CD8 was detected using PercP-labelled anti-human CD8 mouse IgG_1 monoclonal antibodies (Becton Dickinson). Data from 5×10^4 cells were collected, stored and analysed by using Cellquest software (Becton Dickinson). For detection of mbTNF and expression of surface TNFRI and TNFRII, cells were stained with a phycoerythrin-labelled anti-huTNF mouse IgG_1 monoclonal antibody (Pharmingen), FITC-labelled anti-huTNFRI goat polyclonal IgG, FITC-labelled anti-huTNFRII goat polyclonal IgG antibodies, or their respective isotype controls (all from Santa Cruz Biotechnology). CXCR4 and CCR5 expression was measured on $CD8^+$ cells by double-labelling with PercP-labelled anti-human CD8 mouse IgG_1 monoclonal antibody (Becton Dickinson) and either mouse monoclonal antihuCXCR4 (clone 12G5, IgG_{2a}; R&D Systems) or mouse monoclonal antihuCCR5 (clone 45531.111, IgG_{2B}; R&D Systems).

Terminal transferase dUTP nick end-labelling assay (TUNEL assay). After fixation with 3% paraformaldehyde in PBS for 30 min, 5×10^6 cells per sample were washed three times with 0.3% (v/v) Triton X-100 in PBS, and a TUNEL assay was performed[9]. Apoptosis was measured in both uninfected and infected cells isolated from the same donor, and specific apoptosis in the $CD8^+$ T-cell subpopulation was expressed as a percentage of apoptotic cells in the infected culture minus the percentage of apoptotic cells in the uninfected culture.

Fura assay for Ca^{2+} flux determination. Macrophages were scraped from tissue-culture flasks in the presence of 5 mM EGTA/PBS at 4 °C. Cells were washed in PBS and loaded with Fura-2/AM (Calbiochem) in HEPES-buffered saline (HBS; 137 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) at room temperature for 30 min. Cells were washed once in HBS to remove excess Fura-2/AM. Fluorescence emission (at 510 nm) from excitation at 340 and 380 nm was measured in 1-ml suspensions of 10^6 cells ml^{-1} with a model F-2000 fluorescence spectrophotometer (Hitachi Instruments). Using published calibration equations, we found that resting Ca^{2+} was at or below 150 nM (not shown). Chemokines and rgp120 proteins were added as 100× solutions in water.

Patients. PBMCs from HIV-infected subjects were isolated by Ficoll–Hypaque as reported[9]. PBMCs were distributed in six-well plates at 5×10^6 cells per well in RPMI supplemented with 10% FCS. At 24 h after isolation, PBMCs were left untreated or were treated with 20 μg ml^{-1} anti-huTNFRI mouse IgG_1, 20 μg ml^{-1} anti-huTNFRII mouse IgG_{2a}, 10 μg ml^{-1} anti-huTNF mouse IgG_1 (clone 1825), 10 μg ml^{-1} anti-human FasL mouse IgG_{2a} (NOK-2; Pharmingen), and the respective isotype controls (all from R&D Systems). At 18 h after treatment, cells were fixed in 3% paraformaldehyde and analysed for apoptosis by using the TUNEL assay. The $CD8^+$ T-cell subpopulation was characterized by using an anti-huCD8 PerCP-labelled monoclonal antibody.

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The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity

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Cyclin-dependent kinase 5 (Cdk5) and its neuron-specific regulator p35 (refs 1–4) are essential for neuronal migration and for the laminar configuration of the cerebral cortex[5–7]. In addition, p35/Cdk5 kinase concentrates at the leading edges of axonal growth cones and regulates neurite outgrowth in cortical neurons in culture[8]. The Rho family of small GTPases is implicated in a range of cellular functions, including cell migration and neurite outgrowth[9–13]. Here we show that the p35/Cdk5 kinase co-localizes with Rac in neuronal growth cones. Furthermore, p35 associates directly with Rac in a GTP-dependent manner. Another Rac effector, Pak1 kinase[14,15], is also present in the Rac–p35/Cdk5 complexes and co-localizes with p35/Cdk5 and Rac at neuronal peripheries. The active p35/Cdk5 kinase causes Pak1 hyperphosphorylation in a Rac-dependent manner, which results in down-regulation of Pak1 kinase activity. Because the Rho family of GTPases and the Pak kinases are implicated in actin polymerization[16–18], the modification of Pak1, imposed by the p35/Cdk5 kinase, is likely to have an impact on the dynamics of the reorganization of the actin cytoskeleton in neurons, thus promoting neuronal migration and neurite outgrowth.

The p35/Cdk5 kinase and the Rho family of GTPases induce shape changes in fibroblasts (ref. 17 and unpublished data) and have been implicated in neurite outgrowth, axon guidance and cell migration[9–13]. To explore a possible interaction between the p35/Cdk5 kinase and the Rho family of GTPases, we expressed p35 in

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COS-7 cells with either active (V12 or V14, GTP-bound) or inactive (N17, GDP-bound) mutants of Cdc42, Rac or Rho fused to glutathione *S*-transferase (GST). A GTP-dependent association was found between p35 and GST–RacV12 (Fig. 1a) as no detectable p35 bound to GST–RacN17. To determine whether the Rac–p35 complexes also contain Cdk5, a dominant-negative mutant of Cdk5 (Cdk5N144 (ref. 19)) was used, which binds more stably with p35 (ref. 8). In the presence of p35, Cdk5N144 also associated with active RacV12 (Fig. 1b). To verify the binding specificity between p35 and Rac, p35 was coexpressed with myc-tagged RacL61 or with two mutants of the Rac effector domain, RacL61A37 and RacL61K40. RacL61A37 was shown to bind the Pak kinases but had lost its ability to induce lamellipodia, whereas RacL61K40 was shown to induce lamellipodia but no longer associated with the Pak kinases[20,21]. Although a clear association was seen between p35 and RacL61, both the L61A37 and L61K40 Rac mutants lost their ability to bind to p35 (Fig. 1c).

To determine whether the p35–Rac interactions are direct, bacterially produced RacL61 and RacL61K40 were labelled with [^{32}Pγ-GTP], incubated with equal amounts of GST–p35, GST–Cdk5 or GST and examined by Cerenkov counting. On average, a 3.5-fold-stronger binding between [^{32}Pγ-GTP]–RacL61 and GST–p35 than [^{32}γ-GTP]–RacL61 with GST–Cdk5 or GST was seen (Fig. 1d). The RacL61K40 mutant failed to interact with GST–p35. A Pak1 mutant (GST–Pak1R299) was used as a control and bound RacL61 but not RacL61K40, as expected. Together, these observations indicate a direct and specific physical association between p35 and Rac in a GTP-dependent manner, which indicates that the p35/Cdk5 kinase is a downstream target of Rac.

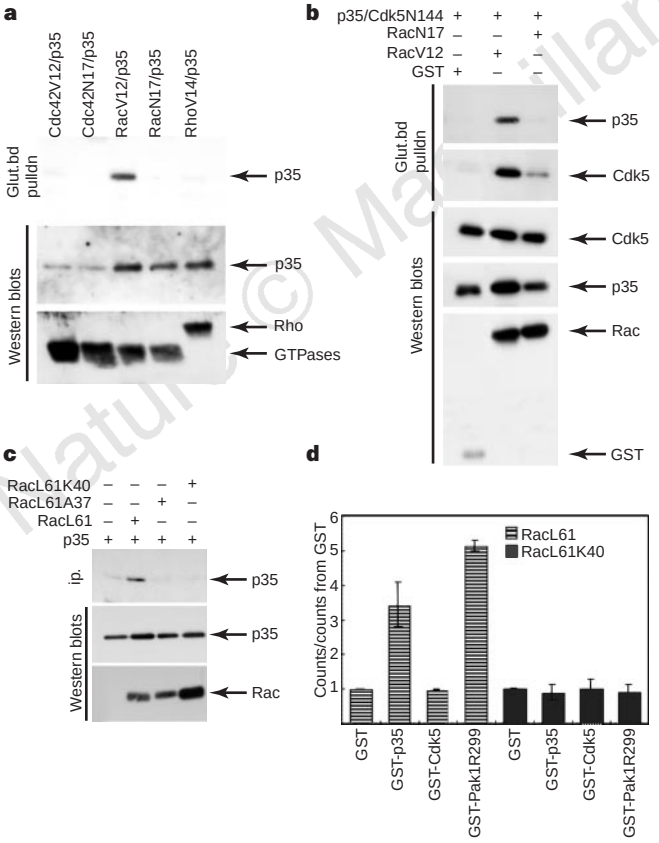

Figure 1 The p35/Cdk5 kinase associates with Rac in a GTP-dependent manner. **a**, p35 western blot of glutathione-bead pulldowns from COS-7 cell lysates expressing p35 and the GTPase mutants (as shown) fused to GST. **b**, As in **a**, including Cdk5N144. **c**, Association between p35, myc–RacL61, myc–RacL61K40 or myc–RacL61A37 was examined in expressing COS-7 cells by immunoprecipitation with antibody 9E10 followed by p35 western blot analysis; lower panels in **a–c** show protein expression levels. **d**, GST–p35, GST–Cdk5, GST–Pak1R299 or GST bound to glutathione beads were incubated with [^{32}Pγ-GTP]–RacL61 or [^{32}Pγ-GTP]-RacL61K40 and examined by Cerenkov counting.

Active Rac has been shown to localize to plasma membranes by carboxy-terminal geranyl-geranylation[22]. To determine the subcellular distribution of p35, lysates obtained from cortices at embryonic day 18 (E18) were fractionated into membrane, cytoplasmic and nuclear pools and analysed by western blotting. The bulk of p35 was associated with the membrane (Fig. 2A), whereas most cdk5 was present in the cytoplasm with a smaller proportion at the membranes. Negligible amounts of p35 and Cdk5 were seen in the nucleus. As a control, the same extracts were examined for the distribution of Rac and SV2, a neuronal membrane marker. To examine if neuronal p35 and Rac associate, p35 immunprecipitations were carried out from cortical lysates of p35$^{+/-}$ and p35$^{-/-}$ mice. A strong interaction was seen between p35 and Rac in lysates obtained from p35$^{+/-}$ cortices, whereas no association was evident in p35$^{-/-}$ cortices (Fig. 2B). In confirmation, p35/Cdk5 association was only seen in p35$^{+/-}$ cortices. To investigate whether p35 and Rac co-localize, we initially examined the subcellular distribution of transfected p35 and Rac in Swiss3T3 fibroblasts by immunocytochemistry. Co-localization of RacV12 and p35 was evident particularly in the most actively ruffling areas and at the peripheries of these cells (see arrows in Fig. 2Ca). In primary neurons, co-localization of p35 and Rac was evident in lamellipodia-rich areas of axonal growth cones (Fig. 2Cc) where Cdk5 was concentrated (Fig. 2Cd). p35 and Rac also co-localized in neuronal soma (Fig. 2Cb). Together, these studies establish that the p35/Cdk5 kinase is likely to be a neuron-specific effector of Rac.

Because Rac is known to have many downstream effectors, we investigated whether the p35/Cdk5 kinase is part of a previously identified pathway. Among the Rac effectors, the Pak kinase family has been extensively characterized[14,15], two members of which, Pak1 and Pak3 (Pakα and β in rats), are highly enriched in brain[23,24]. Upon association with GTP–Rac or Cdc42, the Pak kinases autophosphorylate and become active. When p35 was coexpressed with myc-tagged Pak1 and RacL61, association between p35 and Pak1 was detected, whereas no interactions were evident in the absence of active Rac (Fig. 2D). Coexpression of RacL61K40, Pak1 and p35 did not facilitate trimolecular complex formation (data not shown), which further suggests that the p35-Pak1 association is dependent on active Rac. To determine whether p35/Cdk5, Rac and Pak1 form complexes in neurons, Pak1 immunoprecipitations were done using cortical lysates obtained from wild-type, p35$^{+/-}$ or p35$^{-/-}$ mice, revealing co-immunoprecipitated Cdk5 from wild type, less from p35$^{+/-}$ and negligible amounts from p35$^{-/-}$ cortices (Fig. 2E). The levels of Pak1–Rac association were unaltered. In primary neurons, p35 and Cdk5 co-localize in axonal growth cones[8]. To examine the localization of Pak1, primary neurons were co-immunostained for Pak1 and Cdk5 or Pak1 and Rac. Prominent co-localization of Cdk5 and Pak1 was evident in axonal growth cones (Fig. 2Ce) where, as expected, extensive Pak1 and Rac co-localization was also seen (Fig. 2Cf). Together, these experiments demonstrate that Rac can associate simultaneously with both p35/Cdk5 and Pak1 kinases and that this complex is present in the highly dynamic areas of neurons such as the growing tips of axons.

Upon coexpression with p35, Cdk5 and active Rac, slower mobility species of Pak1 were apparent; these became more prominent with increasing amounts of the p35/Cdk5 kinase (Fig. 3a). The slower-migrating forms of Pak1 were abolished in the absence of active Rac or in the presence of RacL61K40, and they were greatly reduced upon coexpression with Cdk5N144. Treatment of Pak1 immunoprecipitates with phosphatase diminished the slower-migrating species, which showed that these were phosphorylated forms of Pak1 (Fig. 3b). These experiments indicate that the active p35/Cdk5 kinase can induce hyperphosphorylation of Pak1. Densi-

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tometric analysis showed that high concentrations of the p35/Cdk5 kinase result in phosphorylation of ~75% of total Pak1 expressed, indicating that a large proportion of this protein had associated with the p35/Cdk5 kinase. To examine whether the phosphorylated forms of Pak1 were due to autophosphorylation or phosphorylation by the p35/Cdk5 kinase, we used a catalytically inactive mutant of Pak1 (ATP binding site)[16,25]. Inactive Pak1 displayed the same mobility shifts as wild-type Pak1 (Fig. 3c), indicating direct phosphorylation by the p35/Cdk5 kinase. To test whether Pak1 is a substrate of the p35/Cdk5 kinase, purified GST–Pak1R299 was used as an *in vitro* substrate for the recombinant GST–p25/GST–Cdk5 kinase. p25 is an amino-terminally truncated form of p35 that is able to interact with and activate Cdk5 (ref. 1). GST–Pak1R299 was phosphorylated by the p25/Cdk5 kinase whereas no phosphorylation of GST was observed (Fig. 3d). Inhibition of p25/Cdk5 with a specific inhibitor, roscovitine[26], caused the loss of GST–Pak1R299 phosphorylated species.

To compare GST–Pak1R299 phosphorylated in transfected COS-7 cells in the presence of the p35/Cdk5 kinase and RacL61 with GST–Pak1R299 phosphorylated *in vitro* by the p25/Cdk5 kinase, we performed two-dimensional phospho-tryptic peptide analysis. Six distinct GST–Pak1R299 peptides were apparent *in vivo*, whereas the *in vitro* phosphorylated GST–Pak1R299 resolved into one major peptide that perfectly comigrated with one of the strongest-labelled peptides *in vivo* (Fig. 3e, peptide X). Upon longer exposure, another labelled peptide was apparent that also had a labelled counterpart *in vivo* (Fig. 3e, peptide Y). Therefore, it is likely that Pak1 is phosphorylated *in vitro* by the p25/Cdk5 kinase at overlapping sites, but not as extensively as Pak1 phosphorylated *in vivo*. This indicates that the Pak1–Rac–p35/Cdk5 complex causes exposure and phosphorylation of additional sites on Pak1 by the p35/Cdk5 kinase. Alternatively, the presence of yet another kinase in such a complex cannot be ruled out.

To determine the effects of the p35/Cdk5 kinase on Pak1 activity, Pak1 kinase assays were done using histone H4 as an *in vitro* substrate. The Pak1 kinase induction by RacL61 was abolished in the presence of the p35/Cdk5 kinase (Fig. 4a). Titration of p35/Cdk5 revealed progressive inhibition of the activity of Pak1 kinase (Fig. 4b), whereas coexpression of Cdk5N144 with p35 had no inhibitory effects on Pak1 (Fig. 4c), indicating that active p35/Cdk5 kinase downregulates Pak1 activity. The p35/Cdk5 kinase had no effect on a constitutively active Pak1 kinase mutant (Pak1E423)

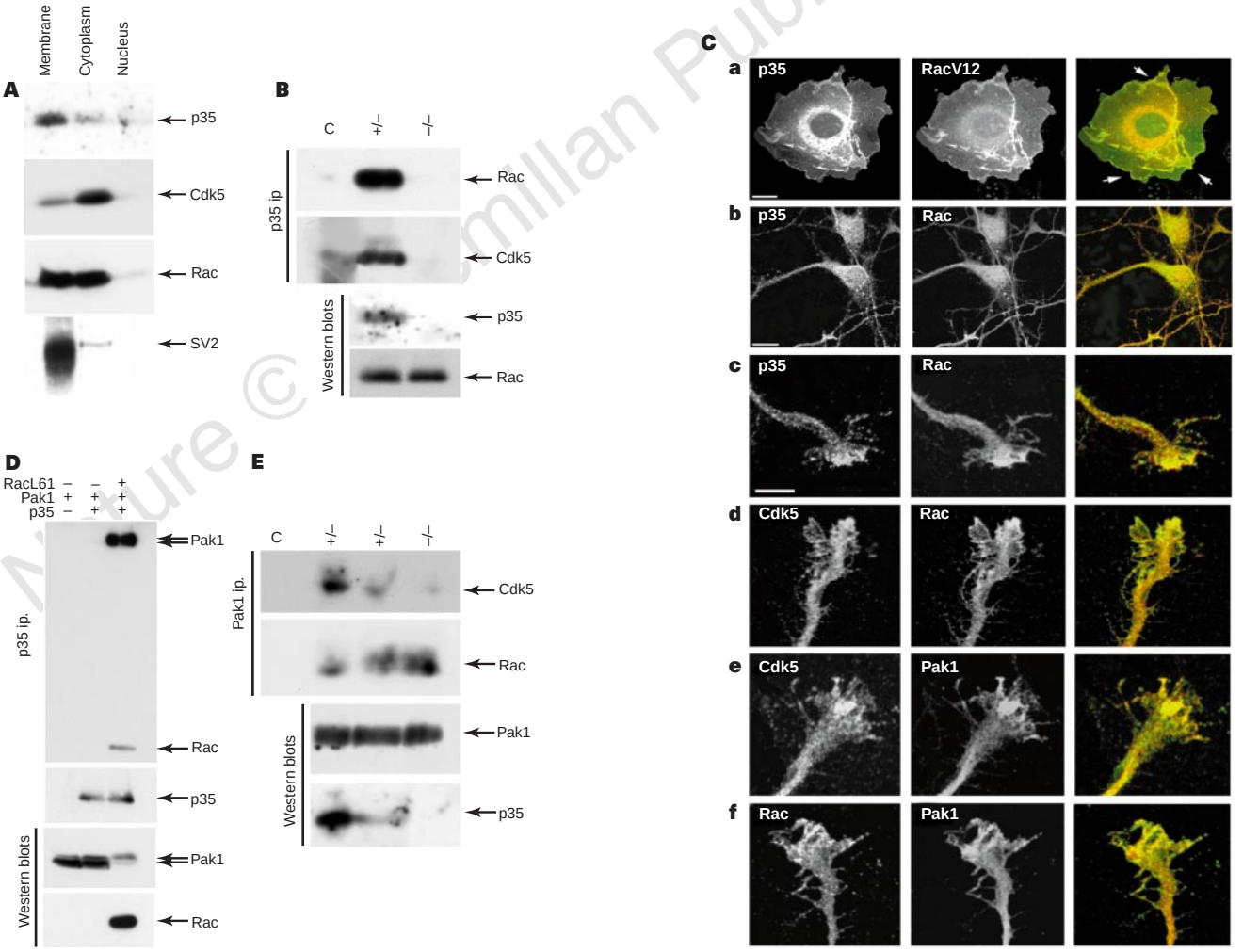

Figure 2 In neurons, p35 co-localizes and associates with Rac and Pak1 at the cell periphery. **A**, p35, Cdk5, Rac and SV2 western blots of fractionated cortical lysates. **B**, p35 immunoprecipitates from $p35^{+/-}$ and $p35^{-/-}$ cortices examined for Rac or Cdk5 association. **Ca**, Swiss3T3 cells expressing p35 (red) and HA-RacV12 (green); **b**, **c**, primary neuronal cultures immunostained for p35 (green) and Rac (red); **d**, Cdk5 (red) and Rac (green); **e**, Cdk5 (green) and Pak1 (red); or **f**, Rac (green) and Pak1 (red). Arrows point to areas of p35 and Rac co-localization. Bars, 10 μm (**a**, **b**) and 5 μm (**c–f**). **D**, Lysates from COS-7 cells expressing as shown, immunoprecipitated with anti-p35 antibodies and immunoblotted with the 9E10 antibody. **E**, As in **B**, but Pak1 immunoprecipitations were subjected to Cdk5 and Rac western blotting; including $p35^{+/+}$ samples. In **B** and **E**, C depicts control immunoprecipitations; in **B**, **D**, **E**, lower panels show protein levels in individual lysates.

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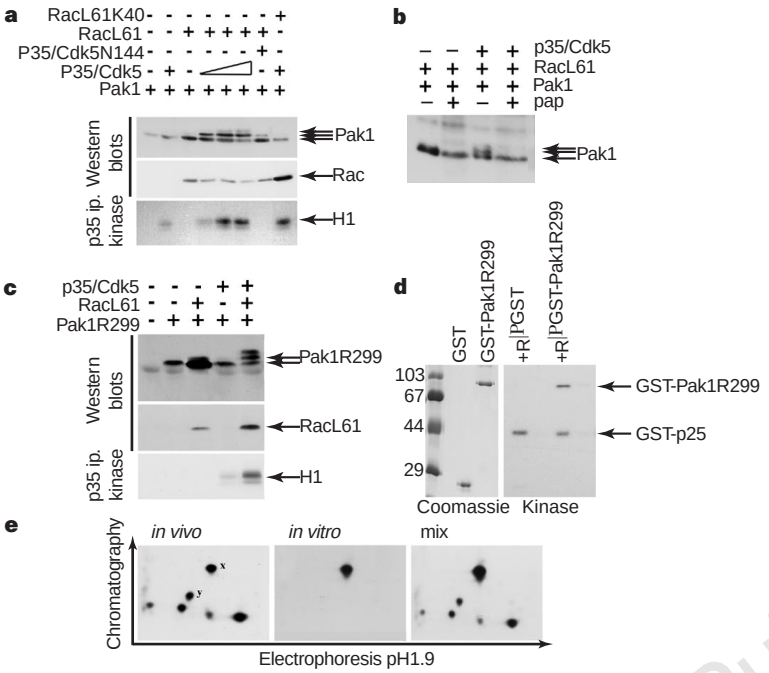

Figure 3 The p35/Cdk5 kinase associates with and phosphorylates Pak1 in a Rac-dependent manner. **a**, Western blots showing the effects of the active or inactive p35/Cdk5 kinase on myc–Pak1 in transfected COS-7 cells. **b**, Pak1 immunoprecipitates from a sample treated (+) or not (–) with potato acid phosphatase (PAP). **c**, As in **a** using the inactive Pak1R299 mutant. A small shift of unknown origin was seen in the presence of RacL61. Active Rac upregulates p35/Cdk5 activity by unknown mechanisms (**a**, **c**). **d**, GST–Pak1R299 or GST were used as substrates of the p25/Cdk5 kinase obtained from baculovirus infected Hi5 cells. The kinase was inhibited with 0.2 μM roscovitine (R). **e**, Comparison of two-dimensional tryptic-phosphopeptide maps obtained from GST–Pak1R299 phosphorylated in COS-7 cells in the presence of p35/Cdk5 and RacL61 (*in vivo*) or phosphorylated *in vitro* by the p25/Cdk5 kinase. X and Y depict co-migrating peptides.

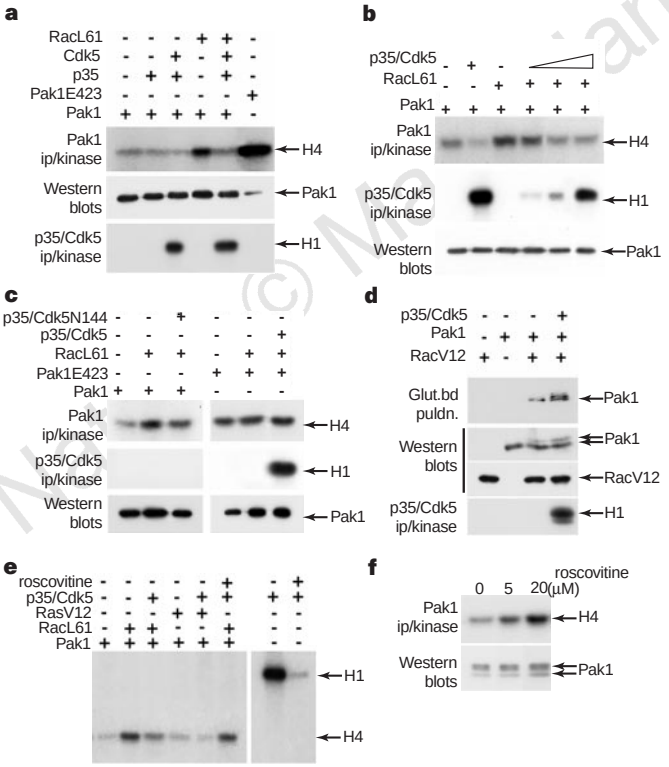

Figure 4 The p35/Cdk5 kinase inhibits Pak1 activity but not its affinity for Rac. **a–c**, Membrane lysates from COS-7 cells expressing as shown were immunoprecipitated with antibody 9E10 to isolate Pak1, and were then subjected to histone H4 kinase assays. **d**, Lysates from COS-7 cells expressing as shown were subjected to glutathione bead pulldown to isolate Rac and examined for Pak1 association by 9E10 western blots. **e**, Set amounts of Pak1 and p35 immunoprecipitates were mixed and examined for Pak1 kinase activity in the presence or absence of RacL61 or RasV12 and the inhibitor roscovitine. **f**, Lysates from primary neurons treated with roscovitine were examined for Pak1 kinase activity. Protein levels were examined by western blotting (**a–d** and **f**) and p35/Cdk5 kinase activity by phosphorylation of histone H1 (**a–e**).

(Fig. 4c). To determine whether the observed decrease in the activity of Pak1 kinase could be a consequence of reduced Rac and Pak1 association after phosphorylation by the p35/Cdk5 kinase, the extent of Pak1 and RacL61 association was compared in the presence or absence of the p35/Cdk5 kinase. Figure 4d shows that Pak1 association with Rac is unaltered, if not increased, when the p35/Cdk5 kinase is present. We further tested if Pak1 kinase activity could be inhibited by the p35/Cdk5 kinase *in vitro*. Purified Pak1 was mixed with recombinant RacL61 and the p35/Cdk5 kinase. Figure 4e demonstrates the stimulation of Pak1 kinase by RacL61, although the stimulated Pak1 activity was reduced by the p35/Cdk5 kinase. This inhibition was alleviated by roscovitine, confirming that the effect was dependent on the catalytic activity of the p35/Cdk5 kinase. To determine whether the endogenous Pak1 kinase is inhibited by p35/Cdk5 in neurons, primary cortical cultures were treated with increasing amounts of roscovitine for 1 h. A progressive rise in Pak1 kinase activity was observed, with increasing amounts of roscovitine (~fourfold at 20 μM concentrations; Fig. 4f). Together, these data indicate that the active p35/Cdk5 kinase serves to inhibit the Pak1 kinase in neurons.

In summary, the interaction of the p35/Cdk5 kinase, Rac and Pak1 and the subsequent modification of Pak1 demonstrate the existence of a neuron-specific signalling complex. The function of the Rho GTPases and Pak1 in the regulation of the actin cytoskeleton and cell shape is now well established[16–18,27,28]. Furthermore, it has been suggested that Pak1-mediated actin reorganization in fibroblasts has two components, only one of which requires kinase activity[16]. We propose that the p35/Cdk5–Pak1–Rac complex serves to inhibit, both temporally and spatially, the activity of Pak1 kinase in neurons, in this way switching Pak1 between an active or inactive state and thus allowing two distinct Pak1 activities on the actin cytoskeleton. The dynamics of this process are provided by the instability of the protein interactions, as they rely on the presence of active Rac that rapidly exchanges GTP for GDP and p35 has a short half-life[29]. Our experiments indicate that the steady-state association between p35/Cdk5, Rac and Pak1 is small, which is due largely to the transient nature of the interactions. We therefore propose that this dynamic protein assembly functions in the rapid regulation of the actin cytoskeleton, which is necessary for neurite outgrowth and the migration of developing neurons. □

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Methods

Transient expression in cells. COS-7 cells were transfected using the calcium phosphate precipitation method and Swiss3T3 using lipofectamine (GIBCO BRL). Cells were collected 24 h after transfection. The experiments used GST-, haemagglutinin- or myc-tagged GTPases, myc or GST-tagged Pak1, p35 and Cdk5 expression constructs.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization with 0.3% triton X-100. Immunostaining was done as described previously[8] using anti-p35 (pAb neu-cyc, pAb N20; Santa Cruz), anti-Cdk5 (mAb DC39, pAb 50; Kinetek Biology Corporation), anti-Pak1 (pAb, N20, Santa Cruz), anti-HA (mAb 12CA5) and anti-Rac (mAb, Upstate Biotechnology) antibodies to detect p35, Cdk5, Pak1 and Rac. This was followed by the use of Texas-red-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies in all cases except those in which p35 immunostaining was performed. In these cases, biotin-conjugated anti-rabbit and Texas-red-conjugated anti-mouse antibodies were used followed by FITC-conjugated streptavidin to amplify the signals. Coverslips were mounted in ProLong antifade (Molecular Probes) and analysed using a Leica or Zeiss confocal microscope.

Protein interactions. For p35/Cdk5–Rac or Pak1–Rac associations, transfected cells were lysed in 20 mM Tris pH 7.2, 2 mM $MgCl_2$, 0.5% NP-40, 150 mM NaCl, 1 mM DTT, protease and phosphatase inhibitors (buffer A). Lysates were incubated with glutathione beads or with 9E10 antibody and protein G sepharose or anti-p35 antibody and protein A sepharose, at 4 °C, washed three times in lysis buffer and resuspended in 1 × sample buffer. For Pak1–Rac–p35/Cdk5, tissues were lysed in STM buffer (10 mM Tris HCl pH 8.0, 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM DTT, protease and phosphatase inhibitors) containing 0.5% NP-40. Immunoprecipitations were made using as a control normal rabbit serum, anti-p35 (pAb neu-cyc, pAb C19; Santa Cruz), or anti-Pak1 (pAb, N20; Santa Cruz) followed by protein A sepharose. The beads were washed in buffer A and resuspended in 1 × sample buffer. *In vitro* interactions were done by incubating 40 μg each of GST–p35, GST–Cdk5 and GST or 10 μg GST–Pak1R299 with glutathione beads. After washing, the samples were halved and each half was incubated with equal amounts of [^{32}Pγ-GTP]-labelled recombinant RacL61 or RacL61K40, from which the GST tags had been removed by thrombin cleavage, as described[20], at 4 °C for 1 h. The beads were washed extensively in buffer A and subjected to Cerenkov counting.

Fractionation experiments. Rat embryo cortices were lysed in STM buffer. Extracts were spun at 600*g* and then at 100,000*g* to obtain cytoplasmic fractions. Membranes were extracted from the 600*g* spin by STM lysis with 0.5% NP-40. Nuclear fractions were the final 600 *g* pellet lysed in RIPA (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM DTT, protease and phosphatase inhibitors) buffer.

Phosphorylation of Pak1. COS-7 cells were transfected with myc–Pak1 and, where appropriate, RacL61. The titration points of the p35/Cdk5 kinase are 0.5 μg, 1 μg and the maximum 2 μg each of p35 and Cdk5. For phosphatase treatment, Pak1 immunoprecipitates were resuspended in 60 μl 0.1 M MES pH 6.0, halved and one half treated with potato acid phosphatase (Sigma) for 15 min at 37 °C. For *in vitro* phosphorylation of Pak1, GST–Pak1R299 was obtained from transfected COS-7 cells by glutathione-bead pulldown, eluted and dialysed against PBS. GST–p25 and GST–Cdk5 were obtained from baculovirus-infected Hi5 insect cells, immobilized on glutathione beads, and subjected to kinase assays as described previously[8], using 1 μg of GST–Pak1R299 as a substrate. The assays were done in the presence or absence of 0.2 μm roscovitine. Two-dimensional tryptic-phosphopeptide mapping was performed comparing GST–Pak1R299 isolated from transfected COS-7 cells coexpressing p35, Cdk5 and RacL61, which had been labelled for 3 h with ^{32}P-orthophosphate, and GST–Pak1R299 phosphorylated *in vitro* by the GST–p25/GST–Cdk5, kinase as described previously[30]. Equal counts of the *in vivo* or *in vitro* samples (determined by Cerenkov counting) were analysed alone or in combination to examine the extent of co-migration between individual peptides.

Kinase assays. RacL61 (10 ng) was co-transfected into COS-7 cells with Pak1 (2 μg) or Pak1E423 (2 μg) and, where appropriate, p35 (2 μg) and Cdk5 (2 μg) or Cdk5N144 (2 μg) expression vectors. In the p35/Cdk5 titration experiments, 0.1 μg, 1 μg and 3 μg each of p35 and Cdk5 were used. For Pak1 kinase assays, membrane fractions were used. p35/Cdk5 kinase assays from lysates of transfected cells were done as described previously[8]. Pak1 kinase assays were done in the presence of 1 mM ATP, 1–2 μCi [^{32}Pγ-ATP], 3 μg histone H4 and kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$) for 10 min at 30 °C. For *in vitro* Pak1 kinase assays, standardized amounts of Pak1 immunoprecipitates were incubated, where appropriate, with standardized amounts of p35/Cdk5 immunoprecipitates and/or 2–5 μg of bacterially produced GTPases at 4 °C. Kinase assays were done in the presence or absence of 0.2 μm roscovitine. For *in vivo* roscovitine treatment, primary neuronal cultures were exposed for 1 h to 5 μM or 20 μM roscovitine, or to DMSO as a control. Endogenous Pak1 was immunoprecipitated from the lysates using a specific antibody and subjected to kinase assays as described.

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