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ORIGINAL ARTICLE TRANSLATIONAL THERAPEUTICS

Combined Inhibition of Fyn and c-Src Protects Hippocampal Neurons and Improves Spatial Memory via ROCK after Traumatic Brain Injury

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Abstract

Our previous studies demonstrated that traumatic brain injury (TBI) and ventricular administration of thrombin caused hippocampal neuron loss and cognitive dysfunction via activation of Src family kinases (SFKs). Based on SFK localization in brain, we hypothesized SFK subtypes Fyn and c-Src, as well as SFK downstream molecule Rho-associated protein kinase (ROCK), contribute to cell death and cognitive dysfunction after TBI. We administered nanoparticle wrapped small interfering RNA (siRNA)-Fyn and siRNA-c-Src, or ROCK inhibitor Y-27632 to adult rats subjected to moderate lateral fluid percussion (LFP)–induced TBI. Spatial memory function was assessed from 12 to 16 days, and NeuN stained hippocampal neurons were assessed 16 days after TBI. The combination of siRNA-Fyn and siRNA-c-Src, but neither alone, prevented hippocampal neuron loss and spatial memory deficits after TBI. The ROCK inhibitor Y-27632 also prevented hippocampal neuronal loss and spatial memory deficits after TBI. The data suggest that the combined actions of three kinases (Fyn, c-Src, ROCK) mediate hippocampal neuronal cell death and spatial memory deficits produced by LFP-TBI, and that inhibiting this pathway prevents the TBI-induced cell death and memory deficits.

Keywords: c-Src; Fyn; ROCK; siRNA; traumatic brain injury

Introduction

Traumatic brain injury (TBI) causes tissue deformation and contusion often associated with intracranial hemorrhage (ICH).[1–5] The primary TBI injury initiates release of many molecules, including thrombin, oxyhemoglobin, reactive oxygen species (ROS), cytokines, and damage-associated molecular pattern molecules (DAMPs) that mediate secondary injury after TBI (left panel in Fig. 1).[1–8] Since many molecules and signaling pathways are implicated in the secondary brain injury post-TBI, blocking a single mediator or pathway may not be clinically effective.[6]

Src family kinases (SFKs) are non-receptor tyrosine kinases that include several family members: Fyn, c-Src, Hck, Lck, and others.[9] SFKs are activated by many trans-membrane receptors including adhesion receptors, tyrosine kinase receptors, G protein-coupled receptors and cytokine receptors.[10] This unique feature of SFKs makes them a point of convergence for these toxic molecules released post-TBI. In addition to our previous studies that showed the increase of thrombin and oxyhemoglobin in cerebrospinal fluid correlates with SFK activation after TBI in rats,[7] other studies reported that high-mobility group protein 1 (HMGB1), a DAMP

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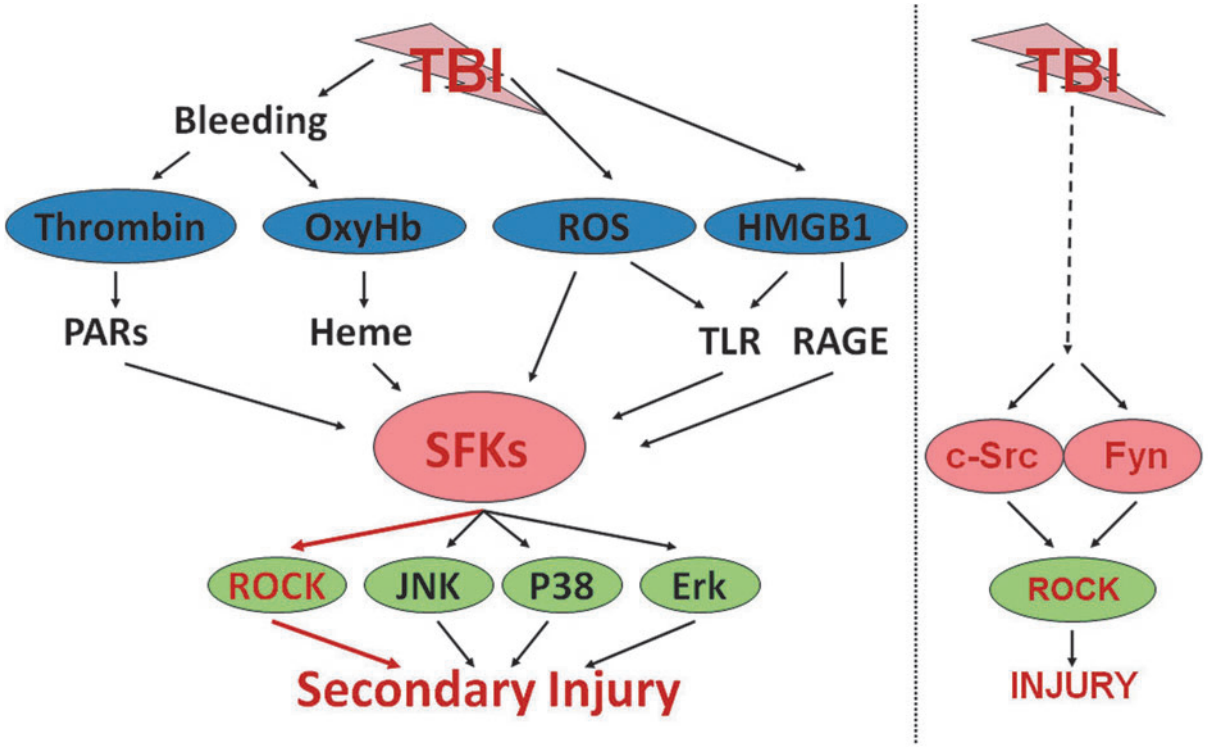

FIG. 1. Diagrams of Src family kinases (SFKs) as a point of convergence for many upstream toxic molecules released post–traumatic brain injury (TBI) and downstream effectors which results in secondary brain injury. Left panel: TBI injury triggers the release of many molecules, including thrombin, oxyhemo¬globin, reactive oxygen species (ROS), cytokines, damage-associated molecular pattern molecules (DAMPs), and others. These molecules activate SFKs through many trans-membrane domains (e.g., adhesion receptors, tyrosine kinase receptors, G protein-coupled receptors and cytokine receptors). The activated SFKs further activate downstream molecules, such as Rho-associated protein kinase (ROCK), Jun N-terminal kinase, P38, Erk, and others, which result in the secondary brain injury after TBI. Right panel: Hypothesis of the signaling pathway of SFK subtypes (Fyn and c-Src) and ROCK, which is proposed to mediate brain injury after TBI. Color image is available online.

released following TBI,[11,12] binds Toll-like receptors and receptor for advanced glycosylated end (RAGE) to activate SFKs (left panel in Fig. 1).[13]

Using the non-specific SFK inhibitor PP2, we reported that inhibiting SFK improved outcomes after TBI and ICH.[7,8] However, it remained unclear which SFK subtypes contribute to secondary brain injury after TBI. Based upon the fact that Fyn and c-Src are highly expressed in hippocampal neurons and other brain regions in adult rodents,[14-16] and Fyn and c-Src have complementary functions,[17,18] we hypothesized that activation of Fyn and/or c-Src contribute to the secondary brain injury following TBI. In addition, SFKs can activate many neurotoxic down-stream signaling pathways including Rho-associated protein kinase (ROCK), Jun, P38, Erk, and others (left panel in Fig. 1).[19–30] Since studying all of these downstream molecules would be challenging, we focused on ROCK because it has been implicated in various types of brain injury[21,31] and there are several inhibitors available.

In this study, we developed the following hypothesis: TBI → Fyn/c-Src activation → ROCK activation → loss of hippocampal neurons → cognitive decline (right panel in Fig. 1). To address this hypothesis, animals received different treatments (e.g., small interfering RNA [siRNA]-scramble, siRNA-Fyn, siRNA-c-Src, combined siRNA-Fyn and siRNA-c-Src, or ROCK inhibitor Y-27632) immediately after TBI. NeuN immunoreactive CA2/3 neurons were examined on Day 1 and Day 16 and cognitive function assessed on Days 12-16 after TBI. The data show that the combined actions of Fyn and c-Src, and the downstream SFK molecule ROCK mediate the hippocampal neuronal death and spatial memory loss following TBI.

Methods

Animals

Sprague-Dawley male rats ($n = 95$ total) weighing 300-350 g were purchased from Envigo, and randomly assigned to experimental and control groups. All experiments and analyses were performed by individuals blinded to the groups. All experimental procedures were performed in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee, University of California at Davis. The manuscript was written to be compliant with the Animal Research: Reporting of In Vivo Experiments guidelines.

Assessment of knocking down efficiency of nanoparticle-siRNA using reverse transcription polymerase chain reaction

Rats ($n = 15$) were divided into five groups (3 rats/group). The siRNA-scramble or siRNA-Fyn/c-Src was dissolved in distilled water at the concentration of 100 μg/5 μL, and incubated with 5 μL nanoparticle (Altogen Biosystems, Las Vegas, NV) at room temperature for 30 min prior to intracerebroventricular (i.c.v.) injection into the right lateral ventricles of the rats. Apart from naïve rats used for blank controls, animals received i.c.v. injections (coordinates: 0.9 mm anterior-posterior, 1.4 mm lateral to the right, and 4.6 mm depth, with respect to bregma) of either 10 μL vehicle control (50% nanoparticle, 5 μL nanoparticle mixed with 5 μL distilled water), 10 μL nanoparticle coupled 100 μg scrambled siRNA controls, 10 μL nanoparticle siRNA-Fyn (100 μg/rat), or 10 μL nanoparticle siRNA-c-Src (100 μg/rat). One day after i.c.v. injections, rats were euthanized, and hippocampi dissected, frozen in liquid nitrogen, and stored at -80°C.

Total RNA was extracted from the brain tissue using mirVana PARIS kit (Lifetech, Carlsbad, CA). Reverse transcription polymerase chain reaction (RT-PCR) was conducted using primers specific for Fyn (Fyn 270 FWD: CTGACGGAGGAGAGGGACG; Fyn 399 REV: GGCTGCGTGGAAGTTGTTGTA) and primers specific for c-Src (c-Src 1224 FWD: GTGCAAAGTGGCCGAC TTTG; c-Src 1363 REV: ACACGTCCGACTTGATG GTG) (Lifetech) according to a standard SYBR Green RT-PCR protocol. 18S ribosomal RNA (rRNA) was used as internal controls for RT-PCR. Statistical differences were determined using analysis of variance (ANOVA) followed by Dunnett's *post hoc* test.

Lateral fluid percussion

Rats were anesthetized with 4% isoflurane (Minrad, New York, NY), intubated, and mounted in a stereotaxic frame. Experimental TBI was produced using a fluid percussion device (VCU Biomedical Engineering, Richmond, VA) as described previously.[7] Briefly, a 4.8-mm diameter craniotomy was performed with a trephine that was centered at 4.5 mm posterior to bregma and 3.0 mm lateral to the sagittal suture on the right parietal bone. A rigid plastic injury tube (modified Luer-Loc needle hub, 2.6 mm inside diameter) was glued over the craniectomy with cyanoacrylate adhesive. After connecting the injury tube to the fluid percussion device, a fluid percussion pulse of 2.15 atm was delivered within 10 sec to achieve moderate TBI.

Treatment groups following TBI in rats

Rats ($n = 39$) were divided into five groups, including sham operation/ siRNA-scramble ($n = 5$), TBI/siRNA-scramble ($n = 10$), TBI/siRNA-Fyn ($n = 8$), TBI/siRNA-c-Src ($n = 8$), TBI/siRNA-Fyn/siRNA-c-Src ($n = 8$). The animals had i.c.v. injections of siRNA-scramble (100 μg/animal, in 10 μL volume with 5 μl distilled water and 5 μL nanoparticle, siRNA-Fyn (100 μg/animal), siRNA-c-Src (100μg/animal), or combined siRNA-Fyn (100 μg/animal) and siRNA-c-Src (100 μg/animal) 1 day prior to TBI or sham surgery in rats. The siRNA-Fyn (Sense, GCUUGUACAGCAUUACUCATT; Antisense, UGAG UAAUGCUGUACAAGCTG), siRNA-c-Src (Sense, GG CUCAUAGAGGACAACGATT; Antisense, UCGUUG UCCUCUAUGAGCCGG) or siRNA-scramble (Ambion, Austin, TX) were wrapped with nanoparticles (Altogen Biosystems) and incubated at room temperature for 30 min prior to being injected into the right lateral ventricles of the rats.

Lateral fluid percussion–induced moderate TBI was performed 24 h after i.c.v. injection of siRNA-nanoparticle complexes. Following spatial memory assessments in the Morris water maze (MWM), the rats were anesthetized and perfused with 4% paraformaldehyde (PFA), and brains were removed for immunohistochemistry.

Treatment with ROCK inhibitor Y-27632 following TBI in rats

Rats ($n = 18$) were divided into three groups (4-6 rats/group) for the acute study: sham operation/vehicle (5% dimethyl sulfoxide [DMSO] in 2 mL saline, intraperitoneally [i.p.], $n = 4$); TBI/vehicle (5% DMSO in 2 mL saline, i.p., $n = 8$); and TBI/Y-27632 (10 mg/kg, i.p., immediately after TBI, $n = 6$). For example, a rat (body weight at 300 g in the group of 10 mg/kg) received i.p. injection of 2 mL saline containing 3.33 mg Y-27632 predissolved in 0.1 mL DMSO. Twenty-four hours after TBI, the rats were anesthetized and perfused with 4% paraformaldehyde, and the brains were fixed for immunohistochemistry.

Rats ($n = 23$) were divided into three groups for the chronic study, including sham operation ($n = 5$), TBI/vehicle ($n = 12$) and TBI/Y-27632 (10 mg/kg, i.p., $n = 7$). The three groups of rats received the same TBI surgery

and treatment (either Y-27632 or vehicle) as in the acute study, except that these animals were sacrificed after spatial memory assessments.

Morris water maze

Spatial memory was assessed using the MWM on Days 12-16 after TBI as described previously.[7] The test apparatus consisted of a large white circular tank (183 cm diameter by 60 cm high) filled with water to a depth of 22 cm. Water temperature was maintained at 24-28 °C. A transparent circular escape platform (12.8 cm diameter, 20 cm high) was placed in a fixed position in the tank 2 cm below the water surface. Four consistent visual cues were located in the test room outside of the maze. Rats were released from one of four starting points (selected randomly on each day for each rat) and allowed 120 sec to find and mount the escape platform. If the rat did not find the platform within 120 sec, the experimenter placed the rat on the platform. The rat remained on the platform for 30 sec before being removed from the maze. The rat received a 4-min inter-trial interval in a warmed holding cage before being returned to the maze for subsequent trials. Rats received a total of four trials per day, one from each starting point, over 5 consecutive days, and probe trials 24 h after the last trial. Mean latency to find the platform was calculated for each day as well as the mean swim speed. Statistical differences were determined using repeated measures ANOVA with assessment days as the repeated variable within subjects followed by Dunnett's *post hoc* test.

Brain processing and immunohistochemistry

After completing probe trials in MWM, rats were anesthetized with 3% isoflurane and transcardially perfused with phosphate-buffered saline (PBS) and then 4% PFA on Day 16 after TBI. After PFA perfusion, the brains were removed, post-fixed 2-6 h, and placed in 30% sucrose in PBS. The fixed rat brains were embedded in frozen section medium and mounted on a microtome. Coronal sections (50 μm) were cut on a freezing microtome. The avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) method was used to perform NeuN (a marker of mature neurons) immunohistochemistry as described previously.[7]

Stereological cell counts

NeuN-stained cells were counted on a microscope (Nikon E600, Nikon, Tokyo) with a motorized stage (Bioprecision2) controlled using computer software (Stereo Investigator™ 8.0) as described previously.[7] Serial sections cut at 50 μm thick were collected from bregma -2.80 mm to approximately bregma -4.16 mm for a total of 30 sections. Every third section was used for NeuN staining for a total of 10 sections per brain. Briefly, the border of CA2/3 of the brain was outlined using a 4× objective. A 100× oil immersion objective (1.4 numerical aperture) with oil condenser was used for cell counting. The NeuN-positive neurons were counted if the soma fell within the counting frame or were on the inclusion line. Estimated numbers of the NeuN-positive neurons in the target brain region were generated by the Stereologer software using the following equation: $N_{obj} = (\sum N)$ (1/SSF) (1/ASF)(1/TSF).[32] In this equation $\sum N$obj indicates the sum of objects sampled in the sections, SSF indicates the section sampling fraction, ASF indicates the area sampling fraction, and TSF indicates the thickness sampling fraction. Statistical differences were determined using an unpaired t-test.

Results

Nanoparticle-based siRNA-Fyn and siRNA-c-Src knock down in hippocampus

The nanoparticle-wrapped siRNAs-Fyn (100 μg/rat, i.c.v.) and siRNA-c-Src (100 μg/rat, i.c.v.) significantly knocked down Fyn and c-Src messenger RNA (mRNA) transcripts to 22% and 43% compared with siRNA-scramble controls ($p < 0.01$, siRNA-Fyn or siRNA-c-Src vs. siRNA-scramble; Fig. 2). In contrast, siRNA-scramble (si-NC) had no effect on hippocampal Fyn and c-Src mRNAs compared with naïve and nanoparticle transfection reagent alone (negative control, TR-alone) controls (Fig. 2).

Effects of combined siRNA-Fyn and siRNA-c-Src on numbers of NeuN$^+$ hippocampal CA2/3 neurons after TBI

Rat brain sections were immunostained using NeuN. In the hippocampus of sham rats, the subdivisions CA1c through CA3c were shaped in a tight U (Fig. 3A), shown as a single layer of densely packed 5 to 6 NeuN$^+$ pyramidal cells deep (Fig. 3B). NeuN$^+$ staining and cell counts showed that the number of CA2/3 NeuN$^+$ cells decreased in the siRNA-scramble treated TBI group compared with sham surgery control (Fig. 3C, 3D, 3K; TBI/siRNA-scramble vs. Sham/siRNA-scramble, $p < 0.01$). Treatment of TBI animals with siRNA-Fyn alone, or siRNA-c-Src alone failed to attenuate CA2/3 NeuN$^+$ cell loss in hippocampus after TBI (Fig. 3E, 3F, 3G, 3H, 3K), while combined administration of siRNA-Fyn and siRNA-c-Src significantly reduced CA2/3 NeuN$^+$ cell loss in hippocampus after TBI ($p < 0.05$, TBI/siRNA-Fyn/ siRNA-c-Src vs. TBI/siRNA-scramble; Fig. 3I, 3J, 3K). Animals that died or lost >20% body weight in TBI/siRNA-scramble, TBI/siRNA-Fyn, and TBI/siRNA-c-Src groups were excluded from this study (Supplementary Table S1).

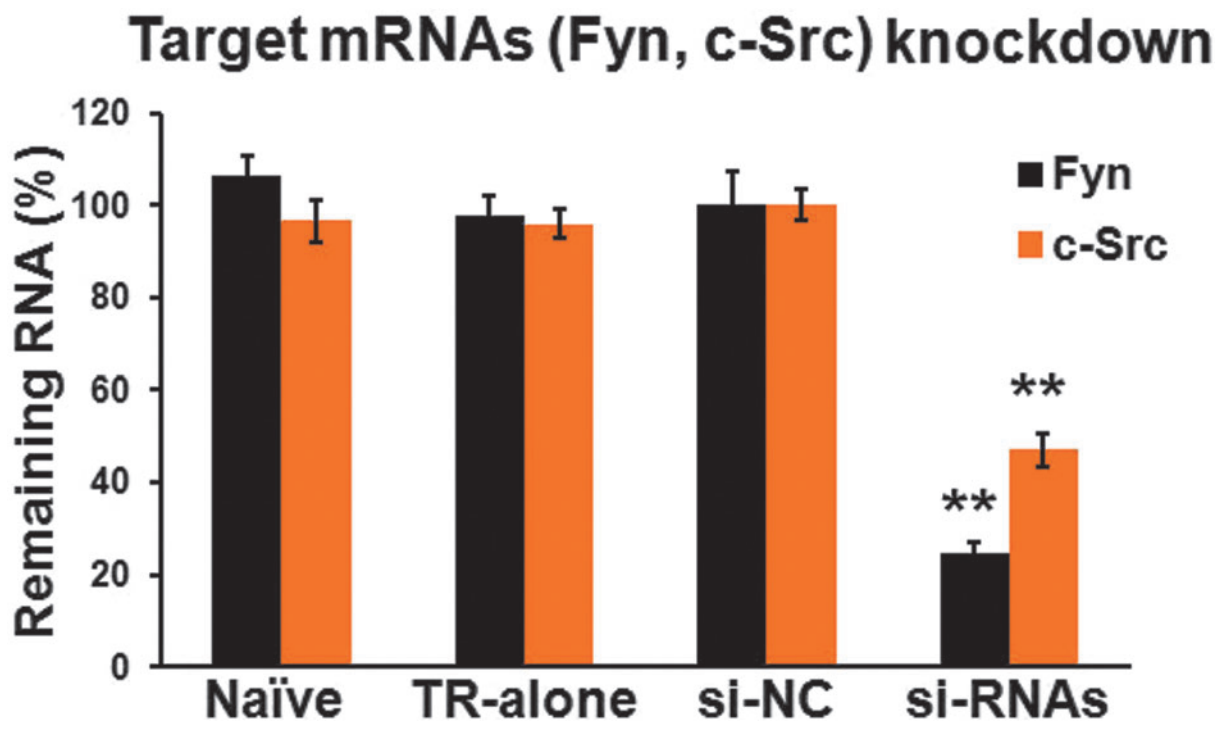

FIG. 2. The nanoparticle-wrapped small interfering RNA (siRNA)-Fyn and siRNA-c-Src significantly knocked down Fyn and c-Src messenger RNAs (mRNAs). Adult Sprague Dawley rats had siRNA-Fyn, siRNA-c-Src, scrambled-siRNA (siNC) or nanoparticle transfection reagent alone (vehicle control, TR-alone) injected into the lateral ventricle. Naïve animals had no cerebral injections. One day later the hippocampus ipsilateral to the injection side was dissected and total RNA extracted from the hippocampus. Reverse transcription polymerase chain reaction for Fyn and c-Src mRNAs was then performed with 18 s ribosomal RNA (rRNA) used as internal controls. $^{**}p < 0.01$ vs. si-NC (analysis of variance followed by Dunnett's *post hoc* test). Color image is available online.

The effects of combined siRNA-Fyn and siRNA-c-Src on spatial memory after TBI

The TBI/siRNA-scramble group had longer latencies to find the hidden platform over the 5 days of testing compared with sham control ($p < 0.01$, TBI/siRNA-scramble vs. Sham/siRNA-scramble; Fig. 4). Treatment with combined siRNA-Fyn and siRNA-c-Src post-TBI improved performance compared with the TBI/siRNA-scramble group ($p < 0.01$, TBI/siRNA-Fyn+siRNA-c-Src vs. TBI/siRNA-scramble; Fig. 4). Neither siRNA-Fyn alone nor siRNA-c-Src alone reduced TBI-induced spatial memory deficits (Fig. 4). In contrast, treatment with combined siRNA-Fyn and siRNA-c-Src decreased swim path length compared with the siRNA-scramble treated TBI group ($p < 0.01$, TBI/siRNA-Fyn+siRNA-c-Src vs. TBI/siRNA-scramble; Supplementary Fig. S1A). There were no significant differences in swim speed and the time spent in the target quadrant between the groups of sham/siRNA-scramble, TBI/siRNA-scramble, TBI/siRNA-Fyn and siRNA-c-Src (Supplementary Fig. S1B, S1C).

The effects of Y-27632 on numbers of NeuN$^+$ hippocampal CA2/3 neurons after TBI

Compared with sham control at 1 day (Fig. 5A, 5B) and 16 days (Fig. 5G, 5H), CA2/3 neurons decreased at 1 day (Fig. 5C, 5D) and 16 days (Fig. 5I, 5J) after TBI. The ROCK inhibitor Y-27632 attenuated TBI-induced CA2/3 neuron loss at both 1 day (Fig. 5E, 5F) and 16 days (Fig. 5K, 5L) after TBI. Quantification showed that the number of NeuN$^+$ cells decreased in the vehicle TBI group compared with sham surgery control ($p < 0.01$, TBI/Vehicle vs. Sham/Vehicle; Fig. 5M). Treatment of TBI animals with Y-27632 increased the number of NeuN$^+$ neuronal cells compared with the vehicle group in the ipsilateral CA2/3 region of hippocampus at 16 days after TBI ($p < 0.05$, TBI/Y-27632 vs. TBI/Vehicle; Fig. 5M). There was high background of 3, 3'-diaminobenzidine (DAB) staining in vehicle-treated TBI group (Fig. 5C, 5D), while only ipsilateral rather than contralateral side stained with high background (Supplementary Fig. S2). Animals that died or lost >20% body weight from TBI/vehicle group were excluded from this study (Supplementary Table S2).

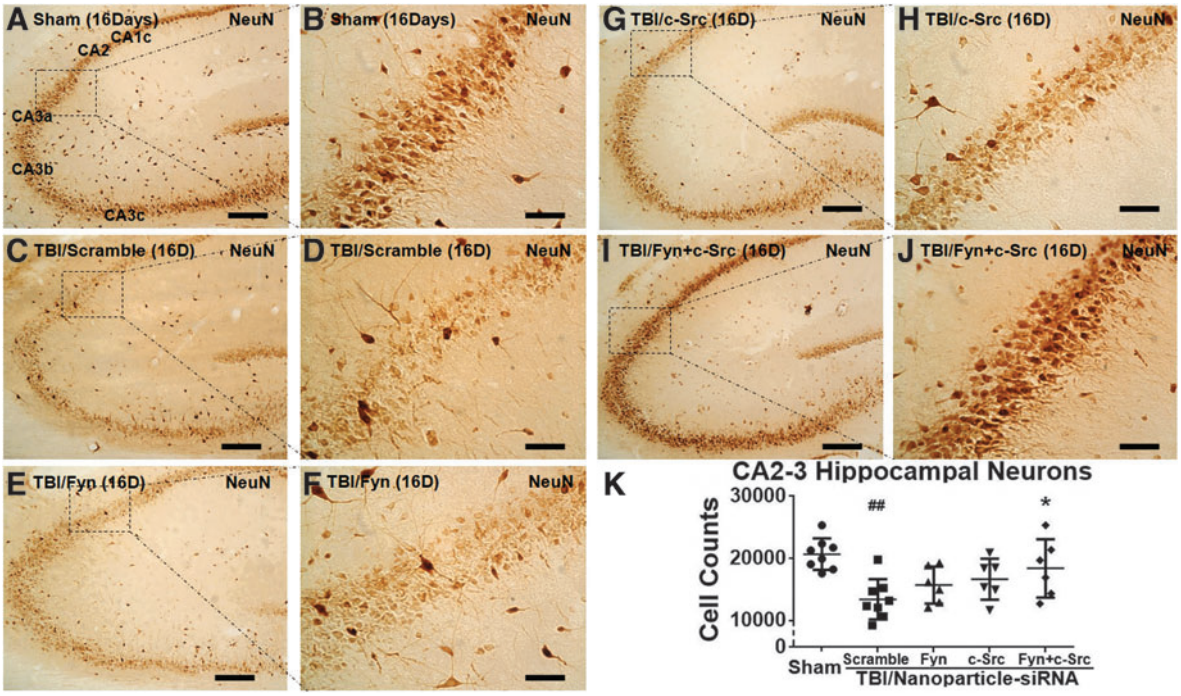

FIG. 3. Combined inhibition of Src family kinase subtypes (Fyn, c-Src) promoted CA2/3 neuron survival at at 16 days after traumatic brain injury (TBI). (**A** and **B**) Subdivisions CA1c through CA3c in the hippocampus of sham rats, shown as a single layer of densely packed 5 to 6 $NeuN^+$ cells (pyramidal neurons) deep. Compared with the sham group, $NeuN^+$ cells decreased in CA2/3 region at day 16 (**C** and **D**) after TBI. Nanoparticle-wrapped small interfering RNA (siRNA) -Fyn or siRNA-c-Src alone failed to attenuate TBI-induced CA2/3 neuron loss after TBI (**E**, **F**, **G**, and **H**). In contrast, combined siRNA-Fyn and siRNA-c-Src prevented TBI-induced CA2/3 neuron loss after TBI (**I** and **J**). Each right panel shows a higher power image of the area within dashed lines in its left panel. **(K)** Quantification of NeuN stained cells in the ipsilateral CA2/3 region of hippocampus between groups at 16 days after TBI. Fewer $NeuN^+$ cells were observed in TBI groups treated with scramble siRNA, siRNA-Fyn, or siRNA-c-Src, as compared with sham surgery control, but more $NeuN^+$ cells were observed in the combined siRNA-Fyn and siRNA-c-Src group compared with the TBI/siRNA-scramble group. Each column represents the mean ± standard error, ## $p < 0.01$ compared with sham group; *$p < 0.05$ compared with TBI/siRNA-scramble group (unpaired t-test). Scale bars: 200 μm (panels A, C, E, G, and I); 50 μm (panels B, D, F, H, and J). Color image is available online.

The effects of Y-27632 on spatial memory after TBI

The TBI/vehicle group had significantly longer latencies to find the hidden platform over the 5 days of testing compared with the sham group ($p < 0.01$, TBI/Vehicle vs. Sham/Vehicle; Fig. 6). Treatment with ROCK inhibitor Y-27632 significantly improved performance compared with the vehicle treated TBI group ($p < 0.05$, TBI/Y-27632 vs. TBI/Vehicle; Fig. 6). There were no statistically significant differences in swim speeds between the groups.

Discussion

Our results demonstrate that inhibiting both Fyn and c-Src (but not either one subtype alone) or inhibiting ROCK prevents loss of hippocampal neurons and reduces cognitive decline after TBI. These data are consistent with our hypotheses "TBI → Fyn and c-Src → ROCK → loss of hippocampal neurons → cognitive decline," and suggest that SFK subtypes (Fyn and c-Src) and ROCK are implicated in the second brain injury after TBI.

This study is an extension of our previous results that showed PP2 improved cell survival and improved spatial memory function following TBI.[7] Since PP2 is a non-specific SFK inhibitor, it remained unclear which SFK subtypes are implicated in the therapeutic efficacy of PP2 to improve outcome after TBI. Therefore, we leveraged the *in vivo* siRNA technology to study which SFK subtypes involve the secondary injury after TBI. In this study we focused on Fyn and c-Src that have many different and specific downstream molecules.

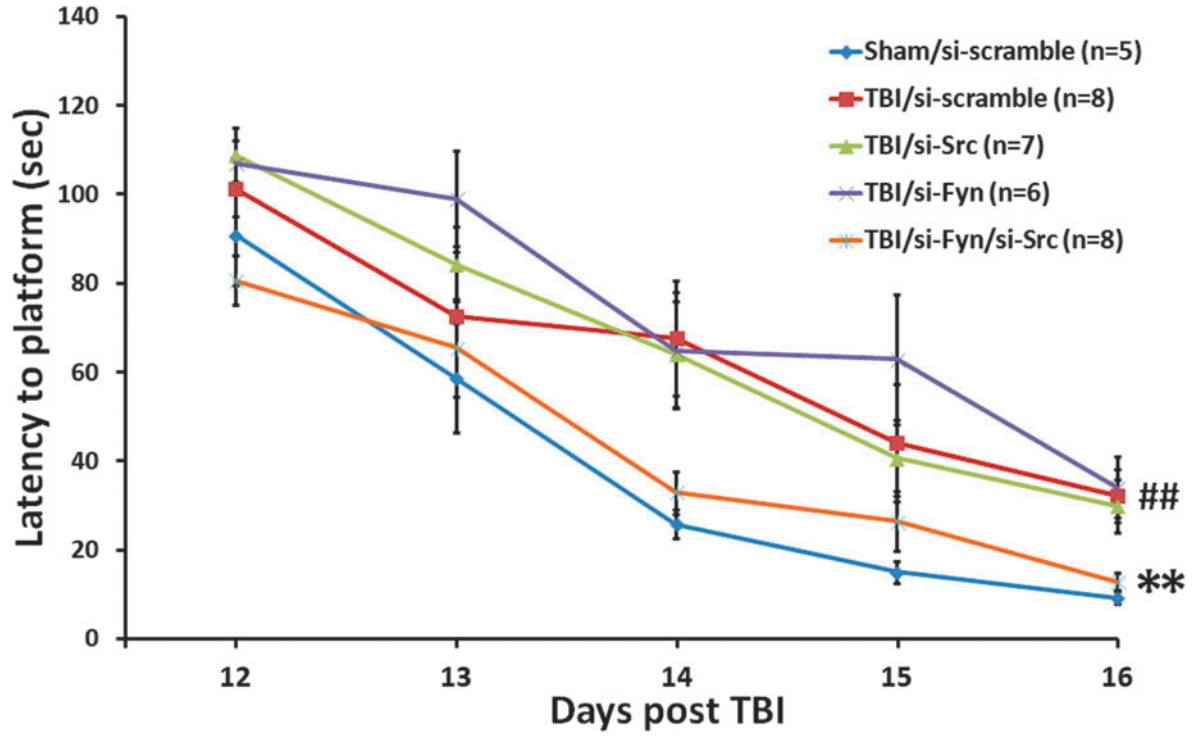

FIG. 4. Combined inhibition of Src family kinase subtypes (Fyn, c-Src) significantly reduced traumatic brain injury (TBI)–induced spatial memory deficits. Spatial memory was examined from 12 to16 days after the experimental TBI using the Morris water maze. The TBI/small interfering RNA (siRNA)-scramble group had significantly longer latencies to find the hidden platform over the 5 days of testing (##$p < 0.01$ vs. sham surgery/siRNA-scramble). Treatment with combined siRNA-Fyn and siRNA-c-Src improved performance compared with the siRNA-scramble treated TBI group (**$p < 0.01$ vs. TBI/ siRNA-scramble). Notably, neither siRNA-Fyn alone or siRNA-c-Src alone reduce TBI-induced spatial memory deficits. The Y-axis shows latency to finding platform (sec). Each point represents the mean ± standard error. Red line: siRNA-scramble-treated TBI (TBI/ siRNA-scramble); green line: siRNA-c-Src alone treated TBI (TBI/siRNA-Src); purple line: siRNA-Fyn alone treated TBI (TBI/siRNA-Fyn); orange line: combined siRNA-Fyn and siRNA-c-Src treated TBI (TBI/siRNA-Fyn/siRNA-c-Src); blue line: sham control (sham/siRNA-scramble) [##$p < 0.01$ vs. sham/siRNA-scramble; **$p < 0.01$ vs. TBI/siRNA-scramble (repeated measures analysis of variance followed by Dunnett's *post hoc* test)]. Color image is available online.

The data show that inhibiting Fyn and Src does not significantly improve learning and memory acquisition in MWM. While we cannot rule out that improvement in MWM acquisition occurred using nonspatial strategies, the most parsimonious explanation is that the reduction in hippocampal cell loss with the combined treatment is likely driving the improvement in MWM acquisition improvement in this study. These data provide supporting evidence that Src and Fyn are implicated in hippocampus-related spatial learning and memory acquisition, but not in nonspatial abilities that do not require hippocampi. Unfortunately, there were no significant differences in probe trial between sham/siRNA-scramble and TBI/siRNA-scramble groups, and thus we could not evaluate if inhibiting Fyn and Src improves memory retention in this study.

ROCK is an important SFK downstream pathway,[19] which plays critical roles and can mediate the death of hippocampal neurons after various types of brain injury in rats.[20,21] Using Y-27632, a non-specific inhibitor of both ROCK1 and ROCK2,[33] we found that Y-27632 protected CA2/3 neurons and improved memory function following TBI in rats. These data suggest Fyn/c-Src phosphorylation of ROCK could mediate the cell death and spatial memory deficits following TBI. We used H_2O_2 to quench the endogenous peroxidase activity prior to

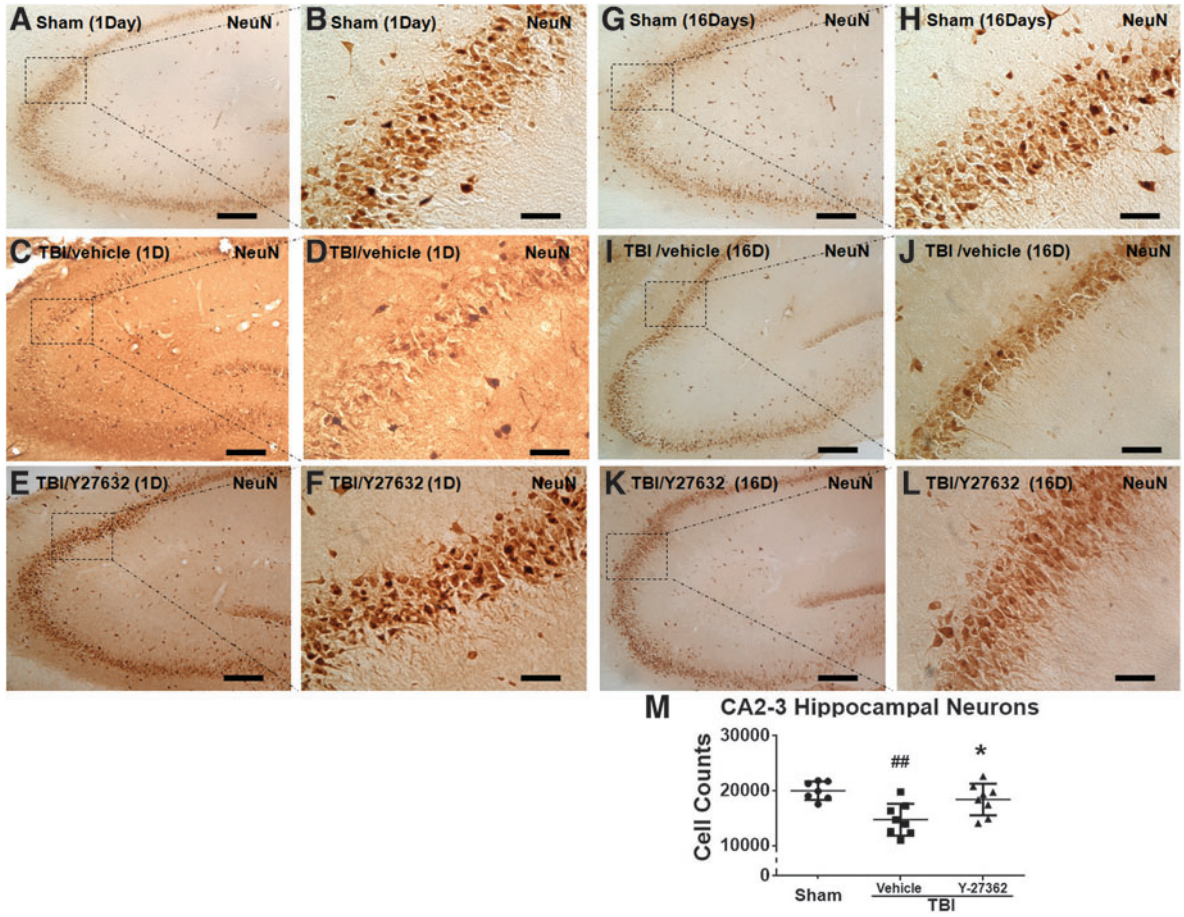

FIG. 5. Rho-associated protein kinase (ROCK) inhibitor Y-27632 improved CA2/3 neuron survival at both 1 day and 16 days after traumatic brain injury (TBI). Compared with the sham groups at 1 day (**A** and **B**) and 16 days (**G** and **H**), there was NeuN$^+$ cell loss in CA2/3 region at 1 day (Panels **C** and **D**) and 16 days (**I** and **J**) after TBI, and the ROCK inhibitor Y-27632 attenuated this TBI-induced CA2/3 neuron loss at both 1 day (**E** and **F**) and 16 days (**K** and **L**) after TBI. Each right panel shows a higher power image of the area within dashed lines in its left panel. **(M)** Quantification of NeuN stained cells in the ipsilateral CA2/3 region of hippocampus between groups at 16 days after TBI. Fewer NeuN$^+$ cells were observed in the vehicle group compared with sham surgery control, but more NeuN$^+$ cells were observed in the Y-27632 group compared with the vehicle group. Each column represents the mean ± standard error, ## $P < 0.01$ compared with sham group; *$p < 0.05$ compared with TBI/vehicle group (unpaired t-test). Scale bars: 200 μm (panels A, C, E, G, I, and K); 50 μm (panels B, D, F, H, J, and L). Color image is available online.

DAB staining, but the high background was still visible in the ipsilateral side. Since contralateral side does not have such background, it indicates the high background was not resulted from staining process but due to samples themselves on ipsilateral side. There might be some other molecules, that were generated in the ipsilateral side at the acute stage while still activated prior to the DAB staining, contributed to the high background. However, the background staining did not compromise the detection and counting of healthy pyramidal neurons in the hippocampus.

Considering these oncogenes/kinases SFKs and ROCK are associated with cell proliferation, cellular repair, and other important cellular processes, silencing of these genes could lead to serious side effects.[27] Therefore, we used acute administration of nanoparticle-based siRNAs (Fyn, c-Src) and Y-27632 to produce a transient decrease of target genes (e.g., Fyn, c-Src, ROCK), and thus avoid side effects caused by long term silencing of these kinases.

We developed a concept of ''aberrant cell cycle disease''[28] that reveals cancers and neurological disorders (including TBI) share a common mechanism- aberrant

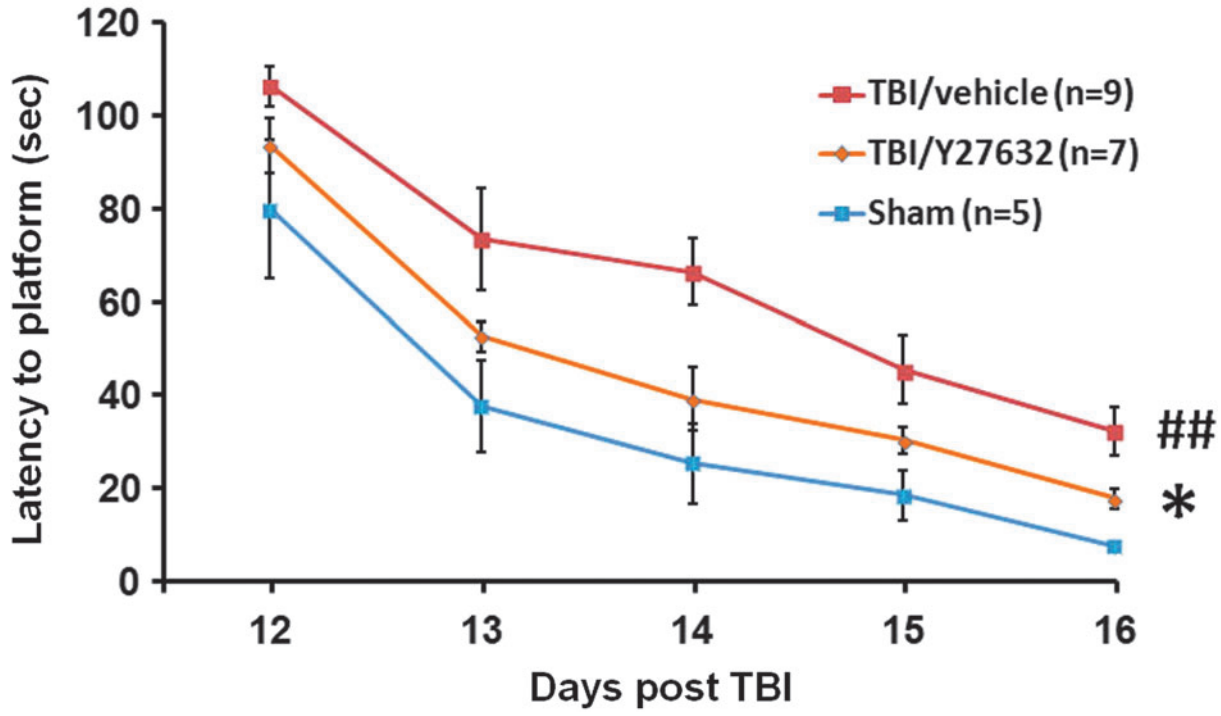

FIG. 6. Rho-associated protein kinase (ROCK) inhibitor Y-27632 reduced traumatic brain injury (TBI)–induced spatial memory deficits. Spatial memory function was examined from 12 to 16 days after the experimental TBI using the Morris water maze. The Y-axis shows latency to finding platform (sec). Each point represents the mean ± standard error. Red line: vehicle-treated TBI (TBI/vehicle); orange line: Y-27632-treated TBI (TBI/Y-27632); blue line: sham control (sham/vehicle). ##$p < 0.01$ vs. sham/vehicle; *$p < 0.05$ vs. TBI/vehicle (repeated measures analysis of variance followed by Dunnett's *post hoc* test). Color image is available online.

cell cycle re-entry, manifested as oncogene/kinase activation and/or tumor suppressor inactivation. This is because oncogene/kinase activation and/or tumor suppressor inactivation are associated not only with tumorigenesis and metastasis in cancers,[34,35] but also with post-mitotic neuronal death and leukocyte infiltration in neurological disorders.[27– 30,36]

This concept is supported by compelling evidence that shows the same oncogene/kinase inhibitor can treat both cancers and neurological disorders, including TBI. There is a long list of such inhibitors: Src inhibitor (PP2), ERK inhibitor (PD98059), mTOR inhibitor (RAD001), CDK inhibitor (roscovitine), and others. For example, several labs reported that Src inhibitor PP2 blocks metastasis and kills cancer cells,[37-39] while we showed that PP2 protects neurons and improves BBB integrity after TBI and other acute brain injury in rats.[7,22,27] Our findings would enrich the list of oncogene/kinase inhibitors that can treat both cancers and neurological disorders with new members (e.g., siRNA-Fyn and siRNA-c-Src, ROCK inhibitor Y-27632), as it had been reported that siRNA-Fyn and c-Src and Y-27632 can treat cancers.

Future studies will need to determine if combined inhibition of Fyn and c-Src protects the BBB and prevents brain edema after TBI. Finally, other SFK members were not tested and will need future study, along with further behavior studies.

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Authors' Contributions

All authors participated in the design, conduct, and/or data analysis of the studies and have read and reviewed the manuscript. DL wrote the manuscript.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2

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