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A Truncated Fragment of Src Protein Kinase Generated by Calpain-mediated Cleavage Is a Mediator of Neuronal Death in Excitotoxicity*[S]

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Background: Abnormal regulation of calpains and Src contributes to stroke-induced brain damage.
Results: The abnormally activated calpains cleave Src to generate a truncated Src fragment capable of directing neurons to undergo cell death.
Conclusion: A new function of Src in neuronal death is discovered.
Significance: Prevention of calpain-mediated cleavage of Src is a potential therapeutic strategy to minimize stroke-induced brain damage.

Excitotoxicity resulting from overstimulation of glutamate receptors is a major cause of neuronal death in cerebral ischemic stroke. The overstimulated ionotropic glutamate receptors exert their neurotoxic effects in part by overactivation of calpains, which induce neuronal death by catalyzing limited proteolysis of specific cellular proteins. Here, we report that in cultured cortical neurons and *in vivo* in a rat model of focal ischemic stroke, the tyrosine kinase Src is cleaved by calpains at a site in the N-terminal unique domain. This generates a truncated Src fragment of ~52 kDa, which we localized predominantly to the cytosol. A cell membrane-permeable fusion peptide derived from the unique domain of Src prevents calpain from cleaving Src in neurons and protects against excitotoxic neuronal death. To explore the role of the truncated Src fragment in neuronal death, we expressed a recombinant truncated Src fragment in cultured neurons and examined how it affects neuronal survival. Expression of this fragment, which lacks the myristoylation motif and unique domain, was sufficient to induce neuronal death. Furthermore, inactivation of the prosurvival kinase Akt is a key step in its neurotoxic signaling pathway. Because Src maintains neuronal survival, our results implicate calpain cleavage as a molecular switch converting Src from a promoter of cell survival to a mediator of neuronal death in excitotoxicity. Besides unveiling a new pathological action of Src, our discovery of the neurotoxic action of the truncated Src fragment suggests new therapeutic strategies with the potential to minimize brain damage in ischemic stroke.

Constitutive activation and expression of Src contribute to formation, survival, and progression of many types of cancer (reviewed in Refs. 1 and 2). In neurons, Src exerts its neurotrophic function by cooperating with Ret, the common receptor of glial cell line-derived neurotrophic factor family ligands to maintain their survival (3, 4). Thus, Src is a key enzyme enhancing survival, growth, and/or proliferation of neuronal and non-neuronal cells. In this paper, we provide evidence that in addition to functioning as a prosurvival enzyme, Src can act as a cell death mediator facilitating neuronal death in excitotoxicity, a key process contributing to brain damage in ischemic cerebral stroke and neurodegenerative diseases.

In ischemic stroke, occlusion of a cerebral artery decreases the supply of oxygen and glucose to brain cells located in the region (referred to as the ischemic core) directly supplied by the artery. Consequently, the cells die in a few minutes (5, 6). However, the area surrounding the ischemic core (referred to as the ischemic penumbra) has a milder deficit of blood supply, due to support from the collateral blood vessels. Neurons in ischemic penumbra receive sufficient oxygen and nutrients to sustain their survival (7, 8). Despite this, if restoration of the blood supply is delayed, neurons in the ischemic penumbra eventually die (5, 6). Thus, these neurons are the targets for neuroprotective therapy to reduce brain damage in stroke patients (5).

One of the major causes of neuronal death in ischemic penumbra is excitotoxicity, which is initiated mainly by overstimulation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate. However, in addition to inducing neuronal death,

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NMDA receptors are involved in maintenance of neuronal survival as well as other physiological functions, accounting for the limited therapeutic potential of NMDA receptor antagonists in reducing stroke-induced brain damage (reviewed in Ref. 9). Thus, deciphering the molecular mechanism governing neuronal death resulting from overstimulation of NMDA receptors will reveal more appropriate molecular targets for therapeutic intervention to reduce brain damage in stroke.

Overstimulation of NMDA receptors leads to calcium overload in the cytosol of the affected neurons. The sustained high cytosolic calcium concentration constitutively activates calpains, which contribute to neuronal death by catalyzing limited proteolysis of specific cellular proteins, some of which are critical for neuronal survival (10). One notable example is the metabotropic glutamate receptor mGluR1α, which plays an essential role in maintaining neuronal survival by activating the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. mGluR1α employs its C-terminal tail to bind and activate PI3K (11). The overactivated calpains abolish the prosurvival function of mGluR1α by cleaving off its C-terminal tail. In addition to blocking prosurvival signaling, calpains can induce neuronal death by aberrantly regulating enzymes, such as caspase-3 and cyclin-dependent kinase 5 (Cdk 5), to initiate prodeath signaling in the affected neurons (12, 13).

Members of the Src family of protein kinases (SFKs),[3] such as Src and Fyn, contribute to brain damage in stroke and Alzheimer disease (14–18). Results of previous studies gave conflicting conclusions on the role of Src and other SFKs in neuronal death in stroke (15–17, 19). Using rodent models of cerebral ischemia and hypoxia-ischemia (15–17), several groups of researchers independently demonstrated that targeted disruption of the *src* gene and inhibitors of SFKs significantly reduce brain damage, suggesting that Src and/or other SFKs contribute to neuronal death in stroke. On the contrary, treatment with inhibitors of SFKs induces cell death of cultured primary cortical neurons (19), suggesting that SFK activity is critical for neuronal survival. The conflicting suggestions arising from these studies are due to our lack of understanding of the role of SFKs in neuronal death. Excitotoxicity, neuroinflammation, and edema resulting from the breakdown of the blood brain barrier and increased vascular permeability are the major contributing factors of neuronal death in stroke patients (6). The main objective of our study reported in this paper is to elucidate the role of Src in neuronal death in excitotoxicity. Our results reveal that Src is aberrantly modified in neurons in response to overstimulation of NMDA receptors. Furthermore, such a modification of Src is a key event contributing to neuronal death in excitotoxicity. Biochemical analyses revealed that this modified form of Src induces neuronal death in part by inhibiting the prosurvival kinase Akt. More importantly, in contrast to the commonly accepted view of Src as a protooncogenic enzyme promoting cell growth and survival, we demonstrate that Src is a key mediator of neuronal death in excitotoxicity. Thus, future investigation of the molecular basis of aberrant modification and the neurotoxic mechanism of Src will identify new targets for therapeutic intervention to reduce brain damage in stroke.

EXPERIMENTAL PROCEDURES

Primary Cortical Neuronal Culture and Treatment with Glutamate, Glutamate Receptor Antagonists, and Calpain Inhibitor—Primary cortical neurons were isolated from mouse embryos collected at day 16 of gestation as detailed in the supplemental material. They were maintained at 37 °C in 5% CO_2 and 95% air in a humidified incubator in the neurobasal medium containing 2.5% B-27, 0.25% GlutaMAX-1, and 1% penicillin and streptomycin. Cells were maintained for 7 days prior to treatment. The cultured neurons were treated with 100 μM glutamate at the seventh day *in vitro* (DIV 7) to mimic excitotoxicity. To examine the effect of other extracellular agents on glutamate-treated neurons, the cultured neurons were pretreated for 30 min with the NMDA receptor antagonist MK801 (50 μM), the GluN2B-containing NMDA receptor antagonist ifenprodil (20 μM), the antagonist for AMPA and kainate receptor 6-cyano-7-nitroquinoxaline-2,3-dione (40 μM), and calpain inhibitor calpeptin (20 μM) prior to treatment with glutamate.

Assays to Monitor Viability of Neurons—Neuronal cell viability in culture was monitored by three different assays. The MTT assay, which measures the rate of enzymatic cleavage of the tetrazolium salt to purple formazan crystal by active mitochondrial reductase in viable neuronal cells, was used to monitor cell viability. The extent of cell death of neurons in culture was monitored also by the activity of lactate dehydrogenase released by the damaged neurons to the culture medium (LDH release assay). In addition to biochemical assays, live and dead neuronal cells were monitored by incubation with calcein-AM and EthD1 (ethidium homodimer-1), which stain live and dead cells, respectively. Fluorescent microscopy reveals the number of live calcein-containing neurons (green) and dead EthD1-stained neurons (red). Details of the cell viability assays are described in the supplemental material.

Calpain Activity Assay—The calpain activity of neurons with and without treatment with 100 μM glutamate was measured by monitoring the rate of cleavage of the fluorogenic substrate Ac-Leu-Leu-Tyr-(7-amino-4-trifluoromethylcoumarin) by calpain in the crude cell lysates as described in the supplemental material.

Transduction of Primary Cortical Neurons with Lentivirus—The genes encoding GFP fusion proteins of full-length Src (Src-GFP), (G2A)Src-GFP carrying the myristoylation-defective G2A mutation, truncated Src lacking residues 1–80 (SrcΔN-GFP), a kinase-dead version of truncated Src (SrcΔN(K/D)-GFP) carrying the K303M mutation, and constitutively active Akt1 mutant (Myr-Akt) were inserted in the pLVX-Tight-Puro vector (Clontech). They were used to generate the lentivirus directing expression of the genes of interest in HEK293FT cells as detailed in the supplemental material. Upon expression, Src-GFP and its mutants contain a Gly-Ser-Gly-Ser linker followed by the enhanced green fluorescence protein fused to the C terminus of Src and mutant Src sequences (20).

[3] The abbreviations used are: SFK, Src family protein kinase; DIV, day *in vitro*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ET-1, endothelin-1; SH2 and -3, Src homology 2 and 3, respectively; LDH, lactate dehydrogenase; Myr, Src myristoylation signal.

Myr-Akt contains a Src myristoylation signal (Myr) and a hemagglutinin epitope fused to its N and C termini, respectively (21). For the lentivirus directing the expression of the transcriptional regulator, it was produced in HEK293FT cells using the pLVX-Tet-on-Advance plasmid (Clontech). For transduction, the cultured neurons (0.5×10^6 cells) at DIV 1 were incubated with a lentivirus generated with PLVX-Tight-Puro plasmids containing the gene of interest and the lentivirus directing the expression of the transcriptional regulator at a multiplicity of infection of ≥20. The neurobasal medium was changed after 24 h. At DIV 5, the recombinant Src proteins were induced by the addition of doxycycline (1 mg/ml). As a control, cells were also transduced with the lentivirus generated by the GFP-pLVX-tight-puro plasmid. Experiments to analyze the effects of expression of the recombinant proteins on the signaling mechanism and survival of the transduced neurons were performed at DIV 7.

In Vitro Calpain Cleavage of Recombinant Src—Purified recombinant Src (100 ng) was digested with different concentrations of calpain 1 (Calbiochem) in the digestion buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT, 30 mM NaCl, 10 mM $CaCl_2$). The reaction mixture was incubated at 30 °C for 45 min. The digested sample was analyzed by Western blotting with anti-Src mAb327 and anti-Src mAb(2–17) antibodies.

Tyrosine Kinase Activity Assay—Crude lysates of neurons were diluted with the dilution buffer (25 mM Hepes, pH 7). Aliquots of the diluted lysates were assayed for Src family kinase activity. For assay of Src kinase activity in the immune complex, Src and Src mutant were immunoprecipitated from the crude neuronal lysate with the anti-Src mAb327 antibody or anti-GFP antibody bound to Protein A-Sepharose beads. The kinase activity was measured by the rate of phosphorylation of the Src-optimal peptide (AEEEAYGEAEAKKKK) as described in the supplemental material.

Subcellular Fractionation—Subcellular fractionation of primary cortical cells was performed based on the method described by Bernocco *et al.* (22). First, cells were washed with ice-cold PBS. Then buffer A (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 5 mM EDTA, 0.015% digitonin, protease and phosphatase inhibitor mixture (Roche Applied Science)) was added to the attached neuronal cells and gently shaken for 30 min at 4 °C. Digitonin from the buffer creates pores in the plasma membrane by solubilizing cholesterol. Therefore, all of the cytosolic proteins were extracted in the solution without rupturing the plasma membrane (23). After incubation for 30 min, the supernatant was collected carefully without detaching neuronal cells. This fraction contains soluble cytosolic proteins. A second extraction was done using buffer B (10 mM PIPES, pH 7.4, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 3 mM EDTA, 0.5% Triton X-100). Cells were detached and lysed in buffer B by rocking for 30 min at 4 °C. The mixture was centrifuged at 8400 × *g* for 10 min. The supernatant contained the Triton X-100-soluble plasma membrane proteins.

Induction of Stroke in Rats by Stereotaxic Injection of Endothelin-1—Male hooded Wistar rats aged 10–12 weeks (300–350 g) were anesthetized with ketamine/xylazine (75:10 mg/kg intraperitoneally) and maintained by inhalation of isoflurane (95% oxygen and 5% isoflurane). A 23-gauge stainless steel guide cannula was stereotaxically implanted into the piriform cortex 2 mm dorsal to the right middle cerebral artery (0.2 mm anterior, −5.2 mm lateral, and −5.9 mm ventral) according to the method of Sharkey *et al.* (24) and as described previously (25). The cannula was secured using acrylic dental cement, and the rat was allowed to recover for 5 days prior to stroke induction. Focal cerebral ischemia was induced in the conscious rat by constriction of the right middle cerebral artery with perivascular administration of endothelin-1 (60 pmol in 3 μl of saline over 10 min). During stroke induction, counterclockwise and/or clockwise circling, clenching, and dragging of the contralateral forepaw were observed, validating the correct placement of the cannula. Stroke severity was scored based on these responses, which have been shown to be a reliable prediction of stroke outcome (25). Thus, this model allows induction of stroke in a fully conscious rat and allows for accurate prediction of stroke outcome in terms of volume of damage and neurological deficits incurred based on observations made during stroke induction. Control rats ($n = 2$) underwent cannula implantation and saline infusion instead of endothelin-1 to demonstrate that saline injection itself does not induce vasoconstriction and cerebral infarction, as described previously (25). Rats were decapitated 3 h after stroke, and forebrains were removed and frozen over liquid nitrogen until further processing. The brain tissues were lysed with ice-cold lysis buffer containing 50 mM Tris, pH 7.0, 1 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 40 mM sodium pyrophosphate, 0.5 mM Na_3VO_4, 50 mM β-glycerophosphate, 0.2 mg/ml benzamidine, 0.1 mg/ml phenymethylsufonyl fluoride, and a mixture of EDTA-free protease inhibitors and phosphatase inhibitors (Roche Applied Science). The detailed procedures of surgical preparation and stroke induction are given in the supplemental material.

Synthetic Peptides—All peptides were chemically synthesized by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based solid phase peptide synthesis chemistry. Their sequences are listed in supplemental Table S3. All except the TAT-Scrambled peptide contain the sequences derived from the segment corresponding to amino acids 1–79 of Src. For the TAT-Src and TAT-Scrambled peptides, they contain a 6-aminohexanoic acid linker connecting the TAT protein transduction domain motif (GRKKRRQRRRPQ) to the Src(49–79) segment or the scrambled sequence with the same amino acid composition as the Src(49–79) segment.

Data Analysis—Densitometric analysis for the quantification of the bands on immunoblots was performed using ImageJ software. Statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 16 (SPSS, Inc., Chicago, IL). The data were reported as the means ± S.D., and the statistical significance was determined by Student's *t* test (two-tailed). $p < 0.05$ was considered statistically significant for all experiments.

RESULTS

A Truncated Src Fragment of ~52 kDa Is Formed in Neurons upon Overstimulation by Glutamate—We used cultured primary cortical neurons treated with glutamate (100 μM) as the

system to investigate the role of Src in neuronal death in excitotoxicity. After isolation from embryonic day 15–16 mouse embryos, the cortical neurons were cultured for 7 days prior to treatment with glutamate. The expression levels of Src and NMDA receptor subunits GluN1, GluN2A, and GluN2B undergo time-dependent increase from day 1 to day 7.[4] In addition, at DIV 7, neurite outgrowths of the cultured neurons are extensive.[4] Cortical neurons at DIV7 were used to study the effects of glutamate overstimulation on neuronal survival.

Treatment of cortical neurons with glutamate at DIV 7 induces a time-dependent decrease in viability and increase in calpain activity (Fig. 1, *A–D*). Because calpains cleave the cytoskeletal protein α-fodrin to form the 145- and 150-kDa proteolytic fragments, the appearance of the α-fodrin fragments of ~150 kDa has been used as a marker of overactivation of calpains in neurons undergoing excitotoxic cell death (26). Fig. 1*E* shows that treatment with glutamate leads to degradation of α-fodrin to form the ~150-kDa α-fodrin fragments, further confirming activation of calpains in the cultured cortical neurons in response to stimulation by glutamate.

Concomitant with the decrease in viability and calpain activation, Src undergoes limited proteolysis to form a truncated fragment of 52 kDa in neurons as early as 5 min after overstimulation with glutamate (Fig. 1, *F* and *G*). The amount of this truncated Src fragment reaches a maximum level at 2 h of treatment. Calpeptin, a cell-permeable calpain inhibitor effectively suppresses formation of this truncated Src fragment (Fig. 1, *F* and *G*). Together, these data indicate that the activated calpain induces proteolysis of Src to form a truncated Src fragment in neurons. Next, we used pharmacological agents to identify the type of glutamate receptors involved in excitotoxicity-induced cleavage of Src in neurons. 6-Cyano-7-nitroquinoxaline-2,3-dione, an AMPA/kainate receptor antagonist, fails to abolish calpain-mediated truncation of Src, whereas the NMDA receptor antagonist MK801 completely blocks the formation of the truncated fragment (supplemental Fig. S1). These results indicate that NMDA receptor is the major glutamate receptor governing calpain-mediated truncation of Src in cultured neurons. Stimulation of the GluN2B subunit-containing NMDA receptors, which are located mostly in extrasynaptic sites, contributes to brain damage in stroke (27). We therefore examined the effect of ifenprodil, an antagonist of the GluN2B subunit-containing NMDA receptor, on neuronal Src truncation by calpain in response to glutamate overstimulation. As shown in supplemental Fig. S1, ifenprodil suppresses formation of the truncated Src, suggesting involvement of GluN2B-containing NMDA receptor in calpain-mediated truncation of Src in neurons.

Calpain Directly Cleaves Src at the Unique Domain to Generate the Truncated Src Fragment in Neurons—To define the cleavage site in Src, we first compared the Western blots of crude neuronal lysates probed with two site-specific anti-Src antibodies: (i) the anti-Src mAb327 antibody with epitope at the SH3 domain, and (ii) the anti-Src mAb(2–17) antibody directing against the segment corresponding to amino acids 2–17 of Src (Fig. 2*A*). Whereas the mAb327 antibody detects both the intact and truncated Src, the anti-Src(2–17) antibody only recognizes full-length Src (Fig. 2*B*), suggesting that calpain cleaves Src in the unique domain. To ascertain if Src is a direct substrate of calpain, we incubated purified recombinant Src with calpain 1 *in vitro* and performed Western blotting of the proteins in the reaction mixture with the two aforementioned antibodies. Fig. 2*C* shows that purified recombinant Src is cleaved by calpain 1 *in vitro* to produce the 52-kDa proteolytic fragment recognized by anti-Src mAb327 antibody but not by the anti-Src mAb(2–17) antibody, indicating that calpain 1 directly cleaves Src at the unique domain. Further subcellular fractionation studies reveal that the truncated Src resides predominantly in cytosol (supplemental Fig. S2), suggesting that calpain-mediated cleavage removes the N-terminal myristoyl moiety that anchors Src to the plasma membrane.

Next, we used a combination of Edman sequencing, mass spectrometry, and biochemical analysis to define the calpain cleavage site in the unique domain of Src. We first determined the N-terminal sequence of the truncated Src generated *in vitro* by calpain 1-mediated cleavage of recombinant Src (supplemental Fig. S3*A*) by Edman sequencing. Despite several attempts, we failed to obtain any sequence information, suggesting that the truncated Src does not have a free N terminus. One possible reason for the lack of free N terminus is that the nascent 52-kDa Src fragment generated by calpain 1-catalyzed truncation contains a glutamine residue at its N terminus, which readily undergoes cyclization. To overcome this technical problem, we employed mass spectrometry to determine the calpain cleavage site. Briefly, purified intact Src and truncated Src fragment (supplemental Fig. 3*A*) were exhaustively digested with trypsin. The tryptic fragments generated were identified by mass spectrometry. Supplemental Fig. S3*B* shows that both intact and truncated Src proteins gave several detectable tryptic fragments derived from the region encompassing amino acid residues 79–461. In addition to these tryptic fragments, mass spectrometric analysis also detected a tryptic fragment LFGGFNSSDTVTSPQR derived from residues 63–78 of the Src sequence only in the tryptic digest of intact Src (supplemental Fig. S3, *B* and *C* , and Tables S1 and S2), suggesting that the calpain cleavage site resides in this segment of the unique domain of Src.

Another approach we used to define the calpain cleavage site in Src is by employing a peptide inhibition assay. We synthesized four peptides (Fig. 3*A* and supplemental Table S3) derived from the unique domain of Src and examined their ability to inhibit calpain 1-catalyzed cleavage of Src *in vitro*. Among them, only the synthetic Src(49–79) peptide, which contains the segment of residues 63–78, could block cleavage of Src by calpain 1 (Fig. 3*B*), providing further support to our claim that the calpain cleavage site resides in this segment (residues 63–78) of the Src unique domain. Indeed, in agreement with the difference in molecular sizes of intact Src (60 kDa) and the truncated Src fragment (~52 kDa), calpain-mediated cleavage at this segment is expected to reduce the molecular size of Src by ~8 kDa.

Because the sequence of the segment encompassing residues 63–78 in the Src unique domain shares little homology

[4] M. I. Hossain, D. C. H. Ng, A. F. Hill, and H.-C. Cheng, manuscript in preparation.

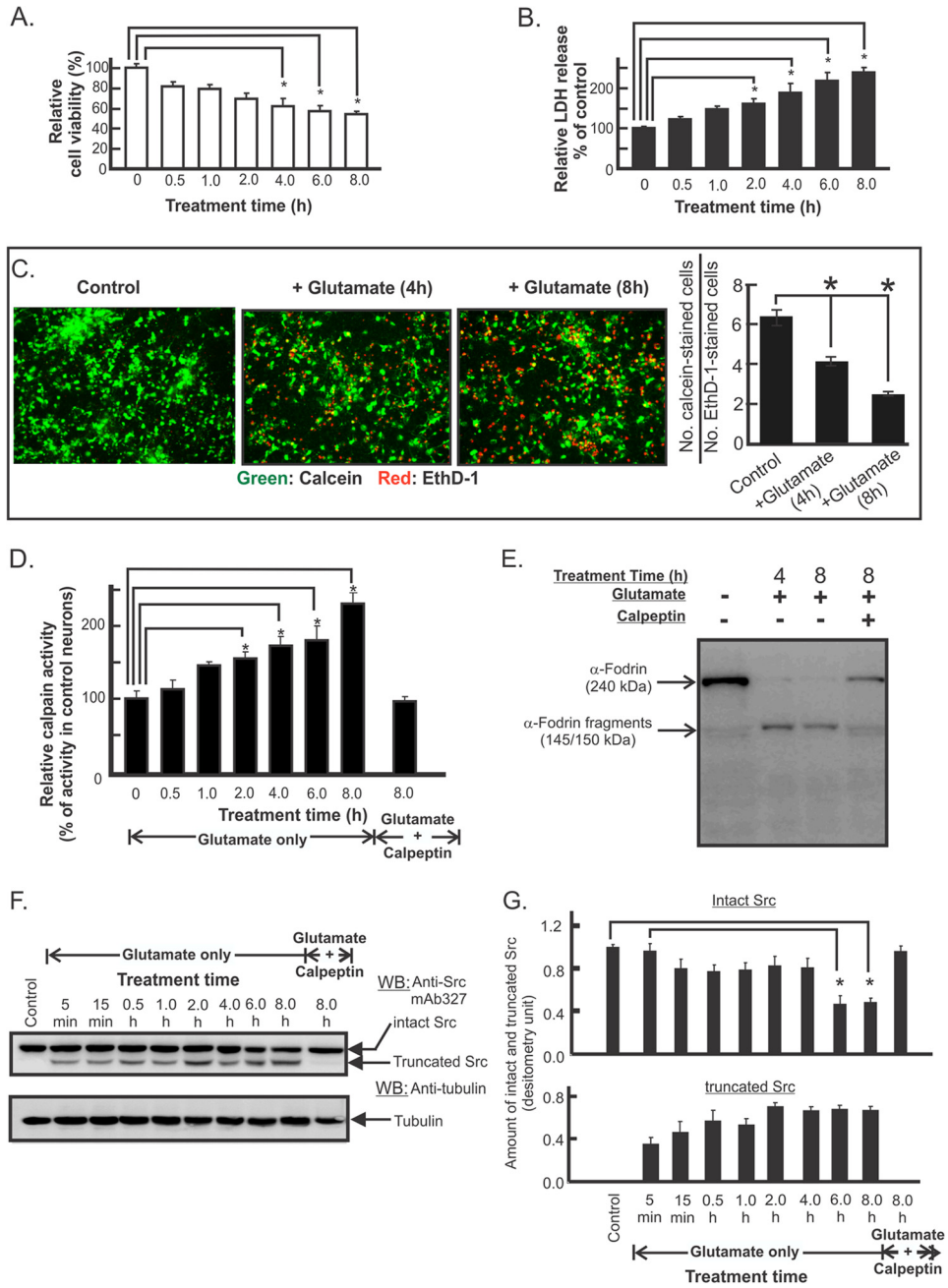

FIGURE 1. **Overstimulation of glutamate receptors of primary cortical neurons induces cell death, calpain activation, and truncation of Src.** Cortical neurons at DIV 7 were incubated with 100 μM glutamate for 8 h. Their viability, their calpain activity, and the structural integrity of neuronal Src and α-fodrin were analyzed at the designated time intervals. *A*, MTT assay to monitor neuronal viability at the designated time points. The amount of the reaction product formazan formed (as measured by absorbance at 570 nm) by the viable neurons in the untreated culture is assigned as 100%. The viability of neurons at different times of treatment is presented as a percentage of the untreated cells (data represented as mean ± S.D. (*error bars*), $n = 5$; *, $p < 0.05$, Student's *t* test). *B*, LDH release assay to monitor the extent of neuronal cell damage. The activity of LDH released to the culture medium from the damaged neurons upon treatment with glutamate at each time point relative to that obtained in the medium of the untreated neurons is presented (data represented as mean ± S.D., $n = 5$; *, $p < 0.05$, Student's *t* test). *C*, the viable neurons were stained with calcein (*green*), and the damaged neurons were stained with ethidium homodimer-1 (*EthD-1*) (*red*). *Control*, untreated neurons. +*Glutamate* (*4 h*) and +*Glutamate* (*8 h*), neurons treated with glutamate for 4 and 8 h, respectively. *Right*, ratio of the number of calcein-stained cells to that of ethidium homodimer-1-stained cells of the control and glutamate-treated neurons (data represented as mean ± S.D., $n = 5$; *, $p < 0.05$, Student's *t* test). *D*, time-dependent changes of calpain activity of primary cortical neurons after treatment with 100 μM glutamate. The calpain activity in the lysates of the untreated neurons is assigned to be 100% (data represented as mean ± S.D., $n = 4$; *, $p < 0.05$, Student's *t* test). *E*, cleavage of neuronal α-fodrin by calpains to form proteolytic fragments of ~150 kDa in neurons treated with glutamate and in the presence or absence of calpeptin (20 μM). *F*, Western blots (*WB*) of lysates from neurons treated with either glutamate alone or glutamate and calpeptin for different times and probed with anti-Src mAb327 antibody or anti-tubulin antibody. The anti-tubulin immunoreactivity signals are used as loading controls. *G*, the immunoreactive signals (in densitometric units) of intact Src (60 kDa) and the truncated Src fragment (52 kDa) (data represented as mean ± S.D., $n = 5$; *, $p < 0.05$, Student's *t* test).

with sequences in other SFK members, such as Fyn and Yes, which are also expressed in neurons, it is logical to predict that cleavage by calpain is an event unique to Src. As shown in Fig. 2, *D* and *E*, although treatment with glutamate induces a time-dependent increase in the amount of the 52-kDa truncated Src fragment in neurons, no cleavage of

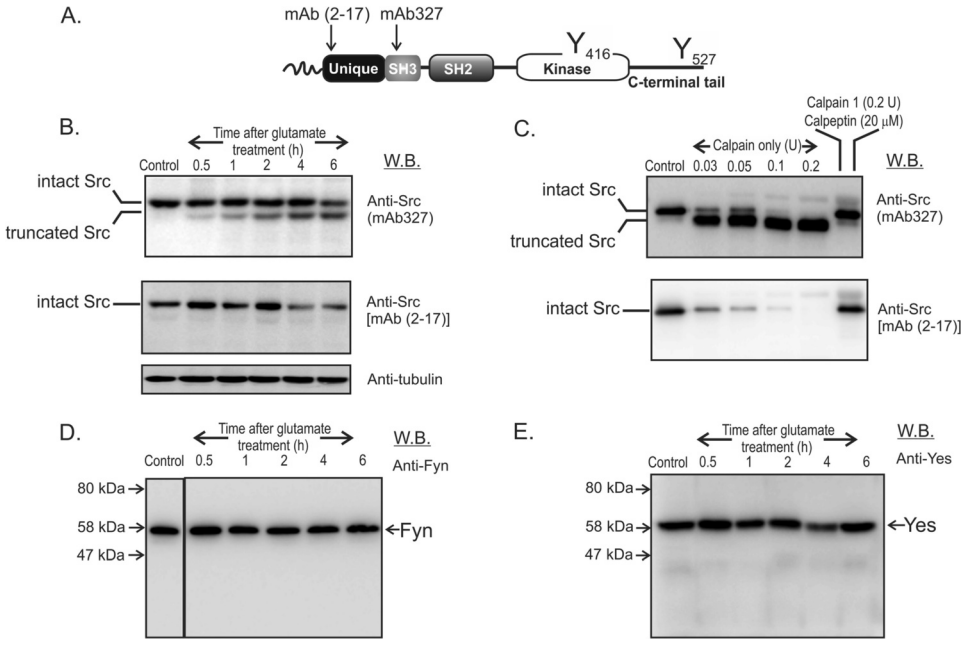

FIGURE 2. **Calpain directly cleaves Src at its unique domain to generate the 52-kDa truncated fragment.** *A*, schematic of Src functional domains. Tyr-416 (Y_{416}) and Tyr-527 (Y_{527}) are the conserved autophosphorylation site in the kinase domain and the C-terminal regulatory tyrosine phosphorylation site, respectively. The locations of epitopes of the two anti-Src antibodies mAb(2–17) and mAb327 are indicated by *arrows*. *B*, Western blots (*W.B.*) of crude lysates of neurons treated with glutamate for different times (0.5–6 h) probed with anti-Src mAb327 and anti-Src mAb(2–17) antibodies. *C*, Western blots of purified recombinant Src treated with different amounts (in activity units) of calpain *in vitro* probed with anti-Src mAb327 and anti-Src mAb(2–17) antibodies. *D* and *E*, Western blots of crude lysates of neurons treated with glutamate for different times (0.5–6 h) and probed with anti-Fyn and anti-Yes antibodies.

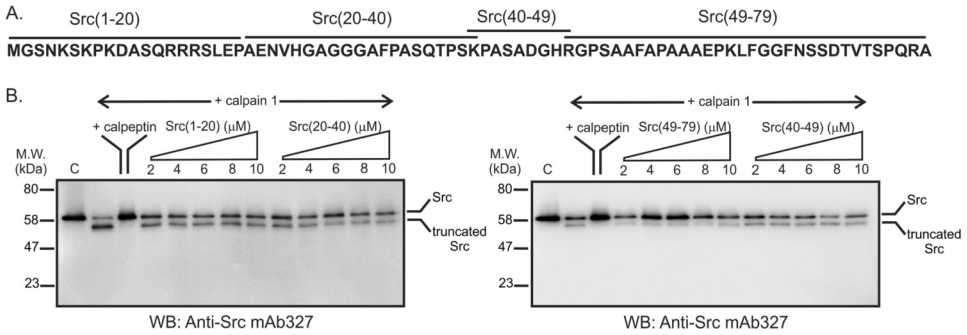

FIGURE 3. **Effects of Src(1–20), Src(20–40), Src(40–49), and Src(49–79) peptides on the calpain 1-mediated cleavage of recombinant Src *in vitro*.** *A*, sequence of the N-terminal myristoylation motif and part of the unique domain of human Src. The segments from which Src(1–20), Src(20–40), Src(40–49), and Src(49–79) were derived are indicated. *B*, the four synthetic peptides at concentrations ranging from 2 to 10 μM were incubated with the reaction mixture containing Src and calpain 1. As a control, calpeptin was added to inhibit calpain 1 activity. Formation of the truncated Src fragment by calpain 1 was monitored by Western blotting (*WB*) with anti-Src mAb327 antibody.

Fyn and Yes was detected, indicating that cleavage by calpain is a unique regulatory property of Src in neurons.

SFK inhibitors significantly reduce brain damage in rodent models of stroke and hypoxia (15–17, 28–31), implying that SFKs are aberrantly activated and that phosphorylation of specific cellular proteins by the activated SFKs contributes to neuronal death in excitotoxicity. We therefore sought to ascertain how glutamate overstimulation affects neuronal Src activity. As shown in supplemental Fig. S4, the activity of intact Src in neurons undergoes changes in two phases following glutamate treatment; an initial phase (0–30 min) of increased activity is followed by a subsequent phase (30 min or longer) of decreased activity that is reduced below basal levels. Because intact Src is involved in maintaining neuronal survival (3, 4, 32), future investigation may focus on examining if the transient activation of intact Src is a cytoprotective event in excitotoxicity.

Cerebral Ischemia Induces Production of a 52-kDa Truncated Src Fragment in a Rat Model of Stroke—We aimed to confirm if excitotoxicity induced by cerebral ischemia can cause truncation of Src in an animal model. The endothelin-1 (ET-1) model of stroke in conscious rats was adopted to investigate the expression of truncated Src following cerebral ischemia with reperfusion. In this model, a guide cannula was stereotaxically implanted in the piriform cortex of the rat (Fig. 4*A*). Following recovery, ET-1 was then applied locally to the middle cerebral artery of the conscious rat to induce focal ischemia with reperfusion that results in reproducible damage to the ipsilateral territory supplied by the middle cerebral artery (Fig. 4*B*). We found that following stroke with a 3-h recovery, a 52-kDa truncated Src fragment appeared predominantly in the ipsilateral side of the cerebral hemisphere (Fig. 4*C*). The results indicate that brain injury caused by middle cerebral artery vasoconstric-

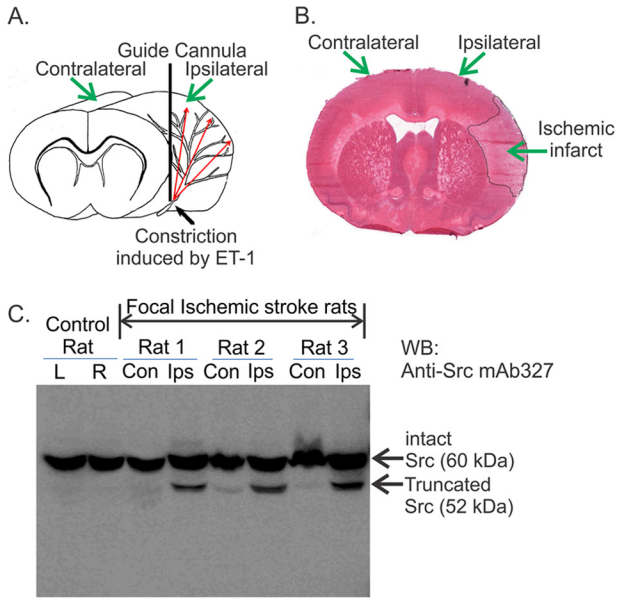

FIGURE 4. **Endothelin 1 (ET-1)-induced cerebral ischemia induces proteolysis of Src kinase in brain cells to form a 52-kDa truncated fragment.** *A*, schematic depicting the ET-1 model of stroke. Vasoconstriction of the middle cerebral artery by applying ET-1 through the guide cannula generates ischemic damage in the parietal, insular, and frontal cortex, as well as in the dorsolateral and corpus striatum. The *red arrows* indicate some of the regions adversely affected by the ET-1 treatment. *B*, hematoxylin and eosin-stained image of a representative brain section 0.2 mm from Bregma close to the site of ET-1 infusion during stroke induction. The ischemic infarct (*dotted black line*) could be seen evolving as soon as 3 h after stroke induction. *C*, anti-Src mAb327 blot of lysate of brain tissues collected in the control rat and three stroke rats. *WB*, Western blot; *Ips*, ipsilateral; *Con*, contralateral; *L*, left hemisphere; *R*, right hemisphere.

tion and subsequent reduction in cerebral blood flow in rats is associated with the formation of a 52-kDa truncated Src fragment similar to that observed in cultured neurons upon overstimulation of glutamate receptors.

Prevention of Calpain-mediated Truncation of Src Alleviates Neuronal Death Induced by Overstimulation of Glutamate Receptors—To investigate if prevention of calpain-mediated truncation of Src in neurons is cytoprotective, we generated a cell membrane-permeable fusion peptide containing the protein transduction domain of the HIV-1 TAT protein at the N-terminal portion, a 6-aminohexanoic acid linker and the Src(49–79) segment capable of blocking cleavage of Src *in vitro* (Fig. 3 and supplemental Table S3). This peptide (referred to as the TAT-Src peptide) inhibited calpain cleavage of recombinant Src *in vitro* (Fig. 5*A*) and blocked calpain-mediated truncation of Src in glutamate-overstimulated neurons (Fig. 5*B*). In contrast, this peptide has no effect on calpain-mediated cleavage of α-fodrin (Fig. 5*C*), suggesting that the TAT-Src specifically blocks calpain-mediated cleavage of Src but not the other calpain substrates in neurons. More importantly, as shown by results of the MTT and LDH assays (Fig. 5, *D* and *E*) and images of neurons after staining with calcein-AM/ethidium homodimer-1 (Fig. 5, *F* and *G*), TAT-Src peptide at 20 μM provides significant protection against neuronal death induced by glutamate overstimulation. In contrast, the TAT-Scrambled peptide containing the TAT-6-aminohexanoic acid segment and the scrambled Src(49–79) segment (supplemental Table S3), however, cannot block calpain-mediated cleavage of Src or protect against glutamate-induced neuronal death (Fig. 5), indicating that the cytoprotective action of the TAT-Src peptide is reliant on the structural integrity of the Src(49–79) segment.

Expression of an Active Recombinant N-terminally Truncated Src Mutant Induces Neurons to Undergo Cell Death—Because the 52-kDa Src fragment generated by calpain-mediated truncation contains the intact SH3, SH2, and kinase domains and the C-terminal tail, it can potentially employ these domains to bind and phosphorylate specific cellular proteins in neurons to facilitate neuronal death. To examine the validity of this notion, we first attempted to isolate the truncated Src fragment in crude neuronal cell lysates and measure its kinase activity and autophosphorylation status. However, although the anti-Src mAb327 antibody readily detects both the intact Src and truncated Src fragment in Western blots, it fails to immunoprecipitate the truncated Src fragment from the crude neuronal lysate (supplemental Fig. S4*B*). A different approach was therefore adopted to monitor the kinase activity and the role of the truncated Src fragment in excitotoxic neuronal death, we generated a lentivirus to direct expression of a recombinant GFP fusion protein of truncated Src mutant (referred to as SrcΔN-GFP) under the control of doxycycline in neurons (Fig. 6*A*). The SrcΔN-GFP protein, with the GFP moiety attached to the C-terminal tail of the truncated Src, lacks the N-terminal segment encompassing the myristoylation motif and unique domain. Thus, it mimics the action of the truncated Src fragment formed by calpain cleavage in neurons upon overstimulation of glutamate receptors. For the control experiments, we transduced neurons with lentivirus directing expression of GFP, GFP fusion proteins of full-length Src (Src-GFP), (G2A)Src-GFP carrying the myristoylation-defective G2A mutation, and the kinase-dead SrcΔN(K/D)-GFP carrying the K303M mutation (Fig. 6*A*).

Fig. 6*A* shows that Src-GFP, SrcΔN-GFP, and its mutant were expressed at levels comparable with that of endogenous Src in the transduced neurons. Both Src-GFP and SrcΔN-GFP were autophosphorylated at Tyr-416 at levels similar to that of intact endogenous Src (Fig. 6*A*). Although the K303M mutation abolishes its kinase activity, the kinase-dead SrcΔN(K/D)-GFP mutant was autophosphorylated at Tyr-416, albeit at a level much lower than those of endogenous Src and Src-GFP (Fig. 6*A*). Autophosphorylation of Src is an intermolecular event (33, 34). Furthermore, Cooper and MacAuley (33) demonstrated trans-autophosphorylation of a kinase-dead Src mutant at Tyr-416 by a catalytically active Src mutant when both mutants were co-expressed in cells. Thus, it is likely that the kinase-dead SrcΔN(K/D)-GFP mutant expressed in the transduced neurons was trans-autophosphorylated by the active endogenous Src.

To determine the activities of the recombinant Src-GFP and mutants, we immunoprecipitated them from crude lysates of the transduced neurons using the anti-GFP antibody and assayed for the kinase activity associated with the immunoprecipitates using the Src-optimal peptide. Fig. 6*B* shows that the kinase-dead SrcΔN(K/D)-GFP exhibited negligible activity, whereas the Src-GFP and SrcΔN-GFP exhibit similar kinase activities. Our data suggest that deletion of the N-terminal myr-

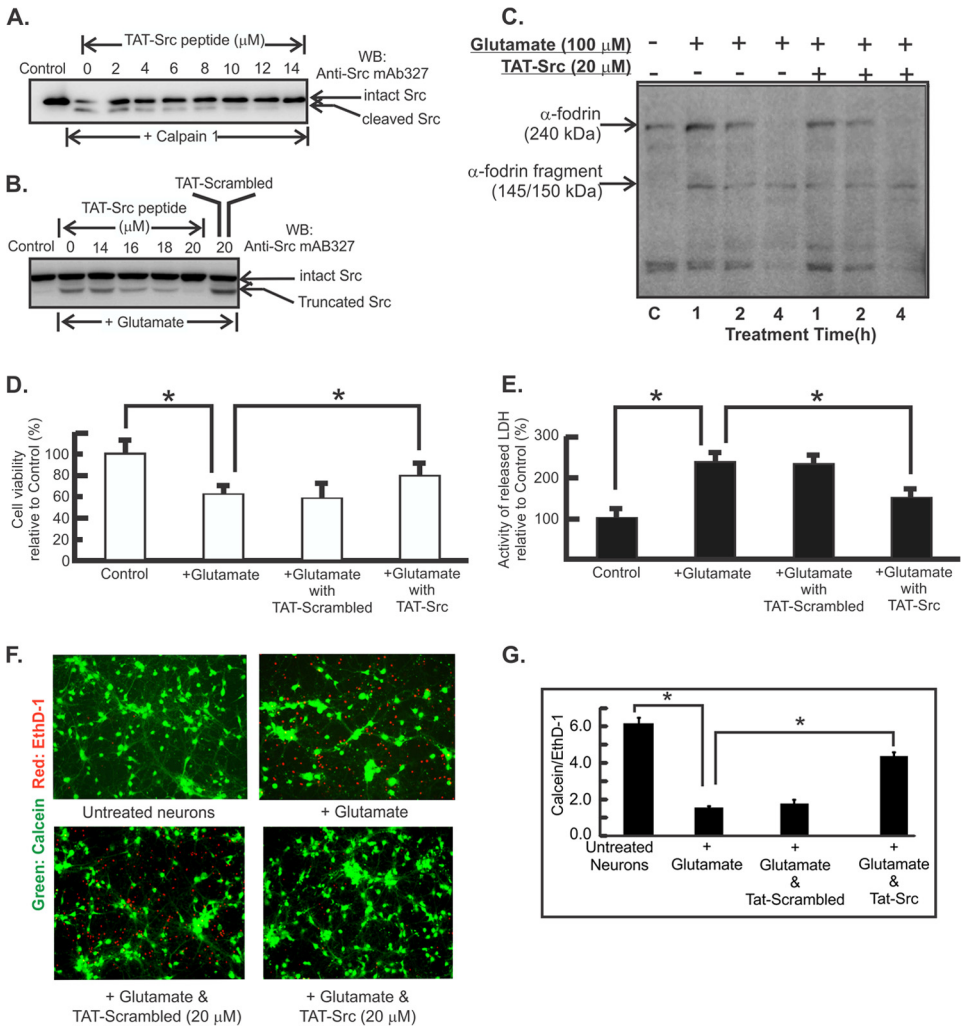

FIGURE 5. **Blockade of calpain-dependent truncation of Src protects against neuronal death in excitotoxicity.** Cultured cortical neurons at DIV 7 were treated with glutamate (100 μM) for 8 h in the presence or absence of TAT-Src peptide or TAT-Scrambled peptide. The neurons were analyzed for calpain cleavage of Src and fodrin and cell viability. *A*, anti-Src Western blot (*WB*) of purified recombinant Src after treatment with calpain *in vitro* in the presence of 0–14 μM Tat-Src peptide (*C*, control). *B*, anti-Src Western blot of lysates of neurons with and without treatment with 100 μM glutamate in the presence of 0–20 μM TAT-Src peptide or 20 μM TAT-Scrambled peptide. *C*, Western blot analysis of the cell lysate to monitor the effect of TAT-Src (20 μM) on calpain cleavage of fodrin in neurons with and without glutamate treatment. *D*, MTT assay measuring viability of neurons after 8-h treatment under different conditions. *E*, LDH release assay to monitor the extent of cellular damage of neurons after 8 h treatment under different conditions. *F*, calcein-AM/ethidium homodimer-1 staining of untreated neurons and neurons treated with glutamate (100 μM) for 15 h in the presence and absence of 20 μM TAT-Src peptide or 20 μM TAT-Scrambled peptide. *Green fluorescence*, calcein-AM-stained viable neurons. *Red fluorescence*, ethidium homodimer-1-stained damaged neurons. *G*, the ratios of calcein-stained cells *versus* the ethidium homodimer-1-stained cells in the control sample and neurons treated with glutamate in the presence or absence of 20 μM TAT-Src peptide or 20 μM TAT-Scrambled peptide (data represented as mean ± S.D. (*error bars*), $n = 5$; *, $p < 0.05$, Student's *t* test).

istoylation motif and the unique domain does not affect the activity of Src in neurons. Thus, we predict that in neurons overstimulated by glutamate, intact Src and the 52-kDa truncated Src fragment generated by calpain cleavage exhibit similar kinase activities. Intriguingly, induced expression of SrcΔN-GFP leads to a significant reduction in cell survival (Fig. 7) and an increase in the number of damaged neurons (Fig. 7*C*, *inset*). In contrast, expression of GFP or Src-GFP has no effect on the viability of the cultured neurons (Fig. 7). These results indicate that SrcΔN-GFP is neurotoxic because its expression alone induces neuronal death. Thus, Src acquires the capability to induce neuronal death when both the myristoylation motif and unique domain are deleted. Similar to SrcΔN-GFP, the 52-kDa Src fragment generated by calpain cleavage in neurons undergoing excitotoxic cell death also lacks the myristoylation motif and a large portion of the unique domain (Fig. 2 and supplemental Fig. S3). Our results strongly suggest that this endogenous 52-kDa Src fragment is a neurotoxic mediator contributing to neuronal death in excitotoxicity.

Presumably, SrcΔN-GFP induces neuronal death by phosphorylating a specific subset of cellular proteins. In light of this, we generated the kinase-dead SrcΔN(K/D)-GFP mutant with the conserved lysine (equivalent to Lys-303 in the intact neuronal Src) essential for ATP binding mutated to methionine. This mutation abolishes the neurotoxic ability of the truncated Src mutant (Fig. 7).

The myristoyl group attached to the N terminus of Src is essential for its targeting to the plasma membrane. Previously, mutation of the conserved Gly-2 essential for myristoylation has been shown to abolish the ability of the constitutively active

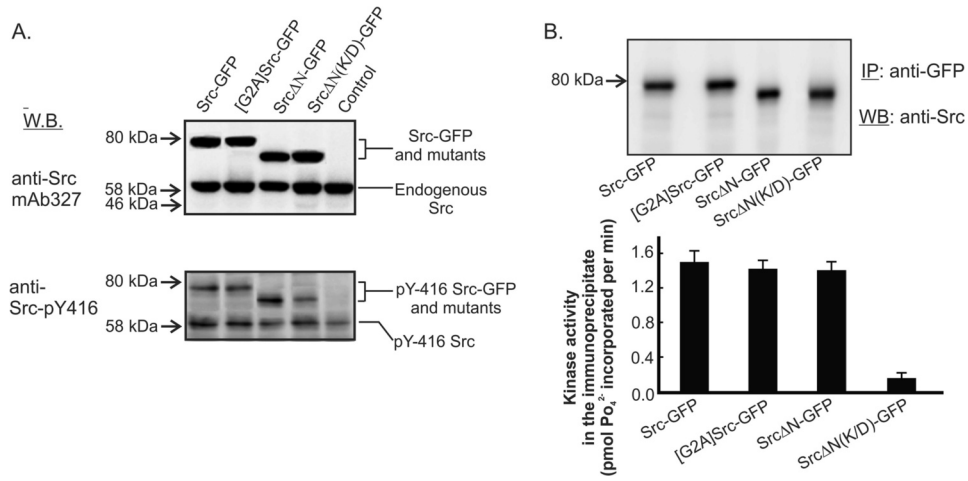

FIGURE 6. **Expression, autophosphorylation levels, and kinase activities of Src-GFP and its mutants in transduced primary cortical neurons.** Primary cortical neurons were transduced with lentivirus directing the expression of Src-GFP, (G2A)Src-GFP, SrcΔN-GFP, and the kinase-dead SrcΔN(K/D)-GFP at DIV 1. Expression of Src-GFP and mutants was induced by doxycycline at DIV 5. *A*, at DIV 7, neurons were collected, and the crude lysates were analyzed by Western blotting (*W.B.*) with anti-Src mAb327 antibody and anti-Src-Tyr(P)-416 antibody, which monitors autophosphorylation of Src family kinases at the consensus site in the activation loop (*e.g.* Tyr-418 of Src or Tyr-424 of neuronal Src). *Control*, untransduced neurons. The kinase-dead SrcΔN(K/D)-GFP mutant was generated by replacement of the conserved Lys-297 (or Lys-303 of neuronal Src) with methionine. *B*, the recombinant Src-GFP and mutants were immunoprecipitated from lysates of the transduced neurons, and the kinase activities associated with the immunoprecipitates were determined. *Error bars*, S.D.

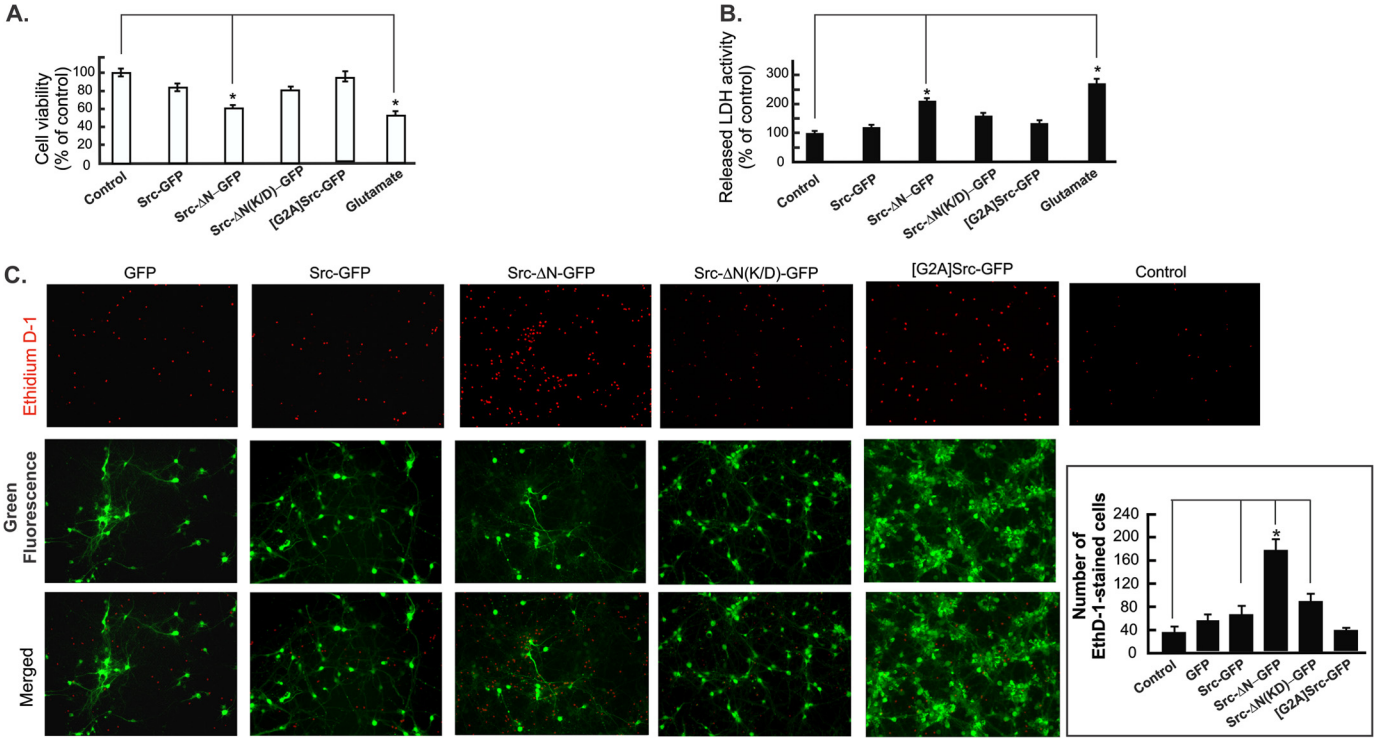

FIGURE 7. **Expression of a truncated Src mutant lacking the myristoylation motif and unique domain induces neuronal death.** Neurons were transduced with lentivirus directing expression of Src-GFP, SrcΔN-GFP, the kinase-dead SrcΔN(K/D)-GFP, and (G2A)Src-GFP mutants at DIV 1. Expression of the recombinant proteins was induced by doxycycline at DIV 5. Effects of their expression were monitored at DIV 7. *A*, MTT assay to monitor the viability of the cultured neurons ($n = 5$; *, $p < 0.05$). The mitochondrial reductase activity of the viable neurons in "Control" is arbitrarily assigned as 100%. *B*, activity of LDH released from the damaged neurons to the cultured medium was monitored as a measure of the extent of neuronal cell death ($n = 5$; *, $p < 0.05$). The activity in control is arbitrarily assigned as 100%. *C*, the effect of expression of Src-GFP, SrcΔN-GFP, SrcΔN(K/D)-GFP, and (G2A)Src-GFP on neuronal survival is monitored by staining with ethidium homodimer-1 (*EthD-1*; *red fluorescence*), which shows the damaged neurons. *Inset*, numbers of ethidium homodimer-1-positive cells in the control and transduced neurons ($n = 5$; *, $p < 0.05$). *Error bars*, S.D.

v-Src mutant to induce cellular transformation of cultured fibroblasts (35, 36). To examine if the cytotoxic activity of the truncated Src fragment is solely due to the removal the myristoyl moiety by calpain, we transduced neurons with the lentivirus that direct the expression of the non-myristoylated (G2A)Src-GFP mutant. As shown in Fig. 6, recombinant (G2A)Src-GFP mutant was expressed at a level similar to that of endogenous Src in neurons, and it was autophosphorylated and exhibited kinase activity. However, in contrast to SrcΔN-GFP, (G2A)Src-GFP does not induce neuronal death (Fig. 7), indicating that removal of just the myristoyl moiety is insufficient to convert Src to a neurotoxic mediator.

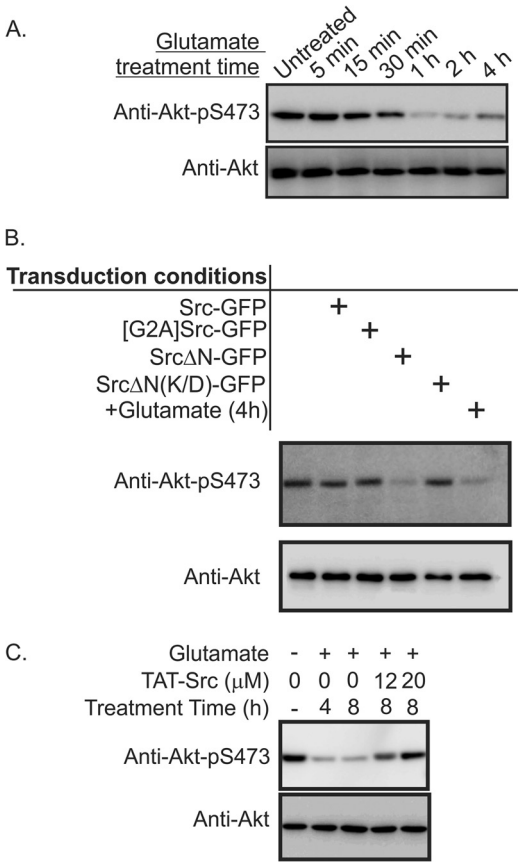

FIGURE 8. **Glutamate overstimulation or expression of the cytotoxic truncated Src fragment induces a significant decrease in Akt phosphorylation at serine 473 in neurons.** *A*, time-dependent changes of Akt phosphorylation in neurons treated with 100 μM glutamate. *B*, effects of expression of Src-GFP, (G2A)Src-GFP mutant, the kinase-dead SrcΔN(K/D)-GFP mutant, and SrcΔN-GFP on neuronal Akt phosphorylation level. Neurons were transduced with the Src-GFP or its mutants at DIV 1 and then induced with doxycycline induction at DIV 5 for 48 h. Crude lysates of the untreated neurons (*Control*) and the transduced neurons were probed with anti-Akt and anti-Akt-Ser(P)-473 antibodies. Akt blots are representative of three separate experiments. *C*, effects of TAT-Src peptide on Akt phosphorylation in glutamate-treated neurons. Crude lysates were prepared from untreated neurons and neurons treated with glutamate for 4 and 8 h in the presence and absence of TAT-Src peptide (12–20 μM). The lysates were probed with anti-Akt and anti-Akt-Ser(P)-473 antibodies.

Akt Inactivation Is Associated with Neuronal Death Caused by Glutamate Treatment and Expression of the Neurotoxic Truncated Src Mutant—The PI3K/Akt signaling pathway is known to maintain neuronal survival. We confirmed earlier findings that glutamate-induced neurotoxicity is associated with reduced phosphorylation at the essential Akt regulatory site (Ser-473 in Akt1; Fig. 8*A*) (37). To test whether calpain-induced Src truncation also caused Akt inactivation, we transduced neurons with GFP fusion proteins of WT-Src, calpain-truncated Src (SrcΔN), and myristoylation signal-mutated Src ((G2A)Src). Fig. 8*C* shows that SrcΔN-GFP, but not Src-GFP and (G2A)Src-GFP, induced a significant reduction of Akt phosphorylation at Ser-473. In addition, the kinase-dead mutant of SrcΔN-GFP (K303M) failed to induce both neurotoxic ability (Fig. 7) and Akt inactivation (Fig. 8*B*). Finally, TAT-Src peptide treatment prevented glutamate-induced inactivation of Akt in neurons (Fig. 8*C*). Together, these data indicate that calpain-induced truncation of Src is a key step in directing neuronal death and inactivation of Akt kinase.

Expression of a Constitutively Active Akt Mutant Reduces the Neurotoxic Action of the Truncated Src Mutant in Neurons—Because the recombinant neurotoxic SrcΔN-GFP inactivates endogenous Akt, we tested whether expression of the constitutively active Akt mutant protects against neuronal death. Luo *et al.* (37) reported that expression of a constitutively active myristoylated mutant of Akt in neurons protects against neuronal death in excitotoxicity. Thus, we generated a lentivirus to direct the expression of myristoylated Akt1 (Myr-Akt) with a myristoylation motif at the N terminus and a hemagglutinin tag at the C terminus. After viral transduction, both SrcΔN-GFP and Myr-Akt were expressed in transduced neurons (Fig. 9*A*) Importantly, Myr-Akt is expressed at a similar level as endogenous Akt. Finally, MTT assay, assay for the activity of LDH released from the damaged neurons, and staining of damaged neurons with ethidium homodimer-1 all revealed that the cell viability of neurons co-expressing Myr-Akt with SrcΔN-GFP is significantly higher than that of neurons expressing SrcΔN-GFP alone (Fig. 9, *B–D*). Our data suggest that the constitutively active Myr-Akt attenuates the neurotoxic effect of SrcΔN-GFP in neurons, and inactivation of Akt is a key step in the neurotoxic pathway of SrcΔN-GFP.

The anti-Akt-Ser(P)-473 blot of the neuronal lysates (Fig. 9*A*) shows that both Myr-Akt and endogenous Akt are phosphorylated at Ser-473 to a similar level regardless of the co-expression of SrcΔN-GFP. However, in neurons expressing SrcΔN-GFP, phosphorylation of endogenous Akt at Ser-473 is much reduced. This data is in agreement with the findings shown in Fig. 8 that neuronal death is associated with reduced phosphorylation of Akt at Ser-473.

DISCUSSION

Overactivated Calpains Target Multiple Protein Substrates to Induce Neuronal Death—Overactivation of calpains contributes to neuronal death in excitotoxicity (38). The overactivated calpains catalyze proteolysis of many cellular proteins, leading to their aberrant regulation or premature degradation (39). In addition to inhibition of calpain cleavage of Src, blocking calpain-mediated cleavage of NCX (sodium-calcium exchanger) (40), STEP (striatal enriched phosphatase) (41), mGluRα-1 (metabotropic glutamate receptor α-1) (11), TRPC6 (transient receptor potential canonical-6) (42), and calcineurin (43, 44) with cell-permeable peptide inhibitors has been documented to provide protection against neuronal death in excitotoxicity. These calpain substrates previously proven to be critical to excitotoxicity can be categorized into two groups. The first group, including NCX, TRPC6, STEP, and mGluRα-1, consists of key signaling proteins in the neuroprotective signaling pathways. Their cleavage by calpain either abolishes their neuroprotective functions or leads to their degradation. The second group contains signaling molecules that acquire the neurotoxic activities upon cleavage by calpain. An example of this group of calpain substrates is the protein phosphatase calcineurin (45–47). Calpain-mediated cleavage irreversibly activates calcineurin by deletion of its autoinhibitory domain. This constitutively active truncated calcineurin induces neuronal death by dephos-

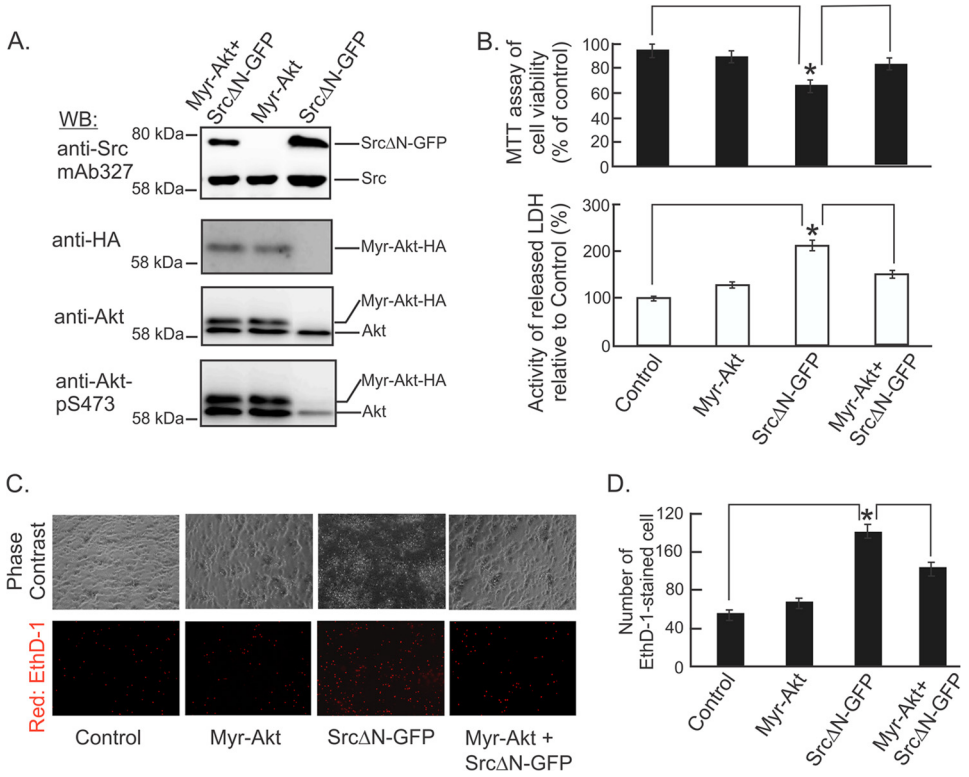

FIGURE 9. **The constitutively active Myr-Akt1 mutant antagonizes the cytotoxic action of SrcΔN-GFP in neurons.** *A*, Western blots (*WB*) of crude lysate of transduced neurons showing the expression of endogenous Src, recombinant SrcΔN-GFP, endogenous Akt and Myr-Akt, and phosphorylation level of Akt and Myr-Akt1 at Ser-473. Because Myr-Akt contains a hemagglutinin tag at its C terminus, its expression was detected by anti-HA blotting. *B*, cell viability of the transduced neurons measured by MTT assay (*top*) and activity of LDH released from the damaged neurons to the cultured medium was monitored as a measure of the extent of neuronal cell death (*bottom*). *C*, phase-contrast and ethidium homodimer-1-stained images of the untreated (*Control*) and the transduced neurons. *D*, number of ethidium homodimer-1-stained cells in the control and transduced neuron (data represented as mean ± S.D. (*error bars*), $n = 5$; *, $p < 0.05$, Student's *t* test).

phorylating BAD, huntingtin, and NFAT (47). Src belongs to the second group of calpain substrates because calpain-mediated cleavage generates the neurotoxic truncated Src fragment capable of directing neuronal death. Future investigation should focus on understanding how the truncated Src fragment and the truncated calcineurin fragment generated by calpain cleavage cooperate to induce neuronal death in excitotoxicity.

The Paradox: Src Contributes to Maintenance of Survival and Death of Neurons—Src plays a key role in maintaining neuronal survival. It mediates the prosurvival action of the thyroid hormone T3 receptor TR1α and glial cell-derived neurotrophic factor receptor by activating PI3K to maintain neuronal survival (3, 4, 32). Furthermore, treatment of cultured cortical neurons with the Src kinase inhibitors PP2 and SU6650 promotes apoptotic cell death (19), supporting the notion that Src kinase activity is critical to maintenance of neuronal survival. In a preliminary study, we transduced primary cortical neurons at DIV 1 with the lentivirus directing expression of Src shRNA to knock down Src expression in neurons. Our data demonstrate that suppression of Src expression induces cell death of the cultured neurons,[4] further supporting the indispensable role of intact Src in maintaining neuronal survival. In light of these findings, our findings presented here therefore reveal a paradox; Src is a promoter of cell survival as well as a mediator of cell death (refer to the model shown in Fig. 10). By acting as the molecular switch converting Src from a cell survival promoter to a neurotoxic enzyme, calpain allows Src to perform these

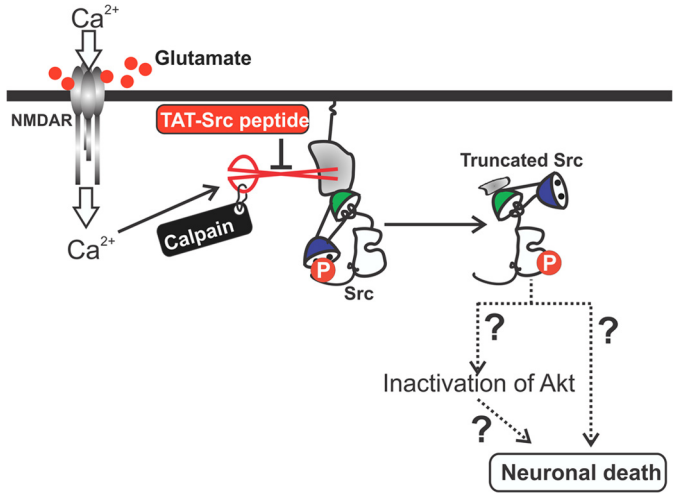

FIGURE 10. **A model depicting the neurotoxic consequence of calpain-mediated cleavage of Src in neurons in excitotoxicity.** The overstimulated NMDA receptor allows massive influx of calcium to overactivate calpain in neurons. The activated calpain cleaves a number of cellular proteins. Calpain-mediated cleavage removes the N-terminal myristoyl group and a significant portion of the unique domain, releasing the 52-kDa truncated Src fragment to the cytosol. This truncated Src fragment facilitates neuronal death in part by inactivating Akt. By blocking calpain cleavage of Src, the TAT-Src peptide alleviates neuronal death induced by overstimulation of glutamate receptors.

paradoxically conflicting functions in neurons. As the cleavage generates the truncated Src fragment residing predominantly in the cytosol, we propose that the truncated Src fragment

exerts its neurotoxic action by phosphorylating specific cellular proteins in cytosol to induce neuronal death.

PI3K maintains neuronal survival by activating Akt (48–50). Recently, Jo *et al.* (51) discovered a cell-permeable small molecule capable of activating Akt in cytosol. Using this compound, they demonstrated in cultured cortical neurons and *in vivo* in a mouse model of stroke that activation of Akt can suppress excitotoxicity and reduces stroke-induced neuronal death (51). In agreement with their findings, we demonstrated that treatment with excess glutamate and expression of the recombinant truncated Src (SrcΔN-GFP) induce a significant decrease in phosphorylation of Akt at Ser-473 in cultured cortical neurons (Fig. 8). Furthermore, the neurotoxic effect of SrcΔN-GFP is significantly reduced by co-expression of the constitutively active Myr-Akt1 mutant in neurons (Fig. 9). Taken together, our results suggest that the truncated Src contributes to neuronal death at least in part by antagonizing the PI3K/Akt prosurvival signaling pathway. Relevant to this, Akt employs the P*XX*P motif near its C terminus to bind to the SH3 domain of Src in both Madin-Darby canine kidney and HEK293 cells. The binding facilitates its phosphorylation by Src and activation by PI3K (52). Schmid and Bohn (53) demonstrated that Src, Akt, and β-arrestin 2 form stable complexes in mouse frontal cortex and cultured mouse cortical neurons, and Src in the complex facilitates Akt activation in response to stimulation of the serotonin receptor 5-HT_{2A}R. Given the significant role played by Akt in maintenance of neuronal survival and our finding that the truncated Src mutant SrcΔN-GFP induces neuronal death and Akt inactivation, future investigation to decipher how calpain cleavage affects the functional interplay between Src and Akt in neurons will shed light on the neurotoxic mechanism of the truncated Src fragment.

Because the kinase-dead K303M mutation abolishes the neurotoxicity of SrcΔN-GFP, it is logical to predict that the 52-kDa truncated Src fragment and SrcΔN-GFP exert their neurotoxic action by phosphorylating specific cellular proteins in neurons. Identifying the cellular proteins that are selectively phosphorylated by the 52-kDa truncated Src fragment and SrcΔN-GFP is an avenue to decipher the neurotoxic mechanism of the truncated Src fragment. Given that the kinase-dead SrcΔN(K/D)-GFP mutant fails to inactivate neuronal Akt, some of these cellular proteins preferentially phosphorylated by the 52-kDa truncated Src fragment and SrcΔN-GFP could be upstream regulators of neuronal Akt.

It is intriguing that (G2A)Src-GFP, albeit lacking the myristoyl group for anchorage to the plasma membrane, is unable to exhibit neurotoxic action when expressed in the cultured cortical neurons. Because the unique domain in this mutant remains intact, our results suggest that the unique domain contains the motifs that constrain the neurotoxic action of Src and that these motifs are removed upon calpain cleavage.

Prevention of Calpain-mediated Cleavage of Src as a Therapeutic Strategy to Reduce Brain Damage in Patients Suffering from Ischemic Stroke—Although Src inhibitors can reduce stroke-induced brain damage in rodent models (16, 17, 54), their use may adversely affect the recovery of brain cells from the damage. Relevant to this, Takadera *et al.* (19) demonstrated that treatment with Src kinase inhibitors induces apoptosis in cultured cortical neurons. Furthermore, Liu *et al.* (55) reported that Src plays a key role in stimulating proliferation of the newborn brain microvascular endothelial cells and perivascular astrocytes, which are essential for resolution of edema and repair of damaged blood brain barrier during the recovery phase of ischemic or hemorrhagic stroke (55). In light of the roles of intact Src in maintaining neuronal survival and repairing the damaged blood brain barrier following stroke, inhibitors suppressing Src kinase activity are not suitable for use to minimize brain damage in patients suffering from stroke because they may adversely affect neuronal survival and the repair of blood brain barrier during the recovery phase of ischemic stroke. Because TAT-Src peptide can alleviate neuronal death by preventing calpain-mediated truncation of Src in neurons (Fig. 4), prevention of calpain cleavage of Src is a better therapeutic strategy than inhibition of Src kinase activity to reduce brain damage in patients suffering from stroke (refer to the model shown in Fig. 10). Calpains play conflicting roles in preventing and initiating cell death. In addition to contributing to neuronal death, calpains are critical for the remodeling process in neural regeneration following stroke (56). Thus, broad spectrum calpain inhibitors are not suitable therapeutics for reducing brain damage in stroke patients because they can inhibit calpains in the neural regenerative process. In light of this, small molecule inhibitors that mimic the TAT-Src peptide to selectively prevent formation of the neurotoxic Src fragment by calpain are better therapeutics for the treatment of stroke patients. Future investigation to elucidate the structural basis of prevention of calpain cleavage of Src by the TAT-Src peptide may benefit the development of these small molecule calpain inhibitors. In light of the failures in recent clinical trials of drugs to protect against brain damage in stroke patients (57, 58), our discoveries illustrate how investigation into the molecular basis of neuronal death in excitotoxicity benefits the search for new molecular targets with the potential to minimize brain damage in ischemic stroke and neurodegenerative diseases.

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