A role of MAP1B in Reelin-dependent Neuronal Migration

The signaling cascades governing neuronal migration are believed to link extracellular signals to cytoskeletal components. MAP1B is a neuron-specific microtubule-associated protein implicated in the control of the dynamic stability of microtubules and in the cross-talk between microtubules and actin filaments. Here we show that Reelin can induce mode I MAP1B phosphorylation, both in vivo and in vitro, through gsk3 and cdk5 activation. Additionally, mDab1 participates in the signaling cascade responsible for mode I MAP1B phosphorylation. Conversely, MAP1B-deficient mice display an abnormal structuring of the nervous system, especially in brain laminated areas, indicating a failure in neuronal migration. Therefore, we propose that Reelin can induce post-translational modifications on MAP1B that could correlate with its function in neuronal migration.

Keywords: cyclin-dependent kinase 5, glycogen synthase kinase 3, microtubule-associated protein, neuronal migration, Reelin

Introduction

Ordered neural migration is an essential step in the organization of brain nuclei and laminated brain regions. Another essential step in neural development is axonal guidance and the formation of neural connections with specific targets. These two processes are controlled by specific guidance cues, including extracellular and membrane-anchored proteins (Tessier-Lavigne and Goodman, 1996; Rice and Curran, 2001). Both in experimental animals and in humans, disruption of ordered neural migration leads to structural and functional defects that are associated with neurological abnormalities including sensory-motor disorders and mental retardation (Feng and Walsh, 2001; Rice and Curran, 2001).

Reelin is an extracellular matrix protein which is essential for the correct migration and positioning of neurons in laminated brain regions such as the cerebral cortex, hippocampus and cerebellum (D’Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). Reelin receptors include the very low density lipoprotein receptor (VLDLR) and the ApoE receptor 2 (ApoER2) (D’Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999). Binding of Reelin to these receptors leads to phosphorylation of the adaptor protein Disabled 1 (mDab1), which may control the activity of the serine/threonine kinase cyclin-dependent kinase 5 (CDK5) through interaction with the regulatory subunits p35 and p39 (Ohshima et al., 1996; Chae et al., 1997; Howell et al., 1999a,b; Walsh and Goffinet, 2000; Keshvara et al., 2001; Ko et al., 2001). It has recently been shown that Reelin activates the tyrosin kinases Fyn and Src to phosphorylate mDab1 (Arnaud et al., 2003; Bock et al., 2003), and that Reelin stimulates serine phosphorylation of glycogen synthase kinase 3 (GSK3), which is believed to decrease GSK3 activity (Beffert et al., 2002). Reelin has also been reported to bind to α3 and β1 integrins, and to cadherin neuronal related (CNR) protocadherins (Senzaki et al., 1999; Dulabon et al., 2000). Since dab1(-/-) and cdk5(-/-) mice show migration abnormalities reminiscent of those in reeler, these genes are believed to be essential for Reelin signaling (Ohshima et al., 1996; Howell et al., 1997; Sheldon et al., 1997; Gilmore et al., 1998; Kwon and Tsai, 1998). Because substrates of CDK5 include cytoskeletal proteins (Ishiguro et al., 1994; Pigino et al., 1997), the Reelin signaling might transduce a signal that regulates the cytoskeleton and cell motility during neuronal migration, in a manner as yet unknown. Indeed, mutations in the microtubule-associated proteins (MAPs) LIS1 and Doublecortin cause severe migration disorders (Hirotsune et al., 1998; Francis et al., 1999; Gleeson et al., 1999; Cahana et al., 2001).

MAP1B is a neuron-specific MAP that is expressed in virtually all developing neurons, both in vivo and in vitro (Avila et al., 1994a). Its function is regulated at the post-translational level by phosphorylation (Avila et al., 1994b). Mode I phosphorylation is mediated by CDK5 and GSK3, being dynamically regulated by functional stimuli such as lysophosphatidic acid (LPA) (Sayas et al., 1999), and axonal regeneration (Gonzalez-Billault et al., 2004). Mode II phosphorylation is catalyzed by casein kinase II and appears to be activated constitutively (Diaz-Nido et al., 1988; Gonzalez-Billault et al., 2004). MAP1B has been implicated in neurite extension, the dynamic stability of microtubules and the cross-talk between microtubules and actin microfilaments, in a mode I phosphorylation-dependent manner (Lucas et al., 1998; Goold et al., 1999; Mack et al., 2000; Gonzalez-Billault et al., 2001).

Map1B-deficient mice have variable degrees of abnormalities in axonal tracts, which are believed to be a consequence of decreased capacity for axonal elongation in these mice (Takei et al., 1997, 2000; Gonzalez-Billault et al., 2000; Meixner et al., 2000; Teng et al., 2001). A role in axonal growth and synaptogenesis has also been proposed for the Drosophila MAP1B ortholog Futsch (Hummel et al., 2000; Roos et al., 2000). In contrast, the involvement of MAP1B in neuronal migration is more controversial (Gonzalez-Billault et al., 2000; Takei et al., 2000; Teng et al., 2001). Here we examine whether MAP1B function can be modified by a canonical signaling pathway controlling neuronal migration, such as Reelin. We show that map1b mutant mice display migration deficits in several brain structures, and also that Reelin may control mode I phosphorylation of MAP1B in a GSK3- and CDK5-dependent manner. These
results suggest that Reelin could modify, through MAP1B function, the neuronal cytoskeleton of migrating neurons.

Materials and Methods

Animals

Generation of map1B mutants (R1/NMR1 background, 129 strain) was made by the gene-trapping approach (Chowdhury et al., 1997). The gene-trapping vector contained a fused chimeric gene composed of neomycine phosphotransferase (neo') and β-galactosidase (β-gal) controlled by the promoter of the endogenous trapped gene (Chowdhury et al., 1997). To genotype the mutant mice, genomic DNA was isolated from mice tails and analyzed by polymerase chain reaction (PCR), using oligonucleotides corresponding to neomycine phosphotransferase gene (neo') contained in the gene-trapping vector (Gonzalez-Billault et al., 2000). Heterozygous animals were bred to obtain homozygous map1B mutants. The genotype of homozygous mutants was indicated by abnormal limb posture (Gonzalez-Billault et al., 2000), and subsequently confirmed by Western blot analyses of spinal cord protein extracts immunostained with anti-MAP1B (monoclonal antibody 125) and anti-β-galactosidase (Promega) antibodies. All animals were treated according to standard, internationally approved protocols.

Histology

Embryos were transcardially perfused at embryonic day (E) 18 with 4% paraformaldehyde. Dissected brains were postfixed in the same fixative, cryoprotected and frozen in dry ice. Coronal sections (40 μm thick) were Nissl-stained or immunostained with the following antibodies: anti-Calretinin antibodies (Swant, Bellinzona, Switzerland) and anti-CSPG. After incubation with biotinylated secondary antibodies and streptavidin-rhodamine complex, sections were analyzed in an epifluorescence microscope. To calculate the density of Cajal–Retzius (CR) cells, we counted the number of calretinin-positive neurons present in the marginal zone of the prospective parietal neocortex (two embryos and eight counts per group) (del Rio et al., 1995).

For BrdU labeling, pregnant females were injected with a single BrdU dose at E10, E11, E13 or E15 (del Rio and Soriano, 1989). Offspring were killed at E18 and processed for the immunohistochemical detection of BrdU (del Rio and Soriano, 1989). The number of BrdU-positive neurons present in 250 μm thick vertical stripes covering the entire thickness of the parietal neocortex was counted (14–16 sections from 2–3 wild-type and mutant embryos per age). To analyze the radial distribution of positive neurons, the number of cells present in the marginal zone, subplate, intermediate zone, subventricular/ventricular zones, as well as in the lower, middle and upper tiers of the cortical plate, were counted. The radial distribution of labeled cells was analyzed as above.

Production of Recombinant Reelin

Reelin-conditioned media were prepared as described (Barallobre et al., 2000; Keshvarya et al., 2001). The plasmid containing full-length Reelin cDNA or the empty vector were transfected into 293T cells using lipofectamine (Gibco-BRL). The next day culture medium was replaced with 10 mM lithium chloride and 20 mM roscovitine to inhibit CDK5 activity (Gonzalez-Billault et al., 2001, 2002). Conditioned media was collected, filtered through 0.22 μm porous membranes and concentrated ~60 times by using Millex GV filters (Millipore). Partially purified recombinant Reelin was a generous gift from Drs T. Curran and D. Benhayon (Memphis, TN). Reelin was diluted with fresh culture medium and added to the neuronal cultures for different periods of time. Partially purified Reelin was used at 2 ng/ml. Control experiments included incubation with conditioned media from control cells. In a few experiments, freshly dissociated cells were incubated with Reelin as described (Howell et al., 1999a). After stimulation, cells were collected in 2× loading sample buffer for SDS-PAGE (0.15 M Tris, pH 6.5, 2-mercaptoethanol 1 mM, SDS 1%, glycerol 10% and bromophenol blue 0.25%), boiled for 10 min and subjected to SDS-PAGE.

Protein Brain Extracts

The brains of reeler and dabl mutant mice were collected at postnatal day (P) 0 and P5. The brains of map1B mutants were also collected at E18. Briefly, the hindbrains and forebrains of mutant mice and control littermates were homogenized in HEPEs 20 mM, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1 mM Ca2+, 10 mM mercaptoethanol, 10% glycerol, 0.5% Tween 20, 1% Triton X-100, 0.2 mM DTT, 10 mM sodium orthovanadate and 5 mM EDTA. The soluble fraction was immunoprecipitated with a GSK3 antibody (Transduction Laboratories). Samples of 10 μg were incubated in a buffer containing 25 mM HEPEs, pH 7.5, 1 mM DTT, 10 mM MgCl2 and a specific GSK3 substrate peptide [G8 (Sayas et al., 1999)] at a final concentration of 0.75 μg/ml in the presence of [γ-32P]ATP. The reaction was stopped with 1% H3PO4. The difference between the kinase activities in the presence or absence of the GSK3 inhibitor LiCl (20 mM) was considered to reflect GSK3 kinase activity (Sayas et al., 1999).

Pharmacological Inhibition of Protein Kinases

Neuronal cultures were prepared and treated with Reelin as above, except that dishes were coated with laminin (20 μg/ml) in some experiments. Cultures were supplemented with 10 mM lithium chloride and 20 mM myo-inositol to inhibit GSK3 activity (Sayas et al., 2002) or with 250 nM roscovitine to inhibit CDK5 activity (Gonzalez-Billault et al., 2001). In some experiments cultures were incubated with both inhibitors.

Expression of Reelin mRNA

Total RNA from map1B mutant and wild-type embryos were subjected to reverse transcription PCR reactions for the detection of Reelin and Actin mRNAs as described (Alvarez-Dolado et al., 1999).
Results

Reelin Stimulates Mode I MAP1B Phosphorylation In Vitro and In Vivo

The response of developing neurons to Reelin is mediated through a complex signaling pathway comprising receptors, adaptor proteins and protein kinases (Walsh and Goffinet, 2000; Rice and Curran, 2001; Arnaud et al., 2003; Bock et al., 2003), which is believed to transduce the Reelin signal into cytoskeletal changes. MAP1B binds microtubules and actin microfilaments, contributing to their stabilization via a process that is believed to depend on type I MAP1B phosphorylation (Pedrotti and Islam, 1996; Pigino et al., 1997; Goold et al., 1999). To examine whether Reelin can induce MAP1B mode I phosphorylation, we treated embryonic cortical neurons (E15–E16) with recombinant Reelin, a paradigm that has been shown to activate the Reelin signaling cascade (Howell et al., 1999a; Keshvara et al., 2001). Reelin-exposed neurons showed a dramatic increase in mode I MAP1B phosphorylation (e.g. ~15-fold at 1 h), which was maintained up to 6 h (Fig. 1A,C). The effects seemed to be specifically induced by Reelin, since 293T-conditioned media, derived from mock-transfected cells, failed to induce an increase in mode I MAP1B phosphorylation (Fig. 1B). Finally, incubation of cultured embryonic forebrain neurons with purified Reelin (2 ng/ml) yielded an increase in mode I MAP1B phosphorylation similar to that observed in the culture forebrain assay (Fig. 1D,E). In contrast, incubation with Reelin supernatant or with purified Reelin failed to induce type II MAP1B phosphorylation (detected with the 125 Mab; data not shown (see also Diaz-Nido et al., 1988)). Finally neither Reelin nor mock transfection altered the expression levels of MAP1B, as detected with the NC19 antibody (Fig. 1A,B,D).

The adaptor protein mDab1 is activated by the Reelin signaling pathway (Howell et al., 1999a; Keshvara et al., 2001). To confirm that the Reelin signaling cascade was activated in our experimental conditions, we have measured the levels of mDab1 phosphorylation by using anti-phosphotyrosine antibodies (Howell et al., 1999a). Embryonic forebrain neurons treated with recombinant Reelin showed a significant increase in mDab1 phosphorylation (Fig. 1F, Reelin). Conversely, a similar treatment with mock transfected cells did not induce any significant change in mDab1 phosphorylation (Fig. 1F, mock). Changes were not due to differential protein expression, since total mDab1 levels were unaltered in all the above-mentioned conditions (Fig. 1F, lower panel).

If Reelin regulates mode I MAP1B phosphorylation in vivo, mice with genetic disruptions of the Reelin signaling pathway may have decreased levels of MAP1B phosphorylation. We first measured mode I MAP1B phosphorylation in protein extracts from Reelin-deficient reeler mice. The forebrains (P0) and hindbrains (P5) of reeler mice showed a 7-fold decrease in the levels of type I MAP1B phosphorylation, compared to wild-type littermates (Fig. 2A,B upper panels). Interestingly, heterozygous reeler mice displayed about a 2-fold reduction, indicating a dose-dependent effect. Subsequently, the forebrains and hindbrains of mDab1(-/-) newborn mice also showed decreased levels of mode I MAP1B phosphorylation (2-fold reduction, Fig. 2A,B lower panels). Together, these results indicate that Reelin stimulates mode I MAP1B phosphorylation in embryonic neurons in vitro, through a signaling pathway that involve mDab1 tyrosine phosphorylation. Thus, we conclude that MAP1B becomes phosphorylated in vitro and in vivo in response to Reelin in a signaling cascade which requires the adaptor protein mDab1. Interestingly, the amount of mode I MAP1B phosphorylation was not totally abolished in both reeler and mDab1-/- mutants, indicating that MAP1B phosphorylation is also modified by other signaling cascades. However, these data suggest that MAP1B phosphorylation can be indeed modified by the Reelin signaling pathway.

Involvement of GSK3 and CDK5 Kinases in MAP1B Phosphorylation Triggered by Reelin

MAP1B is phosphorylated in two different ways by serine/threonine protein kinases: whereas GSK3 and CDK5 induces mode I phosphorylation, casein kinase II leads to mode II phosphorylation (Diaz-Nido et al., 1988; Lucas et al., 1998; Goold et al., 1999; reviewed in Gonzalez-Billault et al., 2004). A recent study reports that Reelin increases serine phosphorylation of GSK3 (Belfert et al., 2002), a kinase that phosphorylates MAP1B in mode I. GSK3 activity is believed to be negatively regulated by serine phosphorylation (Belfert et al., 2002). However, GSK3 is also phosphorylated at its Tyr 216, which is believed to increase GSK3 activity (Sayas et al., 1999). Western
blots of cortical neurons incubated with Reelin supernatant showed increased levels of serine phosphorylation of GSK3 (Fig. 3A, middle panel), in agreement with the above study (Beffert et al., 2002), but also increased tyrosine phosphorylation levels (Fig. 3A, upper panel). All the aforementioned effects were produced without affecting the total GSK3 protein levels (Fig. 3A, lower panel). Because serine and tyrosine phosphorylation levels may have opposite effects on GSK3 activity, we investigated the net effect of Reelin on GSK3 activity. To analyze GSK3 activity we performed an in vitro kinase activity assay, by incubating immunopurified enzyme from control and Reelin-treated neurons, and subsequently we measured its kinase activity on a specific peptide substrate. As shown in Figure 3B, there was a rapid increase in GSK3 enzymatic activity after exposure to Reelin, which lasted up to 6 h (Fig. 3B, filled diamonds). Interestingly, the increased activity of GSK3 after Reelin treatment was significantly inhibited by the addition of lithium chloride to the kinase assay (Fig. 3B, filled squares). Thus, we conclude that Reelin increases the phosphorylation of GSK3 at both serines and tyrosines, which in turn results in a marked increase in GSK3 enzymatic activity.

We have previously shown that a MAP1B phosphoepitope depending on GSK3 activation is increased on addition of Reelin (Fig. 1A). In order to confirm whether GSK3 plays a role in Reelin-induced MAP1B phosphorylation and to identify whether other kinases like CDK5 are responsible for Reelin-induced mode I phosphorylation of MAP1B, we used inhibitors of GSK3 and CDK5. Embryonic forebrain neurons treated with recombinant Reelin in the presence of LiCl (10 mM, a GSK3

Figure 2. Reduced mode I MAP1B phosphorylation in Reeler and Dab1+/−/− mutants. (A) Western blots illustrating mode I MAP1B phosphorylation levels in brain extracts from the forebrain (P0) of reeler, rl+/− and wild-type mice (upper panel), and from mDaβ1+/−/− and wild-type littermates (lower panel). Western blots were incubated with the SMI31 antibody. (B) Histograms showing densitometric analysis of mode I MAP1B phosphorylation in the forebrains and hindbrains of reeler and mDaβ1 mutant brains (mean ± SD; three cases each). The phosphorylation levels of MAP1B protein in the SMI31 epitope were determined as described above. The data was normalized respect to total MAP1B values (NC19), and graphically represented. The data from wild-type brains were referred as 1 relative unit. Note markedly decreased phosphorylation levels in homozygous and heterozygous mutant brains, compared to their wild-type littermates.

Figure 3. Contribution of GSK3 and CDK5 in the type I MAP1B phosphorylation induced by Reelin. (A) Western blots showing P-Tyr-GSK3 (upper panel), P-Ser-GSK3 (middle panel) and GSK3 total levels (lower panel) in cortical cultures treated with Reelin-conditioned media for 0–60 min, showing an increase of both GSK3 tyrosine and serine phosphorylation. (B) Plots illustrating the phosphorylation levels (mean ± SD) of a GSK3-specific peptide substrate in neuronal cultures after exposure to Reelin containing media for 0–6 h, in the presence or absence of LiCl, a GSK3 inhibitor. GSK3 kinase activity is considered to be the difference between the kinase activities in the presence (filled squares) or absence of LiCl (filled diamonds). Note that GSK3 activity increases shortly after Reelin exposition, consistent with an increase in tyrosine phosphorylation of the kinase; and the increased activity lasts for 6 h. (C) Western blot of soluble cell extracts from cortical neurons exposed to Reelin alone for 1 or 6 h, with Reelin and lithium chloride (Li), roscovitine (Ros), or both Li and Ros. Membranes were immunoreacted with the SMI31 MAb to visualize mode I MAP1B phosphorylation, or with the NC19 antibody. (D) Densitometric analysis of the Western blot shown in (C). The data were normalized with respect to the values corresponding to total MAP1B levels (NC19). (E) Densitometric analysis of mode I MAP1B phosphorylation levels detected with the SMI31 MAb, normalized to total MAP1B levels. Embryonic cortical neurons were cultured on laminin-coated plates for 48 h, and then exposed for 1 h to Reelin alone, or to Reelin plus lithium (Li) or to Reelin plus roscovitine (Ros). Inhibition of either CDK5 (Ros) or GSK3 (Li) activities greatly reduces Reelin-induced MAP1B phosphorylation levels.
inhibitor; Lucas et al. 1998) showed a marked decrease in the levels of mode I phosphorylation at 1 and 6 h (Fig. 3C,D). In contrast, incubation with the CDK5 inhibitor roscovitine (250 nM, Calbiochem) does not modify mode I MAP1B phosphorylation (Fig. 3C,D).

Since Reelin actions are believed to be mediated by CDK5, we investigated the contribution of CDK5. It is known that neurons cultured on laminin upregulate p35 expression, thus promoting CDK5 activity (Paglini et al., 1998). We therefore performed similar experiments but with cultured forebrain neurons on laminin. Under these conditions, incubation with roscovitine inhibited mode I MAP1B phosphorylation induced by Reelin (Fig. 3E). Furthermore, inhibition of both GSK3 and CDK5 almost completely abolished mode I phosphorylation of MAP1B (Fig. 3C,D).

Altogether these results strongly suggest that MAP1B phosphorylation can be in part controlled by the Reelin signaling pathway, in a cascade that includes mDab1 tyrosine phosphorylation as well as GSK3 and CDK5 activation.

**Map1B-deficient Mice Show Migratory Deficits**

Because mode I of MAP1B phosphorylation could be induced after treatment with Reelin, we decided to investigate whether MAP1B function could be modified by Reelin. For this reason, in the first set of experiments we investigated if the map1B mutation affected the expression of Reelin. Sections from wild-type and mutant embryos were immunostained with calretinin antibodies to map the distribution of CR cells, the major source of Reelin at embryonic stages (D’Arcangelo et al., 1995; Ogawa et al., 1995; Alcantara et al., 1998). No differences were observed in the distribution or the density of CR cells (18.7 ± 1.2 cells and 19.3 ± 0.9 cells per 250 μm in wild-type and mutant mice, respectively; two embryos and eight counts per group), indicating that MAP1B is not required either for the migration of CR cells or for their settlement in the marginal zone. To confirm that Reelin production was unaltered in mutant embryos, we measured Reelin expression by Western blot and RT-PCR. Again, the data confirmed that the levels of expression of both Reelin transcripts (Fig. 4A) and protein (Fig. 4B) were similar in wild-type and map1B mutant forebrains. Differences in the amount of Reelin mRNA between wild-type, heterozygous and homozygous groups were not significant when normalized against tubulin as an internal control. Taking together, these results indicate that decreased Reelin levels cannot account for the migratory deficits detected in map1B-mutant embryos.

**Figure 4.** Reelin levels are unaltered in map1b mutants. (A) RT-PCR for reelin mRNA showing no significant variations amongst wild-type, heterozygous and homozygous animals. Samples were normalized against actin mRNA internal control. (B) Western blot of brain extracts derived from wild-type and homozygous animals showing no significant variations in the levels of Reelin protein. Samples were normalized against tubulin as an internal control.

Map1B-deficient mice die soon after birth, therefore the phenotype of mutant embryos was analyzed at E18. In agreement with previous studies (Gonzalez-Billault et al., 2000), Nissl-stained sections from map1B mutant embryos revealed structural abnormalities in certain laminated brain regions. Thus, although the neocortex of E18 mutant embryos contained the typical layers, i.e. the marginal zone, the cortical plate and the intermediate zone, the arrangement of postmitotic neurons in the cortical plate appeared disorganized. For instance, map1B-deficient neurons were arranged in a wave-like pattern in the lower cortical plate (corresponding to the prospective layers V and VI) (Fig. 5A,B arrows) — similar, but not indentical to that in reeler mice and mutants of components of the Reelin pathway (Fig. 5C). In other cortical areas, such as the hippocampus, homozygous mutant embryos showed a disorganized or bi-stratified pyramidal layer which was more evident in the CA3 region (Fig. 5D,E). Finally, the cerebella of map1B-deficient embryos were smaller and less foliated than wild-type littermates (Fig. 5F,G). Although Purkinje cells are not still arranged in a monolayer at E18, calbindin-immunostaining showed that mutant Purkinje cells appeared to be more widely distributed than wild-type Purkinje cells, also suggesting a lamination defect (Fig. 5H,I).

To examine migratory deficits in the neocortex of map1B-mutant mice, we pulse injected 5′-bromodeoxyuridine (BrdU) at E10–E15, and the positioning of labeled neurons was recorded in the parietal neocortex of E18 embryos. After E10 injections, labeled neurons in wild-type embryos were consistently found in the subplate and marginal zone, in agreement with earlier studies (Wood et al., 1992; Price et al., 1997) with a few cells located in the lower and upper cortical plate (Fig. 6D). Similar E10 injections in mutant embryos also labeled neurons in the marginal zone and subplate. However, mutant neurons were more widely distributed, and labeled neurons were also present in the intermediate zone, below the subplate (Fig. 6D). After pulses at E11, labeled neurons in wild-type embryos were mainly located in the subplate and in the deep cortical plate (Fig. 6AD). In map1B-mutant embryos, labeled neurons were also present in these layers, but up to 23% of cells accumulated in the intermediate zone (Fig. 6AD). To label cortical plate neurons we administered BrdU at E13 or E15. In wild-type embryos, E13 injections labeled many neurons in the deep and intermediate aspects of the cortical plate, corresponding to layers V and VI (Fig. 6BD). E13-labeled neurons in homozygous mutant embryos were present in layers V and VI, but were also frequent in the intermediate zone (27%) and the
subplate (21%) (Fig. 6B,D). Interestingly, many labeled neurons accumulated below the subplate in the mutant. Finally, E15 injections in wild-type embryos resulted in the accumulation of labeled neurons in the upper tier of the cortical plate (Fig. 6C,D). In contrast, labeled mutant neurons had a wider distribution in the three sublayers of the cortical plate (Fig. 6C,D).

To ascertain whether the preplate splits correctly in map1B mutant embryos, sections were stained with anti-calretinin or calbindin antibodies (CSPG; CS-56 antibody). Calretinin immunostaining showed that CR cells were correctly positioned in both wild-type and mutant mice, in the marginal zone (Fig. 7C,F). Similarly, CSPG immunostaining revealed that the subplate was positioned correctly in map1B mutant embryos (Fig. 7B,E). These results indicate that although the preplate appears to be split correctly into the marginal zone and the subplate in map1B-mutant mice, some cortical plate migrating neurons fail to settle appropriately into their correct cortical layer, suggesting abnormal migration of cortical neurons.

The present BrdU analyses in the neocortex, together with the lamination abnormalities in the pyramidal layer of the hippocampus and the cerebellum, indicate that map1B-deficient mice have migratory deficits that occur post-preplate splitting. Indeed, other post-preplate splitting mutants with neuronal migration defects have been described. Some of them have been related to the the Reelin signaling pathway such as cdk5 (−/−) and p35(−/−) mutant mice (Ohshima et al., 1996; Chae et al., 1997; Gilmore et al., 1998), while others, such as the Double-cortin mutant (Corbo et al., 2002), have not.

**Discussion**

The present study shows migration abnormalities in the neocortex, hippocampus and more faintly in the cerebellum of map1B-deficient mice. No migration deficits have been reported in other strains of map1B-deficient mice which have been generated by distinct gene targeting approaches (Edelmann et al., 1996; Takei et al., 1997; Meixner et al., 2000). In the first mutant line (Edelmann et al., 1996), map1B-deficient animal die around E12, and in other mutant strains the lack of MAP1B gave rise to a mixture of phenotypes (Takei et al., 1997; 2000; Gonzalez-Billault et al., 2000; Meixner et al., 2000). For this reason, interpretation of the phenotypes found in the latter map1B mutant lines had been somewhat controversial. A possible explanation for such discrepancies is the different genetic background among the various lines. It has been shown that genetic background can be responsible for dramatic changes in the phenotype of a mutated gene. For example, with the 129/Sv background, 100% of Pax-2-deficient mice display exencephaly in the midbrain/hindbrain region, whereas with a C57BL/6J background the frequency of this phenotype is reduced to 30% (Schwarz et al., 1997). A similar effect has been described for engrailed-1 knockout mice (Bilovocky et al., 2003). In agreement with this, null map1B mutants (Meixner et al., 2000) and a hypomorph mutant expressing MAP1B alternative transcripts (Takei et al., 2000) sharing the same genetic background, with a C57BL/6J background the frequency of this phenotype is reduced to 30% (Schwarz et al., 1997). A similar effect has been described for engrailed-1 knockout mice (Bilovocky et al., 2003). In agreement with this, null map1B mutants (Meixner et al., 2000) and a hypomorph mutant expressing MAP1B alternative transcripts (Takei et al., 2000) sharing the same genetic background, with a C57BL/6J background the frequency of this phenotype is reduced to 30% (Schwarz et al., 1997). A similar effect has been described for engrailed-1 knockout mice (Bilovocky et al., 2003).

It has been proposed that the Reelin pathway transduces a signal that leads to cytoskeletal rearrangement and changes in cell motility (Walsh and Goffinet, 2000; Rice and Curran, 2001; Kawauchi et al., 2003). The present study showing that MAP1B responds in vitro to extracellular Reelin by increasing mode I phosphorylation, and that Reelin-deficient mice have decreased levels of MAP1B mode I phosphorylation, supports the hypothesis that Reelin can trigger an increase in MAP1B phosphorylation,
Figure 6. Distribution of BrdU-labeled neurons in the parietal neocortex. (A–C) Examples illustrating the distribution of neurons labeled by BrdU at E11 (A), E13 (B) or E15 (C), and analyzed at E18 in wild-type (left) and map1B mutant (right) embryos. Scale bars, 200 μm. (D) Radial distribution of BrdU-labeled neurons (in percentages) in the parietal neocortex of wild-type (black bars) and map1B mutant (white bars) embryos. Postmitotic neurons were labeled at E10, E11, E13 or E15, and were analyzed at E18. Cortical layers comprised the subventricular/ventricular zones (SVZ/VZ), intermediate zone (IZ), subplate (SP), marginal zone (MZ), and the lower, middle and upper tiers of the cortical plate (CP3, CP2, CP1).
the hippocampal pyramidal layer is disrupted, and the cerebellum diffusely positioned in the cortical plate and intermediate zone, than those in the neocortex, hippocampus and cerebellum are less dramatic in MAP1B mutants (del Rio et al., 1999). Netrin-1 is also dependent on Netrin-1 function, is dramatically impaired in neuronal migration and axonal guidance (Tessier-Lavigne and Goodman, 1996). Netrin-1 is also able to induce MAP1B phosphorylation and axonal elongation, are indistinguishable from those of the specific GSK3 inhibitor SB-216763 (Owen and Gordon-Weeks, 2003). Finally, the implication of GSK3 in mode I MAP1B phosphorylation after Reelin treatment is also supported by recent findings that LIS1 could be either an obligatory component of the Reelin pathway that functions downstream of mDab1 or could be a component of just one of several branches that constitute the intracellular response to Reelin (Assadi et al., 2003). LIS1 is another microtubule-associated protein that when mutated produces neuronal migration defects that occur after preplate splitting. Analogously, LIS1 mutants display migration abnormalities that are not identical to the reeler mutant (Hirotsume et al., 1998; Cahan et al., 2001).

Proline-directed protein kinases such as GSK3 and CDK5 have been implicated in mode I MAP1B phosphorylation (Pigino et al., 1997; Lucas et al., 1998; Garcia-Perez et al., 1998; Goold et al., 1999). Our pharmacological experiments with lithium — which blocks GSK3 activity and MAP1B phosphorylation — indicates an essential role of this kinase in Reelin-induced mode I MAP1B phosphorylation. Notably, it has recently been reported that the effects of lithium, specifically upon MAP1B phosphorylation and axonal elongation, are indistinguishable from those of the specific GSK3 inhibitor SB-216763 (Owen and Gordon-Weeks, 2003). Finally, the implication of GSK3 in mode I MAP1B phosphorylation after Reelin treatment is also supported by the increase in GSK3β kinase activity. The analysis of CDK5 contribution was more complex, since in our experiments Reelin-induced mode I phosphorylation was not blocked by roscovitine. This could be due to the fact that neurons were initially cultured on poly-L-lysine, a substrate that does not favor CDK5 activity (Paglini et al., 1998). When embryonic neurons were cultured on laminin, which increases CDK5 activity, roscovitine decreased mode I phosphorylation. Because embryonic neurons in vivo develop in a rich extracellular milieu, composed of many extracellular proteins, and since CDK5 may be essential for the Reelin signaling (Ohshima et al., 1996; Chae et al., 1997; Rice and Curran, 2001; Smith and Tsai, 2002), we propose that both GSK3 and CDK5 cooperate in vivo in the Reelin-induced mode I phosphorylation of MAP1B. In fact, pharmacological blockade of both GSK3 and CDK5 almost

Figure 7. Distribution of subplate cells and Cajal-Retzius cells in the map1B mutant neocortex. (A–F) Comparison of the distribution of subplate cells (B, E: CS-56-immunoreactivity) and Cajal-Retzius cells (C, F: calretinin-immunoreactivity) in the map1B mutant and wild-type parietal neocortex at E18. No major differences were detected. (A) and (D) are sections counterstained with bisbenzimide, to illustrate the pattern of lamination. Abbreviations as in Figure 5.

both in vivo and in vitro. This view is reinforced by the finding that Reelin induces activation of GSK3, one of the enzymes responsible for mode I MAP1B phosphorylation (Sayas et al., 1999). That MAP1B is modified in vivo by Reelin is further supported by the observation that disruption of the mab1 gene, essential for transduction of the Reelin signal (Howell et al., 1997, 1999a; Sheldon et al., 1997), reduces mode I MAP1B phosphorylation. The fact that MAP1B is highly expressed in migrating neurons (Gonzalez-Billault et al., 2000) is also consistent with this protein having a role in Reelin-dependent migration. Nevertheless, the fact that mode I MAP1B phosphorylation is not totally abolished in Reeler and mDab1 mutant mutants suggests that other proteins can modulate MAP1B function. This seems to be case for Netrin-1, an extracellular cue participating in neuronal migration and axonal guidance (Tessier-Lavigne and Goodman, 1996). Netrin-1 is also able to induce MAP1B phosphorylation, and several neuronal tract responding to Netrin-1 effects are mispositioned in MAP1B mutants (del Rio et al., 2004). Moreover, neuronal migration of pontine nuclei neurons, which is also dependent on Netrin-1 function, is dramatically impaired in MAP1B mutants (del Rio et al., 2004).

The migratory deficits observed in map1B mutant embryos in the neocortex, hippocampus and cerebellum are less dramatic than those in reeler mutants. Thus, cortical plate neurons are diffusely positioned in the cortical plate and intermediate zone, the hippocampal pyramidal layer is disrupted, and the cerebellum has foliation abnormalities (Rice and Curran, 2001). However, unlike reeler, the preplate splits correctly into the marginal zone and subplate in map1B-deficient mice, although mutant subplate neurons are ectopically positioned in the intermediate zone. Also the cerebellar phenotype for Purkinje cells is less dramatic than in reeler. Similar variation in the severity of migration deficits has been reported in mutations of several genes which are believed to act downstream of the Reelin pathway. For instance, mice targeted for the cdke5 or p35 genes (Ohshima et al., 1996; Chae et al., 1997; Gilmore et al., 1998; Kwon and Tsai, 1998; Ko et al., 2001) also show a correct splitting of the preplate, but with migration defects in later-generated neurons that should migrate to the cortical and hippocampal plates. Moreover, p35 mutations lead to mild effects in the positioning of neocortical and hippocampal neurons (Chae et al., 1997), which are severed in double p35/p39 mutants (Ko et al., 2001). In contrast, mutations in the dab1 gene or double mutations of the Reelin receptors (ApoER2 and LDLR) show migration deficits almost identical to those in reeler (Howell et al., 1997; Sheldon et al., 1997). These data support the hypothesis that mutation in genes acting progressively more downstream in the Reelin cascade results in progressively less severe phenotypic alterations, which is consistent with the present findings in map1B mutants. It is also possible that other cytoskeletal-associated proteins, with functional redundancy to MAP1B, participate in the Reelin signaling pathway. This notion is also strengthened by recent findings that LIS1 could be either an obligatory component of the Reelin pathway that functions downstream of mDab1 or could be a component of just one of several branches that constitute the intracellular response to Reelin (Assadi et al., 2003). LIS1 is another microtubule-associated protein that when mutated produces neuronal migration defects that occur after preplate splitting. Analogously, LIS1 mutants display migration abnormalities that are not identical to the reeler mutant (Hirotsume et al., 1998; Cahan et al., 2001).
completely abolished type I MAP1B phosphorylation. This is consistent with a synergistic effect for those kinases. In fact, several reports have indicated that many GSK3 substrates must be previously phosphorylated in the –4 position by other protein kinases, including CDK5, to be then modified by GSK3 (Cohen and Frame, 2001). Indeed, the MAP1B phospho-epitope recognized by the SMI31 MAb contains different serines at a –4 position that could be modified by cdk5. Thus, we propose that MAP1B can be phosphorylated by CDK5 to act as a primer to favor GSK3 modification.

It is believed that CDK5 is a downstream effector in the Reelin pathway, although there is no evidence of activation of CDK5 by Reelin (Zukerberg et al., 2000; Smith and Tsai, 2002). In addition, mDab1 is phosphorylated by CDK5 in a Reelin-independent manner (Keshvara et al., 2001). A recent study reports that Reelin regulates serine phosphorylation of GSK3, suggesting an inhibition of enzymatic activity (Beffert et al., 2002). However, GSK3 has two isoforms, α and β, and its activity is downregulated by phosphorylation of serines and upregulated by phosphorylation on tyrosines (Cohen and Frame, 2001; Grimes and Jope, 2001). In contrast, our study shows that Reelin increases phosphorylation of both serines and tyrosines of GSK3, resulting in a marked increase in GSK3 enzymatic activity. This observation is also consistent with that indicating that phosphorylation of MAP1B, a GSK3 substrate, increases upon exposure to Reelin (Fig. 8). Several pathways have been proposed to inhibit GSK3 activity, including the insulin/IGF-I pathway through activation of the PI3K-Akt/PKB pathway (Cohen and Frame, 2001; Grimes and Jope, 2001) and the Wnt/Wingless signaling cascade (Welsh and Proud, 1993; Cook et al., 1996). In contrast, only a few studies report activation of GSK3 by FGF2 or LPA (Sayas et al., 1999, 2002; Hashimoto et al., 2002) by mechanisms that remain largely unknown. We have shown here that the Reelin signaling pathway also activates GSK3 (Fig. 8).

The present analyses in vitro and in vivo indicate that MAP1B can be modified by the Reelin signaling pathway. It is thought that both microtubules and actin filaments, which are concentrated at the leading process of migrating neurons, are essential for neuronal migration (Rivas and Hatten, 1995). MAP1B has been shown to control the dynamic properties of microtubules (Pedrotti and Islam, 1996; Togel et al., 1998). For instance, it has been suggested that cultured map1B-deficient neurons contain fewer microtubules than control cells (Gonzalez-Billault et al., 2001). Moreover, in the absence of MAP1B, the number of dynamic microtubules in the distal part of the axons is much lower, whereas the number and distribution of stable microtubules increase (Gonzalez-Billault et al., 2001).

We also show that mode I MAP1B phosphorylation is activated by Reelin. Mode I phosphorylated MAP1B is upregulated during development and is present in a gradient-dependent manner in axons and neurites, being enriched in the distal part (Riederer et al., 1990; Ulloa et al., 1993, 1994; Mack et al., 2000). At the cellular level, inactivation of GSK3 or CDK5 kinases induces a neural phenotype in vitro that resembles that of map1B-deficient neurons (Pigino et al., 1997; Lucas et al., 1998; Paglini et al., 1998; Goold et al., 1999; Gonzalez-Billault et al., 2002). Moreover, ablation of mode I MAP1B phosphorylation by micro-CALI experiments affects growth cone tuning in cultured neurons (Mack et al., 2000). Finally, it has been reported that overexpression of Wnt7b, a signal that inactivates GSK3, induces a decrease of MAP1B phosphorylation in cortical ex-

Figure 8. Schematic diagram showing the Reelin signaling pathway. The extracellular protein Reelin binds to the VLDLR and ApoER2 receptors. The adaptor protein Dab1 associates with the NPxY motifs in the cytoplasmic domains of these receptors. Reelin binding to their receptors activates Src/Fyn tyrosine kinases, resulting in phosphorylation of Dab1. Furthermore, the Reelin signaling pathway activates a complex array of intracellular cascades including PI3K/Akt, Cdk5 and probably other unknown kinases. These intracellular kinases control GSK3 activity, which, together with CDK5, phosphorylate MAP1B. Mutations in the reelin and dab-1 genes elicit a similar decrease in mode I-MAP1B phosphorylation.

plants, leads to abnormal positioning of cortical neurons (Viti et al., 2003). All these data have led to the suggestion that most functions of MAP1B are regulated by mode-l phosphorylation, which is likely to increase microtubule and actin instability and cross-talk (Goold et al., 1999; Mack et al., 2000).
In conclusion, we propose that MAP1B function can be modified by the Reelin signaling pathway. Absence of MAP1B can to some extent mimic alterations in the Reelin pathway, producing abnormal migration of neurons. Nevertheless, MAP1B function could also be altered by other extracellular cues, acting either independently or in parallel with Reelin. Finally, the increase in MAP1B phosphorylation is dependent on GSK3 and CDK5 protein kinases.

Notes
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