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# Processing of cdk5 Activator p35 to Its Truncated Form (p25) by Calpain in Acutely Injured Neuronal Cells

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**Recently, it was shown that conversion of cdk5 activator protein p35 to a C-terminal fragment p25 promotes a deregulation of cdk5 activity, which may contribute to neurodegeneration in Alzheimer's disease. In this study, we present evidence that calpain is a protease involved in the conversion of p35 to p25. To activate calpain, rat cerebellar granule neurons were treated with maitotoxin (MTX). A C-terminus-directed anti-p35 antibody detected that p35 conversion to p25 paralleled the formation of calpain-generated alpha-spectrin (alpha-fodrin) breakdown products (SBDP's) in a maitotoxin-dose-dependent manner. Two calpain inhibitors (MDl28170 and SJA6017) reduced p35 processing but were unchanged when exposed to the caspase inhibitor carbobenzoxy-Asp-$CH_2OC(=O)$-2,6-dichlorobenzene or the proteasome inhibitors (lactacystin and Z-Ile-Glu(OtBu)Ala-Leu-CHO). p35 protein was also degraded to p25 when rat brain lysate was subjected to *in vitro* digestion with purified $\mu$- and m-calpains. Additionally, in a rat temporary middle cerebral artery occlusion model, p35 processing to p25 again paralleled SBDP formation in the ischemic core. Lastly, in malonate-injured rat brains, the ipsilateral side showed a striking correlation of SBDP formation with p35 to p25 conversion and tau phosphorylation (at Ser202 and Thr205) increase. These data suggest that calpain is a major neuronal protease capable of converting p35 to p25 and might play a pathological role of activating cdk5 and its phosphorylation of tau in Alzheimer's disease.** 

***Key Words:*** **calpain; cdk5; p25; neurodegeneration; Alzheimer's disease.**

One of the major pathological hall marks for Alzheimer's disease (AD) is neurofibrillar tangles (NFT). The major component of these intracellular NFT's is an aggregate of hyperphosphorylated Tau protein (1). Many of the phosphorylated sites of Tau found in AD brains are either proline-directed (Thr-Pro or Ser-Pro), suggestive of the involvement of cyclin-dependent kinase (cdk5) and glycogen synthase kinase 3beta (GSK3b) (2). In fact, the involvement of cdk5 is supported by several studies, which showed that cdk5 co-localized with NFT-containing neurons in AD brains (3, 4). Cdk5 is a ubiquitously expressed protein (5), but its main activator protein (p35) is exclusively expressed in neuronal cells (6, 7). Like other cyclin-dependent kinases, cdk5 activity is tightly controlled by p35. Interestingly, cdk5 is co-purified with an N-terminal truncated form of p35 known as p25, which starts at residue 99 (8). The ability of p25 to lead to overactivation of cdk5 and Tau hyperphosphorylation has been confirmed in transgenic mice overexpressing p25 (driven by a neuronal-specific enolase promoter) (9). Very recently, Patrick *et al.* (10) showed that in Alzheimer's diseased brains, p35 was processed pathologically by an unknown protease and hence lead to overactivation of cdk5 and subsequent tau hyperphosphorylation. It is therefore important to identify the protease involved in the processing of p35 to p25.

Aggregated amyloid beta-peptide has been showed to be neurotoxic by causing oxidative stress and loss of intracellular calcium homeostasis (11). A sustained calcium elevation would be a perfect environment for calpain activation. In fact, activation of both $\mu$- and m-calpains have been shown in AD brains (12–15). Calpain activation is well documented in neuronal apoptosis (17–20) and has been shown to occur during the onset of neuronal apoptosis in Alzheimer's disease (16). It is our hypothesis that calpain might be involved in p35-p25 conversion, therefore, we examine the role of

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calpain in p35 processing in *in vitro, in situ* (cell culture-based) and *in vivo* models.

## MATERIALS AND METHODS

*Rat cerebellar granule neuronal cultures.* All animal procedures conform to internal animal care guidelines and those of the Society for Neuroscience. Cerebellar granule neurons were isolated from 7-day-old rat pups as described previously (18). The cells remain in culture for 7 days *in vitro* before use. At the beginning of an experiment, cultures were washed three times with serum-free DMEM containing 30 mM $K^+$. Cultures were preincubated with appropriate compounds (when used) for 1 h prior to treatment with maitotoxin (for an additional 2 h at 37°C).

*Temporary middle cerebral artery occlusion model.* All procedures were within the guidelines of the Institutional Animal Care and Use Committee of Parke-Davis. Male Sprague–Dawley rats (Charles River Laboratories), weighing 275 to 325 g, were anesthetized with 2% isoflurane balanced with air and oxygen and allowed to breath spontaneously. Body temperature was maintained at 37.5°C throughout the experiment by individual feedback controllers and heating pads. Femoral vein catheters were implanted for infusion of vehicle. Both common carotid arteries were isolated through a small neck incision and occluded with vascular clips (Microserrefine No. 3, FST, Inc.) just before middle cerebral artery occlusion (MCAO). A vertical incision was made between the left eye and ear, the temporalis muscle was partially excised, and a craniotomy was performed at the site where the middle cerebral artery (MCA) meets the rhinal fissure. The dura over the MCA was opened and reflected to allow occlusion of the vessel with a Sundt AVM Micro Clip No. 1 (Codman) at the point where it meets the rhinal fissure. Anesthesia was discontinued until the time of reperfusion. All vascular clips were removed after 3 h of ischemia, and reperfusion of the MCA was verified by examination of the site of occlusion. An absorbable gelatin sponge (Gelfoam, Upjohn) was placed in the area of excised muscle and incisions sutured with 4-O silk. Immediately following MCAO, an infusion of a 1:9 solution of ethanol and polyethylene glycol 300 was administered at a rate of 0.33 ml/kg/h for a total of 9 h. Following infusion, rats were deeply anesthetized with isoflurane and cortical brain samples were removed and frozen in cold isopentance (−40°C) and stored at −80°C.

*Malonate (succinic dehydrogenase inhibitor) injections in rats.* Male Sprague–Dawley rates were weighted and anesthetized with 65 mg/kg pentobarbitol injected ip. Body temperature was maintained at 37.5°C throughout the experiment by individual feedback controllers and heating pads. A midline scalp incision was made from between the eyes to approximately the back of the head to expose the underlying skull. A blunt instrument was used to gently tease the tissues from the bone. This results in a clean and dry skull with adequate exposure to position the burr hole in the right hemisphere. Immediately before infusing malonate into the caudate nucleus, the needle is primed, and the dura torn with a size 5 Dumont forceps (stereotaxic coordinates used was 0.5 mm anterior, 2.6 mm lateral, 4.5 mm ventral). A 26 gauge needle was then lowered, aligned flush to the dura, and very slowly lowered an additional 4.5 mm over 0.5 min. Once in place 3 $\mu$M of malonate in 3 $\mu$L Milli-Q water or vehicle alone (control) was infused with a syringe infusion pump (UMP2, WPI Inc.) over a period of 6 min. The needle was left in place for 6 more minutes and then slowly withdrawn over 5 min. The burr hole was then sealed with bone wax and the skin incision closed with wound clips. The rats were removed from the stereotaxic frame and temperature control continued for about 3 h. When fully awake, there were returned to the animal housing room where they have food and water *ad libitum*. At the appropriate time, animals were euthanized. The ipsilateral and contralateral hemispheres were dissected and quick frozen in −70°C cooled isopentane.

*Preparation of rat brain lysate.* Frozen brain tissue was pulverized using an ice-cooled mortar–pestle over dry ice. About 50 mg of the powdered tissue was resuspended using 0.2 mL of 1% Triton lysis buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton, 5 mM EGTA, 5 mM EDTA). The samples were vortexed thoroughly and place on ice for 30 min, with intermittent vortexing. The lysates were then cleared by centrifugation at 20,000*g* in a microcentrifuge at 4°C for 10 min. Protein concentration assessment and Western blotting was done as described below.

*Purified calpain digestion of brain extract.* Total protein was extracted from naïve rat cerebral cortex by a Triton X-100 method (21). Naïve rat cerebrum extract (30 $\mu$g of protein) was digested with or without 1 $\mu$g of purified calpain in 100 mM Hepes buffer (pH 7.4 at RT), 10 mM DTT, 10% (v/v) glycerol, 5 mM $Ca^{2+}$ or 5 mM EGTA for indicated times. The digestion was halted by the addition of SDS-containing sample buffer for PAGE. Samples were subjected to SDS–PAGE, transferred to PVDF membrane, and probed with the indicated antibodies (alpha-spectrin and p35).

*Protein extraction of cell samples and analysis by Western blotting.* At the end of an experiment, the medium was removed and cells were washed once with TBS/EDTA. 1% Triton lysis buffer with protease inhibitor cocktail (Boehringer Mannheim) (17) was added directly to the wells and the cells were frozen at −50°C overnight. Cells were then thawed on ice and lysates were cleared by centrifugation at 20,000*g* in a microcentrifuge at 4°C for 10 min. The protein concentrations in the samples were measured using a modified Lowry assay (Bio-Rad). Equal amounts of total protein (15 $\mu$g) were loaded on each lane and run on SDS/PAGE [4–20% (w/v) acrylamide] with a Tris/glycine buffer system and then transferred to a PVDF membrane (0.2 $\mu$m) with a Tris/glycine buffer system with a semi-dry electrontransferring unit (Bio-Rad) at 20 mA for 2 h. The blots were probed with an anti $\alpha$-fodrin (spectrin) monoclonal antibody (Affiniti Research Products (UK)), a polyclonal anti-p35 antibody (C-terminus-directed, C19) (Santa Cruz Biotechnologies) or the monoclonal, Phospho-Tau (AT8) antibody (Innogenetics)) overnight. To develop, a biotinylated secondary antibody and streptavidin conjugated with alkaline phosphatase (Amersham) or horseradish peroxidase (Chemicon) followed by a substrate, nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Kirkegaard & Perry Laboratories) or Lumiglo kit (Amersham) was used. Densitometric analysis of Western blots were performed using a color scanner (Umax UC630) and the NIH program Image 1.5.

## RESULTS

### *Cdk5 Activator p35 in Processed to p25 in Maitotoxin-Treated Cerebellar Granule Neurons (CGN)*

Since both $\mu$- and m-calpain have been shown to be activated in human Alzheimer's disease brains, we hypothesize that calpains have been involved in the conversion of p35 to p25. To test this hypothesis, we employed a rat CGN culture system as p35 is only present in neuronal cells. To activate calpain *in situ*, we used various concentrations of maitotoxin, both a potent calcium channel opener and an acute neurotoxin that we previously demonstrated to activate calpain in neurons (17). In our initial experiments, we observed that intact p35 was rapidly degraded in cells treated with 0.01 to 0.1 nM MTX for 3 h (Fig. 1A). We also observed partial accumulation of a fragment of 25 kDa (p25), in MTX-treated cultures (Fig. 1A). By either extending the MTX treatment time or increasing the concentra-

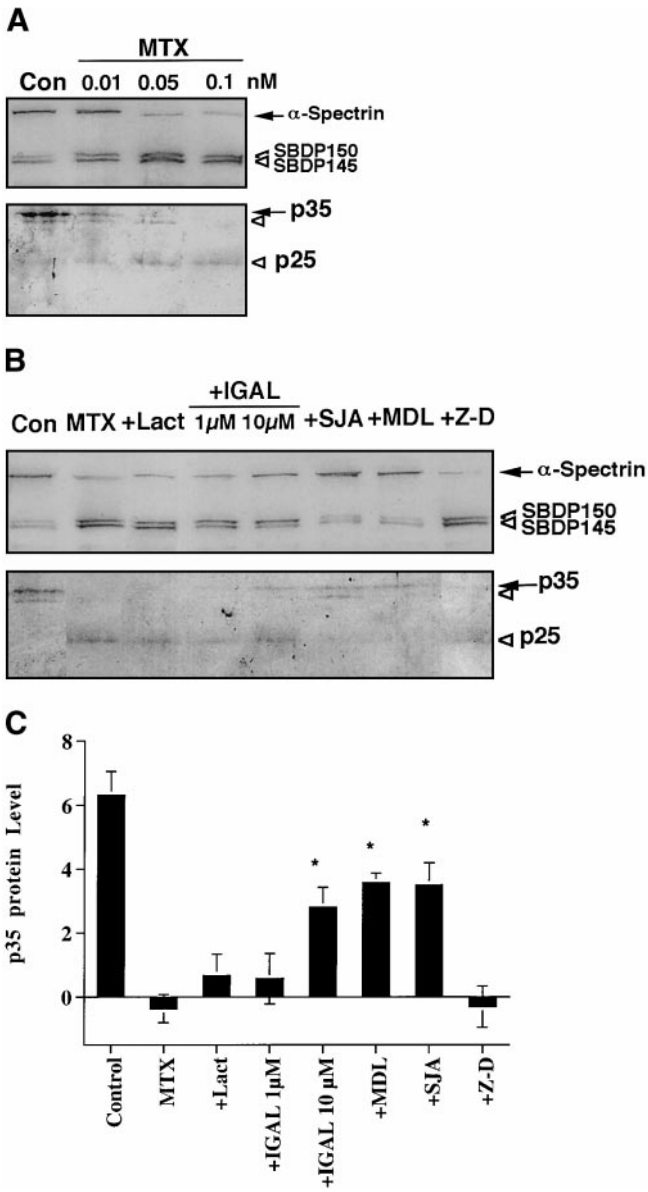


**FIG. 1.** Proteolytic cleavage of p35 to p25 in cerebellar granule neurons is induced by influx of $Ca^{2+}$ and is inhibited by calpain inhibitors. (A) Primary cerebellar granule cultures were treated with various concentrations of maitotoxin (MTX) for 2 h at 37°C. Cell lysates were subjected to Western blotting and probed with either the alpha-spectrin antibody (top) or the p35 C-terminus antibody (bottom). An increase in the appearance of the 150- and 145-kDa spectrin breakdown products and a concomitant increase in the appearance of the p25 cleavage product was evident with increasing concentrations of MTX. (B) Cultures were preincubated with either lactacystin (1 $\mu$M), IGAL (1 or 10 $\mu$M) (proteasome inhibitors), SJA6017 (30 $\mu$M), or MDL28170 (30 $\mu$M) (calpain inhibitors) (all from Calbiochem) or Z-D-DCB (50 $\mu$M, from Bachem) for 1 h prior to treatment with MTX 0.1 nM for 2 h at 37°C. (C) Densitometric quantification of data in (B) as mean ± SEM ($n = 4$). Asterisks indicate data statistical significantly different from MTX alone (Student's $t$ test, $P < 0.05$).

tions of MTX, the level of p25 actually diminished, suggested that it is further degraded (data not shown). The formation of the 150 and 145 kDa $\alpha$-spectrin breakdown product (SBDPs) were also monitored as markers for calpain activity (17). We found that the formation of SBDP150 and SBDP145 strikingly paralleled that of p25 (Fig. 1A).

Since calpain is highly activated in MTX-treated cells (17), we suspected that it might be responsible for p35–p25 conversion. To confirm that, we pretreated the cortical cultures with two cell-permeable calpain inhibitors (30 $\mu$M MDL28170 (MDL) or 35 $\mu$M SJA6017 (SJA)) 1 h before treating with 0.1 nM of MTX. Formation of p25 fragment was blocked by both SJA6017 and MDL28170, in parallel with partial, but significant, recovery of the intact p35 protein (Figs. 1B and 1C). We also did parallel blots for alpha-spectrin, which revealed that both MDL28170 and SJA6017 blocked SBDP formation. In addition, two proteasome inhibitors (lactacystin and Z-Ile-Glu(OtBu)Ala-Leu-CHO (IGAL)) (22) and a pan-caspase inhibitor carbobenzoxy-Asp-$CH_2OC(=O)$-2,6-dichlorobenzene (Z-D-DCB) (17, 18) were employed. Lactacystin (1 $\mu$M) and 1 $\mu$M IGAL were ineffective in blocking either p25 or SBDP's formation. Previously, 1 $\mu$M of IGAL was shown to be an effective concentration for proteasome inhibition in cell culture (22). At higher concentration of IGAL (10 $\mu$M), it was partially effective in protecting both alpha-spectrin and p35, suggesting that it cross-inhibits calpain at higher concentrations, as was previously documented. Caspase inhibitor Z-D-DCB (50 $\mu$M) had no effect on either p35–p25 conversion or alpha-spectrin breakdown (Fig. 1B).

### *p35 Is a Substrate for $\mu$- and m-Calpains in Vitro*

Due to the extreme sensitivity of p35 to degradation in culture, we examined if p35 was equally sensitive to degradation *in vitro.* We observed that upon brief incubation of naïve rat cerebrum lysate (8 min) in a calcium-containing buffer, there was indeed a partial breakdown of p35 to p25, which paralleled the increase of SBDP150 (Fig. 2A). In buffer containing EGTA, no increase of p25 or SBDP150 was observed. We also examined the effects of various protease inhibitors in blocking the calcium-induced p35–p25 conversion. Both calpain inhibitors SJA6017 and MDL28170 were effective in blocking p25 and SBDP150 formation while lactacystin, Z-D-DCB or cathepsin B inhibitor CA-074Me (23) were not effective (Fig. 2A).

To confirm that p35 is a substrate for calpains, we also digested naïve rat brain lysate with both purified $\mu$-calpain and m-calpain (for 8 min). While 8 min incubation in calcium buffer alone produced some level of p25 (lane 2, Fig. 2B), addition of both $\mu$- and m-calpains drastically increased the level of p25. Furthermore, both calpains completely degraded intact p35. The presence of MDL28170 fully restored the intact p35 level and prevented p25 formation (Fig. 2B). Again, both the degradation of p25 pattern completely paralleled that of a-spectrin degradation (Fig. 2B). Taken

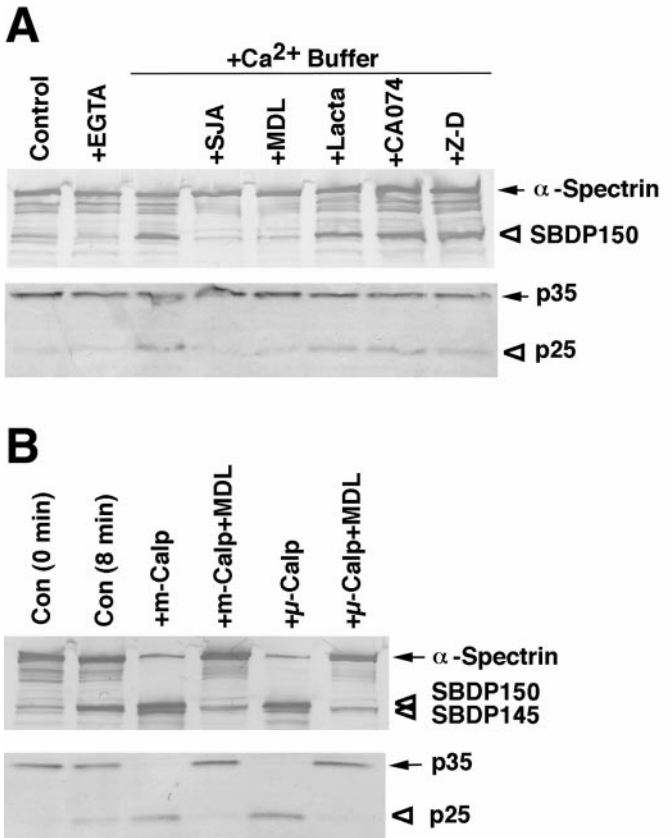


**FIG. 2.** Calpain cleaves p35 to p25 *in vitro.* (A) Rat brain lysate was incubated in $Ca^{2+}$ buffer (3 mM $CaCl_2$) or in the presence of EGTA (5 mM) for 8 min at room temperature. Lysates were analyzed by Western blotting and probed with either the anti-alpha spectrin antibody (top panel) or the anti-p35 C-terminus antibody (bottom panel). Spectrin breakdown to the 150-kDa breakdown product and p35 cleavage to produce the p25 fragment was evident the presence of $Ca^{2+}$. A reduction in the appearance of the spectrin breakdown products and correspondingly the p25 fragment was observed in the presence of calpain inhibitors SJA 6017 (30 $\mu$M) and MDL 28170 (35 $\mu$M). The proteasome inhibitor lactacystin (1 $\mu$M), the cathepsin B inhibitor CA074Me (10 $\mu$M; from Peptide International) (23), and caspase inhibitor Z-D-DCB (50 $\mu$M) did not significantly reduce the appearance of the p25 fragment or the spectrin breakdown products. (B) Rat brain lysate was incubated with 0.5 $\mu$L (units) $\mu$- or m-calpain for 8 min at room temperature in the presence of $Ca^{2+}$. The digested lysates were analyzed by Western blotting and probed with either the anti-alpha-spectrin antibody (top panel) to assess calpain activity or the anti-p35 C-terminus antibody (bottom panel).

together, these data confirm the $\mu$- and m-calpains can convert p35 to p25 *in vitro.*

### *p35–p25 Conversion in Temporary Middle Cerebral Artery Occlusion in Rats*

Previously, calpain has been shown to be activated in various models of cerebral ischemia (24). Here, using an established temporary middle cerebral artery occlusion model (25), we examined the potential occurrence of p35–p25 conversion. In this model, rat cortex was unilaterally subjected to 3 h of ischemia, followed by 6 hours of reperfusion. At which point, cortical brain samples were immediately removed and frozen. We found that when compared to the contralateral counterpart, the ischemic core (at 9 h) showed a drastic loss of intact p35 and a significant increase of the p25 level (Figs. 3A and 3B). Again, the p35–p25 conversion paralleled the breakdown of $\alpha$-spectrin (Fig. 3A), suggestive that both processes were mediated by calpains.

### *p25 Accumulation and Tau Hyperphosphorylation in Malonate-Injected Rat Brains*

Malonate is a mitochondrial oxidative phosphorylation inhibitor as well as a neurotoxin. It has been used *in vitro* and *in vivo* to mimic mitochondrial dysfunction

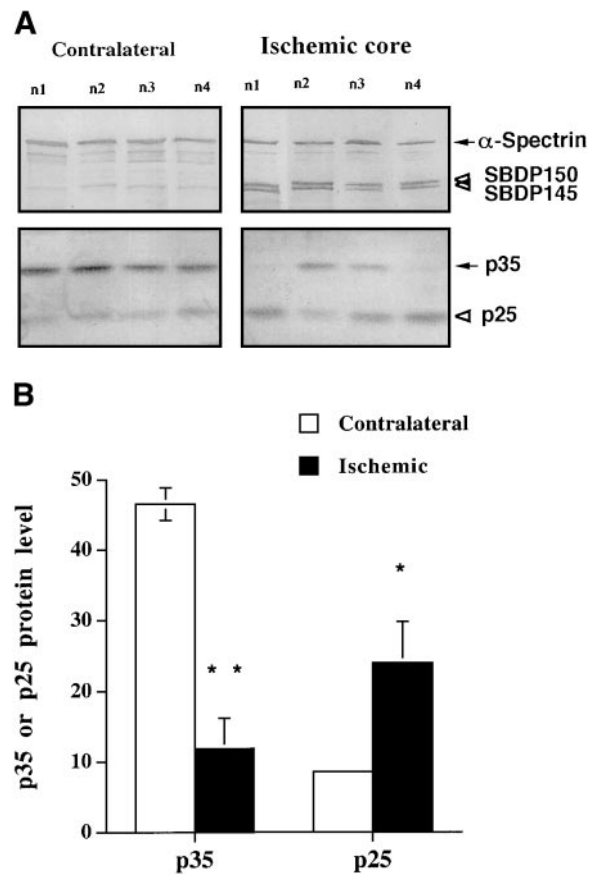


**FIG. 3.** Accumulation of p25 fragment in rat tMCAO brains. (A) Temporary middle cerebral artery occlusion (tMCAO) in rats were performed according to Methods. Following 3 h of ischemia and 6 h of reperfusion, cortical brain samples were removed and preserved at −70°C. Cortical brain samples (20 $\mu$g, $n = 4$) were analyzed by Western blots analysis and probed with anti-spectrin or anti-p35 antibody. Densitometric analysis of intensity of p35 and p25 were done by scanning of Immunoblot and intensity of bands was measured using the NIH image program. (B) Densitometric quantification of data in (A) as mean ± SEM ($n = 4$). Asterisks indicate data statistical significantly different from the Contralateral group (Student's $t$ test, $^*P < 0.05$; $^{**}P < 0.005$).

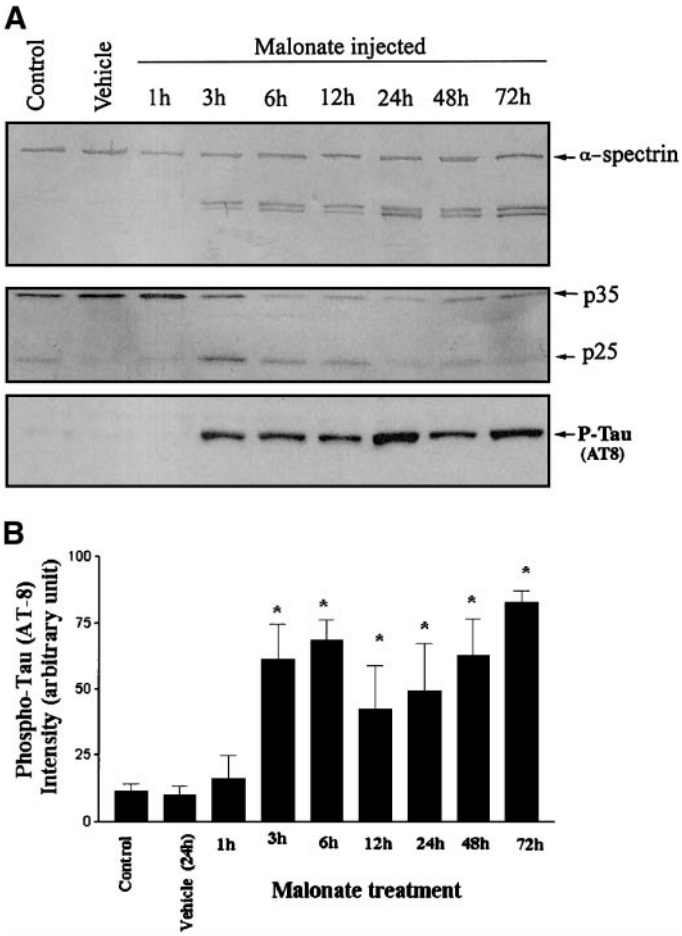


**FIG. 4.** Accumulation of p25 fragment in malonate injected rat brains and corresponding increase in phosphorylation of Tau. Stereotaxic injections of malonate in rat brains was done as outlined in methods. Rats were sacrificed at specific intervals post injection, up to 72 h. The ipsilateral and contralateral hemispheres were dissected and quick frozen. (A) 20 $\mu$g of brain lysates was subjected to Western blot analysis and probed with anti-alpha-spectrin (top panel), anti-p35 C-terminus antibody (middle panel) or the phospho-tau antibody (AT8) (bottom panel), respectively. (B) Densitometric analysis of phospho-tau level (mean ± SEM) after malonate injection ($n = 5$). Asterisks indicate data statistical significantly different from vehicle alone (Student's $t$ test, $P < 0.05$). The autoradiogram was scanned and intensity of bands was measured using the NIH image program.

that is associated with both acute neurological disorders (e.g., stroke) and chronic neurodegeneration (such as Alzheimer's disease) (26, 27). We investigated whether p35 processed to p25 in a malonate-injection model, by monitoring the integrity of both p35 and spectrin over various time points after infusing malonate into the caudate nucleus. For alpha-spectrin breakdown products (SBDP150 and SBDP145) began to accumulate as early as 3 h and maximized by 24 h and remained stable out to 72 h (Fig. 4A, top panel). Interestingly, intact p35 was reduced beginning in 3–6 h post-malonate infusion with a concomitant increase of p25 (Fig. 4A, middle panel). However, in contrast to SBDP's, the accumulation of p25 appeared to be transient. The later post-malonate infusion time points (24–72 h) found p25 protein returning to near basal levels (Fig. 4A). Again, this effect might be due to the possibility that p25 was a subject of further proteolysis by calpain or other proteases, such as proteasome.

Since p25-activated cdk5 has been suggested by Patrick *et al.* (10) to hyperphosphorylate Tau, we investigated if there was a parallel accumulation of phosphorylated Tau (P-Tau) in malonate-injured rat brains. A phospho-Tau antibody AT8 was used, which detects Tau phosphorylated dually at S202 and T205. This antibody has been shown to only detect abnormally hyperphosphorylated Tau in Alzheimer's brains (28, 29). Indeed, there was almost no AT8 P-tau signal in naïve or vehicle-injected rat brains (Fig. 4A). Yet, after 3 h post-malonate injection, there was a significant increase in phosphorylation at the AT8 site on tau when compared to control (lane 1) or the vehicle injected animals (lane 2) ($n$ of 5) (Figs. 4A and 4B).

## DISCUSSION

In this study, we present evidence that calpain is a key protease involved in the conversion of p35 to p25, the potential pathological activator of cdk5 in Alzheimer's disease (10). p25 differs from p35 in that it lacks the N-terminal $Gly_2$ myristylated site. Thus, instead of anchoring onto the plasma membrane like p35, p25 is presumably cytosolic. This might be a critical step is hyperphosphorylated cytosolic Tau and thus the formation of tau aggregate.

In rat cerebellar granule neurons treated with MTX, p35 was rapidly processed to p25, in a calpain-inhibitor-sensitive manner (Figs. 1 and 2). None of the protease inhibitors we have tested appeared to be able to modify this process. Interestingly, the p25 fragment that was generated *in situ* or *in vivo* was not very stable and tended to be further degraded (Figs. 2 and 4). We speculated that calpain, proteasome or another unidentified protease is likely responsible in mediating this event. It is of interest to note that p35 was shown to be degraded by the proteasome pathway in cells (31).

Upon incubation of rat cerebrocortex lysate in a $Ca^{2+}$-containing buffer, p35 was also partially converted to p25 (Fig. 2A), suggesting that the endogenous protease is present in the brain compartment. Also, the calcium-dependency and sensitivity to calpain inhibitors of p25 formation suggests that the endogenous protease is calpain. This hypothesis is consistent with the observation that the p35–p25 conversion was exaggerated when exogenous calpain was added to the brain lysate (Fig. 2B).

In addition, we demonstrated that in two *in vivo* models of acute neuronal injury (tMCAO and malonate injection), p35 was also converted to p25. The pattern of p35–p25 conversion was strikingly similar to the

well established calpain-mediated spectrin breakdown pattern, suggesting that calpain mediated both processes. Conversion of p35 to p25 could be an important step in generating hyperphosphorylated cytosolic Tau and hence the pathogenesis of Alzheimer's disease (10). Consistent with this concept, in the malonate-injured rat brains, in addition to p35–p25 conversion, we did observe a profound and sustained increase of AD-like hyperphosphorylated Tau (Fig. 4). It is consistent with a previous study which indicated that in ischemic rat brain, there is increased cdk5 activity (31). To prove that calpain mediates the increase of Tau phosphorylation indirectly via cdk5 *in vivo*, it would be important in the future to show that a calpain inhibitor can attenuate phosphorylated Tau levels in animals. NFT formation and cdk5 overactivation, by itself, can have a detrimental effect on neuronal survival, leading to the ultimate neuronal death found in AD. In fact, overexpression of cdk5, p25 and Tau leads to apoptosis (10). Loss of intracellular calcium homeostasis has been implicated in Alzheimer' s disease (11, 32). Sustained intracellular calcium elevation in susceptible central neurons could be a result of chronic amyloid beta peptide toxicity or build up of other neurotoxic factors such as glutamate. Both $\mu$- and m-calpain activation has been reported, as evidenced by the presence of their autolytic forms in human AD brains (12–15). Thus, it is conceivable that the amyloid beta-peptide-induced intracellular calcium elevation would set in motion a cascade of calpain mediated p35 to p25 conversion, tau hyperphosphorylation and aggregation, neurofibrillary tangle formation and ultimately neuronal death. This is consistent with the report that cdk5 inhibition as well as calpain inhibition can attenuate amyloid beta-peptide induced neuronal death (19, 20).

In summary, our results suggest that calpain is a major neuronal protease capable of converting p35 to p25 and might play a pathological role of activating cdk5, leading to hyperphosphorylation of tau in Alzheimer's disease.

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