Traumatic Scratch Injury in Astrocytes Triggers Calcium Influx to Activate the JNK/c-Jun/AP-1 Pathway and Switch on GFAP Expression

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Astrocyte activation is a hallmark of central nervous system injuries resulting in glial scar formation (astrogliosis). The activation of astrocytes involves metabolic and morphological changes with complex underlying mechanisms, which should be defined to provide targets for astrogliosis intervention. Astrogliosis is usually accompanied by an upregulation of glial fibrillary acidic protein (GFAP). Using an *in vitro* scratch injury model, we scratched primary cultures of cerebral cortical astrocytes and observed an influx of calcium in the form of waves spreading away from the wound through gap junctions. Using the calcium blocker BAPTA-AM and the JNK inhibitor SP600125, we demonstrated that the calcium wave triggered the activation of JNK, which then phosphorylated the transcription factor c-Jun to facilitate the binding of AP-1 to the GFAP gene promoter to switch on GFAP upregulation. Blocking calcium mobilization with BAPTA-AM in an *in vivo* stab wound model reduced GFAP expression and glial scar formation, showing that the calcium signal, and the subsequent regulation of downstream signaling molecules, plays an essential role in brain injury response. Our findings demonstrated that traumatic scratch injury to astrocytes triggered a calcium influx from the extracellular compartment and activated the JNK/c-Jun/AP-1 pathway to switch on GFAP expression, identifying a previously unreported signaling cascade that is important in astrogliosis and the physiological response following brain injury.

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Introduction

Astrocytes are the most abundant cell type in the central nervous system (CNS). More and more evidence has suggested that they are not "supporting cells" but are indeed multifunctional in brain development, ionic homeostasis, energy metabolism and synaptic signaling (Ransom and Ransom, 2012; Scemes and Giaume, 2006). Under injury and pathological conditions, quiescent astrocytes are rapidly activated and form glial scars, a process called astrogliosis. Astrogliosis is thought to be responsible for a deficit of neuronal repair after injury, because the rapidly formed glial scars create a physical barrier that prevents neuronal regeneration (Miller, 2005; Pekny and Nilsson, 2005; Sofroniew, 2009; Verkhratsky et al., 2012). Therefore, understanding the

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mechanisms of astrogliosis will allow the identification of potential therapeutic targets for reducing glial scar formation and thereby improve neural repair after injury.

One of the most remarkable changes in reactive astrocytes is an increase in glial fibrillary acidic protein (GFAP), followed by cell hypertrophy and hyperplasia (Pekny and Nilsson, 2005). First reported by Eng in 1969 (Eng et al., 1971, 2000), GFAP is an astrocyte specific class III intermediate filament known to provide structural stability and is widely used as a specific marker of mature astrocytes, injury-induced reactive astrocytes, and astroglioma (Eng et al., 2000; Jung et al., 2007). GFAP, however, was found to be more than a pathological and tumor marker. It has been shown to take part in maintaining cell structure (Nawashiro et al., 1998), in the development of white matter and the blood brain barrier (Liedtke et al., 1996), in astrocyte-neuron interaction by modulating synaptic efficacy (McCall et al., 1996), and even in many neurodegenerative diseases such as amyotrophic lateral sclerosis (Yoshii et al., 2011) and Alexander disease (Flint et al., 2012). We have previously shown that GFAP upregulation accompanied hypertrophy and migration of reactive astrocytes after a scratch traumatic injury in primary cultures of astrocytes (Ghirnikar et al., 1994; Yu et al., 1993). Studies on mice deficient in GFAP and vimentin suggested that the upregulation of these astrocytic intermediate filaments is a crucial step in reactive gliosis and glial scar formation (Pekny et al., 1999).

Calcium signals are one of the most universal intercellular and intracellular biological signals which modulate cells, from fertilization, proliferation, and migration, to apoptosis and necrosis. Calcium signals play an important role in the physiology and pathophysiology of astrocytes (Verkhratsky, 2006). Calcium waves in astrocytes were first identified by Smith and colleagues (Cornell-Bell et al., 1990). Since then, their generation was thought to represent a form of glial excitation (Zorec et al., 2012). Calcium waves have been found to be involved not only in astrocyte–astrocyte communication, but also communication between astrocytes and neurons (Fields, 2008). Calcium signals can be induced by many different stimuli, such as glutamate (Cornell-Bell et al., 1990), ATP (Arcuino et al., 2002; Molnár et al., 2011), and mechanical poking (Charles et al., 1991).

In this study, we report that scratch injury can mobilize calcium influx from the extracellular compartment which is then transmitted through gap junctions as calcium waves. Blocking this calcium signal *in vivo* reduces the expression of GFAP and the extent and size of glial scars caused by a stab wound, showing that the calcium signal mediates astrocytic responses to brain injury. The calcium wave might initiate the traumatic signal created by scratch and transmit this signal to the neighboring astrocytes and induce the astrogliosis process. Our results also demonstrate that the calcium signal activates the JNK/c-Jun/AP-1 signaling pathway and switches on GFAP upregulation in astrocytes, an early and essential step in the reactive astrogliosis process.

Materials and Methods

Primary Culture of Astrocytes

Primary cultures of astrocyte were prepared from cerebral cortices of newborn ICR mice (Department of Laboratory Animal Science, Peking University Health Science Center) as reported previously (Chen et al., 2005; Lau and Yu, 2001; Yang et al., 2012). In brief, neopallia freed of meninges and olfactory bulb were cut into small cubes (1 mm^3) in Dulbecco's modified Eagle's medium (DMEM). The tissue was disrupted by vortex for 90 s and the resulting suspension was passed through two sterile nylon Nitex sieves of pore sizes 70 and 10 μm (Spectrum Laboratories). Each dissociated cerebrum was distributed into 35 mm culture dishes (Corning Incorporated) or glass-bottomed cell culture dishes (NEST Biotechnology, China) containing 2 mL DMEM with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific). All cultures were incubated in a Heraus CO_2 incubator (Germany) at 37°C with 5% CO_2 (v/v). Culture medium was changed twice per week and after 2 weeks 7% (v/v) FBS was used thereafter. Confluent cultures used for experiments were at least 4 weeks old and contained at least 95% astrocytes as determined by GFAP staining.

Scratch Wound

Confluent cultures of astrocytes were scratched with a plastic pipette tip according to a preset grid which was equivalent to the removal of approximately 40% of the total cells in the dish to simulate traumatic injury in astrocytes along the scratch wound (Yang et al., 2012; Yu et al., 1993). Immediately following scratch, culture dishes were washed twice with fresh DMEM to remove the debris and incubated with fresh media containing 7% FBS.

Calcium Imaging

Before imaging, cultures were washed once in HEPES-buffered saline (HBS; 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.2) and incubated in HBS containing 5 μM Fluo-3 AM (Biotium) and 0.02% pluronic acid for 20 min at 37°C. Cells were observed with a TCS SP2 confocal microscope (Leica Microsystems, Germany) or an Olympus IX71 microscope (Olympus, Japan). To prevent the influx of extracellular calcium, cells were preincubated for 10 min in Ca^{2+}-free HBS (127 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 5 mM EGTA, 25 mM HEPES, pH 7.2) and experiments were performed in Ca^{2+}-free HBS.

Stab Wound Models

All animal studies were approved by the Institutional Animal Care and Use Committee of Peking University. Sprague Dawley rats (approximately 300 g male) were purchased and maintained in the Laboratory Animal Center of Peking University Health Science Center, and housed in laminal-flow cabinets under specific pathogen-free conditions. Before performing the stab wound, rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.) and the operations were

performed on a stereotaxic apparatus (David Kopf Instruments). The scalp was incised, and a small slit (Bregma from −2 mm to −6 mm, latero-lateral 2.5 mm) made with a dental drill as described (Robinson et al., 2005). Then, 4 μL of 25 μM BAPTA-AM (BAPTA) or DMSO in saline was injected into four points (Bregma from −3 mm to −5 mm, latero-lateral 2.5 mm, at 2 and 4 mm depth from the dura) symmetrically in the areas of the stab in both hemispheres 0.5 h before performing the stab. Each injection was delivered over 2 min, and the needle was left in the same position for an additional 2 min after each injection to avoid reflux along the injection track. Then, a number 23 dissection blade was inserted through the slit to a depth of 5 mm to generate a stab wound. Rats were killed a week after lesion ($n = 3$).

Inhibitor Treatment

Cultures were pretreated with U0126 (blocks the MEK/ERK signal pathway; Promega), SP600125 (inhibits JNK; Sigma-Aldrich), SB203580 (inhibits p38; Sigma-Aldrich), BAPTA (chelates free calcium; EMD Millipore), AG 490 (blocks the JAK/STAT signal pathway; Beyotime, China), or carbenoxolone (CBX, blocks gap junctions; Sigma-Aldrich) for 30 min in serum-free media before scratch injury. During post-scratch incubation, the inhibitor remained in serum-free incubation medium.

Immunofluorescent Staining

For immunocytochemistry, cells were washed three times with ice-cold phosphate buffered saline (PBS) and fixed with 4% Paraformaldehyde (PFA) for 20 min before being permeabilized with 0.3% Triton X100 for 15 min. The cultures were then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature and incubated with primary antibody (anti-GFAP 1:100) at 4°C overnight. Primary antibodies used for immunostaining GFAP were polyclonal rabbit antibody to multiple sclerosis plaque GFAP (Smith et al., 1983). After washing three times with PBS, the cultures were incubated with secondary antibody conjugated with Alexa Fluor 488 (1:200, Invitrogen) for 1 h at room temperature. Hoechst 33342 (2 μg/mL, Sigma-Aldrich) was used to stain nuclei. Finally the cultures were observed with a TCS SP2 confocal microscope (Leica Microsystems, Germany) or a DMI4000 B inverted microscope (Leica Microsystems, Germany).

For immunohistochemistry, brains were removed from rats which had been anesthetized with chloral hydrate, and then transcardially perfused with 300 mL 37°C saline followed by 300 mL 4% PFA in PBS (4°C, pH 7.4). The brains were fixed at 4°C in 4% PFA overnight and cryoprotected by 20% sucrose in PBS at 4°C overnight, then 30% sucrose in PBS at 4°C for 3 days. Brains were frozen on powdered dry ice and cryosectioned (20 μm in thickness). Immunohistochemistry was performed in the same way as the immunocytochemistry described above and observed with a TCS SP2 confocal microscope (Leica Microsystems, Germany) or DMI4000 B inverted microscope (Leica Microsystems, Germany).

Reverse-Transcription Polymerase Chain Reaction and Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent (Life Technologies). RNA (2 μg) was used for RT with MMLV reverse transcriptase (Promega). PCR and real-time PCR were performed according to our previously published method (Chen et al., 2005). Quantitative real-time PCR was carried out with Taqman Mix (Life Technologies) in 20 μL of final volume using a 7500 Real-Time PCR System (Life Technologies). Samples were run for 40 cycles at default thermal cycling conditions for Taqman Real-Time PCR (stage 1: 1 cycle, 50°C for 2 min; stage 2: 1 cycle, 95°C for 10 min; and stage 3: 40 cycles, 15°C for 15 s, 60°C for 1 min). Relative gene expression values were calculated with the $2^{-\Delta\Delta Ct}$ method. GFAP primers and probe were from ABI (Life Technologies). 18S was used as a control (Forward: 5′-AACGAGACTCTGGCATGCTAACT-3′, Reverse: 5′-CGCCACTTGTCCCTCTAAGAA-3′, Probe: 5′-TTACGCGAC CCCCGAGCGG-3′).

Western Blot Analysis

For testing the activities of p-JNK and p-c-Jun, cells were lysed with NP-40 lysis buffer [20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% NP-40, 1 mM Phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail (Roche, Sweden)]. For testing the level of cytoskeleton protein, cells were lysed with RIPA buffer [20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% NP-40, 1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, protease inhibitor cocktail (Roche, Sweden)]. Cells were lysed in their respective lysis buffers for 30 min on ice and centrifuged at 12,000*g* at 4°C for 30 min. Equal amounts of proteins in the supernatant were loaded for each Western blot analysis. Proteins were separated by SDS-PAGE and then transferred to a PVDF membrane (Millipore) for 2 h at 200 mA. Primary antibodies (GFAP, 1:5,000, Cell Signaling Technology; p-JNK, 1:1,000, Cell Signaling Technology; p-c-Jun, 1:500, Santa Cruz Biotechnology; β-actin, 1:5,000, Sigma; GAPDH, 1:5,000, Cell Signaling Technology) were incubated in 3% BSA in Tris-buffered-saline with Tween 20 (TBST) at 4°C overnight and then in HRP-conjugated secondary antibodies (1:5,000, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The signals were detected by chemiluminescence with ECL solution (Santa Cruz Biotechnology).

Plasmid Construction and Luciferase Reporter Assay

The 7X AP-1 binding site sequences (TGACTCA) from the promoter region of GFAP (−1,610 bp relative to translation start) were inserted into pGL3-Basic (Promega). A luciferase reporter assay was performed followed the protocol of the pGL3 Luciferase Reporter Vectors Technical Manual (Promega). Briefly, cells were transfected using 3 μg plasmid and 4 μL Lipofectamine® 2000 (Life Technologies) per 35 mm culture dish. Twenty-four hours after transfection the cells were used to perform experiments.

Quantitative Chromatin Immunoprecipitation Assay

Quantitative chromatin immunoprecipitation (ChIP) analysis for the GFAP promoter region was performed as previously described (Buira et al., 2010) with some modifications. Briefly, ChIP assays were performed on primary cultures of astrocytes. Control and cells 1 h after scratch were fixed with 1% formaldehyde in PBS at 37°C for 10 min before being lysed with lysis buffer [1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitor cocktail (Roche, Sweden)].

The lysed cells were collected into a 1.5 mL prechilled tube and then processed ultrasonically to produce approximately 700 bp DNA fragments. After centrifugation, an aliquot of supernatant was removed from each sample and diluted (1:5) with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1, protease inhibitor cocktail) and used as a loading control for the experiment (input). Supernatants from each sample were diluted with dilution buffer (1:10) and incubated with 5 μg anti-c-Jun antibody for ChIP (Santa Cruz Biotechnology) or rabbit IgG (Sigma-Aldrich) at 4°C overnight. Prewashed protein A beads (10 µL, GE Healthcare) were added and then incubated for 4 h at 4°C. The immunoprecipitant was collected by centrifugation and washed successively with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.1) for 10 min each at 4°C. The protein–DNA complex was then eluted from the beads with elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 10 min. DNA was separated from protein by incubation at 65°C for 6 h and purified with a QIAquick PCR Purification kit (Qiagen, Germany). The binding activity of AP-1 to its binding site on the GFAP promoter was examined by real-time PCR with primers (Forward: 5′-AGGCTGTTGCTATGAC-3′, Reverse: 5′-CTATGCCAA GGTGAGT-3′). Samples were run for 40 cycles (95°C for 30 s, 60°C for 1 min, 72°C for 30 s). Amplification specificity was confirmed by analysis of melting curves.

Statistical Analysis

All analyses were performed using Prism 5.0 statistical software (Graph Pad Software). Significance tests were performed using one-way ANOVA followed by Newman–Keuls *post hoc* test. Values were considered statistically significant at a 5% level of significance (P 0.05). Graphic representations were mean ± SEM.

Results

Scratch-Induced Calcium Waves in Primary Cultures of Astrocytes

In primary cultures of astrocytes, scratch injury triggered an increase of intracellular calcium levels in cells along the edge of the wound, and the calcium signal spread inwards as a wave (Fig. 1A and Supp. Info. Video 1). The calcium wave propagation took about 1 min to travel 600–800 μm (Fig. 1B), with a speed declining from about 47–4 μm/s as the wave moved further away from the wound (Fig. 1C). By comparing the ratio of the fluorescence intensity of the calcium signal in cells before and after scratch and at different distances from the scratch edge, we noticed that the calcium signal in cells along the edge (within 100 μm from the wound) was stronger and lasted longer than in cells further away (more than 100 μm) (Fig. 1D). High-magnification imaging of a single cell demonstrated a polarized calcium signal mobilized from the area near the scratch injury (Fig. 1E and Supp. Info. Video 2).

Scratch-Triggered Calcium Waves Spread Through Gap Junctions

Calcium wave propagation was demonstrated to be mediated through gap junctions using the gap junction inhibitor CBX (Fig. 2A). The CBX effect was dose-dependent, and 200 µM CBX completely blocked the scratch-triggered calcium wave propagation (Fig. 2B). At the same time, we observed a slight increase of the intracellular calcium signal intensity in some cells in the control culture without scratch under CBX treatment. This suggested that CBX may not only be a gap junction inhibitor, but might also induce a release of calcium from intracellular sources; therefore, its blockage of calcium wave propagation is not through inhibition of calcium release (Fig. 2C).

Source of Calcium was from the Extracellular Compartment

BAPTA chelates both intracellular and extracellular calcium ions. BAPTA treatment (10 µM) in astrocytes during scratch could completely inhibit the generation of the scratch-triggered calcium wave (Fig. 3A). The intensity and duration of the calcium signal in cells along the edge of the scratch wound were also significantly reduced (Fig. 3B). In order to elucidate the source of calcium for generating the calcium wave in the scratch-injured astrocytes, experiments were performed in calcium-free HBS. We found that no calcium wave was generated in astrocytes in the absence of extracellular calcium (Fig. 3C). These observations indicated that the calcium signals and waves generated in astrocytes after scratch were from an influx of calcium ions from the extracellular compartment.

Scratch-Induced GFAP Upregulation was Mediated Through the JNK/c-Jun Pathway

We examined the changes of GFAP levels in control and scratched astrocytes in primary cultures by Western blot (Fig. 4A). The levels of GFAP in scratched astrocytes were significantly higher than in the control. Under similar experimental conditions, we observed that scratch wound activated JNK and c-Jun in astrocytes. The phosphorylation of JNK was detected within 5 min after scratch and it reached a peak of about 220% of the basal level at 0.5 h, and then gradually returned to the basal level by 24 h (Fig. 4B).

c-Jun, one of the downstream targets of JNK, was also phosphorylated after scratch with a time course quite similar to JNK phosphorylation. c-Jun phosphorylation reached a peak at 0.5 h and returned to basal levels at 24 h (Fig. 4C). Reverse-transcription polymerase chain reaction and real-time PCR results showed that the inhibition of JNK activity, but not the inhibition of other mitogen-activated protein kinases (MAPKs) or JAK/STAT, suppressed GFAP gene transcription (Fig. 4D). To examine the effect of inhibiting JNK on GFAP

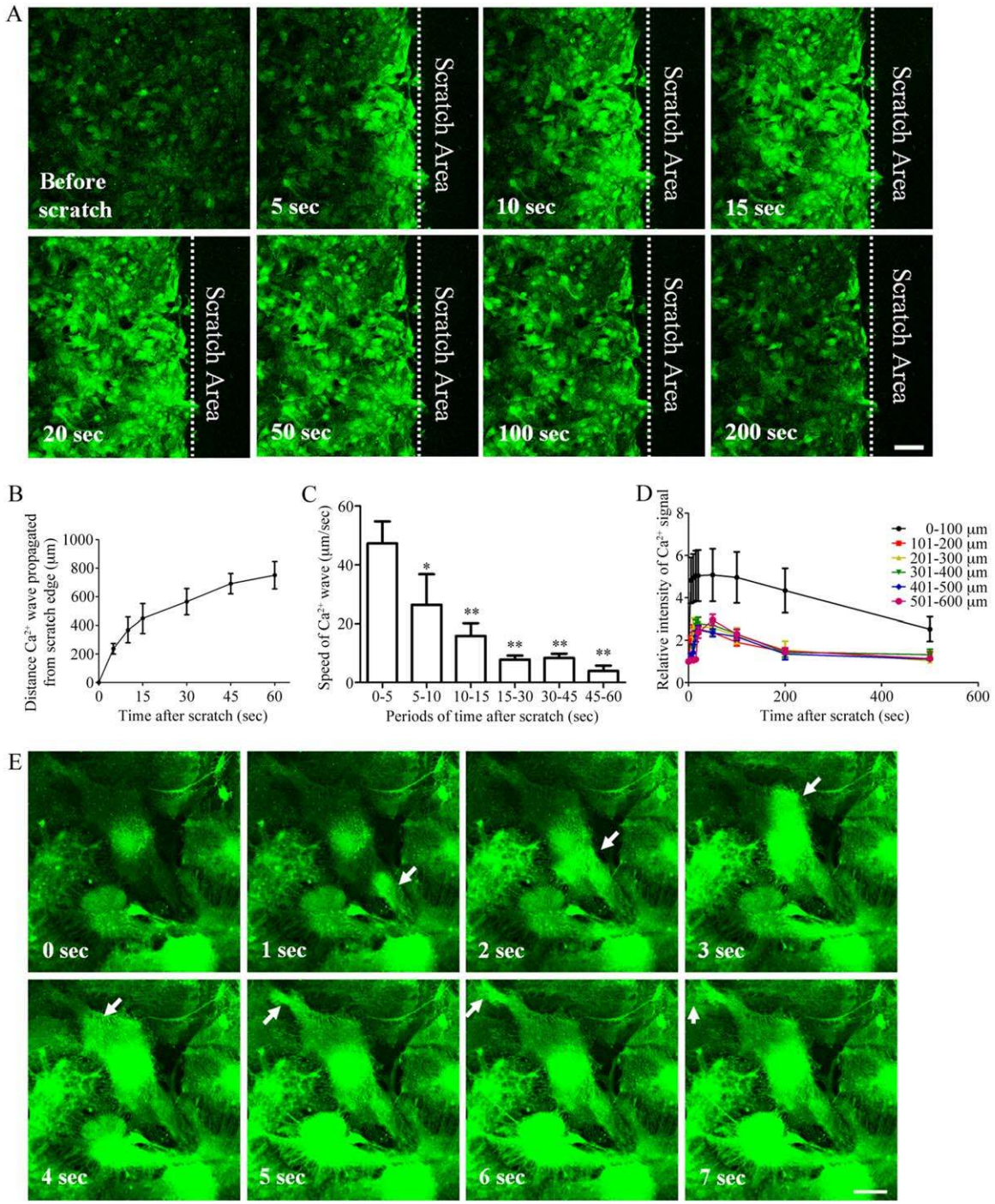

FIGURE 1: Scratch-induced calcium waves in primary cultures of astrocytes. (A) Time lapse recording of a scratch induced calcium wave between astrocytes with the calcium indicator Fluo-3 AM. The time labels reflect the time points after scratch (s). Dotted lines show the scratch edge. Bar = 100 μm. (B) Distance of calcium wave propagation from the scratch edge at different time points after scratch. *n* = 3. (C) Speed of calcium wave propagation after scratch. **P*<0.05, ***P*<0.01 compared with 0–5 s, *n* = 3. (D) Change of calcium signal at different distances from the scratch edge (*n* = 5). (E) Time lapse recording of a scratch-induced calcium wave in single astrocytes with the calcium indicator Fluo-3 AM. The time labels reflect the time points (s) after the calcium signal was transmitted into the cell in the center of the figures. Arrows show the edge of calcium wave propagation. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

levels in reactive astrocytes, Western blots demonstrated a dose-dependent suppression of GFAP protein levels by SP600125 in astrocytes 12 h after scratch (Fig. 4E). The DMSO control did not show any effect on the GFAP RNA or protein levels. We also treated cultures with 20 and 40 μM SP600125 and confirmed the reduction in GFAP protein levels by immunostaining (Fig. 4F).

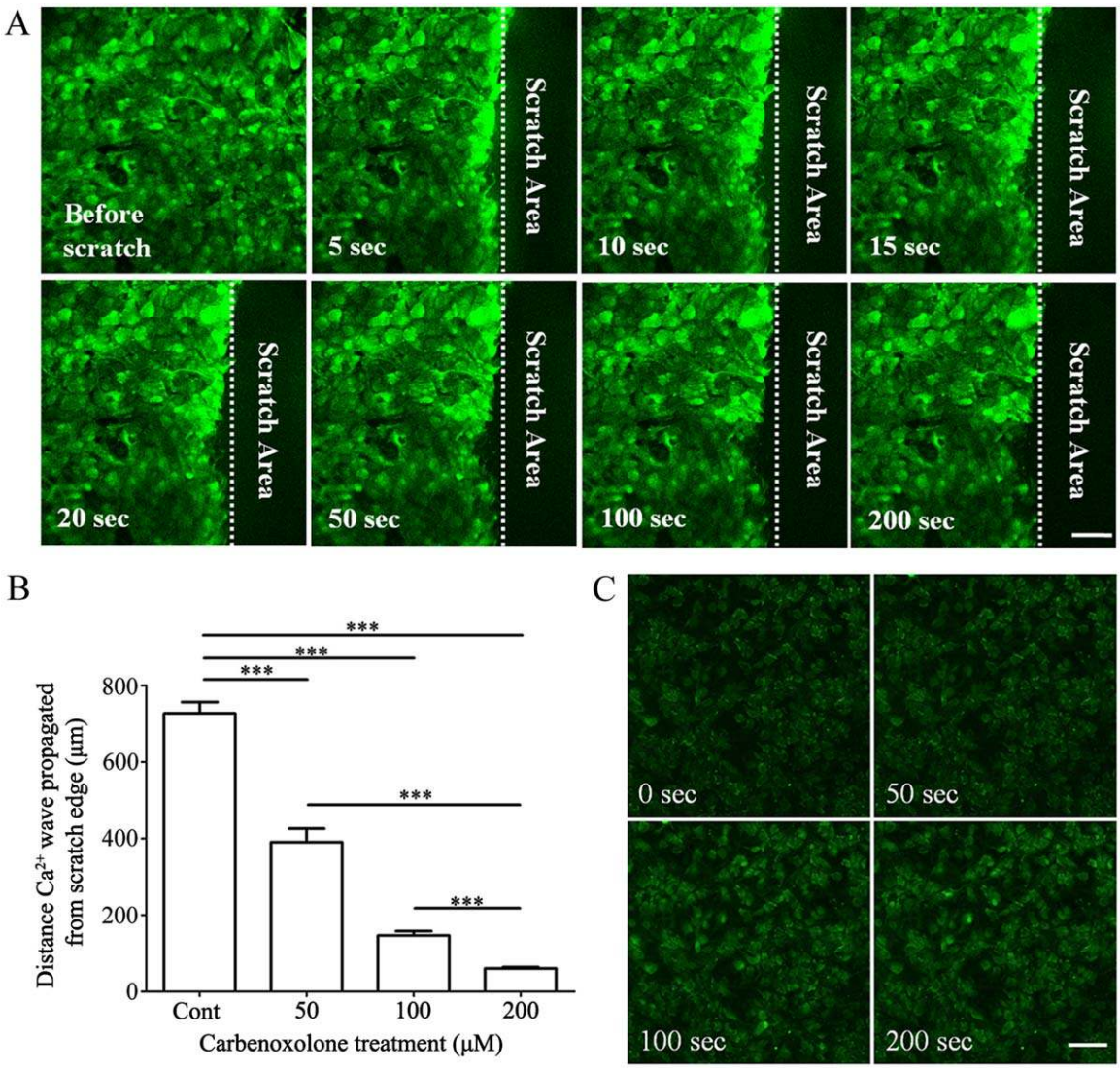

FIGURE 2: Scratch triggered calcium waves spread through gap junctions. (A) Time lapse recording of a scratch induced calcium wave pretreated with 200 μM Carbenoxolone (CBX). The time labels reflect the time points after scratch (s). Dotted lines show the scratch edge. Bar = 100 μm (original videos are in Supp. Info.). (B) Distance of calcium wave propagation from the scratch edge after scratch when astrocytes were pretreated with different concentrations of CBX (n = 3). Cont: control without CBX treatment, ***P<0.001 compared with Cont. (C) Time lapse recording of CBX (100 μM) induced calcium signal in astrocytes. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Calcium Activated JNK/c-Jun to Regulate GFAP Levels

BAPTA at 10 μM inhibited scratch activation of JNK phosphorylation in scratched astrocytes (Fig. 5A). At the same time, phosphorylation of c-Jun was also suppressed and remained at the basal level (Fig. 5B). GFAP levels and the activities of both JNK and c-Jun were not affected by DMSO. The effect of BAPTA on GFAP protein levels in scratched astrocytes was dose dependent, as observed in cultures 12 h after scratch (Fig. 5C). This was detected from the immunostaining of scratched astrocytes, in which GFAP fluorescence intensity was decreased with an increase in BAPTA concentrations (Fig. 5E). GFAP transcription after scratch was also inhibited with BAPTA treatment in a dose-dependent manner, as determined by real-time PCR (Fig. 5D).

The Calcium/JNK/c-Jun Signaling Pathway Switched on GFAP Upregulation Through AP-1

There is an AP-1 binding site in the GFAP gene promoter region at −1,528 bp (Fig. 6A). We examined the AP-1 activity in scratched astrocytes with the 7X AP-1 luciferase reporter assay and observed an increase in AP-1 activity in parallel to the phosphorylation of c-Jun (Fig. 6B). It is interesting to observe that scratch related activation of AP-1 could be inhibited by SP600125 (Fig. 6C). We then examined

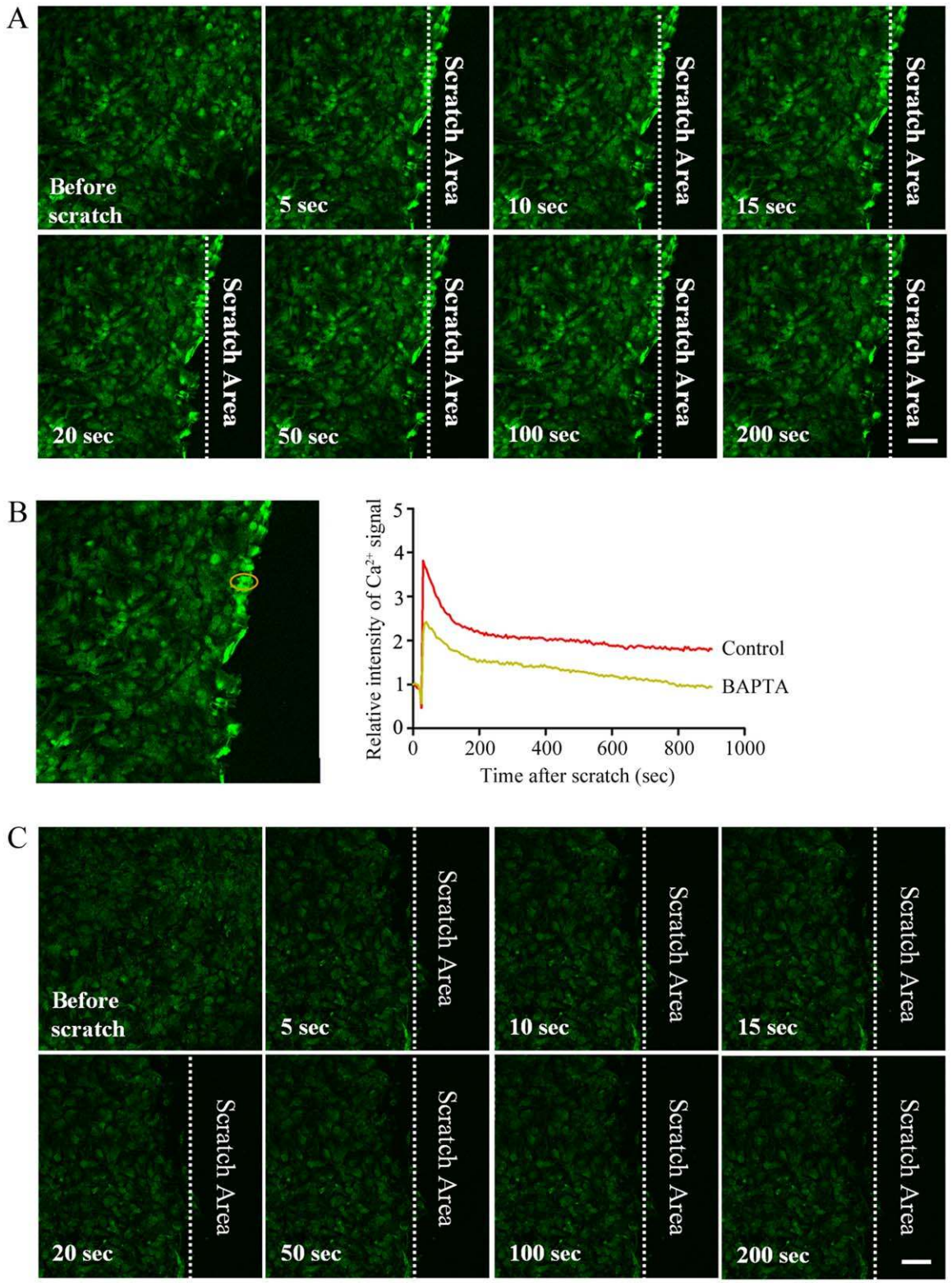

FIGURE 3: The source of calcium was from the extracellular compartment. (A) Time lapse recording of the scratch induced calcium wave between astrocytes pretreated with 10 μM BAPTA with the calcium indicator Fluo-3 AM. The time labels reflect the time points (s) after scratch. Dotted lines show the scratch edge. Bar = 100 μm. (B) Differences of the calcium signal at the scratch edge between astrocytes pretreated with 10 μM or without BAPTA treatment. (C) Time lapse recording of the calcium wave in astrocytes pretreated with Fluo-3 AM and calcium-free medium and scratched in calcium-free medium. Dotted lines showed the scratch edge. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

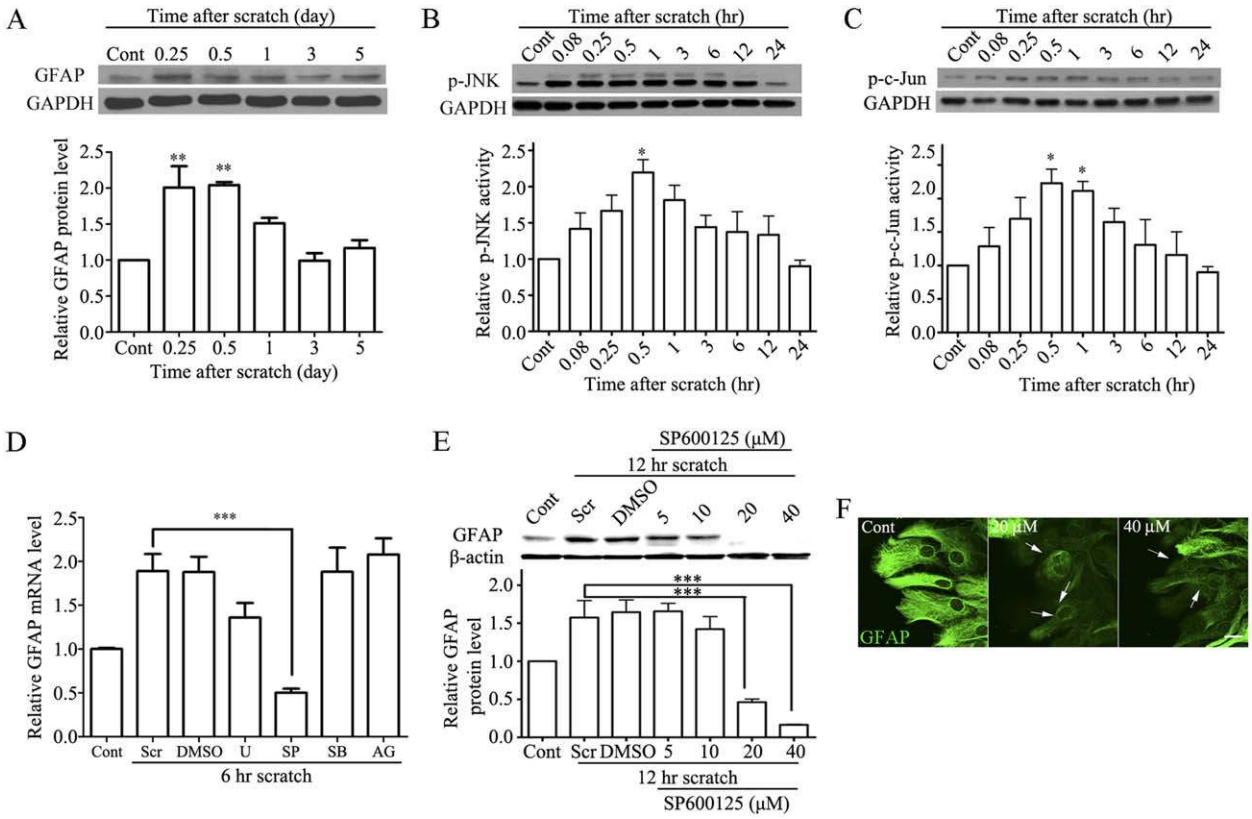

FIGURE 4: Scratch-induced GFAP upregulation was mediated through the JNK/c-Jun pathway. (A) Western blot measurements of GFAP protein levels at different time points after scratch, **P<0.01 compared with Cont, n = 4. (B) Activity of p-JNK at different time points after scratch. Cont: control without scratch, *P<0.05 compared with Cont, n = 4. (C) Activity of p-c-Jun at different time points after scratch. Cont: control without scratch, *P<0.05 compared with Cont, n = 4. (D) Real-time PCR measurements of the levels of GFAP mRNA in astrocytes at 6 h after scratch with treatments of different MAPK inhibitors. Cont: control, Scr: scratch. DMSO: scratch+DMSO treatment, U: scratch+U0126 (20 μM) treatment, SP: scratch+SP600125 (20 μM) treatment, SB: scratch+SB203580 (20 μM) treatment, AG: scratch+AG 490 (3 μM) treatment. **P<0.01 compared with Scr, n = 3. (E) Levels of GFAP protein in astrocytes 12 h after scratch under treatments with different concentrations of SP600125. Cont: control without scratch, Scr: scratch. DMSO: scratch+DMSO treatment, ***P<0.001 compared with Scr, n = 4. (F) Confocal image of GFAP in astrocytes along the scratch edge under treatments with different concentrations of SP600125. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

whether the activated c-Jun could bind to the conserved AP-1 sequence in the GFAP gene promoter region with a ChIP assay. In the control cultures, the binding of c-Jun with the AP-1 sequence was rather weak, suggesting a low transcriptional activity of the GFAP gene by the AP-1 complex. Real-time PCR demonstrated a prominent increase of c-Jun binding with AP-1 in astrocytes 1 h after scratch. The increase in c-Jun/AP-1 binding was reduced by BAPTA and SP600125 treatments (Fig. 6D). These results clearly demonstrated that calcium influx triggered by scratch could activate the JNK/c-Jun signaling pathway and switch on GFAP transcription through enhancing the AP-1 binding to its recognition sequence in the GFAP gene promoter region.

A Calcium Signal was Involved in Astrogliosis in vivo

In the stab wound model, GFAP protein levels in BAPTA treated cortices were compared with the control a week after the stab wound was inflicted. We observed a significant inhibition in scar formation in the BAPTA treated cortex as compared with control (Fig. 7A). By comparing the intensity of GFAP fluorescence, we also observed a lower GFAP staining intensity in astrocytes around the stab wound in the BAPTA treated cortex (Fig. 7B). A reduction in the size of the glial scar area in the BAPTA treated cortex (≈0.16 mm^2) compared with the control (≈0.44 mm^2) was also noted (Fig. 7C). A reduction in cell size of reactive astrocytes in the BAPTA-treated cortex (≈146 μm^2) was also observed as compared with the control cortex (≈369 μm^2) (Fig. 7D) indicating the inhibition of hypertrophy by BAPTA.

Discussion

Astrocyte activation is thought to inhibit neuronal repair after CNS injury due to the rapid formation of a physical barrier—glial scars (Pekny and Nilsson, 2005; Sofroniew, 2009). Upregulation of GFAP is an important and prominent feature

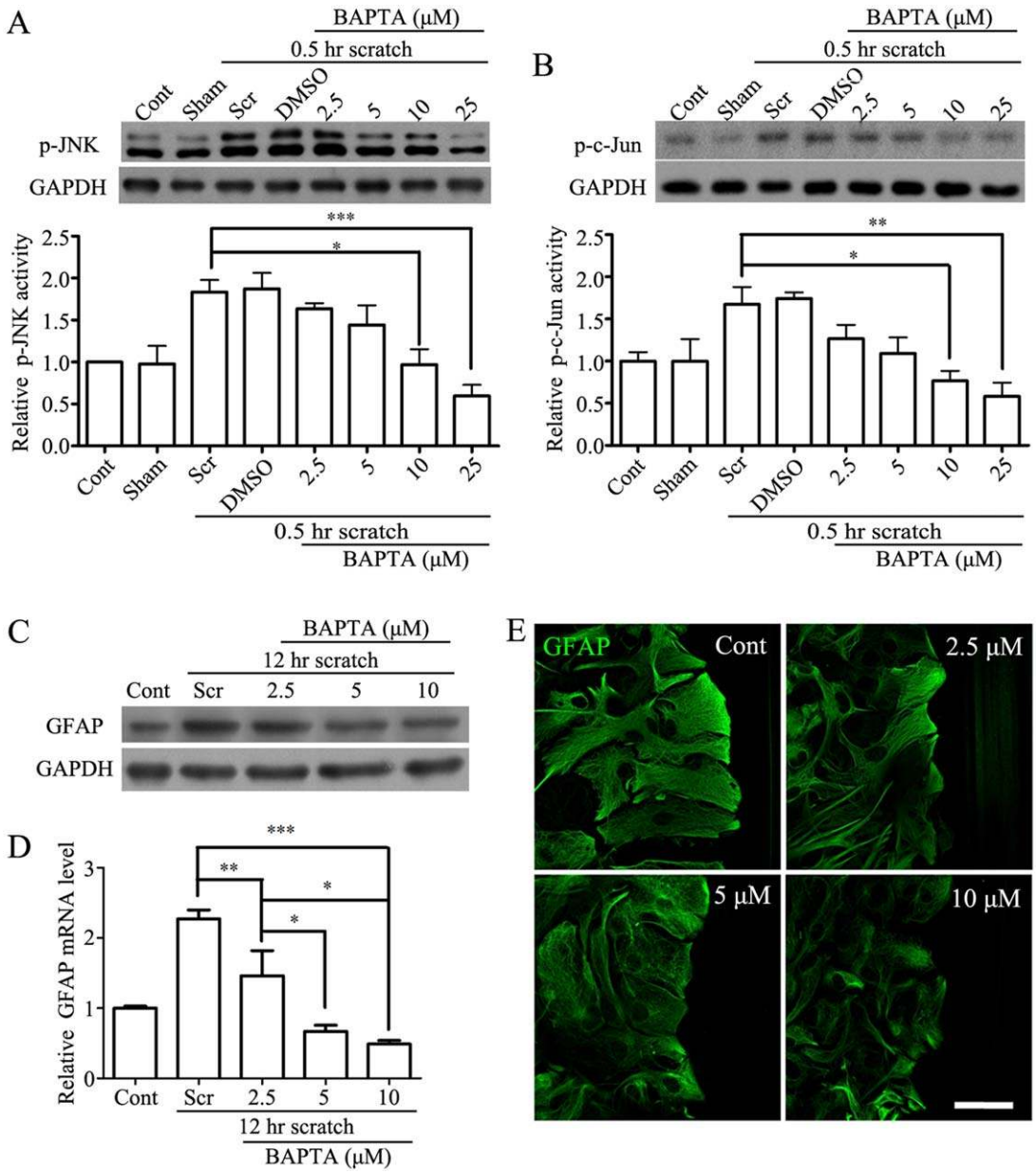

FIGURE 5: Calcium activated JNK/c-Jun to regulate GFAP levels. (A) Activity of p-JNK in astrocytes pretreated with different concentrations of BAPTA at 0.5 h after scratch. Cont: control without scratch; Sham: DMSO treatment without scratch; Scr: scratch; DMSO: DMSO treatment with scratch. *P<0.05, ***P<0.001 compared with Scr, n = 4. (B) Activity of p-c-Jun in astrocytes pretreated with different concentrations of BAPTA at 0.5 h after scratch. Cont: control without scratch; Sham: DMSO treatment without scratch; Scr: scratch; DMSO: DMSO treatment with scratch. *P<0.05, **P<0.01 compared with Scr, n = 4. (C) Level of GFAP protein in astrocytes 12 h after scratch under treatments with different concentrations of BAPTA. Cont: control without scratch, Scr: scratch. (D) Real-time PCR measurements of the levels of GFAP mRNA in astrocytes 6 h after scratch under treatments with different concentrations of BAPTA. Cont: control without scratch, Scr: scratch. *P<0.05, **P<0.01, ***P<0.001, n = 3. (E) Confocal image of GFAP in astrocytes along the scratch edge treated with different concentrations of BAPTA. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of astrocyte activation and astrogliosis (Eng et al., 2000; Yu et al., 1993). The rational design of therapeutic interventions for CNS injury depends critically upon a comprehensive understanding of the process of scar formation, and thus requires an understanding of the mechanism of GFAP upregulation after injury.

In this study, we used a scratch-injury model to simulate traumatic injury. The scratch model was first used to study astrocyte behavior after traumatic injury *in vitro* (Yu et al., 1993). Subsequently other labs used the model to study cell migration (Etienne-Manneville and Hall, 2001) and astrogliosis (MacFarlane and Sontheimer, 1997; Zhu et al., 2007).

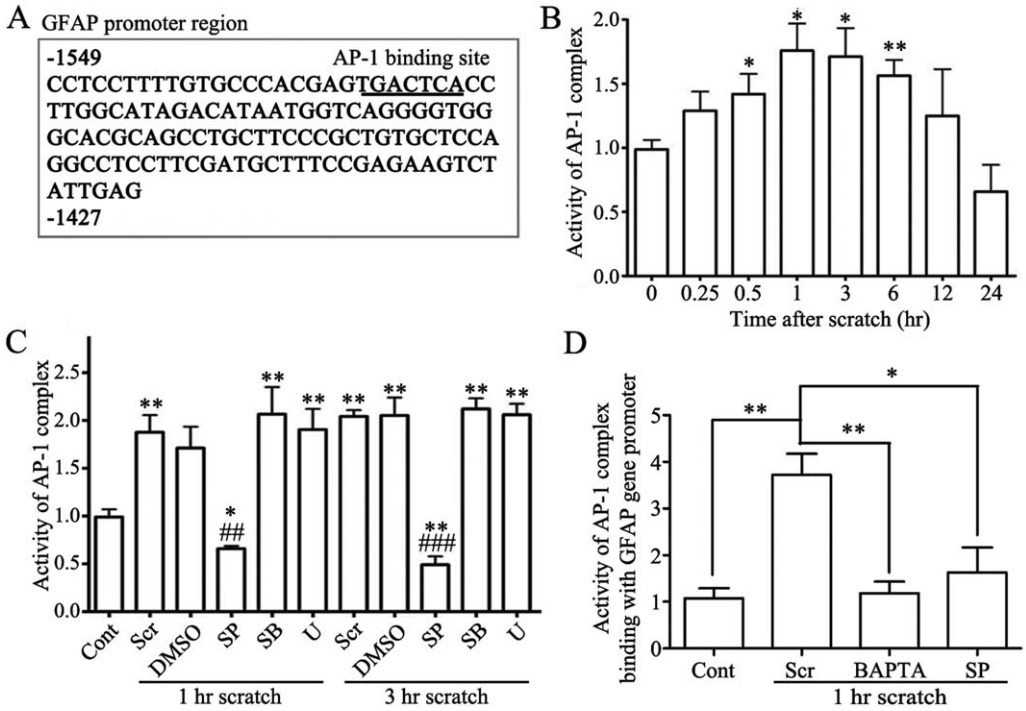

FIGURE 6: The calcium/JNK/c-Jun signaling pathway switched on GFAP upregulation through AP-1. (A) AP-1 binding site on the GFAP promoter region. (B) The activity of the AP-1 transcriptional complex at different time points after scratch. *P<0.05, **P<0.01 compared with 0 h, n = 4. (C) The activity of the AP-1 transcriptional complex from astrocytes after treatments with different inhibitors at 1 h after scratch. DMSO: DMSO treatment, U: U0126 (20 μM), SP: SP600125 (20 μM), SB: SB203580 (20 μM). * P<0.05, **P<0.01 compared with Cont, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared with Scr, n = 4. (D) The AP-1 complex binding to the GFAP promoter from astrocytes after treatment with BAPTA (10 μM) and SP600125 (20 μM) by ChIP. *P<0.05, **P<0.01, n = 4.

The astrocytes along the scratch are traumatically injured and exhibit GFAP upregulation, hypertrophy, proliferation, and cell migration (Ghirnikar et al., 1994; Kornyei et al., 2000; Lanosa and Colombo, 2007; Yu et al., 1993), all of which are main characteristics of astrocyte activation *in vivo* (Sofroniew, 2009; Wu and Schwartz, 1998). Using this model we have proven the importance and necessity of GFAP in the astrocytic hypertrophy and migration that occurs after astrocyte activation (Ghirnikar et al., 1994; Yu et al., 1993). We have also proven that reactive astrocytes release interleukin-1, interleukin-6, tumor necrosis factor alpha, and interferon-gamma after traumatic injury, indicating the role of reactive astrocytes in neuroinflammation (Lau and Yu, 2001). In addition, we have used this model to discover drugs that inhibit astrocyte activation and astrogliosis (Wu and Yu, 2000). We have also revealed the dedifferentiation of astrocytes after traumatic injury (Yang et al., 2012), and have recently used this model to study the effect of AQP5 on astrocytic hypertrophy after injury (Chai et al., 2013). The scratch-injury model is currently widely accepted and used as an *in vitro* astrogliosis model (Ebrahimi et al., 2012; Homkajorn et al., 2010; Hsuchou et al., 2012; Lim et al., 2007; Liu et al., 2011; MacFarlane and Sontheimer, 1997; Malhotra et al., 1997; O'Toole et al., 2007; Perez-Ortiz et al., 2008; Puschmann et al., 2010; Yuan et al., 2012; Zhu et al., 2007) or to induce a traumatic injury (Eng et al., 1997; FaberElman et al., 1996; Huang et al., 2009; Katano et al., 1999; Lu et al., 2013; Pan et al., 2012; Robel et al., 2011; Tomobe et al., 1996) to study the astrocytic response. We therefore believe the scratch model is a suitable and credible model to study astroglisis *in vitro*.

The scratch triggered a calcium influx in astrocytes along the scratch in primary cultures prepared from mouse cortex; the calcium formed a wave and propagated through gap junctions. Here, we report that calcium activated the JNK/c-Jun/AP-1 signaling pathway to switch on GFAP upregulation and resulted in glial scar formation. This phenomenon was confirmed in a stab wound performed in rat brain.

The influx of calcium into astrocytes triggered by scratch became waves to propagate among astrocytes. Astrocytic calcium signals can modulate intracellular calcium levels and the firing patterns of neighboring neurons (Fiacco and McCarthy, 2006; Nedergaard, 1994; Newman and Zahs, 1998; Parpura et al., 1994). Calcium singals and waves revealed many new physiological and pathological roles of astrocytes, thus changing the view of astrocytes from "passive support cells" to "active partner cells" (Ransom and Ransom, 2012). In this study, we reported that traumatic injury triggered an influx of calcium, and the signal transmitted the

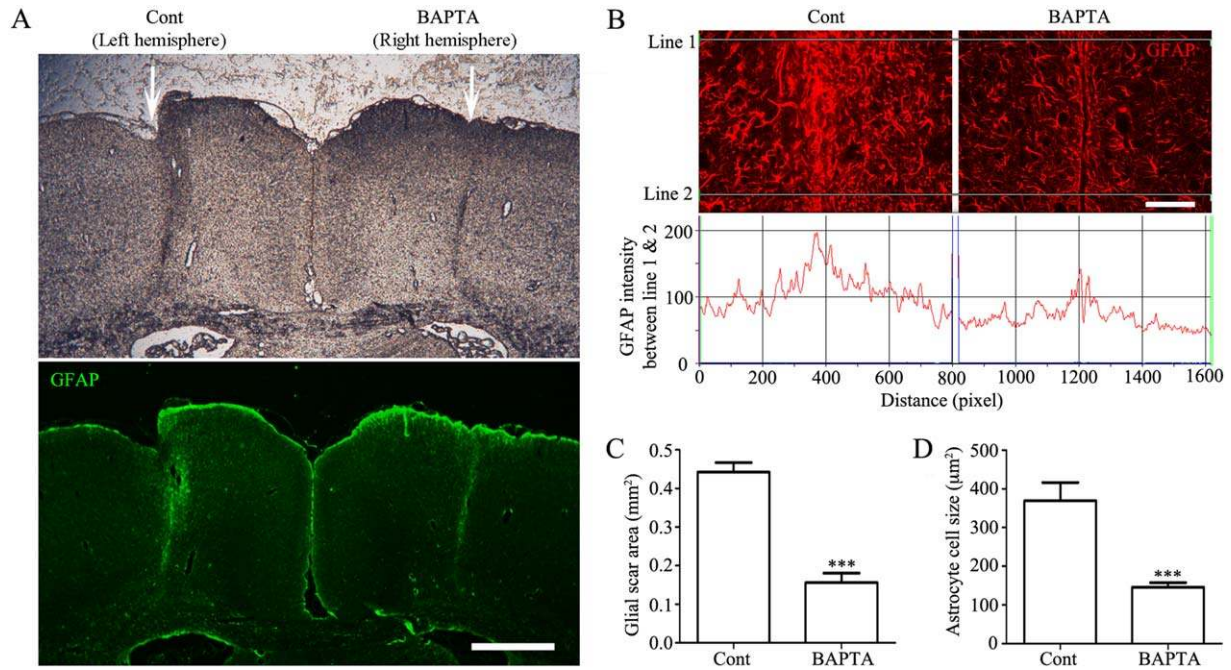

FIGURE 7: A calcium signal was involved in astrogliosis *in vivo*. (A) Brain slice of the stab wound model without (Cont) or with BAPTA pretreatment. Cont (DMSO) and BAPTA (25 μM) pretreatment. Bar = 1 mm. (B) Confocal image of GFAP in a stab wound and the average intensity of GFAP fluorescence in control and BAPTA treatment. Bar = 100 μm. (C) Comparison of the area of the glial scar between cont and BAPTA treatment. ***$P<0.001$, $n = 3$. (D) Comparison of the cell sizes of astrocytes (the area of GFAP staining) around the glial scar between Cont and BAPTA treatment. ***$P<0.001$, $n = 10$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

injury information among astrocytes, which switched on GFAP upregulation and resulted in astrogliosis.

The intense and rapid influx of calcium into astrocytes after scratch indicated that calcium signals may be an early intracellular signal in astrocytes in response to traumatic injury. This is clearly indicated by the polarized mobilization of calcium in a single astrocyte initiated from the injured side (Fig. 1E). Many studies have shown that calcium influx and intercellular calcium waves can be triggered in astrocytes by various means, including various injury related factors like glutamate (Cornell-Bell et al., 1990), ATP (Arcuino et al., 2002; Molnár et al., 2011), bradykinin (Gimpl et al., 1992), chemokine CCL21 (van Weering et al., 2010), and HIV-1 Tat (El-Hage et al., 2005). In this study, calcium influx and waves were triggered by a scratch wound.

The calcium waves triggered by the scratch appeared to spread away from the wounds. The CBX experiments demonstrated that the scratch triggered calcium waves could be completely inhibited. These results supported the hypothesis that calcium mobilized between astrocytes through gap junctions, and thus, astrocytes in primary cultures formed a syncytium (Finkbeiner, 1992; Jin and Chen, 2011). However, we observed an increase of the intracellular calcium concentration in some astrocytes treated with CBX, indicating that blocking gap junctions could elevate intracellular calcium release in some astrocytes. ATP can bind to the P2 receptor to generate calcium waves between astrocytes with no physical contact (Hassinger et al., 1996; Zhang et al., 2007). Blocking the P2 receptor with suramin did not affect the formation of calcium waves in astrocytes after scratch, indicating that ATP was not involved in the generation of calcium waves in astrocytes after scratch (unpublished data).

The source of calcium to generate calcium waves could be from either the extracellular compartment or release from intracellular calcium storage compartments, like endoplasmic reticulum and mitochondria (Scemes and Giaume, 2006). That scratch triggered calcium influx from the extracellular compartment into astrocytes was further confirmed in experiments performed with BAPTA treatment or in calcium-free HBS. Scratch induced an influx of extracellular calcium and generated an elevation of calcium in astrocytes along the scratch, which was then disseminated to neighboring astrocytes as a calcium wave through gap junctions. We also noticed that the mobilization rate and fluorescence intensity of the waves gradually decreased with distance. Whether these changes would have any effect on the degree of astrocyte reactivity to the traumatic scratch injury requires further investigation.

In this study, scratch triggered an initial calcium mobilization from the extracellular compartment and led to the

astrocytes' ultimate response of increased GFAP levels. Astrocytes along the scratch wound showed a more intense and lasting calcium signal which at the same time resulted in these astrocytes having a higher level of GFAP. Blocking the calcium influx obviously inhibited this upregulation. BAPTA treatment in rat brain stab wound also inhibited the increase of GFAP expression and scar formation; this again confirmed the role of calcium in astrogliosis. These results are consistent with a study in spinal cord injury in which blocking calcium influx reduced GFAP increase (Du et al., 1999). A similar correlation was observed in rat brain after systematic injection with kainic acid (Gramsbergen and van den Berg, 1994). Therefore, calcium is very likely one of the early injury signals for traumatic scratch that initiates the signal transduction cascade for the process of activation of astrocytes and an increase in GFAP synthesis.

Calcium is known to act as a second messenger, initiating signal transduction cascades and altering the physiology of the responding cell. To elucidate the mechanism for calcium to induce GFAP upregulation, we examined the MAPK pathway in astrocytes after scratch. This pathway contains many proteins and controls multiple cellular functions. Activation of this pathway promotes cell division and cell migration, and many forms of cancer are associated with aberrations in the pathway. Among the members of the pathway, we identified that inhibition of JNK, but not ERK and p38, suppressed GFAP upregulation after scratch. Blocking calcium influx also inhibited JNK activation in astrocytes after scratch. This is in agreement with the findings of a study in which calcium activated JNK was involved with CaMKII in IL-1β and lipoteichoic acid induced MMP-9 upregulation in astrocytes (Wang et al., 2010; Wu et al., 2009). There is other evidence indicating that JNK might be involved in regulating GFAP synthesis, such as in astrocytes treated with endothelin-1 (Gadea et al., 2008), in brain inflammation (Kaminska et al., 2009), in methamphetamine-induced glial activation (Hebert and O'Callaghan, 2000), and even in human astrocytoma cell line U87 after receptor FPRL1 activation (Kam et al., 2007). Interestingly, we noticed a basal level of JNK activity in control astrocytes before scratch. If we treated the control with SP600125, the GFAP level was further reduced, indicated that the JNK pathway may also be involved in GFAP expression under physiological conditions.

There are other signals and pathways reported to modulate the expression of GFAP under various experimental conditions, such as Nuclear factor I (Cebolla and Vallejo, 2006), DREAM (Cebolla et al., 2008) JAK/STAT (Morga et al., 2009), ERK (Ciccarelli et al., 2004), and IKK/NFκB (Hwang et al., 2010). Among them, JAK/STAT was the most studied signaling pathway mediating GFAP upregulation in astrogliosis induced by metallothioneins (Leung et al., 2010), LPS-induced inflammation (Morga et al., 2009), ciliary neurotrophic factor (Wang et al., 2002), and the dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sriram et al., 2004). ERK, another member of the MAPK family, was also reported to be involved in the GFAP upregulation in astrogliosis, in a manner similar to JNK, in brain inflammation (Kaminska et al., 2009), methamphetamine-induced glial activation (Hebert and O'Callaghan, 2000), and in human astrocytoma cell line U87 after receptor FPRL1 activation (Kam et al., 2007). To our surprise, blocking JAK/STAT with AG 490 or blocking ERK with U0126 did not affect the GFAP expression in astrocytes after scratch. This implies that the scratch triggered calcium influx has a certain order of specificity in activating JNK.

AP-1 is a transcription factor that plays a role in both cell proliferation and differentiation (Raivich and Behrens, 2006). It is known in human (Masood et al., 1993) and mouse (Sarid, 1991) that the GFAP gene has an AP-1 binding site in its promoter region. There are reports that AP-1 mediates the expression of GFAP and many astrocyte-specific molecules during the development of the CNS (Gopalan et al., 2006; Masood et al., 1993), kainate treatment, and inflammation (Kleinman et al., 2008; Pennypacker et al., 1994). In a SNP study of Alexander disease, an additional AP-1 binding site in the GFAP promoter was shown to lead to an increase in GFAP transcription (Bachetti et al., 2010). As a component of AP-1 and a downstream target of JNK, c-Jun's activation enhanced AP-1 binding to the GFAP promoter in scratch-injured astrocytes, defining a new signaling pathway, calcium/JNK/c-Jun/AP-1, that mediated the GFAP upregulation in scratch-activated astrocytes.

In this study, we also confirmed the results of the scratch model with a stab wound model, as in earlier reports (Lu et al., 2013; Robel et al., 2011). Our results showed that blocking the calcium signal inhibited scar formation, hypertrophy, and GFAP upregulation, indicating the importance of the calcium signal on trauma induced astrogliosis *in vivo*. The stab wound model is a classic *in vivo* model that allows the study of brain trauma and astrogliosis in rats (Vijayan et al., 1990), mice (Theodoric et al., 2012), zebrafish (Baumgart et al., 2012), and turtles (Kalman et al., 2013). GFAP upregulation has been found using the stab wound model during astrocyte hypertrophy and proliferation (Moumdjian et al., 1991; Vijayan et al., 1990). This model has also been used to study the effect of trauma induced neuroinflammation on astrogliosis (Ghirnikar et al., 1998), showing that interleukin-1 and chemokines are involved in neuroinflammation and astrogliosis after trauma (Ghirnikar et al., 1996; Glabinski et al., 1996; Lin et al., 2006). Aquaporin-4 has been proven to take part in astrocyte migration and scar

formation in stabbed brain (Lu et al., 2011; Saadoun et al., 2005). Based on the stab wound model, some studies have reported that certain signaling pathways, including ERK (Carbonell and Mandell, 2003; Nicole et al., 2005), Notch (Givogri et al., 2006), and Fyn (Chun et al., 2004), are involved in astrogliosis. Our study is complimentary with a recent paper that reported that the calcium signaling pathway mediated N-cadherin expression in stab wound induced astrogliosis (Kanemaru et al., 2013). Investigation of the regulation and levels of these and other signaling molecules is an important topic for future study.

In summary, we found that scratch traumatically injured astrocytes and triggered a calcium influx from the extracellular compartment along the wound. This influx of calcium formed a calcium wave that propagated through gap junctions. At the same time, intracellular calcium signals activated the JNK/c-Jun/AP-1 signaling pathway and switched on GFAP upregulation.

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