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Neuroprotection with Delayed Calpain Inhibition after Transient Forebrain Ischemia

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Abstract

Objective—Delayed neurodegeneration after transient global brain ischemia offers a therapeutic window for inhibiting molecular injury mechanisms. One such mechanism is calpain-mediated proteolysis, which peaks 24 to 48 hours after transient forebrain ischemia in rats. This study tests the hypothesis that delayed calpain inhibitor therapy can reduce brain calpain activity and neurodegeneration after transient forebrain ischemia.

Design—Prospective randomized placebo-controlled animal trial.

Setting—University research laboratory.

Subjects—Adult male Long Evans rats.

Interventions—Rats subjected to 10-minute transient forebrain ischemia were randomized to intravenous infusion of calpain inhibitor CEP-3453 or vehicle beginning 22 hours after injury. Measurements and Main Results: In a dose-response study, a 60 mg/kg bolus followed by 30 mg/kg infusion was required to reduce post-ischemic brain calpain activity measured by Western blot of hippocampal homogenates at 48 hours after injury. The same dosing protocol decreased degeneration of CA1 pyramidal neurons measured at 72 hours after injury.

Conclusions—These results suggest a causal role for calpains in delayed post-ischemic neurodegeneration, and demonstrate a broad therapeutic window for calpain inhibition in this model.

Keywords

brain; neuron; ischemia; calpain; hippocampus; CA1; neuroprotection; protease inhibitor; neurodegeneration

INTRODUCTION

A key mechanism of neuronal death following transient global brain ischemia is disruption of Ca^{2+} homeostasis [1]. An initial rise in cytosolic Ca^{2+} during ischemia occurs in both selectively vulnerable and ischemia resistant neurons [2–5]. However, a delayed secondary

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increase in intracellular Ca^{2+} occurs only in those neurons destined to die [6,7]. One major effect of intracellular Ca^{2+} overload is the activation of calpains, a family of Ca^{2+} -dependent non-lysosomal neutral cysteine proteases. The proposed physiologic roles of calpains in the brain include regulation of neurite outgrowth [8], long term potentiation [9,10], and synaptic remodeling [11]. More globally, calpains are involved in cell mitosis, migration and differentiation [12]. Under physiologic conditions calpains are activated in response to transient localized increases in cytosolic Ca^{2+} and regulated by calpastatin, an endogenous inhibitor protein. Under ischemic and post-ischemic conditions, intracellular Ca^{2+} overload appears to overwhelm endogenous regulatory systems resulting in pathologic calpain activity.

A dramatic increase in calpain activity has been demonstrated during and after focal and global brain ischemia [13–21]. After transient forebrain ischemia in rats, a bimodal pattern of calpain-mediated α -spectrin degradation is observed in the hippocampus, with transient activity at 1 hour and peak activity occurring between 24 and 48 hours after reperfusion [22]. The same study also found a progressive expansion of the number of neurons displaying calpain activity in the CA1 hippocampus between 24 and 48 hours reperfusion. The calpain activity preceded both dendritic fragmentation and histologic evidence of neurodegeneration consistent with a causal relationship with neuronal death [22].

The objective of this study was to evaluate the neuroprotective effect of calpain inhibitor therapy following transient forebrain ischemia. Our unique approach was to match the timing of therapy with the time course of delayed post-ischemic calpain activity. Initiating therapy 22 hours after reperfusion has the added benefit of exploiting a potentially broad therapeutic window for clinical translation. Our results suggest that this approach is effective, and support the hypothesis that calpains play a causal role in delayed post-ischemic neurodegeneration.

MATERIALS AND METHODS

Reagents

The water-soluble calpain inhibitor CEP-3453 was provided by Cephalon (West Chester, PA). CEP-3453 is the bisulfite addition product of the aldehyde of CEP-3122 [$\text{CH}_3\text{SO}_2\text{-D-(Bn)Ser-Phe-H}$] [23]. Rabbit polyclonal antibody directed at the calpain-derived fragment of α -spectrin (Ab38) was provided by Robert Siman (Univ. of Pennsylvania, Philadelphia, PA). Mouse monoclonal antibody reacting with both intact and calpain-cleaved spectrin (MAB1622) was purchased from Chemicon International (Temulca, CA). Anti-rabbit fluorescent secondary antibody Alexa 568 was purchased from Molecular Probes (Eugene, OR). Hoechst 33258 was purchased from Sigma-Aldrich (St. Louis, MO). Reagents for enhanced chemiluminescence (ECL) were purchased from PerkinElmer (Boston, MA).

Transient Global Forebrain Ischemia Model

Animal experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The animal protocol was approved by our institution's Animal Care and Use Committee. Male Long Evans rats weighing 400 to 500 grams were anesthetized with halothane (3%), 70% N_2O and 30% O_2 by insufflation chamber and then maintained initially by facemask. Rats were orotracheally intubated with a 14 g angiocath and mechanically ventilated with a pressure controlled small animal ventilator (initial settings; rate 80, peak inspiratory pressure 15 cm H_2O , inspiration time 30%). A surgical plane of anesthesia was maintained with halothane (1.0–1.5%) 70% N_2O and 30% O_2 . PE 50 femoral arterial and venous catheters were placed by cut-down using aseptic technique. Temperature was monitored with a needle thermocouple probe inserted below with temporalis muscle. Temperature was maintained between 37.0 °C and 38.0 °C throughout the experimental period and up to 1 hour reperfusion by using a warming pad and overhead lamp. ECG was monitored

by electrocardiographic limb leads. After a 10-minute stabilization period, an arterial blood sample was obtained. Ventilator adjustments were made to maintain the PaCO₂ between 35 and 45 mm Hg.

Transient forebrain ischemia (TFI) was initiated by the combination of bilateral carotid occlusion and hypovolemic hypotension to a mean arterial pressure (MAP) of 30–35 mm Hg. Hypovolemic hypotension was achieved by rapidly withdrawing blood from the femoral arterial cannula and maintained during the ischemic period by withdrawal or infusion of blood through the femoral venous cannula. When a MAP of 30–35 mm Hg was achieved, both carotid arteries were reversibly occluded with surgical aneurysm clips. The duration of ischemia was timed from when the aneurysm clips were placed. After 10 minutes of ischemia, the aneurysm clips were removed and shed blood was reinfused through the femoral venous cannula. Rats were maintained on mechanical ventilation for 1 hour after which the arterial femoral cannula was removed. To enable continuous post-anesthesia intravenous infusion, the proximal end of the femoral venous cannula was passed subcutaneously via trocar and externalized through a skin incision at the nape of the neck. The cannula was then pulled through a dacron mesh anchoring button that was sewn in place. The externalized portion of the cannula was covered with a protective tether attached to the anchoring button.

Rats were then extubated and administered oxygen through a facemask for an additional 30 minutes. Rats were placed in a flat bottom recovery cage and the externalized femoral vein cannula was connected to an infusion swivel mounted above the cage. Intravenous infusion of 0.9% saline at 0.25 ml/kg/hr was initiated to keep the cannula patent until initiation of drug or vehicle therapy. Sham (uninjured) animals were subjected to anesthesia and surgical preparation without bilateral carotid occlusion and hypovolemic hypotension.

Experimental Protocols

Dose-Response Study—Twenty-two hours after TFI, rats were block randomized (n=3/group) to either low-dose calpain inhibitor therapy (CEP-3453, 10mg/kg IV bolus followed by 5mg/kg/hr IV × 26 hours), medium-dose calpain inhibitor therapy (CEP-3453, 30mg/kg IV bolus followed by 15mg/kg/hr IV × 26 hours), high-dose calpain inhibitor therapy (CEP-3453, 60mg/kg IV bolus followed by 30mg/kg/hr IV × 26 hours), or equal volume of drug vehicle (0.9% saline, 0.5 ml/kg IV bolus followed by 0.25ml/kg/hr IV × 26 hours). Sham operated rats were used as controls. Rats surviving to 48 hours were euthanized and hippocampal homogenates were generated for Western blot analysis of brain calpain activity.

Neuroprotection Study—Twenty-two hours after TFI, rats were block randomized (n=6/group) to receive either high-dose calpain inhibitor (CEP-3453, 60 mg/kg IV bolus and 30 mg/kg IV infusion for 50 hours) or vehicle (0.9% saline, 0.5 ml/kg IV bolus followed by 0.25ml/kg/hr IV × 50 hours). Sham operated rats served as controls (n=6). Rats were euthanized 72 hours after injury and brains were processed for immunohistochemistry and fluorescence microscopy.

Western Blots—Following decapitation under general anesthesia brains were rapidly microdissected on an ice-chilled plate. The dorsal hippocampus was homogenized using a 2.0 ml Wheaton dounce homogenizer and a type B pestle in 10:1 vol/wt buffer containing 10 mM Tris (pH 7.4), 10 mM EGTA, 250 mM sucrose, 2 ng/ml aprotinin, 5 ng/ml leupeptin, 2 ng/ml pepstatin, and 1 mM PMSF. Protein concentration was determined by the Bradford assay method. 100 ng samples were mixed 1:1 in 2X loading buffer and separated by SDS-PAGE (7.5% T, 2.5% C) followed by electroblot transfer to nitrocellulose. The nitrocellulose membrane was rinsed in TTBS [20 mM Tris base (pH 7.6), 137 mM NaCl, 0.1% Tween 20] and then preblocked for one hour at room temperature in TTBS containing 5% powdered milk.

Membranes were then probed with anti- α -spectrin primary monoclonal mouse antibody (MAB 1622, Chemicon; 1:5,000) in TTBS for one hour at room temperature. This antibody reacts with both intact α -spectrin and the 145/150 kDa doublet produced by calpain-mediated cleavage. Secondary antibody was HRP-linked anti-mouse IgG (1:10,000, Perkin-Elmer) in TTBS for 30 minutes at room temperature. Blots were developed by ECL (Perkin-Elmer). Band densities were quantified by computer densitometry and analyzed. One-way ANOVA with Scheffe post-hoc analysis were used to compare ischemia-induced change in mean 145/150 kDa densities relative to vehicle treated controls for each drug dose and brain region. Results are expressed as mean $\pm$ standard deviation.

Immunohistochemistry and Epifluorescence Microscopy—Rats were euthanized by perfusion fixation with 4% paraformaldehyde under general anesthesia. Following perfusion fixation, brains were removed and post-fixed overnight in 4% paraformaldehyde followed by serial incubations in 10%, 20% and 30% sucrose until brains sank to the bottom of the vial. Brains were then frozen in dry ice ethanol slurry and 40 micrometer sections generated using a freezing/sliding microtome.

Brain sections were washed in 20mM Tris and 150 mM NaCl (TBS), pH 7.4, and preblocked in 5% normal goat serum containing 0.1% Triton X-100 in TBS (Block). Sections were then incubated overnight at 4°C with primary rabbit polyclonal antibody (Ab38, 1:20,000) diluted in blocking solution. Ab38 specifically detects calpain-cleaved alpha-spectrin [17]. Sections were washed in TBS, and then incubated with anti-rabbit fluorescent secondary antibodies Alexa 568 (Molecular Probes, Eugene, OR) for 60 min at room temperature. All sections were counterstained with Hoechst 33258 (2.5 μ g/ml in TBS, Sigma). The slides were mounted with Fluoromount medium. Negative controls were prepared identically except for omission of the primary antibody. To evaluate the effect of calpain inhibition on caspase activity, a subset of sections were immunolabeled with primary antibody Ab246 which specifically detects the caspase-derived fragment of alpha-spectrin [24].

Calpain activity was quantified by regional Ab38 immunofluorescence in the CA1 sector hippocampus at -2.6 and -3.6 relative to bregma. Epifluorescence images were captured at 100x magnification using Nikon Eclipse E600 epifluorescence microscope with a rhodamine filter (510–560/590 nm), a digital 12 megapixel camera, and a Dell Power Edge 1300 computer. Intensity settings were initially adjusted to minimized background fluorescence and then all images were captured at the same settings. Regional CA1 fluorescence was quantified by computerized densitometry (ImageQuant densitometric software, Molecular Dynamics) and the average of the two regions examined in each animal was calculated. Mean Ab38 fluorescence intensity in the vehicle and CEP-3453 treatment groups was statistically compared using a two-tailed Student's t-test (alpha error 0.05). Results are expressed as mean $\pm$ standard deviation.

Neurodegeneration was quantified by counting normal appearing Hoechst-stained neuronal nuclei in the hippocampal CA1 pyramidal layer at -2.6 and -3.6 relative to bregma. Epifluorescence images using a DAPI filter set were obtained at 200 $\times$ magnification in the focal plane with the greatest number of nuclei. Any nuclei that were out of focus were not counted regardless of morphology. Normal appearing nuclei, defined as rounded with dispersed granular chromatin, were counted by 5 investigators blinded to treatment and mean values were used for analysis. The normal nuclei counts were compared between vehicle and CEP-3453 treatment groups using a two-tailed Student's t-test (alpha error 0.05). Results are expressed as mean $\pm$ standard deviation.

RESULTS

Dose Efficacy of Delayed CEP-3453 Treatment

Western blot analysis of hippocampal homogenates from vehicle treated rats 48 hours after transient forebrain ischemia revealed significant calpain activity as demonstrated by the characteristic 145/150 kDa doublet produced by calpain-mediated cleavage of alpha-spectrin (Fig. 1A). Calpain activity was negligible in sham operated controls using this assay. Delayed CEP-3453 calpain inhibitor therapy at low and medium doses did not diminish ischemia-induced calpain activity. In contrast, delayed administration of CEP-3453 at the highest dose tested (900 mg/kg total dose) had a dramatic effect on post-ischemic calpain activity. At this dose, the ischemia-induced increase in the mean 145/150 kDa doublet density was reduced by $97 \pm 3\%$ ($p < 0.05$, Fig. 1B).

Post-Ischemic Neuroprotection

Relative to sham operated controls, mean CA1 sector Ab38 immunofluorescence increased by $263 \pm 281\%$ in vehicle treated rats and $68 \pm 147\%$ in CEP-3453 treated rats ($p = 0.17$) (Figure 2). Although not statistically significant, this result is consistent with our Western blot data demonstrating CEP-3453-mediated inhibition of delayed post-ischemic calpain activity in hippocampus. As illustrated in Figure 2, calpain-mediated spectrin cleavage is detectable throughout the stratum oriens, stratum pyramidale, stratum radiatum and most prominent in the stratum lacunosum moleculare. There was no detectable increase in Ab246 labeling in the CA1 sector of vehicle or CEP-3453 treated groups relative to sham injured rats (Data not shown). Therefore, we found no evidence that calpain inhibition caused a secondary upregulated caspase activity in this model. Normal CA1 pyramidal layer nuclei averaged $10 \pm 12\%$ of control in vehicle treated rats and $50 \pm 42\%$ of control in CEP-3453 treated rats ($p = 0.047$) (Figure 3).

Discussion

In this study, intravenous administration of the calpain inhibitor CEP-3453 beginning 22 hours after transient forebrain ischemia effectively inhibited calpain-mediated alpha-spectrin degradation in the post-ischemic hippocampus. Dose-response analysis revealed that effectiveness was dependent on a relatively high dose of drug administration (CEP-3453 60 mg/kg IV bolus and 30 mg/kg IV continuous infusion). Analysis of neurodegeneration 72 hours after injury reveals evidence of neuroprotection with high dose therapy in the CA1 sector pyramidal neurons of the hippocampus. This study is significant in that it demonstrates 1) that intravenously administered CEP-3453 effectively inhibits brain calpain activity, 2) the effectiveness of matching calpain inhibitor therapy with the time course of post-ischemic calpain activity, and 3) the broad therapeutic window for calpain inhibition after transient global brain ischemia.

Previous studies have demonstrated neuroprotective effects of post-ischemic calpain inhibitor therapy [15,25–31]. However, the effectiveness of calpain inhibition in these studies was incomplete and may have been limited by the fact that drug administration was not synchronized to the time course of calpain activity in the post-ischemic brain. Following transient forebrain ischemia in rats, a bimodal pattern calpain activity is observed, with an initial minor peak occurring at 1 hour after reperfusion and a major (10-fold greater) secondary peak occurring between 24 and 48 hours after reperfusion [22]. No previous studies have examined either delayed or sustained calpain inhibitor therapy directed at attenuating the secondary peak in calpain activity following transient global brain ischemia.

This study focuses on the role of calpains in delayed post-ischemic neurodegeneration. However, direct and indirect effects of CEP-3453 on other pathologic proteases cannot be completely ruled out. CEP-3453 is the bisulfite addition product of the aldehyde of CEP-3122 [CH₃SO₂-D-(Bn)Ser-Phe-H] [23]. Both compounds have an IC₅₀ of 8.0 nM for μ -calpain in vitro and 0.7 μ M for calpain activity in intact cell culture. [23]; Ming Tao, unpublished data) which is similar to the commonly used calpain inhibitor MDL-28170 [32]. Similar to other calpain inhibitors in this class, CEP-3453 has inhibitory activity against cathepsin B (IC₅₀ 15 nM). The role of cathepsin B in post-ischemic neurodegeneration remains controversial, but there is evidence that lysosomal cathepsins are released secondary to calpain-mediated cleavage of lysosomal membrane proteins [33]. Therefore direct or indirect inhibition of cathepsin activity cannot be ruled out in this study. Although CEP-3453 has no direct effect on caspases, calpain inhibition can indirectly affect caspase activity through multiple mechanisms of cross-talk [34]. However in the CA1 pyramidal neuron population that was the focus of this study, we are unable to detect significant caspase-mediated cleavage of alpha-spectrin without or with CEP-3453 treatment.

A number of potential mechanisms have been identified to explain the neuroprotective effect of delayed calpain inhibition. Transient calcium fluxes induced by ischemia and initial reperfusion occur in most brain cells [2–5]. However, a well-documented phenomenon of delayed secondary calcium overload is closely associated with delayed post-ischemic neurodegeneration [6,7]. The mechanism of delayed secondary neuronal calcium overload remains controversial. The majority of calcium regulatory proteins in the synapse, plasma membrane and endoplasmic reticulum are known calpain substrates. Furthermore, calpain cleavage of these proteins causes dysregulation of function that in most cases could contribute to sustained elevations in cytosolic calcium. Therefore, the potential net effect of pathological calpain activity once triggered is a feed-forward mechanism of cytosolic calcium overload and sustained calpain activity leading to neuronal death. In addition to disruption of the calcium regulatory system, a number of other molecules associated with cell survival/death signaling have been linked to calpain-mediated cell death including BCL family proteins [35–37], caspases [38,39] AIF [40], CRMP3 [41], p25/CKD5 [42,43], and p53 [44,45]. Furthermore calpain-mediated degradation of lysosomal membrane proteins and subsequent cathepsin release has been proposed mechanism of post-ischemic neurodegeneration [46]. The relative role of various calpain substrates in post-ischemic neurodegeneration is an area of ongoing investigation.

In addition to the potential mechanisms of neuroprotection, several key questions must be addressed regarding the therapeutic paradigm in our study. Most importantly, it remains to be determined if delayed calpain inhibitor therapy results in sustained neuroprotection (beyond 72 hours) and functional neuronal survival. Future studies will need to examine more long-term and functional outcomes. Furthermore, the relative contribution of early and late post-ischemic calpain activity to delayed neuronal death needs to be further explored. Perhaps optimal therapy will require inhibition of both. Finally, because the cause of delayed post-ischemic calpain activation remains to be elucidated, the optimal duration of calpain inhibitor will need to be examined.

We have demonstrated for the first time that initiating therapy 22 hours after transient global brain ischemia significantly impacts an important biochemical injury pathway. This novel observation has obvious clinical implications. Although the phenomenon of delayed post-ischemic neuronal death is well accepted, the effectiveness of delayed therapy has not been explored due to the assumption that injury pathways are maximally active during ischemia and early reperfusion. If future studies demonstrate that delayed calpain inhibitor therapy results in sustained functional neuroprotection, the overall approach to brain resuscitation following global ischemic insults such as cardiac arrest will need to be revised.

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References

1. Kristian T, Siesjö BK. Calcium in ischemic cell death. *Stroke* 1998;29(3):705–718. [PubMed: 9506616]
2. Erecińska M, Silver IA. Relationship between ions and energy metabolism: cerebral calcium movements during ischaemia and subsequent recovery. *Can J Physiol Pharmacol* 1992;70 (Suppl):S190–193. [PubMed: 1295670]
3. Silver IA, Erecińska M. Ion homeostasis in rat brain in vivo: intra- and extracellular $[Ca^{2+}]$ and $[H^{+}]$ in the hippocampus during recovery from short-term, transient ischemia. *J Cereb Blood Flow Metab* 1992;12(5):759–772. [PubMed: 1324251]
4. Silver IA, Erecińska M. Intracellular and extracellular changes of $[Ca^{2+}]$ in hypoxia and ischemia in rat brain in vivo. *J Gen Physiol* 1990;95(5):837–866. [PubMed: 2163431]
5. Siemkowicz E, Hansen AJ. Brain extracellular ion composition and EEG activity following 10 minutes ischemia in normo- and hyperglycemic rats. *Stroke* 1981;12(2):236–240. [PubMed: 7233472]
6. Dux E, Mies G, Hossmann KA, Siklós L. Calcium in the mitochondria following brief ischemia of gerbil brain. *Neurosci Lett* 1987;78(3):295–300. [PubMed: 3627566]
7. Zaidan E, Sims NR. The calcium content of mitochondria from brain subregions following short-term forebrain ischemia and recirculation in the rat. *J Neurochem* 1994;63(5):1812–1819. [PubMed: 7931337]
8. Song DK, Malmstrom T, Kater SB, Mykles DL. Calpain inhibitors block Ca^{2+} -induced suppression of neurite outgrowth in isolated hippocampal pyramidal neurons. *J Neurosci Res* 1994;39(4):474–481. [PubMed: 7884824]
9. Lynch G, Baudry M. The biochemistry of memory: a new and specific hypothesis. *Science* 1984;224 (4653):1057–1063. [PubMed: 6144182]
10. Tomimatsu Y, Idemoto S, Moriguchi S, Watanabe S, Nakanishi H. Proteases involved in long-term potentiation. *Life Sci* 2002;72(4–5):355–361. [PubMed: 12467876]
11. Chan SL, Mattson MP. Caspase and calpain substrates: roles in synaptic plasticity and cell death. *J Neurosci Res* 1999;58(1):167–190. [PubMed: 10491581]
12. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* 2003;83(3):731–801. [PubMed: 12843408]
13. Bartus RT, Dean RL, Cavanaugh K, Eveleth D, Carrierio DL, Lynch G. Time-related neuronal changes following middle cerebral artery occlusion: implications for therapeutic intervention and the role of calpain. *J Cereb Blood Flow Metab* 1995;15(6):969–979. [PubMed: 7593358]
14. Bartus RT, Dean RL, Mennerick S, Eveleth D, Lynch G. Temporal ordering of pathogenic events following transient global ischemia. *Brain Res* 1998;790(1–2):1–13. [PubMed: 9593800]
15. Hong SC, Lanzino G, Goto Y, Kang SK, Schottler F, Kassell NF, Lee KS. Calcium-activated proteolysis in rat neocortex induced by transient focal ischemia. *Brain Res* 1994;661(1–2):43–50. [PubMed: 7834383]
16. Neumar RW, Hagle SM, DeGracia DJ, Krause GS, White BC. Brain mu-calpain autolysis during global cerebral ischemia. *J Neurochem* 1996;66(1):421–424. [PubMed: 8522983]
17. Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R. Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci* 1994;14 (6):3934–3944. [PubMed: 8207497]
18. Saido TC, Yokota M, Nagao S, Yamaura I, Tani E, Tsuchiya T, Suzuki K, Kawashima S. Spatial resolution of fodrin proteolysis in postischemic brain. *J Biol Chem* 1993;268(33):25239–25243. [PubMed: 8227089]

19. Yamashima T, Saido TC, Takita M, Miyazawa A, Yamano J, Miyakawa A, Nishijyo H, Yamashita J, Kawashima S, Ono T, et al. Transient brain ischaemia provokes Ca²⁺, PIP₂ and calpain responses prior to delayed neuronal death in monkeys. *Eur J Neurosci* 1996;8(9):1932–1944. [PubMed: 8921284]
20. Yao H, Ginsberg MD, Eveleth DD, LaManna JC, Watson BD, Alonso OF, Loor JY, Foreman JH, Busto R. Local cerebral glucose utilization and cytoskeletal proteolysis as indices of evolving focal ischemic injury in core and penumbra. *J Cereb Blood Flow Metab* 1995;15(3):398–408. [PubMed: 7713997]
21. Yokota M, Saido TC, Tani E, Kawashima S, Suzuki K. Three distinct phases of fodrin proteolysis induced in postischemic hippocampus. Involvement of calpain and unidentified protease. *Stroke* 1995;26(10):1901–1907. [PubMed: 7570746]
22. Neumar RW, Meng FH, Mills AM, Xu YA, Zhang C, Welsh FA, Siman R. Calpain activity in the rat brain after transient forebrain ischemia. *Exp Neurol* 2001;170(1):27–35. [PubMed: 11421581]
23. Chatterjee S, Gu ZQ, Dunn D, Tao M, Josef K, Tripathy R, Bihovsky R, Senadhi SE, O’Kane TM, McKenna BA, et al. D-amino acid containing, high-affinity inhibitors of recombinant human calpain I. *J Med Chem* 1998;41(15):2663–2666. [PubMed: 9667954]
24. Zhang C, Siman R, Xu YA, Mills AM, Frederick JR, Neumar RW. Comparison of calpain and caspase activities in the adult rat brain after transient forebrain ischemia. *Neurobiol Dis* 2002;10(3):289–205. [PubMed: 12270691]
25. Yokota M, Tani E, Tsubuki S, Yamaura I, Nakagaki I, Hori S, Saido TC. Calpain inhibitor entrapped in liposome rescues ischemic neuronal damage. *Brain Res* 1999;819(1–2):8–14. [PubMed: 10082855]
26. Rami A, Agarwal R, Botez G, Winckler J. mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. *Brain Res* 2000;866(1–2):299–312. [PubMed: 10825507]
27. Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD. Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke* 1998;29(1):152–158. [PubMed: 9445345]
28. Li PA, Howlett W, He QP, Miyashita H, Siddiqui M, Shuaib A. Postischemic treatment with calpain inhibitor MDL 28170 ameliorates brain damage in a gerbil model of global ischemia. *Neurosci Lett* 1998;247(1):17–20. [PubMed: 9637399]
29. Hong SC, Goto Y, Lanzino G, Soleau S, Kassell NF, Lee KS. Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* 1994;25(3):663–669. [PubMed: 8128523]
30. Rami A, Kriegelstein J. Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia in vitro and ischemia in vivo. *Brain Res* 1993;609(1–2):67–70. [PubMed: 8508322]
31. Lee KS, Frank S, Vanderklish P, Arai A, Lynch G. Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc Natl Acad Sci U S A* 1991;88(16):7233–7237. [PubMed: 1871130]
32. Mehdi S, Angelastro MR, Wiseman JS, Bey P. Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain. *Biochem Biophys Res Commun* 1988;157(3):1117–1123. [PubMed: 2849930]
33. Yamashima T, Tonchev AB, Tsukada T, Saido TC, Imajoh-Ohmi S, Momoi T, Kominami E. Sustained calpain activation associated with lysosomal rupture executes necrosis of the postischemic CA1 neurons in primates. *Hippocampus* 2003;13(7):791–800. [PubMed: 14620874]
34. Bevers MB, Neumar RW. Mechanistic role of calpains in postischemic neurodegeneration. *J Cereb Blood Flow Metab* 2008;28(4):655–673. [PubMed: 18073773]
35. Choi WS, Lee EH, Chung CW, Jung YK, Jin BK, Kim SU, Oh TH, Saido TC, Oh YJ. Cleavage of Bax is mediated by caspase-dependent or -independent calpain activation in dopaminergic neuronal cells: protective role of Bcl-2. *J Neurochem* 2001;77(6):1531–1541. [PubMed: 11413236]
36. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000;150(4):887–894. [PubMed: 10953012]
37. Takano J, Tomioka M, Tsubuki S, Higuchi M, Iwata N, Itohara S, Maki M, Saido TC. Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J Biol Chem* 2005;280(16):16175–16184. [PubMed: 15691848]

38. Blomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, Mallard C, Hagberg H. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis"? *J Biol Chem* 2001;276(13):10191–10198. [PubMed: 11124942]
39. Ruiz-Vela A, González de Buitrago G, Martínez-A C. Implication of calpain in caspase activation during B cell clonal deletion. *EMBO J* 1999;18(18):4988–4998. [PubMed: 10487751]
40. Cao G, Xing J, Liou AKF, Yin X-M, Clark RSB, Graham SH, Chen J. Critical Role of Calpain I in Mitochondrial Release of Apoptosis-Inducin Factor in Ischemic Neuronal Injury. *J Neurosci* 2007;27(35):9278–9293. [PubMed: 17728442]
41. Hou ST, Jiang SX, Desbois A, Huang D, Kelly J, Tessier L, Karchewski L, Kappler J. Calpain-cleaved collapsin response mediator protein-3 induces neuronal death after glutamate toxicity and cerebral ischemia. *J Neurosci* 2006;26(8):2241–2249. [PubMed: 16495451]
42. Saito T, Konno T, Hosokawa T, Asada A, Ishiguro K, Hisanaga SI. p25/Cyclin-dependent kinase 5 promotes the progression of cell death in nucleus of endoplasmic reticulum-stressed neurons. *J Neurochem*. 2007
43. O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Callaghan SM, Slack RS, Albert PR, Vincent I, Park DS. Differential roles of nuclear and cytoplasmic cyclin-dependent kinase 5 in apoptotic and excitotoxic neuronal death. *J Neurosci* 2005;25(39):8954–8966. [PubMed: 16192386]
44. Atencio IA, Ramachandra M, Shabram P, Demers GW. Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. *Cell Growth Differ* 2000;11(5):247–253. [PubMed: 10845425]
45. Pariat M, Carillo S, Molinari M, Salvat C, Debüssche L, Bracco L, Milner J, Piechaczyk M. Proteolysis by calpains: a possible contribution to degradation of p53. *Mol Cell Biol* 1997;17(5):2806–2815. [PubMed: 9111352]
46. Yamashima T. Ca²⁺-dependent proteases in ischemic neuronal death: a conserved 'calpain-cathepsin cascade' from nematodes to primates. *Cell Calcium* 2004;36(3–4):285–293. [PubMed: 15261484]

Figure 1. Dose-response effect of delayed CEP-3453 therapy following transient forebrain ischemia
A. Western blot of hippocampal homogenates 48 hours following either sham operation (S1–4) or 10 minute transient forebrain ischemia (V1–4 and C1–3). CEP-3453 treated rats were given an initial intravenous drug bolus 22 hours after reperfusion followed by a continuous intravenous infusion for 26 hours (LD, 10mg/kg IV bolus followed by 5mg/kg/hr IV infusion; MD, 30mg/kg IV bolus followed by 15mg/kg/hr IV; HD, 60 mg/kg IV bolus and 30 mg/kg/hr IV infusion). Rats were euthanized at 48 hours and Western blots performed on hippocampal homogenates using primary antibody to alpha-spectrin (MAB 1622) which detects the 145/150 doublet generated by calpain-mediated proteolysis. **B.** Densitometry analysis of Western blots of hippocampal homogenates 48 hours following 10 minutes transient forebrain ischemia.

Graph shows change in density of 145/150 doublet relative to vehicle-treated controls for LD, MD, and HD CEP-3453 treatment. Data is expressed as mean $\pm$ sd. Results demonstrate that delayed administration of high dose CEP-3453 significantly reduces calpain activity in the post-ischemic hippocampus. $*=p<0.05$ relative to vehicle.

A.

B.

Figure 2. Calpain inhibition in the CA1 sector hippocampus with delayed administration of CEP-3453 following transient forebrain ischemia

A. Representative images of CA1 hippocampus 72 hours after sham surgery (sham) or 10 minute TFI treated with drug vehicle or CEP-3453. CEP-3453 treatment consisted of a 60 mg/kg IV bolus at 22 hours post-TFI followed by a 30 mg/kg continuous IV infusion for 50 hours. Sections are immunolabeled for calpain activity (Ab38, red), and counterstained with Hoechst. (blue). B. Densitometry of Ab38 immunofluorescence in the hippocampal CA1 sector 72 hours sham surgery of 10-minute TFI followed by vehicle or CEP-3453 treatment. Data is expressed as mean $\pm$ sd.

Figure 3. Neuroprotection with delayed administration of CEP-3453 following transient forebrain ischemia

A. Representative Hoechst-stained images of CA1 hippocampus 72 hours after sham surgery or 10 minute TFI treated with drug vehicle or CEP-3453. CEP-3453 treatment consisted of a 60 mg/kg IV bolus at 22 hours post-TFI followed by a 30 mg/kg continuous IV infusion for 50 hours. B. Relative quantification of normal appearing Hoechst-stained nuclei in the hippocampal CA1 sector 72 hours 10-minute TFI followed by vehicle or CEP-3453 treatment. Data is expressed as mean $\pm$ sd.