Pak1 Regulates Dendritic Branching and Spine Formation

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ABSTRACT: **The serine/threonine kinase p21-activated kinase 1 (Pak1) modulates actin and microtubule dynamics. The neuronal functions of Pak1, despite its abundant expression in the brain, have not yet been fully delineated. Previously, we reported that Pak1 mediates initiation of dendrite formation. In the present study, the role of Pak1 in dendritogenesis, spine formation and maintenance was examined in detail. Overexpression of constitutively active-Pak1 in immature cortical neurons increased not only the number of the primary branching on apical dendrites but also the number of basal dendrites. In contrast, introduction of dominant negative-Pak caused a reduction in both of these morphological features. The length and the number of secondary apical branch points of dendrites were not significantly different in cultured neurons expressing these mutant forms, suggesting that Pak1 plays a role in dendritogenesis. Pak1 also plays a role in the formation and maintenance of spines, as evidenced by the altered spine morphology, resulting from overexpression of mutant forms of Pak1 in immature and mature hippocampal neurons. Thus, our results provide further evidence of the key role of Pak1 in the regulation of dendritogenesis, dendritic arborization, the spine formation, and maintenance.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 655–669, 2007

Keywords: **Pak1; dendritic branching; spine formation; actin; cytoskeletal dynamics**

INTRODUCTION

During development, migrating neurons initiate formation of dendrites and axons, which then elongate and branch to form complex dendritic arborizations. During maturity, neurons continue to generate new spines and maintain old ones on these dendritic arbors. The regulation of dendritic dynamics is an essential aspect of neuronal structural plasticity and CNS function.

Rho family GTPases including RhoA, Rac1, and Cdc42 are known to be key regulators of the cytoskeletal dynamics underlying structural plasticity. RhoA exerts a negative influence on dendritic development by blocking dendritic initiation and reducing branching and growth. In contrast, Rac1 and Cdc42 promote dendritic initiation and facilitate branching (Redmond and Ghosh, 2001). Furthermore, Rho GTPases signaling cascades have been implicated in the process of spine

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formation. For example, overexpression of constitutively active (CA)-RhoA causes a decrease in spine density and a shortening of spine necks, whereas inhibition of RhoA or Rho kinase results in increased spine length (Tashiro et al., 2000). Moreover, CA-Rac1 transgenic mice exhibit more and smaller spines (Luo et al., 1996), while dominant negative (DN)-Rac1 overexpression results in fewer and longer spines (Nakayama et al., 2000; Tashiro and Yuste, 2004).

The protein kinase p21-activated kinase 1 (Pak1) is an important downstream effector that is bound and activated by Rac1 and Cdc42 (Manser et al., 1994). Pak1 consists of an N-terminal regulatory region, which includes the *C*dc42/*R*ac1 *i*nteraction and *b*inding (CRIB) domain, and a C-terminal kinase domain (Bokoch, 2003). Association of Rac1/Cdc42 with the CRIB domain relieves Pak1 auto-inhibition, resulting in auto-phosphorylation of specific residues including T423, followed by activation of the protein kinase domain. Through this mechanism, Pak1 inducibly regulates actin cytoskeletal dynamics. For instance, microinjection of CA-Pak1 protein into cultured cells promotes rapid formation of filopodia and membrane ruffles (Sells et al., 1997), whereas DN-Pak attenuates the effects of CA-Rac1 or CA-Cdc42 (Zhao et al., 1998b). Furthermore, Pak1 expression induces neurite outgrowth in rat pheochromocytoma cells (Daniels et al., 1998).

Several studies have examined the effect of Pak1 activity on spine morphology. For example, DN-Pak transgenic mice have shorter but larger spines and lower spine density in adulthood (Hayashi et al., 2004). Furthermore, CA-Pak1 expression induces an increase in the number of spines, while kinase dead-Pak1 expression results in reduced spine number in cultured hippocampal neurons (Zhang et al., 2005). However, these studies do not address important aspects of spine formation and maintenance, including the effect of Pak1 on spine size during synaptogenesis and the influence of CA-Pak1 in mature spines. Previously we have shown that Pak1 functions as a downstream effector of Rac1 to regulate dendritic initiation in cortical neurons (Hayashi et al., 2002). Here, we assess the function of Pak1 in dendritic development, define its contribution to length and branching, and further delineate its role in spine formation and maintenance.

MATERIALS AND METHODS

DNA Constructs

Human Pak1 wild type and Pak1 T423E (CA-Pak1) in a pCMV6M expression vector (Sells et al., 1997) were kindly donated by Dr. J Chernoff. The DN-Pak (mouse Pak1 amino acid 67–150) cDNA fragment was amplified as previously described (Hayashi et al., 2002). The EF-myc-CA-Pak1-IRES-EGFP and EF-hemagglutinin (HA)-DN-Pak-IRES-EGFP vectors used for the dendritic structural analysis were generated as described previously (Hayashi et al., 2002). The cDNA of β-galactosidase (Amercham Pharmacia Biotech, Buckinghamshire, UK) was subcloned into a pEF-BOS vector (referred to as β-gal in this paper).

To generate pCAG-EGFP used in the spine formation studies, the cDNA of EGFP from pEGFP-N1 (Clontech, Palo Alto, CA) was inserted into pCAGGS that was kindly provided by Dr. Miyazaki (Niwa et al. 1991). To express both Pak1 mutant and the EGFP in the same cells, bi-expression vectors, pCAG-GFP-CAG-myc-CA-Pak1, and pCAG-GFP-CAG-HA-DN-Pak were generated as follows. To generate pCAG-EGFP-CAG (pCGC) a 2.3-kb fragment, which contained the CAG promoter and the *EcoRI* site, was amplified with pCAGGS by PCR, and inserted into the *HindIII* site downstream of the EGFP gene in pCAG-EGFP. Mutant Pak1 cDNA was subcloned into the *EcoRI* site of the pCGC. These constructs were verified by DNA sequencing, and protein productions were confirmed by Western blot analysis.

Adenoviral Vectors

The adenoviral vector is based on human adenovirus type 5 (Ad5), rendered replication incompetent by deletion of the E1A, E1B, and E3 regions of the genome. The adenoviral vectors AdexCAG-CA-Pak1-IRES-EGFP and AdexCAG-DN-Pak-IRES-EGFP used in the spine maintenance studies were constructed as follows. myc-CA-Pak1 fragment or HA-DN-Pak fragment was prepared as previously described (Hayashi et al., 2002), and inserted into the *SwaI* cloning site of pAdex1pCAG-swaI-IRES-EGFP (Zhao et al., 1998a), named pAdexCAG-CA-Pak1-IRES-EGFP and pAdexCAG-DN-Pak-IRES-EGFP, respectively. The adenoviral vectors, AdexCAG-CA-Pak1-IRES-EGFP and AdexCAG-DN-Pak-IRES-EGFP were derived from pAdexCAG-CA-Pak1-IRES-EGFP and pAdexCAG-DN-Pak-IRES-EGFP using a modification of the COS-TPC method (Miyake et al., 1996). Each clone was checked by restriction enzymatic digestions and PCR for E1A (Zhao et al., 1998a) to confirm the lack of inclusion of no parent adenoviruses (Ad5). Adenoviral vectors were purified and concentrated by double cesium step gradient centrifugation (Hashimoto et al., 1996).

Immunoblot Analysis and *In Situ* Hybridization

The samples of cortices from E16.5, P0 or adult (8 week old) mice were prepared as described previously (Hayashi et al., 2002) and subjected to immunoblot analysis. Rabbit polyclonal anti-αPak (Pak1) antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect Pak1, followed by peroxidase-conjugated secondary antibody. The signal was visualized with an enhanced chemilumines-

cence detection system (Amercham Pharmacia Biotech). The blots were quantitatively analyzed with NIH image or ImageJ. *In situ* hybridization was performed as described previously (Ohshima et al., 2001).

Culture of Dissociated Neurons

E16.5 cortical neurons obtained from pregnant ICR mice (Japan SLC, Shizuoka, Japan) were cultured as described previously (Hayashi et al., 2002), with slight modifications. Briefly, the cerebral cortex of E16.5 mouse was excised, incubated with 0.25% trypsin in PBS for 10 min at 37°C, dissociated, and washed with neurobasal medium (GIBCO BRL, Life Technologies, Rockville, MD) supplemented with penicillin G, and streptomycin. The dissociated cells were cultured on the glass area of poly-L-lysine (Sigma, St. Louis, MO)-coated glass-bottom dishes (Matsunami, Osaka, Japan), at 3.8×10^5 cells/cm^2 in neurobasal medium supplemented with 2% B-27 (Invitrogen Life Tech., Carlsbad, CA), penicillin G, and streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Primary hippocampal neurons were prepared from E18 Wistar rat (Japan SLC, Shizuoka, Japan) or P0 ICR mice (Japan SLC). Hippocampi were excised and treated with 45 U of papain (Worthington, PAPL, Lakewood, NJ), 0.01% DNase I (Boehringer-Mannheim, Indianapolis, IN), 0.02% L-cysteine, 0.02% bovine serum albumin (BSA), and 0.5% glucose in PBS for 10 min at 30°C. After papain treatment, 20% FBS was added to stop the papain reaction. Then, cells were dissociated, washed and plated at a density of 1.1×10^4 cells/cm^2 onto poly-L-lysine-coated glass coverslips (Asahitechno-glass, Tokyo, Japan) in neurobasal medium with 2% B-27 supplement. Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Half the volume of culture medium was changed to fresh neurobasal medium once a week.

Introduction of Plasmid DNAs and Infection of Adenovirus into Neurons

To introduce the vectors that express mutant forms of Pak1 into immature neurons, the *in utero* electroporation method was performed as described previously (Hayashi et al., 2002). At 24 h after *in utero* electroporation, the cortex of each embryo was excised from the rest of the brain. The fluorescent area of the cerebral cortex was dissected out under a fluorescence microscope, and dissociated. Dissociated cells electroporated with mutant Pak1 plasmid DNAs and those with β-gal plasmid were mixed in equal volumes, and cultured on poly-L-lysine-coated glass-bottom dishes at a density of 3.8×10^5 cells/cm^2 in neurobasal medium supplemented with 2% B-27, penicillin G, and streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. As a control for the effect of the reporter gene, neurons transfected with plasmid encoding GFP alone were also co-cultured with β-gal-transfected cells and analyzed in each parameter.

Ca^{2+}-phosphate-mediated transfection was performed to introduce the Pak1 plasmid, pCAG-GFP-CAG-myc-CA-Pak1, pCAG-GFP-CAG-HA-DN-Pak, or pCGC for control, into primary hippocampal neurons at 11 days *in vitro* (DIV) as described previously (Kohrmann et al., 1999). To express mutant Pak1 in mature primary hippocampal neurons, adenovirus, AdexCAG-CA-Pak1-IRES-EGFP, AdexCAG-DN-Pak-IRES-EGFP, or AdexCAG-IRES-EGFP for control was added to the cultures and the medium was replaced with fresh neurobasal medium just before the infection. After 1-h incubation, the infection medium was replaced with the preceding neurobasal medium. After another 2 days of incubation, the cultured neurons were fixed and subjected to analysis.

Immunocytochemistry and DiI Staining

Cultured cortical or hippocampal cells were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffered (pH 7.4) for 10 min at room temperature, washed with PBS three times, permeabilized and blocked with blocking buffer (1% BSA, and 0.1% Triton X-100 in PBS) at room temperature for 1 h. The cells were then incubated with the primary antibody at room temperature for 1 h, followed by Alexa488 (1:5000), Alexa594 (1:3000), or Alexa633 (1:1000) secondary antibody (Molecular Probes, Eugene, OR). The following primary antibodies were used in this study: mouse monoclonal anti-myc antibody (9E10) (1:200, Santa Cruz), mouse monoclonal anti-HA antibody (12CA5) (1:200, Roche), rabbit polyclonal anti-β-galactosidase antibody (1:400, Harmaceuticals, Aurora, OH), rabbit polyclonal anti-GFP antibody (1:500, MBL, Nagoya, Japan), rabbit monoclonal anti-p-Pak1 antibody (1:100, kindly donated by Dr. J. Chernoff) (Sells et al., 2000), mouse monoclonal anti-neuron-specific class III β-tubulin antibody (1:500, CHEMICON, CA), and mouse monoclonal anti-PSD95 antibody (1:100, Upstate Biotechnology). F-actin was visualized by the use of Alexa594-conjugated phalloidin (Molecular Probes). Images were quantitatively analyzed with ImageJ. For the quantitation of mutant Pak1 expression level, fixed cells were incubated with anti-Pak1 antibody, followed by Alexa594 secondary antibody. Stained neurons were imaged by a laser-scanning confocal microscope. Fluorescent intensity by Pak1 staining on transfected neurons or nontransfected ones were analyzed to confirm the expression level of exogenous Pak1.

For 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) labeling of neurons, cells were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffered (pH 7.4) for 10 min at room temperature, washed with PBS three times, and incubated with a suspension of crushed crystals of compound name (DiI, Molecular Probe) in PBS (0.4 mg/mL) at 37°C for 1 min, according to Allison et al. (2000). The labeled cells were washed three times and imaged.

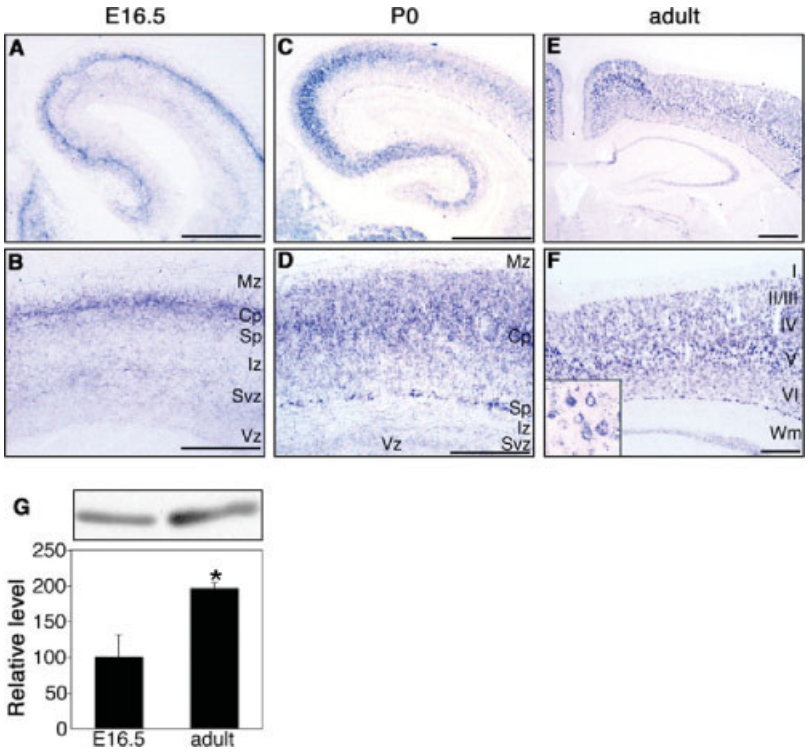

Figure 1

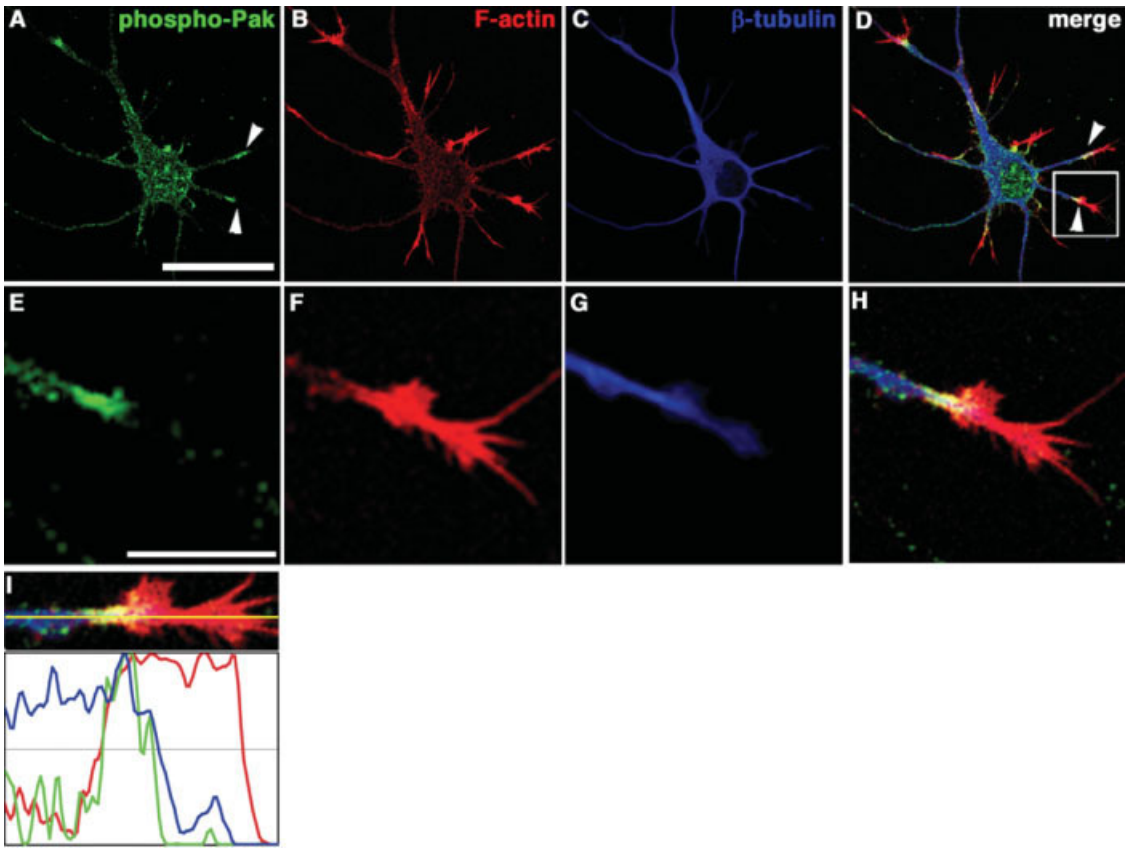

Figure 2

Imaging and Analysis

Cell images were acquired with a laser-scanning confocal microscope based on a Fluoview FV500 scanning unit (Olympus, Tokyo, Japan). EGFP and Alexa488 were excited at 488 nm with an Ar laser and detected through the band-pass filter BA505-525 (Olympus), whereas Alexa594 and DiI were excited at 543 nm with a HeNe (G) laser and detected through the band pass filter BA560IF (Olympus). Alexa633 was excited at 633 nm with the HeNe (R) laser and detected through band pass filter BA560IF (Olympus). Images were stacked with several z-section images acquired at 1 μm intervals, which included all fluorescent-positive areas in the neurons. All GFP-positive or DiI-positive neurons with pyramidal morphology on the dishes were quantitatively analyzed for dendrite or spine morphology with MetaMorph software. In every experiment, pyramidal neurons were defined as cells that exhibited a pyramidal cell body with one major dendrite that gradually tapered from the soma (typical apical dendrites *in vivo*). The main shaft of each apical dendrite was defined as the major projection from the cell body, and a primary apical branch, previously referred to as "initiation of secondary apical dendrite" in our work, was defined as branch on this main shaft. A secondary apical dendrite was defined as a dendrite that emerged from the main shaft and secondary apical branching was defined as branching from the secondary apical dendrite. The basal dendrite was defined as the dendrite which extended from the cell body in the opposite direction from the major apical dendrite. *n* in dendrite analyses shows the number of neurons examined. All parameters analyzed were measured for each neuron. These experiments were performed blindly.

For all spine analyses, the region of the apical dendrite after the first branch point was selected. The spine length was measured from the base of the dendrite to the tip of the protrusion, and maximum spine head diameter was used to define spine width. These experiments were performed blindly. Data were shown as means ± SEM. Statistically significant differences between CA-Pak1 or DN-Pak and the control (Student's *t* test, $p < 0.05$) are indicated by asterisks in the bar graphs.

RESULTS

Pak1 Is Expressed in Mouse Brain During Development and at Maturity

In our previous study, the expression pattern of Pak1 during development was examined (Hayashi et al., 2002). To determine if Pak1 contributes to structural plasticity at neuronal maturity as well as during development, further analyses of Pak1 expression in brain were conducted. Expression of the kinase by *in situ* hybridization analysis was evaluated in E16.5 embryos (embryonic day 16.5), newborn (postnatal day 0, P0 mice), and adult (8 week old) mice [Fig. 1(A–F)]. Pak1 was expressed in both the cerebral cortex and the hippocampus at all ages examined [Fig. 1(A–F)]. At E16.5 and P0 Pak1 was strongly expressed in the cortical plate, but not in the ventricular zone [Fig. 1(A–D)]. High Pak1 expression was observed throughout the cerebral cortex of adult mice, especially in pyramidal neurons of layer V and in the pyramidal cell layer of the hippocampus [Fig. 1(E,F)]. Thus, Pak1 was expressed early in cortical neurons, and these cells continued to express the kinase as they migrated and matured. To assess how

Figure 1 Analysis of endogenous Pak1 expression in mouse brain. A–F: *In situ* hybridization of Pak1 at E16.5 (A and B), P0 (C and D), and in an adult (E and F) mouse brain. The area circumscribed by the square in F shows the cells in layer V (five-times magnification). Scale bar in A, C, and E: 500 μm; B, D, and F: 200 μm. Mz, marginal zone; Cp, cortical plate; Sp, subplate; Iz, intermediate zone, Svz, sub-ventricular zone; Vz, ventricular zone; I–VI, laminar I–VI of cerebral cortex; Wm, white matter. G: protein levels of Pak1 in the embryo and adult cerebral cortex. Twenty micrograms of homogenate protein of each cortex was subjected to SDS-PAGE and analyzed by immunoblotting with anti-Pak1 antibody. Quantification of multiple experiments is shown. The level of Pak1 protein in E16.5 was normalized to 100. Each bar represents a mean ± SEM. Statistical significance is indicated by asterisks: $*p < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2 Analysis of phospho-Pak localization in cortical neurons. A–H: The primary cultured cortical neurons at 5 DIV were immunostained with anti-phospho-Pak (pT423 Pak, green) (A, D, E, H), phalloidin-Texas Red for F-actin (red) (B, D, F, H), and anti-neuronal class III β-tubulin (TuJ1) (blue) (C, D, G, H). The area circumscribed by the square in D showed image E–H. Arrowheads in A and D show phospho-Pak accumulation, which was at the base of the growth cone. The tips of stained neurons were quantitatively analyzed to show phospho-Pak localization (I). Scale bar in A: 50 μm; in E: 10 μm. Images A–D or E–H are at the same magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mRNA expression and distribution were reflected in Pak1 protein levels, immunoblot analysis of dissected cortical tissue homogenates was also conducted [Fig. 1(G)]. Cortex from adult mice contained twice as much Pak1 than cortex from E16.5 embryos. Taken together these data suggest that Pak1 functions in cortical neurons both during development and at maturity.

Phospho-Pak Localizes at the Base of Dendritic Growth Cone in Mouse Cortical Neurons

Previously we showed that total Pak1 is expressed ubiquitously in the cytoplasm, and the strongest signal for phospho-Thr423 Pak occurs at the distal ends of dendrites on cultured cortical neurons at 5 DIV [Hayashi et al., 2002 and Fig. 2(A,E)]. To more carefully analyze phospho-Pak localization, cortical neurons stained for activated Pak were also counterstained for cytoskeletal element using the actin microfilament binding protein phalloidin and an antibody to the neuron-specific microtubule marker, neuronal class III β-tubulin [Fig. 2(B–D, F–H)]. Activated Pak co-localized with microtubules in the cell soma and dendritic processes (blue) but not the nucleus [Fig. 2(D,H)]. Interestingly, quantitative analysis showed that while phospho-Pak was not detected in the tip of the dendritic growth cones labeled by phalloidin (red), it did colocalize with F-actin and β-tubulin at the base of these structures [Fig. 2(I)]. These findings further implicate Pak activity in the process of dendritogenesis.

Pak1 Regulates the Numbers of Basal Dendrites and Primary Apical Branching

To investigate the functions of Pak1 in dendritic development, mutant forms of Pak1 were introduced into embryonic neuronal progenitors by *in utero* electroporation, and the effects on dendritic morphology, including number, length, and branching (as was previously examined, Hayashi et al., 2002), were quantitated in primary cultures. Recombinant DNA plasmids that expressed β-gal or myc-tagged CA-Pak1 together with GFP, via an internal ribosomal entry site (IRES) element, were used to transfect cells attached to the lateral ventricle of E15.5 embryonic brains. After 24 h, the targeted cells were harvested and β-gal- and CA-Pak1-transfected cells were combined for culturing. Neurons were fixed at 5 DIV and immunostained with anti-myc and anti-β-galactosidase or anti-Pak1 antibodies. The level of Pak1 was 2.5-times higher in transfected GFP-positive cells than in untransfected GFP-negative neurons (data not shown, $p < 0.001$). As a control for the effect of the reporter gene, neurons transfected with plasmid encoding GFP alone were also co-cultured with β-gal-transfected cells, and no significant difference was appreciated in all parameters (data not shown). In contrast, neurons expressing CA-Pak1 appeared to have more dendrites [Fig. 3(B)] than those expressing β-gal [Fig. 3(A)].

Counting the number of branch points on both apical and basal dendrites of neurons expressing CA-Pak1 or β-gal revealed an increase in the number of total branch points on apical dendrites of neurons with CA-Pak1 (CA-Pak1: 6.02 ± 2.67, β-gal: 4.81 ± 2.52, $p < 0.05$) [Fig. 3(C)]. Further subclassification of the branch points unveiled an interesting difference. Primary branch points were defined as those occurring on the main dendritic shaft [arrow in Fig. 3(B)], while secondary branch points included all subsequent branching [arrowhead in Fig. 3(B)]. In response to CA-Pak1 expression, number of primary branch points on apical dendrites was significantly increased (CA-Pak1: 5.58 ± 0.21; β-gal: 4.62 ± 0.23, $p < 0.005$) [Fig. 3(D)], but the number of secondary branch points was not statistically altered

Figure 3 Effect of CA-Pak1 on dendritic development. Cortical neurons electroporated with β-galactosidase or CA-Pak1 were cultured in the same dish and immunostained at 5 DIV (A and B). Neurons electroporated with CA-Pak1 were compared to those with β-gal in regards to total number of branch points on apical dendrites (C), the number of primary branch points on apical dendrites (D), the number of secondary and following branch points on apical dendrites (E), total dendritic length per apical dendrite (F), the average length of the main shaft (G), the average length per secondary apical dendrite (H), the number of basal dendrites (I), the number of branch points on basal dendrites (J), and the average length per basal dendrite (K). Scale bar in B: 50 μm. Images A and B are at the same magnification. The arrow and the arrowhead in B show primary and secondary branching, respectively. Each bar represents a mean $\pm$ SEM. Statistical significance is indicated by asterisks: $^*p < 0.05$, $^{**}p < 0.01$. Each culture was repeated three times independently and the representative data are shown (Control $n = 50$ neurons counted; CA-Pak1 $n = 53$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(CA-Pak1: 0.30 ± 0.05, β-gal: 0.35 ± 0.06) [Fig. 3(E)]. In addition, there was no change in the total length of the apical dendrite (CA-Pak1: 274.2 ± 15.3 μm, β-gal: 254.2 ± 15.4 μm) [Fig. 3(F)], the average length of the main shaft of the apical dendrite (CA-Pak1: 58.9 ± 5.0 μm, β-gal: 58.7 ± 2.7 μm) [Fig.

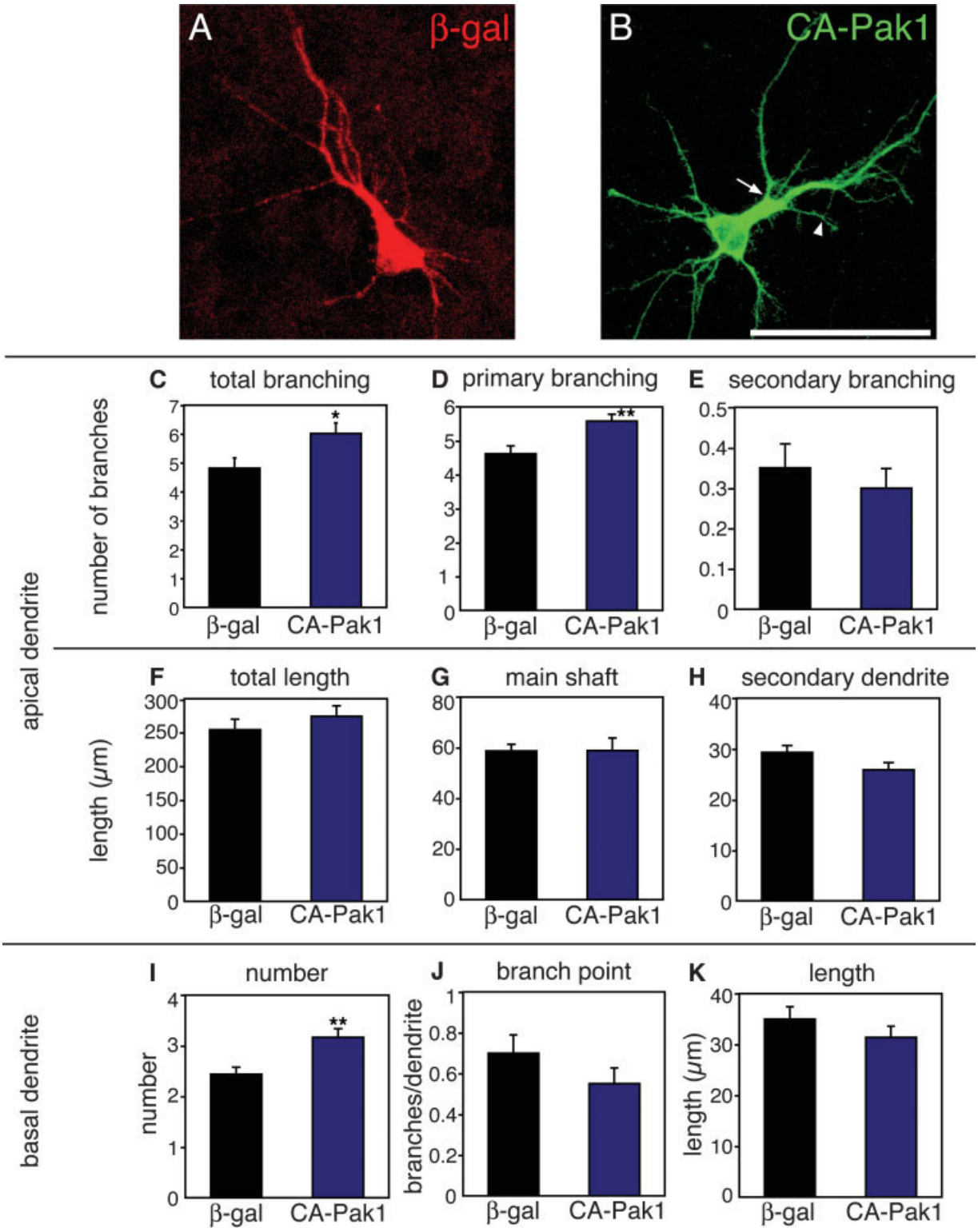

Figure 3

3(G)], or the average length of secondary apical dendrites (CA-Pak1: 25.9 ± 1.4 μm, β-gal: 29.3 ± 2.2 μm) [Fig. 3(H)]. While, the number of basal dendrites was increased in neurons expressing CA-Pak1 (CA-Pak1: 3.17 ± 0.17, β-gal: 2.44 ± 0.14, $p < 0.005$) [Fig. 3(I)], there was no difference in the number of branch

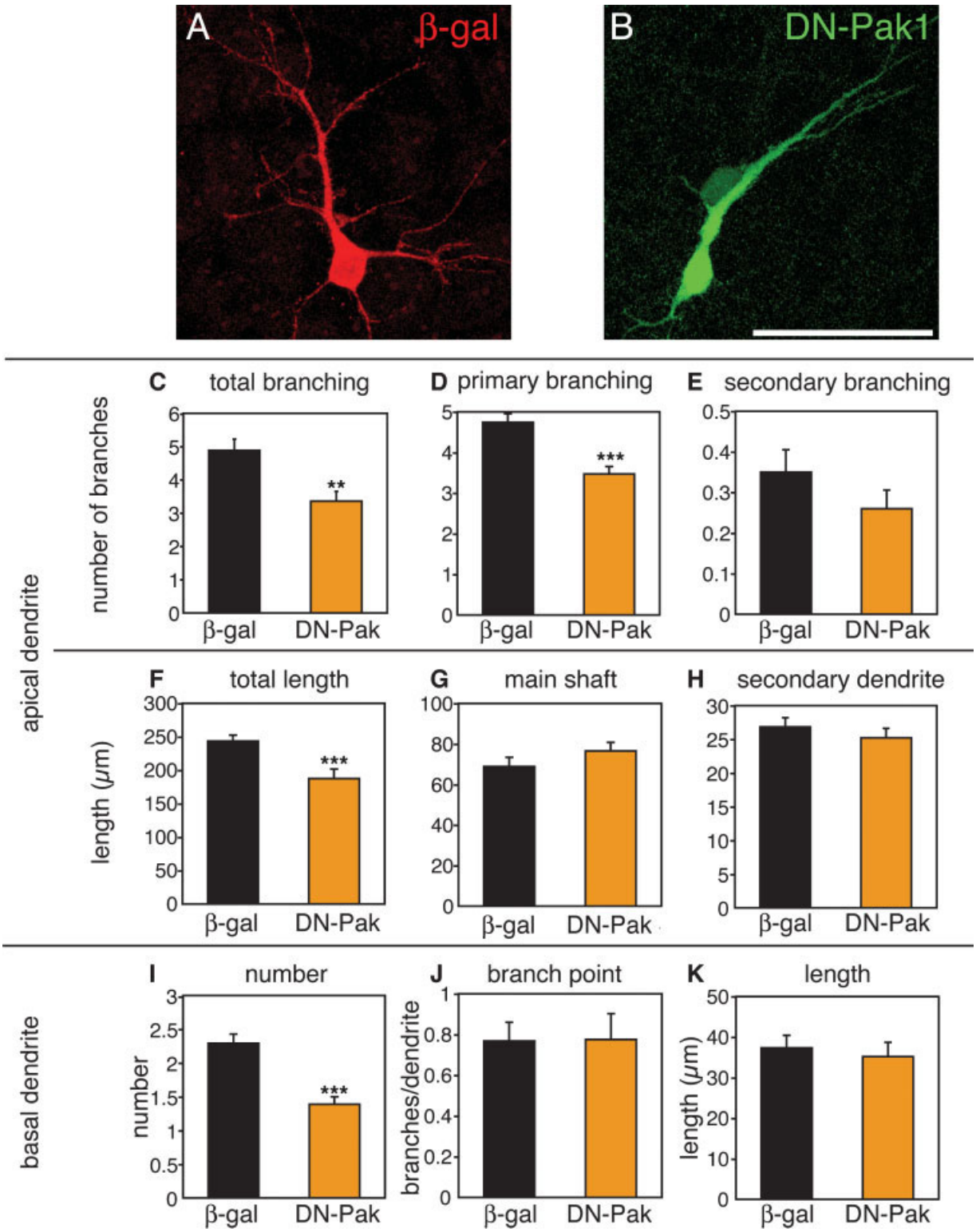

Figure 4

points per basal dendrite (CA-Pak1: 0.55 ± 0.08, β-gal: 0.70 ± 0.09) [Fig. 3(J)] and the average length per basal dendrite (CA-Pak1: 31.4 ± 2.2 μm, β-gal: 35.0 ± 2.4 μm) [Fig. 3(K)]. These data suggest that Pak1 activity is associated with sprouting or the emergence of new dendrites, as indicated by the increase in the number of basal dendrites and primary apical branch points in response to CA-Pak1 expression.

In the reverse experiment, embryonic neuronal progenitors were transfected with β-gal or with DN-Pak, which inhibits Pak1, Pak2, and Pak3 activity, and cultured to 5 DIV. In contrast to CA-Pak1, expression of DN-Pak resulted in cultured cells that clearly had fewer dendrites [Fig. 4(A,B)]. Statistical analysis revealed that DN-Pak-positive neurons had a decreased number of total branch points on apical dendrites when compared to neurons electroporated with β-gal (DN-Pak: 3.36 ± 2.34, β-gal: 4.88 ± 2.43, $p < 0.01$) [Fig. 4(C)]. Subclassification of the branch points revealed that the number of primary branch points was decreased (DN-Pak: 3.47 ± 0.18, β-gal: 4.74 ± 0.21, $p < 0.001$) [Fig. 4(D)], but not the number of secondary branch points (DN-Pak: 0.26 ± 0.05, β-gal: 0.35 ± 0.06) [Fig. 4(E)]. Despite the decrease in total dendritic length of apical dendrites in neurons expressing DN-Pak (DN-Pak: 187.6 ± 14.3 μm, β-gal: 243.7 ± 8.8 μm, $p < 0.001$) [Fig. 4(F)], there was no difference in the average length of the main shaft of apical dendrites (DN-Pak: 76.6 ± 4.1 μm, β-gal: 68.9 ± 4.6 μm) [Fig. 4(G)] or the average length per secondary apical dendrite (DN-Pak: 25.2 ± 1.4 μm, β-gal: 26.9 ± 1.4 μm) [Fig. 4(H)].

Expression of DN-Pak also resulted in a decreased number of basal dendrites (DN-Pak: 1.39 ± 0.11, β-gal: 2.26 ± 0.14, $p < 0.001$) [Fig. 4(I)], but not in the number of branch points per basal dendrite (DN-Pak: 0.78 ± 0.13, β-gal: 0.77 ± 0.09) [Fig. 4(J)] or the average length per basal dendrite (DN-Pak: 35.2 ± 3.6 μm, β-gal: 37.4 ± 3.1 μm) [Fig. 4(K)]. These results indicate that the expression of DN-Pak, similar to CA-Pak1, strongly influenced the number of basal dendrites and the primary branch points, but had no effect on apical dendritic length and secondary branching of apical dendrites.

Pak1 Is Localized at Dendritic Spines in Hippocampal Neurons

Several studies have demonstrated the involvement of Pak1 in spine formation (Penzes et al., 2003; Hayashi et al., 2004; Zhang et al., 2005). However, the relative contribution of Pak1 to the structural maintenance of mature spines remains unclear. To address this question, the localization of Pak1 in mature hippocampal neurons was assessed. Cultured mouse hippocampal neurons at 20 DIV were immunostained with anti-Pak1 antibody [Fig 5(A,D–H), green], phalloidin [Fig 5(B,D,F,H), red], and anti-PSD95 antibody, which localizes specifically to dendritic spines [Fig 5(C,D,G,H), dark blue]. Pak1 was localized throughout the cytosol, even within spines [Fig. 5(A,E)]. Pak1 co-localized with F-actin in throughout [Fig. 5(D,F,H), yellow], and with PSD95 in dendritic spines [Fig. 4(D,G,H), light blue], suggesting that Pak1 functions in mature spines.

Pak1 Regulates Formation of Spines

To further evaluate the contribution of Pak1 to spine formation, dual gene promoter expression vectors were engineered to express either CA-Pak1 or DN-Pak, together with high levels of GFP. Each of these plasmids, as well as a control plasmid expressing only GFP, was used to transfect primary cultured hippocampal neurons at 11 DIV. At 18 DIV, the cultures were stained with anti-GFP antibody and anti-PSD95 as a marker for spines. There were no differences in PSD95 localization between control, CA-Pak1, or DN-Pak transfected neurons (data not shown). Initial observation revealed that neurons expressing CA-Pak1 appeared to have smaller spines in comparison

Figure 4 Effect of DN-Pak on dendritic development. Cortical neurons electroporated with β-galactosidase or DN-Pak were cultured in the same dish and immunostained at 5 DIV (A and B). Neurons electroporated with DN-Pak were compared to those with β-gal in regards to total number of branch points on apical dendrites (C), the number of primary branch points on apical dendrites (D), the number of secondary and following branch points on apical dendrites (E), total dendritic length per apical dendrite (F), the average length of the main shaft (G), the average length per secondary apical dendrite (H), the number of basal dendrites (I), the number of branch points on basal dendrites (J), and the average length per basal dendrite (K). Scale bar in B: 50 μm. Images A and B are at the same magnification. Each bar represents a mean ± SEM. Statistical significance is indicated by asterisks: $^{**}p < 0.01$, $^{***}p < 0.001$. Each culture was repeated three times independently and the representative data are shown (control $n = 50$ neurons counted; DN-Pak, $n = 64$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

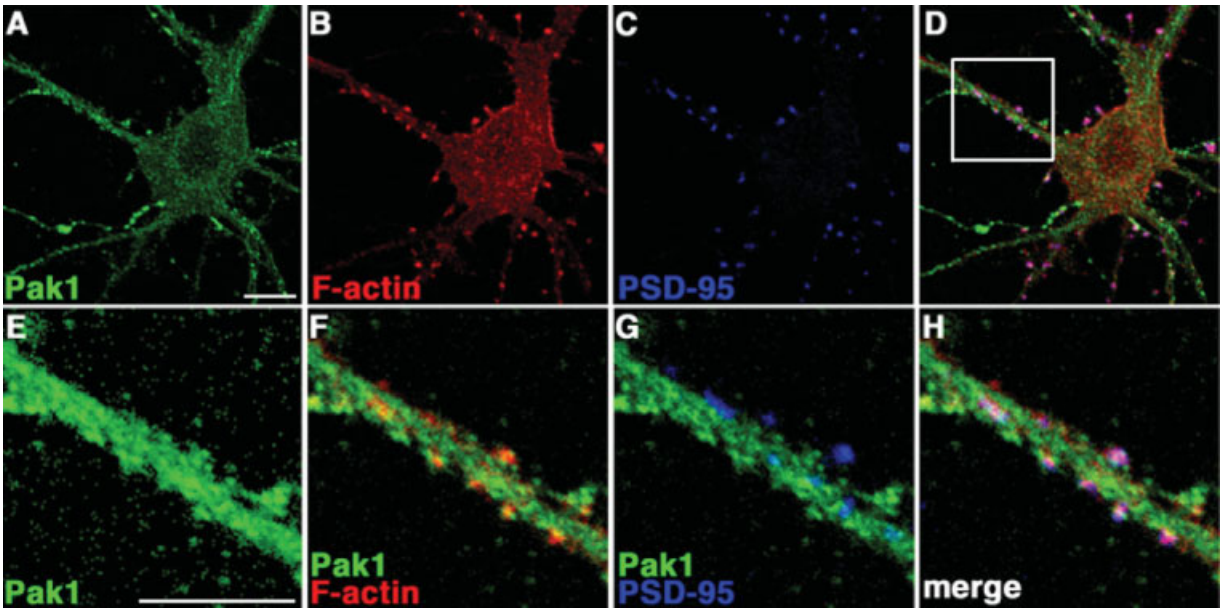

Figure 5

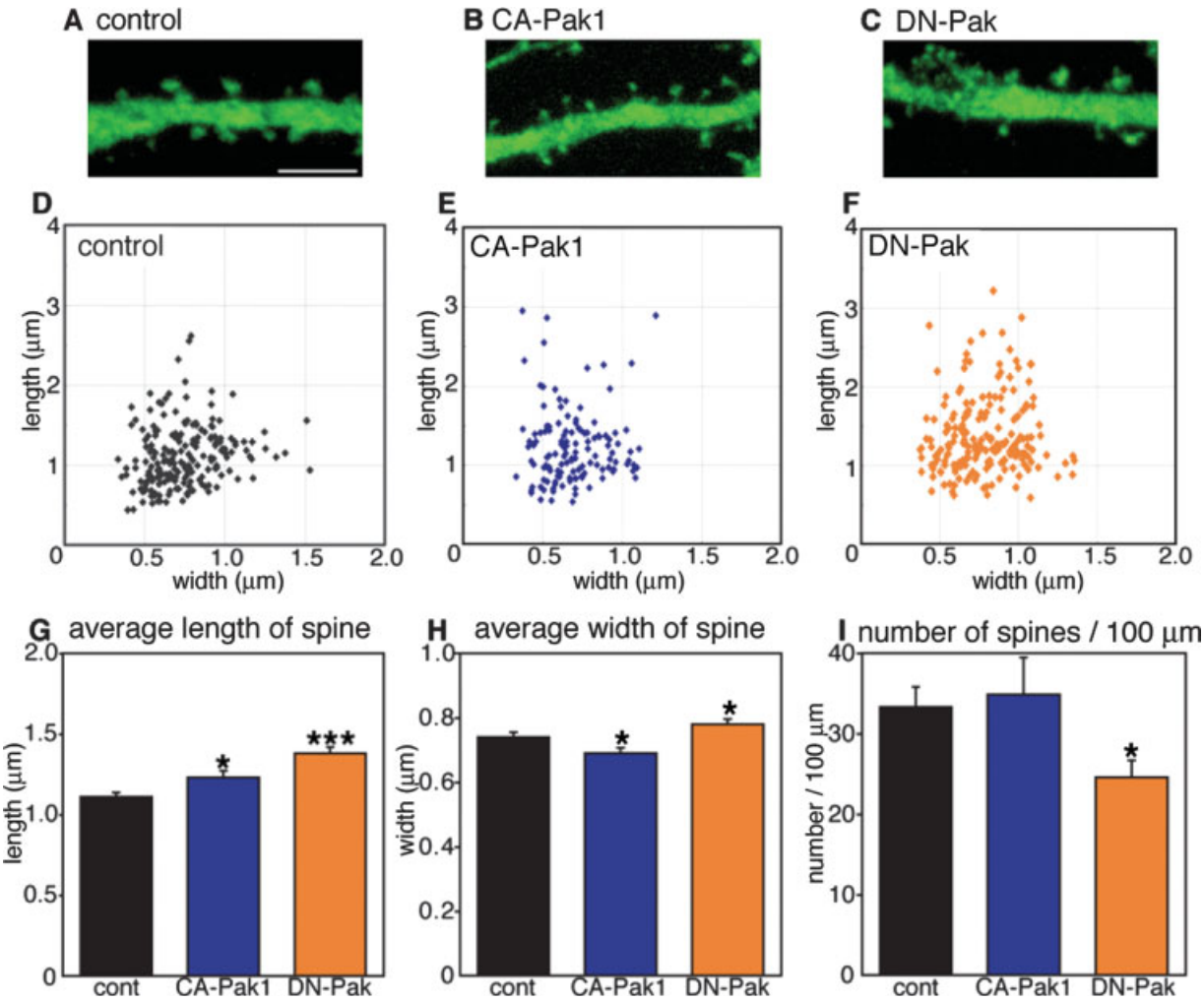

Figure 6

to controls [Fig. 6(A,B)]. In contrast, neurons with DN-Pak appeared to have slightly larger spines [Fig. 6(C)].

To quantify the effect of Pak1 on spine morphology, the length of spines and the width of spine heads were measured using confocal microscopic images. As shown in the plots of spine length *versus* head width [Fig. 6(D–F)], expression of CA-Pak1 caused a shift toward spines with less width. On the other hand, expression of DN-Pak caused a diffusion of the population toward longer spines with larger heads, in comparison to controls. Quantitation showed that spines with CA-Pak1 were significantly longer and exhibited narrower heads in comparison to controls (length: CA-Pak1 1.23 $\pm$ 0.04 μm, control 1.11 $\pm$ 0.03 μm, $p < 0.05$; width: CA-Pak1 0.69 $\pm$ 0.02 μm, control 0.74 $\pm$ 0.02 μm, $p < 0.05$) [Fig. 6(G,H)]. In contrast, DN-Pak expression induced longer spines with larger heads (length: DN-Pak 1.38 $\pm$ 0.04 μm, $p < 0.001$; width: DN-Pak 0.78 $\pm$ 0.02 μm, $p < 0.05$) [Fig. 6(G,H)]. Furthermore, expression of DN-Pak caused a significant reduction in spine density (control 33.3 $\pm$ 2.5 spines/100 μm, DN-Pak 24.6 $\pm$ 2.1 spines/100 μm, $n = 38$, $p < 0.05$), while CA-Pak1 caused no such change (CA-Pak1 34.9 $\pm$ 4.6 spines/100 μm) [Fig. 6(I)]. These data further implicate Pak1 in the formation and shape of dendritic spines.

Pak1 Regulates the Maintenance of Spine Morphology

Since histological and immunoblot analyses demonstrated that Pak1 is most abundant in the adult brain [Fig. 1(G)], we examined the involvement of Pak1 in the maintenance of spine morphology. Adenoviral vectors were used to introduce mutant forms of Pak1 into mature hippocampal neurons to circumvent reduced transfection efficiency. The bidirectional lipophilic fluorescent tracer, DiI, was employed to facilitate clear resolution of spine morphology. At 21 DIV, primary cultured hippocampal neurons were infected with recombinant adenovirus expressing either CA-Pak1, DN-Pak, or GFP. Cultures were then fixed at 23 DIV, and the neurons were labeled with DiI and imaged.

As with developing neurons, mature hippocampal neurons expressing CA-Pak1 had smaller spines [Fig. 7(A,B)], and those expressing DN-Pak had larger spines when compared to control cells expressing GFP [Fig. 7(C)]. Plotting spine length *versus* spine width showed that expression of CA-Pak1 caused spines to shorten and exhibit narrower heads, and that expression of DN-Pak caused spines to exhibit widen heads [Fig. 7(D–F)]. Quantitative analysis revealed that spines on neurons with CA-Pak1 were shorter and narrower in width in comparison to controls (length: CA-Pak1 0.98 $\pm$ 0.02 μm, control 1.13 $\pm$ 0.02 μm, $p < 0.001$; width: CA-Pak1 0.66 $\pm$ 0.01 μm, control 0.93 $\pm$ 0.02 μm, $p < 0.001$) [Fig. 7(G,H)]. In contrast, DN-Pak caused spines to lengthen (length: DN-Pak 1.27 $\pm$ 0.03 μm, $p < 0.001$; width: DN-Pak 0.95 $\pm$ 0.02 μm) [Fig. 7(G,H)]. Furthermore, neurons with CA-Pak1 had a higher density of spines, while neurons with DN-Pak had a lower density of spines in comparison to control neurons (control 36.0 $\pm$ 2.4 spines/100 μm, CA-Pak1 47.9 $\pm$ 2.1 spines/100 μm, $p < 0.05$; DN-Pak 30.2 $\pm$

Figure 5 Immunocytochemistry of Pak1 in hippocampal neurons. Primary cultured hippocampal neurons at 20 DIV were immunostained with anti-Pak1 (green) (A, D–H), phalloidin-Texas Red for F-actin (red) (B, D, F, H), and anti-PSD-95 (blue) (C, D, G, H). The area circumscribed by the square in D showed Images E–H. Scale bars in A and E: 10 μm. Images A–D or E–H are at the same magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6 Pak1 functions in dendritic spine development. CA-Pak1 or DN-Pak with GFP was expressed in hippocampal neurons at 11 DIV. The culture was fixed at 18 DIV and stained with anti-GFP antibody, followed by Alexa 488 secondary antibody. A–C: Images of the spines in neurons with GFP alone (control, A), CA-Pak1 and GFP (B), or DN-Pak and GFP (C). Scale bar in A: 5 μm. Images A–C are at the same magnification. D–F: Plots of the distribution of spine length *versus* spine head width in neurons with GFP alone (control, D), CA-Pak1 and GFP (E), or DN-Pak and GFP (F). G–I: Quantitation of spine length (G), spine head width (H), and spine density (I) in neurons with GFP alone (cont, black bar), CA-Pak1 and GFP (blue bar), or DN-Pak and GFP (orange bar). Each bar represents a mean $\pm$ SEM. Statistical significance is indicated by asterisks: $*p < 0.05$, $***p < 0.001$. Each culture was repeated three times independently (Control, $n = 179$ spines counted from 37 neurons; CA-Pak1, $n = 127$ spines from 31 neurons; DN-Pak, $n = 197$ from 41 neurons). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

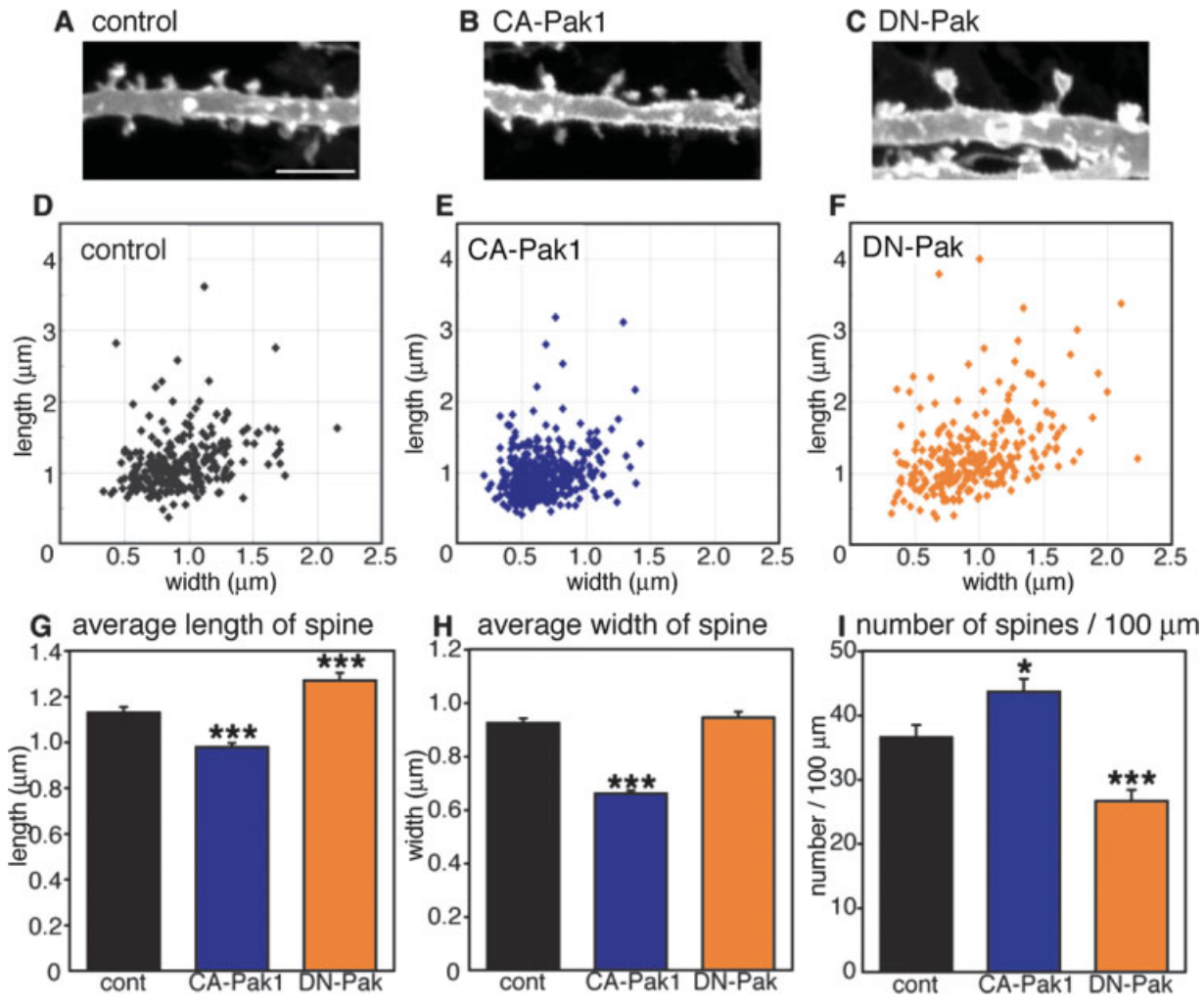

Figure 7 Pak1 functions in the maintenance of dendritic spine morphology. Hippocampal neurons at 21 DIV were infected with adenovirus vectors expressed CA-Pak1 or DN-Pak with GFP. The culture was fixed at 23 DIV and labeled with DiI. A–C: Images of the spines of neurons with GFP alone (control, A), CA-Pak1 and GFP (B), or DN-Pak and GFP (C). Scale bar in A: 5 μm. Images A–C are at the same magnification. D–F: Plots of the distribution of spine length *versus* spine head width in neurons transfected with GFP alone (control, D), CA-Pak1 and GFP (E), or DN-Pak and GFP (F). G–I: Quantitation of spine length (G), spine head width (H), and spine density (I) in neurons with GFP alone (cont, black bar), CA-Pak1 and GFP (blue bar), or DN-Pak and GFP (orange bar). Each bar represents a mean ± SEM. Statistical significance is indicated by asterisks: $*p < 0.05$, $***p < 0.001$. Each culture was repeated three times independently (Control, $n =$ 272 spines counted from 38 neurons; CA-Pak1, $n = 390$ spines from 37 neurons; DN-Pak, $n = 263$ from 41 neurons). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2.0 spines/100 μm, $p < 0.001$) [Fig. 7(I)]. These results suggest that Pak1 regulates the maintenance of spine morphology as well as its formation.

DISCUSSION

Pak1 was originally identified as a Ser/Thr kinase, which acts downstream of Rac1/Cdc42 in the brain (Manser et al., 1994). Recent *in vitro* and *in vivo* studies have begun to uncover the function of Pak1 in neurons (Hayashi et al., 2002; Hayashi et al., 2004; Penzes et al., 2003; Zhang et al., 2005). The studies reported here demonstrate that while Pak1 does not affect dendritic length, the branching of basal dendrites, or secondary branching on apical dendrites, it does regulate primary branching of apical dendrites and the number of basal dendrites. Furthermore, our

present findings directly implicate Pak1 in the regulation of dendritic spine formation and maintenance.

Involvement of Pak1 in Dendritogenesis

Pak1 is expressed in cortical neurons upon migration and elongation of their axons and dendrites. In addition, active Pak1 accumulates at the base of dendritic growth cone in close association with microtubule and actin cytoskeletal components, which is probably correlated with previous studies to have shown that Pak1 regulates both actin and microtubule dynamics (Sells et al., 1997; Edwards et al., 1999; Wittmann et al., 2004). Expression of CA-Pak1 in cortical neurons increased the number of basal dendrites and primary apical branch points, while expression of DN-Pak did the opposite. However, neither CA-Pak1 nor DN-Pak had a significant effect on other aspects of dendritic arborization, such as dendritic length, secondary apical branching, or basal branching. These results indicate that Pak1 regulates the numbers of basal dendrites and the first branching of apical dendrites. In other words, when Pak1 become active, it promotes initiation of dendrites and first branching, but not the extension of dendrites. In contrast, when Pak1 is inactive, it may prevent these dendritogenic processes.

Pak1 Controls Sprouting of Branches from the Main Dendrite but not Further Arborization

Apical dendritic arborization may be categorized into either primary branching, in which neurite processes extend from the main shaft, or secondary branching, in which neurite processes extend from the secondary dendrite. As part of this study, the role of Pak1 in these two distinct processes was assessed. Expression of mutant forms of Pak1 affected the number of primary branching on apical dendrites, but neither secondary branching nor the branching of basal dendrites were altered. As evidenced by this distinct effect of expression of mutant Pak1, the mechanism of primary branching must be different from that of secondary branching. In contrast, the lack of effect of mutant Pak1 expression on secondary apical branching and basal branching may indicate a shared mechanism. Primary apical branching is the equivalent of what has been previously described as "sprouting branching," while secondary apical branching and basal branching are equivalent to "bifurcation" (Whitford et al., 2002). Thus, Pak1 regulates sprouting branching, but not bifurcation.

Involvement of Pak1 in Spine Formation and Maintenance

Expression of Pak1 mutants had a dramatic effect on the morphology of hippocampal neuronal spines both during development and at maturity. Expression of DN-Pak resulted in bigger but fewer, while expression of CA-Pak1 resulted in smaller but more spines. Furthermore, spines on immature neurons expressing CA-Pak1 had longer and thinner spines, implying that Pak1 also regulates spine maturation. The lack of effect of CA-Pak1 on spine density in developing neurons may be due to the elevated level of endogenous Pak1 activity in spines at this stage. In concordance with previous reports that active Pak1 localized at spines (Penzes et al., 2003; Hayashi et al., 2004), total Pak1 also localized to spines in mature hippocampal neurons, suggesting that its involvement in spine function or maintenance. Indeed, mature neurons expressing mutant Pak1 change the spine shape and density. In support of these findings, neurons of transgenic mice, which express DN-Pak via the α-CamKII promoter, have been shown to have larger spines and decreased spine density (Hayashi et al., 2004). These studies indicate that proper activation of Pak1 is essential for the formation, maturation, and stability of dendritic spines. One unanswered question is how Pak1 affects the localization of neurotransmission machinery at the spine. The localization of PSD95 was unaffected by the expression of mutant forms of Pak1. Nonetheless, further studies using approaches such as electron microscopic analysis may be necessary to define the relationship between Pak1 activity and the recruitment of the PSD constituents required to mediate neurotransmission.

Rho-GTPases have been broadly implicated in dendrite and spine formation and maintenance, which are essential aspects of structural plasticity. For example, transgenic mice that express CA-Rac1 in Purkinje cells have smaller and more numerous spines (Luo et al., 1996). Moreover, expression of DN-Rac1 in pyramidal neurons of rat or mouse hippocampal slice results in a decreased number of spines (Nakayama et al., 2000; Tashiro and Yuste, 2004). These results further support the premise that Pak1 is a downstream effector of Rac1 in dendritic spine formation. Possible upstream and downstream molecules such as PIX and LIM-kinase have also been suggested to be involved in the regulation of spine morphology (Parnas et al., 2001; Meng et al., 2002; Park et al., 2003). Interestingly, defects in the genes encoding these molecules as well as Pak3 have been identified in some families of mental retardation patients (Chelly and Mandel, 2001). Here, we have

studied the role of Pak1 in dendrite and spine formation and maintenance in primary cultured neurons. It will be important to study the role of Pak1 in dendrite and spine regulation and maintenance in intact brain tissue in the future to further understand the regulatory mechanism of structural plasticity as well as the pathophysiology of neurological disorders.

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