Dual role of Src kinase in governing neuronal survival

M. Iqbal Hossain\textsuperscript{1}†§, Ashfaqul Hoque\textsuperscript{1}†, Guillaume Lessene\textsuperscript{2}, M. Aizuddin Kamaruddin\textsuperscript{1}, Percy W.Y. Chu\textsuperscript{1}, Ivan H.W. Ng\textsuperscript{1,3}, Sevgi Irtegun\textsuperscript{1}, Dominic C.H. Ng\textsuperscript{1}, Marie A. Bogoyevitch\textsuperscript{1}, Antony W. Burgess\textsuperscript{2}, Andrew F. Hill\textsuperscript{1} and Heung-Chin Cheng\textsuperscript{1}*

From \textsuperscript{1}Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria 3010, Australia; \textsuperscript{2}Divisions of Chemical and Structural Biology, Walter and Eliza Institute for Medical Research, Parkville, Victoria 3010; \textsuperscript{3}Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia

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† These authors contributed equally to the work described in this manuscript.

*To whom correspondence should be addressed. E-mail: heung@unimelb.edu.au

§ Present address: Department of Physiology, University of Melbourne
Abstract

**Background:** Src-family kinases (SFKs) are involved in neuronal survival and their aberrant regulation contributes to neuronal death. However, how they control neuronal survival and death remains unclear. **Objective:** To define the effect of inhibition of Src activity and expression on neuronal survival. **Results:** In agreement with our previous findings, we demonstrated that Src was cleaved by calpain to form a 52-kDa truncated fragment in neurons undergoing excitotoxic cell death, and expression of the recombinant truncated Src fragment induced neuronal death. The data confirm that the neurotoxic signaling pathways are intact in the neurons we used for our study. To define the functional role of neuronal SFKs, we treated these neurons with SFK inhibitors and discovered that the treatment induced cell death, suggesting that the catalytic activity of one or more of the neuronal SFKs is critical to neuronal survival. Using small hairpin RNAs that suppress Src expression, we demonstrated that Src is indispensable to neuronal survival. **Additionally, we found that neuronal death induced by expression of the neurotoxic truncated Src mutant, treatment of SFK inhibitors or knock-down of Src expression caused inhibition of the neuroprotective protein kinases Erk1/2, or Akt.** **Conclusions:** Src is critical to both neuronal survival and death. Intact Src sustains neuronal survival. However, in the excitotoxic condition, calpain cleavage of Src generates a neurotoxic truncated Src fragment. Both intact Src and the neurotoxic truncated Src fragment exert their biological actions by controlling the activities of neuroprotective protein kinases.

(248 words)
INTRODUCTION

The involvement of Src-family kinases (SFKs) in neuronal survival has been well documented (Hossain et al., 2012). Five SFK members Src, Fyn, Yes, Lyn and Lck are widely expressed in neurons (Bae et al., 2012; Lowell and Soriano, 1996; Maness, 1992; Summy et al., 2003). Among them, Src mediates neurotropic signaling by the common receptor Ret of the glial cell line-derived neurotrophic factor (GDNF) (Encinas et al., 2001). In addition, Src forms complexes with phosphatidylinositol-3-kinase (PI3K) and the 3, 3’, 5-tri-iodothyronine (T3) receptor in primary cortical neurons and is implicated in mediating the neuroprotective actions of the T3 receptor by activating the neurotrophic PI3K in this complex (Cao et al., 2009). Thus, Src and other neuronal SFKs are considered to be key enzymes in signaling pathways sustaining neuronal survival.

Paradoxically, Src and Fyn are known to contribute to brain damage in ischemic stroke, intracerebral hemorrhage and Alzheimer’s disease (Haass and Mandelkow, 2010; Ittner et al., 2010; Liu and Sharp, 2011; Paul et al., 2001). Targeted disruption of the Src gene, and treatment with several SFK inhibitors significantly reduce brain damage in rodent models of cerebral ischemia and hypoxia-ischemia, suggesting that Src and/or other SFK members in neurons contribute to neuronal death in stroke (Paul et al., 2001). On the contrary, treatment with inhibitors of SFKs induces cell death in cortical neurons (Takadera et al., 2012), suggesting that SFK activity is critical for neuronal survival as well. Thus, these conflicting reports concerning the role of Src and other SFKs in neurons suggest that neuronal SFKs participate in maintaining neuronal survival in physiological conditions as well as contributing to neuronal death in pathological conditions. Presumably, neuronal SFKs are aberrantly regulated to exert their
neurotoxic function. Indeed, we recently demonstrated in vivo and in cultured primary neurons undergoing excitotoxic cell death that Src is cleaved by calpain to form a 52-kDa fragment (Hossain et al., 2013). More importantly, we provided evidence that the 52-kDa Src fragment contributes to neuronal death and so our results indicate that calpain cleavage is a key step in the conversion of Src to a mediator of cell death in neurons undergoing excitotoxic neuronal death (Hossain et al., 2013).

Extending our ongoing efforts to elucidate the mechanism of these apparently conflicting functions of neuronal SFKs, we employed pharmacological and small hairpin RNA (shRNA) knockdown approaches to define the role of Src in survival of cultured primary cortical neurons. Here we report that SFK inhibitors induce neuronal death, suggesting that at least one of the SFKs expressed in neurons is critical to cell survival. As Src was reported to mediate the neurotrophic signals of GDNF and T3 receptors, we sought to define the contribution of Src to neuronal survival. Specifically, we used shRNAs to suppress neuronal Src expression and found that suppression of Src expression alone is sufficient to induce neuronal death, confirming that Src is critical for neuronal survival. In addition to inducing cell death, treatment with SFK inhibitors and knockdown of Src expression cause inactivation of the extracellular signal regulated kinase 1 and 2 (Erk1/2). Relevant to this, the neurotoxic Src fragment induces neuronal death by inhibiting Akt (Hossain et al., 2013). As Erk1/2 and Akt are critical for neuronal survival (Bading, 2013; Howitt et al., 2012; Jo et al., 2012; Kim et al., 2005; Lai et al., 2011; Miyawaki et al., 2009; Bading, 2013 #237), our data suggest that Erk1/2 relays the pro-survival signals of Src and Akt antagonizes the neurotoxic signals of truncated Src fragment in excitotoxicity. Our results suggest sustaining the neuroprotective Src/Erk1/2/Akt signaling
pathways as a potential strategy to maintain neuronal survival in acute neurological conditions and chronic neurodegenerative diseases.
RESULTS

Expression levels of Src and NMDA receptor subunits undergo time-dependent increase in cultured primary cortical neurons

Both extrasynaptic and synaptic NMDA receptors play important roles in governing neuronal survival (Hardingham and Bading, 2010), and Src is an important upstream regulatory kinase controlling NMDA receptor activity by forming protein complexes with NMDA receptor and phosphorylating receptor subunits (Kaufman et al., 2012; Salter and Kalia, 2004; Zhang et al., 2007). Since both Src and NMDA receptors interplay to regulate neuronal survival (Hossain et al., 2013), we first analyzed the expression levels of Src and the various subunit of NMDA receptors in primary cortical neurons and the morphology of the neurons at different times after they were cultured in vitro. As shown in Figure 1A, Src and NMDA receptor subunits GluN1, GluN2A and GluN2B are detectable as early as day 1 in vitro (DIV 1). Their expression levels undergo a time-dependent increase from DIV 1 to 7. At DIV 7, extensive neurite outgrowths and many synapses between neighboring neurons have been formed, features that are typical of differentiated neurons. Immunofluorescence analysis of the endogenous Src and the various NMDA receptor subunits in neurons at DIV 7 shows that the subunits of the NMDA receptors are present in cell bodies and dendrites (Figure 1B). These data indicate that at DIV7, the cortical neurons are differentiated and suitable for mechanistic studies for neuronal survival.
The signaling pathways governing cell death and aberrant regulation of Src are intact in cortical neurons at DIV 7

Over-stimulation of the cultured neurons at DIV 7 with 100 µM of glutamate induces cell death (Figure 2A), indicating that the signaling pathways mediating the excitotoxic signals originating from glutamate receptors are well developed. The neurotoxic effect of glutamate was blocked by the NMDA receptor antagonist MK801 but not by the AMPA and kainate receptor antagonist 6-cyno-7-nitroquinoxaline-2,3-dine (CNQX) (Figure 2A), suggesting that the neurotoxic effect of glutamate is mediated by NMDA receptor. In agreement with this notion, over-stimulation by NMDA induces neuronal death. There are two major types of NMDA receptors: (i) the extrasynaptic NMDA receptors which are enriched in GluN2B subunit and (ii) the synaptic NMDA receptors enriched in GluN2A subunit. Previous studies revealed that signals emanating from the over-stimulated extrasynaptic NMDA receptors are neurotoxic (Hardingham and Bading, 2010; Karpova et al., 2013; Kaufman et al., 2012; Milnerwood et al., 2010; Okamoto et al., 2009; Rusconi et al., 2011; Zhang et al., 2011; Zhou et al., 2013). In agreement with these findings, the neurotoxic effect of glutamate was suppressed by the GluN2B-containing NMDA receptor antagonist Ifenprodil (Figure 2A). In our previous study, we discovered that over-stimulation of glutamate receptors leads to sustained activation of calpains which catalyze the cleavage of Src to form a neurotoxic truncated Src fragment of ~52-kDa. As shown in Figure 2B, stimulation of cultured cortical neurons with glutamate and NMDA induces the formation of a ~52-kDa truncated Src fragment. The formation of this fragment is suppressed when the cells were treated with glutamate and the calpain inhibitor calpeptin, indicating that formation of the Src fragment by limited proteolysis of Src by calpain in neurons over-stimulated by glutamate and NMDA. Furthermore, formation of this truncated fragment was abolished in neurons treated
with glutamate in the presence of MK801 or Ifenprodil but not in those treated with glutamate and CNQX, suggesting that the generation of truncated Src fragment is induced in response to over-stimulation of the extrasynaptic NMDA receptors of the cultured cortical neurons.

**Truncated fragment of Src kinase generated by calpain is neurotoxic**

Results of our previous study indicate that the 52-kDa Src fragment generated by limited proteolysis by calpain contains the intact SH3, SH2, kinase domains and the C-terminal tail but lacks the N-terminal segment containing fatty acid acylation motif and unique domain. To find out its function, we generated a lentivirus directed expression of a recombinant GFP fusion protein of truncated Src mutant (referred to as Src-ΔN-GFP) under the control of doxycycline in neurons as shown in Figure 3A. For the control experiments, we transduced neurons with lentivirus directing expression of GFP and GFP fusion proteins of full-length Src kinase (Src-GFP). Previously, it has been confirmed that PI3K/Akt signaling pathway is essential in maintaining neuronal survival (Luo et al., 2003), we therefore monitored phosphorylation level of Akt at Ser-473 upon transduction of Src-ΔN-GFP in primary cortical neurons. Immunoblot data revealed that Src-ΔN-GFP significantly reduced phosphorylation of Akt at Ser-473 (Figure 3A) and subsequently decreased neuronal viability and survival (Figure 3B & C). Staining of dead cells with EthD-1 further demonstrated that Src-ΔN-GFP significantly increased number of dead cells compared to that of either GFP and Src-GFP transduced primary cortical neurons (Figure 3D). Thus, in agreement with our previous report (Hossain et al., 2013) these data indicate that truncated fragment generated by calpain-mediated cleavage in primary cortical neurons during excitotoxicity mediates neuronal death by suppressing, in part, activity of Akt.
More importantly, the signaling pathways mediating the neurotoxic action of the truncated Src fragment are intact in the cultured neurons we used in our study.

_Treatment with SFK inhibitors induce cell death of primary cortical neurons_

For the pharmacological approach to inhibit SFKs, we first used two known chemical inhibitors of SFKs, PP2 and SU6656 to treat primary cortical neurons followed by examination of their effects on neuronal viability. Treatment with the inhibitors decreases Src kinase activity in primary cortical neurons as suggested by decreased phosphorylation at Src-pY416 (Supplemental Figure S1A) and kinase activity assay using Src optimal peptide (Supplemental Figure S1B). We further found that treatment of cortical neurons with SU6656 or PP2 for 8 hours induced a significant reduction in cell viability as assed by the MTT assay (Supplemental Figure 1C). This decrease in cell viability correlated with the increase in the activity of LDH released from damaged neurons as a result of the treatment of neurons with either of these two SFK inhibitors (Supplemental Figure S1D).

As both SU6656 and PP2 are broad spectrum protein kinase inhibitors (Bain et al., 2007), their neurotoxic effects as depicted in Supplemental Fig. S1 could be caused by inhibition of SFKs as well as inhibition of other protein kinases in neurons. To further ascertain the role of SFKs in neuronal survival, we examined the effect of treatment of neurons with a more selective SFK inhibitor A419259 on their survival (Meyn et al., 2006; Wilson et al., 2002). Fig. 4A and B shows that treatment of neurons with 0.3 – 5 μM of A419259 for 24 h significantly reduced neuronal survival. Since the treatment also led to significant reduction in autophosphorylation of neuronal Src, the reduction in neuronal viability was likely a result of inhibition of SFKs in neurons.
Taken together, our results of treatment of neurons with the chemically distinct SFK inhibitors suggest that the activity of one or more of the neuronal SFKs is critical for maintenance of neuronal survival. Since treatment of SFK inhibitors did not cause the formation of the neurotoxic 52-kDa truncated Src fragment (Figure 4C and Supplemental Figure S1A), our data suggest that the SFK inhibitors induced neuronal death exclusively by inhibition of the activity of intact SFKs in neurons.

**Suppression of Src expression induces cell death in cultured primary cortical neurons**

In addition to Src, other SFKs co-expressed in neurons may be inhibited upon treatment of the neurons by the cell membrane-permeable SFK inhibitors. Among the SFKs co-expressed in neurons, Src has been implicated in mediating the pro-survival signaling pathways emanating from glial cell line-derived neurotrophic factor receptor Ret and the nuclear receptor of thyroid hormone 3,3’,5’-tri-iodothyronine (TR1α) (Encinas et al., 2001; Encinas et al., 2004). These observations prompted us to investigate the effect of knockdown of Src expression on the viability of primary cortical neurons. As shown in Figure 5A, neuronal Src expression was suppressed by ~70% in neurons transduced with lentivirus directing the expression of two Src-specific small hairpin RNAs, shRNA1 and shRNA2. In contrast, the non-silencing shRNA (ns shRNA) had no effect on Src expression. Transduction with the shRNA1- or shRNA2-lentivirus also led to significant changes in the phenotypes of the neurons – notably, the number of intact cell bodies and the degree of axonal connections were reduced. In contrast, the transduced neurons and neurons transduced with the lentivirus directing expression of the non-silencing shRNA were properly developed with well-connected synapses and much longer axons (Figure
5B). Results of both MTT and LDH assays demonstrated that the reduced expression of Src is associated with reduced cell survival (Figure 5C). This was further confirmed by the results of live/dead cell staining of the neurons with Calcein AM and Ethidium homodimer-1 (EthD-1), namely that the transduction with the shRNA1- or shRNA2-lentivirus leads to a significant increase in the number of dead cells (stained by EthD-1) and reduction in the number of live cells stained by Calcein-AM (Figures 5D and 5E). Taken together, our results indicate that Src is essential for survival of the cultured primary cortical neurons.

**Inhibition of SFK activity or knockdown of Src expression induces Erk1/2 inhibition**

It is well documented that stimulation of synaptic NMDA receptor can initiate neuroprotective signals that protect neurons against apoptosis (Hardingham et al., 2001; Hardingham and Bading, 2010; Wu et al., 2001). Erk1/2 are key mediators of the synaptic NMDA receptor neuroprotective signaling pathway. To test a prediction that Src maintains neuronal survival at least in part by sustaining Erk1/2 in the active state, we examined the effect of treatment with SFK inhibitors and knockdown of Src expression on the Erk1/2 activation state in neurons.

We initially intended to monitor the phosphorylation status of Erk1/2 in neurons at DIV1 after their transduction with the Src shRNA lentivirus at DIV1. Since the majority of the transduced neurons were dead and degraded (Fig 5), they were not suitable for use for Western blots. In light of this, we examined the time course of the change in cell viability of neurons transduced by the Src shRNA2 at DIV 1-6. Our results reveal significant reduction in cell viability in all transduction conditions with neurons transduced at DIV1 exhibiting the lowest level of cell viability (Supplemental Figure S2).
Figure 6 shows the effects of down-regulation of Src in neurons transduced with the lentivirus expressing the Src-shRNA2 at DIV2. Figure 6A shows that the transduced neurons expressing the Src-shRNA exhibited a significant reduction in expression of neuronal Src. However, transduction of the neurons with lentivirus expressing the non-silencing shRNA and treatment of the neurons with 1 µM A419259 for 24h did not alter the Src expression level. Figure 6B reveals that inhibition of Src kinase activity or knockdown of Src kinase protein reduces phosphorylation of neuronal Erk1/2 at Thr-183 and Tyr-185 in the activation loop. From the ratios of the densitometric unit of the anti-pErk (T183/Y185) signal to that of anti-Erk signal, it is obvious that treatment with either SFK inhibitors or knockdown of Src expression significantly decreases neuronal Erk1/2 phosphorylation at these sites (Figure 6B). Since Erk1/2 activated by phosphorylation of Thr-183 and Tyr-185 by their upstream kinase MEK, our results indicate that Src is involved in the maintenance of the active state of Erk1/2. More importantly, our results suggest Erk1/2 operates down-stream of Src to mediate the pro-survival signals of intact Src in neurons.

PI3K and its downstream effector kinase Akt play significant roles in protecting neurons against cell death induced by ischemic stroke (Baba et al., 2009; Chan et al., 1999; Miyawaki et al., 2009; Namikawa et al., 2000; Yamaguchi et al., 2001), suggesting that PI3K and Akt are key enzymes maintaining neuronal survival. We therefore examined the effect of knockdown of Src expression and treatment with the SFK inhibitor A419259 on Akt phosphorylation. Fig. 6C shows down-regulation of Src expression and treatment with A419259 did not cause inactivation of Akt/ Own results suggest that under the conditions we performed our experiments, Akt is not involved in the neuroprotective signaling mechanism of intact Src and other neuronal SFKs.
DISCUSSION

Src family kinases (SFKs) are at the center point of many cell-surface signaling events, such as the pathways activated by receptor tyrosine kinases (PDGFR, EGFR, and FGFR etc.), G-protein coupled receptors (GPCRs), cytokines, immune cell receptors as well as integrins and other cell adhesion molecules (Parsons and Parsons, 2004). Thus, they are important for transmitting a diverse range of cellular signals. Furthermore, SFKs not only play important role in proliferation and differentiation of neuronal cells (Ingraham et al., 1989; Maness et al., 1990), but also act as critical regulators in synaptic transmissions (Grant et al., 1992; Umemori et al., 2003; Zhao et al., 2000), axonal movement (Morse et al., 1998), and modulation of synaptic plasticity (Takasu et al., 2002). In developing CNS, SFKs are responsible for neuronal differentiation and neurite outgrowth (Beggs et al., 1994; Ignelzi et al., 1994), while in developed CNS they are mainly involved in regulating the activity of ionotropic glutamate receptors, such as the NMDA and AMPA receptors, as well as voltage-gated ion channels, including potassium channels and calcium channels. Although targeted deletion of the Src gene causes osteoporosis in mice, the Src-deficient mice did not exhibit obvious neurological abnormalities and the effect of knockdown of Src on neuronal survival has not been studied in detail (Soriano et al., 1991). In spite of the lack of obvious neurological abnormalities in the Src-deficient mice, Ignelzi et al. reported that cerebellar neurons derived from these mice exhibited impaired neurite outgrowth in response to stimulation by the neural cell adhesion molecule L1 (Ignelzi et al., 1994), suggesting that Src performs a specific and non-redundant function in the L1-dependent neurite outgrowth process.
Src family kinases participate in a wide array of signaling events in CNS through their interactions with many cellular proteins. Src is implicated to mediate the pro-survival action of the thyroid hormone T3 receptor TR1α and GDNF receptor by activating phosphatidylinositol-3 kinase (PI-3K) to maintain neuronal survival (Cao et al., 2009; Encinas et al., 2001; Encinas et al., 2004). In these previous studies, the authors employed SFK inhibitors capable of inhibiting all SFKs as well as a number of non-SFK kinases to define the roles of Src. Although these results suggest the significance of Src in neuronal survival, more detailed mechanistic studies using more specific approaches to inhibit Src and the SFKs are required to confirm this. In our study, in addition to using two commercially available and widely used SFK inhibitors, we employed two specific shRNAs against Src to address the functional role of full length Src protein in neuronal survival, and demonstrated that suppression of Src expression significantly reduces neuronal survival. Together, our data indicates that among SFKs, Src itself plays a critical role in maintenance of neuronal survival.

In search of possible signaling mechanism of neuronal death upon Src inhibition or Src knockdown, we found a significant decrease of phosphorylation of Akt at Ser-473. Relevant to our finding of Akt as a key component of the pro-survival pathway of Src in neurons, it has been reported that both the stimulated GDNF receptor Ret and the T3-stimulated thyroid hormone receptor TR-1 maintain neuronal survival by sustaining the PI-3K and Akt in their active states (Cao et al., 2009; Encinas et al., 2004). Results from previous studies using non-neuronal cells, such as HEK293 cells and Neuro2a cells, treated with SFK inhibitors have provided evidence suggesting SFKs as an obligatory components mediating the activation of PI-3K/Akt by the stimulated Ret and TR-1. Thus, our results presented here support these findings and highlight
that Src is critical for neuronal survival. In light of these findings, Src may maintain neuronal survival at least in part by sustaining Akt in its active state. In addition to cooperating with Akt, Src also maintains neuronal survival by regulating mitochondrial function. Src is targeted to the mitochondria where it phosphorylates NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2) of the mitochondrial respiratory complex I, and the phosphorylation contributes to neuronal survival (Ogura et al., 2012). Thus, future investigations of the neuroprotective mechanism of Src should include defining how phosphorylation of this complex I protein cooperates with activation of Akt to mediate the pro-survival signals of Src in neurons.

If Src is a physiological pro-survival enzyme in neurons, how could Src and other neuronal SFKs contribute to ischemic stroke-induced neuronal death and brain damage in animal models? We postulate that Src is aberrantly regulated under pathological conditions and this aberrantly regulated form of Src loses its pro-survival function and gains neurotoxic activity (Hossain et al., 2011; Hossain et al., 2013). Src undergoes limited proteolysis by calpain at the unique domain to form a 52-kDa truncated product in platelets and neurons (Feder and Bishop, 1990; Hossain et al., 2013; Oda et al., 1993), and undergoes activation and S-nitrosylation of Cys-498 in rat brain after cerebral ischemia and reperfusion (Akhand et al., 1999; Hossain et al., 2011; Rahman et al., 2010; Tang et al., 2012). A major event leading to neuronal death in ischemic stroke and neurodegeneration is excitotoxicity, in which NMDA receptors of neurons were over-stimulated by excessive concentration of glutamate. The over-stimulated NMDA receptors allow a massive influx of calcium into neurons, which causes over-activation of calpain. We demonstrated that Src in neurons over-stimulated by glutamate is cleaved by calpain to form a 52-kDa fragment. More importantly, this truncated Src fragment induces neuronal death in part by inhibiting Akt
Thus, our previous results and results presented in this study indicate that Src is a key regulator of neuronal survival and a mediator of neuronal death. In neurons undergoing excitotoxic cell death, calpain cleavage converts Src from a promoter of neuronal survival to a mediator of neuronal death. Src kinase inhibitors were shown to reduce brain damage in mouse models of ischemic and hemorrhagic stroke (Hou et al., 2007; Liu and Sharp, 2011), presumably by inhibiting the kinase activity of the neurotoxic truncated Src fragment generated by calpain cleavage. However, the use of these inhibitors may also induce adverse effects by suppressing the pro-survival signals of intact Src and other neuronal SFKs. Nevertheless, results of our studies indicate that further investigation into pro-survival signaling mechanism of intact Src and the neurotoxic mechanism of the truncated Src fragment may benefit development of therapeutic strategies for treatment of stroke patients.
Experimental Procedures

Materials

Neurobasal medium, DMEM, Opti-MEM reduced serum medium, B-27 supplement, GlutaMAX-I, and RPMI 1640 medium were purchased from GIBCO (Rockville, MD, USA). The specific SFK inhibitor A419259, penicillin, streptomycin, NMDA, glutamate, SU6656, PP2, DMSO, DNase, trypsin and trypsin inhibitor, L-glutamine, sodium pyruvate, and non-essential amino acids were from Sigma (St Louis, MO). The lactate dehydrogenase (LDH) activity assay kit was from Promega. Src-specific monoclonal antibody (mAb327) that recognises the Src SH3 domain was a gift from Drs Don Fujita, Jeffrey Bjorge and Joan Brugge. Phospho-Src (pSrc-416) antibody, recognizing the phosphorylated consensus autophosphorylation site (corresponding to Tyr-416 of chicken Src or Tyr-418 of mammalian Src) in the activation loop of all Src-family kinases was from BD Bioscience. Antibodies against NMDA receptors (anti-GluN1, anti-GluN2A and anti-GluN2B), Akt and Erk1/2, the phosphospecific pS473 antibody directing at the phosphorylated Ser-473 of Akt and the phosphospecific pErk1/2 antibody directing at the phosphorylated Thr-183 and Thr-185 were from Cell Signaling Inc. Anti-Tubulin antibody was purchased from Abcam. The lentivirus packaging plasmid psPAX2 and envelope plasmid pMD2.G were from Addgene. Lentiviral pLVX-Tight-Puro and pLVX-Tet-On Advanced plasmids were purchased from Clontech. The HEK293FT cell line for generating the lentivirus, OptiPrep, Lipofectamine 2000, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, Calcein AM and Ethidium homodimer-1 (EthD-1) and poly-D-lysine were purchased from Invitrogen. A set of lentiviral vectors pLKO.1 containing the five genes encoding the shRNA for silencing the expression of mouse neuronal Src (Catalog Number:
RMM4534-NM_001025395) was from Open Biosystems. Cell culture dishes and plates were from Nunc.

*Primary cortical neuronal culture*

All experiments involving animals were approved by the Animal ethics committee, The University of Melbourne and were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia. Pregnant mice (C57BL/6) were group-housed (4 mice to a cage) in the Animal Facility in Bio21 Institute at The University of Melbourne under a 12-hour light/dark cycle. All efforts were made to minimize suffering of animals. The animals were provided access to drinking water and standard chow *ad libitum* and monitored daily prior to the experiments. Mouse primary cortical neuronal culture was performed based on the previously described method (Hossain et al., 2013). Pregnant mice (n = 20/22, weight 37.7 ± 0.4 gram, P < 0.0001, gestational day 15-17) were euthanized by CO₂ asphyxiation and the embryos (n = 7/8 per mouse) were collected by Cesarean section. Embryos obtained from mice at Day 15–16 of gestation were used to prepare primary cortical neuron culture. Briefly, the cortical regions of the embryonic brains were aseptically dissected, free of meninges and dissociated in Suspension Buffer [250 ml of Hank’s balanced salt solution (HBSS), 1.16 mM MgSO₄, 3.0×10⁻³ mg/ml BSA]. They were subjected to trypsin digestion at 37 °C for 5 min in Trypsin Digestion Buffer (20 ml Suspension Buffer containing 0.04 mg/ml DNase and 0.2 mg/ml trypsin). Tryptic digestion was stopped by the addition of 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) to the cell suspension and centrifuged (1000×g, 5 min at 23 °C). The pelleted sample was subjected to mechanical trituration in Trypsin Inhibitor Buffer for 30 s. Cells were collected by centrifugation (1000×g, 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 at 37 °C. Cells were plated to a density of 5 x 10^5 cells per well in 24-well plates previously coated with 0.1 mg/ml sterile poly-D-lysine. 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. At 5 days in vitro (DIV), half of the medium was replaced with fresh medium. Cells were allowed to grow till the seventh day (DIV 7) in culture prior to treatment with Src kinase inhibitors, glutamate, calpeptin and glutamate receptor antagonists.

**Western Blotting**

Equal amount of proteins were loaded in each well of a SDS-PAGE gel and separated using running buffer [25 mM Tris, 192 mM glycine, 10% (w/v) SDS] for approximately 80 minutes at 150 V, and then transferred onto a PVDF membrane at constant voltage (100 V) for 60 minutes using Western transfer buffer [25 mM Tris, 192 mM glycine, 10% (w/v) SDS, 20% (v/v) methanol]. The membrane was then blocked with 5% (w/v) non-fat dry milk in Tris buffered saline with Tween 20 (TBST) (0.2 M Tris–HCl, pH 7.4, 1.5 M NaCl, and 0.1% Tween 20). For detection of phospho-specific proteins, the membrane was blocked with 1% (w/v) BSA in TBST. After blocking, the membrane was washed three times (10 min each) with TBST and then probed with primary antibodies overnight at 4 °C and 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.

**MTT assay of neuronal viability**

Primary neurons isolated from fetal mouse cortex (5×10^5 cells per well) were cultured in 24-well plates as described in the previous sections. After treatment with various pharmacological agents (as indicated in Figure 2A), the cells in each well were monitored for their viability by the MTT assay. Briefly, MTT was dissolved in RPMI 1640 medium without phenol red at a stock concentration of 5 mg/ml and filtered using 0.22 µm syringe filter to remove insoluble residues as described previously (Shioda et al., 2007). MTT solution equal to 10% (v/v) of the volume of culture medium (0.5 mg/ml final concentration) was added per well of cells. After incubation at 37 °C for 30 min, the culture medium was removed by aspiration and dried for 10 min. An aliquot of 200 µl DMSO was added to dissolve the formazan crystals formed from reduction of MTT by the mitochondrial reductase of live neurons. To determine the amount of formazan formed, 100 µl mixtures from each well of cells were transferred to a 96-well microtiter plate (Falcon) and the absorbance at 570 nm was measured using FLUROstar Optima (BMG) plate reader. Cell viability was compared as a percentage of the untreated cells (control).

**Assay of LDH released from damaged neurons**

The activity of LDH released from the damaged neurons to the culture medium is a measure of neuronal death. The LDH activity assay was performed in the dark according to the
manufacturer’s protocol. Briefly, 50 μl of culture medium from each well of the culture plate was transferred 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 and was stopped by adding 50 μl of 1 mM acetic acid. The absorbance of whole mixture was measured in triplicate at a wavelength of 490 nm using FLUROstar Optima (BMG) plate reader. The release of LDH was calculated as a percentage of the untreated control.

**Monitoring neuronal survival by Calcein-AM and Ethidium homodimer-1 staining**

Calcein acetoxymethyl (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 all neurons. It is converted by the intracellular esterases of live neurons to form the intensely fluorescent Calcein (excitation wavelength, ~495 nm; emission wavelength, ~515 nm). In brief, primary cortical neurons were cultured in Neurobasal medium without phenol red. The cultured neurons were stained with Calcein-AM and EthD-1 in Neurobasal medium (final concentrations: 2 mM and 0.5 mM of Calcein-AM and EthD-1, respectively) and incubated for 30 min in a CO₂ incubator. After staining, the cells were washed twice with Neurobasal medium. Live and dead cells were visualized using a fluorescence microscope (Leica DMI6000 B).
**Generation of the plasmids for the lentivirus production**

The gene of full length neuronal Src (Src-GFP) with a glycine-Serine-Glycine-Serine (GSGS) linker and a Green Fluorescence Protein (GFP) tag linked to its C-terminal tail was synthesized and inserted in the kanamycin-resistant vector, pMK-RQ (kanR) (GeneArt, Invitrogen). The GSGS-linker was added to minimize the impact of GFP on the regulatory properties and function of Src. Sandilands et al. examined Src-GFP expressed in SYF<sup>−/−</sup> cells and found that it could be inactivated by phosphorylation by CSK and the presence of the GSGS linker and the GFP tag did not affect its activity and function (Sandilands et al., 2004). To facilitate cloning of the synthetic Src-GFP gene in the pLVX-puro vector for lentivirus production, BamHI and EcoRI restriction sites were introduced at the 5’ and 3’-ends of the synthetic genes.

To generate the Src-ΔN-GFP mutant, the Src-ΔN-GFP cDNA was amplified from the pMK-RQ-Src-GFP plasmid. Src-ΔN-GFP lacking the N-terminal segment (residues 1-75) of mouse neuronal Src Sequence (NCBI Reference Sequence: NM_009271.3) was generated with the forward primer (ATCGGGATCCGCCACCATGCAGAGCTGGACCTCTGGCAGG) and the reverse primer for GFP (from 5’- 3’ ends: ATCGGAATTCTCATTTGTACAGCTCGTCCATG). The forward prime also includes the ATG sequence (underlined) as the start codon. The resultant recombinant Src mutant lacks the N-terminal fatty acid acylation motif and the unique domain and contains an additional methionine residue preceding Gln-76 of the original full-length mouse neuronal Src. The cDNAs encoding Src-GFP and Src-ΔN-GFP were subcloned into the lentiviral vector, pLVX-Tight-Puro via the BamHI and EcoRI restriction sites. The resultant pLVX-Tight-Puro-Src-GFP and pLVX-Tight-Puro-Src-ΔN-GFP plasmids were used for lentivirus production. Using a similar
procedure, the gene encoding GFP was introduced into the pLVX-Tight-Puro vector to generate the pLVX-Tight-Puro-GFP plasmid.

**Lentivirus production**

The HEK293FT cells, used for the production of lentivirus, were grown at 37 °C in DMEM complete media supplemented with 10 % foetal 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 they were >80% confluent.

For suppression of Src expression in neurons, the lentivirus was generated by transfecting HEK293FT cells with a mixture of three plasmids and Lipofectamine 2000 (Life Technologies – Invitrogen). In brief, 2.5 µg pMD2.G and 6.5 µg psPAX2 vectors were mixed with 3 µg of pLKO.1 plasmids containing shRNAs against mouse *Src* gene. Each reaction mixture was diluted in 1.5 ml Opti-MEM reduced serum media and incubated for 5 min. 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 (80 % confluence) grown in a 10 cm tissue culture dish. The cells were cultured 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 μm syringe filter. To concentrate the lentivirus, the filtrate was placed in a centrifuge tube containing OptiPrep (~4 ml) as the cushion. The sample was centrifuged at 50,000 × g for 2 h using SW32Ti rotor (Beckman Coulter). After centrifugation, a layer containing the lentiviral particles located between the medium and OptiPrep 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 re-suspended in 20-150 μl of ice-cold PBS (pH 7.4) and stored as 10 μl aliquots at -80 °C.

For expression of recombinant Src-GFP, SrcΔN-GFP and GFP, 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 GFP. 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 Advanced plasmid (3 μg).

**Knockdown of endogenous Src in primary cortical neurons**

For transduction, the cultured neurons (0.5 × 10^6 cells) at DIV 1 to DIV6 were incubated with each of the 5 shRNA lentiviruses at a multiplicity of infection (MOI) ≥ 20. The Neurobasal media was changed after 24 hours. As a control, cells were also transduced with the lentivirus generated by the pLKO.1 plasmid containing non-silencing shRNA (ns shRNA). The effect of transduction of neurons with the shRNA lentiviruses at DIV1 to DIV6 on cell viability is presented in Supplemental Figure S2. For the results presented in Figure 6, neurons were
transduced at DIV2. Experiments to analyze the effects of knockdown of the endogenous Src on the signaling mechanism and survival of the transduced neurons were performed at DIV 7.

Expression of recombinant Src-GFP, Src-\(\Delta N\)-GFP and GFP in cultured cortical neurons

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 Advanced plasmid and (ii) the lentivirus generated with the pLVX-Tight-Puro plasmid containing the gene encoding Src-GFP, Src-\(\Delta N\)-GFP and/or GFP. After incubation at 37 °C in the presence of 5% CO\(_2\) overnight, the culture medium was replaced with fresh medium. Expression of proteins was induced by the addition of doxycycline (1 \(\mu g/ml\)) at DIV5. Experiments to monitor the effect of the recombinant proteins were performed 48 h (i.e. at DIV7) after doxycycline induction.

Immunofluorescence

For immunofluorescence studies, primary cortical neurons were grown on poly-D-lysine coated coverslips for seven days. Cells were washed twice with ice-cold phosphate buffered saline (PBS) then fixed in 4 % (w/v) paraformaldehyde for 20 min at room temperature. Cells were washed with PBS followed by permeabilization using 0.2% (v/v) Triton X-100 in PBS for another 20 min. The permeabilized cells were washed with PBS twice before blocking with 10% (v/v) fetal calf serum in PBS for 1 hour. Cells were washed with PBS and incubated with antibodies against and the three subunits of NMDA receptor (1:250) for 2 h in room temperature. After incubation with primary antibodies cells were washed with PBS twice and incubated with the secondary antibody (anti-rabbit FITC-green for the NMDA receptor subunits) [1:500 in 0.1%
(w/v) BSA in PBS] in the dark for 1 hour at room temperature. Nuclei were visualized using diamidino-2-phenylindole (DAPI) diluted in PBS (1:15000). Finally cells were washed with PBS twice and mounted onto a glass slide using Biomeda Gel/Mount (ProSciTech, Australia). Cells were visualized and images were captured using Leica TCS SP2 confocal microscope with a 100 x 1.35 NA objective (Wetzler, Hassen, Germany).

**Kinase activity assay**

The assay involves a specific peptide substrate of SFK (the Src-optimal peptide of the sequence AEEEIYGEFEAKKKK) that is selectively phosphorylated by SFKs. The assay was carried out at 30 °C for 30 minutes in a 25 µl 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 equal amount of protein from either control or Src kinase inhibitor treated cell lysates. The reaction mixture was vortexed for 1 sec to mix the contents and transferred in a rack kept in a water bath at 30 °C to run the phosphorylation reaction for 30 minutes. The reaction was then stopped by adding 20 µl of 50% (v/v) acetic acid. An aliquot of the reaction mixture was applied to the Whatman P81 phosphocellulose filter paper squares. The filter papers were then washed with 5 × 400 ml of phosphoric acid (0.5% v/v). The papers were washed extensively in phosphoric acid to remove the non-specifically bound [γ-³²P] ATP while the radioactively phosphorylated peptide remained bound to the filter papers (Roskoski, 1983). The final wash was with acetone to facilitate drying of the filter papers. The radioactivity associated with the dried filter papers was determined by scintillation counting. The controls (as blank) containing substrate peptide and ATP but no enzyme (cell lysate) were included to measure the radioactivity from [γ-³²P] ATP non-specifically bound to the filter papers. The
second set of controls (as background) containing [γ-\(^{32}\)P] ATP and the cell lysate but no peptide substrate to assess the amount of the radioactivity contributed from the radioactively labelled cellular proteins phosphorylated by the endogenous protein kinases in the cell lysate.

**Data analysis**

Densitometry analyses for the quantification of the bands on Western blots were done using Image J software (http://rsb.info.nih.gov/ij/). Statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 16 (SPSS, Inc, Chicago, Illinois, USA). The data was reported as the mean ± SD and the statistical significance was determined by parametric procedure as student’s t-test (two-tailed). p < 0.05 was considered statistically significant for all experiments.
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FIGURE LEGENDS

Figure 1. Expression levels of Src and NMDA receptor subunits in cultured primary cortical neurons from Day 1 to Day 7 in culture.

Expression levels of Src, and GluN1, GluN2A and GluN2B subunits of NMDA receptor in cultured cortical neurons were monitored by Western blotting and immunofluorescence. Expression levels of Src, GluN1, GluN2A and GluN2B at different days of culture in vitro (DIV) were determined by Western blotting. The anti-Tubulin Western blots were used as the loading controls. The ratio of densitometric units of anti-Src, anti-GluN1, anti-GluN2A and anti-GluN2B to anti-Tubulin signals at different DIV (Data are represented as mean ± SD and the p values were calculated by Student’s t-test; n = 5, *p ≤ 0.001). A. Expression of Src in neurons. Left: Western blots showing the time-dependent changes of expression level of Src and Tubulin. The ratio of densitometric units of anti-Src to anti-Tubulin signals is shown below the blots. Right: Immunofluorescence of Src in neurons at DIV 7. B. Expression of NMDA receptor subunits in neurons. Western blots showing the time-dependent changes of expression level of GluN1, GluN2A and GluN2B and Tubulin. The ratio of densitometric units of anti-GluN1, anti-GluN2A and anti-GluN2B to anti-Tubulin signals is shown below the blots. Right: Immunofluorescence of NMDA receptor subunits GluN1, GluN2A and GluN2B in neurons at DIV 7. The images of GluN1, GluN2A and GluN2B are merged with the DAPI-stained images.

Figure 2. Effects of over-stimulation of glutamate receptors on neuronal viability and Src integrity.

Cultured cortical neurons at DIV 7 were treated with NMDA (100 µM) alone for 4 h, glutamate (100 µM) alone or in the presence of CNQX (50 µM), MK801 (50 µM) or Ifenprodil (20 µM) for 4 h. A. Neuronal viability determined by MTT assay. The viability of untreated cultured neurons was assigned as 100 %. (Data are represented as mean ± SD, n=5 different cultures, * p<0.05, Student’s t test). B. Western blots of Src and Tubulin in untreated cultured neurons and neurons subjected to different treatment conditions: (i) NMDA for 4 h, (ii) glutamate for 4 h, (iii) glutamate and CNQX for 4 h, (iv) glutamate and MK801 for 4 h, glutamate and Ifenprodil for 4 h and (v) glutamate and calpeptin (20 µM) for 4 h. Proteolysis of Src is observed in neurons treated with NMDA and glutamate.

Figure 3. Effects of expression of recombinant truncated Src (Src-ΔN-GFP) in primary cortical neurons.

Primary cortical neurons were transduced with lentivirus directing expression of either GFP or Src-ΔN-GFP or Src-GFP at DIV 1. Expressions of the recombinant proteins were induced by doxycycline at DIV 5. Effects of their expression were monitored at DIV 7. A. Representative immunoblot showing expression of endogenous and recombinant Src and their effects on the phosphorylation level of Akt at Ser-473. Quantification data at right panel indicates densitometry of Akt and p-Akt level (Data are represented as mean ± SD and the p values were calculated by Student’s t-test; n = 5 different culture *p ≤ 0.001). B. MTT assay to monitor the viability of the cultured neurons (mean ± SD, n = 5; * p< 0.05, student t-test). C. The activity of LDH released from the damaged neurons to the culture medium was monitored as a measure of the extent of
neuronal cell death (mean ± SD, n = 5; * p< 0.05, student t-test). D. The effect of expression of GFP, Src-ΔN-GFP and Src-GFP on neuronal survival is monitored by staining with Calcein AM (green) and Ethidium homodimer-1 (EthD-1; red fluorescence), which stain live (intact) and damaged neurons, respectively. Inset, numbers of EthD-1-positive cells in the control (GFP) and neurons transduced with either Src-ΔN-GFP or Src-GFP (mean ± SD n = 5; * p< 0.05, student t-test).

**Figure 4. Effects of treatment of the selective SFK inhibitor A419259 on neuronal viability.**

**A.** Effect of treatment of cultured neurons with varying concentrations (0 – 5 µM) for 24 h on neuronal viability monitored by MTT assay. **B.** Effect of treating the neurons with 1 µM of A419259 for varying durations (1 – 24 h) on cell viability. **C.** Autophosphorylation status of Src kinase in primary cortical neurons treated with 1 µM of A419259 for 24 h. Data are represented as mean ± SD; n = 3.

**Figure 5. Knockdown of Src kinase with shRNAs decreases neuronal viability.**

Primary cortical neurons at DIV1 were transduced with lentivirus encoding shRNA1 or shRNA2 which can specifically knockdown the expression of Src in cells. The untransduced neurons were used as the control. Neurons transduced with the lentivirus encoding the non-silencing shRNA (ns shRNA) were used as the negative control. At DIV 7, the neurons were harvested and analyzed for the expression of Src and Tubulin, and cell morphology. **A.** Western blots of the crude lysates of the untransduced neurons (control) and the transduced neurons probed with the anti-Src mAb327 antibody and anti-Tubulin antibody. Left Panel: Ratios of Src/Tubulin level in the untransduced and transduced cells. Data represented as mean ± SD, n = 3 *p < 0.05, student’s t-test. **B.** Phase contrast photomicrographs of the untransduced and transduced neurons showing improper neuronal differentiation and death in shRNA1 and shRNA2 treated neurons. **C.** Right: Cell viability determined by the MTT assay (data presented as mean ± SD, n = 5 *p ≤0.0004, Student’s t-test). Left: Assay of the activity of lactate dehydrogenase (LDH) released from the control and transduced neurons (data presented as mean ± SD, n = 5, *p ≤0.0003, Student’s t-test). **D.** Staining of the control and shRNA transduced neurons with Calcein-AM and Ethidium homodimer-1 (EthD-1) which stain live and dead cells, respectively. **E.** Quantification of live and dead cells in control and Src knockdown primary cortical neurons. (Data are presented as mean ± SD, n =5 *p ≤0.0004, Student’s t-test).

**Figure 6. Effects of SFK inhibitors and Src knockdown on the phosphorylation status of neuronal Erk1/2 and Akt.**

**A.** Representative Western blots to monitor the levels of Src, Erk1/2, pErk1/2 (T183 and Y185), Akt and pAkt (S473) in lysates of control neurons, neurons treated with the SFK inhibitors and neurons in which expression of Src was suppressed by shRNA. For Src knockdown, neurons were transduced with shRNA2 lentivirus at DIV 2. At DIV 7, the transduced neurons were harvested for analysis. For SFK inhibitors, neurons at DIV 7 were treated with 1 µM A419259 for 24 h. **B.** Ratios of the immunoreactive signals of Erk1/2 and pErk1/2. **C.** Ratios of the immunoreactive signals of Akt and pAkt (S473) (Data represented as mean ± SD; n = 3)
Figure 7. Src is both a promoter of neuronal survival and an executor of neuronal death.

Under normal physiological conditions, intact Src promotes neuronal survival. It exerts its neurotrophic effect in part by activating the pro-survival kinase Erk1/2. Under excitotoxic conditions, over-stimulation of NMDA receptor by glutamate leads to massive influx of calcium into the cytosol. The sustained increase in cytosol calcium concentration causes over-activation of calpain which cleaves Src to form the truncated Src fragment. The Src fragment induces neuronal death in part by inhibiting Akt. The question marks (?) indicate unknown mechanisms.

Supplemental Figure S1. Effects of treatment of SFK inhibitors on neuronal viability.

A. Autophosphorylation status of Src kinase in primary cortical neurons treated with SU6656 (10 μM) and PP2 (20 μM) for 8 h. B. Assay of Src kinase activity in lysates of untreated neurons and neurons treated with SU6656 or PP2 (data represented as mean ± SD; n = 5, *p ≤ 0.001). Cultured neurons were treated with glutamate (100 μM), SU6656 (10 μM) and PP2 (20 μM) for 8 h. Their viability and integrity were assessed by C. MTT assay and D. LDH assay. Data are represented as mean ± SD; n = 5, *p ≤ 0.001.

Supplemental Figure S2. Effect of transduction of cultured neurons with the Src shRNA at DIV1 to DIV6 on cell viability.

Neurons were transduced with shRNA2 lentivirus at DIV1, DIV2, DIV4 and DIV6. At DIV 7, the transduced neurons were harvested for analysis with MTT assay to monitor cell viability.
Figure 1

A. Days in culture | WB:
---|---
58 kDa | Anti-Src
1.6 | Anti-Tubulin
1.2 | 
0.8 | 
0.4 | 
0.4 | Src/tubulin

B. Days in culture | WB:
---|---
175 kDa | Anti-GluN1
1.6 | Anti-Tubulin
1.2 | 
0.8 | 
0.4 | GluN1/tubulin

175 kDa | Anti-GluN2A
1.6 | Anti-Tubulin
1.2 | 
0.8 | 
0.4 | GluN2A/tubulin

175 kDa | Anti-GluN2B
1.6 | Anti-Tubulin
1.2 | 
0.8 | 
0.4 | GluN2B/tubulin

Images:
- DAPI
- GluN1
- GluN2A
- GluN2B

Scale bars: 10 μm
Figure 2

A. Relative cell viability (% of control)

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>NMDA</th>
<th>Glu</th>
<th>CNQX</th>
<th>MK801</th>
<th>Ifenprodil</th>
<th>Calpeptin</th>
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B. WB:

- anti-Src
- anti-Truncated Src
- anti-Tubulin

NMDA, Glu, CNQX, MK801, Ifenprodil, Calpeptin

$\rho < 0.05$
Figure 3.
Figure 4

A. Cell viability (% of control)

Concentrations of A419259 (μM)

treatment time: 24 h

B. Cell viability (% of control)

Duration of treatment
with A419259 (1 μM)

C. MW (kDa)

Control, A419259 24h

pY418-Src

WB: Anti-pY418 Src

Src

WB: Anti-Src
Figure 5

A. 

WB:
- Anti-Src: 58 kD
- Anti-Tubulin

B.

Control  ns shRNA  shRNA1  shRNA2

C.

Relative cell viability (%)

D.

Calcein AM
- Control  shRNA1  shRNA2  ns shRNA

E.

Calcein AM / EthD-1
- Control  shRNA1  shRNA2  ns shRNA
Figure 6
Supplemental Figure S1
Supplemental Figure S2
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Author/s:
Hossaina, M I; Hoquel, A; Lessene, G; Kamaruddin, MA; Chu, PWY; Ng, IHW; Irtegun, S; Ng, DCH; Bogoyevitch, MA; Burgess, AW; Hill, AF; Cheng, H-C

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