even when Reelin signaling is totally ablated (by *dab1*-/-) means that p35 (and, by inference, Cdk5) functions independently of Reelin. However, Ohshima *et al*. (2001) also found that *dab1*–/+ *p35*-/- cerebellar Purkinje cells were more disordered than in *p35*-/-, while *dab1*–/+ has no phenotype. This could be due to the aforementioned Dab1-independent role of p35, or could be due to interdependence between Dab1 and p35. Beffert *et al*. (2004) found that mutation of *p35*-/- and *apoer2*-/- singly impairs hippocampal development slightly, but double mutation caused a Reeler-like defect. However, *p39*-/- and *vldlr*-/-, each of which singly has little effect on the hippocampus, had no greater effect in combination. It is difficult to exclude that the effects in the hippocampus are not simply additive. More informatively, double mutation of *p35*-/- and *vldlr*-/- or *apoer2*-/- caused invasion of cortical plate neurons into the marginal zone, which is not seen with any single mutant.

Since neither *vldlr*-/- nor *apoer2*-/- totally blocks Reelin signaling, these results suggest that decreased Reelin signaling makes a worse phenotype when p35 is missing. There was also a requirement for p35 or p39 for Reelin-induced LTP in hippocampal cultures. Interpreting these experiments, in which pathways are partly or fully inactivated by homozygous mutation of a component, is difficult, and may mean that the pathways are parallel and converge downstream, or that the components interact.

To test whether Reelin and Cdk5 signaling might intersect directly, several investigators have used biochemical approaches. Initially, Keshvara *et al*. (2002) found that Cdk5 could phosphorylate Dab1 (p80 splice form) at one or more sites near the C-terminus, including serine 491 (S491). There was reduced tyrosine phosphorylation of Dab1 in *cdk5*-/- brain samples, but this may be due to altered access of migrating neurons to Reelin in the mutant cortex since Reelin stimulated Dab1 tyrosine phosphorylation equally in cultured *cdk5*-/- and wild-type neurons. Also, Dab1 was phosphorylated at S491 equally in control and Reeler brains. Similarly, Beffert *et al*. (2004) found no effect of *p35*-/- mutation or Cdk5 inhibitors on Reelin-induced Dab1 tyrosine phosphorylation in neurons, nor was there an effect of Reelin on phosphorylation of Cdk5 substrates. However, Ohshima *et al*. (2007) obtained somewhat different results. In transfected tissue culture cells, Cdk5 inhibited Fyn-mediated p80 tyrosine phosphorylation, and Reelin was more active in inducing Dab1 tyrosine phosphorylation in *cdk5*-/- than control neurons. It is not clear why these results differ from those of Beffert *et al*. (2004) and Keshvara *et al*. (2002), except that Ohshima *et al*. (2007) used neurons from *cdk5*-/- and *cdk5*+/+ animals in a *reelin*-/- background, to remove possible effects of Reelin exposure *in vivo*.

If C-terminal phosphorylation of Dab1 by Cdk5 has any effect on development, it may be subtle. Expression of the p45 splice from of Dab1, which lacks the normal C-terminus of p80, rescues the *dab1*-/- phenotype (Herrick and Cooper, 2002). This protein is relatively highly expressed, implying increased stability, but still undergoes Reelin-stimulated tyrosine phosphorylation and degradation. The phenotypic rescue is not perfect, and phenotypes are revealed when gene dosage of the Dab1 p45 allele is reduced. Therefore, the normal C-terminus of Dab1 p80 is not required, but promotes normal brain development. The novel C-terminus of Dab1 p45 may also have a function: Ohshima *et al*. (2007) found a Cdk5 phosphorylation site in the C-terminus of p45, and provided evidence that Cdk5 may stimulate p45 tyrosine phosphorylation and its degradation. However, these results have not been confirmed in neurons.

3.5 Ubiquitin–Proteasome System

Following Reelin-induced tyrosine phosphorylation, Dab1 is targeted for degradation by the ubiquitin–proteasome system (UPS) (Arnaud *et al*., 2003a; Bock *et al*., 2004). This mechanism probably underlies the observed increases in Dab1

protein levels in embryonic brains that have mutations in *reelin*, both *vldlr* and *apoer2*, or both *src* and *fyn*, and in the *dab1 5F* knockin mouse (Arnaud *et al*., 2003b; Howell *et al*., 2000; Kuo *et al*., 2005; Rice *et al*., 1998; Trommsdorff *et al*., 1999). Reelin-induced degradation in neuron cultures requires SFK activity and the Reelin receptors (Arnaud *et al*., 2003a; Bock *et al*., 2004). Since Dab1 tyrosine phosphorylation always correlates with increased SFK activity, the requirement of Dab1 phosphorylation for Dab1 degradation could be explained if the UPS was activated by SFKs. However, in neurons that contain both phosphorylated and nonphosphorylated Dab1, only the phosphorylated Dab1 is degraded (Arnaud *et al*., 2003a), implying that phosphorylation tags Dab1 for recognition by the UPS.

A direct role for Dab1 ubiquitination or degradation in Reelin signaling has been suggested (Rice *et al*., 1998). For example, codegradation of Dab1-associated molecules may relay a signal. However, this is currently a matter of conjecture. What is clear is that changes in Dab1 level due to the UPS do affect the ability of neurons to respond to Reelin challenge. Neurons from Reeler mice contain more Dab1 protein and more phosphorylated Dab1 after Reelin challenge, than controls (Howell *et al*., 1999a). Neurons that were pretreated with Reelin have less Dab1 and less phosphorylated Dab1 after repeat Reelin challenge (Bock *et al*., 2004). Strikingly, the UPS provides a main mechanism for removing phosphorylated Dab1 from stimulated neurons: inhibiting the UPS allows Dab1 to remain phosphorylated, and SFKs to remain active, for many hours longer than normal (Arnaud *et al*., 2003a). This points to the UPS as a major negative regulator of Reelin signaling.

3.6 Phosphatases

Phosphotyrosine phosphatases (PTPs) are potentially involved at several steps in the Reelin response, including reversal of Dab1 tyrosine phosphorylation and activation of SFKs. When SFK inhibitors are added to Reelin-stimulated neurons, Dab1 rapidly loses its phosphotyrosine, implying the existence of PTPs that dephosphorylate Dab1 (L. Arnaud, personal communication). In addition, initial activation of SFKs by Reelin implies a switch of phosphotyrosine from the C-terminal inhibitory site to the activation loop of the SFK, again involving one or more PTPs. One candidate PTP that may be involved in SFK activation in neurons is the transmembrane receptor PTP, PTPα (also called RPTPα). This PTP was shown to activate Src by dephosphorylating its C-terminal tyrosine (Zheng *et al*., 1992). Brains and fibroblasts of mice lacking PTPα have decreased Src and Fyn activity (Ponniah *et al*., 1999; Su *et al*., 1999), and close examination reveals lamination defects in the hippocampus and impaired NMDA receptor phosphorylation and decreased LTP. These results suggest that PTPα may be involved in SFK activation in response to Reelin, but this has not been studied directly.

4 Summary and Conclusions

In summary, the core signaling pathway illustrated in Fig. 13.1 mediates almost all known effects of Reelin, but the events occurring downstream of SFK activation and Dab1 tyrosine phosphorylation remain to be critically evaluated. Directed tests for genetic interactions between mutations in Reelin pathway components and Cdk5 or Lis1 components suggest ways that Reelin signaling could impinge on other pathways implicated in neuron migrations. On the other hand, biochemical approaches, some unbiased, have suggested alternative mechanisms, illustrated in Fig. 13.2. It is quite possible, even likely, that SFK-Dab1 will be a branch point in the signaling pathway, and different aspects of the Reeler phenotype may be mediated by different branches of the pathway. Critical analysis in the mouse, and hopefully in more genetically amenable vertebrates, will be needed to fully understand how Reelin regulates brain development and function.

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Chapter 14
The Relationship of Oxytocin and Reelin in the Brain

George D. Pappas and C. Sue Carter

Contents

1 Introduction 217
2 Oxytocin and Oxytocin Receptors 217
3 Oxytocin and Reelin Haploinsufficient Reeler Mouse Model 218
4 Oxytocin and Autism 219
5 Metabolism, Experience, and Neuropeptides 222
6 Summary 222
References 223

1 Introduction

Oxytocin (OT) is a small (nine amino acid) peptide, synthesized primarily in the paraventricular and supraoptic nuclei of the hypothalamus. Classically associated with functions such as birth and lactation, OT can also influence social behavior, the hypothalamus–pituitary–adrenal (HPA) axis, and may have a role in the regulation of neural development.

2 Oxytocin and Oxytocin Receptors

OT receptors (OTRs) consist of several transmembrane receptors of the G protein family, distributed throughout the nervous system, especially in hippocampal and hypothalamic nuclei. OTRs also tend to increase initially in the postnatal period

G. D. Pappas
Department of Psychiatry, Psychiatric Institute, College of Medicine, University of Illinois at Chicago, 1601 West Taylor Street MC912, Chicago, IL 60612
e-mail: gdpappas@uic.edu

C. S. Carter
Department of Psychiatry, Psychiatric Institute, College of Medicine, University of Illinois at Chicago, 1601 West Taylor Street MC912, Chicago, IL 60612

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and, at least in rats, may decline in adulthood in areas such as the cortex (Tribollet *et al*., 1989; Shapiro and Insel, 1990), possibly supporting the hypothesis that the effects of OT may differ between developing and adult animals.

OT has been implicated in cellular proliferation; stem cells treated with OT proliferate and begin to express OTRs (Green *et al*., 2001). Both OT and reelin are particularly significant during development, and the absence of either may interfere with normal brain development. In addition, reelin is critical to the development of the GABAergic system which modulates the release of OT (Liu *et al*., 2005).

3 Oxytocin and Reelin Haploinsufficient Reeler Mouse Model

Availability of the reelin haploinsufficient (+/−) reeler mouse (HRM) provides a model for examining the role of reelin in the development of the OT system and especially in the expression of the OTR. In rodents, OT expression increases during the immediate postnatal period. Serum levels of OT are low in certain types of autism (Green *et al*., 2001). It has been reported that OT infusion may reduce repetitive symptoms in autism spectrum disorder (Hollander *et al*., 2003).

To date, there has been one published study directly examining possible interactions between OTR and reelin (Liu *et al*., 2005). In this study, we used immunocytochemistry and *in situ* hybridization in the haploinsufficient (+/−) (HRM) versus wild-type (+/+; WTM) adult mice to quantify OTR abundance in the piriform cortex, the neocortex, the hippocampus, and the retrosplenial cortex. Light microscopy of central nervous system (CNS) sections from the normal WTM brain revealed OTR mRNA and protein to be abundant throughout the cortical regions of the brain, including the neocortex, allocortex, and archicortex. In the +/− HRM, the number of OTR-positive cells in the same cortical areas as those studied in the WTM are 25 to 50% decreased from the wild-type mice (Fig. 14.1, 14.2).

Both reelin and OT have been implicated in autism and in schizophrenia, since marked deficits in OTRs and reelin occur in specific cortical regions (Liu *et al*., 2005). The neocortex and retrosplenial area of the cortex are implicated in both memory and emotion (Carter, 2003). In addition, reductions in OTR binding were apparent in most areas of the hippocampus (archicortex), including the dentate gyrus of mice. These findings on the distribution of OTR in the cortex are consistent with our earlier findings on the downregulation of reelin in these same brain regions (Pappas *et al*., 2001, 2003). Preliminary analyses of other regions containing OTRs, including the reticular thalamus and the central and basolateral amygdala, do not show striking differences between the wild-type (+/+) and the reeler (+/−) mice (Liu *et al*., 2005). In general, cortical and hippocampal deficits of either reelin or OTRs might be expected to be associated with reductions in memory and learning, especially in the cortex of the social environment. Many studies have implicated OT in social memory (see Gimpl and Fahrenholz, 2001). In mice, both maternal experience associated with increases in endogenous OT and treatment of virgin mice with intraventricular injection of OT were associated with improved

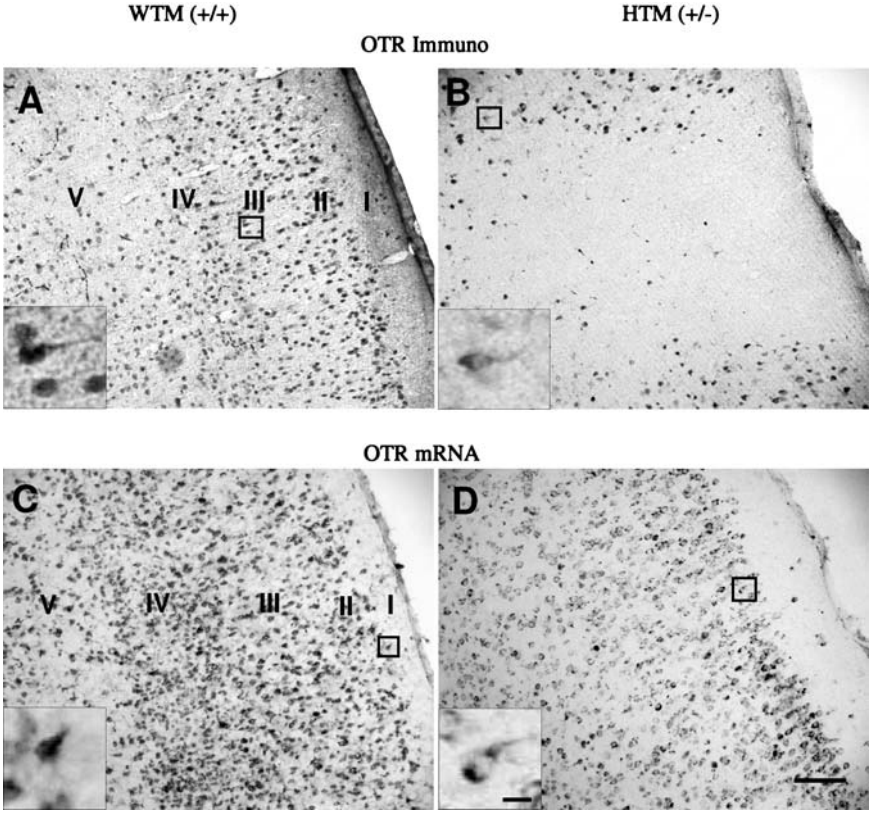

Fig. 14.1 Oxytocin receptor (OTR) **(A, B)** and OTR mRNA **(C, D)** in the motor cortex. Immunolabeling in the wild-type mice (WTM) showed reactivity throughout layers II to V **(A)**, while the haploinsufficient reeler mice (HRM) have lower OTR immuno-reactivity **(B)**. WTM (+/+) show abundant mRNA activity **(C)**, but a lower level of OTR mRNA in the HRM (+/-) **(D)**. Scale bar equals100μm and in inset equals 20μm. (Liu, Pappas, and Carter, 2005; reprinted with permission www.ingentaconnect.com/content/maney/nres)

spatial memory, probably as a result of activity in the hippocampus (Tomizawa *et al*., 2003).

4 Oxytocin and Autism

Reelin and OT play a role in regulating affect and mood. Downregulation of reelin has been correlated with schizophrenia (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Costa *et al*., 2001). It has been proposed that mice haploinsufficient for reelin (+/−) offer an animal model that may also have relevance for the features of autism (Liu *et al*., 2005; Tueting *et al*., 2006). Preliminary studies of social behavior in

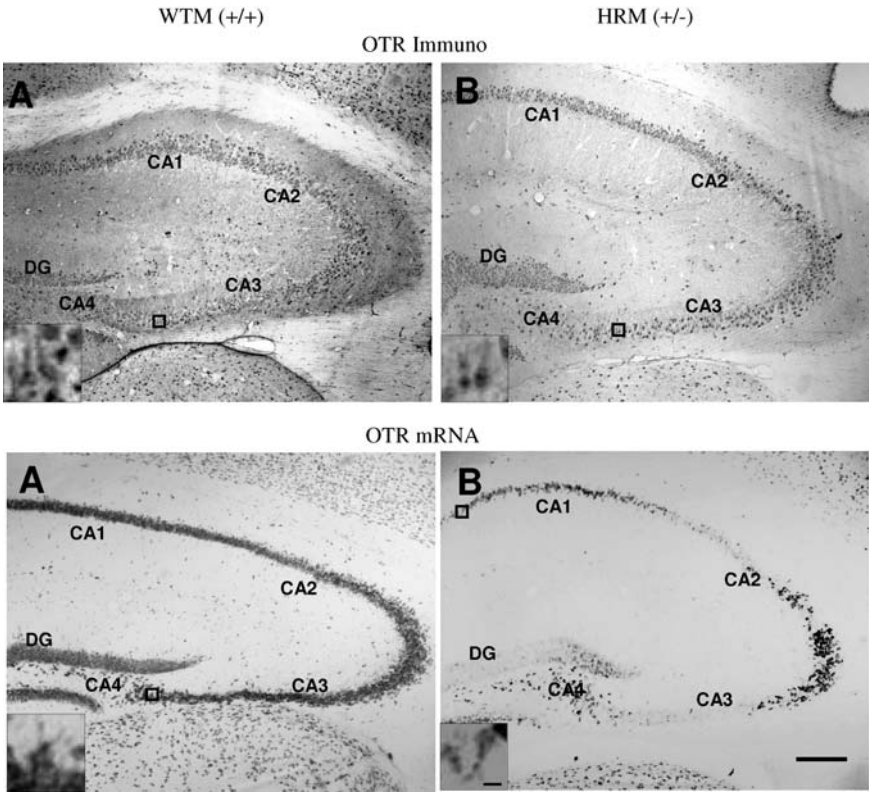

Fig. 14.2 Oxytocin receptor (OTR) **(A, B)** and OTR mRNA **(C, D)** in the hippocampus and dentate gyrus. OTR immunoreactivity is expressed in cells in CA1, 2, 3 and 4 and the dentate gyrus **(A)** in the wild-type mice (WTM). There is a significant change in OTR immuno-reactivity in the haploinsufficient mice (HRM) **(C)**. OTR mRNA also is localized to the cells of CA1, 2, 3 and 4 in the WTM (+/+) **(C)**. However, in the HRM (+/-) reduction are seen in mRNA localization in the hippocampus. All cellular areas of the hippocampus and dentate gyrus show diminished expression of OTR mRNA, with the possible exception of a small portion of CA3 and the hilus of the dentate gyrus **(D)**. Scale bar equals 200μm and in inset equals 10μm. (Liu, Pappas, and Carter, 2005; reprinted with permission www.ingentaconnect.com/content/maney/nres)

(+/−) reeler mice, with levels of reelin that are about half those seen in normal animals, reveal reductions in social interactions and a failure to show social recognition (Doueriri and Guidotti, unpublished data).

We have observed dramatic reductions in OTRs or mRNA for the OTRs (especially in cortical–hippocampal area) in (+/−) reeler mice (Liu *et al*., 2005). The origins of this difference remain to be studied. However, the promoter region of the reelin gene has CpG islands susceptible to epigenetic methylation (Chen *et al*., 2002). Hypermethylation of the reelin gene promoter results in a decrease in the transcription of reelin mRNA, due to chromatin and promoter remodeling (Dong *et al*., 2005). The OTR also contains CpG islands and is susceptible to epigenetic

regulation (Kimura *et al*., 2003). Hypermethylation and gene silencing, induced by developmental events, such as exposure to neuropeptides, differential maternal stimulation (Meaney and Szyf, 2005), or viral infections (Shi *et al*., 2003), might influence subsequent reelin expression. In rats, the offspring of mothers exposed to high levels of maternal care experienced reduced reactivity to stressors, as well as increased expression of the hippocampal glucocorticoid receptor gene in later life. More recently, these authors have reported that treatment of adult mice with a histone deacetylase inhibitor reversed the effects of low levels of maternal stimulation on reelin (downregulation), while the effects of high levels of early postnatal maternal care (upregulation of reelin) were reversed by methionine, capable of increasing methylation of the gene for reelin (Weaver *et al*., 2006).

Most studies of reelin-deficient mice have been conducted in males, and thus little is known regarding sex differences in the consequences of reduction in reelin. However, Purkinje cell loss in reelin-deficient mice, compared to the wild-type, was observed only in males. In that study it was reported that "females were spared" (Hadj-Sahraqui *et al*., 1996). A recent report dealing with Purkinje cell development reports that at ages between P10 and P18, Purkinje cell numbers were decreased in male reelin-deficient mice, while wild-type male and female littermates, and female mice with only one functional gene for reelin (+/rl) displayed normal Purkinje cell numbers. These investigators hypothesize that "reelin haploinsufficiency may be compensated by estrogens" (Assenza *et al*., 2005). It is also possible that OT alone or in conjunction with estrogen plays a role in the sexually dimorphic consequences of reelin deficiency.

Reelin also regulates GABA. Genes for a subset of the GABA receptors are found on the 15q11-13 loci, another autism susceptibility locus (AUTSL) (Cook *et al*., 1998), also involved in Prader Willi syndrome, and exhibit an associated deficit in OT. GABA plays a role in many aspects of brain function, especially under conditions when inhibitory processes are required. Functional interactions among OT, reelin, and GABA could be of critical importance to the features of autism.

In addition to OT, another neuropeptide, arginine vasopressin (AVP), has also been implicated in the autism spectrum disorder (ASD) (Insel *et al*., 1999; Young *et al*., 2002; Leckman and Herman, 2002; Winslow, 2005; Welch and Ruggiero, 2005). Changes in either OT or AVP or their receptors could be capable of influencing the features of ASD. Relevant to possible mechanisms through which OT or AVP might influence autism, is the fact that the effects of these neuropeptides on brain and behavior are sexually dimorphic, especially during the course of development (Carter, 2003; Bales *et al*., 2004a,b, 2006; Yamamoto *et al*., 2004; Bielsky *et al*., 2005; Thompson *et al*., 2006). We hypothesize here that AVP, which has a unique role in males, must be present in optimal levels to be protective against ASD. AVP is androgen dependent, and males are more sensitive to AVP, especially during development. Either excess AVP or disruptions in the ASD system could play a role in development of the traits of autism. In contrast, OT, in some cases more abundant in females, normally may be protective.

The capacity of OT or AVP to modify either reelin, the OTR, or AVPRs would have life-long consequences for physiology and behavior. Deficiencies or atypical

expression of reelin/GABA, through the primary actions of reelin, or through changes in systems directly dependent on OT or AVP, might confer vulnerability to certain features of autism. Such changes could be manifest as increases in anxiety or low levels of sociality, as well as cognitive deficits resulting from changes in the organization of laminar brain areas.

5 Metabolism, Experience, and Neuropeptides

Research on neuropeptides, such as OT and AVP, tends to focus on the expression of either the peptides themselves or their receptors. However, the functional availability of these compounds depends on their dynamic synthesis and degradation. For example, peptidases and proteases regulate the production and degradation of both OT and AVP (Mitsui *et al*., 2004; Tsujimoto and Hattori, 2005). These enzymes are regulated by genetics and differ between males and females but can also be affected by diet, salt intake, and stress-related changes in anxiety or trauma across the life span (Maes *et al*., 1998, 1999, 2001). Of particular interest are prolyl endopeptidases (PEP); these enzymes regulate the metabolism of various peptides, including OT and AVP. Changes in PEPs may have regionally specific effects; for example, inhibition of PEP produces an increase in AVP in the septal nucleus (Miura *et al*., 1995). Because AVP in the septal area is sexually dimorphic, individual or sex differences in the effects of enzymes such as PEP, especially in this region, offer a possible substrate for individual differences in behavioral reactivity.

Other sources of possible variance in these systems could come from individual differences in lifestyle across the life span. Exercise and fitness have been shown to modulate the autonomic actions of OT, with increased responsivity to OT as a function of increased fitness (Michelini *et al*., 2003). As another example, diet might have the capacity to influence enzymes that regulate neuropeptides, such as OT and AVP, as well as steroid production (Cameron, 1991). The consequences of diet and exercise, even in early life, might be sexually dimorphic and individually variable.

6 Summary

It is almost impossible to consider the interrelationship of reelin and OT without their role in the development of ASD.

Both reelin and OT play a role in regulating affect and mood. Downregulation of reelin has been strongly correlated with schizophrenia and autism (Fatemi *et al*., 2005), and it is proposed that reelin haploinsufficient (+/−) reeler mice may serve as a model for neural deficits seen in both schizophrenia and autism (see Liu *et al*., 2005).

Any theory regarding the causes of ASD might be able to account for the striking male-bias in the occurrence of these disorders. Sex differences in the central regulation and expression of OT and AVP may help in understanding the features

of ASD. However, differentiating the possible roles of OT, AVP, and reelin in the features of ASD is not simple.

OT and AVP have some shared functions; for example, both peptides, administered exogenously, can promote positive social interactions and pair bond formation (Cho *et al*., 1999). In general, centrally active AVP seems to be associated with increased vigilance, anxiety, arousal, and activation, while OT has behavioral and neural effects associated with reduced anxiety, relaxation, growth, and restoration (Carter, 1998; Uvnas-Moberg, 1998). In addition, OT may protect the central and autonomic nervous system against overreactivity or even shutdown, especially in the face of extreme challenges (Porges, 2001).

Exaggerated activity or abnormal activity in systems that rely on AVP, possibly due to increased exposure to androgens (Baron-Cohen, 2002), would be consistent with several features of ASD. Sexually dimorphic effects of OT and AVP, including actions that extend beyond the nervous system to influence metabolic or immune reactions, also might be critical links to uncovering the mechanisms underlying the causes and effects of ASD.

OT is estrogen-dependent and in some cases is higher in females (Carter, 2003). OT can regulate responses to stressors and inflammation and also can be inhibited by stressful experiences. Of potential relevance to ASD is the fact that AVP in the extended amygdale–lateral septal axis of the nervous system is sexually dimorphic (higher in males) (Carter, 2003). In addition, males appear to be more sensitive than females, especially during development, to the actions of AVP. There are several examples in which females appear to be remarkably insensitive to AVP or its absence. Insensitivity to AVP or a lack of dependence on this peptide might be protective in females against the features of ASD. Females might also be protected either directly or indirectly by OT. Experience associated with reductions in fear and an increased sense of safety or trust would be expected to be protective in ASD and related disorders that are characterized by high levels of anxiety (Porges, 2001; Corbett *et al*., 2006). Sexually dimorphic differences in coping mechanisms, including the willingness to use social interactions to reduce anxiety, could be another mechanism through which males and females might differ in the expression of the features of ASD. Knowledge of natural ways to stimulate the release of endogenous OT or to inhibit "excess" AVP might be protective against the development of the features of ASD, perhaps even remediating the expression of ASD-like behaviors in later life. However, we cannot, at this point, exclude the possibility that disruptions in systems that rely on AVP might also increase the vulnerability to ASD.

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Chapter 15
Reelin and Thyroid Hormone

Manuel Álvarez-Dolado

Contents

1 Introduction 227
2 Developmental Regulation of *Reelin* and *Dab1* by T3 228
3 Mechanism of T3 Action 230
4 Molecular Mechanism of T3 Control on *reelin* Expression 231
5 Biological Implications of *Reelin* Control by T3 232
6 Conclusions 232
References 233

1 Introduction

Thyroid hormone [3,5,3'-triiodothyronine (T3) and thyroxine (T4)] is essential for proper brain development. In humans, the lack of adequate T3 levels during the perinatal period leads to cretinism, a syndrome associated with mental retardation and neurological deficits, such as ataxia, spasticity, and deafness (for review, see Legrand, 1984; Dussault and Ruel, 1987; Braverman and Utiger, 2000; Bernal, 2005a). These alterations are due to misregulation of the gene expression controlled by T3 through its interaction with nuclear receptors, which act as ligand-modulated transcription factors (Muñoz and Bernal, 1997; Forrest and Vennström, 2000; Yen *et al*., 2006).

In experimental animals, hypothyroidism causes an array of morphological abnormalities in the neonatal brain (Dussault and Ruel, 1987; de Long, 1990). An important alteration is the reduction in myelination, as a consequence of downregulation of the major myelin proteins, and disruption of oligodendrocyte differentiation (Rodríguez-Peña, 1999; Billon *et al*., 2002). Additional effects of T3 deficiency are a marked delay in neuronal migration, and alterations of neuronal size, packing density, and dendritic morphology (Patel *et al*., 1976; Legrand, 1984; Berbel *et al*.,

M. Álvarez-Dolado
Cellular Regeneration Laboratory, Centro de Investigación Príncipe Felipe (CIPF), Avenida Autopista del Saler 16-3, Valencia 46013, Spain
e-mail: mdolado@cipf.es

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1993, 2001; Lucio *et al*., 1997). Thus, for instance, migration of granule neurons from the external toward the internal granule layer of the cerebellum is retarded, and defects in the positioning of Purkinje cells are observed (Patel *et al*., 1976; Lauder, 1979; Legrand, 1984; Clos and Legrand, 1990). Furthermore, in the cerebral cortex, there are significant abnormalities in lamination, reflecting migration defects (Berbel *et al*., 1993, 2001; Lucio *et al*., 1997).

In the last two decades, a number of genes with a putative role in these alterations have been identified as regulated by T3. They include those coding for cytoskeletal and extracellular matrix proteins (tau, actin, tenascin-C), neurotropins and their receptors, cell adhesion molecules (N-CAM, L1, TAG-1), transcription factors, and intracellular signaling proteins (RC3, Rhes) (Bernal, 2002, 2005a). Among these genes, *reelin* is of special relevance, given its direct implication and key role in processes, such as cell migration and neuronal positioning.

2 Developmental Regulation of *Reelin* and *Dab1* by T3

Reelin-deficient mice manifest many of the features observed in the hypothyroid brain (Goffinet, 1980; Derer, 1985; Schiffmann *et al*., 1997; Alcántara *et al*., 1998). This led A. Muñoz's and E. Soriano's groups to perform a complete developmental study of *reelin* and *dab1* expression in hypothyroid rats (Álvarez-Dolado *et al*., 1999). Quantification by Northern blot showed that *reelin* expression is decreased by 60% in the hypothyroid cortex at postnatal day 0 (P0). More detailed studies using *in situ* hybridization and immunohistochemistry evidenced a complex regulation of *reelin* expression by T3 (Álvarez-Dolado *et al*., 1999). In general, the *reelin* regional and laminar expression patterns are not altered during hypothyroidism. However, both the number of labeled neurons, and their intensity of labeling in the hippocampus and layers I and V/VI of the neocortex, are significantly lower than those in control rats, particularly at P0 (Fig. 15.1A,B). At P5, these differences in the expression levels are weaker than at previous stages. The number of reelin-positive neurons in cortical layer I and hippocampus is not affected; in contrast, in layers II–VI it decreases in hypothyroid rats. At later stages, the pattern of expression in the cortex and hippocampus of hypothyroid rats gradually becomes equal to that in controls. Consistent with the mRNA expression pattern, immunolocalization of Reelin protein in hypothyroid brains shows a marked deficiency in the neocortex and hippocampus at E18–P0 (Fig. 15.1C,D). This deficiency tends to disappear with development. Collectively, the data show that *reelin* expression levels are decreased at perinatal stages in the cortex and hippocampus of hypothyroid rats, whereas they appear to reach normal levels at later postnatal stages.

Strikingly, in the cerebellum and olfactory bulb, hypothyroidism modifies the *reelin* expression in a completely different way (Álvarez-Dolado *et al*., 1999; Manzano *et al*., 2003). At E18–P0, both *reelin* transcripts and immunoreactivity levels are lower in the cerebellum of hypothyroid rats. In contrast, no remarkable differences are observed in the primordium of the olfactory bulb at these ages.

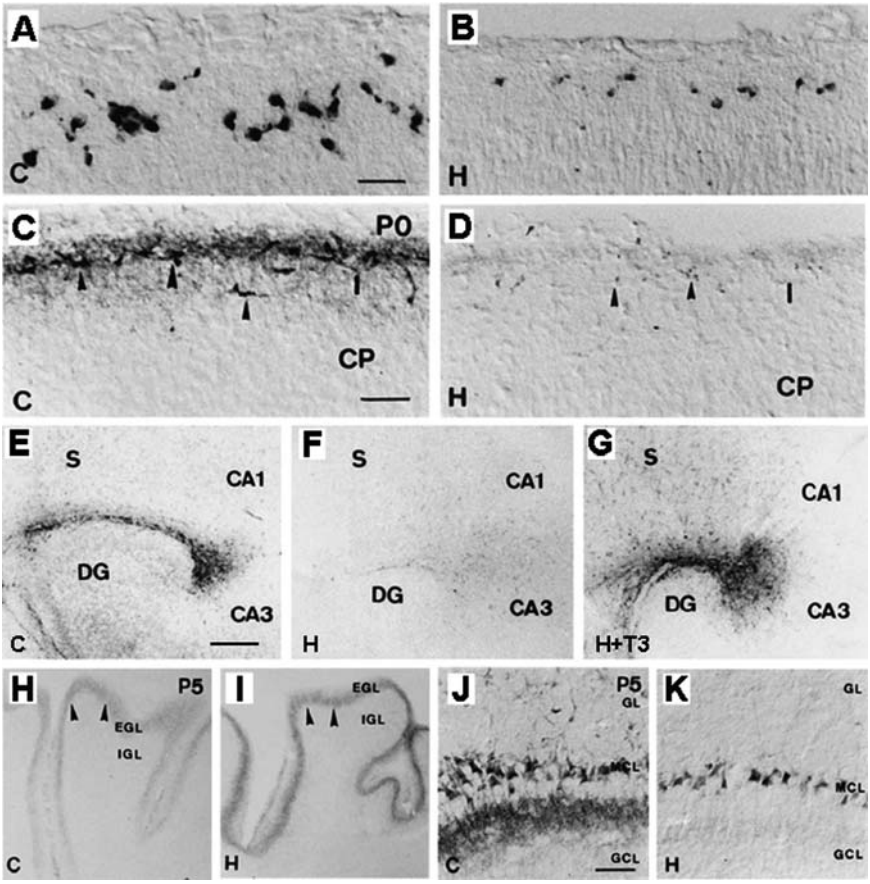

Fig. 15.1 Effects of hypothyroidism on *reelin* RNA and protein expression in the neonatal brain. (**A, B)** Pattern of *reelin* RNA expression in the neocortex of control (**A**) and hypothyroid (**B**) rats at P0. (**C, D)** Photomicrographs showing the distribution of CR50 antibody immunostaining in layer I of control (**C**) and hypothyroid rats (**D**) at P0. Some CR50-positive Cajal-Retzius cells are indicated by arrowheads. Note the decreased staining in hypothyroid animals. Cortical layers are indicated to the right. (**E–G)** Reelin expression detected by CR50 immunostaining in hippocampal organotypic slice cultures. (**E**) Slice from euthyroid rats incubated for 6 days in standard serum. (**F**) Slice from hypothyroid rats incubated for 6 days in thyroid-depleted serum. (**G**) Slices from hypothyroid rats incubated for 6 days in T3/T4-depleted serum supplemented with 500 nM T3. Note that the reduced expression levels in hypothyroid slices are rescued by T3 treatment. (**H–K)** Patterns of Reelin distribution in the cerebellum (**H**, **I**) and olfactory bulb (**J**, **K**) of control (**H**, **J**) and hypothyroid (**I**, **K**) rats at P5. Note the increased Reelin levels in the hypothyroid cerebellum and the opposite in the olfactory bulb. Abbreviations: C, control; CA3, CA1, hippocampal subdivisions CA3 and CA1; CP, cortical plate; DG, dentate gyrus; EGL, external granule cell layer; GCL, granule cell layer; GL, glomerular cell layer; H, hypothyroid; I, cortical layer I; IGL, internal granule cell layer; MCL, mitral cell layer; ML, molecular layer; S, stratum lacunosum-moleculare. Scale bars: **A**, 40 μm (applies to **A–D**); **E**, 200 μm (applies to **E–I**); **J**, 50 μm (applies to **J** and **K**). (Figure modified from Álvarez-Dolado *et al*., 1999. © *The Journal of Neuroscience*) (*See Color Plates*)

At P5–P15, *reelin* mRNA and immunolabeling are prominent in both the EGL and the IGL of the cerebellum, where, contrarily to previous ages, their levels are clearly elevated in hypothyroid rats (Fig. 15.1H,I). In contrast, in the olfactory bulb, decreased levels of RNA and protein are noticed at P5 in hypothyroid brains (Fig. 15.1J,K), whereas no changes are detected at P15.

Alteration of reelin levels affects the expression of other proteins implicated in the same signaling pathway, such as Dab1. Though in hypothyroid rats the synthesis of *dab1* transcripts is unaltered, the Dab1 immunoreactivity is higher than that of controls in the cortex and hippocampus at E18–P0. Conversely, at P5 the levels of Dab1 are lower in hypothyroid than in control rats. This alteration in Dab1 expression is also observed in the cerebellum. Finally, at later developmental stages (P15, P25), no differences are seen between controls and hypothyroid rats. The results indicate that the levels of Dab1 are inversely correlated with those of Reelin in hypothyroid rats (Álvarez-Dolado *et al*., 1999). This is consistent with the reported abnormal accumulation of Dab1 protein, but normal *dab1* mRNA levels, in *reeler* mutant mice (Rice *et al*., 1998).

The complex pattern of *reelin* and *dab1* expression during hypothyroidism is a consequence of the intricate mechanism of action exerted by T3 and its receptors in the brain. I will discuss the complexity of the T3 system and its implications on the control of *reelin* expression in the following sections.

3 Mechanism of T3 Action

T3 controls gene expression by interacting with its nuclear receptors (Oppenheimer and Schwartz, 1997; Forrest and Vennström, 2000; Yen *et al*., 2006), a family of proteins with several functional domains, especially the ligand- and the DNA-binding domains (Mangelsdorf *et al*., 1995; Weiss and Ramos, 2004). There are two receptor genes, designated TRα and TRβ, that encode nine protein products generated by alternative splicing and differential promoter usage. Of the nine, only three isoforms (α1, β1, β2) have an intact T3-binding domain and are able to interact with DNA-specific nucleotide sequences (T3REs: thyroid response elements) present in target genes (Lazar, 1993; Muñoz and Bernal, 1997). The physiological role of the other six nonreceptor proteins remains to be solved (Gauthier *et al*., 2001).

Expression of T3 receptors in the brain starts around E12–14 and is predominantly located in neurons but has also been detected in oligodendrocytes, astrocytes, and microglia (Lima *et al*., 1998, 2001; Rodríguez-Peña, 1999). TRα1 isoform is widely expressed throughout the brain and accounts for 70–80% of total T3 binding capacity (Ercan-Fang *et al*., 1996). The TRβ gene is also expressed in the brain, with a more discrete pattern of expression (Forrest *et al*., 1990). The current view is that the different physiological roles of each receptor depend on their particular patterns of temporal and regional expression (Forrest *et al*., 1990; Forrest and Vennström, 2000). They can regulate gene transcription through the activation of positive T3REs or repression of negative T3REs. In general, TRα1 receptor acts as

a base expression controller whereas TRβ isoforms exert a more specific and timely regulation. There are examples of differential gene regulation in the same cells, specifically exerted through TRα and not TRβ receptor (Manzano *et al*., 2003).

Their function as upregulators or downregulators of gene expression may also depend on the interaction with other nuclear receptors and transcription cofactors (Nishihara *et al*., 2004; Moore and Guy, 2005). Their functional interaction as heterodimers with other nuclear receptors, such as retinoid X receptor (RXR) and retinoic acid receptor (RAR), is essential for understanding the mechanism of gene control by T3 (Muñoz and Bernal, 1997; Forrest and Vennström, 2000).

Finally, to add more complexity, T3 levels are not equal in all brain areas. They depend on the activity of deiodinases that convert T4 into T3 (Bianco *et al*., 2002; Bernal, 2005a), and membrane transporters that control T4/T3 flux through the blood–brain barrier and choroid plexus (Dickson *et al*., 1987; Abe *et al*., 2002; Bernal, 2005b). In addition, several studies have indicated posttranscriptional regulatory effects of T3 on mRNA stabilization, processing, and translation, or on posttranslational mechanisms (Aniello *et al*., 1991).

4 Molecular Mechanism of T3 Control on *reelin* Expression

The precise mechanism of T3 action on *reelin* expression, whether transcriptional or posttranscriptional, remains to be determined. The lack of a strict correlation between the changes in RNA and protein levels supports the idea that *reelin* expression is regulated by T3 at both levels. Location of T3REs in the promoter or intronic sequences of the *reelin* gene remains to be confirmed. This would provide direct evidence of the effects of T3 at the transcriptional level. Nonetheless, the fact that T3 treatment of hypothyroid organotypic brain slices *in vitro* restores the normal *reelin* expression levels strongly suggests a direct effect of this hormone (Fig. 15.1E–G) (Álvarez-Dolado *et al*., 1999). In addition, *reelin* expression levels are specifically restored in the hypothyroid cerebellum after GC-1 treatment, a specific TRβ agonist (Manzano *et al*., 2003).

However, other indirect *in vivo* mechanisms cannot be ruled out. For instance, BDNF has been found to negatively regulate the expression of *reelin* (Ringstedt *et al*., 1998). Previous studies indicated that BDNF expression is diminished at P15 and later ages in the cerebellum of hypothyroid rats (Neveu and Arenas, 1996), although the levels in the cortex and hippocampus remain unchanged (Álvarez-Dolado *et al*., 1994). Therefore, the increased expression of *reelin* in the hypothyroid cerebellum at P5–P15 may be secondary to the modulation of BDNF levels by T3 in this region.

The differences observed in the effect of the hormone in distinct brain regions, especially in the cerebellum, suggest that T3 may cooperate with locally acting factors, or that hormone action is modulated by region- or cell-specific proteins. Thus, we should consider the physiological levels of T3 in each region, based on the expression and activity of deiodinases and transporters. Interestingly, a strong

correlation between *reelin* expression levels and the presence of different deiodinase subtypes in the cerebellum has been reported (Verhoelst *et al*., 2005). Finally, alterations in the expression pattern of other extracellular matrix proteins and cell adhesion molecules (tenascin-C, L1, TAG-1, N-CAM) during hypothyroidism may also affect *reelin* expression (Iglesias *et al*., 1996; Álvarez-Dolado *et al*., 1998, 2000, 2001).

5 Biological Implications of *Reelin* Control by T3

Given the drastic phenotype caused by the lack of Reelin and its important role during brain development, the finding that T3 influences *reelin* expression has been of fundamental importance in better understanding the basis of the alterations that occur in the hypothyroid brain during development.

Reelin and Dab1 are critical for neuronal migration which, in turn, is responsible for lamination and precise cellular localization during CNS development. These processes are severely affected by hypothyroidsm. An abnormal laminar distribution has been reported in the auditory cortex of hypothyroid rats, including an increased number of neurons in layers V/VI, a concomitant decrease in layers II to IV, and the abnormal presence of neurons in the subcortical white matter (Berbel *et al*., 1993; Lucio *et al*., 1997). These cytoarchitectonic abnormalities most probably reflect migration defects in the cortex. Also, it has been shown that iodine deficiency causes an impaired maturation of hippocampal radial glial cells, which are involved in neuronal migration (Martínez-Galán *et al*., 1997). Additionally, hypothyroidism affects the migration of cells from germinative zones toward the olfactory bulb and caudate putamen, as well as the migration of granule neurons from the external toward the internal granule layer of the cerebellum (Patel *et al*., 1976; Lu and Brown, 1977; Legrand, 1984). As a result, the precise timing to establish appropriate neuronal connections is disrupted, and there is a decrease in the number and density of synaptic contacts. Finally, ectopic localization of Purkinje cells is a typical abnormality found in the hypothyroid cerebellum, which remarkably also occurs to much higher extent in *reeler* mice (Mariani *et al*., 1977; Legrand, 1984; Miyata *et al*., 1997). This array of abnormalities is very likely a consequence of the reduction in Reelin content reported in the hypothyroid brain during the perinatal period.

6 Conclusions

The finding that T3 regulates *reelin* expression has been essential in better understanding the basis of the alterations that occur in the hypothyroid brain during development. It explains most of the observed anomalies and has helped to find other molecules regulated by T3 that are implicated in neuronal migration. Further efforts should be devoted to finding other hormones and factors that may be implicated in the mechanisms governing *reelin* expression in the brain.

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Chapter 16
A Tale of Two Genes: Reelin and BDNF

Thomas Ringstedt

Contents

1 Introduction 237
2 BDNF and the Neurotrophins 238
2.1 BDNF Is a Survival Factor for Cajal-Retzius Cells 238
2.2 BDNF Regulates Reelin Expression 239
2.3 BDNF Regulates Neocortical Cell Migration Independent of Reelin 240
3 Reelin and BDNF Mediate the Effects of Epileptic Seizures 241
4 Reelin and BDNF Promote Neuronal Plasticity 241
5 Reelin and BDNF Are Involved in Mental Disorders 243
6 Overlap Between the Epigenetic Regulation and Signal Transduction of BDNF and Reelin 244
References 245

1 Introduction

BDNF is a survival factor for the Cajal-Retzius cells in the marginal zone, which are an important source of Reelin in the neocortex. BDNF is also a negative regulator of Reelin expression in both Cajal-Retzius cells and GABAergic cells in the cortical plate. BDNF and Reelin act in parallel to regulate many processes during neural development and maintenance, including cell migration and neural plasticity. Frequently, BDNF and Reelin have opposite influences on the processes they regulate, suggesting that BDNF-induced downregulation of Reelin is involved. Reelin is an important regulator of neural migration during neocortex formation. BDNF seems to influence this process both directly and indirectly via regulation of Reelin expression. Moreover, epileptic seizures increase BDNF levels while decreasing Reelin levels, and BDNF and Reelin seem to have opposite roles in mediating the effects of the seizures. Mental disorders, in particular schizophrenia, involve alterations in BDNF and Reelin expression. Again, the changes are mainly opposite, and a

T. Ringstedt
Neonatal Unit, Karolinska Institutet, Astrid Lindgren Children's Hospital, Q2:07, SE-171 76, Stockholm, Sweden
e-mail: thomas.ringstedt@ki.se

S. H. Fatemi (ed.), *Reelin Glycoprotein: Structure, Biology and Roles in Health and Disease.* 237

negative regulation of Reelin by BDNF has been suggested. In contrast, hippocampal LTP is promoted by both Reelin and BDNF signaling. Finally, there is overlap in the epigenetic regulation and signaling pathways of BDNF and Reelin.

2 BDNF and the Neurotrophins

In the 1950s, a diffusible factor that increased innervation of internal organs by promoting neuronal survival was isolated. It was named *nerve growth factor* (NGF) (Cohen and Levi-Montalcini, 1957). A similar, but distinct protein was purified from pig brain in 1982, and was named *brain-derived neurotrophic factor* (BDNF) (Barde *et al*., 1982). This was the birth of the NGF family of neurotrophic factors, or the neurotrophins. Since then, two more factors have been added to the family in mammals: neurotrophin 3 (NT-3) (Hohn *et al*., 1990) and neurotrophin 4 (NT-4) (Hallbook *et al*., 1991). The neurotrophins bind to the Trk family of tyrosine kinase receptors: NGF to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC (but also to a certain extent to TrkA and TrkB). They also bind to the P75 neurotrophin receptor (P75NTR) with equal affinity. The original concept of neurotrophins as target derived survival factors for innervating neurons, still holds. Competition for neurotrophic factors weeds out ill-positioned neurons during the period of naturally occurring cell death. Interestingly, neurotrophin homologues are not found in invertebrates. It is therefore possible that the plasticity inferred by an extrinsic regulation of neuronal survival (as opposed to cell-intrinsic regulation) has coevolved with higher neuronal complexity. While the neurotrophin roles as survival factors in the peripheral nervous system occur during embryonic development, brain neurons mostly seem to develop neurotrophic dependency postnatally, if at all. In addition to promoting survival, neurotrophins are now known to affect neuronal differentiation, maturation, migration, axonal guidance, and plasticity. BDNF in particular stands out as an important regulator of these processes, often paralleling the effects of reelin.

2.1 BDNF Is a Survival Factor for Cajal-Retzius Cells

The Cajal-Retzius (CR) cells are early born cells that are part of the embryonic preplate, marginal zone, and hippocampus. During development, they are the primary producers of Reelin in the neocortex and the hippocampus. It has long been believed that the Reelin produced by CR cells is essential for correct lamination of the neocortex by regulating positioning of migrating neurons (D'Arcangelo *et al*., 1995; Ogawa *et al*., 1995; Super *et al*., 2000), although this has lately been put in doubt (Yoshida *et al*., 2006). The later fate of the CR cells is unclear. It has been suggested that they die or differentiate into other cell types, but at least a subgroup of this (probably) heterogeneous cell population remains in the adult brain (Meyer *et al*., 1999; Riedel *et al*., 2003).

BDNF is only weakly expressed in the mouse brain before birth, but the expression increases rapidly during the first weeks of life (Friedman *et al*., 1991; Timmusk

et al., 1994) and remains high during adulthood. BDNF mutant mice investigated at postnatal day 18 (P18, about their longest survival time) display significantly reduced CR cell numbers in the marginal zone/layer I compared to wild-type littermates (Ringstedt *et al*., 1998). Thus, BDNF acts as a survival factor for CR cells in the postnatal brain neocortex. However, CR cells in murine hippocampal brain slices exceed their *in vivo* survival time and remain up to 14 days after explantation (the longest experimental period), regardless of whether the slices are derived from wild-type or BDNF mutant embryos, suggesting that BDNF is not a survival factor for hippocampal CR cells (Marty *et al*., 1996).

2.2 BDNF Regulates Reelin Expression

BDNF is also a negative regulator of Reelin expression. In wild-type mice, Reelin expression by the marginal zone CR cells decreases during the first 3 postnatal weeks, inversely correlated with the postnatal rise in BDNF expression. In BDNF mutant mice, CR cell Reelin expression remains constant from birth until P11. At P18 (when the survival effect of BDNF becomes evident), Reelin levels in the remaining CR cells drop to wild-type levels. Conversely, BDNF treatment of dissociated cultures of cortical neurons reduces Reelin levels. In transgenic mice, nestin-driven overexpression of BDNF in the brain (hereafter referred to as nestin-BDNF), reduces CR cell Reelin expression to 50% of wild-type (at embryonic day E18.5) (Ringstedt *et al*., 1998). Reelin expression by GABAergic cells in the cortical plate is highly reduced or absent in the nestin-BDNF mice. Expression of the downstream effector of Reelin, Dab1, increases (Alcantara *et al*., 2006), probably as a direct effect of the reduced Reelin level (Howell *et al*., 1999). BDNF further increases expression of calretinin in neocortical (Alcantara *et al*., 2006) and hippocampal (Marty *et al*., 1996) CR cells.

In addition to the reduced Reelin expression, nestin-BDNF mice display polymicrogyria at E18.5 (Ringstedt *et al*., 1998; Alcantara *et al*., 2006), and the normal bilayered organization of the neocortical marginal zone, with CR cells close to the pial membranes and GABAergic neurons in the inner part, is disturbed. Beginning at E16, the CR cells are organized in ectopic clusters spaced by empty stretches. At E18.5 the CR cells are enlarged and their axons project abnormally deep into the neural cortex (Alcantara *et al*., 2006). The CR cell clusters occupy the sulci of the polymicrogyria, while GABAergic neurons are found in the gyri. The laminar distribution of cells in the cortical plate is altered as revealed by BrdU labeling. However, only the migration of late born (E14 to E16) cells is affected. These are present in increased proportions in the interstitial and marginal zones of nestin-BDNF mice. BrdU labeling of early born cells (E11) revealed that unlike the Reeler mouse, the preplate is split in nestin-BDNF mice. Interestingly, positioning of BrdU-labeled cells differs between gyri and sulci, and the sulci contains 33% less BrdU-positive cells. Together with the asymmetric presence of CR and GABAergic cells in sulci and gyri, this hints at the polymicrogyria being shaped by differentiated

migration in areas influenced by CR or GABAergic cells, respectively (Alcantara *et al*., 2006). Interestingly, a conditional knockout of the β1 integrin receptor, which can act as a Reelin receptor (Dulabon *et al*., 2000), also results in CR cell clustering and polymicrogyria (Graus-Porta *et al*., 2001; Magdaleno and Curran, 2001). In sum, these studies suggest that Reelin and BDNF interact to regulate cortical plate development.

2.3 BDNF Regulates Neocortical Cell Migration Independent of Reelin

The alternate distribution of CR and GABAergic cells in the marginal zone of the nestin-BDNF embryos indicates that tangential migration is affected, since the GABAergic interneurons (Ang *et al*., 2003), and at least the majority of the CR cells (Yoshida *et al*., 2006), enter via tangential migration. Tangential migration of interneurons is independent of Reelin signaling (Pla *et al*., 2006), and Reelin is not essential for CR cell migration, since CR cells are distributed normally along the marginal zone of Reeler mice (mutant for Reelin) (Derer, 1985). Intraventricular injection of BDNF in E13 mice results in altered cell positioning only 2 days later, a period that might be too short for the occurrence of changes in cell migration due to reduced Reelin expression. Also, the CR cells seem unaffected, although Reelin levels have not been investigated (Ohmiya *et al*., 2002). Application of NT-4 to cortical slice cultures or intraventricular injection in E14 mouse embryos produces a phenotype related to that observed in the nestin-BDNF mice (Brunstrom *et al*., 1997). Increased numbers of both CR and GABAergic cells enter the marginal zone, probably via tangential migration from the ganglionic eminence and/or cortical hem. NT-4-induced clustering of CR-like cells resembles that observed in the nestin-BDNF mice, but whether NT-4 also affects Reelin expression has not been investigated. In parallel, BDNF does not induce increased cell number in the marginal zone when used at the same dose as NT-4 (20 ng/ml), nor when used at 10-fold higher doses. Only extremely high doses (1 mg/ml) of BDNF are sufficient to increase marginal zone cell number (Brunstrom *et al*., 1997). NT-4 shares the TrkB receptor with BDNF, but downstream signaling can still proceed differently (Minichiello *et al*., 1998). However, both BDNF and NT-4 are equally potent in inducing lateral migration of GFP-labeled cells from E14–E16 ganglionic eminence explants into the interstitial and marginal zone of isochronic cortical explants (Polleux *et al*., 2002). This was demonstrated to be a direct effect, mediated by the PI3-kinase pathway, one of the pathways known to be induced by neurotrophins. Chemotaxic stimulation of embryonic cortical neurons by BDNF or NT-4 has also been demonstrated *in vitro* (Behar *et al*., 1997). BDNF increases neocortical expression of axon guidance receptors Robo1 and Robo2 (Alcantara *et al*., 2006), which also are involved in cell migration (Andrews *et al*., 2006). Both BDNF and NT-4 are expressed at low levels in the embryonic neocortex, although NT-4 expression precedes that of BDNF (Friedman *et al*., 1991; Timmusk *et al*., 1993). In conclusion, BDNF regulates cell

migration in the neocortex both directly, and indirectly via regulation of the expression of Reelin and other potential mediators of cell migration.

3 Reelin and BDNF Mediate the Effects of Epileptic Seizures

The hippocampal CR cells synthesize and secrete Reelin, which is an important regulator of hippocampal development (Del Rio *et al*., 1997; Frotscher *et al*., 2003). However, Reelin expression remains in the hippocampus, even after the disappearance of CR cells (Haas *et al*., 2000). Granule cell dispersion (GCD), a widening of the dentate gyrus granule cell layer, has been reported after mesial-temporal lobe epilepsy in humans (Houser, 1990). Mimicking epilepsy in rodents by kainic acid-induced seizures results in GCD. A similar phenotype is observed in Reeler mice and in mice mutant for the ApoER2 and VLDLR Reelin receptors (Rakic and Caviness, 1995; D'Arcangelo *et al*., 1999). Reelin expression is downregulated after seizures, before GCD occurs. GCD is also induced after experimentally induced downregulation of Reelin expression by the blocking antibody CR-50, indicating that GCD is regulated by the altered Reelin levels after epileptic seizures. Thus, in addition to its function during hippocampal development, Reelin seems to have a role in maintaining hippocampal integrity throughout adult life (Heinrich *et al*., 2006). BDNF, on the other hand, is upregulated after kainic acid-induced seizures. If seizure induction is followed by antisense block of BDNF synthesis, or K252a block of Trk receptors, GCD does not occur (Guilhem *et al*., 1996). Given that BDNF is a negative regulator of Reelin, it is conceivable that BDNF at least in part is responsible for the downregulation of Reelin expression and induction of GCD after epileptic seizures. There is a strong link between BDNF and epileptic seizures. BDNF protein and mRNA levels are elevated in the temporal lobe of human epileptic brains (Takahashi *et al*., 1999; Murray *et al*., 2000). Experimental induction of seizures in rats by lesions (Isackson *et al*., 1991) or kindling (Ernfors *et al*., 1991) increases BDNF mRNA levels in many brain regions, including hippocampus and neocortex. Long-term administration of BDNF to rat hippocampus results in spontaneous seizures in 25% of the animals (Scharfman *et al*., 2002). Conditional knockout of BDNF or its TrkB receptor in neurons results in a mild impairment (BDNF) or complete abolishment (TrkB) of kindling-induced epileptic seizures in mice (He *et al*., 2004). Thus, TrkB signaling by BDNF and other neurotrophins is part of the epileptogenic process.

4 Reelin and BDNF Promote Neuronal Plasticity

Both BDNF and reelin have been implicated in plasticity, the modulation of synaptic strength, in hippocampus and neocortex. Induction of hippocampal long-term potentiation (LTP), a plasticity event essential for memory formation, is completely

blocked in hippocampal slices by the addition of a general antagonist against LDL receptors, including the reelin ApoER2 and VLDL receptors (Bu and Schwartz, 1998). Mice mutant for ApoER2 or VLDLR display memory formation deficits (in contextual fear conditioning) (Weeber *et al*., 2002). Addition of Reelin results in an immediate enhancement of LTP in wild-type hippocampal slices, but not in slices from ApoER2 or VLDLR mutant mice (Weeber *et al*., 2002). Reelin-enhanced LTP is mediated through interaction with postsynaptic NMDA receptors: a splice variant of ApoER2 causes phosphorylation of the NMDA receptor subunits NR2A and NR2B in the postsynaptic density of excitatory synapses (Beffert *et al*., 2005). The expression of this ApoER2 splice variant is triggered by behavioral activity (Beffert *et al*., 2005). Similarly, BDNF (and other neurotrophins) are induced by neuronal activity (Ernfors *et al*., 1991), and BDNF expression in the hippocampus parallels the ability to undergo LTP. Addition of BDNF to hippocampal slice cultures promotes LTP induction (Figurov *et al*., 1996), while LTP is impaired in hippocampal slices from BDNF mutant mice (Korte *et al*., 1995). Although there is some controversy about the site of BDNF action (Xu *et al*., 2000), BDNF seems to have a robust postsynaptic effect on LTP (Kovalchuk *et al*., 2002). Like Reelin, BDNF potentiates the NMDA response to glutamate, but by phosphorylation of the NR1 subunit (Suen *et al*., 1997; Levine *et al*., 1998).

In addition to its role in hippocampus, BDNF promotes LTP in the visual cortex (Akaneya *et al*., 1997; Jiang *et al*., 2001). Another form of plasticity in the visual cortex of higher mammals is the formation of ocular dominance columns. Axons from the visual system that enter layer IV of the visual cortex have their terminals segregated into eye-specific ocular dominance columns during postnatal development. Addition of exogenous BDNF or NT-4 (Cabelli *et al*., 1995), or a TrkB antagonist (Cabelli *et al*., 1997) inhibits this process in cats. Blocking one eye during development (monocular deprivation, MD) alters the size of the ocular dominance columns in favor of the active eye, but only if MD occurs during a certain time-window: the critical period for ocular dominance plasticity. Dark-rearing animals can delay this time-window into adulthood. Transgenic overexpression of BDNF in mouse postnatal neocortex shortens the critical period for ocular dominance plasticity and accelerates maturation of the visual cortex (Huang *et al*., 1999). Contrary to wild-type mice, dark-rearing the BDNF-overexpressing mice does not delay the plasticity window, indicating that BDNF overexpression can replace the influence of visual experience (Gianfranceschi *et al*., 2003). In a recent study that used differential display to compare gene expression in the visual neocortex of dark- and light-reared cats and mice, the Reelin signaling pathway gene Dab1 was found to be differently regulated. Dab1 expression was high 5 weeks postnatally in light-reared cats, but low in dark-reared cats. The reverse was true 20 weeks postnatally. Thus, Dab1 expression coincides with the peaks of plasticity in light- and dark-reared animals, respectively, indicating a role for Reelin signaling in visual cortex plasticity (Yang *et al*., 2006). BDNF expression normally increases after eye opening and during the critical period for ocular dominance plasticity, but this increase in BDNF expression is, like the critical period, delayed in dark-reared animals. Thus, the peaks in BDNF and Dab1 expression can be expected to overlap

in the visual cortex. Interestingly, increased Dab1 expression has been shown to correlate with low Reelin levels (Howell *et al*., 1999). It is therefore possible that the change in Dab1 expression is caused by the negative regulation of Reelin expression by BDNF.

5 Reelin and BDNF Are Involved in Mental Disorders

Altered levels of Reelin and BDNF have been reported in the brains and sera of patients with schizophrenia or autism. Reelin has been reported to be expressed at lower levels in the hippocampus (Impagnatiello *et al*., 1998; Fatemi *et al*., 2000; Guidotti *et al*., 2000; Knable *et al*., 2004) and temporal and prefrontal neocortex (Impagnatiello *et al*., 1998; Guidotti *et al*., 2000) of schizophrenic patients, than in normal controls. BDNF, on the other hand, has been reported to display an increased expression in the hippocampus (Takahashi *et al*., 2000; Iritani *et al*., 2003) and neocortex (Takahashi *et al*., 2000; Durany *et al*., 2001; Iritani *et al*., 2003) of schizophrenic patients. Its receptor, TrkB, was reported to be downregulated in the hippocampus and prefrontal cortex (Takahashi *et al*., 2000). Although a reduced BDNF expression in hippocampus also has been reported (Durany *et al*., 2001; Knable *et al*., 2004), the schizophrenic change in expression seems to be quite opposite that described for Reelin. A negative regulation of Reelin by BDNF in schizophrenic patients has, therefore, been suggested (Takahashi *et al*., 2000). However, the increase in BDNF levels may also be due to a defective secretion, a hypothesis that is supported by observations of decreased serum levels of BDNF in schizophrenic patients (Karege *et al*., 2002; Toyooka *et al*., 2002; Iritani *et al*., 2003). Interestingly, levels of the unprocessed form of Reelin are increased in the blood of schizophrenic patients (Fatemi *et al*., 2001). That BDNF might be part of the etiology of schizophrenia is supported by the association of a BDNF polymorphism (C270T) with schizophrenia (Szekeres *et al*., 2003). Associations between Reelin polymorphisms and autism have been described (Persico *et al*., 2001; Serajee *et al*., 2006). Reduced levels of Reelin (Fatemi *et al*., 2005b) have been described in the cerebellum and parts of the neocortex of autistic subjects. BDNF, on the other hand, displays increased levels in the basal forebrain of autistic patients (Perry *et al*., 2001), and BDNF hyperactivity has also been proposed as a cause of autism (Tsai, 2005). However, the lack of overlap makes a direct regulation of Reelin levels by BDNF unlikely as a cause of the described alterations. Both higher (Miyazaki *et al*., 2004) and lower (Hashimoto *et al*., 2006) serum levels of BDNF in autistic patients have been reported.

Reelin levels are also lower in the prefrontal cortex, hippocampus, and cerebellum of subjects with bipolar disorder (Fatemi *et al*., 2000, 2005a; Guidotti *et al*., 2000; Knable *et al*., 2004). No observations of altered BDNF levels in the brains of bipolar patients have been reported, but intracerebral administration of BDNF may have antidepressant effects in animals (Siuciak *et al*., 1997). There are also associations between BDNF polymorphisms, cognitive ability (Egan *et al*., 2003), neuroticism

(Sen *et al*., 2003), and bipolar disorder in human subjects (Neves-Pereira *et al*., 2002; Sklar *et al*., 2002).

6 Overlap Between the Epigenetic Regulation and Signal Transduction of BDNF and Reelin

Epigenetic modifications of DNA are self-perpetuating modifications of DNA and histone proteins that affect transcription. The polygenic nature of epigenetic modifications makes this an interesting concept from a psychiatric perspective. Hypermethylation of the Reelin promoter in the brain of schizophrenic patients has been reported (Abdolmaleky *et al*., 2005). DNA methylation is an epigenetic modification that decreases transcription by interfering with transcription factors. Both the Reelin and the BDNF promoters are cytosine methylated by DNA (cytosine-5) methyltransferase (DNMT), and can undergo acute changes in methylation status (Levenson *et al*., 2006). Furthermore, activation of the PKC signaling pathway decreases Reelin methylation in the hippocampus (Levenson *et al*., 2006). The PKC signaling pathway is important for hippocampal plasticity, and can be activated by BDNF/TrkB signaling.

There is a large overlap in the processes regulated by Reelin and BDNF. It is therefore not surprising that they signal in part via convergent pathways. Reelin signaling via the ApoER2 or VLDL receptors proceeds via dimerization and tyrosine phosphorylation of Dab1, which allows it to interact with SH2 domain proteins, among them phosphatidylinositol 3-kinase (PI3K) (Beffert *et al*., 2002; Bock *et al*., 2003). BDNF binds to and dimerizes TrkB, thereby inducing tyrosine phosphorylation. Phosphorylated TrkB also interacts with SH2 domain proteins and stimulates PI3K signaling via the SH2 adapter protein Shc (Bibel and Barde, 2000). Furthermore, BDNF can activate cyclin-dependent kinase 5 (CDK5) (Tokuoka *et al*., 2000; Wang *et al*., 2006), which acts on the cytoskeleton. CDK5 acts in parallel with Reelin to regulate cell positioning (Ohshima and Mikoshiba, 2002; Beffert *et al*., 2004), but has also been shown to phosphorylate Dab1 (Keshvara *et al*., 2002) and modulate Reelin signaling (Ohshima *et al*., 2007). BDNF and Reelin signaling also converge in regulation of the activity-regulated cytoskeletal-associated protein (Arc). Arc mRNA is present in dendrites where it is locally translated and involved in synaptic stabilization (like during LTP). BDNF promotes translation (Yin *et al*., 2002) and transcription (Ying *et al*., 2002) of Arc mRNA, while Reelin via the $\alpha 3\beta 1$ integrin receptor promotes Arc mRNA translation (Dulabon *et al*., 2000; Dong *et al*., 2003).

In sum, BDNF and Reelin act in parallel during brain development and maintenance. The reports so far suggest multiple interactions at several different levels, such as through the use of convergent signaling pathways and through the regulation of CR cell survival and Reelin expression by BDNF. Yet further investigation is needed to elucidate the precise nature of the cross-talk between Reelin and BDNF. Their ability to regulate cell positioning and plasticity in the brain makes them likely candidates in the etiology of mental illness.

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