

Reelin Signals through Phosphatidylinositol 3-Kinase and Akt To Control Cortical Development and through mTor To Regulate Dendritic Growth[▽]

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Reelin is an extracellular matrix protein with various functions during development and in the mature brain. It activates different signaling cascades in target cells, one of which is the phosphatidylinositol 3-kinase (PI3K) pathway, which we investigated further using pathway inhibitors and in vitro brain slice and neuronal cultures. We show that the mTor (mammalian target of rapamycin)-S6K1 (S6 kinase 1) pathway is activated by Reelin and that this depends on Dab1 (Disabled-1) phosphorylation and activation of PI3K and Akt (protein kinase B). PI3K and Akt are required for the effects of Reelin on the organization of the cortical plate, but their downstream partners mTor and glycogen synthase kinase 3 β (GSK3 β) are not. On the other hand, mTor, but not GSK3 β , mediates the effects of Reelin on the growth and branching of dendrites of hippocampal neurons. In addition, PI3K fosters radial migration of cortical neurons through the intermediate zone, an effect that is independent of Reelin and Akt.

Reelin, the large glycoprotein that is defective in reeler mutant mice, is secreted by neurons such as cortical Cajal-Retzius cells and directs the organization of target neurons in the cortical plate (CP) and other structures (18, 40). The role of Reelin is not limited to architectonic development. It is present in the adult brain, particularly in GABAergic interneurons in the cortex and hippocampus (58), and regulates the growth and branching of dendrites in vivo (48) and in vitro (55). Reelin affects synaptic plasticity and memory (7) and the migration of gonadotropin-releasing hormone neurons (13) and may be implicated as a susceptibility factor in psychoses (73), which indicates a wide array of functions.

With some exceptions (13, 61), actions of Reelin require binding to two receptors of the lipoprotein receptor family, VLDLR and ApoER2 (72). This triggers tyrosine phosphorylation of the intracellular adaptor Disabled-1 (Dab1) by kinases of the Src family, particularly Src and Fyn (4, 9, 43, 46). Several signaling molecules respond to Reelin, but most events are incompletely characterized and not integrated into a coherent picture. Identified partners of Reelin signaling include Lis1 (2), the adaptor Nck β (60), Crk scaffolding proteins (3, 14, 35), and Dab2IP, a Ras GTPase-activating protein (33). Previous studies showed that phosphorylated Dab1 recruits the p85 α subunit of phosphatidylinositol 3 kinase (PI3K), and that Reelin triggers the phosphorylation of Akt (protein kinase B) and glycogen synthase kinase 3 β (GSK3 β) in cultured cortical neurons (6, 10). The effects of Reelin on GSK3 β may be context dependent: whereas GSK3 β activity and phosphorylation of the Tau microtubule-associated protein are both increased in Reelin-deficient mice (30, 56), Reelin induces Map1b phosphorylation through activation of GSK3 β and Cdk5 (27).

Although PI3K and Akt are activated in response to Reelin, their role and that of downstream partners remain poorly understood. Studies of mutant mice are not really contributive because of the probable redundancy and embryonic lethality of simple or multiple gene inactivations (11, 22, 26, 29). In other systems, Akt stimulates mammalian target of rapamycin (mTor) through the tuberous sclerosis complex 1/2 (TSC1/2) and Rheb (Ras homolog enriched in brain). Rheb binds to and regulates the mTor-Raptor-mLST8 complex (mTORC1), whereas its action on the mTor-Rictor-mLST8-Sin1 complex (mTORC2) is less clear (49). mTORC1 activates ribosomal S6 kinase 1 (S6K1) by phosphorylation at Thr389 (12). S6K1 phosphorylates mTor at Ser2448, an event previously attributed to Akt (15, 32). The mTORC2 complex phosphorylates Akt at Ser473, thereby increasing its activity, which is required for signaling to some but not all Akt targets (28, 34, 37, 65).

In the present work, we investigated further the role of the PI3K/Akt pathway in Reelin signaling. Inasmuch as mutant mice are not fully contributive because of lethality or genetic redundancy, we used chemical inhibitors that target all members of one enzyme family in living embryonic brain slices and dissociated neurons in culture. We show that Reelin activates mTor and S6K1 in a Dab1-, PI3K-, and Akt-dependent manner. However, whereas PI3K and Akt are necessary for positioning neurons in the CP, mTor (mTORC1 and mTORC2), S6K1, and GSK3 β are not. This indicates that the phosphorylation of Akt at Ser473 (by mTORC2) is not important for this function and that other Akt targets remain to be identified. Interestingly, PI3K, Akt, and mTor mediate the effects of Reelin on the growth and branching of dendrites in hippocampal neurons, whereas GSK3 is dispensable. We also found that PI3K plays an additional role in promoting radial neuronal migration, an action that is independent of Reelin and Akt.

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MATERIALS AND METHODS

Neuronal and slice culture. Animal procedures were carried out in accordance with institutional and European guidelines and ratified by competent animal

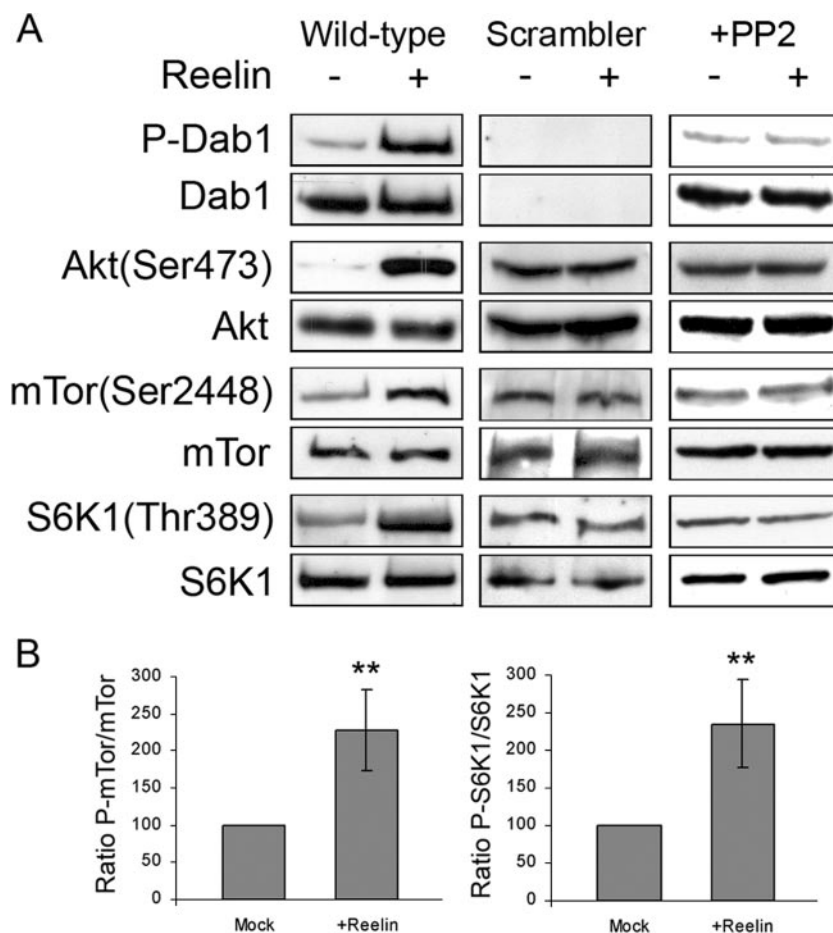


FIG. 1. Reelin-induced phosphorylation of mTor and S6K1 depends on Dab1 phosphorylation by Src kinases. Cortical neurons from wild-type or Dab1 mutant (Scrambler) brains were cultured for 3 days and stimulated for 20 min with Reelin (+, supernatant from transfected cells) or supernatant from mock-transfected cells (–). Wild-type neurons were stimulated in the presence or absence of the Src kinases inhibitor PP2 (20 μ M). (A) Cell lysates were analyzed by Western blotting to estimate the phosphorylation of the indicated proteins. (B) Comparison of mTor and S6K1 phosphorylation in Reelin- and mock-stimulated neurons. Bars are standard errors of the means from four independent experiments. **, significantly different using Student's paired *t* test ($P < 0.01$).

ethics committees. Brains from fetuses at embryonic day 18 (E18) (for hippocampus) or E16 (for cortices) were collected in cold Hanks solution without Ca^{2+} and Mg^{2+} supplemented with 0.6% glucose (CMF-HBSS-G; Lonza) and dissociated as described previously (42). Cells were plated in 12-well plates on coverslips coated with poly-L-Lysine (Sigma) at a density of 1×10^5 cells per dish (hippocampal neurons) or 1.5×10^6 cells per dish (cortical neurons) and were cultured in Dulbecco modified Eagle medium-F12 medium supplemented with B27 and penicillin-streptomycin (Invitrogen). Hippocampal neurons treated with LY294002 were cultured at 2×10^5 cells per dish and treated with inhibitor only at 24 h after plating to counteract the toxic effect of PI3K inhibition. The slice culture system was described previously (41, 43, 78).

Pathway inhibitors. The following inhibitors were used: LY294002 (PI3K inhibition), tricinibine (TCBN) and Akt inhibitor IV (Akt inhibition), rapamycin (mTor inhibition), and PP2 (Src kinase inhibition) and its inactive isomer PP3. GSK3 inhibition was carried out using thiazolidinone 8 (TDZD-8), LiCl, or SB415286. Apart from SB415286, which was from Tocris, the inhibitors were from Calbiochem.

Production of recombinant proteins. HEK293T cells cultured in Dulbecco modified Eagle medium (Lonza) with 10% fetal bovine serum were transfected with the Reelin cDNA construct pCrl, kindly provided by T. Curran (19), using Lipofectamine 2000 (Invitrogen). After 24 h, the medium was replaced with a serum-free medium, which was collected 2 days later and stored at 4°C in the presence of a protease inhibitor cocktail (Complete, Roche). Prior to use, the supernatants were concentrated using Biomax columns with 30,000-molecular-weight-cutoff filters (Millipore, Bedford, MA) to reach the approximate concen-

tration of 400 pM, which was estimated as described previously (42), and dialyzed against culture medium by drop dialysis (Millipore VSWP02500).

Histology and immunohistochemistry. Slices were fixed in Bouin's fluid for 2 hours prior to embedding in paraffin. Sections were stained with hematoxylin-eosin (HE) or by immunohistochemistry. Tbr1 (a gift from R. Hevner) was used to label early-generated neurons. Detection of apoptotic cells was carried out with an antibody against the cleaved form of caspase 3 (Cell Signaling 9661), and PP splitting was monitored with antibody CS-56 (Sigma) directed against chondroitin sulfate. For immunohistochemistry, sections were deparaffinized, incubated with 3% H_2O_2 for 30 min, boiled in sodium citrate buffer, blocked for 30 min in 5% normal goat serum in phosphate-buffered saline (pH 7.4), and incubated with primary antibodies overnight. Detection was carried out with an avidin-biotin-peroxidase kit (Vectastain ABC; Vector Laboratories) using diaminobenzidine as the chromogen.

BrdU labeling and immunohistochemistry. To study cell proliferation in cultured slices, bromodeoxyuridine (BrdU) (Sigma) was added after 1 day in vitro, at 20 $\mu\text{g}/\text{ml}$, for 30 min. After three washes, slices were cultured for 4 hours longer. To study inside-out layering, BrdU (20 $\mu\text{g}/\text{mg}$) was administered to pregnant mice by intraperitoneal injection 2 hours before preparation of embryonic brain slices. Anti-BrdU (Becton Dickinson 347580) immunohistochemistry was performed as described above, with an additional incubation with 2 N HCl before blocking.

Western blotting and immunoprecipitation. Slices were lysed for 10 min at 4°C in NP-40 buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.08% Na_3VO_4 , 0.1% NaF, 1 mM phenylarsine oxide, 25 mM NaPP_i, 80

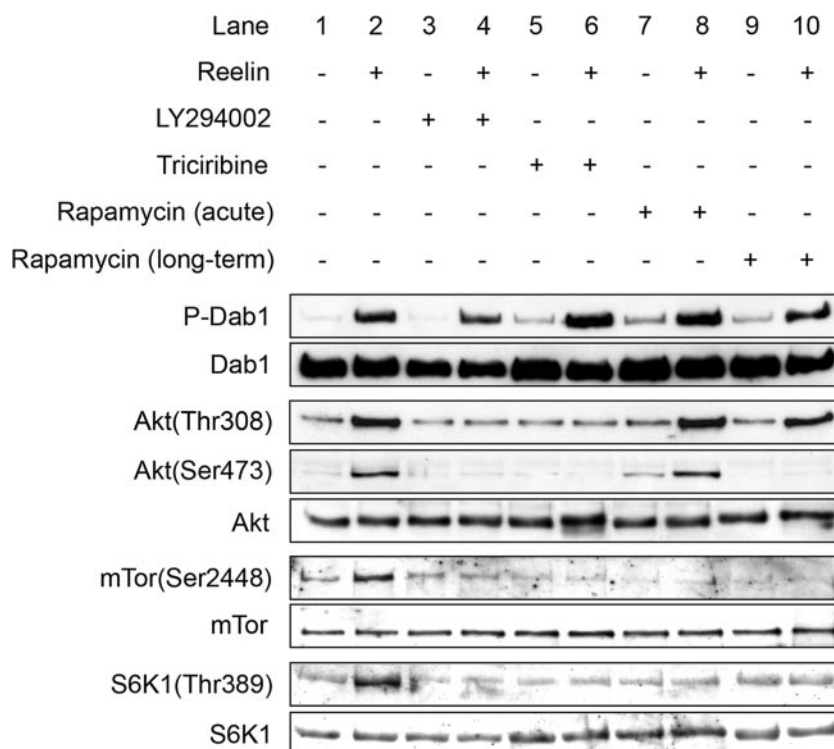


FIG. 2. Reelin-induced phosphorylation of mTor and S6K1 depends on PI3K and Akt. Cortical neurons were cultured for 3 days and stimulated for 20 min with control supernatant (lanes 1, 3, 5, 7, and 9) or with Reelin (lanes 2, 4, 6, 8, and 10). Cultures were incubated with LY294002 (50 μ M) (lanes 3 and 4), TCBN (1 μ M) (lanes 5 and 6), rapamycin (200 nM) for 30 min (acute, lanes 7 and 8), or rapamycin (200 nM) for 24 h (long-term, lanes 9 and 10) before and during exposure to Reelin. Cell lysates were analyzed by Western blotting to estimate the phosphorylation of the indicated proteins.

mM β -glycerol phosphate, 0.1 μ M okadaic acid, and 2 mM proteinase inhibitor with EDTA (Complete; Roche). Lysates were clarified by centrifugation at $14,000 \times g$ for 10 min at 4°C , and the protein concentration was measured by the Bradford method. Samples corresponding to 30 μ g protein were analyzed on 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membrane (Protran BA 85; BioScience) by electroblotting (Invitrogen). Membranes were blocked with 5% low-fat milk and 0.01% Tween 20 in phosphate-buffered saline for 30 min and incubated overnight at 4°C with antibodies. After washing, secondary horseradish peroxidase-conjugated antibodies (DAKO) were applied for 35 min, and membranes were washed, treated with the SuperSignal West Pico chemiluminescent substrate (Pierce), and exposed to Hyperfilm ECL (Amersham Biosciences). The following antibodies were used: anti-Akt (Santa Cruz sc-1618), phospho-Akt (Ser473 and Thr308) (Cell Signaling Technology 9271 and Santa Cruz sc-16643), anti-GSK3 β and -phospho-GSK3 β (Ser9) (Cell Signaling Technology 9332 and 9336), anti-Tau and -phospho-Tau (Ser396) (Biosource 44752G and AHB0042), anti-mTor and -phospho-mTor (Ser2448) (Cell Signaling Technology 2972 and 2971), and anti-S6 kinase and -phospho-S6 kinase (Thr389) (Santa Cruz sc-230 and sc-11759). For the Dab1 phosphorylation assay, 35 μ g total protein was incubated with a rabbit polyclonal antibody raised against a C-terminal peptide of Dab1 overnight at 4°C , followed by an incubation with protein A-agarose beads (Roche) for 2 h. The beads were washed three times with NP-40 buffer. Proteins were eluted by boiling for 2 min in polyacrylamide gel electrophoresis loading buffer and analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membrane, and Dab1 was detected with a mouse monoclonal anti-Dab1 antibody (E1) or with an antiphosphotyrosine monoclonal antibody (4G10; UBI). Experiments were carried out at least in triplicate.

Labeling of VZ neurons for visualization of migration. Whole brains from animals at E14 were removed, and Cell Tracker Green CMFDA (Molecular Probes C-2925) was injected in the ventricles at a concentration of 5 μ M. Brains were then incubated for 15 min at 37°C and sliced at 300 μ m. Comparable coronal slices were selected and cultured for 6, 18, 32, or 48 h before fixation with 3.7% paraformaldehyde. Fixed slices were cut with a vibratome at 30 μ m,

mounted in Vectashield (Vector Laboratories), and photographed under a fluorescence microscope.

Data analysis. Quantitative analyses were performed using the Scion Image J software (<http://rsb.info.nih.gov/ni-image/>), and images were edited with Photoshop (Adobe).

RESULTS

Reelin activates mTor and S6K1 via Dab1 phosphorylation, PI3K, and Akt. When added to wild-type cultured neurons, Reelin stimulated the phosphorylation of Dab1 and Akt (Ser473 and Thr308), as shown previously (4, 6). Here, we demonstrate that Reelin also induces phosphorylation of mTor (Ser2448) and S6K1 (Thr389) (Fig. 1). However, Reelin was unable to stimulate the phosphorylation of mTor and S6K1 when it was added to neurons from Dab1-deficient mice or in the presence of the Src inhibitor PP2, which blocks Dab1 phosphorylation (Fig. 1). The phosphorylation of mTor and S6K1 was also prevented by inhibition of PI3K with LY294002, by inhibition of Akt with TCBN, or in the presence of the mTORC1 inhibitor rapamycin (30 min, acute incubation) (Fig. 2, lanes 3 to 8). Akt phosphorylation at Ser473 by mTORC2 is insensitive to acute, but inhibited by prolonged, rapamycin treatment (23, 65, 66). When neurons were exposed to rapamycin for 24 h and stimulated by Reelin, Akt phosphorylation at Ser473 (but not at Thr308) was indeed inhibited (Fig. 2, lanes 9 and 10). Together, these results indicate that Reelin activates S6K1 through the mTORC1 complex and induces

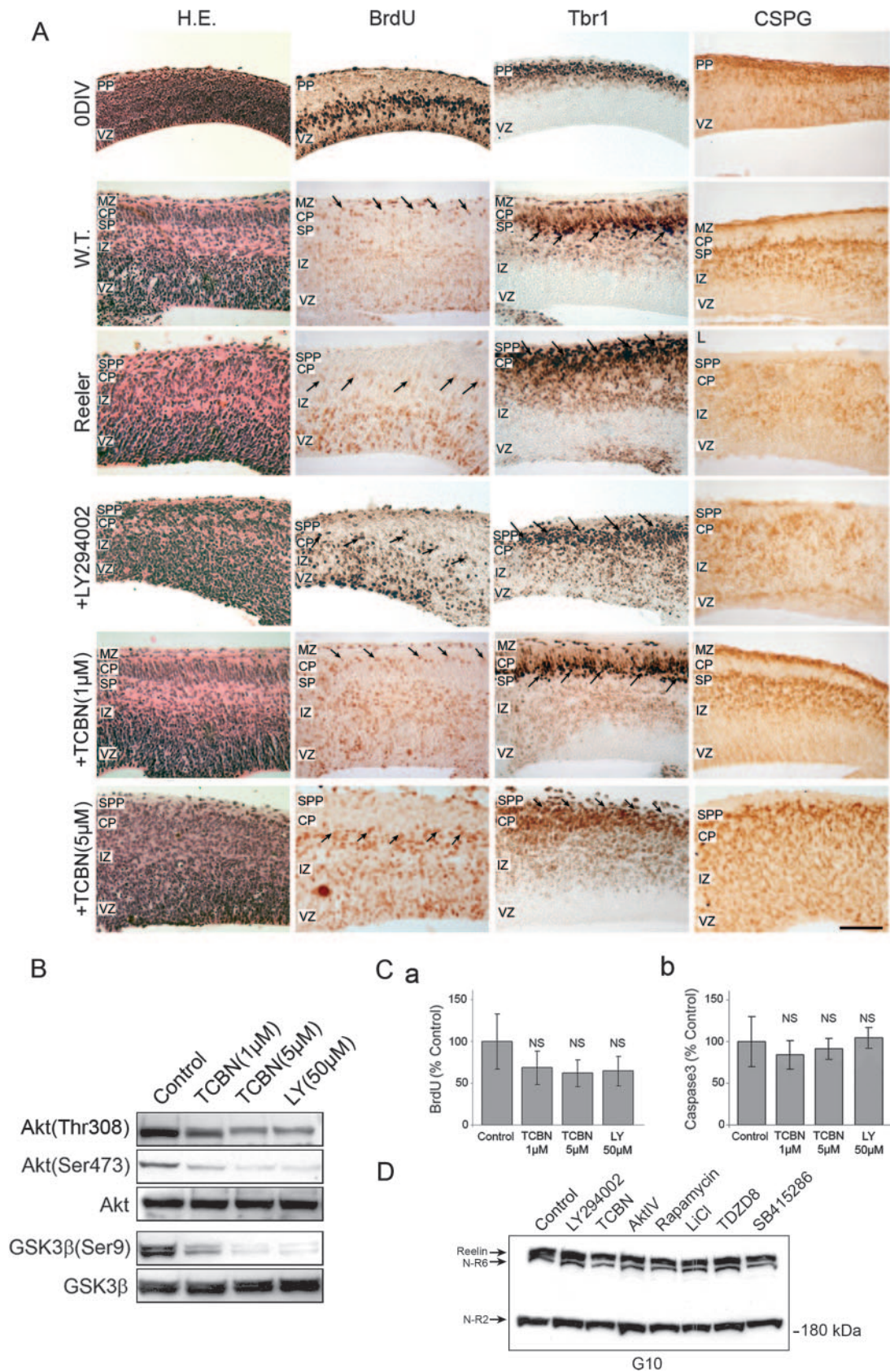


FIG. 3. CP development is perturbed following inhibition of PI3K or Akt. (A) Photomicrographs of sections stained with HE for appreciation of histological organization, immunolabeled for BrdU and Tbr1 to visualize the inside-out layering, and immunolabeled for chondroitin sulfate

phosphorylation of Akt Ser473 through mTORC2 and that these events are all dependent on Dab1 phosphorylation and activation of the PI3K/Akt pathway.

PI3K and Akt, but not mTor or GSK3 β , are required for the effects of Reelin on CP formation. We studied the signaling components PI3K, Akt, mTor, and GSK3 β using specific cell-permeant inhibitors and the organotypic brain slice culture assay (42, 43). Slices were prepared at E13, at the preplate (PP) stage, prior to the formation of the CP and PP splitting (Fig. 3A). After 2 days in vitro, the telencephalic layers and a laminar CP developed in wild-type slices. Neurons labeled with BrdU 2 hours before slice preparation were able to migrate past older, Tbr1-positive neurons, demonstrating a normal inside-to-outside layering. PP splitting, monitored by chondroitin sulfate proteoglycan labeling, occurred normally. On the other hand, Reeler slices exhibited the defects seen in vivo: poorly organized CP with outside-to-inside maturation, formation of a superplate instead of a marginal zone (MZ), and defective PP splitting.

We first confirmed the previously reported role of PI3K in brain slice development (10). As illustrated in Fig. 3A, addition of LY294002 (50 μ M) to wild-type slices resulted in abnormal cortical maturation that proceeded from outside to inside, with formation of a superplate and defective PP splitting, like in reeler slices. We then assessed the role of Akt by culturing slices in the presence of TCBN, which blocks all Akt isoforms (Akt1, Akt2, and Akt3) by targeting an Akt activator different from PI3K and phosphoinositide-dependent protein kinase 1 (76). The activity of the inhibitor was monitored by examining the phosphorylation of Akt at Thr308 and Ser473 (two sites dependent on PI3K activation) and of the Akt substrate GSK3 β at Ser9 (Fig. 3B). In the presence of TCBN (5 μ M), the inhibition of Akt and the phenotypic alterations in slices were comparable to those obtained with the PI3K inhibitor (Fig. 3A and B). We observed similar results with the structurally different Akt IV inhibitor (44) (not shown). In order to rule out interfering effects of PI3K and Akt inhibition on cell proliferation or apoptosis, slices cultured with and without LY294002 or TCBN (1 or 5 μ M) were pulsed briefly with BrdU to label proliferating cells, and an antibody against activated caspase 3 was used to detect apoptotic cells. As shown in Fig. 3C, inhibition of PI3K (LY294002, 50 μ M) or Akt (TCBN, 1 μ M or 5 μ M) seemed to decrease cell proliferation (but not statistically significantly because of high variability) and did not affect cell death at E13, whereas earlier stages were more affected (not shown). This moderate decrease in cell proliferation cannot account for the CP phenotype, since it is observed

when slices are treated with 1 μ M TCBN, a concentration that did not fully inhibit Akt (Fig. 3B) and did not perturb CP development (Fig. 3A). Together, these results suggest that PI3K and Akt are required during CP development. They cannot be attributed to interference of inhibitors with Reelin expression, since levels of Reelin were unaffected when slices were treated with the different compounds used (Fig. 3D).

mTor is an important partner in the PI3K/Akt pathway, and our results above show that Reelin activates this enzyme. In brain slices incubated with the specific mTor inhibitor rapamycin, the phosphorylations of S6K1 at Thr389 (substrate of mTORC1), mTor at Ser2448 (substrate of S6K1), and Akt at Ser473 (substrate of mTORC2) were all inhibited (Fig. 4A). However, this had no effect on radial migration, CP development, and PP splitting (Fig. 4A). This indicates that neither Reelin-induced activation of the mTor-S6K1 pathway nor phosphorylation of Akt on Ser473 is not necessary for CP formation.

GSK3 β , another key Akt target, is activated under Reelin stimulation by phosphorylation at Tyr216 (27), suggesting that it may be involved in the Reelin signal. Although homozygous GSK3 $\beta^{-/-}$ mice die during fetal development, some do survive long enough to visualize early CP formation, which occurs normally, unlike in reeler mice (Fig. 4B). A redundancy between GSK3 β and GSK3 α was recently shown (20). Since GSK3 α is also expressed in the embryonic brain, this may account for the normal phenotype of the GSK3 $\beta^{-/-}$ brain. We thus examined CP formation in slices treated with inhibitors that block both GSK3 β and GSK3 α , namely LiCl, SB415286, and TDZD-8 (5, 16, 50, 59, 70). Active concentrations were defined by estimating phosphorylation of Tau at Ser396, a site phosphorylated by both GSK3 enzymes (8, 36, 70) (Fig. 4C). At biochemically effective doses (10 mM LiCl, 56 μ M TDZD-8, or 6 μ M SB415286), these compounds did not affect radial neuronal migration, CP formation, or PP splitting (Fig. 4C [only results with SB415286 are shown]). Other studies showed that Reelin increases phosphorylation of GSK3 β at Ser9 (6), which inhibits the enzyme, and that basal GSK3 β activity is increased in reeler brains (30, 56), indicating that observations on GSK3 β -deficient mice and experiments with inhibitors may not be fully relevant. If inhibition of GSK3 is a crucial element of Reelin signaling, incubation with GSK3 inhibitors might be able to rescue the reeler phenotype in slices. However, incubation with LiCl, SB415286, or TDZD-8 did not affect the phenotype of cultured reeler slices (not shown), further showing that GSK3 α and/or GSK3 β is not

proteoglycan (CSPG) to monitor the splitting of the PP. The first row illustrates slices at the initiation of culture (zero days in vitro [DIV]). The following rows illustrate wild-type (W.T.) slices, reeler slices, and wild-type slices treated with LY294002 (50 μ M) or TCBN (1 μ M or 5 μ M) and cultured for 2 days. BrdU was injected 2 h before slicing. Arrows point to BrdU- or Tbr1-positive cells within the CP and allow visualization of inside-out CP layering. VZ, ventricular zone; SP, subplate; SPP, superplate. Bar, 100 μ m. (B) After 2 days in vitro, slice lysates were analyzed by Western blotting to estimate efficiency of the inhibitors, by comparing the phosphorylation of Akt and Gsk3 β . LY, LY294002. The two bands for GSK3 β are due to alternative splicing (51). (C) Slices prepared at E13 were cultured with LY294002 (50 μ M), TCBN (1 μ M or 5 μ M), or control medium and subjected to a short pulse of BrdU. Numbers of BrdU-positive cells in ventricular and subventricular zones (a) and of apoptotic cells, positive for activated caspase 3 (b), are expressed as a percentage of the control value. Error bars are standard errors of the means from four experiments. NS, not significant using Student's paired *t* test. (D) Reelin signal detected in Western blots in supernatants from slices cultured for 2 days in vitro in the presence of the following inhibitors: LY294002 (50 μ M), TCBN (5 μ M), AktIV (2 μ M), rapamycin (200 nM), LiCl (10 mM), TDZD8 (56 μ M), and SB415286 (6 μ M).

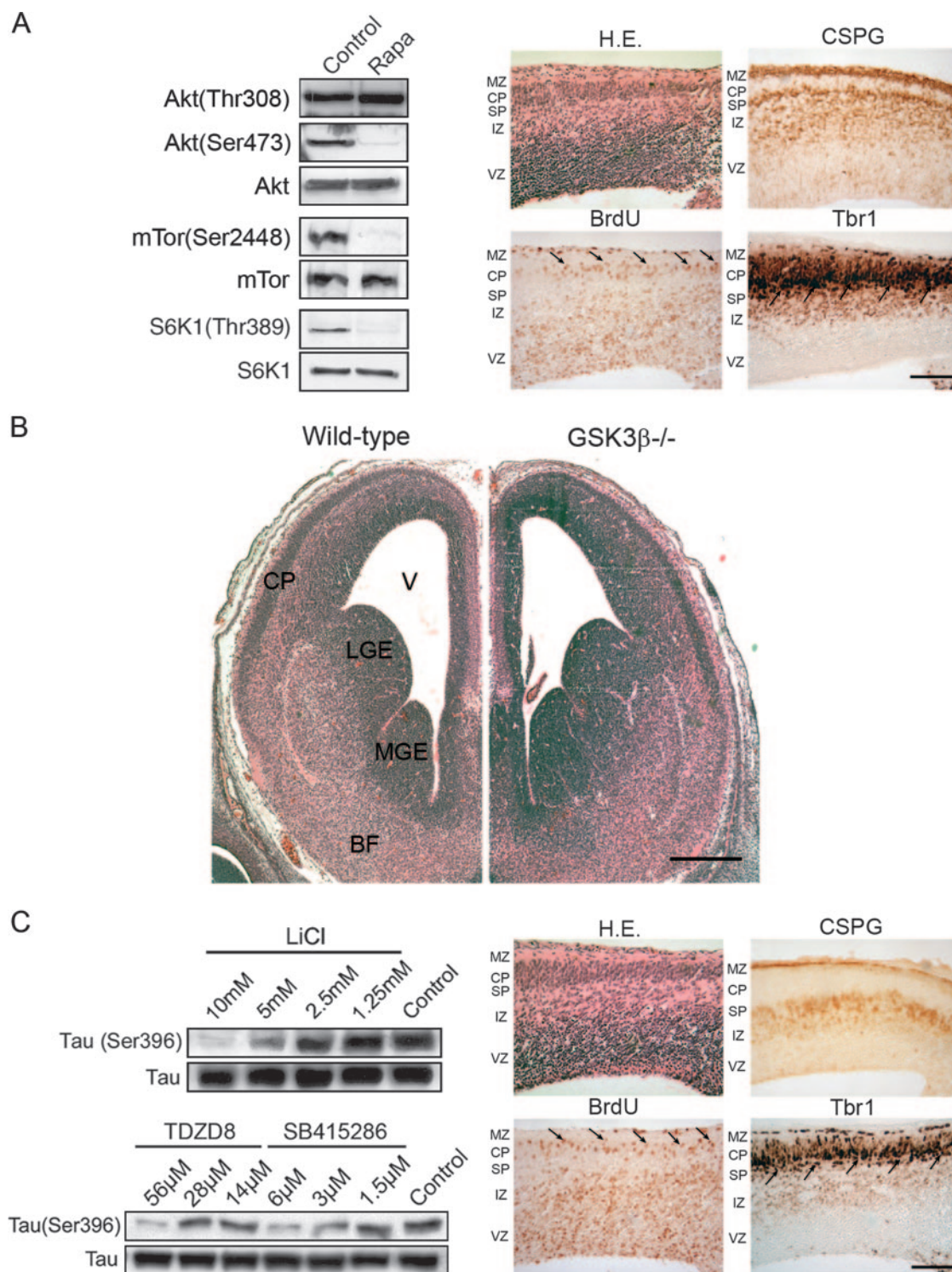


FIG. 4. GSK3 and mTor activities are not involved in CP development. (A) E13 brain slices were cultured in control medium or in the presence of rapamycin (200 nM). After 2 days in vitro, slice lysates were analyzed by Western blotting to estimate phosphorylation of Akt, mTor, and S6K1. The presence of rapamycin had no effect on CP formation (HE stain), PP splitting (chondroitin sulfate proteoglycan [CSPG] labeling), or inside-out layering (BrdU and Tbr1 labeling). SP, subplate; VZ, ventricular zone. Bar, 100 μ m. (B) Montage of HE-stained coronal sections in the forebrains of wild-type and GSK3 $\beta^{-/-}$ mutant embryos at E14. V, lateral ventricle; BF, basal forebrain; LGE and MGE, lateral and medial ganglionic eminences, respectively. Bar, 300 μ m. (C) E13 brain slices were cultured in control medium or in presence of inhibitors of GSK3 (LiCl, TDZD8, and SB415286). After 2 days in vitro, slice lysates were analyzed by Western blotting to estimate phosphorylation of Tau. The presence of LiCl (10 mM), TDZD8 (56 μ M), or SB415286 (6 μ M) (histology is shown for SB415286 only) had no effect on CP formation (HE stain), PP splitting (CSPG labeling), or inside-out layering (BrdU and Tbr1 labeling). Bar, 100 μ m.

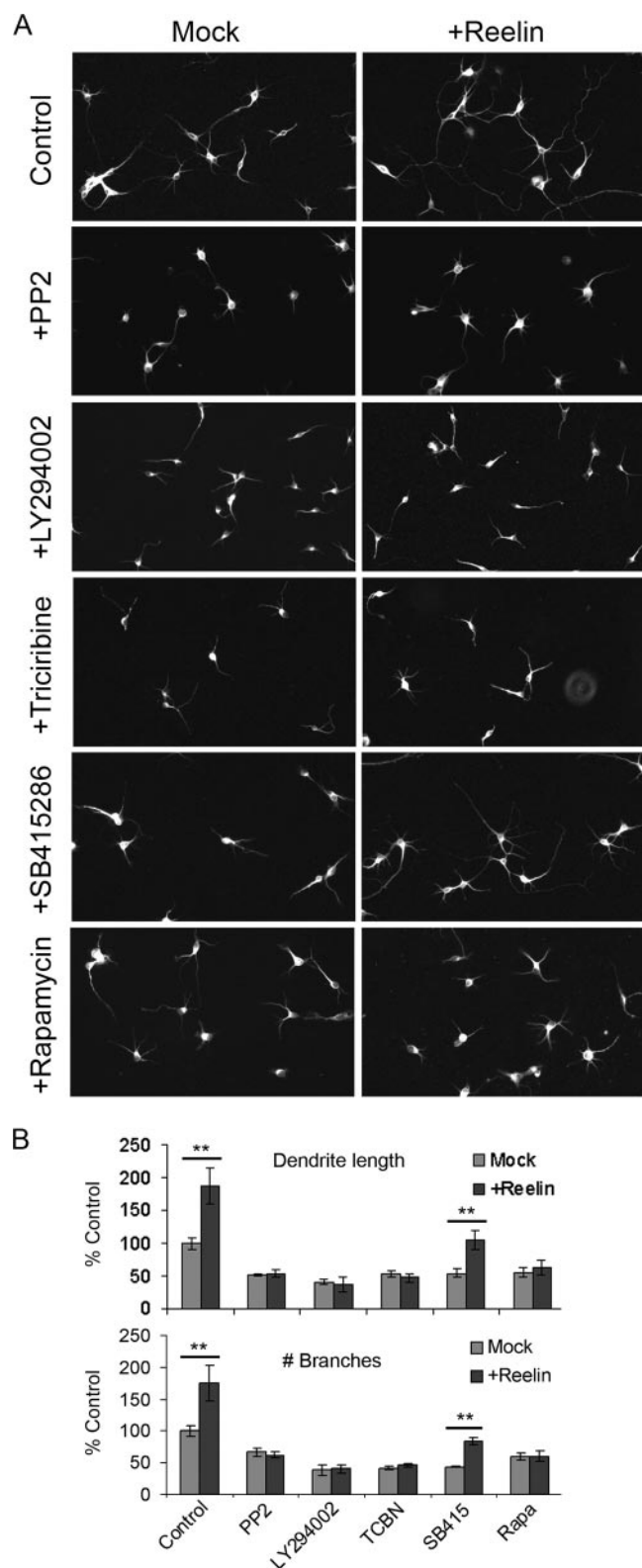


FIG. 5. The trophic effect of Reelin on dendrites is dependent on Src kinases, PI3K, Akt, and mTor but not on GSK3. E18 hippocampal neurons from reeler brains were cultured for 5 days in control medium or in the presence of inhibitors (PP2 at 10 μ M, LY294002 at 50 μ M [added 24 h after plating], TCBN at 1 μ M, SB415286 at 6 μ M, or rapamycin at 200 nM). The medium was complemented with Reelin or

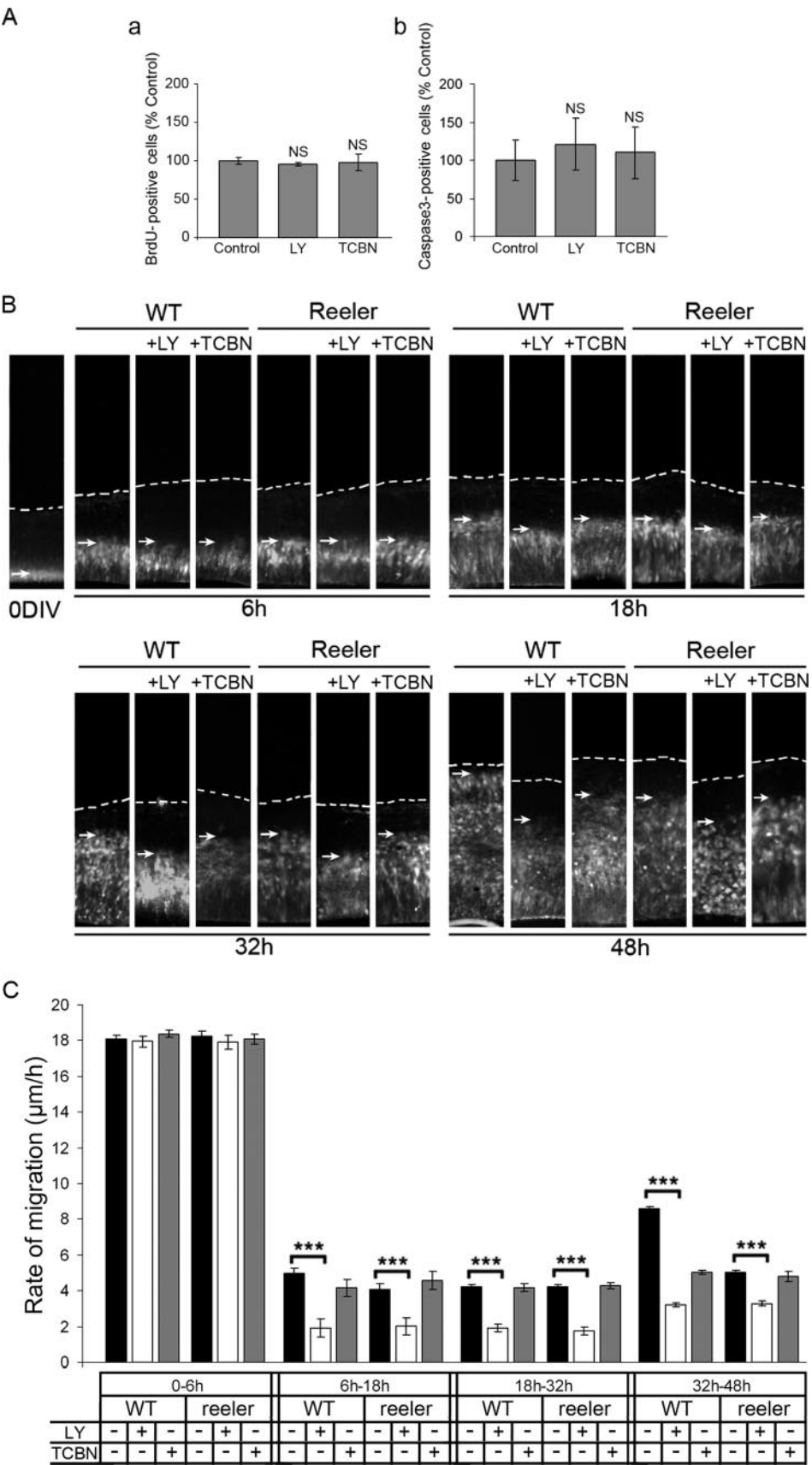
primarily involved in CP formation and radial migration, even though they are modulated by Reelin signaling.

PI3K, Akt, and mTor are involved in Reelin-stimulated dendritic growth and branching. Because Reelin regulates directly hippocampal dendritic growth (55), we assessed the role of the PI3K pathway in this process. First, we confirmed that Reelin is able to stimulate dendrite outgrowth and branching in our culture conditions (Fig. 5). We then compared dendritic development in hippocampal neurons stimulated with Reelin and control supernatants, in the presence of inhibitors of PI3K, Akt, mTor, and GSK3. As control, we blocked Reelin signaling by the Src inhibitor PP2. As shown previously (55), PP2 inhibited dendrite growth, and this could not be overcome by the addition of Reelin (Fig. 5), whereas PP3, the inactive isomer of PP2, had no effect (not shown). In the absence of Reelin, inhibition of PI3K, Akt, mTor, or GSK3 perturbed dendritic growth, as previously described (39, 63, 70). Interestingly, whereas Reelin failed to influence dendrite growth and branching in the presence of inhibitors of PI3K, Akt, or mTor, it was able to stimulate the formation of dendrites in the presence of GSK3 inhibitors (Fig. 5). These results suggest that Src kinases, PI3K, Akt, and mTor are all required for the trophic action of Reelin on dendrites. On the other hand, although GSK3 is involved in neurite outgrowth in some models (17), it is not involved in the effects of Reelin.

PI3K regulates radial neuronal migration independently of Reelin and Akt. As many cellular events depend on the PI3K pathway, we tested whether PI3K or Akt inhibition could influence the migration of neurons before they enter and integrate into the CP. Cells in ventricular zones of E14 wild-type and reeler brains were labeled with the Cell Tracker Green CMFDA. We verified that treatment with the inhibitors LY294002 and TCBN did not affect cell proliferation and survival during the migration assay (Fig. 6A).

Slices cultured with and without LY294002 or TCBN were processed after 6, 18, 32, and 48 h in vitro. In the absence of inhibitors, neurons migrated at comparable speeds in wild-type and reeler slices. The speed was the highest during the first 6 hours (18.05 ± 0.25 μ m/hour for wild type and 18.26 ± 0.24 μ m/hour for reeler) and decreased when neurons migrated through the intermediate zone (IZ) (4.98 ± 0.24 μ m/hour for wild type and 4.04 ± 0.37 μ m/hour for reeler after 6 to 18 h.; 4.22 ± 0.10 μ m/hour for wild type and 4.24 ± 0.11 μ m/hour for reeler after 18 to 32 h) (Fig. 6B and C). Reeler neurons did not enter the nascent CP and failed to reach the MZ, whereas several wild-type neurons migrated through the developing CP and reached the border of the MZ after 48 h of culture (Fig. 6B). In the presence of LY294002, in both wild-type and reeler slices, the migration speed was unaffected during the first 6 h (wild type, 17.95 ± 0.30 μ m/hour; reeler, 17.89 ± 0.40 μ m/hour).

mock supernatant. (A) MAP2 immunofluorescence. (B) Comparison of MAP2-labeled dendrites was performed by measuring total dendrite length and the number of branches. Data are expressed as percentage of the mock-treated neurons in control condition. Bars are standard errors of the means based on analysis of 60 neurons in three independent experiments. Asterisks indicate a significant difference ($P < 0.01$) between Reelin- and mock-treated dendrites, using Student's paired *t* test.



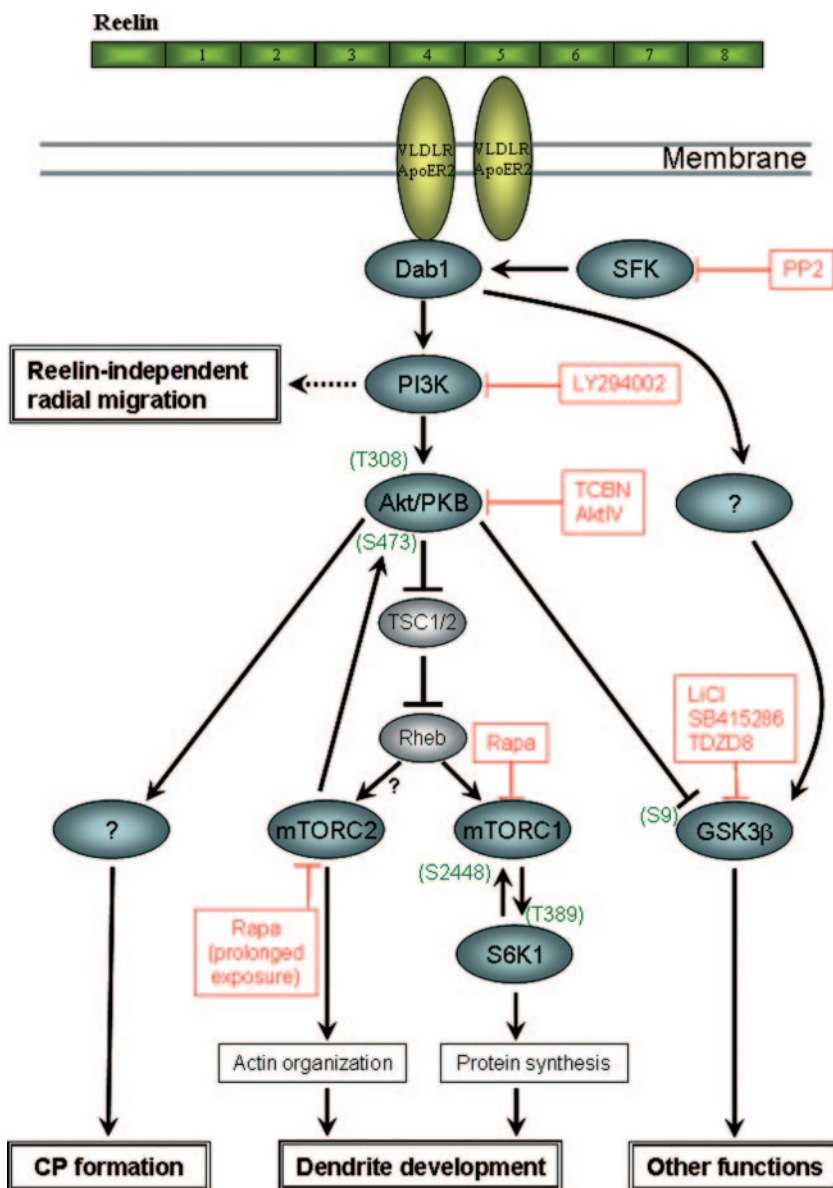


FIG. 7. Schematic view of the effects of Reelin on PI3K pathways. The inhibitors used in the study are boxed in red. The sites phosphorylated under Reelin stimulation are indicated in green.

hour), but it was significantly reduced after 18 h (wild type, $1.91 \pm 0.51 \mu\text{m}/\text{hour}$; *reeler*, $2.01 \pm 0.46 \mu\text{m}/\text{hour}$ after 6 to 18 h) or later (wild type, $1.94 \pm 0.22 \mu\text{m}/\text{hour}$; *reeler*, $1.76 \pm 0.23 \mu\text{m}/\text{hour}$ after 18 to 32 h). On the other hand, inhibition

of Akt by 5 μ M TCBN in wild-type and reeler slices had no effect in the rate of radial migration (wild type, 18.38 ± 0.17 μ m/hour from time zero to 6 h, 4.25 ± 0.45 μ m/hour from 6 to 18 h, 4.26 ± 0.21 μ m/hour from 18 to 32 h, and 5.03 ± 0.13

FIG. 6. PI3K inhibition decreases migration speed in wild-type and reeler slices independently from Akt. (A) Brain slices prepared at E14 were cultured in the presence of LY294002 (LY) (50 μ M) or TCBN (5 μ M) or in control medium and subjected to a short pulse of BrdU. Numbers of BrdU-positive cells in ventricular and subventricular zones (a), and of apoptotic cells, positive for activated caspase 3 (b), are expressed as percentages of control values. Error bars are standard errors of the means from four experiments. NS, not significant using Student's paired *t* test. (B) Ventricular zone cells of E14 wild-type (WT) or reeler brains were labeled with the Cell Tracker green CMFDA compound. Slices prepared at the same coronal level were cultured in control medium or in presence of 50 μ M LY294002 (LY) or 5 μ M TCBN. Slices were fixed and processed for histology after the indicated times. The dotted line indicates the pial surface, and the arrows indicate the front of fluorescent cells. DIV, days in vitro. (C) Comparison of migration rates in the six situations at the different time points. Data are means $\pm$ standard errors of the means. ***, significantly different ($P < 0.001$) using Student's paired *t* test.

$\mu\text{m}/\text{hour}$ from 32 to 48 h in culture; reeler, $18.09 \pm 0.28 \mu\text{m}/\text{hour}$ from time zero to 6 h, $4.57 \pm 0.49 \mu\text{m}/\text{hour}$ from 6 to 18 h, $4.29 \pm 0.19 \mu\text{m}/\text{hour}$ from 18 to 32 h, and $4.78 \pm 0.28 \mu\text{m}/\text{hour}$ from 32 to 48 h in culture) (Fig. 6B and C). These results indicate that PI3K inhibition decreases the speed of radial migration in the IZ in a Reelin-independent manner and that this function of PI3K does not depend on Akt activation and is presumably mediated by other PI3K targets.

DISCUSSION

Signaling events induced by Reelin downstream from Dab1 phosphorylation by Src family kinases remain poorly understood. Partners of Reelin signaling include Lis1 (2), the adaptor Nck β (60), Crk scaffolding proteins (3, 14, 35), and Dab2IP, a GTPase-activating protein for Ras (33). In cultured neurons and brain slices subjected to Reelin signaling, phosphorylated Dab1 binds p85 α , activates PI3K (6, 10), and regulates phosphorylation of Akt and GSK3 β (6, 27). Thus far, genetic redundancy and/or early lethality of multiple mutations has hampered analysis of the PI3K pathway *in vivo* (11, 22, 26, 29). Here, we show that Reelin activates the mTor-S6K1 pathway in a Dab1-, PI3K-, and Akt-dependent manner and that different elements in this complex pathway mediate effects of Reelin on CP formation and dendritic growth. Furthermore, we find that PI3K influences radial neuronal migration independently of Akt and of Reelin signaling. Our results are summarized in Fig. 7, which will serve as a guide to the discussion.

We found that during cortical development, normal activity of PI3K and Akt is required for PP splitting and CP layering, two events regulated by Reelin, in which neither mTor, S6K1, nor GSK3 takes part. We show that Reelin induces phosphorylation of Akt at Thr308, which is important for its effects on CP formation and hippocampal dendritic growth. This phosphorylation is performed by phosphoinositide-dependent protein kinase 1 and is requisite for its activation (1). On the other hand, phosphorylation of Akt at Ser473 is not necessary for CP development, because the CP develops normally in slices exposed to prolonged rapamycin treatment that blocks the mTORC2 complex and Akt phosphorylation at Ser473. Recent studies showed that Ser473 phosphorylation is similarly dispensable for a number of activities of Akt (28, 37), such as phosphorylation of TSC2 or GSK3 β , hinting that these two Akt substrates might be downstream targets in CP development. This is not the case, however, since phosphorylation of TSC2 induces activation of mTORC1, and we show that this complex is not involved in CP development. Similarly, inhibition of GSK3 has no effect on CP development and does not modify the reeler phenotype in slices. However, phosphorylation of GSK3 β by Akt inhibits its activity, and it would thus be interesting to assess whether transgenic mice with constitutive activation of GSK3 β exhibit a reeler-like phenotype.

The lack of implication of GSK3, mTor, and S6K1 in Reelin signaling to CP development raises the question of the Akt effectors involved. Among the many Akt substrates, three appear to be particularly worth considering. Phospholipase C γ 1 binds to phosphorylated Dab1 (3) and is phosphorylated by Akt, thereby regulating cell motility (75). p27Kip1, another direct substrate of Akt (24, 47, 68), is involved in radial neu-

ronal migration and CP development, independently from its role in cell cycle regulation (45, 53). PAK1, a kinase phosphorylated by Cdk5 (54), is also a substrate of Akt implicated in cell migration (77). Clearly, further investigation is needed to define which Akt substrates are involved in Reelin signaling to cortical neurons.

Reelin stimulates the growth and branching of dendrites in a lipoprotein receptor- and Dab1-dependent manner (55, 57), and our results show that inhibition of PI3K, Akt, and mTor, but not GSK3, prevents this effect. Thus, in contrast to the situation during CP development, mTor is necessary for the trophic actions of Reelin on dendrites, which may be explained by several mechanisms. Reelin may activate the mTORC2 complex, thereby stimulating phosphorylation of Akt at Ser473 (34, 65), and this could modulate some targets different from those involved in CP development. The mTORC2 complex could also modulate cytoskeletal dynamics through Rho and Rac (38, 64). Finally, Reelin activates the mTORC1-S6K1 pathway, thus stimulating protein synthesis, which may account for trophic activity. A similar action on protein synthesis was recently proposed to explain effects of Reelin on synaptic plasticity (21).

In contrast to its effects on CP maturation and dendritic growth, the action of PI3K on radial migration is Reelin and Akt independent. Neurons from wild-type and reeler mice migrate at comparable speeds until they reach the CP, and PI3K inhibition hampers radial migration similarly in both genotypes, whereas inhibition of Akt has no effect. Another study showed a decreased rate of migration of Dab1 (scrambler) mutant neurons (62). Several explanations might account for this apparent discrepancy. First, we studied neurons at E14, when glia-guided locomotion predominates, whereas Sanada et al. (62) studied early neurons at E13, which mostly migrate towards the PP by somal translocation. Second, they used Dab1-defective tissues, whereas we examined Reelin mutant brains, and some Dab1-independent effects of Reelin have been described (13). Finally, the culture systems and data acquisition in the two studies are very different.

In normal conditions, following a relatively rapid start, neurons slow down in the IZ and subplate, to accelerate again in the CP. The effect of PI3K inhibition is not detectable until they reach the IZ, and it cannot be attributed to delayed action of the inhibitor, which is detected by Western blotting after less than 1 hour. Other studies have shown that migrating neurons slow down when they reach the IZ, where they change their morphology to adopt a so-called multipolar migration. Multipolar neurons seem to lose their polarity towards the pia and move in various directions before they ultimately resume their radial movement towards the CP (52, 69). Cellular polarization during migration involves the accumulation of phosphatidylinositol(3,4,5)-tris-phosphate, the principal product of PI3K, at the leading edge (25, 74). Inhibition of PI3K may thus make it more difficult for migrating cells to adapt their polarity in the complex environment of the IZ.

Our results show clearly that PI3K is involved in both CP development and radial migration. Other studies indicate that the defect of CP development that we observed is indeed dependent on Reelin and not secondary to the altered radial migration. First, the p85 α subunit of PI3K binds to phosphorylated Dab1 (10), and Reelin signaling activates PI3K. Fur-

thermore, PI3K inhibition generates a reeler-like phenotype in brain slices, which is clearly different from that generated by mutations that decrease the rate of radial migration without affecting PP splitting and/or inside-out layering, such as in *Lis1*, $\alpha 3$ integrin, or *map2*^{-/-} *map1b*^{-/-} mutant mice (31, 67, 71). Reciprocally, inhibition of Src family kinases (43, 46) or of Akt (this study) perturbs formation of the CP without affecting radial migration directly.

In conclusion, our results show that Reelin activates several partners of the PI3K/Akt signaling pathway, whereas some components of the pathway influence neuronal development independently of Reelin. Different targets downstream from Akt mediate the effects of Reelin on CP development and on dendritic growth, indicating that the various functions of this protein are presumably mediated by a complex network of biochemical signaling mechanisms, most of which remain to be identified.

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