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Neuroscience Research 57 (2007) 210–219
Neuroscience Research
www.elsevier.com/locate/neures

Constitutive activation of neuronal Src causes aberrant dendritic morphogenesis in mouse cerebellar Purkinje cells

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Received 31 August 2006; accepted 19 October 2006
Available online 29 November 2006

Abstract

Src family tyrosine kinases are essential for neural development, but their *in vivo* functions remain elusive because of functional compensation among family members. To elucidate the roles of individual Src family members *in vivo*, we generated transgenic mice expressing the neuronal form of c-Src (n-Src), Fyn, and their constitutively active forms in cerebellar Purkinje cells using the L7 promoter. The expression of the constitutively active n-Src retarded the postnatal development of Purkinje cells and disrupted dendritic morphogenesis, whereas the wild-type n-Src had only moderate effects. Neither wild-type nor constitutively active Fyn over-expression significantly affected Purkinje-cell morphology. The aberrant Purkinje cells in n-Src transgenic mice retained multiple dendritic shafts extending in non-polarized directions and were located heterotopically in the molecular layer. Ultrastructural observation of the dendritic shafts revealed that the microtubules of n-Src transgenic mice were more densely and irregularly arranged, and had structural deformities. In primary culture, Purkinje cells from n-Src transgenic mice developed abnormally thick dendritic shafts and large growth-cone-like structures with poorly extended dendrites, which could be rescued by treatment with a selective inhibitor of Src family kinases, PP2. These results suggest that n-Src activity regulates the dendritic morphogenesis of Purkinje cells through affecting microtubule organization.

Keywords: Dendrogenesis; Microtubule; Src; Fyn; L7 promoter; Transgenic mouse

1. Introduction

Neural development is regulated by a variety of extracellular cues that are processed *via* specific receptors and downstream intracellular signaling pathways. The Src family of tyrosine kinases (SFKs) are non-receptor protein tyrosine kinases, that are abundantly expressed in the nervous system (Maness, 1992) and have been implicated in diverse neural-cell signaling pathways that contribute to neurogenesis, differentiation, migration, neurite extension, and synaptic transmission (Beggs et al., 1994; Ignelzi et al., 1994; Morgan et al., 2000; Zhao et al., 2003; Kalia et al., 2004). SFKs are membrane-localized *via* an amino-terminal fatty-acid modification and functionally cooperate with various membrane receptors to relay extracellular signals into the cells (Thomas and Brugge, 1997). Of the eight SFK members, c-Src, Fyn, c-Yes, Lyn, and Lck are highly expressed in the central nervous system and there are neural isoforms of c-Src (n-Src) and Fyn (FynB) (Martinez et al., 1987; Cooke and Perlmutter, 1989; Zhao et al., 1991; Umemori et al., 1992; Bare et al., 1993; Chen et al., 1996; Omri et al., 1996). The expression of n-Src is restricted to neuron and is temporally augmented at the onset of neural differentiation, implicating the role for n-Src in early stages of neuronal development. In contrast, Fyn is widely expressed in the nervous system and maintained at relatively high levels in mature tissues, suggesting that Fyn plays roles in maintenance and/or higher order function of the nervous system (Sudol et al., 1988; Wiestler and Walter, 1988). However, despite the abundant expression of Src in developing neurons, Src-knockout mice show no overt nervous system phenotype (Soriano et al., 1991). Fyn-knockout mice also exhibit only

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0168-0102/$ – see front matter
doi:10.1016/j.neures.2006.10.007

moderate defects in hippocampal architecture and function (Grant et al., 1992) and in myelination (Umemori et al., 1994). Nonetheless, double mutations of Src and Fyn cause severe defects in prenatal development and lamination of the cortex and cerebellum (Kuo et al., 2005). These *in vivo* studies suggested that Src and Fyn play essential roles in neural development, but failed to define the precise function of each SFK member due to functional compensation.

In vitro studies using neuronal cultures from mutant mice have identified potential functions for SFKs. Neurite outgrowth dependent on the neural-cell adhesion molecule L1 is specifically inhibited in Src-deficient neurons (Ignelzi et al., 1994), and NCAM-140 dependent neurite outgrowth is inhibited in Fyn-deficient neurons (Beggs et al., 1994). Fyn has been reported to have specific functions in the post-synaptic density (Grant et al., 1992), AMPA- or NMDA-receptor signaling (Grant, 1996; Narisawa-Saito et al., 1999; Rong et al., 2001), myelination signaling (Umemori et al., 1994; Sperber et al., 2001), and Reelin signaling (Arnaud et al., 2003). c-Src has been implicated in the regulation of microtubule organization and growth-cone migration (Maness et al., 1988; Maness and Matten, 1990; Lim and Halpain, 2000). However, these roles have not been evaluated *in vivo*.

To address the functions of Src and Fyn *in vivo*, we generated conventional transgenic mice that over-express n-Src, Fyn, or their constitutively active forms under the control of the L7 promoter, which is highly specific for Purkinje cells (Barski et al., 2000; Tomomura et al., 2001; Zhang et al., 2001). Purkinje-cell development was retarded in transgenic mice for the constitutively active n-Src and was accompanied by aberrant dendritic morphogenesis, whereas Fyn transgenic mice did not exhibit any overt phenotype. These observations indicate that n-Src functions *in vivo* in the dendritic morphogenesis in mouse Purkinje cells.

2. Materials and methods

2.1. Generation of transgenic mice

To induce heterologous gene expression specifically in cerebellar Purkinje cells, we used PCR mutagenesis to generate a modified murine *L7* gene, in which all ATG sequences within the original exons were eliminated (Smeyne et al., 1995). To generate transgenic constructs, murine *n-src* or human *fyn* cDNAs were inserted into the fourth exon of the modified *L7* gene (Fig. 1B). The *Not* I fragment from pBS-*L7src* or pBS-*L7fyn* was then microinjected into the pronuclei of fertilized eggs of BDF1 mice. To express constitutively active c-Src and Fyn, *srcY535F* and *fynY531F*, in which the regulatory tyrosine residues were replaced by phenylalanine, were introduced into the transgenes. These mutant mice were maintained in an inbred C57BL/6 genetic background. Mouse genotypes were determined by PCR of DNA samples prepared from tail clippings. The mice used for this study were handled in strict adherence with local governmental and institutional animal-care regulations.

2.2. Antibodies

Anti-Fyn and anti-actin were purchased from Santa Cruz Biotechnology, Inc. Anti-Src was from Oncogene Research Products. Anti-Src [pY418] and Anti-Src [pY529] were from BioSource International. Anti-phosphotyrosine (4G10) was from Upstate Biotechnology. Monoclonal anti-calbindin D-28K was from SIGMA. Rabbit polyclonal anti-calbindin D-28K was from CHEMICON International, Inc. Biotinylated anti-mouse IgG and biotinylated anti-rabbit IgG were from Vector Laboratories, Inc. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from Zymed Laboratories, Inc. Alexa594-conjugated anti-rabbit IgG, Alexa488-conjugated anti-rabbit IgG, and Alexa594-conjugated anti-mouse IgG were from Molecular Probes, Inc.

2.3. Immunoprecitpitaion and immunoblotting

Mouse cerebellar tissues were homogenized in 10 vol. (w/v) of ODG buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 5% glycerol, 2% *n*-octyl-β-D-glucoside, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor). The solubilized homogenates were centrifuged for 30 min at 20,000 × *g* to obtain clear supernatants (cerebellar lysates). For immunoblotting, protein samples separated by SDS-PAGE were transferred to a nitrocellulose membrane under semi-dry conditions. The membranes were blocked with T-TBS (Tris-buffered saline, pH 7.4, containing 0.1% Tween-20), or T-TBS containing 1% bovine serum albumin or 4% skim milk. Membranes were then incubated with primary antibodies for 2 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactivity was visualized with an enhanced chemiluminescence system (PerkinElmer Life Sciences).

2.4. Histological analysis

Mice were anesthetized with sodium pentobarbital and perfused transcardially with saline containing 0.1% sodium nitrate, followed by a fixative consisting of 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). After further fixation in the same solution for 1 day at 4 °C, samples were equilibrated in 30% sucrose in PBS for cryoprotection and the brain was cut into 10 μm-thick sagittal cryosections. Some sections were stained with cresyl violet. For immunohistochemical studies, sections were blocked with 1% normal goat serum in supermix solution (50 mM Tris–HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.5% Triton X-100) and incubated for 3 h at room temperature with primary antibodies diluted in the supermix solution. After washes, the sections were incubated with biotin-conjugated secondary antibodies for 3 h at room temperature. The signals were developed with diaminobenzidine using a Vectastain ABC Kit (Vector Lab, USA). Some specimens were also stained with hematoxylin. For double-labeling immunofluorescence studies, sections were blocked with 1% normal goat serum in the supermix solution, incubated for 3 h at room temperature with the primary antibodies, incubated for 3 h at room temperature with Alexa594- or FITC-conjugated secondary antibodies, and observed using an Olympus BX60 fluorescence microscope.

2.5. Electron microscopy

Ten-day-old mice were perfused transcardially with a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in PBS under deep anesthesia with sodium pentobarbital. Two control and three L7-SrcY535F mice were examined. Each cerebellum was removed and parasagittal sections (100 μm thick) were prepared on a Vibratome. Sections were osmicated and embedded in epoxy resin. Ultrathin sections were prepared and stained with lead citrate and uranyl acetate and observed under a Hitachi H-600 transmission electron microscope or a Technai G-2 FEI electron microscope.

2.6. Primary cultures of cerebellar cells

The cerebellar dissociated cell culture was prepared essentially as described previously (Furuya et al., 1998). In brief, the cerebella of neonatal mice were removed and transferred to ice-cold 0.25% glucose/Eagle's minimal essential medium. After removal of the meninges, the cerebella were washed in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (BSS; SIGMA) and incubated for 13 min at 37 °C in 500 μl of trypsin solution (0.1% trypsin and 500 U/ml DNase I in Ca^{2+}/Mg^{2+}-free Hanks' BSS). The cerebella were then washed three times in Ca^{2+}/Mg^{2+}-free Hanks' BSS by centrifugation at 900 rpm for 3 min. The

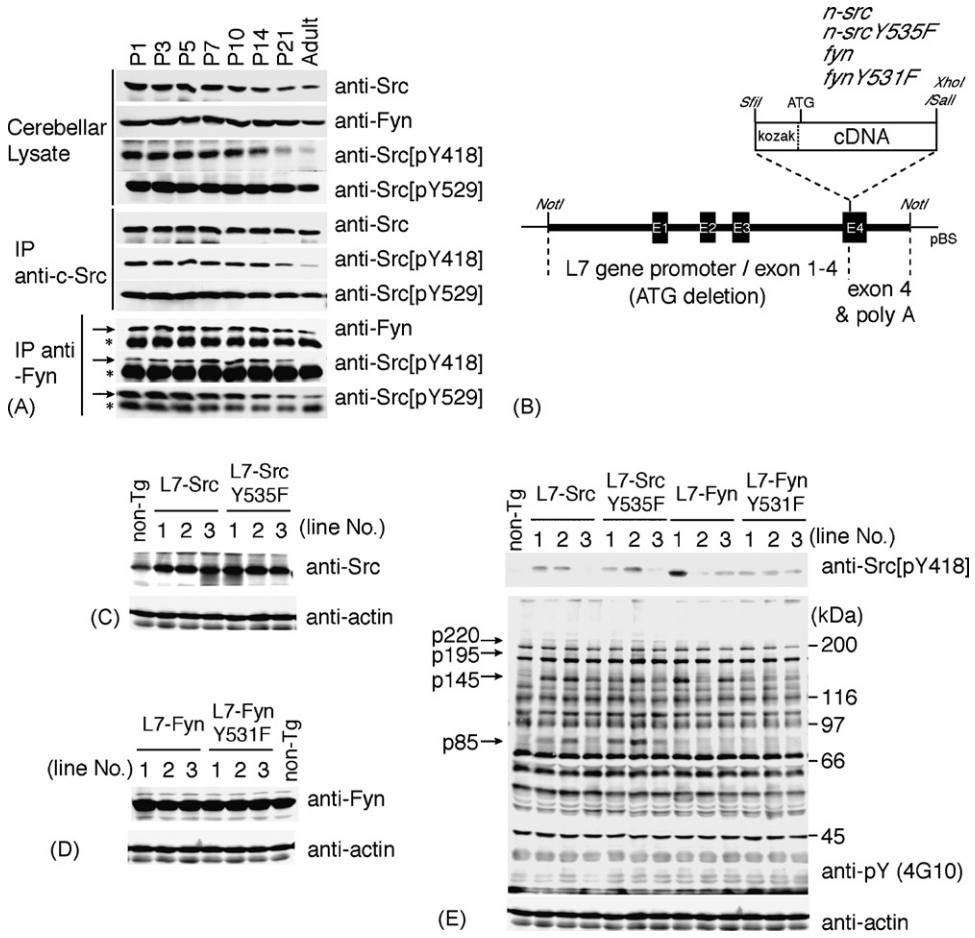

Fig. 1. Generation of L7-mediated n-Src and Fyn transgenic mice. (A) Expression and activity of c-Src and Fyn during cerebellar development. Whole cerebellar lysates prepared at the indicated stages of development were subjected to immunoblot analysis with anti-Src, anti-Fyn, anti-Src [pY418] and anti-Src [pY529] antibodies (upper four panels). c-Src and Fyn were immunoprecipitated from cerebellar tissue lysates, and probed with anti-Src, anti-Fyn, anti-Src [pY418] and anti-Src [pY529] antibodies (lower six panels). Arrows indicate the positions of Fyn and asterisks denote those of immunoglobulin. (B) Construction of transgenes. The cDNAs encoding n-Src, n-SrcY535F, Fyn, and FynY531F were inserted into exon 4 of the *L7* gene in which potential initiation codons had been disrupted. (C) Expression of n-Src in transgenic (Tg) lines. Whole cerebellar lysates from the transgenic lines were subjected to immunoblot analysis with anti-Src (upper panel) and anti-actin (lower panel). (D) Expression of Fyn in transgenic lines. Whole cerebellar lysates from the transgenic lines were subjected to immunoblot analysis with anti-Fyn (upper panel) and anti-actin (lower panel). (E) Tyrosine phosphorylation of cerebellar proteins in transgenic lines. Whole cerebellar lysates from the indicated transgenic lines were subjected to immunoblot analysis with anti-Src [pY418] (upper panel), anti-phosphotyrosine (4G10) (middle panel), and anti-actin (lower panel). The locations of p85, p145, p195, and p220 are indicated by arrows.

trypsin-treated cerebella were dissociated in 500 μl of DNase solution (500 U/ml DNase I and 12 mM $MgSO_4$ in Ca^{2+}/Mg^{2+}-free Hanks' BSS) by trituration with a Pasteur pipette. After complete dissociation, the cell suspensions were centrifuged at 1000 rpm. for 5 min and the cell pellets were resuspended in seeding solution (DMEM/F-12 containing 10% fetal bovine serum [FBS]). The cell suspension (100 μl) was plated at a concentration of 4×10^6 cells/ml on a cover slip (12 mm in diameter) coated with poly-L-lysine (MW > 300,000; SIGMA) and incubated at 37 °C in 5% CO_2/95% air. After a 1 h incubation, 900 μl of serum-free culture medium (DMEM/F-12 medium containing 100 μM putrescine, 30 nM sodium selenite, 3.9 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 nM progesterone, 10 ng/ml insulin, 100 μg/ml transferrin, 0.5 ng/ml tri-iodothyronine) was added to each well and incubated at 37 °C in 5% CO_2/95% air. At 5 days *in vitro* (DIV), half of the medium was replaced with 1% FBS/culture medium containing test reagents. At 12 DIV, the cultures were analyzed by immunocytochemistry.

2.7. Immunocytochemistry

To stain Purkinje cells, dissociated cerebellar cells were fixed with ice-cold 4% paraformaldehyde in PBS at 12 DIV. After fixation, the cells were washed three times with PBS for 5 min each at room temperature and incubated with anti-calbindin D-28K and anti-Src [pY418] antibodies in the supermix solution overnight at 4 °C. Cells were then washed three times in PBS for 5 min each at room temperature. The cells were incubated with Alexa594-conjugated anti-mouse IgG and Alexa488 conjugated anti-rabbit IgG in supermix solution for 3 h at room temperature and washed three times for 10 min with PBS. An Olympus BX60 fluorescence microscope was used for observations.

3. Results

3.1. Expression and activity of Src and Fyn during cerebellar development

An immunoblot analysis of whole cerebellar lysates revealed that both Src (c-Src and n-Src) and Fyn kinases are highly expressed in the developing cerebellum from postnatal day 1 (P1) to P10, and then downregulated upon maturation (P14 to adult) (Fig. 1A). To assess the activity states of these kinases, the immunoprecipitated proteins were probed with

antibodies that recognize the autophosphorylated tyrosine (pY418) and the phosphorylated negative regulatory site (pY529). The ratio of pY418/pY529 (active to inactive form) substantially decreased with cerebellar maturation for both Src and Fyn, indicating that the kinase activity was also down-regulated during cerebellar development (Fig. 1A). These observations indicate that functional activation of Src and Fyn coincide with postnatal cerebellar development, potentially including dendritic morphogenesis of Purkinje cells, and that downregulation of Src function may correlate with cerebellar maturation.

3.2. Generation of L7 promoter-mediated SFK transgenic mice

To examine the roles of n-Src and Fyn in neural development, we generated transgenic mice that over-express these SFKs in Purkinje cells under the control of the L7 promoter (Fig. 1B). The transgenic lines obtained were L7-Src for animals overexpressing n-Src, L7-SrcY535F for animals overexpressing a constitutively active n-Src in which Tyr was replaced by Phe at the negative regulatory site (n-SrcY535F), L7-Fyn for animals overexpressing Fyn, and L7-FynY531F for animals overexpressing constitutively active FynY531F. We expected that these lines would enable us to evaluate the cell-autonomous effects of n-Src or Fyn expression on postnatal Purkinje cell development because the L7 promoter directs gene expression specifically in Purkinje cells starting at early postnatal stages (Barski et al., 2000; Tomomura et al., 2001; Zhang et al., 2001). The specificity of the L7 promoter was confirmed in a control experiment using a GFP reporter transgene (data not shown). Mice overexpressing each of the kinases were born healthy and grew into adults. No apparent defects in motor coordination have been observed thus far in any of the transgenic lines.

An immunoblot analysis of the cerebellar lysates showed that there was a significant increase in Src protein levels in the transgenic mice (Fig. 1C), whereas Fyn over-expression was barely detectable probably due to high levels of endogenous Fyn in cells other than Purkinje cells (Fig. 1D). However, we confirmed the over-expression of Fyn in Purkinje cells by immunostaining cerebellar sections with anti-Fyn antibody (data not shown). Immunoblot analysis with anti-Src [pY418], which cross-reacts with various activated SFKs including Fyn, detected the activated forms n-Src and Fyn (Fig. 1E, upper panel), indicating that these molecules were functionally expressed in these mice. Consistent with the increased SFK activity, tyrosine phosphorylation of some cellular proteins was elevated (Fig. 1E, middle panel). In the cerebella of adult L7-Src and L7-SrcY535F mice, proteins of 85 kDa (p85), 145 kDa (p145), 195 kDa (p195), and 220 kDa (p220) had elevated tyrosine phosphorylation. In L7-Fyn and L7-FynY531F mice, p145 was highly phosphorylated to an extent similar to that in n-Src transgenic mice, but tyrosine phosphorylation of p85, p195, and p220 was not elevated. These observations demonstrate that there are functional differences between n-Src and Fyn.

3.3. Characterization of L7-mediated n-Src and Fyn transgenic mice

To examine the effects of n-Src and Fyn over-expression on cerebellar organization, we analyzed tissue sections stained with cresyl-violet. In L7-Fyn and L7-FynY531F mice, there were no detectable defects in cerebellar organization (Fig. 2A–C). The morphologies of Purkinje cell bodies and dendrites were also normal (data not shown). In L7-Src and L7-SrcY535F mice, however, some Purkinje cells were heterotopically localized in the molecular layer of the cerebellum. The extent of the defect was much greater in L7-SrcY535F than in L7-Src mice (Fig. 2D and E), suggesting that the defects correlated with the level of Src activity. The aberrant Purkinje cells were observed more frequently in the posterior region of the cerebellum (Fig. 2F and G), probably due to the differential expression patterns of the L7 promoter. Similar phenotypes were observed in two additional L7-Src lines and two additional L7-SrcY535F lines (data not shown). We further confirmed the expression of activated n-Src in Purkinje cells by immunostaining cerebellar sections with anti-Src [pY418] and anti-calbindin D-28K, a specific marker for Purkinje cells. In adult L7-SrcY535F mice, strong signals for anti-Src [pY418] were obtained in the cell bodies and dendritic shafts of Purkinje cells (Fig. 2I), whereas only weak signals were obtained in the non-transgenic mice (Fig. 2H).

3.4. Characterization of abnormal Purkinje cells in L7-SrcY535F mice

To further characterize the defects observed in L7-SrcY535F mice, we immunostained Purkinje cells at various developmental stages, from P3 to adult, for calbindin D-28K. At P3 to P5, normal immature Purkinje cells did not appear polarized; short dendrites extended in all directions and the cell bodies aligned to form the Purkinje-cell layer (Fig. 3A and C). At these stages, no significant defects were observed in L7-SrcY535F mice (Fig. 3B and D).

At P7, normal Purkinje cells became polarized and the immature dendrites began to converge into a single dendritic shaft from which thin, complex dendritic branches extended. The alignment of the Purkinje cells was almost completed by this stage (Fig. 3E and O). In P7 L7-SrcY535F mice, however, a substantial population of the Purkinje cells appeared less polarized and retained multiple dendritic shafts with poorly developed branches extending in random directions. Some Purkinje cells still remained in the molecular layer (Fig. 3F and O).

From P10 to adult, normal Purkinje cells developed a more complex network of dendrites to complete dendritic morphogenesis (Fig. 3G, I and K). In L7-SrcY535F mice, most Purkinje cells developed complex dendritic branches similar to normal cells, although there was some delay in development (Fig. 3H, J and L). During these stages, the numbers of aberrant Purkinje cells decreased (Fig. 3O), suggesting that some compensatory mechanism might operate to reconstruct Purkinje cell organization. However, some populations of aberrant Purkinje cells with

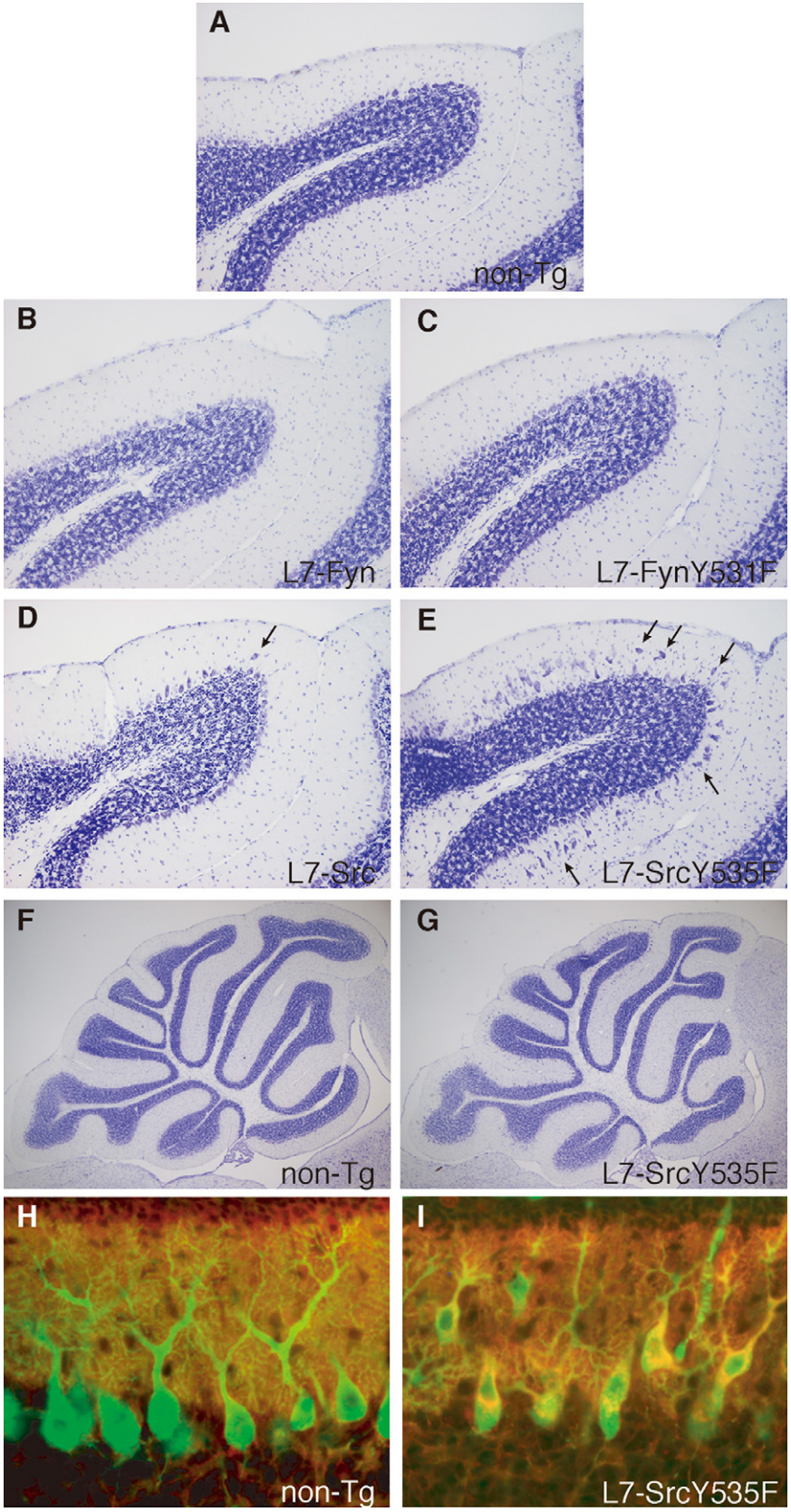

Fig. 2. Cerebella of n-Src and Fyn transgenic mice. Sections of cerebellar lobule VI in mice of the non-Tg (A), L7-Fyn (B), L7-FynY531F (C), L7-Src (D), and L7-SrcY535F (E) were stained with cresyl violet. Arrows indicate heterotopic Purkinje cells in the molecular layer. Lower magnification views of non-Tg (F) and L7-SrcY535F (G) lines are also shown. Purkinje-cell sections of non-Tg (H) and L7-SrcY535F (I) P10 mice containing Purkinje cells were double-stained with anti-calbindin D-28K (green) and anti-Src [pY418] (red).

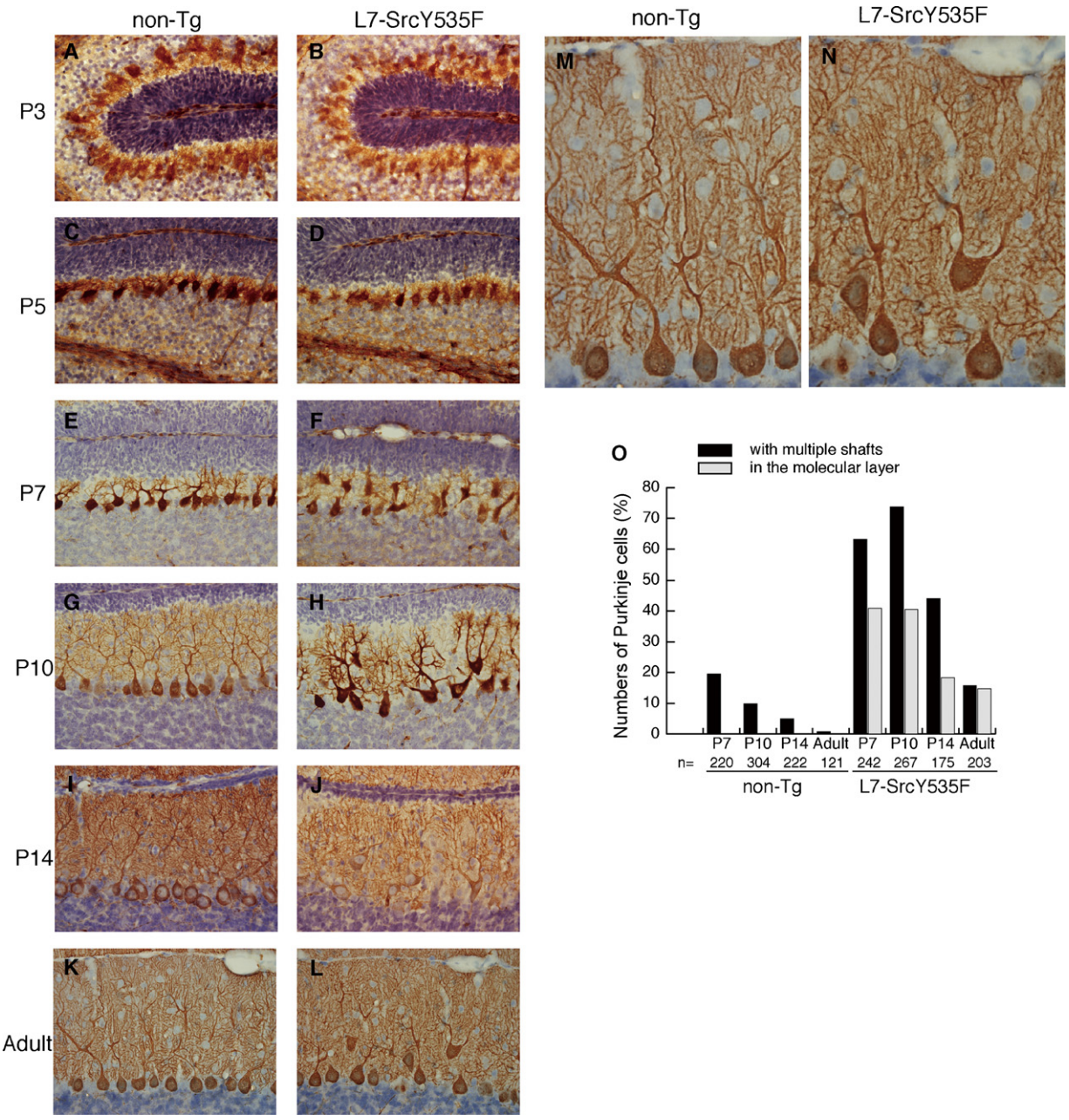

Fig. 3. Characterization of aberrant Purkinje cells of L7-SrcY535F mice. Purkinje cells in cerebellar lobules VIII and IX of non-Tg (A, C, E, G, I, and K) and L7-SrcY535F (B, D, F, H, J, and L) at the indicated developmental stages from P3 to adult were stained with hematoxylin, followed by immunostaining with anti-calbindin D-28K. Higher magnification views of adult tissues of non-Tg (M) and L7-SrcY535F (N) mice are also shown. (O) Percentages of Purkinje cells with multiple dendritic shafts (black bars) and heterotopically localized in the molecular layer (gray bars) in lobules VIII and IX. *n*, number of Purkinje cells observed.

multiple dendritic shafts and heterotopic positioning were still present in the cerebella of adult L7-SrcY535F mice (Fig. 3M–O). Dendrite extension was also abnormal in these cells. The dendrites of normal Purkinje cells extend in the same plane and do not show crossover patterns. In contrast, there were many irregular crossovers between the dendrites of the transgenic Purkinje cells, suggesting that the direction of dendrite extension was not properly regulated in these mice.

To further characterize the aberrant Purkinje cells of n-Src transgenic mice, dendrites in the molecular layer of the cerebellar cortex of mice at P10, the stage at which transgenic Purkinje-cell abnormalities were most prominent, were examined at the ultrastructural level. The dendritic shafts of the Purkinje cells were identified by the presence of hypolemmal cisternae, a specialized form of endoplasmic reticulum situated just below the plasma membrane of Purkinje-cell dendrites. At low magnification, we could see that the density and arrangement of microtubules in the dendrites were substantially different in control and L7-SrcY535F mice. The density of microtubules was much higher in L7-SrcY535F mice (Fig. 4D) than in controls (Fig. 4A). At higher magnification, it was apparent that the arrangement of microtubules in L7-SrcY535F mice was irregular and microtubules were intertwined and curved (Fig. 4E), in contrast to their almost parallel arrangement in the control

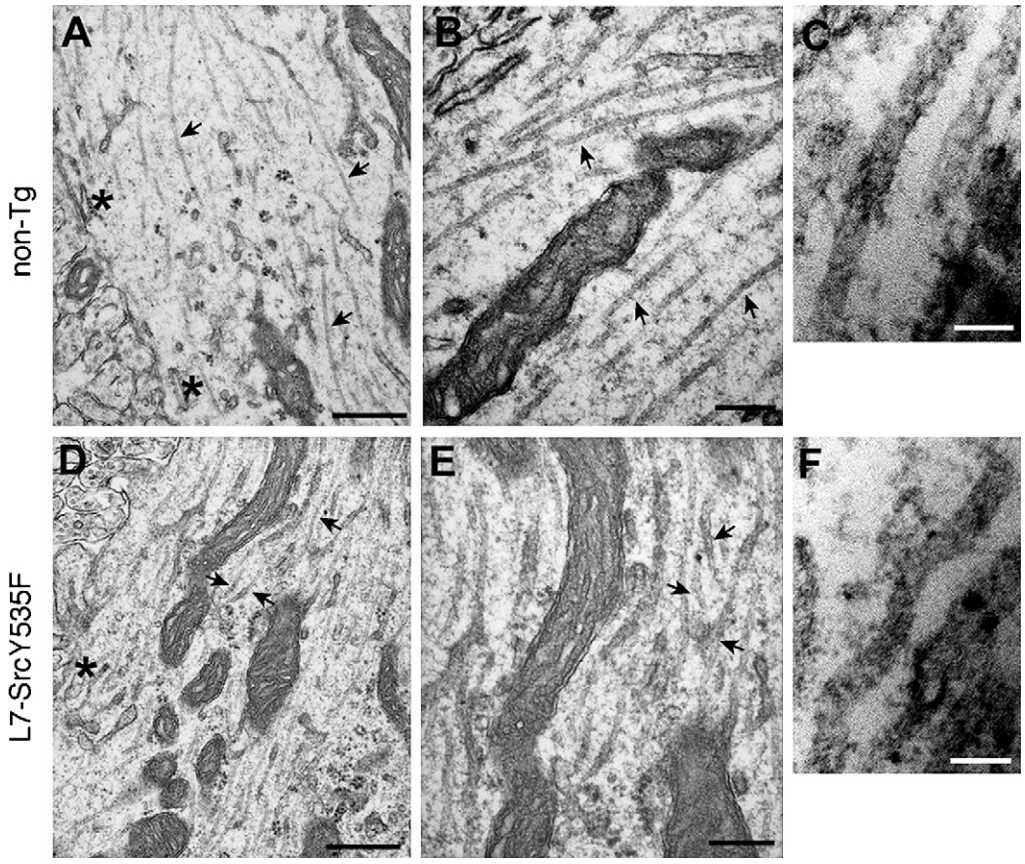

Fig. 4. Ultrastructural analysis of aberrant Purkinje cell dendrites. In non-Tg cells (A–C), the microtubules (25 nm in diameter) are straight and arranged in parallel. In the L7-SrcY535F cells (D–F), the density of microtubules was much higher, they were irregularly arranged, and deformities in microtubule shape were evident at higher magnification. The arrows in A, B, D, and E indicate microtubules. The asterisks in A and D indicate hypolemmal cisternae. Scale bars: 500 nm in A and D, 200 nm in B and E, and 50 nm in C and F.

(Fig. 4B). There were no significant differences between the control and L7-SrcY535F mice with respect to the morphology of mitochondria and the rough endoplasmic reticulum. No overt abnormalities were found in the synaptic organization of L7-SrcY535F mice (not illustrated). Further magnification highlighted the structural deformities of the microtubules in L7-SrcY535F mice (Fig. 4F), in contrast to the almost straight microtubules in the control (Fig. 4C). These observations show that the expression of constitutively active n-Src affects the assembly of microtubules in Purkinje cells.

3.5. Characterization of L7-SrcY535F Purkinje cells in primary culture

To confirm the defects caused by n-Src expression *in vitro*, we prepared dissociated cerebellar cell cultures. At 12 DIV, normal Purkinje cells extended dendrites with numerous branches (Fig. 5A–C). In these cells, we were unable to detect signals for anti-Src [pY418], indicating that SFKs were in an inactive state in normal cells (Fig. 5a–c). In L7-SrcY535F Purkinje cells, however, approximately 67% of cells were strongly positive for anti-Src [pY418] (Fig. 5d–f), indicating that n-Src was functionally overexpressed even in culture. Some populations of L7-SrcY535F Purkinje cells developed abnormally thick dendritic shafts and large growth-cone-like structures with poorly extended dendrites (Fig. 5D–F, arrowheads). These abnormalities were frequently observed in Purkinje cells expressing relatively high levels of n-SrcY535F activity (Fig. 5d–f). To further confirm the contribution of n-Src activity to the defects in dendrogenesis, we treated Purkinje cells with a universal SFK inhibitor, PP2. In non-transgenic cells, PP2 treatment caused a reduction in the area of the dendritic tree and thickness, suggesting that a basal level of SFK activity is required for dendrite extension. PP2 treatment of L7-SrcY535F Purkinje cells suppressed the defects caused by n-Src expression and conferred a morphology similar to that of non-transgenic cells (Fig. 5J–L).

4. Discussion

To address the *in vivo* functions of n-Src and Fyn, we generated conventional transgenic mice in which these SFKs or their constitutively active forms were over-expressed in cerebellar Purkinje cells. Transgenic mice expressing constitutively active n-Src exhibited apparent defects in Purkinje-cell development, accompanied by impaired dendritic morphogenesis. The onset of the defects coincided with the increase in n-Src expression during postnatal dendrogenesis of Purkinje cells. Some Purkinje cells with non-polarized multiple dendritic shafts failed to integrate into the Purkinje-cell layer

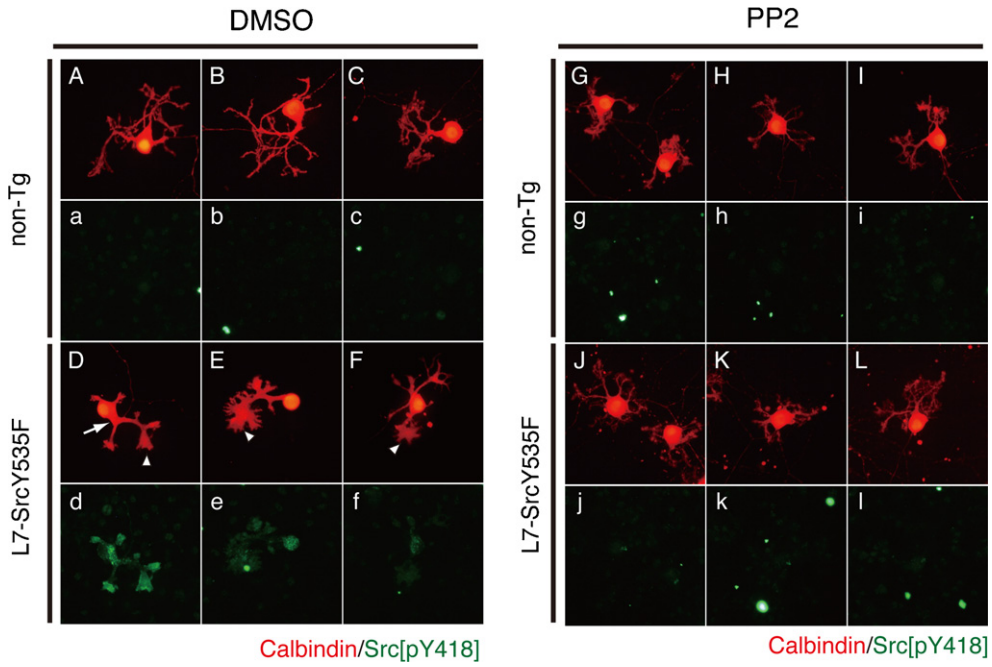

Fig. 5. Characterization of Purkinje cells in primary culture. Primary cultures of cerebellar cells were prepared from neonatal non-Tg (A–C, a–c, G–I, g–i) or L7-SrcY535F (D–F, d–f, J–L, j–l) mice. DMSO (A–F, a–f) or 10 μM PP2 (G–L, g–l) was added to the cultures at 5 DIV and the cells were immunostained with anti-calbindin D-28K (A–L, red) or anti-Src [pY418] (a–l, green) at 12 DIV. An arrow indicates an abnormally thick dendritic shaft (D). Arrowheads show expanded growth-cone-like structures with few dendritic protrusions (D–F).

and were retained in the molecular layer. This unusual positioning of Purkinje cells in n-Src transgenic mice may be due to the non-polarized extension of dendrites, which may sterically interfere with the alignment of Purkinje cell bodies in the cell plate. The constitutive activation of n-Src could affect signaling by a cell polarity determinant, thereby resulting in the extension of non-polarized multiple dendritic shafts, although further investigation would be needed to identify such a cell polarity determinant. On the other hand, Src has been implicated in the regulation of cell adhesion and migration as well as in cytoskeletal organization (Thomas and Brugge, 1997). Thus, it is also possible that the deregulated activation of these pathways might cause the delayed migration of Purkinje cell bodies in n-Src transgenic mice. Indeed, we observed that Purkinje cells from n-Src transgenic mice developed large growth-cone-like structures in primary culture, which might affect the cell adhesion and/or migration of Purkinje cells in the molecular layer.

An ultrastructural analysis revealed that the arrangement of microtubules was impaired in n-Src transgenic Purkinje cells. The microtubule density was higher in transgenic mice than in controls, and some microtubules were misaligned and randomly bent, possibly due to a structural deformity. Src is involved in the regulation of microtubule assembly by directly phosphorylating tubulin (Maness and Matten, 1990), and Src associates with microtubule-associated protein 2 (MAP2) (Lim and Halpain, 2000) and with γ-tubulin (Kukharskyy et al., 2004). These findings suggest that Src regulates microtubule organization by directly modulating the microtubule components, although the precise function of Src in these mechanisms remains to be clarified. The findings also suggest that the aberrant dendritic morphogenesis in Purkinje cells over-expressing n-Src is due to this abnormal microtubule organization. Recently, it has been shown that Src activates the phosphatidylinositol 3-kinase (PI3K)-mediated pathway to regulate neurite extension in a neural-cell line (Pan et al., 2005). PI3K is involved in the cell signaling underlying neurite morphogenesis, including polarization, elongation, guidance, and axon branching (Gallo and Letourneau, 1998; Ming et al., 1999). A number of signaling molecules with the potential to regulate the cytoskeleton, such as Akt, integrin-linked kinase (ILK), and GSK-3β, are downstream mediators of PI3K (Cantley, 2002). Thus, it is possible that excessive activation of the PI3K pathway in n-Src transgenic mice may affect the arrangement of microtubules. Furthermore, Src is located upstream of small GTPases, such as Rac, Rho, and Cdc42, that play critical roles in cytoskeletal organization (Takai et al., 2001; Sato et al., 2005). The contribution of the small GTPase-mediated pathway should be extensively examined to elucidate the function of n-Src in dendritic morphogenesis. To define the pathway mediated by n-Src, we are now in the process of identifying n-Src-specific substrate proteins in Purkinje cells.

Src activity is precisely regulated during dendritic morphogenesis of Purkinje cells. The amount and activity of Src protein are both dramatically elevated in the developing cerebellum, but they are substantially down-regulated with maturation, suggesting that Src activity should be turned off when neural development is completed, and that the failure to down-regulate Src would cause a delay in development and aberrant dendritic morphogenesis, as was observed in this study. Negative regulatory systems, including Csk-mediated inactivation (Nada et al., 1991; Nada et al., 1993) and ubiquitination-mediated

degradation (Harris et al., 1999), have been implicated in the down-regulation of Src function. To fully understand how neural development is regulated by Src, the mechanisms that precisely control Src activity need to be clarified.

Acknowledgements

We thank members of the Genome Information Research Center (Osaka University) for the generation of transgenic lines, T. Yamamoto for the generous gift of human *fyn* cDNA, and Y. Wada for the maintenance of transgenic lines. This work was supported by a grant-in-aid for Scientific Research of Priority Areas, Cancer, from the Ministry of Education, Culture, Sports, Science and Technology, and by a health science research grant from the Research on Advanced Medical Technology, nano-001 to N.M.

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