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Neurotoxicity induces cleavage of p35 to p25 by calpain

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Cyclin-dependent kinase 5 (cdk5) and its neuron-specific activator p35 are required for neurite outgrowth and cortical lamination^{1–3}. Proteolytic cleavage of p35 produces p25, which accumulates in the brains of patients with Alzheimer's disease⁴. Conversion of p35 to p25 causes prolonged activation and mislocalization of cdk5. Consequently, the p25/cdk5 kinase hyperphosphorylates tau, disrupts the cytoskeleton and promotes the death (apoptosis) of primary neurons. Here we describe the mechanism of conversion of p35 to p25. In cultured primary cortical neurons, excitotoxins, hypoxic stress and calcium influx induce the production of p25. In fresh brain lysates, addition of calcium can stimulate cleavage of p35 to p25. Specific inhibitors of calpain, a calcium-dependent cysteine protease, effectively inhibit the calcium-induced cleavage of p35. *In vitro*, calpain directly cleaves p35 to release a fragment with relative molecular mass 25,000. The sequence of the calpain cleavage product corresponds precisely to that of p25. Application of the amyloid β -peptide A β (1–42) induces the conversion of p35 to p25 in primary cortical

neurons. Furthermore, inhibition of cdk5 or calpain activity reduces cell death in A β -treated cortical neurons. These observations indicate that cleavage of p35 to p25 by calpain may be involved in the pathogenesis of Alzheimer's disease.

The open reading frame of p35 does not contain introns², so alternative splicing cannot account for the generation of p25. Internal initiation of translation of p35 messenger RNA is also unlikely to produce p25 because there is no internal methionine near the beginning of the p25 sequence. Proteolytic cleavage is, therefore, the most likely mechanism for conversion of p35 to p25 (Fig. 1a).

Despite extensive efforts to identify p25 in the mouse, only full-length p35 was detectable during embryonic development and in the adult (data not shown). We next sought to determine whether p25 could be produced *in vivo* under certain experimental conditions. We found that 4 h of focal ischaemia, induced by middle cerebral artery occlusion in mice, produced p25 in the ipsilateral cortex but not in the control contralateral cortex (Fig. 1b). The conversion of p35 to p25 caused it to relocalize to the cytoplasm (Fig. 1c), as reported previously⁴.

To investigate the mechanism of the conversion of p35 to p25 further, we tested for conditions that would induce the appearance of p25 in cultured primary cortical neurons. Treatment with hydrogen peroxide stimulated cleavage of p35 to p25 in primary neurons (Fig. 1d). Other insults such as treatment with the excitatory amino-acid glutamate also caused the production of p25 in cortical neurons at high concentrations of glutamate (Fig. 1e). An increase in intracellular calcium levels, caused by the calcium ionophore ionomycin, stimulated efficient conversion of p35 to p25 (Fig. 1f). These results indicate that neurotoxicity induces cleavage of p35 to p25 and suggest a role for calcium in this process.

To identify the protease that cleaves p35 to p25, we sought to recapitulate the proteolytic cleavage event. In fresh mouse brain lysates, 1 mM Ca²⁺ efficiently stimulates the cleavage of p35 (Fig. 2a). The cleavage product is likely to be p25, as it has a relative molecular mass of 25K and co-migrates with recombinant p25 expressed in COS-7 cells (lane 1). Also, like p25, it is specifically recognized by the p35 carboxy-terminal-specific antibody, but not by the p35 amino-terminal-specific antibody (see Supplementary Information).

p35 contains no obvious consensus sequences for cleavage by known proteases. To identify the protease activated by calcium, we tested protease inhibitors with different specificities for their effectiveness in inhibiting the calcium-stimulated p35 conversion. Calpeptin and calpain inhibitor II, which inhibit the calcium-dependent cysteine protease calpain, completely inhibited p35 cleavage (Fig. 2b, lanes 3–4), whereas the general cysteine protease inhibitor leupeptin partially inhibited p35 cleavage (lane 8). A titration of four calpain-specific inhibitors shows that 10 nM calpeptin, 100 nM calpain inhibitor I, 100 nM calpain inhibitor II and 5 nM calpastatin effectively inhibit p35 conversion (Fig. 2d and Supplementary Information), consistent with the reported median inhibitory concentration (IC₅₀) values for these inhibitors⁵. The lack of effect of the cdk5 inhibitor roscovitine indicates that cdk5 activity may not be necessary for cleavage to occur (Fig. 2b, lane 9).

m-calpain and μ -calpain are the two main isoforms of calpain in the brain⁶. The two calpains differ in their calcium requirements but have similar substrate specificities. μ -calpain requires 3–50 μ M calcium for half-maximal activity, whereas m-calpain requires 0.2–1 mM calcium for activity. To determine whether calpain was indeed activated in the conditions tested *in vitro*, we examined the cleavage of a well characterized calpain substrate, non-erythroid α -spectrin (also known as α -fodrin)⁵. One millimolar calcium, which stimulated conversion of p35 to p25 in mouse brain lysates, also led to cleavage of endogenous spectrin into the characteristic 145K and 150K fragments, indicating that calpain was activated (Fig. 2c). Furthermore, spectrin cleavage was inhibited by calpeptin,

calpain inhibitor I and leupeptin, showing that calpain activation correlates tightly with p35 cleavage.

To investigate further whether calpain cleaves p35 in brain lysates, we fractionated mouse brain lysates by glycerol gradient centrifugation and examined whether the p35 cleavage activity co-segregated with calpain activity. We incubated the glycerol gradient fractions with either purified p35 or spectrin; both p35 cleavage activity and calpain activity completely co-segregated in fractions 8–10 (Fig. 3a, b). Depletion of m-calpain from brain lysates using an m-calpain-specific antibody significantly reduced the Ca²⁺-induced cleavage of p35 (Fig. 3c–e), further indicating that conversion of p35 to p25 may lie downstream of calpain activation.

To determine whether calpain directly cleaves p35, we incubated purified calpain with purified p35. Both m-calpain and μ -calpain cleaved p35 to a 25K fragment that co-migrates with p25 on SDS-PAGE (Fig. 3f). A titration of purified m-calpain and μ -calpain shows that 0.002 unit of m-calpain and 0.006 unit of μ -calpain cause half-maximal cleavage of p35 (Fig. 3g, h). To verify that the 25K fragment produced by calpain cleavage is indeed p25, we subjected p35 purified from a baculovirus expression system to calpain cleavage. The 25K calpain cleavage product was micro-sequenced from its N terminus. The first five amino acids from the N terminus of this fragment are AQPPP, which precisely matches the N terminus of p25 previously purified from bovine brain lysates⁷ (Fig. 3i).

To understand the mechanism for p35 cleavage in primary cortical neurons, we tested whether an increase in intracellular calcium is required for p35 conversion. H₂O₂ or ionomycin did not

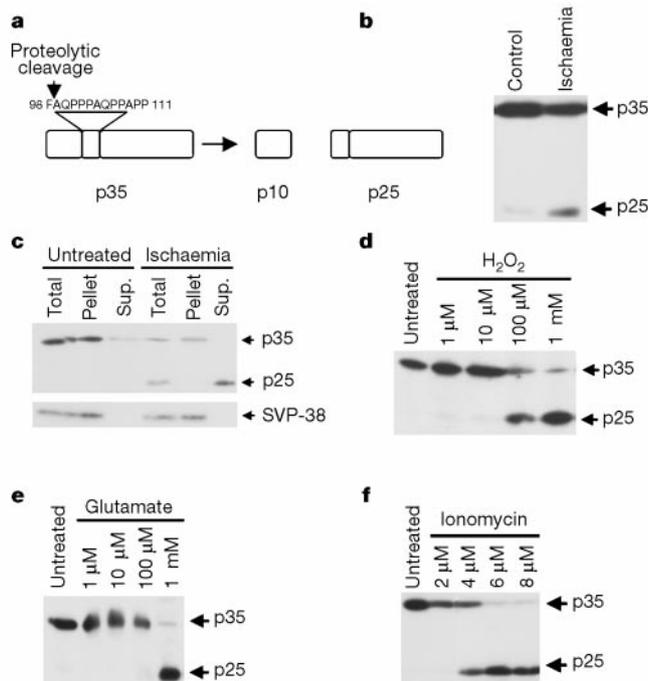


Figure 1 Neurotoxicity induces cleavage of p35 to p25. **a**, The cdk5 activator p35. Proteolytic cleavage of p35 between residues 98 and 99 liberates the neurotoxic 25K C-terminal fragment termed p25. **b**, Ischaemia induces conversion of p35 to p25. Western blot of p35 in the ipsilateral and control contralateral mouse brain tissue after 4 h of focal ischaemia. **c**, Distribution of p35 and p25 in fractionated brain lysates. The supernatant contains cytoplasmic proteins; the pellet corresponds to proteins associated with the membrane, cytoskeleton and nucleus. Synaptophysin (SVP-38) was used as a membrane marker. **d**, Oxidative stress induces p35 conversion. Neurons were treated with increasing amounts of H₂O₂, lysed and assayed for p35 cleavage. **e**, Glutamate induces p35 conversion. Neuronal cultures were treated with increasing concentrations of glutamate and assayed for p35 cleavage. **f**, Ionomycin induces p35 conversion. Rat cortical neurons were treated with the indicated amount of ionomycin and assayed for p35 cleavage.

stimulate conversion of p35 to p25 in neurons that were incubated in calcium-free medium (Fig. 4a). Similarly, removing calcium with either EGTA or BAPTA-AM also prevented conversion of p35. These results indicate that calcium is necessary for conversion of p35. The addition of calpeptin, which inhibits calpain activity in neurons (as indicated by the reduction of spectrin cleavage (Fig. 4c), also inhibited the cleavage of endogenous p35 to p25 caused by H₂O₂ (Fig. 4b). Similarly, conversion of p35 to p25 induced by ionomycin was completely reversed by increasing concentrations of calpeptin (Fig. 4d, e). Together, these experiments indicate that activation of calpain by neurotoxic processes through calcium influx is both necessary and sufficient for conversion of p35 to p25 in cortical neurons.

As p25 is found to accumulate in brains of Alzheimer's disease patients but not in normal brains, and because it is present in neurons containing neurofibrillary tangles⁴, we were interested in determining whether the amyloidogenic peptide A β (1–42) can induce conversion of p35 to p25. The A β peptides are primary constituents of the amyloid plaques found in the brains of patients with Alzheimer's disease. They have been shown to aggregate and cause neuronal death⁸. Treatment of primary cortical neurons with 20 μ M A β (1–42) peptide resulted in conversion of p35 to p25 and breakdown of endogenous spectrin from 280K to the 150K and 145K products (Fig. 5a, b). The same concentration of a control peptide, A β (42–1), induced neither conversion of p35 to p25 nor cleavage of spectrin. These results indicate that, in Alzheimer's

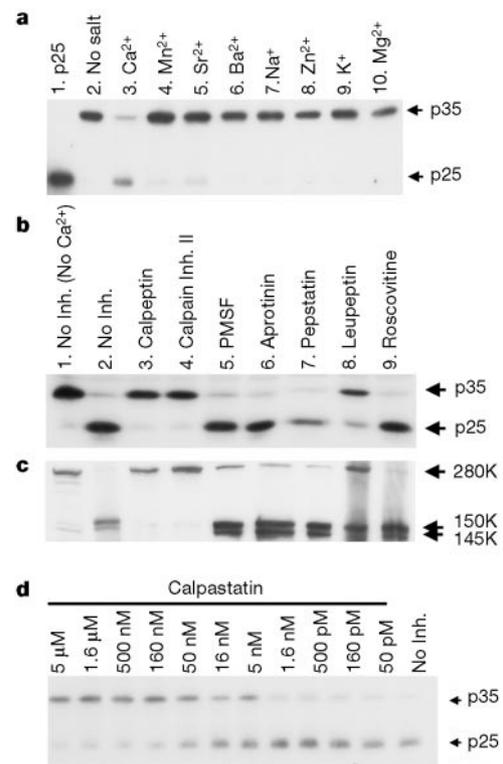


Figure 2 Ca²⁺ is necessary for p35 cleavage *in vitro*. **a**, Ca²⁺ induces p35 cleavage in mouse brain lysates. Western blot of p35 showing the effects of adding different chloride salts (1 mM) to mouse brain lysates. Lane 1, COS cell lysates transfected with recombinant p25 (control). **b**, Calpain inhibitors can inhibit conversion of p35 to p25. Calcium chloride was added to mouse brain lysate to stimulate p35 cleavage in the presence of the following inhibitors: 2 μ M calpeptin (lane 3), 5 μ M calpain inhibitor II (lane 4), 1 mM PMSF (lane 5), 1 μ g μ l⁻¹ aprotinin (lane 6), 1 μ g μ l⁻¹ pepstatin (lane 7), 1 μ g μ l⁻¹ leupeptin (lane 8), 10 μ M roscovitine (lane 9). **c**, Calpain activation correlates with p35 cleavage activity. Samples as in **b** were probed with an antibody against the endogenous calpain substrate non-erythroid α -spectrin. **d**, Mouse brain lysates treated with 5 mM calcium chloride and the indicated amount of calpastatin was assayed for p35 conversion.

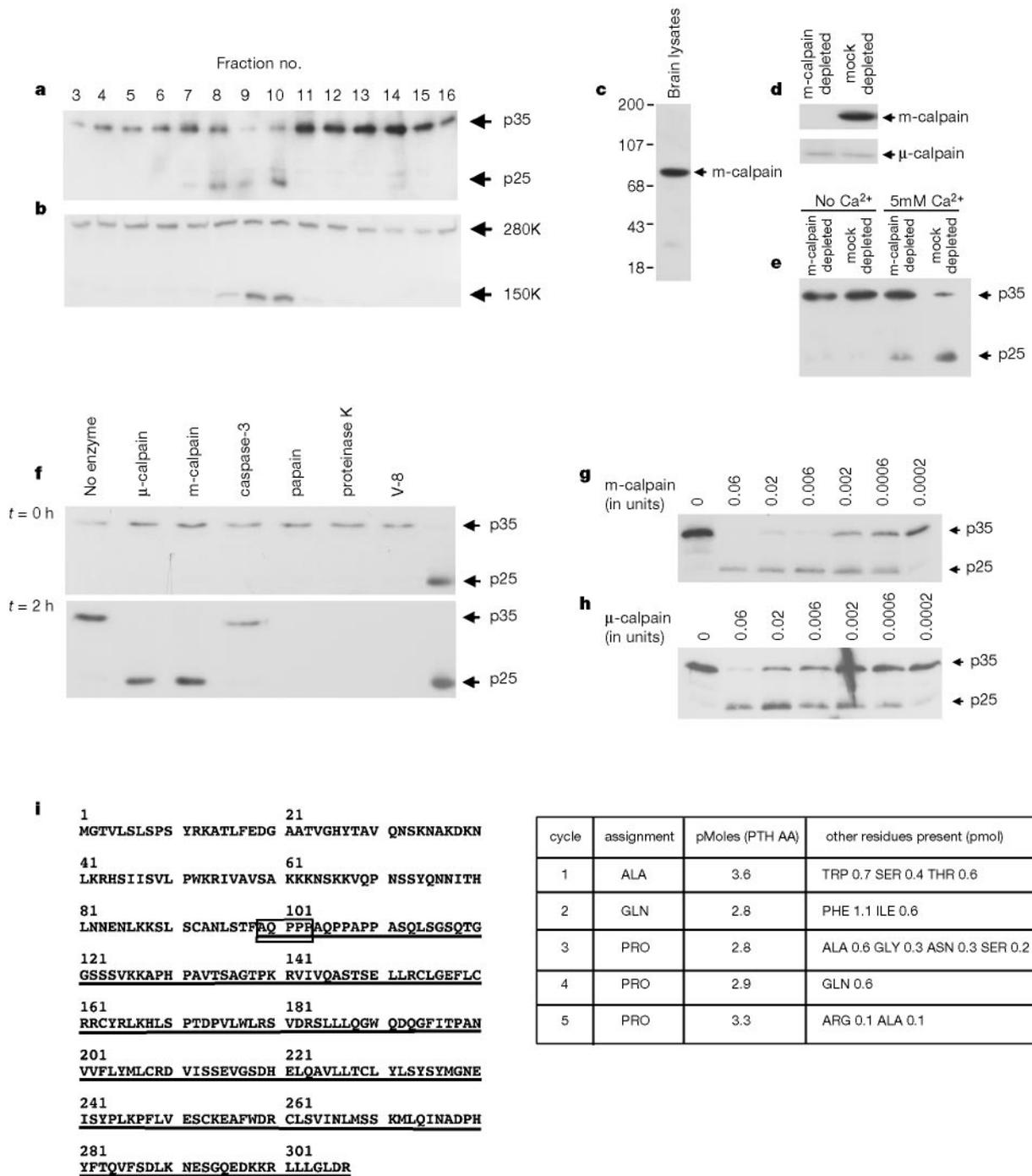


Figure 3 Calpain directly cleaves p35 to p25. **a**, Calpain activity co-fractionates with p35-cleaving activity. Mouse brain lysate was fractionated by a glycerol gradient. Each fraction was incubated with p35 immunoprecipitated from mouse brain lysates for 2 h at 25 °C to assay for p35 cleavage activity. **b**, The same fractions as in **a** were incubated with inactivated mouse brain lysate (as a source of spectrin) and assayed for spectrin cleavage activity. **c**, Western blot on rat brain lysates showing specificity of the m-calpain antibody. **d**, Western blots showing the levels of m-calpain and μ-calpain in rat brain lysates after immunodepletion. Endogenous m-calpain was completely depleted, whereas μ-calpain levels remained the same. **e**, Immunodepleting m-calpain from rat brain lysates substantially reduces the p35-cleavage activity. Rat brain lysates depleted 3 times with the m-calpain specific antibody was treated with 5 mM Ca²⁺ and assayed for p35

cleavage. **f**, Calpain cleaves p35. p35 immunoprecipitated from mouse brain lysates was treated with the proteases as indicated. Western blot of p35 before (upper panel) and after (lower panel) protease treatment. **g**, Decreasing concentrations of m-calpain was incubated with p35 purified from baculovirus-infected insect cells for 30 min at 30 °C and assayed for p35 cleavage. **h**, as in **g**, but with μ-calpain. **i**, The calpain cleavage product has the same sequence as p25. The entire sequence of p35 is shown, the sequence for p25 is underlined. Recombinant p35 from baculovirus-infected insect cells was purified and treated with m-calpain. The calpain cleavage product was microsequenced. The boxed area shows the first five N-terminal residues of the cleavage product obtained from protein microsequencing. The table shows the concentrations of residues present in each sequencing cycle in pmol.

cycle	assignment	pMoles (PTH AA)	other residues present (pmol)
1	ALA	3.6	TRP 0.7 SER 0.4 THR 0.6
2	GLN	2.8	PHE 1.1 ILE 0.6
3	PRO	2.8	ALA 0.6 GLY 0.3 ASN 0.3 SER 0.2
4	PRO	2.9	GLN 0.6
5	PRO	3.3	ARG 0.1 ALA 0.1

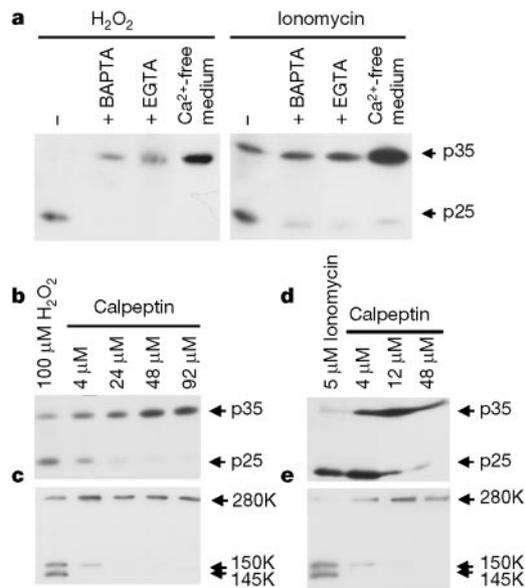


Figure 4 Conversion of p35 to p25 in primary cortical neurons is mediated by calpain and requires Ca^{2+} . **a**, Left panel, primary cortical neurons were treated with 1 mM H_2O_2 and 30 μ M BAPTA-AM, 5 mM EGTA or phosphate-buffered saline (PBS). Right panel, neurons treated with 4 μ M ionomycin were incubated with 500 μ M BAPTA-AM, 5 mM EGTA or PBS and assayed for p35 conversion. **b**, H_2O_2 -induced p35 conversion is mediated by calpain. 100 μ M H_2O_2 was added to neurons treated with increasing amounts of the

calpain inhibitor calpeptin, and the samples were assayed for p35 cleavage. **c**, as in **b**, but samples were assayed for cleavage of spectrin. **d**, The calpain inhibitor calpeptin can inhibit ionomycin-stimulated conversion of p35 to p25. 5 μ M ionomycin was added to cultures treated with increasing amounts of calpeptin, as indicated, and assayed for p35 cleavage. **e**, as in **d**, but cleavage of spectrin was assayed.

disease, the abundant amyloid plaques in the brain can cause calpain activation and conversion of p35 to p25.

$A\beta(1-42)$ induces cell death in cell-culture models. As $A\beta(1-42)$ causes conversion of p35 to p25, and the p25/cdk5 kinase induces cell death in cultured primary cortical neurons⁴, we were interested in determining whether the p25/cdk5 kinase is involved in $A\beta(1-42)$ peptide-induced cell death. Cells treated with 10 μ M $A\beta(1-42)$ showed markedly reduced survival compared with mock-treated neurons (Fig. 5c). We found that 10 μ M of the cdk5 inhibitor butyrolactone significantly inhibited neuronal cell death caused by $A\beta(1-42)$. Similarly, 20 μ M of the calpain inhibitor calpeptin considerably reduced $A\beta(1-42)$ -induced neuronal death. Inhibiting cdk5 activity by expressing a cdk5 antisense construct in hippocampal neurons also inhibits cell death caused by $A\beta(1-40)$ ⁹. Together, these results indicate that active cdk5 is involved in $A\beta$ -induced cell death.

The tight regulation of p35 allows cdk5 to be activated in a

temporally and spatially specific manner. In contrast, p25 causes mislocalization and constitutive activation of cdk5. The cleavage of p35 to p25 by calpain represents a new regulatory mechanism whereby proteolytic cleavage deregulates a protein kinase. By cleaving p35, calpain does not destroy cdk5 activity; rather, it alters the properties of cdk5 such that the p25/cdk5 kinase causes collapse of the cytoskeleton and cell death. Thus, whereas p35/cdk5 activity is necessary for proper development and other functions of the mature nervous system, p25/cdk5 activity is detrimental to neurons.

Altered calcium homeostasis is a convergence point of various aetiological factors relevant to the pathogenesis of Alzheimer's disease. In particular, loss of calcium homeostasis has been implicated in causing tau hyperphosphorylation and neuronal apoptosis¹⁰⁻¹³. This study, showing cleavage of p35 to p25 by calpain, suggests one mechanism by which calcium can activate a tau-phosphorylating kinase. Consistent with our model, activated m-calpain and μ -calpain are both abnormally upregulated in the

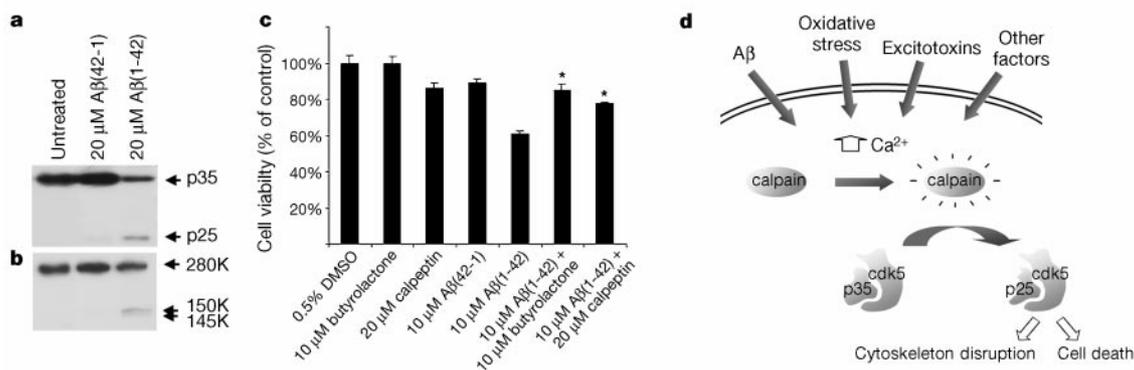


Figure 5 $A\beta(1-42)$ induces conversion of p35 to p25; inhibition of cdk5 or calpain reduces $A\beta(1-42)$ -induced cell death. **a**, $A\beta(1-42)$ causes generation of p25. Primary cortical neurons treated with 20 μ M of $A\beta(1-42)$ or $A\beta(42-1)$ for 4 days were assayed for p35 cleavage. **b**, as in **a**, but samples were assayed for spectrin cleavage. **c**, Inhibition of $A\beta(1-42)$ -induced cell death. Four-day-old primary cortical neurons were treated with

either 0.5% DMSO, 10 μ M butyrolactone, 20 μ M calpeptin, 10 μ M $A\beta(1-42)$ or 10 μ M $A\beta(42-1)$. Cell viability after a 24-h treatment was assayed with MTT and compared with untreated neurons. Data are averages (\pm s.e.) of four independent experiments. Asterisk, $P < 0.01$ compared with $A\beta(1-42)$ treated neurons. **d**, Neurotoxic processes activate the protease calpain, which results in p25 production and deregulation of cdk5.

brains of patients with Alzheimer's disease^{14–17}. In addition, active m-calpain accumulates in neurofibrillary tangles in Alzheimer's disease brains¹⁴.

The amyloid hypothesis proposes that the A β peptides, perhaps acting with other influences such as excitotoxins, free radicals and oxidative stress, cause an increased or unregulated entry of calcium into affected cells, which ultimately leads to the activation of a tau-phosphorylating kinase¹⁸. Our observations indicate that cdk5 may be one of the kinases 'activated' by amyloid- β peptide through calpain-mediated conversion of p35 to p25 (Fig. 5d). Given the potentially deleterious role of cdk5 in Alzheimer's disease, the calpain-mediated p35 cleavage pathway may serve as a target for pharmacological intervention. \square

Methods

Chemicals and antibodies

p25 antibody was raised against the whole protein and purified against glutathione S-transferase (GST)-p25. Non-erythroid α -spectrin antibody was purchased from Chemicon. p35 C-19, p35 N-20 and μ -calpain polyclonal antibodies were purchased from Santa-Cruz. The anti-rat m-calpain antibody is a gift from J. Elce. PMSF, Pepstatin A, aprotinin, leupeptin, MTT, glutamate and all metal chlorides were purchased from Sigma. Purified calpain I, purified calpain II, calpastatin, calpeptin, calpain inhibitor I, calpain inhibitor II, BAPTA-AM, butyrolactone and ionomycin were purchased from Calbiochem. H₂O₂ was purchased from Fisher Scientific. A β (1–42) and A β (42–1) were purchased from Bachem.

Primary cortical neuronal cultures

E17–E19 pregnant Long Evans rats were purchased from Harland Sprague–Dawley. Embryos were surgically removed and their cortices were dissected and cultured as described³. Cortical cultures were grown in basal growth medium on 6-well plates coated with laminin and poly-D-lysine. Treatments with H₂O₂ and glutamate were performed 10 d after plating for 5 h. Treatment with ionomycin was performed 4 d after plating for 5 h. In experiments where calpeptin, EGTA, or BAPTA-AM were used, the drugs were added 30 min before challenges were applied.

Glycerol gradient

A 11-ml 10–25% glycerol gradient was set up in ELB buffer (50 mM Tris pH 7, 0.1% NP-40, 250 mM NaCl). We layered 300 μ l of fresh mouse brain lysate on top of the gradient and centrifuged at 40K r.p.m. for 26 h. We collected 17 600- μ l fractions. We incubated 10 μ l of each fraction at 25 °C for 2 h with either purified p35 or frozen and thawed mouse brain lysates in a reaction buffer (5 mM CaCl₂, 5 mM cysteine, 150 mM imidazole, pH 7.5) to a final volume of 100 μ l. The product from each reaction was electrophoresed on a 12% gel and analysed as described in the text.

Protein microsequencing

Recombinant p35 and His-tagged cdk5 were produced by baculovirus and the p35/cdk5 complex was purified through a Ni²⁺ column. We treated 1 μ g of the purified p35/cdk5 complex with 2 units of purified m-calpain (Calbiochem) at 30 °C for 30 min. The reaction was stopped by 1% SDS. The entire reaction was electrophoresed on a 15% acrylamide gel and transferred to a 0.2- μ m PVDF membrane (BioRad). The membrane was stained with Coomassie Brilliant Blue (BioRad), the 25K cleavage product was excised, and 1/3 of the excised band was sequenced on Applied Biosystems Model 494 Precise Protein Sequencer with Model 140C Microgradient Delivery System and Model 785A Programmable Absorbance Detector.

Ischaemia

To induce focal ischaemia, adult mice (C57BL/6), weighing 16–20 g, were anaesthetized initially with 1.5% isoflurane and thereafter maintained in 1.0% isoflurane in 70% N₂O and 30% O₂. An 8.0 nylon monofilament suture coated with a silicone/hardener mixture (Heraeus Kulzer) was inserted into the right common carotid artery. The suture was advanced 9–10 mm from the insertion site through the internal carotid artery, occluding the middle cerebral artery (MCA). Mice all woke up hemiplegic and were killed with isoflurane 4 h after the induction of ischaemia. Global ischaemia was induced for 1 h as described¹⁹. Generation and fractionation of brain lysates was as described²⁰.

Cell viability assay

Primary cortical neurons were plated on 96-well plates. Four days after plating, neurons were incubated with 20 μ M A β (1–42) (from a stock of 1 mg ml⁻¹ in ddH₂O) with or without 10 μ M butyrolactone (from 2.8 mM stock in DMSO) or 20 μ M calpeptin (from 2 mM stock in DMSO) for 24 h. Cell viability was measured by treating neurons with MTT (Sigma) at a final concentration of 0.5 mg ml⁻¹ for 1 h. After MTT treatment, cell medium was replaced with 100 μ l DMSO and the optical density of each well at 495 nm was determined using a microtitre plate reader.

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LAG-3 is a putative transcriptional activator in the *C. elegans* Notch pathway

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Notch signalling controls growth, differentiation and patterning during normal animal development^{1,2}; in humans, aberrant Notch signalling has been implicated in cancer and stroke^{3,4}. The mechanism of Notch signalling is thought to require cleavage of the receptor in response to ligand binding⁵, movement of the receptor's intracellular domain to the nucleus^{6,7}, and binding of that intracellular domain to a CSL (for CBF1, Suppressor of