Development/Plasticity/Repair

Absence of Fyn and Src Causes a Reeler-Like Phenotype

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Nonreceptor protein tyrosine kinases of the Src family regulate the survival, proliferation, differentiation, and motility of many cell types, but their roles in brain development are unclear. Biochemical and *in vitro* experiments implicate Src and Fyn in the Reelin-dependent tyrosine phosphorylation of Dab1, which controls the positioning of radially migrating neurons in many brain regions. However, genetic evidence that either Src or Fyn mediates Reelin-dependent migrations *in vivo* has been lacking. Here, we report that, although Src is dispensable and although the absence of Fyn causes an intermediate phenotype, the combined absence of Src and Fyn almost abolishes tyrosine phosphorylation of Dab1 and causes defects in the fetal cortex and cerebellum very similar to those of *dab1* mutants of the same age. Neurogenesis is not detectably affected, but the layering of neurons in the cortex is inverted, and the formation of the Purkinje plate is impaired. This implies that Src and Fyn are needed for Reelin-dependent events during brain development.

Key words: **cerebellum; cortex; reelin; Fyn; Dab1; Src; tyrosine phosphorylation**

Introduction

The Src family of nonreceptor protein tyrosine kinases has been highly conserved over metazoan evolution and plays key roles in relaying signals that regulate cell proliferation, differentiation, and motility (Brown and Cooper, 1996). At least three Src-family kinases (SFKs)—Src, Fyn, and Yes— have been detected in the developing mammalian brain (Cotton and Brugge, 1983; Martinez et al., 1987; Sudol et al., 1988; Cooke and Perlmutter, 1989; Zhao et al., 1991; Umemori et al., 1992). Targeted knock-outs of *src* and *yes* in the mouse do not lead to obvious defects in brain development (Soriano et al., 1991; Stein et al., 1994; Lowell and Soriano, 1996), although *src* is required for cultured cerebellar neurons to extend neurites on certain surfaces (Ignelzi et al., 1994). In contrast, *fyn* gene disruption causes obvious phenotypes in the developing and adult brain. There are increased numbers of granule cells in the dentate gyrus and pyramidal neurons in the CA3 region of the temporal hippocampus (Grant et al., 1992), the dendrites of pyramidal neurons of layer V of the medial cortex are misoriented (Sasaki et al., 2002), and late-generated cortical neurons are found in deeper layers than normal (Yuasa et al., 2004). These defects likely reflect a requirement for Fyn in neurons or their progenitors during development. In addition, Fyn is important for other aspects of brain function. Cultured neurons require *fyn* for neurite extension on specific extracellular ligands (Beggs et al., 1994), whereas oligodendrocytes require Fyn for myelination (Umemori et al., 1994; Sperber et al., 2001). In mature neurons, Fyn is involved in the regulation of NMDA and AMPA receptors (Grant, 1996; Kojima et al., 1997; Miyakawa et al., 1997; Narisawa-Saito et al., 1999), and *Fyn* mutant mice exhibit defects in learning and memory (Grant et al., 1992; Kojima et al., 1997).

Src-family kinases share a common mechanism of activation and can phosphorylate many of the same substrate proteins in cells, thus there is the potential for redundancy. Indeed, although individual knock-outs of *src*, *fyn*, or *yes* do not impact the survival of embryos or pups, 85–90% of *src fyn* double mutants die perinatally, and all *src fyn yes* triple mutants die early in gestation, suggesting that these Src-family kinases can partially compensate for each other *in vivo* (Stein et al., 1994; Klinghoffer et al., 1999). Compensation is also suggested by the observation that Src and Fyn kinase activities are slightly increased in *fyn* and *src* mutant neonatal brains, respectively (Grant et al., 1995). Specific developmental phenotypes caused by combined mutations of *fyn* and *src* or *yes* in the nervous system have not been described previously.

The mammalian neocortex has characteristic laminations, containing different neuron types arranged in stereotypical patterns. Layer formation requires proper migrations of projection neurons from their origins in the neuroepithelium that lines the ventricles. Layering is disrupted by mutations that either affect the migratory ability of the neurons or their ability to sense the position at which they should cease migration (Walsh and Goffinet, 2000; Rice and Curran, 2001; Gupta et al., 2002). In particular, mutations in Reelin pathway genes cause distinctive abnormalities in many laminated brain regions, known as the Reeler phenotype. Reelin is a secreted protein, made by Cajal-Retzius neurons of the marginal zones of the neocortex and hippocampus and in the nuclear transitory pathway and external granule layer of the cerebellum (D'Arcangelo et al., 1995; Rice et al., 1998). In the cortex, *reln* mutations prevent splitting of the preplate and cause inversion of the cortical plate. In addition, the radial glia, which serve both as neuronal precursors and as guides for migration, show abnormal end feet and branching, and the

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projection neurons in the cortical plate are misoriented. In the cerebellum, *reln* mutations prevent proper migrations of the Purkinje cells, and an inner granule cell layer fails to form.

The combined mutation of *vldlr* and *apoER2*, or the single mutation of *dab1*, also causes a Reeler phenotype. The gene products Vldlr, ApoER2, and Dab1 are expressed by radial glia and migrating cortical plate cells of the neocortex and hippocampus and by Purkinje cells of the cerebellum. Vldr and ApoER2 are receptors that bind to Reelin through their extracellular domains and to Dab1 through their cytoplasmic domains (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999b). Dab1 is an intracellular protein that becomes tyrosine phosphorylated, and then degraded, in response to Reelin (Rice et al., 1998; Hiesberger et al., 1999; Howell et al., 1999a; Arnaud et al., 2003b; Bock et al., 2004). An allele of Dab1 that lacks the phosphorylated tyrosine residues behaves as a null allele (Howell et al., 2000).

Despite the importance of tyrosine phosphorylation in Reelin signaling, there have been no reports of a Reeler phenotype resulting from mutations in tyrosine kinase genes. However, biochemical and genetic evidence implicates Src-family kinases in Reelin-induced Dab1 phosphorylation. Inhibitors of Src-family kinases reduce Dab1 tyrosine phosphorylation *in vitro* (Arnaud et al., 2003a; Bock and Herz, 2003) and inhibit preplate splitting and formation of a normal cortical plate in cortical slice cultures, although layer inversion was not documented (Jossin et al., 2003). In addition, *fyn* mutant neurons show reduced Dab1 tyrosine phosphorylation and degradation in response to Reelin (Arnaud et al., 2003a; Bock and Herz, 2003). Additional reductions in Dab1 tyrosine phosphorylation and degradation occur if *src* gene dosage is reduced in *fyn* homozygotes, suggesting partial redundancy (Arnaud et al., 2003a).

Here, we provide genetic evidence suggesting that *fyn* and *src* are partly redundant members of the Reelin pathway. Although postnatal *src fyn* double homozygous mutant mice cannot be analyzed, because of perinatal lethality (Stein et al., 1994), the development of the *src fyn* double-mutant fetal brain strongly resembles that of *dab1* mutants. Combined with previous results showing that Src-family kinases are required for biochemical events in Reelin signaling, the new data provide genetic evidence implicating Fyn and Src in the Reelin signaling pathway.

Materials and Methods

Animals and tissue preparation. Animals were derived from an *src fyn yes* breeding colony in the mixed C57BL/6 × 129Sv strain background and genotyped as described previously (Arnaud et al., 2003a). Embryos were removed from timed pregnant dams and cerebral hemispheres were dissected for protein assays and neuron cultures. For protein analysis, embryonic day 16.5 (E16.5) embryo cerebra were frozen on dry ice and stored at −80°C until use. Neuron cultures were prepared essentially as described previously (Herrick and Cooper, 2002; Arnaud et al., 2003a). For histology, embryo heads were fixed in 4% paraformaldehyde, the brains were dissected out and soaked overnight in 4, 10, 20, and 30% sucrose, and equilibrated and embedded in OCT on dry ice. Sections (8–12 μm thick) were stored frozen until use.

Protein analysis. Brain samples and neuron cultures were lysed in neuron radioimmunoprecipitation assay buffer (Arnaud et al., 2003a) for Western blotting or in NP-40 lysis buffer (1% NP-40, 100 mM NaCl, 50 mM NaF, 25 mM HEPES, pH 7.4, 10% glycerol, 2 mM EDTA, and protease and phosphatase inhibitors) for immunoprecipitation. Protein concentrations were normalized using Bradford protein assay (Bio-Rad, Hercules, CA). Dab1 was immunoprecipitated using rabbit anti-Dab1 (B3; a kind gift from Brian Howell, National Institute of Neurological Disorders and Stroke, Bethesda, MD), focal adhesion kinase (FAK) with affinity-purified rabbit antibody (catalog #sc-558; Santa Cruz Biotechnology, Santa Cruz, CA), and p190 Rho GTPase-activating protein (GAP) with mouse clone D2D6 (Upstate Biotechnology, Lake Placid, NY). Mixed protein A and protein G agarose (Santa Cruz Biotechnology) was used for precipitation. Protein samples were analyzed on 8% polyacrylamide gels, as described previously (Arnaud et al., 2003a). Blots were probed with mouse anti-phosphotyrosine 4G10 (Upstate Biotechnology), rabbit anti-Src-family kinases (recognizing Src, Fyn, and Yes; SRC-2; Santa Cruz Biotechnology), mouse anti-βIII tubulin (TuJ1; Covance, Princeton, NJ), rabbit anti-FAK pTyr397 (catalog #44-624; Biosource International, Camarillo, CA), or the same antibodies used for immunoprecipitation. Dab1 tyrosine phosphorylation stoichiometry was determined using ImageJ to quantify Western blot signals from blots that probed first for phosphotyrosine and then stripped and reprobed for Dab1 protein. Phosphorylation level was divided by Dab1 protein level to calculate phosphorylation stoichiometry, relative to wild type. Means, SEs, and numbers of determinations are shown.

Immunohistochemistry. Slides were rehydrated with PBS for 5 min and blocked with 5% goat serum in PBS and 0.1% Tween 20 at room temperature for 1 h. A 20 min boil with 0.01 M sodium citrate, pH 6.0, was used for antigen retrieval. Slides were incubated with primary antibodies overnight at 4°C, washed three times with PBS, and then incubated with secondary antibodies for 2 h at room temperature, washed, incubated with 4′,6′-diamidino-2-phenylindole dihydrochloride (2.5 μg/ml; Sigma, St. Louis, MO) in PBS for 5–10 min, washed, and mounted with ProLong Gold (Invitrogen, Carlsbad, CA).

The following primary antibodies were used in 5% goat serum in PBS plus 0.1% Tween 20: mouse anti-chondroitin sulfate proteoglycan (CSPG; diluted 1:100; Sigma), rabbit anti-calretinin (1:1000; Chemicon, Temecula, CA), rabbit anti-calbindin (1:400; Chemicon), guinea pig anti-Brn1 (1:500; kind gift from Robert J. McEvilly, University of California, San Diego, CA), rabbit anti-Tbr1 (1:1000; kind gift from Robert Hevner, University of Washington, Seattle, WA), rabbit anti-Cux1 (1:400; kind gift from Chris Walsh, Harvard Medical School, Boston, MA), mouse anti-reelin (1:400; kind gift from André M. Goffinet, University of Louvain Medical School, Brussels, Belgium), and rabbit anti-Dab1 B3 (1:400).

The following secondary antibodies were used at 1:400 in 20% goat serum in PBS plus 0.1% Tween 20: Alexa Fluor 488 anti-mouse (Invitrogen), Alexa Fluor 568 anti-rabbit (Invitrogen), and FITC anti-guinea pig (Jackson ImmunoResearch, West Grove, PA). Images were collected by epifluorescence with MetaMorph software (Universal Imaging, Downingtown, PA) and levels adjusted in Photoshop (Adobe Systems, San Jose, CA). The distribution of neurons was quantified by dividing the thickness of the cortex, excluding the ventricular zone, into nine bins of equal thickness, and counting the labeled cells in each bin. For each genotype, the mean and SE of counts from replicate sections from two embryos from different litters were calculated, except for Cux1, in which data are based on sections from a single embryo.

Results

Fyn- and Src-dependent protein phosphorylation in the developing cortex

To investigate the roles of Src and Fyn in brain development, we generated a breeding colony of *src*+/−*fyn*−/− mice and set up timed matings. Because most, but not all, *src*−/−*fyn*−/− pups die perinatally (Stein et al., 1994), we were concerned that surviving pups might represent a subpopulation with less severe phenotype. Therefore, we recovered embryos at E16.5 or E18.5 for analysis.

We first studied the levels of Dab1 protein and tyrosine phosphorylation in embryonic cortex and in cultured cortical neurons (Fig. 1). Protein extracts were prepared from cortices dissected from littermate E16.5 embryos from a *src*+/−*fyn*−/− intercross and from two wild-type E16.5 embryos from a different litter, and were analyzed by Western blotting (Fig. 1*a*). Tyrosine phosphorylation of some proteins (Fig. 1*a*, asterisks) was reduced by homozygous mutation of *fyn* (lane 2) relative to controls (lanes 1 and 7). Phosphorylation was further reduced by heterozygous

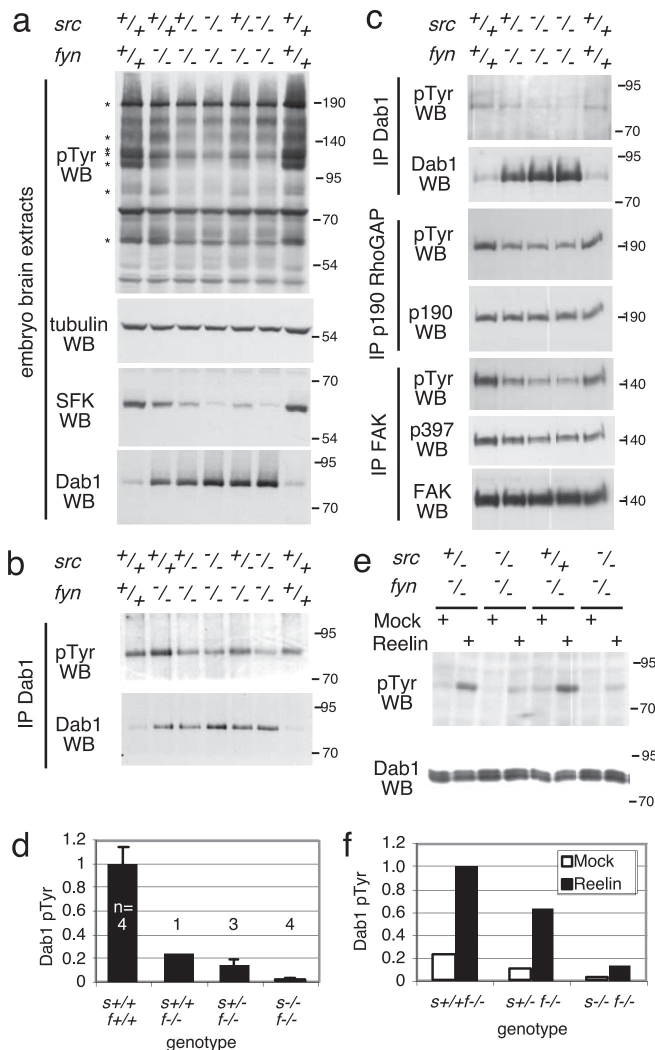

Figure 1. Expression levels and tyrosine phosphorylation of Dab1 in mutant embryonic brain and neuron cultures. ***a***, ***b***, Protein extracts were prepared from E16.5 embryo brains. *fyn*−/− littermate embryos with various *src* genotypes were obtained from a *src*+/−*fyn*−/− intercross. Wild-type embryo brains were from another litter. ***a***, Protein samples were analyzed directly by immunoblotting for phosphotyrosine, neuron-specific βIII tubulin, Src-family kinases, and Dab1. The asterisks indicates proteins whose phosphorylation was reduced by *src fyn* mutation. Note the reduction in Src-family kinase expression and the increase in Dab1 protein levels as *src* and *fyn* gene dosages were reduced. ***b***, Dab1 was immunoprecipitated from the protein extracts in ***a*** and immunoblotted for phosphotyrosine and Dab1. Relative phosphorylation levels were calculated after densitometry. ***c***, Protein extracts from independent E16.5 embryo brains were analyzed by immunoprecipitation of Dab1, p190 Rho GAP, and FAK and immunoblotted with the indicated antibodies. Mutation of *src* and *fyn* caused decreases in tyrosine phosphorylation of Dab1, p190, and FAK, a smaller decrease in FAK autophosphorylation (pTyr397), and increases in Dab1 protein level, with no effect on protein levels of p190 or FAK. ***d***, Number of experiments, mean, and SE of the stoichiometry of Dab1 tyrosine phosphorylation from different experiments. ***e***, Neurons from littermate *fyn*−/− embryos of various *src* genotypes were cultured for 5 d and stimulated for 15 min with Reelin-containing or mock supernatants. Samples were analyzed by immunoblotting with antibodies to phosphotyrosine and Dab1. ***f***, Stoichiometry of tyrosine phosphorylation of Dab1 after *in vitro* stimulation with Reelin. Both basal and Reelin-stimulated tyrosine phosphorylation of Dab1 were greatly decreased in *src*−/−*fyn*−/− (*s*−/−*f*−/−) neurons. WB, Western blot; IP, immunoprecipitation.

(lanes 3 and 5) or homozygous (lanes 4 and 6) mutation of *src* in the *fyn*−/− background. Probing for neuron-specific βIII-tubulin (TuJ1) showed equal loading and probing with an antibody that recognizes many SFKs showed the expected reductions when *fyn* and *src* were mutated. As expected, if Dab1 degradation depends on tyrosine phosphorylation, protein levels of Dab1 were strongly increased by mutation of *fyn* and were further increased by additional mutation of *src*. Dab1 tyrosine phosphorylation was then assessed by immunoprecipitating Dab1 and blotting for phosphotyrosine (Fig. 1*b*,*c*). Although homozygous mutation of *fyn* reduced Dab1 phosphorylation to ∼20% of control (Arnaud et al., 2003a) (Fig. 1*d*), heterozygous and homozygous mutation of *src* reduced the stoichiometry to ∼14 and 3% of control, respectively (Fig. 1*d*). We also assessed the tyrosine phosphorylation of two other proteins: p190 Rho GAP and FAK (Fig. 1*c*). Tyrosine phosphorylation of p190 Rho GAP was reportedly reduced in *fyn*−/− and reduced further in *src*−/−*fyn*−/− neonatal brains (Brouns et al., 2001). P190 is also a substrate for the unrelated tyrosine kinase Arg (Hernandez et al., 2004). We detected a major reduction in p190 tyrosine phosphorylation attributable to *fyn* mutation and a slight additional reduction attributable to *src* mutation (Fig. 1*c*). FAK activation involves autophosphorylation at Y397, followed by SFK-mediated phosphorylation at other sites (Schlaepfer et al., 1999). Accordingly, we detected stronger decreases in overall FAK tyrosine phosphorylation than in phosphorylation at Y397, when *fyn* alone or *fyn* and *src* were mutated (Fig. 1*c*). These results show that primarily Fyn, and secondarily Src, is required for high-level phosphorylation of p190, FAK, and Dab1 in developing cortex.

To determine the effect of Reelin on Dab1 tyrosine phosphorylation in the absence of Fyn and Src, neurons were prepared from individual embryos derived from *src*+/−*fyn*−/− intercrosses. After genotyping, selected cultures were stimulated with Reelin *in vitro*, and the phosphorylation of Dab1 was measured. As shown in Figure 1*e*, all cultures have high levels of Dab1 protein, as expected when Fyn is absent. Slight additional increases in Dab1 protein level were detected as one or both copies of *src* were deleted. However, there was a striking decrease in both the basal and Reelin-stimulated levels of Dab1 tyrosine phosphorylation in *src*+/−*fyn*−/− neurons (lanes 1–2) relative to *fyn*−/− neurons (lanes 5–6), and an additional decrease in *src*−/−*fyn*−/− neurons (lanes 3–4 and 7–8). *Src*−/−*fyn*−/− neurons had only one-seventh the basal and Reelin-stimulated levels of Dab1 tyrosine phosphorylation as *fyn*−/− neurons. Residual Reelin-induced tyrosine phosphorylation of Dab1 in *src*−/−*fyn*−/− neurons is presumably attributable to other SFKs, such as Yes or Lyn.

Effects of double mutation of *fyn* and *src* on cortical development: expression of Reelin and Dab1

We examined the structure of the developing cortex in embryos derived from *src*+/−*fyn*−/− intercrosses at E16.5 and E18.5. For comparison, we used embryos of the same ages derived from *dab1*+/− intercrosses. To control for regional variation, we compared regions of dorsomedial cortex near the hippocampus.

Fyn mutation, or *src fyn* double mutation, did not significantly affect Reelin expression, which in all cases was restricted to scattered neurons (presumably Cajal-Retzius neurons) in the marginal zone (Fig. 2). However, as expected from Western blots (Fig. 1), Dab1 levels were increased throughout the developing cortical plate, subplate, and ventricular zone in *fyn* and *src fyn* mutants (Fig. 2). The Dab1 antibody was specific, because staining of *dab1* mutant cortex was strongly reduced (Fig. 2).

Nissl staining revealed no significant differences in the brain size, cortex thickness, or total number of nuclei in control, *fyn*−/−, *src*−/−, or *src*−/−*fyn*−/− E18.5 embryos (supplemen-

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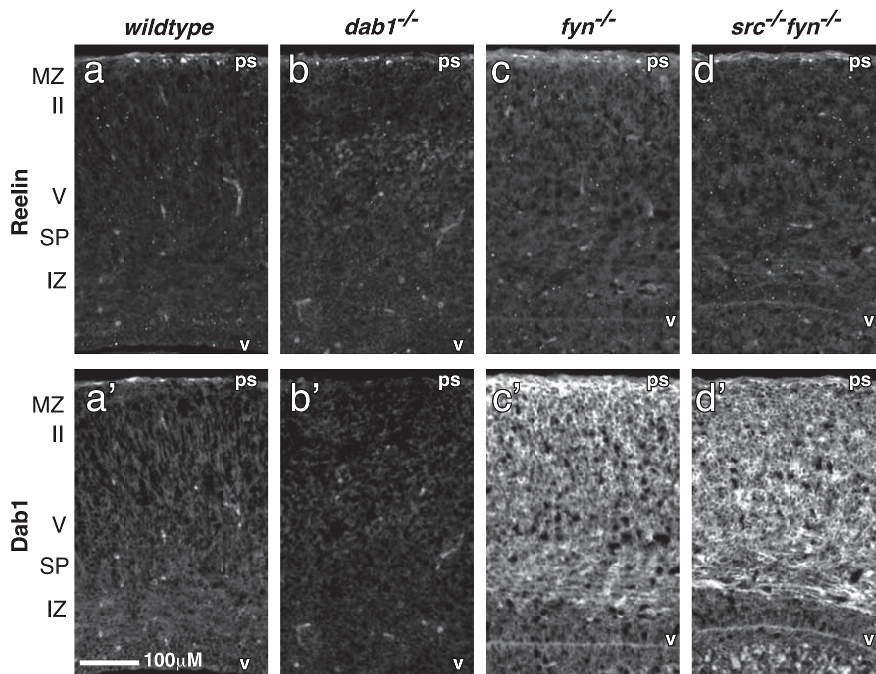

Figure 2. Dab1 staining is increased in the *src*−/−*fyn*−/− neocortex. Coronal sections of E18.5 neocortices were double stained with antibodies against Reelin and Dab1. The separate channels are shown. All images were captured using the same exposure time, and levels were adjusted equally. ***a–d***, Reelin was expressed equally by scattered neurons in the marginal zone across all genotypes. However, Dab1 staining was absent in *dab*−/− (***b'***) and elevated in *fyn*−/− (***c'***) and *src*−/−*fyn*−/− (***d'***) neocortices relative to wild-type (***a'***) neocortex. ps, Pial surface; MZ, marginal zone; II and V, layers II/III and V/VI of the cortical plate, respectively; SP, subplate; IZ, intermediate zone; v, ventricle.

tal Figs. S1, S2, available at www.jneurosci.org as supplemental material). In addition, we used antibodies to phosphohistone H3 to identify dividing cells at E16.5. The number and distribution of dividing cells in *src*−/−*fyn*−/− cortex was similar to that in control (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). This suggests that symmetric and asymmetric divisions in the neuroepithelium do not require Src or Fyn. However, there clearly were differences in neuron positions, because there was a cell-poor marginal zone in control, but not in *dab1*, *fyn*, or *src fyn* mutant embryo cortices, and lamination was not clear in *dab1* and *src fyn* mutants (supplemental Figs. S1, S4, available at www.jneurosci.org as supplemental material).

Preplate splitting in *src fyn* mutant embryos

One of the earliest defects detected in the Reeler neocortex is abnormal splitting of the preplate (Angevine and Sidman, 1961; Sheppard and Pearlman, 1997). The preplate is composed of Cajal-Retzius neurons, which migrate into the marginal zone from either local or distant origins (Hevner et al., 2003; Takiguchi-Hayashi et al., 2004), and subplate neurons, which migrate radially from the neocortical ventricular zone to lie below Cajal-Retzius neurons. Subsequent divisions in the neocortical ventricular zone give rise to cortical plate neuroblasts, which enter the subventricular zone and may go through one or two divisions before migrating outward along or between radial glia guides. Each neuron migrates almost to the pial surface, passing between the subplate neurons and stopping short of the Cajal-Retzius neurons in the marginal zone. The preplate is thus split into subplate and Cajal-Retzius neurons. In the Reeler mouse, subplate neurons remain close to the Cajal-Retzius neurons and are displaced outward above the developing cortical plate to form a "superplate."

We used antibodies to CSPG, which is secreted by subplate neurons (Sheppard et al., 1991; Sheppard and Pearlman, 1997), to visualize the subplate. As shown in Figure 3, the subplate was readily detected in E18.5 control embryos and was absent in *dab1*−/− embryos (Fig. 3*a*,*b*). CSPG staining was more dispersed in *fyn*−/− cortex (Fig. 3*c*) and was weak in *src*−/−*fyn*−/− cortex (Fig. 3*d*). Similar results were obtained at E16.5 (Fig. 3*e*,*f*) and at E18.5 with antibodies to calretinin, which marks cells and axons in the subplate and marginal zone (Fonseca et al., 1995) (Fig. 3*g*,*h*). The defects in preplate splitting likely contribute to the hypercellularity of the marginal zone in mutant embryos (supplemental Figs. S1, S4, available at www.jneurosci.org as supplemental material).

Cortical inversion in *src fyn* mutant embryos

Because each cortical plate neuron stops migrating when it reaches the edge of the marginal zone, the outer, shallower layers of wild-type cortex contain younger neurons than the inner, deeper layers (Rakic, 1972; Caviness and Sidman, 1973; Caviness, 1982). In the Reeler mutant mouse, this inside-out layering order is inverted (Caviness and Sidman, 1973; Caviness, 1982). The later-born neurons seem to have difficulty passing their earlier siblings, which are in abnormally close contact with radial glia fibers (Pinto-Lord et al., 1982). The cortex is also inverted in *dab1* mutants, and *dab1* mutant neurons remain closely associated with radial glia fibers (Gonzalez et al., 1997; Howell et al., 1997; Sheldon et al., 1997; Sanada et al., 2004).

Layering of the cortical plate in *fyn src* mutant cortex was assessed using layer-specific markers at E18.5. Tbr1 is expressed in the preplate and cortical plate, most abundantly in the subplate and layer VI cortical plate neurons (Hevner et al., 2001) (Fig. 4*a*). In *dab1* mutants, as shown before (Herrick and Cooper, 2002), Tbr1+ neurons migrate excessively and are found in the marginal zone and upper cortical plate (Fig. 4*b*). In *src fyn* double-mutant cortex, Tbr1+ neurons were present in superficial layers of the cortex and marginal zone (Fig. 4*d*). This suggests that *src fyn* mutation, like *dab1* mutation, causes early-born cortical plate neurons to migrate excessively into the Reelin-containing marginal zone.

We used Brn1 and Cux1 as markers for late cortical plate neurons. Brn1 is expressed throughout the cortical plate but at highest levels in layers II–IV (McEvilly et al., 2002). Similarly, Cux1 is most strongly expressed by a subset of layer II–IV neurons (Feng and Walsh, 2004; Nieto et al., 2004). It is not clear whether Cux1 and Brn1 are always coexpressed. By E18.5 in wild-type cortex, most Brn1+ and Cux1+ neurons have settled in the upper cortical plate (Fig. 4*e*). In *dab1* mutant cortex, Brn1 and Cux1 were expressed at high levels in the lower cortical plate, as well as in the intermediate zone (Fig. 4f), although some neurons in the intermediate zone were Brn1 and Cux1 positive and were presumably in transit (Fig. 4*e*). This is consistent with cortical plate inversion. In *src fyn* double-mutant cortex, Cux1+ and

Brn1+ neurons were excluded from the marginal zone and upper cortical plate and were positioned in the lower cortical plate, as in the *dab1* mutant (Fig. 4*h*). The cortical phenotypes were quantified and are shown in Figure 4*i*–*k*. The results show that *src fyn* mutation causes layer inversion similar to, but not as pronounced as, *dab1* mutation. Although only five E18.5 and four E16.5 *src fyn* double-mutant embryos were analyzed using Tbr1 and Brn1, layer inversion was found in all cases. This is statistically significant ($p < 0.005$; χ^2 test).

The *fyn* mutant phenotype was more subtle. Tbr1+ neurons were positioned above the residual subplate in a broader region than in controls (Fig. 4*c*). *Fyn*−/− Tbr1+ neurons were not detected in the upper layers or marginal zone. This suggests that, although most Tbr1+ neurons responded to Reelin and positioned appropriately, some may have migrated excessively before settling. Cux1+ neurons were found in a broad region, including the marginal zone and entire cortical plate down to the top of the residual subplate (Fig. 4*g*). Thus, some late-born Cux1+ neurons have overmigrated into the marginal zone, some are positioned correctly in the upper cortical plate, some are mispositioned in the lower cortical plate, and yet others are in transit in the intermediate zone. Brn1+ neurons were also broadly scattered, but a majority were positioned, as in *dab1* mutant cortex, at the bottom of the cortical plate (Fig. 4*g*). This suggests a partial Reeler phenotype, with most early cortical plate neurons positioned correctly. Some late cortical plate neurons may be trapped below excessively adherent early cortical plate neurons, whereas others successfully migrated into the upper cortical plate but failed to stop at the Reelin-containing marginal zone.

In contrast to *fyn* mutant cortex, the positions of Tbr1+ and Brn1+ neurons in *src*−/− cortex were normal (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Thus, Src is only needed for cortical lamination when Fyn is absent.

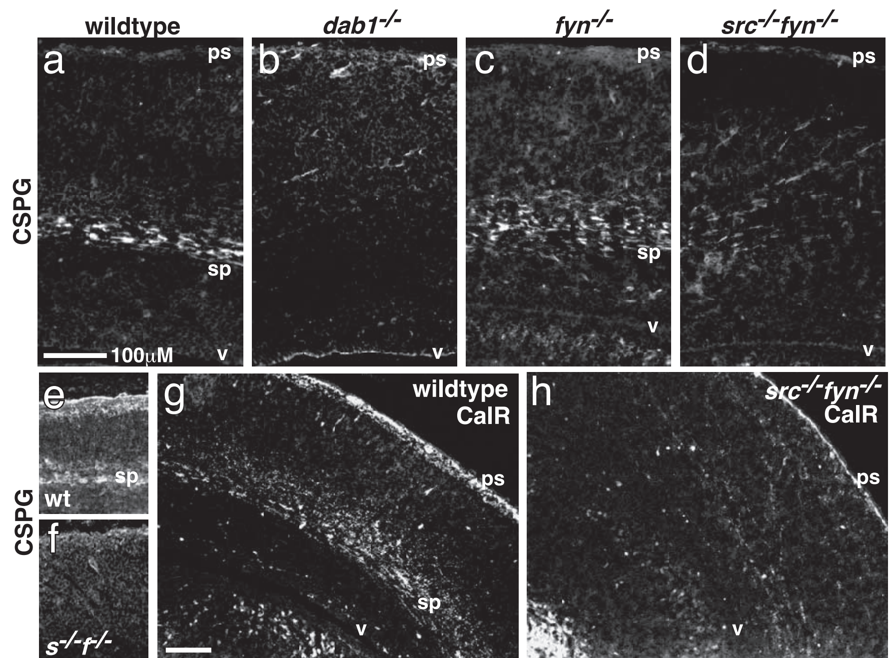

Figure 3. The *src*−/−*fyn*−/− mutant shows defects in preplate splitting. ***a***, ***b***, CSPG antibody stained the subplate in wild-type E18.5 neocortex (***a***) and faintly stained the superplate and scattered subplate cells in *dab1*−/− (***b***). ***c***, ***d***, Subplate staining was dispersed in *fyn*−/− (***c***), and CSPG+ cells were dispersed through the cortical plate in *src*−/−*fyn*−/− (***d***). ***e***, ***f***, CSPG staining at E16.5 in wild-type (***e***) and *src*−/−*fyn*−/− (***f***) showed the same pattern seen at E18.5. ***g***, ***h***, Calretinin staining was detected in the subplate of wild type (***g***) but stained fibers and cells throughout the *src*−/−*fyn*−/− cortex (***h***). Scale bars: ***a*** (for ***a–f***), ***g*** (for ***g***, ***h***), 100 μm. ps, Pial surface; sp, subplate; v, ventricle; CalR, calretinin.

Cerebellar Purkinje cell migrations require Src and Fyn

Cerebellar Purkinje cells arise between E11 and E13 and migrate outwards along radial glia to lie below the external granule layer. In Reeler mutants, the Purkinje cells move away from the ventricular zone but do not reach the Purkinje plate (Rice and Curran, 2001). Although the primary defect is unknown, Purkinje cells appear to be obstructed by disorganized radial glia in the intermediate zone (Yuasa et al., 1993). Purkinje cells can be detected with antibodies to calbindin (Jande et al., 1981) or Dab1 (Gallagher et al., 1998; Rice et al., 1998). At E18.5, calbindin staining revealed a Purkinje layer in wild-type embryos, with characteristic gaps corresponding to future parasagittal stripes (Fig. 5*a*). In both *dab1* and *src fyn* mutants, most Purkinje cells were misplaced in clusters between the ventricular zone and the external granule layer, although a partial Purkinje layer was detected in *src fyn* cerebellum (Fig. 5*b*,*c*). Similar results were obtained with Dab1 staining (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). Thus, the *dab1* and *src fyn* double mutation cause similar defects in Purkinje cell migration in the cerebellum.

Discussion

Although mutational activation of Src-related kinases can have dramatic effects on cell biology, ranging from malignant transformation to induction of differentiation, loss-of-function mutations cause a phenotype in only a subset of the cells in which the genes are normally expressed. Here, we report that *src*−/− embryo brains seem normal, whereas *fyn*−/− embryo brains have a complex phenotype, with scattering of both early and late cortical neurons into inappropriate layers. However, the phenotype is clearly distinct from *dab1*, and, by implication, from Reeler. In contrast, mutation of both *src* and *fyn* reveals a phenotype that is similar to, but less severe than, the *dab1* null and Reeler phenotypes. The marginal zone is hypercellular, and the cortical plate is inverted (Fig. 4). This phenotype correlates with a virtual absence of Reelin-induced Dab1 tyrosine phosphorylation and Dab1 degradation in cultured *src*−/−*fyn*−/− neurons and in fetal cortex (Fig. 1). These results provide genetic evidence that *src/fyn*, together with *reelin*, *vldlr/apoer2*, and *dab1*, are involved in the Reelin signaling pathway.

Src fyn double-mutant fetal brains are subtly different from those of *dab1* mutants. There is a residual subplate, the cortical plate is not as clearly inverted, and a rudimentary Purkinje plate is detected. The incomplete phenotype is consistent with the slight residual response of cultured *src*−/−*fyn*−/− neurons to Reelin stimulation and is likely attributable to other Src-family kinases. *Yes* and *lyn* are also expressed at low levels in developing forebrain (Sudol et al., 1988; Zhao et al., 1991; Umemori et al., 1992) and are candidates for phosphorylation of Dab1 when Fyn and Src are absent. However, brain development cannot be studied in *src*−/−*fyn*−/−*yes*−/− mutant embryos because they die too

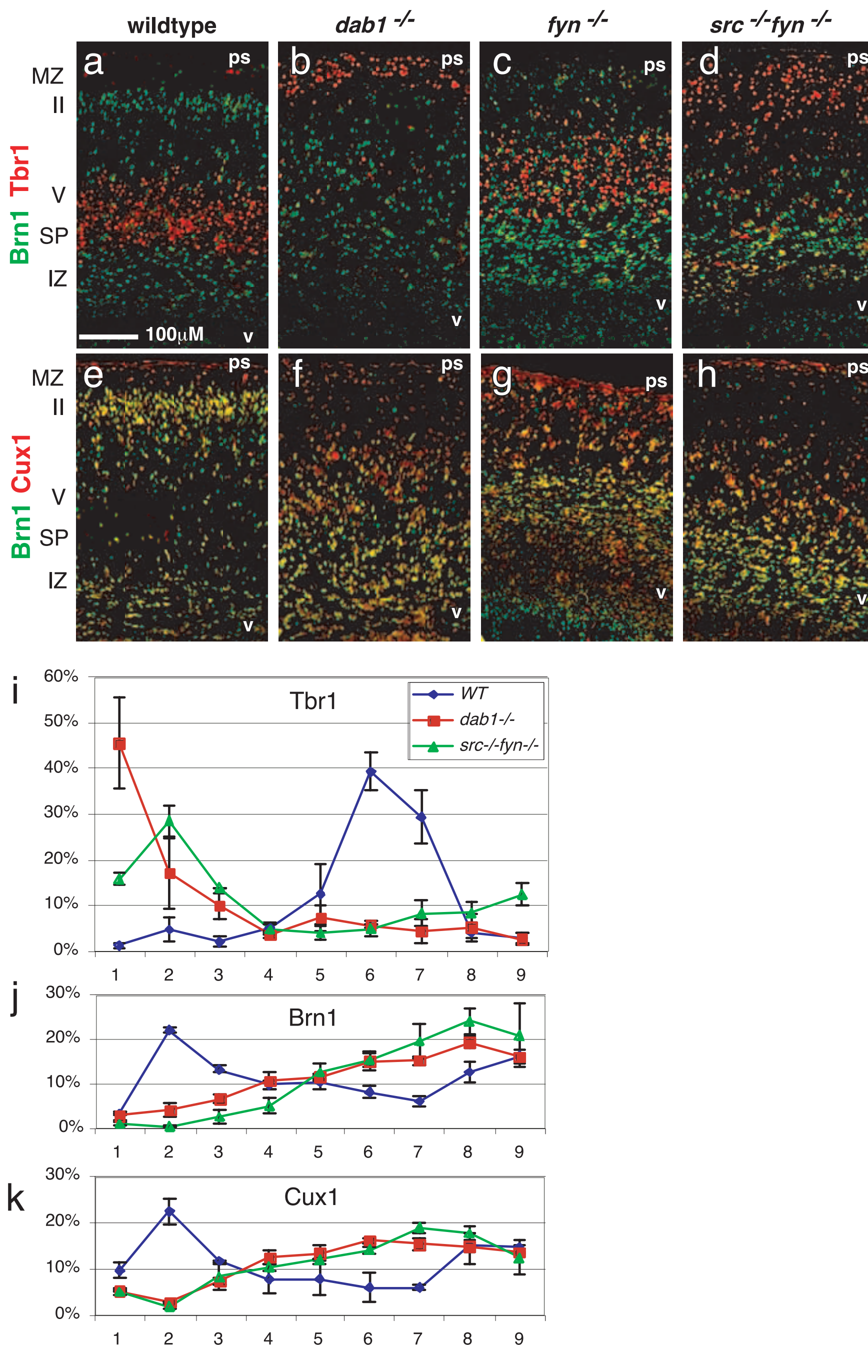

Figure 4. Cortical lamination is inverted in the *src*−/−*fyn*−/− mutant. ***a–d***, E18.5 cortices stained with antibodies to Tbr1 (red) and Brn1 (green). ***e–h***, Staining with antibodies to Cux1 (red) and Brn1 (green). Tbr1 marks subplate and early-born cortical plate neurons, whereas Cux1 and Brn1 mark overlapping populations of late-born cortical plate neurons. Note approximate layer inversion in *dab1*−/− and *src*−/−*fyn*−/− relative to wild type. ***i–k***, Quantification of phenotypes. The percentage of Tbr1+ (***i***), Brn1+ (***j***), and Cux1+ (***k***) in different layers from marginal zone (bin 1) to the top of the ventricular zone (bin 9) was calculated based on replicate sections from one (Cux1) or two (Tbr1 and Brn1) embryos of each genotype. Tbr1 and Brn1 staining of a total of five E18.5 and four E16.5 *src*−/−*fyn*−/− cortices showed that all had an inverted cortical plate. ps, Pial surface; v, ventricle; WT, wild type.

early (Klinghoffer et al., 1999). *Src*−/−*fyn*−/−*lyn*−/− mutant embryos have not been generated.

Adult *fyn*−/− mouse cortex has a clear marginal zone and normally placed early cortical plate neurons (assessed by BrdU labeling on E13) (Yuasa et al., 2004) (T. Herrick and J. A. Cooper, unpublished results). However, layer II–III neurons (detected using BrdU on E16 or using antibodies to calmodulin-regulated kinase II) are deeper than normal, suggesting that Fyn is important for these neurons to migrate to the marginal zone (Yuasa et al., 2004). Our analysis of *fyn*−/− fetal cortex suggests that preplate splitting is incomplete, that some early cortical plate neurons are shallower, and that later cortical plate neurons are found in both shallower and deeper positions than normal. These differences may be attributable to strain background or to changes in neuron position during postnatal development.

One surprising outcome of this study is that neuron numbers and cell proliferation in the cortex were not detectably altered when Fyn and Src were absent, although there was an overall reduction in tyrosine phosphorylation of many cell proteins. The role of Src-family kinases in proliferation of other cell types is controversial. It is clear that *lck* gene disruption causes reduced numbers of thymocytes, but this appears to be because of a block before a proliferative developmental stage (Molina et al., 1992). Disruption of other SFK genes generally does not cause decreases in cell numbers (Lowell and Soriano, 1996). For example, *src* disruption does not interfere with osteoclast numbers, but does interfere with their function in resorbing bone (Boyce et al., 1992). In an extreme case, mutation of *src*, *hck*, *fgr*, and *lyn* does not inhibit blood platelet production, but the platelets are defective (Obergfell et al., 2002; Lowell, 2004). In addition, immortalized fibroblasts that lack *src*, *fyn*, and *yes* proliferate and respond to growth factors (Klinghoffer et al., 1999). However, primary fibroblasts fail to proliferate in response to growth factors if SFK activity is inhibited (Broome and Courtneidge, 2000). Thus, SFKs are required for proliferation of some cell types under some conditions. Our results do not exclude a requirement for low-level SFK activity in neuronal progenitors for their symmetric and asymmetric divisions, but we do suggest that SFK activity can be greatly reduced (to $<$5% of normal, judging from Dab1 phosphorylation) without compromising proliferation of neuronal precursors and production of postmitotic neurons.

Another conclusion of this study is that neurons can migrate in the absence of Src and Fyn. *Src*−/−*fyn*−/− Cajal-Retzius, as well as subplate and cortical plate neurons all migrate successfully away from their origins. In many cell types, Src-family kinases are important for cytoskeletal remodeling and cell movements in response to integrin and other signals from the exterior (Boyce et al., 1992; Klinghoffer et al., 1999; Obergfell et al., 2002; Lowell, 2004). We and others have observed that tyrosine phosphorylation of p190 Rho GAP and FAK is inhibited in *fyn*−/− and *src*−/−*fyn*−/− embryo cortex (Grant et al., 1995; Brouns et al., 2001). In fibroblasts, p190 and FAK are

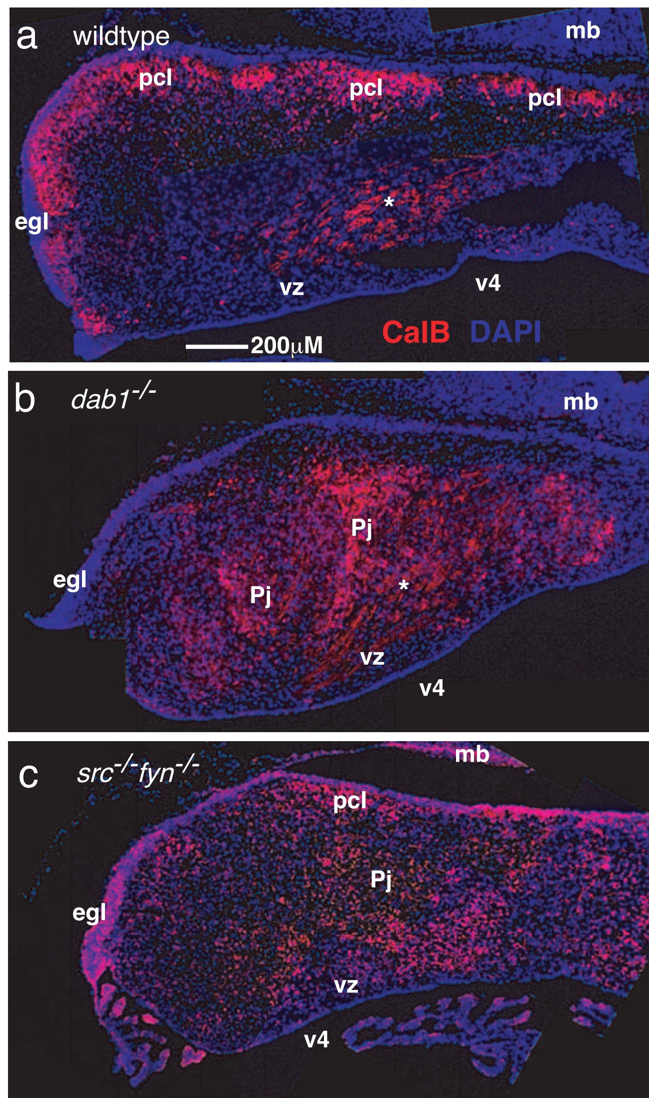

Figure 5. Purkinje cell migration defects in *src*−/−*fyn*−/− cerebellum. Coronal sections of E18.5 cerebellum were stained with antibody to calbindin and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) to show nuclei. Each figure is a montage of high-power images. ***a***, Purkinje cells in the wild-type cerebellum had largely migrated out to form the Purkinje cell layer. ***b***, In the *dab1*−/− cerebellum, Purkinje cells were located in clusters deep in the cerebellum. ***c***, A partial phenotype was seen in the *src*−/−*fyn*−/− cerebellum, where a vestigial partial Purkinje cell layer had formed, but most Purkinje cells were in clusters deep in the cerebellum. In all cases, an external granule layer could be detected. mb, midbrain; pcl, Purkinje cell layer; Pj, ectopic Purkinje cells; egl, external granule layer; vz, ventricular zone; v4, fourth ventricle. The asterisks indicate fiber tracts.

both important for integrin signaling and cell movement (Ilic et al., 1995; Parsons and Parsons, 1997; Schlaepfer et al., 1999; Arthur et al., 2000; Kulkarni et al., 2000). *p190* deletion inhibits axon guidance and fasciculation (Brouns et al., 2001). Serine phosphorylation of FAK is important for radial migrations (Xie et al., 2003), and FAK is required for normal cortical lamination (Beggs et al., 2003), but it is not known whether FAK tyrosine phosphorylation is required. In addition, FAK and Src-family kinases are involved in axon guidance decisions (Liu et al., 2004). However, Src kinase inhibitors do not inhibit neuronal migration in cortical slices (Jossin et al., 2003). Although SFKs are important for organization of the actin cytoskeleton, cortical neuron migrations are heavily reliant on microtubules (Walsh and Goffinet, 2000; Gupta et al., 2002; Xie et al., 2003). Together, these results suggest that neurons migrate independently of Src-family-catalyzed phosphorylation events.

To date, the Reeler phenotype results whenever mutations are made in components acting in the signaling cascade. Thus, mutations in Reelin, its receptors Vldlr and ApoER2, the substrate Dab1, and, as shown here, the tyrosine kinases Fyn and Src, all cause a Reeler-like phenotype. When homozygous mutations at different levels of the cascade have been combined, there are no additional phenotypes, implying that the pathway is linear (Howell et al., 1999a). However, genetic interactions have not been apparent when transheterozygotes of *reelin* and *dab1* are prepared (B. Howell and Cooper, unpublished results). The pathway seems to be robustly buffered from variation, presumably by negative feedback loops such as the regulation of Dab1 protein levels after Reelin stimulation. It will be interesting to see whether other components in the signaling cascade, working downstream of activated Src-family kinases and Dab1, are also required for normal neuron positioning.

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