Neurobiology of Disease

Interaction of Cellular Prion and Stress-Inducible Protein 1 Promotes Neuritogenesis and Neuroprotection by Distinct Signaling Pathways

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Understanding the physiological function of the cellular prion (PrP^c) depends on the investigation of PrP^c-interacting proteins. Stress-inducible protein 1 (STI1) is a specific PrP^c ligand that promotes neuroprotection of retinal neurons through cAMP-dependent protein kinase A (PKA). Here, we examined the signaling pathways and functional consequences of the PrP^c interaction with STI1 in hippocampal neurons. Both PrP^c and STI1 are abundantly expressed and highly colocalized in the hippocampus *in situ*, indicating that they can interact *in vivo*. Recombinant STI1 (His_6-STI1) added to hippocampal cultures interacts with PrP^c at the neuronal surface and elicits neuritogenesis in wild-type neurons but not in PrP^c-null cells. This effect was abolished by antibodies against either PrP^c or STI1 and was dependent on the STI1 domain that binds PrP^c. Binding of these proteins induced the phosphorylation/activation of the mitogen-activated protein kinase, which was essential for STI1-promoted neuritogenesis. His_6-STI1, but not its counterpart lacking the PrP^c binding site, prevented cell death via PKA activation. These results demonstrate that two parallel effects of the PrP^c–STI1 interaction, neuritogenesis and neuroprotection, are mediated by distinct signaling pathways.

Key words: **cellular prion protein; MAPK; neuritogenesis; neuroprotection; PKA; STI1**

Introduction

Cellular prion protein (PrP^c) is a ubiquitous, glycosylphosphatidylinositol-anchored protein, the physiological functions of which are still under discussion. Several biological roles for PrP^c have been proposed, such as protection against oxidative insults, neuronal adhesion, cell differentiation, and survival (Brown and Sassoon, 2002; Martins et al., 2002). Components of various signal transduction pathways, including the Src-related family member $p59^{Fyn}$, phosphatidylinositol-3-kinase (PI3 kinase)/Akt, protein kinase A (PKA; cAMP-dependent protein kinase), and mitogen-activated protein kinases (MAPKs), have been suggested to mediate roles of PrP^c in neuronal survival and neurite outgrowth (Chiarini et al., 2002; Chen et al., 2003; Santuccione et al., 2005).

PrP^c is constitutively expressed in neurons and is abundant in regions such as the olfactory bulb, hippocampus, and synaptic neuropil in close spatiotemporal association with synapse formation. The localization of PrP^c in elongating axons suggests a role in axon growth (Sales et al., 1998, 2002). Interestingly, interaction of PrP^c with the extracellular matrix protein laminin has been shown to promote neuritogenesis and maintenance of neurites (Graner et al., 2000a,b).

Furthermore, strong evidence for a neuroprotective PrP^c function derived from our description of a putative PrP^c 66 kDa ligand (Martins et al., 1997), which was later identified as the stress-inducible protein 1 (STI1) (Chiarini et al., 2002; Zanata et al., 2002). STI1 was described in *Saccharomyces cerevisiae*, in which it was shown to mediate the heat shock response of heat shock protein 70 (Hsp70) genes (Nicolet and Craig, 1989). Murine STI1 has 97% amino acid identity with its human homolog designated Hop (Hsp70/Hsp90-organizing protein). STI1, as well as Hop, interacts with Hsp70 and Hsp90 at its N and C termini (van der Spuy et al., 2000; Carrigan et al., 2004). This interaction facilitates transfer of substrates from Hsp70 to Hsp90 and is important for proper protein folding and maturation (Hernandez et al., 2002).

Interaction of PrP^c with either His_6-STI1 or a peptide mimicking the PrP^c binding domain of STI1 prevented programmed

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cell death of undifferentiated postmitotic retinal cells (Chiarini et al., 2002; Zanata et al., 2002). Engagement of PrP^c increased intracellular cAMP and activated the MAPK pathway in retinal tissue. However, the neuroprotective effect required the activity of PKA rather than MAPK (Chiarini et al., 2002).

We have proposed that PrP^c is part of a multiprotein complex that modulates various cellular functions, depending on both protein combination and cell type (Martins et al., 2002). Hence, it is important to dissect the cell signaling and biological significance of the association of PrP^c with each one of its partners and in different cell types.

We have therefore examined the functional responses and signaling pathways induced by the interaction of STI1 with PrP^c in hippocampal neurons, particularly the roles of the cAMP/PKA and MAPK pathways. Our data indicate that PrP^c and STI1 colocalize in the hippocampus and that the interaction of STI1 with PrP^c has pronounced effects on both neuritogenesis and survival in hippocampal neurons. The neuritogenesis was found to be dependent only on MAPK activity, whereas cAMP-dependent PKA mediates neuroprotection.

Materials and Methods

Materials

Mouse recombinant STI1 (His_6-STI1) and PrP^c (His_6-PrP^c) were purified as described previously (Zanata et al., 2002). Peptides corresponding to mouse STI1 amino acid sequences pepSTI1$_{230–245}$ (230-ELGNDAYKKKDFDKAL-245) and pepSTI1$_{422–437}$ (422-QLEPTFIKGYTRKAAA-437) were chemically synthesized by Neosystem (Strasbourg, France). Polyclonal antibodies anti-STI1 (Zanata et al., 2002) and anti-pepSTI1$_{230–245}$ raised in rabbits were obtained from Bethyl Laboratories (Montgomery, TX), whereas anti-PrP^c was produced in PrP^c-null mice (Chiarini et al., 2002). The monoclonal antibody 6H4 against amino acids 144–152 of human PrP^c was purchased from Prionics (Zurich, Switzerland), and anti-His-Tag-HRP and anti-HisTag antibodies were from Invitrogen (San Diego, CA) and Amersham Biosciences (Piscataway, NJ), respectively. The following chemical inhibitors for different signaling proteins were used: MAP/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenyltio)butadiene (U0126), PKA inhibitor KT5720, PKC inhibitors bisindolylmaleimide (Bim) and chelerethrin chloride (Chel), and phosphodiesterase inhibitor 3-isobutyl-1methylxantine (IBMX; Sigma, St. Louis, MO). The inhibitors were from Calbiochem (La Jolla, CA), except for U0126, which was from Promega (Madison, WI). The PKA activator (forskolin) was purchased from LC Laboratories (Woburn, MA).

Immunohistochemistry

Embryonic day 17 (E17) mouse embryos were fixed with formalin and embedded in paraffin. Sections (3–4 μm) were deparaffinized, rehydrated, and subjected to epitope retrieval by microwave heating in 10 mM citrate buffer, pH 6.4. Additional treatment was made with 50 mM glycine, 0.02 g/L Triton X-100, 0.5 g/L nonfat dry milk, and 1.5 g/L nonimmune goat serum (Martins et al., 1999). Endogenous peroxidase was blocked with 0.3 g/L hydrogen peroxide. Sections were then incubated overnight at 4°C with the primary antibody anti-PrP^c (1:1000) (Chiarini et al., 2002) or anti-STI1 (1:250) (Zanata et al., 2002) diluted in PBS plus 0.5 g/L nonfat dry milk and 1.5 g/L normal goat serum, followed by incubation for 60 min at 37°C with EnVision Labeled Polymer peroxidase (Dako, High Wycombe, UK). Color was developed using DAB (3,3′diaminobenzidine tetrahydrochloride; Sigma) and counterstained with hematoxylin. Sections were visualized in an Olympus (Melville, NY) IMT2-NIC microscope.

Immunofluorescence

Tissue. Brains from E17 wild-type and PrP^c-null mice were removed and immediately frozen in liquid nitrogen. Cryostat sections (3 μm) were fixed in ice-cold acetone for 30 min, air dried, rehydrated, and blocked with TBS (20 mM Tris and 150 mM NaCl) containing 0.1% Triton X-100, 10% normal goat serum, and 50 μg/ml anti-mouse IgG at room temperature for 1 h. Brain sections were then incubated at room temperature for 16 h with anti-PrP^c mouse serum (1:250) (Chiarini et al., 2002) and anti-STI1 rabbit serum (1:100) (Zanata et al., 2002) in TBS and 0.1% Triton X-100 with 1% normal goat serum. After washing, anti-mouse Alexa-568 (1:3000; Molecular Probes, Eugene, OR) and anti-rabbit FITC (1:1000; PharMingen, San Diego, CA) were added to the slices and incubated in the same buffer for 1 h at room temperature.

Cultured cells. Hippocampal neurons (1×10^5 cells) were plated on glass coverslips coated with poly-L-lysine, washed with PBS, and fixed for 20 min at room temperature with 4% paraformaldehyde and 0.12 M sucrose in PBS. For permeabilization, the cells were incubated with 0.2% Triton X-100 in PBS for 5 min at room temperature. After rinsing with PBS, cultured cells were treated for 1 h at room temperature with blocking solution containing 5% bovine serum albumin (BSA; Sigma) in PBS. For PrP^c and STI1 staining, cells were incubated at room temperature for 1 h with anti-PrP^c (1:100) and anti-STI1 (1:100) antibodies (Chiarini et al., 2002) diluted in 1% BSA in PBS. The reaction proceeded by incubation with secondary antibodies [anti-rabbit Cy3 (1:3000; Amersham Biosciences) or anti-mouse Alexa-488 (1:500; Molecular Probes)], followed by permeabilization for 4′,6-diamidino-2-phenylindole (DAPI) staining. After additional washes, the coverslips were mounted on slides using Fluoromount (Southern Biotechnology, Birmingham, AL). Immunolabeled cells were imaged with a Bio-Rad (Hercules, CA) Radiance 2100 laser scanning confocal system running the software Laser Sharp 3.0, coupled with a Nikon (Melville, NY) microscope (TE2000-U). Argon (488 nm) and green HeNe (543 nm) lasers were used to excite the fluorophores. Image processing was done with Photoshop (Adobe Systems, San Jose, CA).

Flow cytometry assay

A total of 10^6 hippocampal neurons from wild-type mice were preincubated in the absence or presence of (7.5×10^{-6} M) of recombinant His_6-STI1 in blocking solution (0.5% BSA in PBS) for 1 h at 4°C. Cells were washed and incubated with anti-His-Tag antibody (1:300; Amersham Biosciences), followed by anti-mouse IgG conjugated to R-phycoerythrin (1:200; Dako), both for 1 h at 4°C. Analyses were performed using a Becton Dickinson (Mountain View, CA) FACScan cytometer, and data acquisition from 10,000 cells was done with the Consort 32 system Lysis II software (Becton Dickinson).

His tag pull down

A total of 8×10^6 neurons from wild-type embryos cultured on poly-L-lysine were incubated with His_6-STI1 (1.2×10^{-6} M) for 20 min at 37°C, the medium was removed, and the cells were lysed with ice-cold (PBS) 0.5% NP-40 plus Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and centrifuged for 10 min at $6000 \times g$. The protein extract was incubated with 50 μl of Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 3 h at room temperature. The beads were then washed with 1% NP-40 plus 20 mM imidazole. Bound material was eluted with Laemmli buffer at 100°C and analyzed by immunoblotting using anti-STI1 (1:10,000; Bethyl), anti-PrP^c (1:1000), or anti-His-Tag-HRP (1:5000; Invitrogen) antibody.

Alexa Fluor 488 STI1 labeling

His_6-STI1 (1 mg) labeling was performed using the Alexa Fluor 488 labeling kit (Molecular Probes), according to the manufacturer's instructions. Wild-type hippocampal neurons (4×10^5 cells) were incubated with His_6-STI1 $Alexa_{488}$ (1.2×10^{-6} M) for 1 h at 4°C, washed with PBS, and fixed for 20 min at room temperature with 4% paraformaldehyde and 0.12 M sucrose in PBS. Immunofluorescence reaction with anti-PrP^c (1:100), followed by secondary antibody incubation (anti-mouse; 1:3000), was done as described above.

Immunoblotting analysis

Purified proteins (His_6-STI1 and His_6-STI1Δ230–245) or protein extracts prepared from wild-type (*Prnp*$^{+/+}$) and PrP^c-null (*Prnp*$^{0/0}$) mice hippocampal cells (Bueler et al., 1992) in Laemmli buffer were resolved in 10% SDS-PAGE, followed by immunoblotting with polyclonal antibody anti-STI1 (1:10,000) or anti-pepSTI1$_{230–245}$ (1:5000) (Zanata et al.,

2002). Protein loading control was performed with a polyclonal antibody to actin (1:200; Sigma). Rabbit nonimmune purified IgG was used as the immunoblotting negative control.

Neuritogenesis assays

Primary hippocampal cultures were obtained from E17 brains of either wild-type (*Prnp*$^{+/+}$; a strain generated by crossing F1 descendants from mating 129/SV and C57BL/6J) or PrP^{c}-null (*Prnp*$^{0/0}$) (Bueler et al., 1992) mice. The hippocampal structure was aseptically dissected in HBSS (Invitrogen) and treated with trypsin (0.06%) in HBSS for 20 min at 37°C. The protease was inactivated with 10% FCS in Neurobasal medium (Invitrogen) for 5 min. After three washes with HBSS, cells were mechanically dissociated in Neurobasal medium containing B-27 supplement (Invitrogen), glutamine (2 mM; Invitrogen), penicillin (100 IU), and streptomycin (100 μg/ml; Invitrogen). The cells (4×10^4 cells) were plated onto coverslips (13 mm) coated with 5 μg/ml poly-L-lysine (Sigma) and treated with recombinant His$_6$-STI1 and His$_6$-STI1Δ230–245; peptides pepSTI1$_{230–245}$ and pepSTI1$_{422–437}$; anti-PrP^{c} (6H4; 6 μg/ml); or anti-pepSTI1$_{230–245}$ (1:200) for 16 h at 37°C and in a 5% CO_2 atmosphere. For analysis of signal transduction mechanisms, cells were preincubated for 1 h with specific inhibitors U0126, KT5720, Bim, and Chel dissolved in dimethylsulfoxide (DMSO) before stimulation of neuritogenesis by His$_6$-STI1 for 16 h. Control cultures received the same concentration of DMSO (0.2%). The cells were fixed with 4% paraformaldehyde in 0.12 M sucrose in PBS for 20 min at room temperature, washed three times with PBS, and stained with hematoxylin.

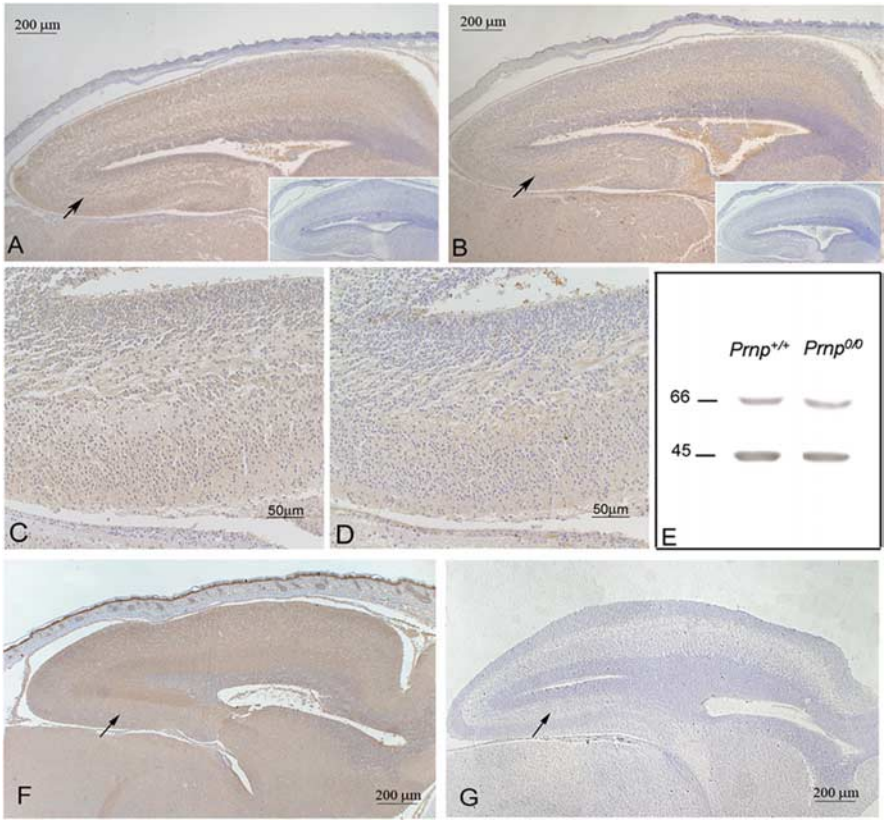

Figure 1. STI1 and PrP^{c} are expressed in the hippocampus. ***A–D***, ***F***, ***G***, Histological brain sections from wild-type (*Prnp*$^{+/+}$; ***A–D***) and PrP^{c}-null (*Prnp*$^{0/0}$; ***F***, ***G***) mouse embryos (E17) were immunostained for STI1 (***A***, ***C***, ***F***) or PrP^{c} (***B***, ***D***, ***G***) and counterstained with hematoxylin. Higher magnifications of the indicated areas (arrows) in ***A*** and ***B*** are shown in ***C*** and ***D***, respectively. Irrelevant antibody controls lack specific signals (***A***, ***B***, insets). The hippocampus is indicated by arrows in ***F*** and ***G***. ***E***, Brain homogenates from wild-type (*Prnp*$^{+/+}$) and PrP^{c}-null (*Prnp*$^{0/0}$) mice were immunoblotted using antibodies against STI1 (66 kDa) and actin (45 kDa).

Morphometric analyses were done using Image J software (National Institutes of Health, Bethesda, MD) and Neuron J plug in. The following parameters were measured: percentage of cells with neurites (cells with neurites of any size/total number of cells), number of neurites per cell (total number of neurites/number of cells with neurites), mean length of neurites per cell (total length of neurites/number of cells with neurites), and percentage of cells with neurites >30 μm, which represents three or more times the average cell body (number of cells with neurites >30 μm/total number of cells). Approximately, a total of 300 cells per sample were analyzed. Data are presented as mean ± SD from at least three independent experiments.

Construction of STI1Δ230–245

A recombinant PCR technique (Ausubel et al., 1993) was used to delete the PrP^{c} binding site of STI1. We amplified cDNA fragments using pTRC-His A STI1 (Zanata et al., 2002) with internal primers 5′-AAAGAGAAGAAGCATTATGAC-3′ and 5′-GTCATAATGCTTCTT-CTCTTT-3′ and external primers 5′-CCGCTCGAGGAGCAGGT-GAATGAGCTAAAGGA-3′ and 5′-CGGGGTACCTCACCGAATTGC-GATGAGACCC-3′. The PCR fragments were cloned between the *Xho*I and *Kpn*I restriction sites in the same vector. Sequencing analysis was done to check for the deletion. The expression and purification of this protein followed the same protocol as described previously (Zanata et al., 2002).

Overlay assay

The indicated amounts of recombinant His$_6$-PrP^{c} and BSA were adsorbed onto nitrocellulose membrane. The membrane was incubated with 5% nonfat milk in TBST (TBS and 0.05% Tween 20) for 1 h at room temperature and washed, followed by incubation with either wild-type His$_6$-STI1 (4 μg/ml) or mutant His$_6$-STI1Δ230–245 (4 μg/ml) for 16 h at 4°C. After extensive washing with TBST, membranes were incubated with polyclonal antibody anti-STI1 (1:10,000) (Zanata et al., 2002) for 2 h at room temperature, followed by a second incubation with peroxidase-coupled anti-rabbit IgG. The reaction was developed using enhanced chemiluminescence (Amersham Biosciences).

Kinase assays

p44/42 MAPK phosphorylation. Phosphorylation assays were done using the PhosphoPlus p44–42 MAPK (Thr202/Tyr204) antibody kit (Cell Signaling, Beverly, MA) according to the manufacturer's instructions. Briefly, primary hippocampal cell cultures (10^6 cells) from either *Prnp*$^{+/+}$ or *Prnp*$^{0/0}$ were plated on dishes pretreated with poly-L-lysine and stimulated with His$_6$-STI1 (3.5×10^{-7} M) for different incubation periods, rinsed once with ice-cold PBS, and lysed in Laemmli buffer. For assaying MAPK phosphorylation, cell extracts were subject to SDS-PAGE, followed by immunoblotting with anti-phospho-MAPK and anti-total MAPK antibodies (Cell Signaling). The bands obtained after x-ray film exposure to the membranes were analyzed by densitometric scanning and quantified using the Scion (Frederick, MD) Image software. Values represent the ratio between phospho-MAPK (p42 plus p44) and total MAPK (p42 plus p44) for each sample. Untreated *Prnp*$^{+/+}$ or *Prnp*$^{0/0}$ values were set as 1.0, and the others are relative to it.

p44/42 MAPK activity. The p44/42 MAPK assay kit (Cell Signaling) was used for estimating the activity of MAPK in primary hippocampal cultures treated with His$_6$-STI1 (3.5×10^{-7} M). Cells were disrupted in lysis buffer and centrifuged for 10 min at 4°C, and the supernatant was transferred to a fresh tube. Active MAPK was immunoprecipitated from 80 μg of total protein in each sample using an immobilized phospho-p44/42 MAPK monoclonal antibody (Cell Signaling). MAPK activity was evaluated by incubation with Elk-1 substrate, followed by electrophoresis and immunoblotting with anti-phospho Elk-1 (Cell Signaling).

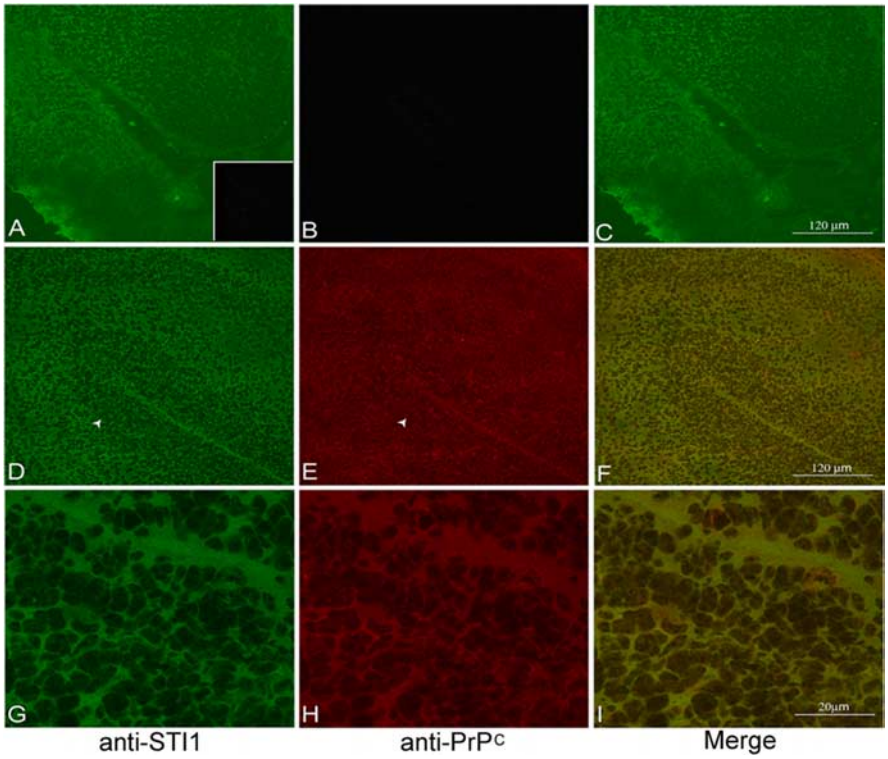

Figure 2. Colocalization of STI1 and PrP^c in the hippocampus. Histological brain sections of *Prnp*$^{0/0}$ (***A–C***) and *Prnp*$^{+/+}$ (***D–I***) mouse embryos (E17) were immunostained for STI1 (***A***, ***D***, ***G***) and PrP^c (***B***, ***E***, ***H***). The hippocampal structure marked by the arrowheads in ***D*** and ***E*** is shown at a higher magnification in ***G*** and ***H***. ***C***, ***F***, and ***I*** are merged images of immunostaining for STI1 (green) and PrP^c (red). There is an extensive colocalization (yellow) of the two proteins in the developing hippocampus. Preimmune rabbit serum displayed no specific staining (***A***, inset).

PKA activity. Primary hippocampal neurons (1.5×10^6cells) cultured onto poly-L-lysine were preincubated with 100 μM IBMX (Sigma) for 1 h at 37°C and treated with or without PKA inhibitor (KT5720; 6×10^{-8} M) for 1 h at 37°C. His$_6$-STI1 (1.2×10^{-6} M) or forskolin (10 μM) was incubated for 20 min at 37°C. The cells were washed with PBS and homogenized with ice-cold extraction buffer (150 mM NaCl, 20 mM $MgCl_2$, 1% Triton X-100, and 25 mM Tris-HCl, pH 7.4) plus Complete Protease Inhibitor Cocktail (Roche). Cellular debris was removed by centrifugation at 6000 × *g* for 10 min. The PKA activity was determined by γ-ATP incorporation to a PKA-specific substrate provided by the PKA assay system kit (Upstate Biotechnology, Lake Placid, NY). The reaction was performed according to the manufacturer's instructions.

Cell death assay

Primary hippocampal cultures (4×10^4cells) from wild-type mice were grown onto poly-L-lysine-coated coverslips and preincubated with His$_6$-STI1 (1.2×10^{-6} M) or control buffer (TBS) for 2 h, followed by staurosporine for 16 h. Cell cultures treated with signaling enzyme inhibitors received KT5720, U0126, Bim, or Chel 1 h before incubation with His$_6$-STI1. The cell cultures were fixed with 4% paraformaldehyde plus 0.12 M sucrose in PBS, pH 7.4, for 20 min and stained with propidium iodide (Sigma) in PBS and 0.1% Triton X-100 for 20 min. Cell death induced by staurosporine (Favata et al., 1998) was detected as condensed and pyknotic profiles. At least three independent experiments were done, and three microscopic fields were counted in each group.

Statistical analysis

The results represent the mean ± SD of at least three independent experiments. Two-way ANOVA with a Tukey's HSD *post hoc* test was used for neuritogenesis, cell death assays, and experiments with inhibitors. The phospho-MAPK statistical analyses were done using single-mean Student's *t* test.

Results

Expression and colocalization of STI1 and PrP^c in the hippocampus

STI1 is expressed in a variety of neurons and glia during neural development as well as in the adult nervous system, suggesting potential roles for this protein during development, plasticity, and regeneration (G.N.M. Hajj, R.M.P.S. Castro, T. Takiishi, M.H. Lopes, Z.S.P. Cook, and V.R. Martins, unpublished data). We compared the expression of STI1 and PrP^c in the embryonic brain. Sections from *Prnp*$^{+/+}$ (E17) mouse brain showed strong immunoreactivity for both STI1 (Fig. 1*A*,*C*) and PrP^c (Fig. 1*B*,*D*) in the cerebral cortex and developing hippocampus (nonspecific staining with the respective irrelevant antibodies are shown in the insets). STI1 also showed high levels of expression in *Prnp*$^{0/0}$ mouse brain (Fig. 1*F*), whereas PrP^c was not detected (Fig. 1*G*), demonstrating the specificity of the PrP^c staining. STI1 levels were examined in brain homogenates from *Prnp*$^{+/+}$ and *Prnp*$^{0/0}$ mice using SDS-PAGE, followed by immunoblotting. Figure 1*E* shows that STI1 levels are similar in *Prnp*$^{+/+}$ and *Prnp*$^{0/0}$ mice brain, indicating that STI1 expression seems to be independent of PrP^c.

When viewed through confocal fluorescence microscopy, brain sections from *Prnp*$^{+/+}$ mice confirmed an abundant concentration of both STI1 (Fig. 2*D*,*G*) and PrP^c (Fig. 2*E*,*H*) in the cortex and hippocampus. Figure 2*A–C* depicts brain sections from *Prnp*$^{0/0}$ mice, in which only STI1 (Fig. 2*A*) was detected. The distribution of both PrP^c and STI1 in the hippocampus is relatively uniform, and immunolabeling is colocalized (Fig. 2*F*,*I*), suggesting that both proteins can interact in this region of the brain.

In dissociated and permeabilized *Prnp*$^{+/+}$ hippocampal neurons, PrP^c and STI1 can be visualized in the cell body, perinuclear region, and along neurites, with a consistent colocalization (Fig. 3*Ah*). In unpermeabilized cells, PrP^c was distributed along the plasma membrane of both the cell body and neurites (Fig. 3*Aa*). STI1, in contrast, was detected mainly on the surface of the cell soma (Fig. 3*Ab*), where it colocalizes with PrP^c (Fig. 3*Ad*).

Previous studies have identified the involvement of PrP^c in neuritogenesis. To evaluate whether PrP^c-STI1 could play a role in hippocampal neurons, we added recombinant His$_6$-STI1 to these cell cultures. Flow cytometry demonstrated that recombinant His$_6$-STI1 binds to the surface of hippocampal neurons (Fig. 3*B*). Additionally, pull-down experiments were performed to confirm His$_6$-STI1 binding to PrP^c. Entire cells were treated with His$_6$-STI1, followed by lysis and incubation of the extract with Ni-NTA agarose. Resin-bound His$_6$-STI1- and STI1-associated proteins were assayed by immunoblotting with anti-STI1, anti-His-Tag, or anti-PrP^c antibodies. Recombinant His$_6$-STI1 binds to cellular PrP^c (Fig. 3*C*), whereas PrP^c did not associate with the resin in the absence of His$_6$-STI1 (Fig. 3*C*, Control).

Cultured hippocampal neurons were incubated with His$_6$-STI1Alexa$_{488}$ and immunostained with anti-PrP^c antibodies. Co-

localization data confirmed that His$_6$-STI1 binds to PrP^c, particularly at the soma surface (Fig. 3*D*). Thus, these data indicate that recombinant His$_6$-STI1 specifically binds resident PrP^c at the cell surface.

STI1 promotes neuritogenesis

Based on the STI1 and PrP^c colocalization in hippocampal neurons and in our previous description that interaction of PrP^c with laminin induced neuritogenesis (Graner et al., 2000a), we investigated whether STI1 may promote neuritogenesis through the engagement of PrP^c. Primary hippocampal cultures from *Prnp*$^{+/+}$ or *Prnp*$^{0/0}$ mouse embryos (E17) were treated either with recombinant wild-type His$_6$-STI1 or with pepSTI1$_{230–245}$ (the STI1 peptide that corresponds to the PrP^c binding site at STI1). Treatments led to a substantial increase in the proportion of wild-type cells with neurites, as assessed through the counting either of processes of all sizes (Fig. 4*A*) or of processes >30 μm (Fig. 4*B*). In contrast, no differences were seen in either the number of neurites per cell (Fig. 4*C*) or the mean length of neurites per cell (Fig. 4*D*). These results indicate that STI1 enhanced only the neuritogenic response of hippocampal cells (percentage of responsive cells) and did not alter the final neurite characteristics (number or length). Conversely, *Prnp*$^{0/0}$ neurons did not extend neurites in response to His$_6$-STI1 or pepSTI1$_{230–245}$ (Fig. 4*A*,*B*).

To characterize whether neuritogenesis promoted by STI1 depends on its specific interaction with PrP^c, we constructed an STI1 deletion mutant lacking residues 230–245 (His$_6$-STI1Δ230–245), therefore eliminating the domain previously characterized as containing the PrP^c binding site (Zanata et al., 2002). Figure 5*A* shows that His$_6$-STI1Δ230–245 has the expected molecular weight and is recognized by the anti-STI1 serum (lane 1), but not by an antibody that recognizes the deleted residues (anti-pepSTI1$_{230–245}$, lane 3). In contrast, wild-type His$_6$-STI1 is recognized by both antibodies (lanes 2, 4). An overlay experiment was performed to test the binding capacities of the wild-type and mutant His$_6$-STI1 to PrP^c. His$_6$-PrP^c and BSA were immobilized onto a nitrocellulose membrane, incubated with wild-type His$_6$-STI1 (lane 6) or His$_6$-STI1Δ230–245 (lane 5), and immunoreacted with the anti-STI1 antibody. This experiment confirmed that wild-type His$_6$-STI1 binds PrP^c (lane 6), whereas mutant His$_6$-STI1Δ230–245 does not (lane 5). The graph in Figure 5*B* shows that His$_6$-STI1Δ230–245 was unable to promote neuritogenesis in wild-type neurons, thus demonstrating that the PrP^c binding domain on STI1 is necessary to induce the neuritogenic response.

We further tested the effect of blocking the PrP^c–STI1 interaction in wild-type cells. Hippocampal neurons treated with His$_6$-STI1 in the presence of antibodies against PrP^c or pepSTI1$_{230–245}$ presented lower neuritogenic response compared with His$_6$-STI1-treated cells (control). To irrelevant control mouse IgG or preimmune rabbit serum (Fig. 5*C*). Therefore, blocking neuronal PrP^c with a specific antibody reproduced the results observed in *Prnp*$^{0/0}$ neurons. This indicates that the data obtained with PrP^c-null neurons were attributable to the absence of PrP^c, rather than to any random events.

Together, the results demonstrate that a specific interaction of STI1 with PrP^c promoted neuritogenesis and that the PrP^c binding domain of STI1 is both necessary and sufficient for inducing this effect.

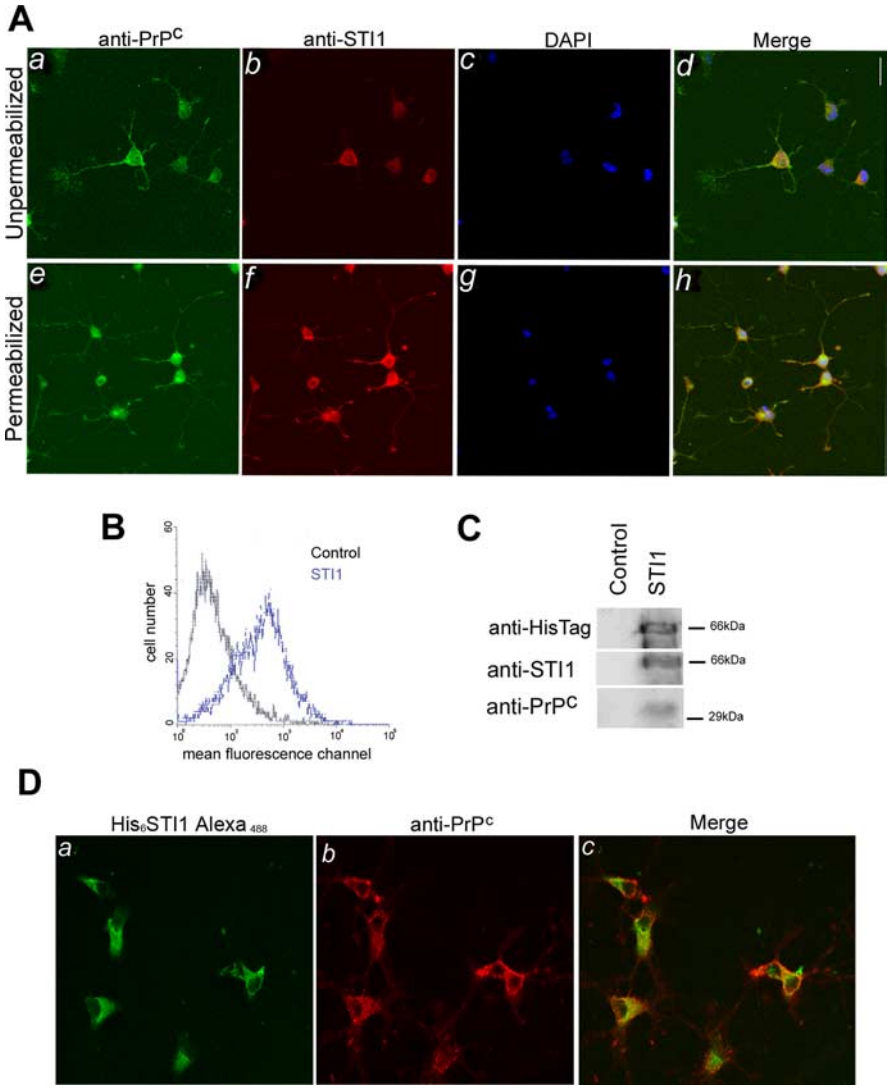

Figure 3. Endogenous and recombinant STI1 colocalize with PrP^c on the surface of hippocampal neurons. ***A***, Cultured, unpermeabilized, or permeabilized hippocampal neurons from *Prnp*$^{+/+}$ mice were immunostained for PrP^c (***a***, ***e***) and STI1 (***b***, ***f***). Cell nuclei were labeled with DAPI (***c***, ***g***). In unpermeabilized cells, colocalization was observed at the surface of the cell soma (***d***). Permeabilized cells show extensive colocalization of both proteins in the cell body, perinuclear region, and neurites (***h***). ***B***, *Prnp*$^{+/+}$ primary neuronal cultures were either treated with (blue curve) or without (black curve) His$_6$-STI1, followed by incubation with an anti-His-Tag antibody. Flow cytometry analysis detected His$_6$-STI1 at the neuronal surface. ***C***, Primary neuronal cultures were either treated with (STI1) or without (Control) His$_6$-STI1. Cells were washed and lysed, and the extracts were incubated with Ni-NTA-agarose. The bound material was eluted off the beads and analyzed by immunoblotting using anti-HisTag, anti-STI1, and anti-PrP^c antibodies. ***D***, Hippocampal cultures were treated with His$_6$-STI1-Alexa$_{488}$ (***a***) and immunostained with anti-PrP^c antibody (***b***). Extensive colocalization of both proteins was observed on the cell surface (***c***).

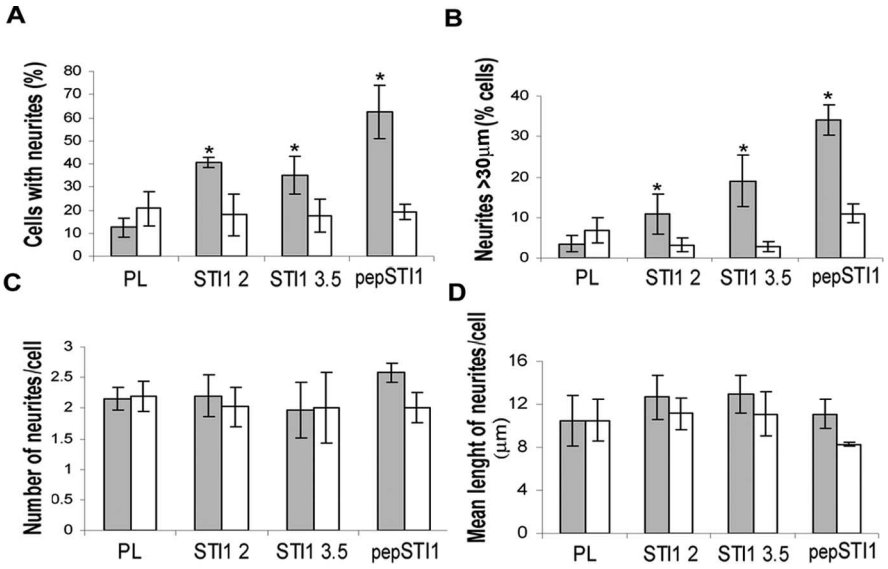

Figure 4. STI1 and its PrP^c binding domain induce PrP^c-dependent neuritogenesis. Cultured hippocampal neurons from *Prnp*$^{+/+}$ (filled bars) or *Prnp*$^{0/0}$ (open bars) were grown on poly-L-lysine and incubated with His$_6$-STI1 at 2×10^{-7} M (STI1 2) or 3.5×10^{-7} M (STI1 3.5), or with 1.6×10^{-5} M of STI1 peptide$_{230-245}$ (pepSTI1). Neurons were fixed and stained, and the neurites were quantified. Values represent the means of at least three independent experiments. Error bars represent SD. *$p < 0.005$ versus poly-L-lysine (ANOVA and Tukey's *post hoc* test). ***A***, Percentage of cells with neurites of any size. ***B***, Percentage of cells with neurites >30 μm. ***C***, Number of neurites per cell. ***D***, Mean length of neurites per cell. PL, Poly-L-lysine.

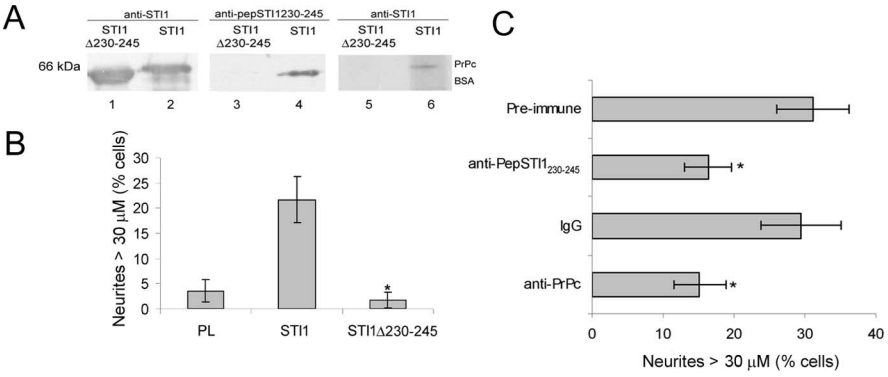

Figure 5. STI1-induced neuritogenesis depends on the PrP^c binding site and PrP^c expression. ***A***, Wild-type His$_6$-STI1 (lanes 2, 4) and His$_6$-STI1Δ230–245 (lanes 1, 3) were immunoblotted with anti-STI1 (lanes 1, 2) and anti-pepSTI1$_{230-245}$ antibodies (lanes 3, 4). Lanes 5 and 6 show the overlay assay, in which BSA (2 μg) and His$_6$-PrP^c (1 μg) were adsorbed onto a membrane that was incubated with His$_6$-STI1 (lane 6) or His$_6$-STI1Δ230–245 (lane 5), followed by immunoblotting with anti-STI1 antibody. ***B***, The graph shows neuritogenesis of primary hippocampal neurons from *Prnp*$^{+/+}$ embryos treated with either His$_6$-STI1 or His$_6$-STI1Δ230–245. *$p < 0.005$, statistically significant compared with His$_6$-STI1-treated neurons (ANOVA and Tukey's *post hoc* test). ***C***, Hippocampal neurons were incubated with 3.5×10^{-7} M His$_6$-STI1, followed by treatment with anti-pepSTI1$_{230-245}$ serum or its preimmune control. Neurons in the presence of His$_6$-STI1 were also treated with anti-PrP^c monoclonal antibody (6H4) or control mouse IgG. Values represent the mean of at least three independent experiments. *$p < 0.005$ versus control (ANOVA and Tukey's *post hoc* test). Error bars represent SD. PL, Poly-L-lysine.

MAPK activity is required for neuritogenesis induced by STI1–PrP^c interaction

We showed previously that engagement of PrP^c with a peptide mimicking the PrP^c binding site of the STI1 molecule produced the activation of both the cAMP/PKA and MAPK pathways (Chiarini et al., 2002). However, the protection against programmed cell death of retinal neurons produced by engagement of PrP^c required the activity of PKA rather than MAPK. Interestingly, MAPKs have been implicated in neuritogenesis stimulated by cell adhesion molecules and neurotrophic factors (Doherty et al., 2000; Huang and Reichardt, 2001).

To evaluate whether MAPK activation is required for the STI1-PrP^c neuritogenic response, we cultured hippocampal neurons in the presence of U0126, a specific inhibitor of the MAPK kinases MEK-1 and MEK-2, which blocks ERK1/2 phosphorylation (Favata et al., 1998). In addition, we tested a set of specific inhibitors of other signal transduction pathways.

The MAPK inhibitor significantly decreased the percentage of cells with neurites (Fig. 6*A*) and the percentage of cells with neurites >30 μm (Fig. 6*B*), whereas the mean number of neurites per cell (Fig. 6*C*) and the mean neurite length per cell (Fig. 6*D*) remained unaltered. Cresyl violet staining indicated no significant cell death among cells treated with U0126 (data not shown). Conversely, neither KT5720, a cell-permeable selective inhibitor of PKA, nor specific PKC inhibitors Chel and Bim (Audesirk et al., 1997) had any effect on neuritogenesis induced by His$_6$-STI1 (Fig. 6*A*,*B*). Furthermore, these drugs did not alter the number of neurites or their length (Fig. 6*C*,*D*).

To verify whether the STI1–PrP^c interaction induces a MAPK cascade in hippocampal neurons, immunoblotting assays were performed to determine the phosphorylation/activation status of the MAPK proteins (Fig. 7*A*, pMAPK). Immunoblotting against total MAPK (Fig. 7*A*, MAPK) was performed as a protein loading control. The relative levels of MAPK phoshorylation were quantified in *Prnp*$^{+/+}$ (Fig. 7*B*) and *Prnp*$^{0/0}$ (Fig. 7*C*). *Prnp*$^{+/+}$ neurons treated with His$_6$-STI1 presented a rapid increase in the phosphorylation status of both the p42 and p44 forms of MAPK, whereas no effect was observed in *Prnp*$^{0/0}$ cells.

The functional activity of p42/p44 MAPK was determined by assessing its ability to phosphorylate a specific recombinant substrate, Elk1, which was then visualized by immunoblotting (Fig. 7*A*, pElk-1). His$_6$-STI1 also induced p42/p44 MAPK activation in *Prnp*$^{+/+}$ neurons but not in *Prnp*$^{0/0}$ neurons (Fig. 7*A*). A higher basal phosphorylation and activity levels of MAPK were found in *Prnp*$^{0/0}$ compared with wild-type cells (Fig. 7*A*), confirming our previous data in newborn mouse retina (Chiarini et al., 2002), as well as those of other authors in adult brain and cerebellum (Brown et al., 2002).

A substantial increase in MAPK phosphorylation was observed in *Prnp*$^{+/+}$ neurons but not in *Prnp*$^{0/0}$ cells treated with pepSTI1$_{230-245}$. Conversely, His$_6$-STI1Δ230–245, which has no effect in neuritogenesis, was also unable to induce MAPK phosphorylation either in *Prnp*$^{+/+}$ or in *Prnp*$^{0/0}$ neurons (Fig. 7*D*).

These results show that neuritogenesis induced by interaction of PrP^c and STI1 depends on MAPK rather than the PKA or PKC pathways.

STI1 promotes neuroprotection in hippocampal neurons through cAMP-dependent protein kinase

We then tested whether STI1 induces neuroprotective responses in hippocampal neurons similar to retinal explants (Chiarini et al., 2002; Zanata et al., 2002). Primary hippocampal cultures from mouse embryos (E17) were treated with staurosporine (*Streptomyces staurospores*), a nonselective protein kinase inhibitor (Ruegg and Burgess, 1989) that is often used as a general inducer of apoptosis (Nicotera and Orrenius, 1998).

Hippocampal neurons from *Prnp*$^{+/+}$ mice were sensitive to cell death induced by staurosporine in a dose-dependent manner (Fig. 8*A*). Treatment with His$_6$-STI1 rescued *Prnp*$^{+/+}$ neurons, depending on the presence of the PrP^c binding site of STI1, because the mutated His$_6$-STI1Δ230–245 had no effect on cell survival (Fig. 8*B*).

We have found previously that His$_6$-STI1, pepSTI1$_{230–245}$, and another PrP^c-engaging peptide prevented programmed cell death of undifferentiated postmitotic retinal cells induced by anisomycin, through activation of PKA (Chiarini et al., 2002; Zanata et al., 2002). To address whether the PKA pathway is also involved in STI1-induced neuroprotection of hippocampal neurons, we tested the effects of specific signaling inhibitors. The neuroprotective effect of His$_6$-STI1 was abrogated by the PKA inhibitor (KT5720), whereas PKC (Bim or Chel) and MAPK (U0126) inhibitors had no effect (Fig. 8*B*). In nonstimulated neurons (absence of STI1), inhibitors had no effect on staurosporine-induced cell death (supplemental Fig. 9, available at www.jneurosci.org as supplemental material).

The activity of PKA was also assessed to confirm its involvement in the neuroprotection stimulated by His$_6$-STI1. Incubation of *Prnp*$^{+/+}$ hippocampal cells with His$_6$-STI1 was followed by an increase in PKA activity, comparable to that obtained with forskolin, a potent activator of PKA (Fig. 8*C*). In contrast, treatment with His$_6$-STI1Δ230–245 was unable to induce PKA activation. The PKA inhibitor (KT5720) strongly inhibited the His$_6$-STI1-stimulated PKA activity.

These results demonstrate the involvement of PKA in neuroprotection induced by the interaction of STI1 with PrP^c in hippocampal neurons, similar to our previously described results in retinal tissue (Chiarini et al., 2002), whereas neither MAPK nor PKC are required for this effect.

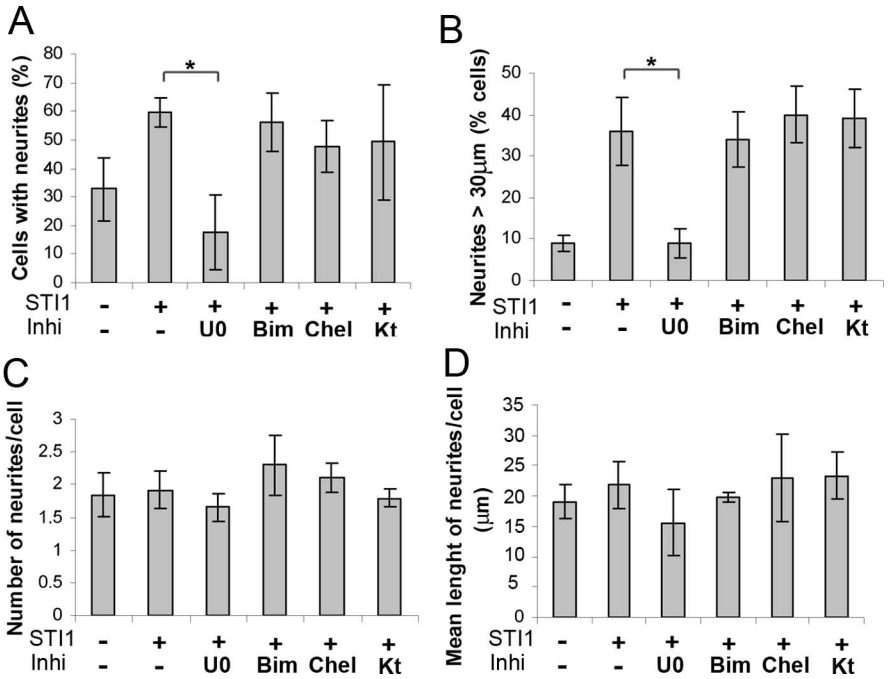

Figure 6. The MAPK pathway is required for neuritogenesis induced by STI1. *Prnp*$^{+/+}$ hippocampal neurons were plated on poly-L-lysine, pretreated for 1 h with inhibitors (2.5×10^{-5} M U0126, 5×10^{-7} M Bim, 10^{-7} M Chel, or 6×10^{-8} M KT5720), and incubated with His$_6$-STI1 (3.5×10^{-7} M) for 16 h. Cells were fixed and stained with hematoxylin, and neurites were quantified. Values represent the mean and SD of at least three independent experiments. ***A***, Percentage of cells with neurites of any size. ***B***, Percentage of cells with neurites >30 μm. ***C***, Number of neurites per cell. ***D***, Mean length of neurites per cell. *$p < 0.005$ versus control (ANOVA and Tukey's *post hoc* test). Inhi, Inhibitors; U0, U0126; Kt, KT5720.

Discussion

This investigation showed that (1) the cellular prion protein (PrP^c) and its binding partner STI1 colocalize in hippocampal neurons, (2) interaction of STI1 with PrP^c mediated by their cognate binding domains induces both neuritogenesis and neuroprotection of hippocampal neurons in culture, and (3) neuritogenesis induced by STI1 is mediated by the MAPK pathway, whereas neuroprotection induced by the same protein depends on a PKA pathway. These data add to the growing body of evidence that PrP^c is involved in neurotrophic signaling within the CNS and helps clarify its mechanisms.

It is becoming increasingly clear that PrP^c has a role in neuronal differentiation. Previous studies from our group showed that PrP^c is the main cellular receptor for a peptide at the γ1-laminin chain. This specific interaction between laminin and PrP^c induced neuritogenesis in mouse hippocampal neurons (Graner et al., 2000a).

We also showed the participation of PrP^c in neurite adhesion and maintenance through its interaction with laminin (Graner et al., 2000b). PrP^c has been proposed to be a cell-surface adhesion protein, and its early developmental distribution resembles that of adhesion molecules (Endo et al., 1989). Recent studies using a time-controlled transcardiac perfusion cross-linking procedure showed that proteins involved in cell adhesion and neurite outgrowth can be found in the molecular microenvironment of PrP^c in the living brain (Schmitt-Ulms et al., 2001, 2004). Furthermore, the laminin receptor, required for cell differentiation and growth, was identified as a cell-surface binding partner of PrP^c (Gauczynski et al., 2001; Hundt et al., 2001). PrP^c also interacts with glycosaminoglycans expressed on the cell surface (Shyng et al., 1995; Rieger et al., 1997; Pan et al., 2002). This is particularly relevant for brain development, because both laminin and glycosaminoglycans are developmentally regulated and contribute to axon growth and the formation of fiber tracts (Reichardt and Tomaselli, 1991). The abundance of PrP^c in elongating axons suggests a role for the protein in axon growth (Sales et al., 2002), which is supported by data from *Prnp*$^{0/0}$ mice, in which the hippocampal mossy fiber projection is disorganized (Colling et al., 1997).

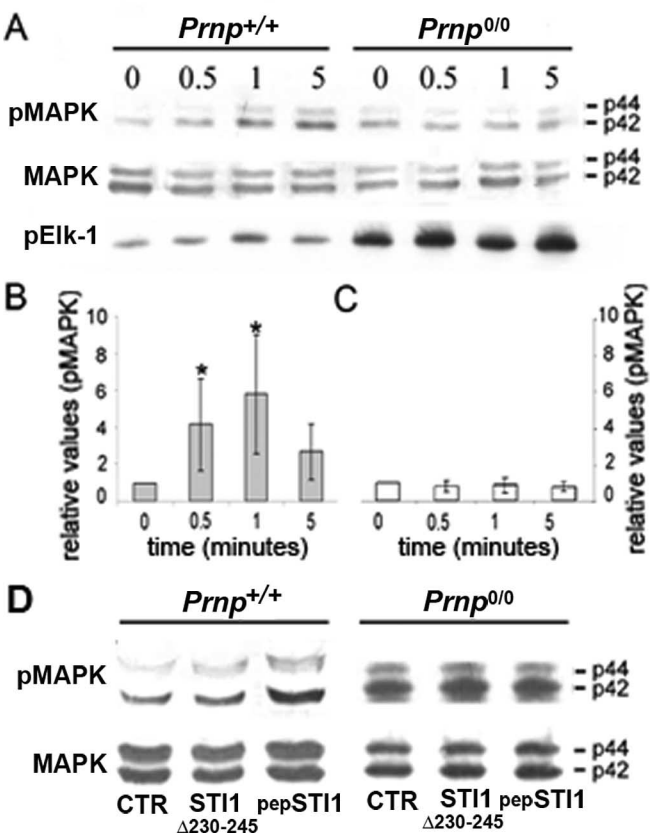

Figure 7. STI1 activates the MAPK pathway. ***A***, Hippocampal neurons from *Prnp*$^{+/+}$ and *Prnp*$^{0/0}$ mice were grown on poly-L-lysine and treated with His_6-STI1 (3.5×10^{-7} M) for 0.5, 1, or 5 min. Cell extracts were subjected to SDS-PAGE, followed by immunoblotting using anti-phospho-MAPK antibody. After stripping, the blots were reprobed with anti-total MAPK. MAPK activity was measured by phospho-MAPK immunoprecipitation, incubation with Elk-1 substrate, SDS-PAGE, and immunoblotting using anti-phospho-Elk-1. ***B***, ***C***, *Prnp*$^{+/+}$ (***B***) and *Prnp*$^{0/0}$ (***C***) immunoblots performed for MAPK phosphorylation and total MAPK were quantified by densitometry. The graphs represent the ratio between phospho-MAPK and total MAPK. $^*p < 0.05$ versus respective untreated cells (single-mean Student's *t* test). ***D***, *Prnp*$^{+/+}$ and *Prnp*$^{0/0}$ hippocampal neurons treated with His_6-STI1Δ230–245 (3.5×10^{-7} M) or pep-$STI1_{230-245}$ (1.6×10^{-5} M) for 1 min. Cell extracts were subjected to SDS-PAGE, followed by immunoblotting using anti-phospho-MAPK antibody. After stripping, the blots were reprobed with anti-total MAPK. pMAPK, Phospho-MAPK; pElk-1, phospho-Elk-1; CTR, control, untreated cells.

PrP^c activation by antibody cross-linking induces a signaling pathway that involves the tyrosine kinase p59fyn in a cell line capable of neuron-like differentiation (Mouillet-Richard et al., 2000). The p59fyn kinase has also been implicated in modulating axonal guidance of olfactory axons that express high levels of PrP^c throughout life and mediate neuronal cell adhesion molecule (NCAM)-induced neurite outgrowth (Morse et al., 1998; Sales et al., 1998;Beggs et al., 1997; Kolkova et al., 2000). Accordingly, recent findings show that PrP^c interacts directly with the NCAM, leading to stabilization of the latter in lipid rafts where fyn is activated, and induces NCAM-dependent neuritogenesis (Santuccione et al., 2005). Nevertheless, the PrP^c binding site at the NCAM was not characterized.

Various studies provided evidence that PrP^c is implicated in neuroprotection. PrP^c has been reported to protect human primary neurons against Bax-induced cell death, either from its location at the cell surface through an unidentified signaling pathway or from the cytosol (Bounhar et al., 2001; Roucou et al., 2003). It was reported recently that several signal transduction pathways involved in survival are activated in mouse primary cerebellar granule neurons grown in PrP-coated tissue culture plates. In this preparation, homophilic PrP^c interaction led to activation of PKA, Src-related tyrosine kinases, PI3 kinase/Akt, and MAPK/ERK kinases. Among downstream targets, increased Bcl-2 levels and decreased Bax levels were observed, consistent with PrP^c-triggering survival signals (Chen et al., 2003).

Investigation of PrP^c-interacting proteins is an important tool to understand not only cellular signaling triggered by, but also the biological function(s) related to, PrP^c. This approach is more likely to reveal physiological functions than antibody-mediated cross-linking (Mouillet-Richard et al., 2000; Hugel et al., 2004; Monnet et al., 2004).

Accordingly, we have demonstrated that interaction of PrP^c with its ligand STI1 protein activates a PKA-dependent signaling pathway to rescue retinal cells from induced apoptosis (Chiarini et al., 2002; Zanata et al., 2002). Recently, Onodera and colleagues (Sakudo et al., 2005) showed that PrP^c cooperates with STI1 to regulate superoxide dismutase activity and consequently to modulate cell survival.

The current experiments provided clear evidence that neuritogenesis induced by STI1 depends on the expression of PrP^c and that MAPK family members are required for this effect. In turn, neuroprotection induced by STI1–PrP^c interaction in hippocampal neurons, similar to our previous studies in retinal cells, is mediated by a PKA signaling pathway.

It is interesting to note that the concentration of His_6-STI1 required to promote neuroprotection is higher than the one necessary to induce neuritogenesis. This can be attributable to the activation threshold of the distinct MAPK and PKA signaling pathways involved in each phenomenon.

Therefore, we propose a model in which PrP^c interacts with STI1 and transduces both a survival or protective signal through PKA and a neuritogenesis/differentiation signal through the MAPK pathway in neuronal cells (supplemental Fig. 10, available at www.jneurosci.org as supplemental material). The PrP^c–STI1 complex may be formed by proteins either in the same cells (supplemental Fig. 10*A*, available at www.jneurosci.org as supplemental material) or in distinct cells (supplemental Fig. 10*B*, available at www.jneurosci.org as supplemental material). In our previous study, we speculated that STI1 could act as soluble neurotrophic factor (Chiarini et al., 2002) interacting with PrP^c. STI1 has indeed been found released by certain tumor cell lines (Eustace and Jay, 2004), glial cells, and in lower amounts by neurons in culture (our unpublished results). Thus, there is a third possibility in which either astrocytes or neurons secrete STI1, which then may act as a paracrine or autocrine factor in neuronal differentiation (supplemental Fig. 10*C*, available at www.jneurosci.org as supplemental material).

The significance of the high levels of STI1 colocalized with PrP^c at the surface of the cell soma rather than in neurites is not clear. The lack of detectable immunoreactivity to STI1 in neurites may be attributable either to low amounts of the protein at the surface or to a true differential distribution. This, as well as the relationships between STI1 and NCAM (Santuccione et al., 2005), both of which are involved in neuritogenesis mediated by PrP^c, remain to be addressed.

The results presented here advance a new biological function for STI1, support a significant role for PrP^c as a response mediator in both neuritogenesis and neuroprotection promoted by STI1, and provide additional insight into the molecular basis of STI1-induced intracellular signaling. The roles of PrP^c in neuro-

nal survival and neuritogenesis may be germane to loss-of-function components of the pathogenesis of prion diseases. Finally, the characterization of PrP^{c} ligands with neurotrophic activity is relevant both for developmental neurobiology as well as for pathology. These molecules may represent new targets for therapeutic intervention in neurodegenerative conditions.

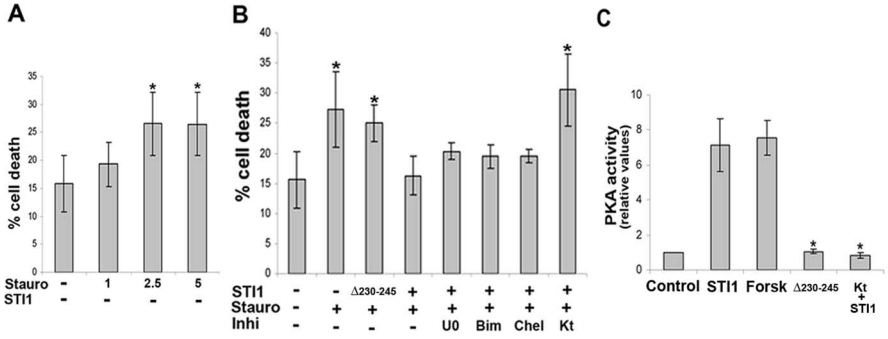

Figure 8. STI1 rescues hippocampal neurons from staurosporine-induced cell death through the PKA signaling pathway. ***A***, Hippocampal neurons from *Prnp*$^{+/+}$ mice were plated on poly-L-lysine, either pretreated with or without His$_6$-STI1 (1.2×10^{-6} M) for 1 h, and incubated with increasing concentrations of staurosporine (1, 2.5, and 5×10^{-8} M) for 16 h. ***B***, hippocampal neurons from *Prnp*$^{+/+}$ mice were plated on poly-L-lysine, either pretreated for 1 h with 1.2×10^{-6} M His$_6$-STI1 or His$_6$-STI1Δ230–245 or not treated. Cells were then treated with the inhibitors (5×10^{-5} M U0126, 5×10^{-7} M Bim, 10^{-7} M Chel, or 6×10^{-8} M KT5720) for 1 h. Cell death was induced by treatment with 2.5×10^{-8} M staurosporine for an additional 16 h. The cells were fixed and stained with propidium iodide, and the percentage of pyknotic profiles, indicating cell death, was estimated. ***C***, *Prnp*$^{+/+}$ neuronal cultures were treated with His$_6$-STI1 and His$_6$-STI1Δ230–245 for 20 min, and the PKA activity was analyzed. Forskolin was used as positive control, and the activity of the PKA inhibitor (KT5720) was also tested. Values represent the mean and SD of at least three independent experiments. *$p < 0.05$ versus control (ANOVA and Tukey's *post hoc* test). Stauro, Staurosporine; Inhi, inhibitors; U0, U0126; Kt, KT5720; Forsk, forskolin.

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