

# Regulation of c-Src Activity in Glutamate-induced Neurodegeneration\*

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c-Src is heavily expressed in the brain and in human neural tissues. Our pursuit for characterization of the neuroprotective mechanisms of tocotrienols led to the first evidence demonstrating that rapid c-Src activation plays a central role in executing glutamate-induced neurodegeneration. It is now known that Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. Here, we sought to examine the mechanisms that regulate inducible c-Src activity in glutamate-challenged HT4 neural cells and primary cortical neurons. Knockdown of c-Src protected cells against glutamate-induced loss of viability. Consistently, microinjection of siRNA against c-Src protected cells against glutamate. Using overexpression and knockdown approaches, we noted that SHP-1 may be implicated in glutamate-induced c-Src activation. Following such activation, Cbp and caveolin-1 were phosphorylated and associated with Csk. Csk was translocated to the membrane where it down-regulated glutamate-induced c-Src activity by catalyzing the inhibitory phosphorylation of a tyrosine residue in c-Src. Findings of this study present a new paradigm that addresses the regulation of c-Src under neurodegenerative conditions.

c-Src is the product of the Src gene and has been found both overexpressed and highly activated in a number of human cancers. The relationship between c-Src activation and cancer progression is significant. Furthermore, c-Src is thought to play a role in the acquisition of the invasive and metastatic phenotype. Thus, c-Src represents a major focus in cancer research (1). The Src protein was the first tyrosine kinase to be discovered. Today, tyrosine kinase-dependent signaling has been implicated in a wide variety of pathways that regulate mammalian cell function. Beyond cancer, c-Src is now known to regulate several aspects of cellular function including osteoclastic bone resorption (2) and gap junction (3). c-Src is heavily expressed in the brain (4) and in human neural tissues (5). Differentiating rodent neurons express high levels of c-Src. In neurons and astrocytes, c-Src is present at 15–20 times higher levels than that found in fibroblasts. The specific activity of the c-Src protein from neuronal cultures is 6–12 times higher than that from the astrocyte

cultures, suggesting a key function of this protein in neurons (6). Initially, c-Src was identified as being important in growth cone-mediated neurite extension and synaptic plasticity (7) and in neuronal differentiation (8). Targeted disruption of c-Src, however, did not cause any abnormality in the brain (4). Our pursuit for characterization of the neuroprotective mechanisms of tocotrienols led to the first evidence demonstrating that rapid c-Src activation plays a central role in executing glutamate-induced neurodegeneration (9, 10). Consistently, it was demonstrated in a subsequent report that Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke (11). Further support of our claim that c-Src is a key player in neurodegeneration is provided by the observation that the Src family kinase inhibitor PP2<sup>2</sup> reduces focal ischemic brain injury (12).

Glutamate toxicity is a major contributor to neurodegeneration. It includes excitotoxicity and an oxidative stress component also known as oxytosis (13, 14). Oxytosis is thought to be a component of excitotoxicity in mature neurons *in vivo* (14). Murine HT hippocampal neural cells lacking intrinsic excitotoxicity pathways have been used as standard models to characterize the oxidant-dependent component of glutamate toxicity (15–19). Our studies on glutamate-induced death of HT4 neural cells led to the first observation that inducible c-Src activity is implicated in neurodegeneration (10). In this study we sought to examine the mechanisms that regulate inducible c-Src activity in glutamate-challenged HT4 neural cells.

## EXPERIMENTAL PROCEDURES

**Materials**—The following materials were obtained from the source indicated: L-glutamic acid monosodium salt; dimethyl sulfoxide (Sigma); and PP2 and PP3 (EMD Biosciences, San Diego, CA). For cell culture, Dulbecco's modified Eagle's medium, fetal calf serum, and penicillin and streptomycin were purchased from Invitrogen. Culture dishes were obtained from Nunc (Denmark).

**Cell Culture**—Mouse hippocampal HT4 neural cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> as described previously (10, 20).

**Primary Cortical Neurons**—Cells were isolated from the cerebral cortex of rat fetuses (Sprague-Dawley, day 17 of gestation)

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<sup>2</sup> The abbreviations used are: PP, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; LDH, lactate dehydrogenase; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CA, constitutively activated; PBS, phosphate-buffered saline; SH, Src homology; Cbp, Csk-binding protein; Csk, C-terminal Src kinase.

as described previously (20, 21). After isolation from the brain, the cells were counted and seeded on culture plates at a density of  $1.5\text{--}2 \times 10^6$  cells/well of 12-well plates (22). The cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum,  $40 \mu\text{M}$  cystine, and antibiotics ( $100 \mu\text{g/ml}$  streptomycin,  $100 \text{ units/ml}$  penicillin, and  $0.25 \mu\text{g/ml}$  amphotericin). Cultures were maintained at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  and 95% air in a humidified incubator. All of the experiments were carried out 24 h after plating (20, 21).

**Glutamate Treatment**—Immediately before experiments, the culture medium was replaced with fresh medium supplemented with serum and antibiotics. Glutamate ( $10 \text{ mM}$ ) was added to the medium as an aqueous solution (10, 23, 24).

**Cell Viability**—The viability of cells in culture was assessed by measuring the leakage of lactate dehydrogenase (LDH) (23) from cells to medium 18–24 h following glutamate treatment using the *in vitro* toxicology assay kit from Sigma. The protocol has been described in detail in previous reports (23). In brief, LDH leakage was determined using the following equation: % of total LDH leaked = (LDH activity in the cell culture media/total LDH activity), where total LDH activity = LDH activity in cell monolayer + LDH activity of detached cells + LDH activity in the cell culture medium (10, 20, 21, 25).

**Microinjection**—The cells ( $0.1 \times 10^6$ /plate) were grown in 35-mm plates for microinjection 24 h prior to injection. As previously described (21), microinjection was performed using a micromanipulator Femtojet B 5247 and Injectman NI 2 (Eppendorf, Hamburg, Germany) with 80 hPa pressure and 0.2 s of time. The compensation pressure during injection was 40 hPa. The glass micropipettes (Sterile femtotip I, Eppendorf) used for injection were with  $0.5\text{-}\mu\text{m}$  inner and  $1\text{-}\mu\text{m}$  outer diameter. As indicated in the respective figure legend, control siRNA and Src siRNA were co-injected with QDot streptavidin conjugate with the emission maximum near 605 nm (Invitrogen). QDot-streptavidin conjugate was used as a fluorescent marker to recognize injection site. After 6 h of injection, the cells were treated with glutamate for 24 h. Calcein AM (Invitrogen) was used for 15 min to stain live cells. Digital images were collected using a specialized phase contrast as well as fluorescent Zeiss Axiovert 200M microscope suited for imaging cells growing in routine culture plates. The sample stage was maintained at  $37^\circ\text{C}$ , and the sample gas environment was maintained exactly as in the culture incubator (21).

**siRNA Delivery and Analysis of Genes and Proteins**—HT4 cells ( $0.1 \times 10^6$  cells/well in 12-well plate) or primary cortical neurons ( $1.5\text{--}2 \times 10^6$  cells/well in 12-well plate) were seeded in antibiotic-free medium 24 h prior to transfection. DharmaFECT<sup>TM</sup> 1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO) was used to transfect cells with  $100 \text{ nM}$  siRNA pool (Dharmacon RNA Technologies) for 72 h as described previously (25). For controls, siControl nontargeting siRNA pool (mixture of 4 siRNA, designed to have  $\geq 4$  mismatches with the corresponding gene) was used. HT4 cells were harvested and seeded for treatment with glutamate as indicated in the respective figure legends. After 12 h of seeding, the culture medium was changed, and the cells were treated as described in the respective figure legends. For quantification of mRNA and protein expression, the samples were collected after 72 h

of siRNA transfection. For the primary neurons, the media were changed, and the neurons were treated with glutamate after 72 h of transfection. Total RNA was isolated from cells using the Absolutely RNA<sup>®</sup> Miniprep kit (Stratagene, La Jolla, CA). The abundance of mRNA for c-Src, Csk, and SHP-1 were quantified using real time PCR using SYBR green-I. The following primer sets were used: m\_GAPDH F, 5'-ATG ACC ACA GTC CAT GCC ATC ACT-3'; m\_GAPDH R, 5'-TGT TGA AGT CGC AGG AGA CAA CCT-3'; m\_c-Src F, 5'-TCC ACA CCT CTC CGA AGC AA-3'; m\_c-Src R, 5'-CAT GCT GAT GGC CTG TGT CA-3'; m\_CSK F, 5'-AAG GGG GAG TTT GGA GAT GTG A-3'; m\_CSK R, 5'-AAT CAC ACC CAG CAG CTG GA-3'; m\_SHP-1 F, 5'-GGG CAC CAT CAT CGT CAT TGA TAT-3'; m\_SHP-1 R, 5'-CAG CGG GAG GGT ACG TGA TAT T-3'; r\_GAPDH F, 5'-TAT GAC TCT ACC CAC GGC AAG TTC A-3'; r\_GAPDH R, 5'-CAG TGG ATG CAG GGA TGA TGT TCT-3'; r\_SHP-1 F, 5'-CAG GGT CAC GCA CAT CAA GGT TAT-3'; and r\_SHP-1 R, 5'-CCG AGT GGC ATA GTA AGG CTG C-3'.

After protein extraction, the protein concentrations were determined using BCA protein reagents. The samples ( $20\text{--}40 \mu\text{g}$  of protein/lane) were separated on a 10% SDS-polyacrylamide gel electrophoresis and probed with anti-c-Src (Upstate Biotechnology, Inc.) or anti-Csk (BD Bioscience, San Jose, CA) antibodies. To evaluate the loading efficiency, the membranes were probed with anti- $\beta$ -actin antibody.

**Overexpression of Src in SHP-1 Knockdown Cells**—Transfection of cells with siRNA was performed as described above. Following siRNA delivery, the cells were transfected with a eukaryotic expression vector containing mouse Src (wild type or constitutively activated) cDNA under the control of a cytomegalovirus promoter (Upstate Biotechnology, Inc.) as reported previously (10). The kinase-activating (constitutively activated (CA)) mutation (*srcY529F*) is a substitution of phenylalanine for tyrosine at position 529. The kinase-inactivating mutation (*srcK297R*) is a substitution of arginine for lysine at position 297 (10). Lipofectamine 2000 (Invitrogen) was used to carry out the transfection. After 24 h of Lipofectamine treatment, the transfection reagent was replaced with regular cell culture medium. The cells were maintained in regular culture condition for 24 h to allow for protein expression. At this point, the cells were harvested and seeded for treatment with glutamate. After 8 h of seeding, the culture medium was changed, and the cells were treated as described in the legend to Fig. 10.

**SHP-1 Overexpression**—Wild type SHP-1 cDNA and SHP-1CS (catalytically inactive SHP-1 variant) in pSVL vector were obtained from Dr. R. Siraganian. Following 18 h of seeding, HT4 cells were transfected with plasmid empty pcDNA3.1, SHP-1, and SHP-1CS using Lipofectamine 2000 transfection reagent (Invitrogen) as described previously (20, 25). After 3 h of Lipofectamine treatment, the transfection reagent was replaced with regular cell culture medium. The cells were maintained in regular culture condition for 24 h to allow for protein expression. At this point, the cells were harvested and seeded for treatment glutamate. After 12 h of seeding, the culture medium was changed, and the cells were treated as described in the figure legend. To assess the level of SHP-1 expression, HT4 cells were harvested 24 h after transfection, seeded for 12 h, and

## c-Src in Neurodegeneration

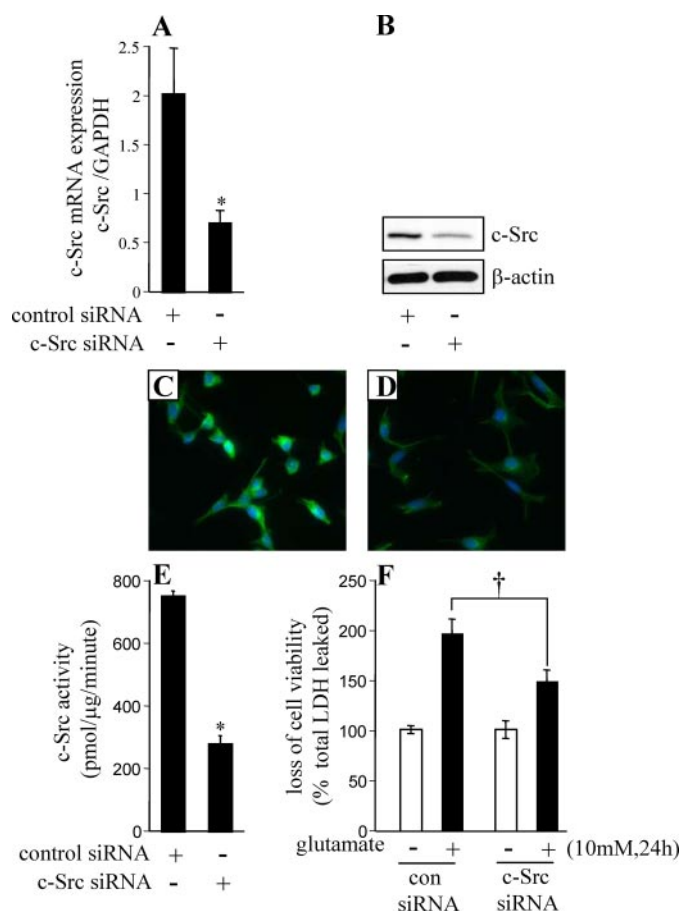
collected for analysis of protein expression by Western blot. The protein concentrations were determined using BCA protein reagents. The samples (20  $\mu\text{g}$  of protein/lane) were electrophoresed on a 10% SDS-polyacrylamide gel and probed with anti-SHP-1 (Upstate Cell Signaling Solutions, Lake Placid, NY). To evaluate the loading efficiency, the membranes were stripped and reprobed with anti- $\beta$ -actin antibody (Sigma).

**Immunoprecipitation and Immunoblots**—For immunoprecipitation, HT4 cells ( $0.5 \times 10^6$  cells/well) were seeded in six-well plates. To inhibit protein-tyrosine phosphatase activity, the cells were treated with 0.2 mM sodium orthovanadate (Sigma) for 10 min. Next, the cells were either treated or not with PP2 or PP3 followed by challenge with glutamate for different time interval under standard culture conditions as indicated in the respective figure legends. During harvest, the cells were washed with ice-cold phosphate-buffered saline (pH 7.4) and lysed with 0.20 ml of lysis buffer (Cell Signaling Technology, Inc. Beverly, MA). Protein concentration was determined using the BCA protein assay kit. The cell lysates (500  $\mu\text{g}$ ) were incubated with 1  $\mu\text{g}$  of immunoprecipitating antibody for overnight at 4 °C and then incubated at 4 °C with 20  $\mu\text{l}$  of anti-rabbit IgG beads (TrueBlot Ig IP beads; eBioscience). Immunoprecipitated complexes were washed four times with lysis buffer (centrifugation at  $1000 \times g$  at 4 °C for 5 min), recovered in 40  $\mu\text{l}$  of 2 $\times$  Laemmli buffer with 50 mM of fresh dithiothreitol and boiled for 10 min. Next, equal volume of samples was loaded onto SDS-PAGE gel and immunoblot.

**Src Kinase Activity**—Kinase activity was performed using the KineActive Src assay kit (Active Motif, Carlsbad, CA). Twenty  $\mu\text{g}$  of total protein was used per sample to measure enzyme activity.

**Immunocytochemistry**—The cells ( $0.5 \times 10^6$ /plate) were seeded in 35-mm plates 24 h before being challenged by glutamate. To inhibit protein-tyrosine phosphatase activity, the cells were treated with 0.2 mM sodium orthovanadate (Sigma) for 10 min and then activated with glutamate for 30 min. The cells were washed with ice-cold PBS thrice and then fixed in 10% buffered formalin for 20 min. Next, the cells were washed thrice with PBS followed by permeabilization using 0.1% Triton X-100/PBS for 15 min. Next, the cells were washed thrice with PBS and incubated with 10% goat serum (Vector Laboratories) for 1 h at room temperature. The cells were then incubated with phospho-caveolin-1 (1:100, in 10% goat serum) overnight in 4 °C. The cells were washed with PBS thrice and then incubated caveolin-1 (1:100, in 10% goat serum) for 2 h in 4 °C. After incubation with primary antibodies, the cells were washed with PBS thrice and incubated with an Alexa-fluor rabbit (red) for caveolin-1 and Alexa-fluor mouse (green) for phospho-caveolin-1 for 1 h at room temperature. After three washes with PBS and incubation with 4',6'-diamino-2-phenylindole (1:10,000) for 2 min, the cells were washed with PBS and mounted in gel mount (aqueous mount; Vector Laboratories) for microscopic imaging.

**Total Membrane Preparation**—Cells ( $2 \times 10^6$ /plate) were seeded in 100-mm plates for membrane isolations. The cells (five plates/sample) were harvested for total membrane preparation. Total membranes were prepared as described previously (20). After washing with ice-cold PBS, the cells were harvested by scraping. The samples were spun at  $700 \times g$  for 10 min at



**FIGURE 1. c-Src knockdown attenuated glutamate-induced cell death.** Transfection with c-Src siRNA decreased c-Src mRNA (A) and protein (B) expression. B, Western blot. C and D, immunocytochemistry. Blue, 4',6'-diamino-2-phenylindole-stained nuclei; green, c-Src protein. C, cells transfected with control siRNA. D, cells transfected with control c-Src siRNA. E, c-Src activity. F, cells with c-Src knockdown were more resistant to glutamate-induced loss of cell viability. †, lower than glutamate control siRNA. The results are the means  $\pm$  S.D.  $p < 0.05$ .

4 °C. Buffer (10 ml) containing 20 mM NaHEPES (pH 7.4), 250 mM sucrose, 2 mM EGTA, 1 mM sodium azide, 100  $\mu\text{M}$  phenylmethylsulfonyl fluoride, and 1  $\mu\text{M}$  protease inhibitor mixture (Sigma) was added to the cell pellet. The samples were homogenized using a motor-driven homogenizer (15 strokes) at 4 °C. The samples were then spun at  $760 \times g$  for 3 min at 4 °C. After centrifugation, the supernatant was collected and spun at  $190,000 \times g$  for 1 h at 4 °C. The resulting total membrane pellet was resuspended in the above-mentioned buffer, and the samples were stored at 80 °C. The protein concentrations were determined using BCA protein reagents (20).

**Statistical Analyses**—The data are reported as the means  $\pm$  S.D. of at least three experiments. Difference between two means was tested by Student's *t* test. Comparisons between multiple groups were made by analysis of variance.  $p < 0.05$  was considered statistically significant.

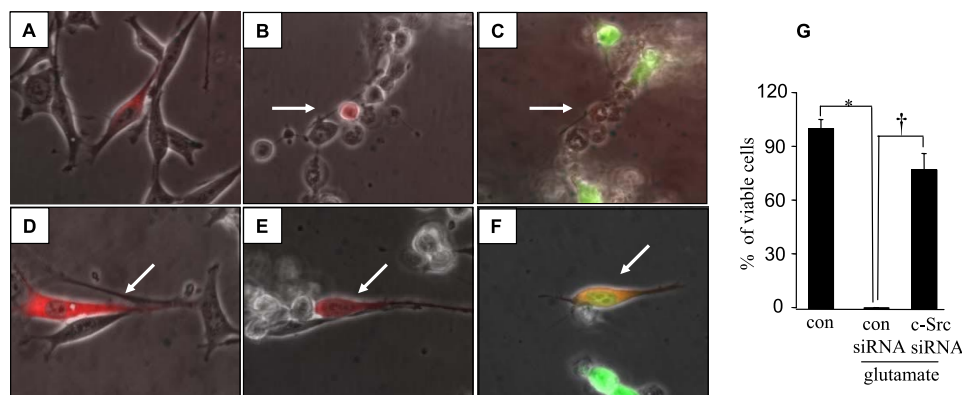
## RESULTS

Our previous claims implicating c-Src activity in glutamate-induced death of HT4 cells was largely based on studies using pharmacological inhibitors (10). Pharmacological inhibitors are often known to suffer from a lack of specificity. To address

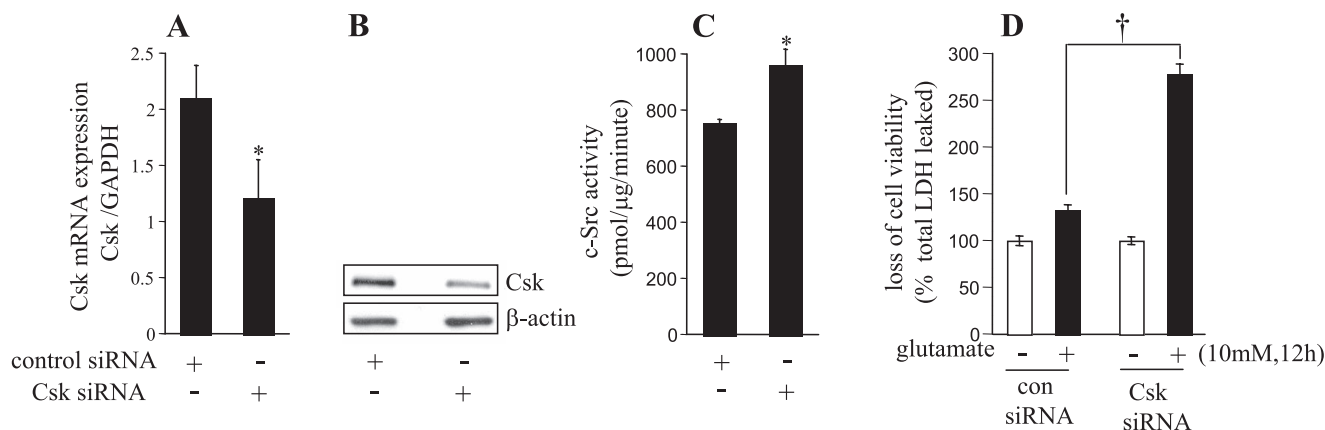
the issue more specifically, we adopted the RNA interference approach to knockdown c-Src. The siRNA approach utilized successfully lowered c-Src mRNA and protein expression in HT4 cells (Fig. 1, A–D). To test whether c-Src knockdown influenced the functional state of c-Src enzyme, an activity assay was performed. c-Src knockdown cells showed significantly lower c-Src activity (Fig. 1E). Experiments testing the effect of c-Src knockdown on glutamate-induced loss of cell viability demonstrated that specific down-regulation of c-Src abundance and activity in the cell protected the cells (Fig. 1F). These observations are consistent with our previous findings using pharmacological inhibitors of c-Src establishing that indeed c-Src contributes to glutamate-induced neurodegeneration (10). To expand on this finding, an additional approach for c-Src siRNA delivery was chosen. A single-cell microinjection technique was employed to deliver c-Src siRNA in specific cells grown on a culture plate. Next, all of the cells in the culture were challenged with glutamate. Although glutamate treatment killed all other cells in the plate, c-Src siRNA clearly protected the cells from glutamate-induced neurodegeneration (Fig. 2). Csk is an endogenous inhibitor of the Src family protein-ty-

rosine kinases (26). To test whether the loss of Csk activity may potentiate glutamate-induced neurodegeneration, Csk knockdown was performed utilizing a siRNA approach (Fig. 3, A and B). Transfection of cells with Csk siRNA significantly lowered the expression of Csk mRNA (Fig. 3A) as well as protein (Fig. 3B) expression. In cells with Csk knockdown, the activity of c-Src was significantly increased (Fig. 3C). Under the given culture conditions, >90% of the cells die after 24 h of glutamate treatment (not shown). Cell viability was only minimally influenced after 12 h of glutamate treatment (Fig. 3D). Csk knockdown clearly potentiated glutamate-induced cell death. The loss of cell viability at 12 h after glutamate treatment was significantly higher in Csk knockdown cells compared with cells transfected with corresponding scrambled control siRNA (Fig. 3D). These results demonstrate that Csk-dependent maintenance of c-Src in the “locked” inactive format protects against glutamate-induced neurodegeneration. Compromised Csk activity sensitized cells to glutamate-induced neurodegeneration. In this context we note that the effect of Csk knockdown on c-Src activity is modest (Fig. 3C) compared with the more extreme effects of Csk siRNA on protection from glutamate-induced death (Fig. 3D). This implies that other Src family kinases might be mediating the effects observed upon Csk knockdown.

Csk is mainly cytoplasmic and c-Src is predominantly membrane-associated (27). In resting cells, Csk is constantly targeted to the plasma membrane where it causes sustained inhibition of c-Src activity (28). Caveolin-1 is one adaptor protein that recruits Csk to the plasma membrane (29–31). To function as an adaptor protein for Csk, caveolin-1 is phosphorylated at a tyrosine 14 residue by Src family kinases c-Src and Fyn (29–31). We observed that challenging cells with glutamate resulted in phosphorylation of caveolin-1 at tyrosine 14. The phosphorylation was rapid and



**FIGURE 2. Microinjection of siRNA against c-Src to the cytosol of neural cells protected against glutamate-induced loss of cell viability.** Control scrambled siRNA (A) or c-Src siRNA (D) was injected into the cytoplasm. After 6–8 h, the cells were challenged with glutamate for 24 h. The cells injected with c-Src siRNA were live even after 24 h of glutamate exposure (E), whereas control siRNA injected cells were dead (B). C and F, calcein AM vital stain. C, calcein-positive cells rounding up as they die. F, elongated viable calcein positive cell (arrow). G, live and dead cell were scored in three independent experiments. \*, lower than control (no glutamate); †, higher than glutamate-challenged cells treated with control siRNA. The results are the means  $\pm$  S.D.  $p < 0.05$ .



**FIGURE 3. Csk knockdown potentiated glutamate-induced cell death.** Transfection with siRNA against Csk decreased Csk mRNA (A) and protein (B) expression. C, c-Src activity in Csk knockdown cells were significantly higher. D, cells with Csk knockdown were more sensitive to glutamate-induced loss of viability. The results are the means  $\pm$  S.D.  $p < 0.05$ . con, control.

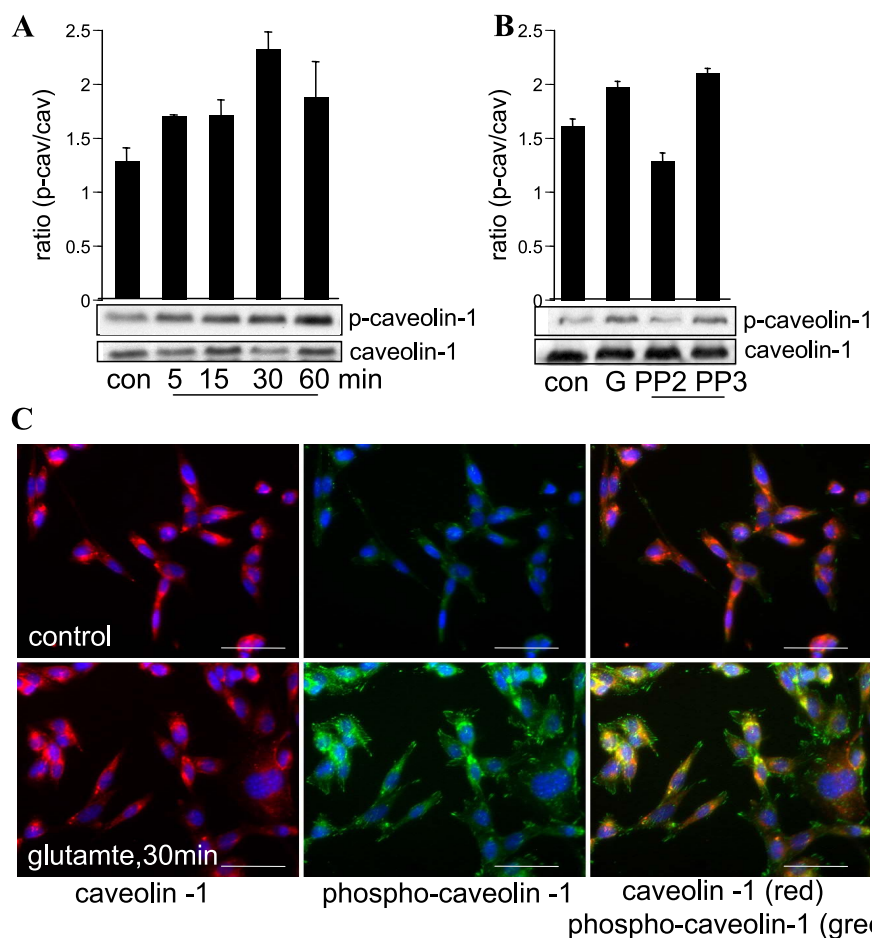


FIGURE 4. **Glutamate-induced phosphorylation of caveolin-1 at tyrosine 14.** *A*, Time course of glutamate-induced phosphorylation of caveolin-1. *B*, glutamate-induced (30 min) phosphorylation of caveolin-1 at tyrosine 14 was sensitive to the c-Src inhibitor PP2 but not to the corresponding control PP3. *C*, control and glutamate-treated cells were immunostained for caveolin 1 (red) and phospho-caveolin 1 (green; 400 $\times$ ). The cells were seeded 24 h before glutamate challenge, and the medium was changed before the cells were challenged with glutamate.

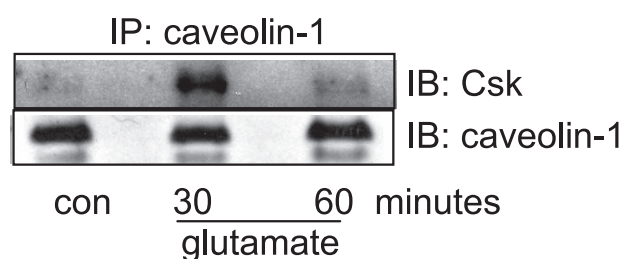


FIGURE 5. **Glutamate-induced association of Csk and caveolin-1.** After 24 h of seeding, the cells were challenged with glutamate for 30 or 60 min and protein was extracted. The cell lysates were subjected to immunoprecipitation with caveolin-1 antibody. The immunoprecipitates (IP) were subjected to SDS-PAGE and subjected to immunoblotting (IB) for the detection of Csk. con, control.

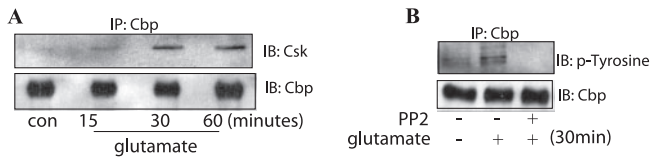
transient (Fig. 4A). Glutamate-induced tyrosine phosphorylation of caveolin-1 was sensitive to the c-Src inhibitor PP2 but not to its corresponding control PP3 (Fig. 4B). These observations constitute suggestive evidence that glutamate-induced phosphorylation of tyrosine 14 in caveolin-1 is caused by c-Src. To confirm our finding that glutamate can actually serve as a trigger for caveolin phosphorylation in neural cells, we sought to employ an additional microscopic approach. Dual immunostaining of

HT4 cells clearly revealed that glutamate induces phospho-caveolin-1 formation, whereas caveolin-1 protein expression remains unaffected (Fig. 4C). These results establish glutamate as an inducer of caveolin-1 phosphorylation in neural cells. To investigate the association of Csk with caveolin-1 in response to glutamate challenge, caveolin-1 was immunoprecipitated from cells either challenged with glutamate or not. Next, the immunoprecipitate was subjected to immunoblot using Csk. The same membrane was also immunoblotted using antibody against caveolin-1 to ensure equivalent loading of lanes. Association of caveolin-1 with Csk was noted after 30 min of glutamate challenge (Fig. 5). This time point coincides with the time point of caveolin-1 phosphorylation following glutamate challenge (Fig. 4).

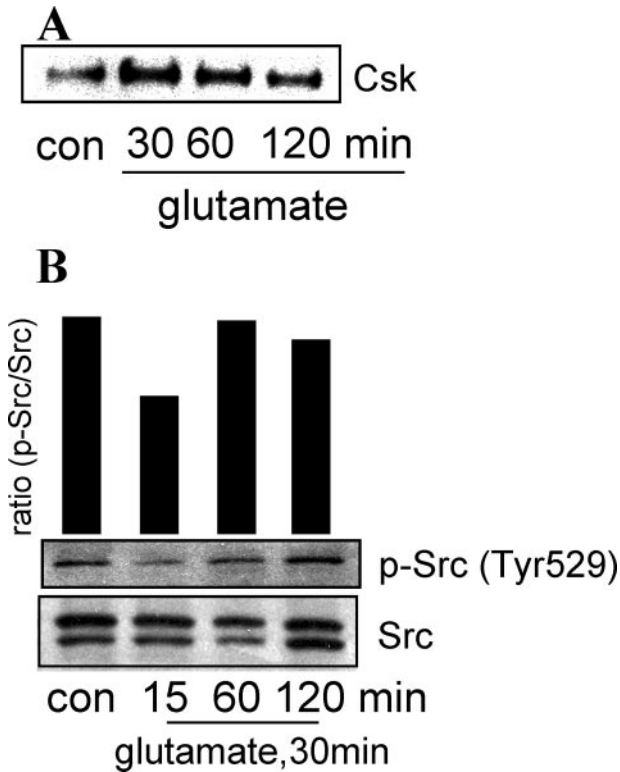
The Csk-binding protein (Cbp/PAG) is a ubiquitously expressed transmembrane protein. Its functions include suppression of c-Src activity through recruiting Csk. Cbp specifically binds to the SH2 domain of cytoplasmic Csk and recruits Csk to the membrane (27, 32). We sought to test whether membrane localization of Csk in response to glutamate challenge involves Cbp.

To investigate the association of Csk with Cbp in response to glutamate challenge, Cbp was immunoprecipitated from cells either challenged with glutamate or not. Next, the immunoprecipitate was subjected to immunoblot using Csk. The same membrane was also immunoblotted using antibody against Cbp to ensure equivalent loading of lanes. Association of Cbp with Csk was evident after 30 min of glutamate challenge (Fig. 6A). This time point coincides with the time point of caveolin-1 phosphorylation and Csk-caveolin-1 association following glutamate challenge (Fig. 4 and 5). Binding of Cbp with Csk is associated with tyrosine phosphorylation of Cbp (33). We observed that glutamate-induced tyrosine phosphorylation of Cbp was abrogated in the presence of the c-Src inhibitor PP2 (Fig. 6B). These findings indicate a role of c-Src in the glutamate-induced tyrosine phosphorylation of Cbp.

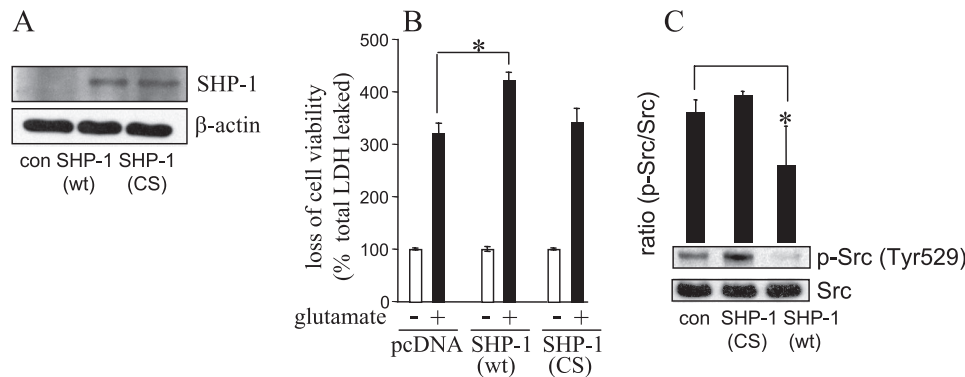
Csk localizes to the membrane and down-regulates c-Src activity (28). To understand how glutamate-induced c-Src activation in neural cells (10) is restrained, we sought to study the kinetics of Csk recruitment to the plasma membrane. Csk was detected in resting cell membrane (Fig. 7A). Upon challenge of cells with glutamate, Csk was rapidly recruited to the membrane. Peak membrane localization was noted at 30 min after



**FIGURE 6. Glutamate-induced association of Csk and Cbp.** After 24 h of seeding, the cells were challenged with glutamate, and protein was extracted. The cell lysates were subjected to immunoprecipitation with antibody against Cbp. *A*, immunoprecipitates (*IP*) were subjected to SDS-PAGE and subjected to immunoblotting (*IB*) for the detection of Csk. *B*, glutamate-induced tyrosine phosphorylation of Cbp. Cbp protein shown in *lower panel* was subjected to immunoblotting using anti-phosphotyrosine (*p-Tyrosine*) antibody. Glutamate-induced tyrosine phosphorylation of Cbp was inhibited in the presence of the c-Src inhibitor PP2. *con*, control.



**FIGURE 7. Glutamate-induced translocation of Csk to the plasma membrane and changes in inhibitory phosphorylation of c-Src.** After 24 h of seeding, the cells were challenged with glutamate and membranes were isolated. *A*, membrane fractions were subjected to SDS-PAGE followed by Western blotting to detect Csk. *B*, glutamate-induced loss of inhibitory phosphorylation of c-Src followed by regain of that phosphorylation. *con*, control.



**FIGURE 8. SHP-1 potentiated glutamate-induced loss of neural cell viability.** *A*, overexpression of wild type (*wt*) or catalytically silent (*CS*) SHP-1 in cells. *B*, overexpression of *wt* but not *CS* SHP-1 potentiated glutamate-induced cell death. *C*, SHP-1 overexpression lowered inhibitory phosphorylation of c-Src. The results are the means  $\pm$  S.D. \*,  $p < 0.05$ . *con*, control.

glutamate challenge. This time point coincides with the time point of caveolin-1 phosphorylation, Csk-caveolin-1 binding, and Csk-Cbp binding following glutamate challenge (Figs. 4–6). At the membrane, Csk is known to phosphorylate c-Src at the tyrosine 529 residue to maintain c-Src in the locked inactive configuration (34). Cytosolic protein extracts were immunoblotted for tyrosine 529-phosphorylated c-Src in response to glutamate challenge. Treatment of cells with glutamate resulted in a rapid dephosphorylation of the tyrosine 529 residue. This effect was transient. Phosphorylation of the tyrosine 529 residue was re-established in 1 h following glutamate insult (Fig. 7*B*). This observation is consistent with the finding that Csk is rapidly recruited to the membrane following binding to caveolin-1 and Cbp (Figs. 4–8*A*).

The findings described represent the first evidence explaining the mechanisms that are involved in the negative regulation of c-Src in response to activation by glutamate. The one piece in the puzzle that remained a complete unknown is the mechanism responsible for tyrosine 529 hypophosphorylation in response to glutamate challenge as noted in Fig. 7*B*. Our efforts to identify a phosphatase for the tyrosine 529 residue in c-Src led to SHP-1 as a candidate. The SH2 domain-containing phosphatase SHP-1 is a well studied classical nonreceptor tyrosine phosphatase that is known to preferentially dephosphorylate c-Src at its inhibitory phosphotyrosine site (35, 36). To test the significance of SHP-1 in glutamate-induced death of HT4, the cells were transfected to overexpress either wild type or catalytically inactive SHP-1 (Fig. 8). Overexpression of wild type, but not catalytically silent, SHP-1 potentiated glutamate-induced death, suggesting the contribution of SHP-1 in facilitating glutamate-induced neural cell death (Fig. 8*B*). Furthermore, we noted that overexpression of wild type but not catalytically silent SHP-1 dephosphorylate c-Src at its inhibitory tyrosine 529 phosphotyrosine site (Fig. 8*C*). These results indicate that SHP-1 is capable of activating c-Src and potentiating neural cell death. To further examine the significance of SHP-1 in glutamate-induced neural cell death, the siRNA-dependent knockdown approach was employed. Transfection of both HT4 cells as well as primary cortical neurons with SHP-1 directed siRNA resulted in lowering of SHP-1 mRNA abundance in the cells (Fig. 9, *A* and *C*). Importantly, SHP-1 knockdown cells were protected against glutamate-induced neural cell death (Fig. 9, *B* and *D*). The results were more clear-cut in primary neurons than in HT4 cells where despite near-complete SHP-1 knockdown only modest protection was noted. It is likely that HT4 cells have a SHP-1-independent component of cell death pathway when challenged with glutamate. In primary neurons, however, SHP-1 knockdown was associated with nearly complete neuroprotection against glutamate (Fig. 9*D*). Taken together, these observations lead to the conclusion that SHP-1 contributes to glutamate-induced

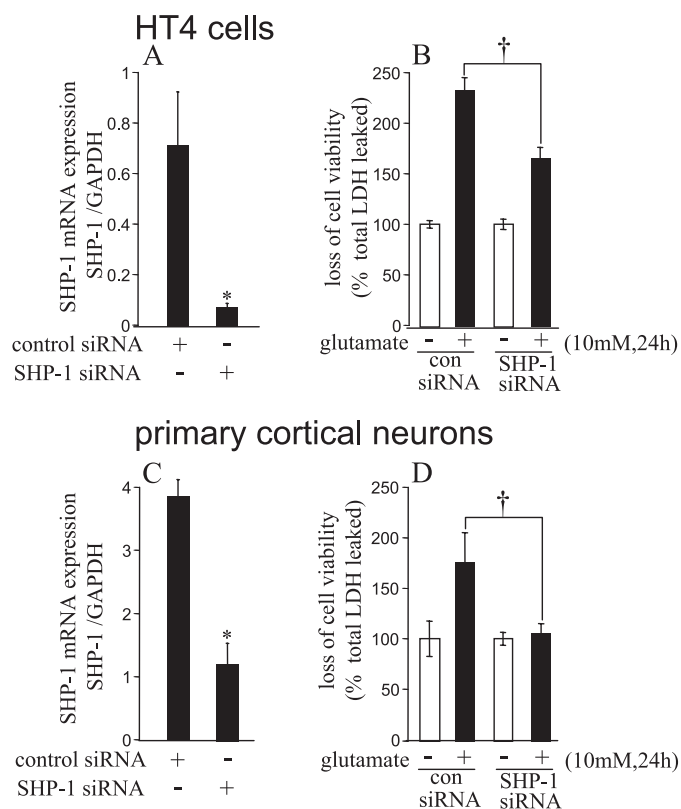


FIGURE 9. SHP-1 knockdown attenuated glutamate-induced death of HT4 cells and primary cortical neurons. A and B, HT4 cells; C and D, primary cortical neurons. A and C, transfection with SHP-1 siRNA decreased SHP-1 mRNA expression. B and D, SHP-1 knockdown attenuated glutamate-induced loss of cell viability. The results are the means  $\pm$  S.D. \* $\dagger$ ,  $p < 0.05$ . con, control.

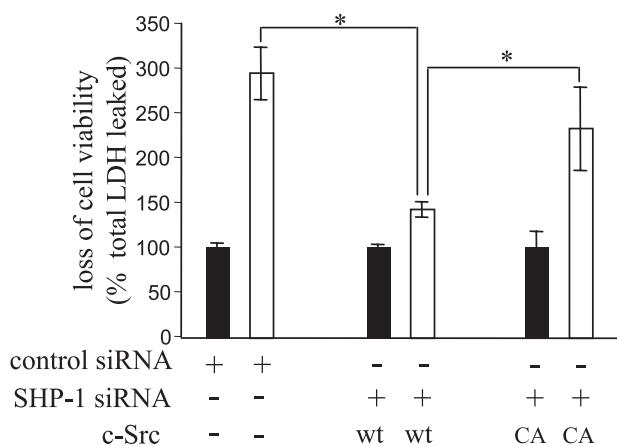


FIGURE 10. Expression of constitutively active c-Src abrogated the neuroprotection against glutamate challenge caused by SHP-1 knockdown. Glutamate challenge (10 mM, 24 h; open bars) caused loss of HT4 cell viability, which was attenuated in the cells with SHP-1 knockdown. Such neuroprotective effect of SHP-1 knockdown was significantly lost in cells expressing constitutively active (CA) c-Src. The results are the means  $\pm$  S.D. \* $\dagger$ ,  $p < 0.05$ . wt, wild type.

neural cell death. In the cell, SHP-1 has numerous substrates in addition to c-Src. Thus, we sought to test whether the neuroprotective effects of SHP-1 knockdown is dependent on c-Src. Of note, the neuroprotective effect of SHP-1 knockdown (Fig. 9) was abrogated in HT4 cells expressing CA-c-Src (Fig. 10). CA-c-Src is generated by a mutation (*srcY529F*) that substitutes tyrosine 529 with phenylalanine. Thus, CA-c-Src does not

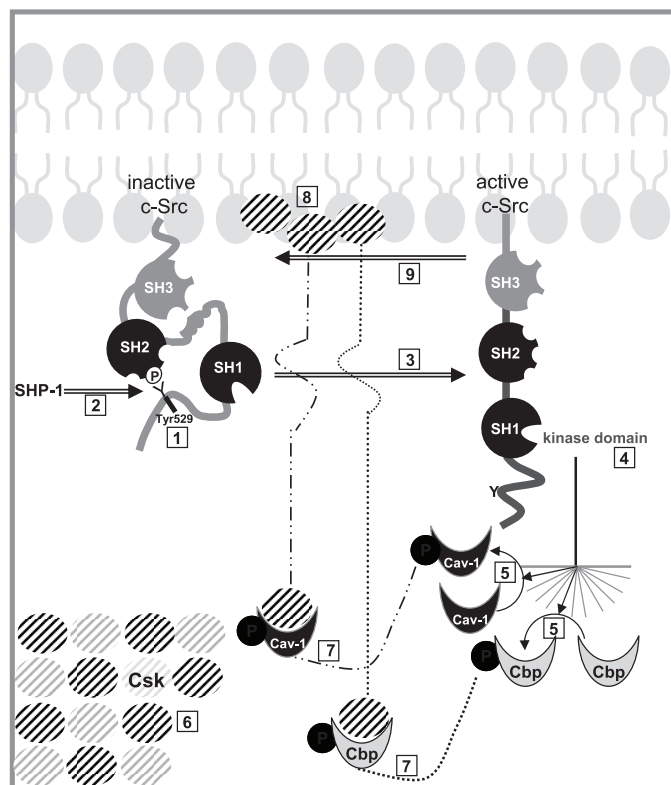


FIGURE 11. Regulation of c-Src in response to glutamate challenge in neural cells: a novel paradigm. Event 1, under resting conditions c-Src is maintained under locked or inactive condition by inhibitory tyrosine phosphorylation. Event 2, in minutes after glutamate treatment SHP-1 unlocks and activates c-Src by dephosphorylating the inhibitory tyrosine phosphorylation. Events 3 and 4, c-Src assumes an unlocked configuration and is now active. Event 5, among other substrates, c-Src phosphorylates caveolin-1 and Cbp. Event 6, under resting conditions Csk is primarily cytoplasmic. Event 7, phosphorylated Cbp and caveolin-1 facilitates membrane localization of Csk. Event 8, Csk accumulates near the membrane. Event 9, Csk re-establishes the inhibitory tyrosine phosphorylation of c-Src, thus lowering c-Src activity. Events 1–5 and 7–9 are in chronological order.

lend itself to regulation of activity by SHP-1 or other such agents targeting tyrosine 529 hypophosphorylation. The observations of this study and those reported previously (10) elucidate the pathways that are involved in the regulation of c-Src in glutamate-challenged HT4 cells (Fig. 11). Under resting conditions c-Src is maintained under locked or inactive condition by inhibitory phosphorylation of tyrosine 529. In minutes after glutamate treatment SHP-1 unlocks and activates c-Src by dephosphorylating tyrosine 529. This regulation is not functional in CA-c-Src where tyrosine 529 is replaced by phenylalanine resulting in constitutively active c-Src activity. In wild type cells, following glutamate-induced activation of SHP-1, c-Src assumes an “unlocked” active configuration. Once active, within minutes c-Src phosphorylates caveolin-1 and Cbp. Under resting conditions, Csk is primarily cytoplasmic in distribution. Phosphorylated Cbp and caveolin-1 facilitates rapid membrane localization of Csk. As a result, Csk accumulates near the membrane where it re-establishes the inhibitory tyrosine phosphorylation of c-Src, thus lowering c-Src activity. All of these processes, *i.e.* the transient turning of c-Src in response to glutamate, may be viewed as being early events and are expected to occur within the first 1–2 h of glutamate treatment (Fig. 11).

## DISCUSSION

Our efforts to characterize the potent neuroprotective effects of a poorly understood nontocopherol form of natural vitamin E led to the first observation directly implicating c-Src in the oxytosis form of neural cell death (10). The phosphorylation of proteins on tyrosine residues, initially believed to be primarily involved in cell growth and differentiation, is now recognized as having a critical role in regulating a wide range of cellular functions. In the adult animal, the brain exhibits one of the highest levels of tyrosine kinase activity (37). Current work highlights the significance of tyrosine phosphorylation in neurodegeneration. Tyrosine phosphorylation has been implicated in the specific form of neurodegenerative disorder called tauopathy (38). Tyrosine phosphorylation is also known to sensitize neural cells to receptor-mediated death (39). Src family kinases have been implicated in such neurodegenerative process (40). The findings of this study have led to a new paradigm addressing the regulation of c-Src under conditions of oxytosis (13), a component of excitotoxicity in mature neurons *in vivo* (14). Indeed, based on the study of oxytosis, we have been effective in identifying strategies to minimize stroke-related neurodegeneration *in vivo* (10, 20, 21, 25). In addition, our previous observation implicating c-Src in oxytosis has led to independent observations demonstrating that c-Src is indeed involved in neurodegeneration *in vivo* (11, 12).

SHP-1, a cytoplasmic tyrosine phosphatase, is best known for its abundance and function in the hematopoietic system (41). SHP-1 is also found in the nervous system, but its functional significance in the central nervous system is only beginning to be elucidated (42). The results of this study support that SHP-1 facilitates glutamate-induced death of HT4 neural cells. This observation is consistent with the report that mice deficient in SHP-1 are less susceptible to focal cerebral ischemia (42). SHP-1 is also known to be responsible for neuronal death during development. Mice lacking SHP-1 had increased numbers of sympathetic neurons during the period of naturally occurring neuronal cell death, and when cultured, these neurons survived better than wild type neurons in the absence of nerve growth factor (43). RNA interference targeting SHP-1 attenuates myocardial infarction in rats, suggesting that targeting SHP-1 down-regulation may prove to be helpful not only in the brain but also in the heart (44). Activation of the cellular Src tyrosine kinase depends upon dephosphorylation of a C-terminal inhibitory tyrosine phosphorylation site (26). Src isolated from human platelets and Jurkat T cells is preferentially dephosphorylated at its inhibitory phosphotyrosine site by the SHP-1 tyrosine phosphatase (35). Activation of Src by SHP-1 has been also noted in HEK293 cells. Pull-down experiments with different glutathione *S*-transferase-SHP-1 fusion proteins revealed efficient interaction of Src-generated phosphoproteins with the SHP-1 catalytic domain rather than with the SH2 domains. Phosphopeptides that correspond to good Src substrates were efficiently dephosphorylated by SHP-1 *in vitro*. Phosphorylated optimal Src substrate AEEEIpYGEFEA (where pY is phosphotyrosine) and a phosphopeptide correspond-

ing to a recently identified Src phosphorylation site in p120 catenin, DDLDPY<sup>296</sup>GMMSD, were excellent SHP-1 substrates. Docking of these phosphopeptides into the catalytic domain of SHP-1 by molecular modeling supports an efficient interaction between SHP-1 and Src (36).

Csk has been reported as a protein-tyrosine kinase in neonatal rat brain that is capable of inhibiting c-Src activity by specifically phosphorylating a C-terminal inhibitory tyrosine phosphorylation site (45). Csk is ubiquitously expressed and cytosolic in localization because they lack fatty acid acylation domain for anchoring to the plasma membrane. Given that c-Src is membrane-anchored, Csk needs to be translocated to the membrane to inhibit c-Src. Membrane translocation of Csk is mainly mediated by binding to membrane proteins and signaling adaptor proteins via their SH2, SH3, and kinase domains (27). The results of this study suggest that Cbp and caveolin-1 are involved in translocating Csk to the plasma membrane shortly after glutamate challenge. In this way, the Cbp and caveolin-1 pathways are responsible for switching off glutamate-induced SHP-1-dependent c-Src activation. Our observation is consistent with previous reports demonstrating that in brain cells, Csk is translocated to the plasma membrane with the aid of a lipid raft-associated membrane protein known as Cbp. Cbp is phosphorylated at tyrosine 314. Because Cbp resides in the lipid raft of the plasma membrane, it is also called PAG (phosphoprotein associated with glycosphingolipid-enriched microdomain) (46). Caveolae play important roles in both signaling transduction and internalization of several G protein-coupled receptors. However, the role of caveolae in cellular signaling remains unclear. Caveolin-1 represents a principal component of caveolae. It is phosphorylated on tyrosine 14 by a Src kinase pathway. Phosphocaveolin-1 but not caveolin-1 with mutation at tyrosine 14 binds to Csk and translocates Csk to the membrane (31). During oxytosis, glutamate challenge is known to cause oxidative stress (13, 14, 25). Oxidative stress is a trigger for the phosphorylation of caveolin-1 at tyrosine 14 (30).

In sum, the findings of this study represent the first evidence toward a new paradigm that addresses the regulation of c-Src under neurodegenerative conditions. SHP-1 has been identified as a key regulator of glutamate-inducible c-Src activation. Importantly, glutamate-induced c-Src activation is rapid and transient. Cbp- and Cav-1-dependent mechanisms facilitate Csk-mediated silencing of glutamate-induced activated c-Src. Although the current study focuses on glutamate-induced neurodegeneration, given the pleiotropic nature of the involvement of c-Src in neurodegeneration, it is reasonable to hypothesize that the pathways identified in this work are relevant to a wider range of cytotoxicity involving c-Src. Given that c-Src activity is now known to be critical in regulating neurodegeneration *in vivo*, it is important that the specific mechanisms modulating c-Src activity during neural cell death be characterized. Such understanding will help develop specific neuroprotective agents targeting the c-Src pathway. Findings of this study support the consideration of SHP-1 inhibitors as neuroprotective agents.



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## **Regulation of c-Src Activity in Glutamate-induced Neurodegeneration**

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