Inhibition of Src Family Kinases and Non-Classical Protein Kinases C Induce a Reeler-Like Malformation of Cortical Plate Development

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During development, most cortical neurons migrate to the cortical plate (CP) radially. CP development is abnormal in reeler and other mutant mice with defective Reelin signaling. Reelin is secreted by Cajal-Retzius cells and binds to the very low density lipoprotein receptor and apolipoprotein E receptor type 2 receptors on the surface of CP cells, inducing tyrosine phosphorylation of the intracellular Dab1 adapter. As with Reelin receptors, the identification of Reelin signaling partners is hampered by genetic redundancy. Using a new in vitro embryonic slice culture system, we demonstrate that chemical inhibitors of Src family kinases and Abl, but not inhibitors of Abl alone, generate a reeler-like malformation and that inhibitors of protein kinases C induce a malformation of cortical development that is also reminiscent of reeler. Our observations demonstrate a key role for these enzymes in radial migration to the cortical plate, possibly via interference with Reelin signaling.

Key words: cortex; development; slice; Reelin; tyrosine kinase; protein kinase C

Introduction

The cerebral cortex develops according to a finely tuned sequence that includes the proliferation of neuronal precursors in ventricular zones (VZs), followed by migration of postmitotic neurons to form architectonic patterns (Nadarajah and Parnavelas, 2002). The earliest cortical neurons, generated at embryonic day (E) 10.5–11.5 in mice, migrate over short distances and form a loose horizontal network called preplate. Cells that are born later assume a different, radial orientation and settle in a densely packed, laminar structure called the cortical plate (CP). The condensation of the CP splits the preplate into two cell contingents that settle in the external marginal zone (MZ) and in the subplate (SP), a cell-poor zone between the CP and the intermediate zone of migration. Most neurons migrate toward the CP radially from telencephalic VZs and are guided by cytoplasmic processes of radial neuroepithelial–glial cells (Rakic, 1972; Rakic and Caviness, 1995; Malatesta et al., 2000; Rakic, 2000, 2002; Noctor et al., 2001). Others originate in ganglionic eminences and reach the CP by tangential migration (Parnavelas et al., 2002). In the CP, progressively younger neurons migrate radially through previously deposited layers and pile up at superficial levels, resulting in the so-called “inside to outside” gradient of CP maturation (Lambert de Rouvroit and Goffinet, 1998). Among the regulators of cortical development identified in human and rodents (Sun et al., 2002), the Reelin pathway plays a key role. Reelin-deficient (reeler) mice are characterized by three anomalies of early cortical development: (1) the MZ is diminutive, and the CP is loose and populated with obliquely oriented neurons; (2) the preplate is not split but instead all of its cells are displaced at subpial levels; and (3) the gradient of cortical maturation is grossly inverted, directed from outside to inside. Reelin is an extracellular glycoprotein secreted in the MZ by Cajal-Retzius cells (D’Arcangelo et al., 1997). It binds to the very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor type 2 (ApoER2), two members of the lipoprotein receptor family expressed on the surface of CP cells (Hiesberger et al., 1999; Trommsdorff et al., 1999), and this results in tyrosine phosphorylation of the intracellular adapter Dab1 (Howell et al., 1999, 2000; Rice and Curran, 1999), and this results in tyrosine phosphorylation of the intracellular adapter Dab1 (Howell et al., 1999, 2000; Rice and Curran, 1999, 2001; Herrick and Cooper, 2002). Defective Reelin signaling results in Dab1 hypophosphorylation coupled with a drastic up-regulation of protein levels (Hiesberger et al., 1999; Howell et al., 1999). The VLDLR and ApoER2 proteins are essentially redundant, and their role as Reelin receptors was identified only after production of double mutant mice (Trommsdorff et al., 1999). Genetic redundancy presumably hampers the identification of other signaling molecules implicated in radial migration and Reelin signaling, such as the kinase(s) responsible for Dab1 phosphorylation, although Fyn and Src (Arnaud et al., 2003; Bock and Herz, 2003) and the phosphatidyl inositide 3 kinase (PI3K) Akt/PKB pathway (Beffert et al., 2002; Bock et al., 2003) may be involved.

Contrary to tangential migration (Anderson et al., 1997; Par-
navales et al., 2000), few in vitro systems have been adapted to the analysis of radial migration (Hemmendinger and Caviness, 1988; Gotz and Bolz, 1992; Anton et al., 1996). A cortical imprint method (Anton et al., 1996) allows estimation of some parameters but does not preserve the structure of the tissue, and slice culture systems described to date are limited by a progressive disorganization of the tissue with blurring of architectonic boundaries. Here we describe an in vitro model of early CP development that allowed us to assess the role of different signaling pathways in radial migration and to identify new putative partners of the Reelin signaling cascade.

Materials and Methods
Animal procedures were performed according to guidelines for the proper use of laboratory animals and were ratified by the competent institutional Animal Ethics Committees. Normal mice of the BALB/c strain and homozygous reeler mice (Orleans allele) of mixed, predominantly BALB/c background were kept in standard conditions. Pregnancies were dated by detecting vaginal plugs, and the day of the plug was noted E0.5. Pregnant mice were killed by cervical dislocation, and the day of the plug was noted E0.5. Pregnant mice were killed by cervical dislocation, and embryos were transferred into cold HBSS under sterile conditions.

For S-phase labeling, bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) dissolved in 0.9% NaCl was injected intraperitoneally (20 μg/g) into the pregnant mouse at defined time points before it was killed. Labeling of neurons at the time of culture was performed by injecting the mouse 1 hr before the beginning of the experiment or by administering a BrdU pulse of 30 min in the culture medium (20 μg/ml). In some experiments, double labeling was performed by injection of BrdU followed by injection of tritiated thymidine (THY) (Amersham Biosciences, Arlington Heights, IL; TRK758, 5 μCi/ml) or administration of a 30 min tritiated thymidine pulse in vitro (1 μCi/ml). For vibratome sectioning, the whole fetal brain at E12.5, E13.5, or E14.5 was embedded in 4% low melting agarose (Promega, Madison, WI) prepared in DMEM-Hank’s F12 medium with glutamine, glucose, and HEPES (BioWhittaker, Walkersville, MD) and glued on a vibratome support using cyanoacrylate. Sections (300 μm thick) were cut in the coronal plane. Care was taken to avoid damage to the pial surface, because this resulted in overmigration of cells in the meninges during culture. In all experiments, a slice was processed for histology immediately after sectioning to verify the developmental status before culture. For culture, sections were laid on collagen-coated polytetrafluoroethylene membranes (Transwell-COL; Costar, catalog #3494) in 12-well plates, and medium was added to a level covering the section but not more. The culture medium was DMEM-F12 supplemented with B27 (1:50), G5 (1:100), penicillin, and streptomycin [all from BioWhittaker or Invitrogen (Gaithersburg, MD)], but without serum additive. We found it important to culture slices under increased oxygen concentrations (Edgar and Price, 2001; Miyata et al., 2002) and used water-saturated 95% O2-5% CO2, with a modular incubator chamber (Billups-Rothenberg, Del Mar, CA). Under those conditions, an organized CP consistently appeared after 1 d in vitro (DIV) and continued to develop after 2 DIV. When the culture was extended to 3 DIV or more, the thickness of the CP did not increase further. Therefore, we concluded that formation of the CP in vitro was best observed after culturing E13.5 or E14.5 slices for 2 DIV. After culture, slices were fixed in Bouin’s fluid for 2 hr before embedding in paraplast and sectioning at 8 μm thickness. We found it essential to section each slice serially, because CP development proceeded best in the center (100–200 μm), whereas the external parts (~100 μm) almost never contained a dense organized CP, presumably because the tri-dimensional structure of the tissue, particularly the radial glial scaffold, was disturbed in the periphery of the slices during preparation. Sections were stained with hematoxylin eosin to examine their general histology and with anti-BrdU antibodies (Becton Dickinson, Mountain View, CA) to visualize incorporation of BrdU into DNA. Tritiated thymidine was revealed by autoradiography using the Hyperco LM1 emulsion (Amersham Biosciences). Immunostaining for reelin was performed using antibody G10, and radial glia were revealed with the Rat401 anti-nestin antibody (Calbiochem, La Jolla, CA). Antibodies against myristoylated, alanin-rich C-kinase substrate (MARCKS) and phosphoMARCKS were from Calbiochem, and anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY).

Results
CP development in vitro recapitulates development in vivo
Vibratome slices (300 μm) were prepared at E12.5 or E13.5, when the CP is not yet developed (E12.5) or incipient (E13.5), and cultured as described in Materials and Methods. After 2 DIV, a dense CP populated with radial neurons appeared in the external field of the telencephalon, bracketed between an external MZ and an inner subplate layer. The intermediate zone of migration and ventricular zone of neuronal proliferation were clearly visible (Fig. 1). The external MZ contained sparse, large horizontal neurons that resembled Cajal-Reetzius cells (Frotscher, 1998) and were Reelin positive (Fig. 1, inset). The relationship between the time of neuron generation and CP development was studied by labeling neuronal cohorts with BrdU or THY, or both, as shown in Figure 2. Injection of pregnant mice with BrdU on the day before they were killed resulted in labeling of a large proportion of CP neurons (Fig. 2A, B). In contrast, when slices were submitted to a short THY pulse after sectioning, only a few THY-positive neurons were found in the CP after 2 DIV (Fig. 2C), and this number did not increase when the culture was extended further. When BrdU was added to the culture on the second DIV, no labeled neurons were detected in the CP (Fig. 2D, E), indicating that most cells that settle in the CP complete their last S-phase in vivo, before slice preparation. To assess whether the CP develops in vitro after an inside to outside sequence, double-labeling experiments were performed as follows. Pregnant mice were injected with BrdU on E12.5 and again with THY on the next morning. Slices were prepared 5 hr after THY administration. After 2 DIV, adjacent sections were stained for histological analysis, revealed with anti-BrdU antibodies, and processed for THY autoradiography (Fig. 3A–C). To estimate the distribution of both cell cohorts, the telencephalic wall was divided into radially superposed compartments (excluding meninges and submeningeal space) as illustrated in Figure 4A, and THY- and BrdU-positive cells were counted in each segment. The results (Fig. 4B) show that BrdU-positive cells were distributed in all layers of the cortical ribbon but were more concentrated in the inner than the outer tier. Conversely, THY-positive neurons were present at every level of the CP but heavily labeled cells were preferentially located in its superficial aspect. Thus a significant proportion of late-migrating, younger neurons were able to pass through the layer formed by the previous cell cohort, showing that CP maturation proceeded from inside to outside. Another feature of normal CP development is the splitting of the preplate by the CP. To
its neurons settle the normal CP, the preplate is not split by the developing CP but assume an oblique orientation and are less densely packed than in Dab1 adapter) generate a unique malformation: CP neurons as-

tered (Caviness, 1976, 1982; Lambert de Rouvroit and Goffinet, 1998). In double-labeling experiments, THY-

morphological features identical to those of the reeler CP expected above, showed that BrdU-positive neurons were dispersed throughout the whole cortical field, whereas THY-labeled cells were located in the deep part of the cortex, in a continuum with migrating cells in the intermediate zone (Fig. 3E,F, 4B). This showed that the reeler CP developed from outside to inside in vitro, as it does in vivo. The morphology of radial glial guides is abnormal in reeler mice (Derer, 1979; Hunter-Schaedle, 1997; Forster et al., 2002). To assess whether this was also seen in reeler slices after culture, sections were stained for the intermediate filament protein nestin, a radial fiber marker. As shown when comparing normal and reeler slices (Fig. 6A,B), reeler radial glial cells were normal in the VZ and intermediate zone but became quite distorted and formed enlarged and branched fibers in the external tiers of the field, when they crossed the cortical plate and superplate. Altogether, these results indicated that the main fea-
tures of the reeler phenotype were produced in vitro.

In vitro development of the reeler CP

As mentioned in the Introduction, among the mutations that perturb cortical development, those that affect the Reelin signaling pathway (Reelin, its receptors VLDLR and ApoER2, and the Dab1 adapter) generate a unique malformation: CP neurons assume an oblique orientation and are less densely packed than in the normal CP, the preplate is not split by the developing CP but its neurons settle “en bloc” in the external field where they form a superplate, and the gradient of cortical maturation is grossly inverted (Caviness, 1976, 1982; Lambert de Rouvroit and Goffinet, 1998). To confirm that the formation of the CP observed in vitro mirrors the situation in vivo, experiments were performed using slices from homozygous Reelin-deficient (reeler) embryos. When E13.5 reeler slices were cultured for 2 DIV, a CP developed with morphological features identical to those of the reeler CP in vivo (Fig. 3D). CP neurons differentiated and migrated normally, but their general orientation was oblique rather than radial. The MZ was poorly defined, some cell poor plexiform zones developed within the CP, and the inner border of the CP, the frontier between cortex and subcortex where the subplate normally appears, was barely identified (Caviness, 1982). Double-labeling experiments of reeler slices with BrdU and THY, performed as de-
ters that affect the Reelin signal-

Inhibition of Src family kinases

Src kinases form a family of at least eight members that are implicated in many cell processes, including cell migration (Blume-Jensen and Hunter, 2001). Like Reelin deficiency, inactivation of Fyn or Src results in upregulation of Dab1 protein levels, suggesting that these kinases play a role in Reelin signaling (Arnaud et al., 2003; Bock and Herz, 2003). To study that question further, we tested whether inhibition of Src family kinases with the well validated inhibitor PP2 (Hanke et al., 1996) (Calbiochem) would perturb neuronal migration and architectonic development in embryonic slices. Experiments performed at the concentrations of 1, 10, and 50 μM PP2 and the inactive control compound PP3 (Calbio-

chem) showed clear effects of PP2 on CP development at 10 μM, a concentration known to inhibit completely Dab1 phosphoryla-
tion in neuronal culture (Arnaud et al., 2003; Bock and Herz, 2003) (our unpublished observations). As shown in Figure 3G, in the presence of PP2 but not the inactive PP3 (data not shown), the CP was loosely organized, with oblique neurons and inter-
pose fiber layers, a phenotype almost identical to that observed in reeler embryos (Caviness, 1976, 1982; Lambert de Rouvroit and Goffinet, 1998). In double-labeling experiments, THY-

labeled neurons migrated in the intermediate zone as in normal animals, but they did not cross early-born, BrdU-labeled cortical cells and instead settled at progressively deeper levels, indicating outside to inside CP maturation (Figs. 3H,I, 4). Furthermore, preplate splitting, studied as described above, failed to occur (Fig. 5C).

The nestin-positive radial glial fibers were normal in the VZ and intermediate zone but became somewhat wavy, enlarged, and distorted in the cortical plate and MZ (Fig. 6C), in a pattern quite evocative of that seen in reeler mice and in slices from reeler embryonic brains.

The activity of PP2 was demonstrated by inhibition of the Reelin-induced tyrosine phosphorylation of Dab1 (Howell et al., 1999) in slices (Fig. 7A). Interestingly, incubation with PP2 re-
resulted in a drastic upregulation of Dab1 protein concentration (Fig. 7A), a biochemical hallmark of defective Reelin signaling (Hiesberger et al., 1999; Arnaud et al., 2003; Bock and Herz, 2003). Thus incubation in the presence of PP2 induced a reeler-like malformation. In addition to Src kinases, PP2 inhibits Abl; however, STI571 (Gleevec), which inhibits Abl but not Src family members, did not have the effects of PP2 (data not shown).

Role of protein kinases C
Protein kinases C (PKC) are central signaling proteins implicated in a multitude of cellular processes (Newton, 2001). They form a family of at least 10–12 isozymes grouped into three classes, namely conventional PKC (α, γ, and alternatively spliced βI and βII), novel PKC (δ, ε, η, ι, θ), and atypical PKC (ξ, η, ζ). In addition, PKC μ and ρ are considered to form a fourth class or a distinct family named protein kinase D (Newton, 2001). Their prominent role in intracellular signaling prompted us to investigate whether they might be involved in radial migration, possibly by modulating the reelin signal.

Embryonic brain slices were cultured in the presence of the following PKC inhibitors (all from Calbiochem): bisindolylmaleimide 1 (BIM1), Ro318220, and Gö6976. At concentrations known to inhibit many PKC isoforms in cells, BIM1 (5 μM), Ro318220 (1 μM), and Gö6976 (0.3 μM) all blocked phosphorylation of MARCKS, a major PKC substrate, demonstrating that they were active in our culture conditions (Fig. 7B). BIM1 and Ro318220 induced a malformation of the developing CP in slices that was very similar to that obtained with PP2. As shown in Figure 3J, the CP became disorderly, with blunted borders and poorly defined MZs and SPs. Double-labeling experiments with BrdU followed by THY revealed that BrdU-positive cells settled at all levels of the CP, whereas THY-positive elements were deposited at its inner border, in continuity with migrating cells in the intermediate zone, indicating that CP maturation proceeded from outside to inside (Figs. 3K,L, 4). BIM1 also inhibited preplate splitting (Fig. 5D). Nestin-positive radial glial fibers were normal in the VZ and intermediate zone but abnormally branched and thickened in the cortical plate and MZ (Fig. 6D), as in reeler or PP2-treated slices. Thus, like PP2, the large-spectrum PKC inhibitors BIM1 and Ro318220 induced a cortical malformation with many morphological features of the reeler anomaly. Unlike PP2, however, incubation of slices with BIM1 did not induce an upregulation of Dab1 protein levels and did not influence Dab1 phosphorylation (Fig. 7B). Furthermore, analysis of the radial distribution of THY- and BrdU-labeled cells (Figs. 3, 4) showed that more cells were dispersed below the cortical plate than in reeler or PP2-treated slices, indicating that the migration defect induced by PKC inhibitors might be more severe. To define further the PKC family implicated, the compound Gö6976, a canonical inhibitor of classical PKCs but not of the other two families (Martiny-Baron et al., 1993), was tested at various concentrations covering the range of active concentrations in cell cultures. No malformation of CP development was produced, suggesting that the effects observed with BIM1 and Ro318220 are caused by inhibition of the novel or atypical families of calcium-independent PKCs. As an additional argument, BIM1 was active in our assay at 5 μM, a concentration that blocks all PKC forms, but not at 1 μM, a concentration that blocks only classical and novel PKCs (Uberall et al., 1997; Mao et al., 2000). This indicated that the effects observed may be caused principally by atypical PKCs. Because several PKC inhibitors are also active...
against glycogen synthase 3β (GSK3β) (Davies et al., 2000), the GSK3β inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD) (Martinez et al., 2002) was tested; at concentrations of 5 and 20 μM, TDZD did not influence CP development (data not shown).

Discussion

Radial migration and CP development in vitro

Few in vitro systems have been adapted to the analysis of radial migration (Hemmendinger and Caviness, 1988; Gotz and Bolz, 1992; Anton et al., 1996). The main interest of the present culture system is that slices are prepared at the preplate stage, before any condensation of the CP, so that the development of the CP in vitro proceeds ab initio and is not simply the extension in vitro of a process initiated in vivo. The hallmarks of early normal cortical plate development, namely preplate splitting, the differentiation of the marginal zone and of Cajal-Retzius cells, and the condensation of the CP with radially organized neurons that settle in the CP from inside to outside, all occur reproducibly in culture after 2 d. As an additional validation, we showed that early cortical development in slices prepared from reeler embryos mimics development in reeler mice in vivo. CP neurons assume an oblique orientation and are packed less densely than in the normal CP, the preplate is not split by the developing CP but its neurons settle en bloc in the external field where they form a superplate, and the gradient of cortical maturation is grossly inverted (Caviness, 1976, 1982; Lambert de Rouvroit and Goffinet, 1998). Furthermore, the anomalies of radial glial fibers in reeler slices are reminiscent of those described in vivo (Derer, 1979; Hunter-Schaedle, 1997; Forster et al., 2002).

All technical features of slice preparation and culture mentioned in Materials and Methods are useful, but the most
significant is the incubation in an oxygen-enriched atmosphere (Miyata et al., 2002), which is found to be critical for cell survival in the depth of the slices. This is particularly important because the CP develops poorly in the superficial 50–100 μm of the slices, whereas a normal CP appears consistently in the ~200 μm central zone. We tentatively attribute this phenomenon to distortions of the architecture of the tissue, especially of the radial glial scaffold, during slice preparation. Although this system allows a reproducible development of the CP within 2 DIV, a limitation remains that very few neurons born in vitro migrate to the CP, which is thus populated by neurons that have already completed their last S-phase in vivo, before slice preparation. The reasons for this are unknown and probably many, because some unidentified factor or factors are probably lacking in the culture environment.

Role of Src family kinases and non-classical PKCs

PP2, an inhibitor of the Src family kinases, produced an anomaly morphologically indistinguishable from reeler in terms of absence of preplate splitting, formation of a loose CP with an intermediate plexiform layer, outside to inside cortical maturation, and distortion of radial glial fibers. The similarity with reeler is further that a double mutation generates the overt neuronal migration phenotype produced in vitro, suggesting genetic redundancy. Our system provides a relatively simple way to study some molecular mechanisms of cortical development and to screen for potential deleterious effects of pharmaceuticals or xenobiotics. It suggests further that a “chemical genomics” (Peterson and Mitchison, 2002) approach of cortical development could be considered.

References
