Supplemental Information

A Truncated Fragment of Src Protein Kinase Generated by Calpain-Mediated Cleavage is A Mediator of Neuronal Death in Excitotoxicity

Hossain, M.I. et al.

Supplemental Experimental Procedures

Materials

Materials include Neurobasal medium, DMEM, Opti-MEM reduced serum media, B-27 supplement, GlutaMAX-I, and RPMI 1640 from Gibco (Rockville, MD, USA). Penicillin, streptomycin, glutamate, DMSO, DNAase, trypsin and trypsin inhibitor, L-glutamine, sodium pyruvate, and non-essential amino acid were purchased from Sigma (St Louis, MO). Calpain 1 and calpeptin (calpain inhibitor) from ICN Biomedicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and poly-D-lysine were from Invitrogen (Carlsbad, CA, USA). Lactate Dehydrogenase (LDH) assay kit was from Promega. Two Src-specific monoclonal antibodies (mAbs) used in the studies, mAb327 which recognizes the SH3 domain and mAb(2–17) directed against amino acids 2-17 of Src was purified as previously described (1). The hybridoma producing the mAb327 antibody was a gift from Dr. Joan Brugge. Phospho-Src (pSrc-416) antibody, recognizing the phosphorylated tyrosine-416 of human Src, phosphorylated tyrosine-416 of chicken Src as well as the consensus autophosphorylation site in the activation loop of all other Src-family kinases was purchased from BD Bioscience. The monoclonal anti-Fyn antibody against a fragment containing residues 1-132 of Fyn, was purchased from BD Transduction Laboratories. Antibodies against NMDA receptors (anti-GluN1, anti-GluN2A and anti-GluN2B), Lyn kinase and HA tag were from Cell Signaling Inc. Anti-tubulin antibody was purchased from Abcam. Calpain activity assay kit was from BioVision. Protein A–Sepharose was from Amersham Pharmacia Biotech. Recombinant Src expressed in Spodoptera frugiperda 9 (SF9) cells was purified by sequential chromatography onto an immunoaffinity column and a cation exchange column as described previously (2). NMDA receptor antagonist MK801, the antagonist specific for the GluN2B-containing NMDA receptor ifenprodil and AMPA receptor antagonist CNQX were purchased from Sigma-Aldrich. Lentiviral pLVX-Tight-puro and pLVX-Advance plasmids were purchased from Clontech. The lentivirus packaging plasmid psPAX.2 and envelope plasmid pMD2.G were from Addgene. The HEK293FT cell line for generating the lentivirus, Opti-prep media and Lipofectamine 2000 were purchased from Invitrogen.

Primary cortical neuronal culture

Primary cortical neuronal culture was prepared using mouse embryos collected at Day 15-Day16 of gestation. Briefly, the cortical regions of the embryonic brains were aseptically dissected, free of meninges and dissociated in Suspension Buffer [250 ml of Hanks balanced salt solution (HBSS), 1.94 ml of 150 mM MgSO₄, 0.75 g BSA]. They were then subjected to trypsin digestion.
at 37 °C for 5 min in trypsin digestion buffer (20 ml Suspension Buffer, 80 µl DNase (10 µg/µl), and 4 mg trypsin). To stop the trypsin digestion, dilute trypsin inhibitor buffer [16.8 ml of suspension buffer and 3.2 ml of trypsin inhibitor buffer (20 ml suspension buffer, 80 µl DNase, 200 µl 150 mM MgSO₄ and 10.4 mg trypsin inhibitor)] was added to the cell suspension and centrifuged at 1000×g for 5 min. at 23 °C. The pelleted sample was subjected to mechanical trituration in trypsin inhibitor buffer for 30 seconds. Cells were collected by centrifugation at 1000×g for 5 min at 23 °C. Finally, the cells were re-suspended in Neurobasal medium supplemented with 10% fetal calf serum, 0.25% GlutaMAX-I and 1% penicillin and streptomycin which was pre-warmed to 37 °C. Cells were plated to a density of 5 × 10⁵ cells per well in 24-well plates previously coated with 0.1 mg/ml sterile poly-D-lysine in H₂O. The cultures were maintained at 37 °C in 5% CO₂ and 95% air in a humidified incubator. After 24 h, the initial Neurobasal medium was replaced with the medium containing 2.5 % B-27, 0.25 % GlutaMAX-I and 1 % penicillin and streptomycin. Half of the medium was replaced with fresh medium at day 5. Cells were allowed to grow till the seventh day in culture prior to treatment with glutamate.

Induction of stroke in rats by stereotaxic injection of endothelin-1

Surgical Preparation
All experiments were performed in accordance with the guidelines of the National Health & Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Experimental Purposes, which complies with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. Male Hooded Wistar rats (340 – 360 g) were anesthetized by intraperitoneal injection of ketamine (75 mg/kg)/xylazine (10 mg/kg) and maintained throughout surgery by inhalation of isoflurane (95 % oxygen and 5 % isoflurane) via a stereotaxic nose cone attachment. 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. (3) and described previously (4). The cannula was secured with dental acrylicate cement and a small screw inserted into the skull. The scalp was closed with sutures and the animals housed individually and allowed to recover for 5 days prior to stroke induction.

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 (American Peptide Company; 60 pmol in 3 µl saline over 10 min) via a 30-gauge injector needle that protruded 2 mm beyond the end of the previously implanted guide cannula (4). Rats were placed in a clear plexiglass box for observation during endothelin-1 injection. During endothelin-1 injection, counter-clockwise circling, clenching and dragging of the contralateral forepaw exhibited by the rats validate correct placement of the cannula and stroke induction (4). Stroke severity was scored 3 to 5 based on these responses at the time of stroke, 5 being the most severe. Behavioral changes in response to stroke occurred within 2 to 10 min of the commencement of the endothelin-1 infusion and were observed and rated over 60 min. Rats that continued to circle beyond 60 min following endothelin-1 infusion were ranked as having a stroke rating of 5 (the most severe). Rats that did not display any behavioral change over 60 min were deemed not to have suffered a stroke and were excluded from the study. Sham injected rats underwent cannula implantation but did not receive endothelin-1 injection. Rectal temperatures were taken with a thermistor
probe, prior to stroke and at 30- or 60-min intervals for 3 h after stroke. Rats were decapitated 3 h after stroke induction and the forebrain removed and frozen in liquid nitrogen until further processing. Proteins in the frozen tissue were extracted by homogenization with the ice-cold Lysis Buffer [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 Na3VO4, 50 mM β-glycerophosphate, 0.2 mg/ml benzamidine, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), EDTA-free protease inhibitors and phosphatase inhibitors cocktail (Roche, Indianapolis, IN, USA)]. After centrifugation at 10,000×g for 10 min, supernatant was collected its protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). The supernatant was stored at -20 °C.

Cell viability assay

MTT assay
MTT was dissolved in RPMI medium 1640 without phenol red at a stock concentration of 5 mg/ml and filtered using 0.45 μm to remove insoluble residues as described previously (5) MTT solution equal to 10% (v/v) of the volume of culture medium (0.5 mg/ml final concentration) was added to each well of glutamate treated and untreated cell and the plate was incubated at 37 °C for 30 min. The culture medium was then removed by aspiration and dried for 10 min. An aliquot of 200 μl DMSO was then added to dissolve the formazan formed in each well. From there, 100 μl was transferred to the well of 96-well microtitre plate (Falcon) and the absorbance was measured in triplicate at a wavelength of 570 nm using FLUROstarOptima (BMG) plate reader. Cell viability was compared as a percentage of the untreated cells.

LDH assay
LDH is a stable cytoplasmic enzyme that is released into the cytoplasm upon plasma membrane damage. The LDH assay, therefore, is a measure of membrane integrity. The assay was performed in accordance with the manufacturer’s protocol. Briefly, 50 μl of culture medium from each well of culture plate was transfer to 96 well microtitre plates (Falcon). 100 μl of LDH assay mixture containing equal volume of LDH assay substrate solution, LDH Assay dye solution and LDH assay cofactor was then added to each well. The reaction was allowed to proceed at room temperature for 30 min in the dark. The reaction was stopped by adding 50 μl of acetic acid (1mM). The absorbance was measured in triplicate at a wavelength of 490 nm using FLUROstarOptima (BMG) plate reader. LDH release was compared as a percentage of the untreated control.

Calcein-AM and ethidium homodimer-1 staining
This is a two-color fluorescent assay in which calcein acetoxyethyl (Calcein-AM) and ethidium homodimer-1 (EthD-1) are used to visualize live and damaged neurons, respectively. The disrupted cell membrane of damaged neurons allows entry of EthD-1, which upon binding to nucleic acids, produces red fluorescence (excitation wavelength, ~495 nm; emission wavelength, ~635 nm). The membrane-permeable calcein-AM enters neurons and is converted by the intracellular esterase of live cells to form the intensely fluorescent calcein (excitation wavelength, ~495 nm; emission wavelength, ~515 nm). In brief, primary cortical neurons were cultured in neurobasal media without phenol red. The cultured neurons were treated with either glutamate or glutamate and TAT-Src peptide or TAT-scrambled peptide for different time intervals. Neurons were incubated with 2 μM calcein-AM and 0.5 μM EthD-1 for 30-40 min in incubator. For neurons expressing the GFP-fusion proteins of Src and its mutants, were stained
with EthD-1 only. After staining, the cells were washed twice with neurobasal medium. Live and dead cells were visualized under fluorescence microscope (Leica DMI6000 B) and their numbers were determined.

**Calpain activity assay**

Calpain activity of glutamate treated and untreated neurons were measured using a fluorogenic calpain substrate Ac-Leu-Leu-Tyr-AFC (7-amino-4-trifluoromethyl coumarin). The intact substrate emits blue light ($\lambda_{\text{max}} = 400$ nm). But upon cleavage by calpain, the free AFC emits a yellow-green fluorescence ($\lambda_{\text{max}} = 505$ nm), which can be quantified by fluorometry. The assay was performed according to the manufacturer’s instructions (BioVision). In brief, lysates (with ~100 µg of proteins) from glutamate treated and untreated cortical neurons collected at the time points indicated were diluted in 85 µl extraction buffer. 10 µl of the 10 x calpain activity assay buffer (BioVision) was added. Two microliter of calpain 1 in 85 µl extraction buffer was used as the positive control and the lysates of neurons treated with glutamate in the presence of the calpain inhibitor was used as the negative control. To start the reaction, 5 µl fluorogenic calpain substrate (BioVision) was added to each reaction mixture and incubated at 37 °C for 1 h in the dark. Fluorescence (excitation at 400 nm and emission at 505 nm) was assessed by using FLUOROstarOptima (BMG) plate reader. Calpain activity in the neuronal lysate was presented as percentage of that of the untreated cell lysate.

**Proteomics analysis of recombinant Src and the truncated Src fragments generated in vitro by treatment with calpain 1**

Purified recombinant Src (1-2 µg) was incubated in vitro with purified calpain 1 [1 units, Abcam (ab91019)] for 1 h at 23 °C in the presence and absence of calpeptin (20 µM). Proteins in the reaction mixtures were separated by SDS-PAGE. After staining with Coomassie blue, the gel slices corresponding to intact Src (~60 kDa) and truncated Src fragment (~52 kDa) were excised from the gel. Gel pieces were diced into smaller pieces and destained in 50 mM triethyl ammonium bicarbonate (TEAB) / 50% acetonitrile. The gel pieces were then incubated at room temperature in 200ul of 20mM Tris(2-carboxyethyl)phosphine (TCEP) / 50mM TEAB and then 100mM iodoacetamide / 50mM TEAB to alkylate the protein. The sample was then briefly washed with 200 µl of 50mM TEAB and then dehydrated in 100% acetonitrile for 5 min and then dried further under vacuum for 10 min. The gel was then rehydrated with 40uL of 50 mM TEAB containing 250 ng proteomics grade trypsin (Sigma Chemicals) and incubated overnight at 37°C. Tryptic peptides were separated and analyzed by liquid chromatography-tandem MS (LC-MS/MS) using an Agilent 1100 dual pump, nano LC linked to an Agilent 1100 ion trap XCT Plus mass spectrometer fitted with an Agilent HPLC-Chip CUBE source. Peptides were injected onto a 40 nl Zorbax 300SB-C18 trapping column at 4ul/min, and then separated by switching the trap column inline with the separation column (Zorbaz 300SB-C18, 43mm x 0.075mm) and running an 8 min gradient of 5% to 50% acetonitrile/ 0.1% formic acid at 400 nl/min. The resulting MSMS spectra were analyzed using the Mascot search engine (6). Parameters use were, Enzyme: None and/or Trypsin, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Peptide Mass Tolerance : ± 1 Da, Fragment Mass Tolerance: ± 0.6 Da, Missed Cleavages: 1, Instrument type : ESI-TRAP.
Synthesis and purification of peptides

All synthetic peptides were synthesized by Fmoc [N-(9-fluorenyl)methoxycarbonyl]–based solid phase peptide synthesis chemistry on a CEM Liberty automated microwave peptide synthesizer (North Carolina, USA). At the completion of synthesis, peptides were cleaved from the resin by treatment with TFA/triisopropylsilane/water (95:2.5:2.5) and isolated by precipitation with cold diethyl ether. Crude peptides were purified on an Agilent Zorbax C18 reversed-phase HPLC column in 0.1% TFA/water buffer with a linear acetonitrile gradient. The molecular weight of all peptides was confirmed by electrospray mass spectrometry on an Agilent QTOF LC/MS instrument and peptide purity (>95%) was confirmed by analytical reversed-phase HPLC. For the TAT-Src and TAT-Scramble peptides, Fmoc-6-aminohexanoic acid (Fmoc-ahx) was used in the synthesis. The peptides used in the studies are listed in Table S3.

Lentivirus production

Insertion of the gene of interest into the lentiviral vector pLVX-Tight-puro

The mouse neuronal Src-GFP gene was synthesized by Invitrogen-Geneart and inserted into the pMK-RQ vector. It was subcloned into the lentiviral vector pLVX-Tight-puro (Clontech) at the BamH1 and EcoRI restriction sites. The full-length nSrc is termed as Src-GFP. The truncated Src (without unique domain) is indicated as Src-ΔN-GFP and was also subcloned into the pLVX-Tight-puro vector. The G2A mutation on Src-GFP was done by PCR reaction and K303M mutation for kinase dead version of Src-ΔN-GFP was introduced by site-direct mutagenesis on pLVX-Tight-puro-Src-ΔN-GFP.

The gene encoding the constitutively active Akt1 with a Src myristoylation signal fused to the N-terminus and a HA tag fused to C-terminus (Myr-Akt) was introduced in a pCMV5 vector as described previously (7). BamH1 and EcoRI sites were introduced by PCR reaction using GATCGGATCCGCCACCATGGGGAGCAGCAAGAGCAAG as the forward primer and ACGGAATTCACTAGAAGCGTAATC as the reverse primer. The PCR product containing the Myr-Akt gene was subcloned into the lentiviral vector pLVX-Tight-puro.

Culture of HEK 293FT cell

The HEK293FT cells, used for the production of lentivirus, were grown at 37 °C in DMEM complete media supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acid and 100U/ml penicillin–streptomycin and incubated in the presence of 5 % CO2. Cells were maintained in geneticin (G418) (500 µg/ml). They were passaged when >80 % confluent.

Production of Lentivirus

Lentivirus was generated by transfecting HEK293FT cells with mixture containing three plasmids and lipofectamine 2000. In brief, for generation of the lentivirus, 2.5 µg pMD2.G and 6.5 µg psPAX2 vectors were mixed with 3 µg of pLVX-Tight-puro plasmid containing the gene encoding GFP-fusion protein of Src or Src mutants or active Akt. For generation of the lentivirus directing the expression of the mutant Tet repressor protein (TetR), 2.5 µg pMD2.G and 6.5 µg psPAX2 were mixed with the pLVX-Tet-on-Advance plasmid. Each mixture was diluted in 1.5 ml Opti-MEM reduced serum media and incubated for five minutes. In a 15-ml falcon tube 36 µl Lipofectamine 2000 was mixed gently with 1.5 ml of Opti-MEM reduced serum media. The
plasmid mixtures and diluted lipofectamine 2000 were mixed and incubated for 20 min at room temperature. Each plasmid/lipofectamine 2000 mixture was added to the HEK293FT cells (70-80 % confluance) grown in a 10 cm petri dish and incubated in 5% CO2 at 37 °C overnight. The original medium was replaced with fresh medium 18-20 h after transfection. The supernatant containing the first batch of the lentivirus was collected 24 h after replacement of the medium. This step was repeated and the second batch of lentivirus was collected after 24 h. The two batches of lentivirus were combined and filtered with a 0.22 μ filter. To concentrate the lentivirus, the filtrate was placed in a centrifuge tube containing Opti-prep (~4 ml) at the bottom as the cushion. The sample was centrifuged at 50,000 x g for 2 h using SW32Ti rotor (Beckman-Coutler). After centrifugation, a layer containing the lentiviral particles located between the medium and opti-prep was collected and placed in a 50-ml Falcon tube. Culture media was added to the tube to top up the volume to 50 ml. Second centrifugation was done at 5000 x g overnight at 4 °C. The pellet containing the lentiviral particles was resuspended in 20-150 μl of ice-cold PBS (pH 7.4) and stored as 10 μl aliquots at –80 °C.

**Transduction of primary cortical neuron with lentivirus**

Cultured primary cortical neurons at Day 1 in culture (DIV 1) were transduced with two types of lentivirus: (i) the lentivirus generated with the pLVX-Tet-on Advance plasmid and (ii) the lentivirus generated with the pLVX-Tight-puro plasmid containing the gene encoding the GFP-fusion protein of Src or its mutants or active Akt. After incubation at 37 °C in the presence of 5% CO2 overnight, the culture medium was replaced with fresh medium. Expression of proteins was induced by the addition of doxycycline (1 mg/ml) at DIV 5. Experiments to monitor the effect of the recombinant proteins were conducted 48 h (i.e. at DIV 7) after doxycycline induction.

**Lysis of cells**

Cells were lysed in 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 Na3VO4, 50 mM β-glycerolphosphate, 0.2 mg/ml benzamidine, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), EDTA-free protease inhibitors and phosphatase inhibitors cocktail (Roche, Indianapolis, IN, USA). After centrifugation at 10,000×g for 10 min, supernatant was collected and protein concentration was determined using Bradford assay (Bio-Rad, Hercules,CA, USA). The supernatant was stored at –20 °C.

**Preparation of neuronal cell lysates for Western blotting**

Equal amount of protein was loaded in each well of 10% SDS-PAGE gel and separated using running buffer [25 mM Tris-HCl, 192 mM glycine, 10 % (w/v) SDS] for approximately 1.2 h at 150 V, and then transferred onto PVDF membrane. The membrane was then blocked with 5% (w/v) non-fat dry milk in Tris buffered saline with Tween20 (TBST) (0.2 M Tris–HCl, pH 7.4, 1.5 M NaCl, and 0.1 % Tween20). After blocking and washing with TBST, the membrane was probed with the primary antibody overnight. The membrane was again washed with TBST three times before probing with horseradish peroxidase-conjugated secondary antibodies (Chemicon, Australia) for 1 h at 25 °C. Protein bands were visualized using chemiluminescence (ECL,
Amersham Biosciences) according to manufacturer’s instruction. Images were taken using Fuji film Las-3000.

**In vitro** calpain cleavage of recombinant Src

Recombinant Src (100 ng) was digested with calpain 1 (Abcam) at different concentrations in calpain digestion buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT, 30 mM NaCl, 10 mM CaCl₂). The reaction mixture was incubated at 30 °C for 45 min. After digestion reaction was stopped by adding 5×SDS sample buffer, resolved onto a 10 % SDS-PAGE, and transferred to polyvinylidene difluoride for immunoblotting.

**Immunoprecipitation**

For immunoprecipitation, ~5 × 10⁵ primary cortical cells were used. In brief, cells were washed with 1 x PBS (ice-cold) and then lysed with lysis buffer. Total protein concentration was determined using Bradford reagent. Crude lysate containing ~500 µg of total proteins supernatant was incubated overnight with either 2.5 µg anti-Src mAb327 antibody or anti-GFP antibody at 4 °C. Immune complexes were collected using 50 µl of Protein A-Sepharose after 4 h of incubation at 4 °C. The immune complexes immobilized on the Protein A-Sepharose were washed three times with wash buffer prior to analysis with kinase activity assay and Western blotting.

**Kinase activity assay**

For Src kinase activity assay immune complexes from control, glutamate treated and Src-GFP and Src-ΔN-GFP induced neuronal cell lysates were suspended in 25 mM HEPES buffer (pH 7.4). The assay involves a specific peptide substrate of SFK (the Src-optimal peptide: AEEEA YGEAEAKKKK) which is selectively phosphorylated by SFKs. The assay was carried out at 30°C for 30 min in a 25µl volume containing the assay buffer (20 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 1 mM MnCl₂, and 50 µM Na₃VO₄), with 25 µM [γ⁻³²P] ATP and 50 µM Src optimal peptide substrate and either equal amount of protein from control or glutamate treated cell lysates from different time points. Phosphorylation of the peptide was allowed to proceed at 30 °C for 30 min. The reaction was then stopped by the addition of 20 µl of 50 % (v/v) acetic acid. An aliquot of the reaction mixture was spotted to the filter paper square. The filter papers were processed as described previously (8). Radioactivity incorporated in the filter paper square was a measure of kinase activity.

**Subcellular fractionation**

Subcellular fractionation of primary cortical neuronal cells was performed based on the method described by Bernocco et al (9). Neuronal cells were first washed with ice cold PBS. Buffer A (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 5 mM EDTA, 0.015 % digitonin and protease and phosphatase inhibitor cocktail (Roche) was added to the attached neuronal cells and gently agitated for 30 min at 4 °C. Digitonin in the buffer creates pores in the plasma membrane by solubilizing cholesterol, releasing the cytosolic proteins without rupturing
the plasma membrane. After incubation for 30 min, the supernatant corresponding to the cytosolic fraction was collected. Care was taken not to perturb the attached permeabilized neurons. The attached permeabilized neurons were incubated with buffer B (10 mM PIPES pH 7.4, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 3 mM EDTA, 0.5 % Triton X-100) for 30 min at 4 °C. The mixture was centrifuged at 8400×g for 10 min. The supernatant was collected as plasma membrane rich proteins.
### Supplemental Tables

**Table S1** Parameters of the peptide fragments derived from tryptic digestion of intact Src identified by LC-MS/MS. The peptides in red fonts were not observed in the tryptic digest derived from truncated Src. It is noteworthy that LFGGFNSSDTVTSPQR is located in the region not covered by the identified tryptic peptides derived from truncated Src (Refer to Table S2).

| Sequences of peptides derived from intact Src | precursor m/z | peptide mass | peptide charge | calculated peptide mass | mass delta | peptide score | peptide expect value |
|-----------------------------------------------|---------------|--------------|----------------|-------------------------|------------|--------------|---------------------|
| GSLLDFLK                                      | 446.82        | 891.6254     | 2              | 891.5066                | 0.1189     | 56.16        | 0.08                |
| LDSGGFYIYTSR                                  | 608.51        | 1215.0054    | 2              | 1214.5932               | 0.4123     | 100.46       | 3.20E-06            |
| WTAPEAALYGR                                   | 617.99        | 1233.9654    | 2              | 1233.6142               | 0.3513     | 86.71        | 7.40E-05            |
| SLQQLVAYYSK                                   | 650.72        | 1299.4254    | 2              | 1298.687                | 0.7384     | 74.14        | 0.0013              |
| TQFNSLQQLVAY                                  | 706.59        | 1411.1654    | 2              | 1410.7143               | 0.4511     | 82.81        | 0.00018             |
| SPEAFLEAQVM(ox)K                              | 747.23        | 1492.4454    | 2              | 1492.7232               | -0.2777    | 36.84        | 15                  |
| LFGGFNSSDTVTSPQR                              | 857.31        | 1712.6054    | 2              | 1711.8166               | 0.7889     | 101.77       | 5.10E-06            |
| LQIVNTEGDWWLAI                                | 898.51        | 1795.0054    | 2              | 1794.8689               | 0.1365     | 62.62        | 0.02                |
| SDVWSFGLTTELTTK                               | 905.14        | 1808.2654    | 2              | 1808.956                | -0.6906    | 92.31        | 2.10E-05            |
| AGPLAGGVTFVALDYESR                            | 1044.19       | 2086.3654    | 2              | 2086.0371               | 0.3283     | 115.54       | 1.10E-07            |
| LPQLVDMAAQIASGMAYVER                          | 721.82        | 2162.4382    | 3              | 2162.0864               | 0.3518     | 24.13        | 2.80E+02            |

**Table S2** Parameters of the peptide fragments derived from tryptic digestion of the truncated Src identified by LC-MS/MS.

| Sequences of peptides derived from truncated Src | precursor m/z | peptide mass | peptide charge | calculated peptide mass | mass delta | peptide score | peptide expect value |
|------------------------------------------------|---------------|--------------|----------------|-------------------------|------------|--------------|---------------------|
| GSLLDFLK                                      | 447.18        | 892.3454     | 2              | 891.5066                | 0.8389     | 60.58        | 0.01                |
| WTAPEAALYGR                                   | 618.15        | 1234.2854    | 2              | 1233.6142               | 0.6713     | 79.34        | 0.00014             |
| SLQQLVAYYSK                                   | 650.56        | 1299.1054    | 2              | 1298.687                | 0.4184     | 72.47        | 0.00069             |
| TQFNSLQQLVAY                                  | 706.6         | 1411.1854    | 2              | 1410.7143               | 0.4711     | 65.6         | 0.0033              |
| SPEAFLEAQVM(ox)K                              | 746.87        | 1491.7254    | 2              | 1492.7232               | -0.9977    | 29.26        | 15                  |
| TQFNSLQQLVAYYSK                               | 895.92        | 1789.8254    | 2              | 1788.9046               | 0.9208     | 116.84       | 2.70E-08            |
| SDVWSFGLTTELTTK                               | 905.66        | 1809.3054    | 2              | 1808.956                | 0.3494     | 108.12       | 2.00E-07            |
| AGPLAGGVTFVALDYESR                            | 1044.19       | 2086.3654    | 2              | 2086.0371               | 0.3283     | 132.56       | 7.40E-10            |
| LPQLVDMAAQIASGMAYVER                          | 722.02        | 2163.0382    | 3              | 2162.0864               | 0.9518     | 33.92        | 10                  |
| LVQLYAYVSEPIYIVYTEMSK                         | 859.1         | 2574.2782    | 3              | 2573.3338               | 0.9443     | 23.85        | 1.10E+02            |
Table S3 Synthetic peptides used to define the calpain cleavage site in Src and block calpain-mediated cleavage of Src in neurons. Ahx: 6-aminohexanoic acid as a linker connecting the Tat protein transduction domain motif (GRKKRRQRRRPQ) to the Src(49-79) segment or the scrambled fragment with the same amino acid composition as the Src(49-79) segment.

| Name                  | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| Src(1-20)             | MGSNKSKPKDASQRRRSLEP                                                     |
| Src(20-40)            | PAENVHGAGGAFPASQTPSK                                                     |
| Src(40-49)            | KPASADGHRC                                                              |
| Src(49-79)            | GRSAAFAPAAAEPKLFGNFSSDTVTSPQRA                                           |
| Tat-ahx-Src(49-79)    | GRKKRRQRRRPQ-ahx-GRSAAFAPAAAEPKLFGNFSSDTVTSPQRA                          |
| Tat-ahx-scrambled     | GRKKRRQRRRPQ-ahx-GSFESFSKANALADAQPRPTAQPFPFTAGSGA                       |
Supplemental Figures

Fig. S1 Effects of antagonist of AMPA- and NMDA-receptors, and calpain inhibitor on calpain-mediated truncation of Src in neurons over-stimulated with glutamate

A. Neurons were pretreated with 40 μM of CNQX (AMPA/Kainate receptor antagonist), 20 μM MK801 (NMDA receptor antagonist), or calpain inhibitor calpeptin (20 μM) for 30 min prior to stimulation with 100 μM glutamate. B. Neurons were pretreated with 40 μM of CNQX, 20 μM MK801, or 20 μM of ifenprodil (antagonist of GluN2B-containing NMDA receptor) for 30 min prior to stimulation with 100 μM of glutamate.
Fig. S2 Distribution of intact and truncated Src in the cytosol and plasma membrane of neurons subjected to treatment with glutamate at different time points
Western blot analysis of cytosolic fraction (panel A) and plasma membrane fraction (panel B) of the lysates of neurons subjected to treatment with glutamate (100 μM) for varying times with anti-Src mAb327 antibody. GAPDH and Na⁺/K⁺ ATPase were used as loading controls of cytosol and plasma membrane. Panels C and D. Time-dependent changes of intact and truncated Src (expressed as densitometric units). Concentration of calpeptin used: 20 μM.
Fig. S3  Proteomics analysis of tryptic fragments derived from intact Src and the 52 kDa truncated Src generated by calpain 1-catalyzed proteolysis of Src

A. SDS-PAGE of reaction mixtures of in vitro proteolysis of recombinant Src by calpain 1 in the presence and absence of Calpeptin. The bands corresponding to intact Src (~60 kDa) and truncated Src (~52 kDa) were excised for proteomics analysis. B. Sequences of identified tryptic fragments generated from intact Src and truncated Src fragment. C. Sequence coverage of identified tryptic fragments generated from intact and truncated Src. The parameters of the tryptic fragments identified are presented in Table S1 and S2. In panels B and C, the three peptides in red fonts were found in the tryptic digest of intact Src but not in the digest derived from the truncated Src. In panel C, the identified peptide fragments derived from both the intact and truncated Src are in bold and underlined.
Fig. S4 Time dependent changes in activity and autophosphorylation at Tyr-416 of intact Src in neurons treated with glutamate for varying periods

A. Kinase activity of Src immunoprecipitated by the anti-Src mAb327 antibody. The kinase activity was monitored by measuring the rate of phosphorylation of the Src-optimal peptide (data are presented as mean ± SD, n = 3, *p ≤ 0.006; Student’s t-test). B. Even though both intact and truncated Src exist in lysates of neurons treated with glutamate, anti-Src mAb327 can only immunoprecipitate intact Src (upper panel). The extent of autophosphorylation at Tyr-416 was monitored by Western blotting with pY416 antibody. C. Ratio of densitometric units of the anti-pY416 signal to the densitometric units of anti-Src signal (data are presented as mean ± SD, n = 3).
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Author/s:
Hossain, MI; Roulston, CL; Kamaruddin, MA; Chu, PWY; Ng, DCH; Dusting, GJ; Bjorge, JD; Williamson, NA; Fujita, DJ; Cheung, SN; Chan, TO; Hill, AF; Cheng, H-C

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