ORIGINAL ARTICLE

Depolarization Induces NR2A Tyrosine Phosphorylation and Neuronal Apoptosis

Yong Liu, Jing-Zhi Yan, Ying-Hui Gu, Wei Wang, Yan-Yan Zong, Xiao-Yu Hou, Guang-Yi Zhang

ABSTRACT: ***Background:*** Cytosol Ca^{2+} overload plays a vital role in ischemic neuronal damage, which is largely contributed by the Ca^{2+} influx through L-type voltage-gated calcium channels (L-VGCCs) and N-methyl-D-aspartate (NMDA) type glutamate receptors. In this article, L-VGCCs were activated by depolarization to investigate the cross-talk between NMDA receptors and L-VGCCs. ***Methods:*** Depolarization was induced by 20 minutes incubation of 75 mM KCl in cultured rat cortical neuron. Apoptosis-like neuronal death was detected by DAPI staining. Tyrosine phosphorylation of NMDA receptor subunit 2A (NR2A), interactions of Src and NR2A were detected by immunoblot and immunoprecipitation. ***Results:*** Depolarization induced cortical neuron apoptosis-like cell death after 24 hours of restoration. The apoptosis was partially inhibited by 5 mM EGTA, 100 μM Cd^{2+}, 10 μM nimodipine, 100 μM genistein, 20 μM MK-801, 2 μM PP2 and combined treatment of nimodipine and MK-801. NR2A tyrosine phosphorylation increased after depolarization, and the increase was inhibited by the drugs listed above. Moreover, non-receptor tyrosine kinase Src bound with NR2A after depolarization and restoration. The binding was also inhibited by the drugs listed above. ***Conclusions:*** The results indicated that depolarization-induced neuronal death might be due to extracellular Ca^{2+} influx through L-VGCCs and subsequently Src activation-mediated NR2A tyrosine phosphorylation.

RÉSUMÉ: La dépolarisation induit la phosphorylation de la tyrosine NR2A et l'apoptose neuronale. ***Contexte :*** La surcharge en Ca2+ du cytosol joue un rôle vital dans le dommage neuronal ischémique, auquel contribue grandement l'influx de Ca2+ à travers les canaux calciques voltage-dépendants de type L (CCVD-L) et les récepteurs N-méthyl-D-aspartate (NMDA) du glutamate. Dans cet article, nous décrivons l'activation des CCVD-L par dépolarisation pour étudier la modulation conjointe entre les récepteurs NMDA et les CCVD-L. ***Méthode :*** Une dépolarisation a été induite dans des neurones corticaux de rat en culture par une incubation de 20 minutes en présence de KCl à 75 mM. La coloration par DAPI a permis de détecter une mort neuronale ressemblant à de l'apoptose. La phosphorylation de la tyrosine de la sous-unité 2A du récepteur NMDA (NR2A) et les interactions de Src et NR2A ont été détectées par immunotransfert et immunoprécipitation. ***Résultats :*** La dépolarisation a induit une mort cellulaire des neurones corticaux ressemblant à l'apoptose après 24 heures de restauration. L'apoptose était partiellement inhibée par l'EGTA à 5 mM, le Cd2+ à 100 μM, la nimodipine à 10 μM, la génistéine à 100 μM, le MK-801 à 20 μM, le PP2 à 2 μM et par la nimodipine associée au MK-801. La phosphorylation de la tyrosine de NR2A a augmenté après la dépolarisation et cette augmentation était inhibée par les produits énumérés ci-haut. De plus, la tyrosine kinase de la famille Src non liée au récepteur se liait avec NR2A après la dépolarisation et la restauration. La liaison était également inhibée par les produits énumérés ci-haut. ***Conclusions :*** Ces résultats indiquent que la mort neuronale induite par dépolarisation pourrait être due à l'influx de Ca2+ extracellulaire à travers les CCVD-L et subséquemment à la phosphorylation de la tyrosine NR2A médiée par l'activation de Src.

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Calcium influx induced intracellular Ca^{2+} overload has been thought to be involved in brain ischemic insults[1,2]. Two principal sites for Ca^{2+} influx are the L-type voltage-gated calcium channels (L-VGCCs) and the N-methyl-D-aspartate (NMDA) type glutamate receptors. The pharmacological, electrophysiological and molecular characteristics of L-VGCCs and NMDA receptors presented in central neuronal system (CNS) have been well studied[3-5]. Previous studies reported that brain ischemia induced neuronal depolarization and release of glutamate, which induced over activation of L-VGCCs and NMDA receptors and excessive influx of Ca^{2+} through the two types of ion channels[2,6,7]. The overload of Ca^{2+} led to neuronal injury after brain ischemia and reperfusion.

Our previous works reported that cross-talk between the L-VGCCs and the NMDA receptors might play an important role in brain ischemic insults *in vivo*, mediated by tyrosine phosphorylation of L-VGCC α1C subunits and NMDA receptor 2A (NR2A) subunits through Src family kinases[8-10]. Both tyrosine phosphorylation of α1C subunits and NR2A subunits increased after brain ischemia and reperfusion, and the increase of NR2A tyrosine phosphorylation was inhibited by a L-VGCC antagonist[9]. Jiang et al showed the role of NMDA receptors in cortical cultures following incubation of glutamate induced apoptosis-like cell death[11]. This study examins the role of L-VGCCs in brain ischemia.

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The aim of the present work was to identify whether L-VGCCs could modulate NMDA receptor function *in vitro* and to investigate whether L-VGCC openness could lead to neuronal apoptosis, and the possible signal transduction pathway in high concentration KCl incubation of cultured rat cortical neurons.

Materials and Methods

Neuronal Culture

Cortical neuronal cultures were prepared from 18-day-old embryos of Sprague–Dawley rat as described before[11]. Briefly, neocortex was meticulously isolated in ice-cold high-glucose Dulbecco's modified Eagle medium (h-DMEM) (Gibco, Grand Island, NY, USA). Cortical cells were dissociated by trypsinisation (0.25% (w/v) trypsin and 0.02% (w/v) Ethylene diamine tetraacetic acid (EDTA) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution), at 37°C for 15 minutes (min), followed by gentle triturating in plating medium (h-DMEM supplemented with 10% fetal bovine serum and 10% horse serum, Gibco). Cells were seeded onto poly-L-lysine (Sigma, St. Louis, MO, USA) -coated wells or coverslips at a density of 1×10^5 cells per cm^2 and incubated at 37°C in 5% CO_2 atmosphere. After 24 hours (h), cultural medium was replaced by Neurobasal Medium supplemented with B-27 (Gibco) and 0.5 mM glutamine and then half-replaced twice every week. Cultures were used after two weeks *in vitro* (DIV), when the cells were vulnerable to high concentration KCl depolarization and verified 95% neurofilaments positive by immunostaining.

Drug treatment

Cultured cortical neurons of fifteen to eighteen days *in vitro* (DIV15-18) were exposed to 75 mM KCl for 20 min, during which the medium was changed into low-glucose Dulbecco's modified Eagle medium (l-DMEM) (Gibco). For some studies, 5 mM Ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA), 100 μM Cd^{2+}, 10 μM nimodipine, 100 μM genistein (Sigma), 20 μM MK-801 (RBI) and combination of nimodipine and MK-801 were added in feeding medium 20 min before and during high concentration KCl exposure, respectively. Two μM PP2 or PP3 were added seperately, 20 min before KCl exposure to the end of restoration. For restoration, at the end of KCl exposure, the cultures were rinsed three times with l-DMEM, and the original feeding medium absent of drugs was restored. All drugs were made as 200× stocks in water, excepted for nimodipine and genistein that were made as 500× stocks in dimethyl sulfoxide (DMSO, Sigma), PP2 and PP3 in 10% DMSO. Vehicle controls were treated with vehicle 0.5% water, 0.2% or 0.02% DMSO and 75mM KCl in l-DMEM. Sham controls were treated only with l-DMEM.

Detection of apoptosis-like cell death

Nuclear condensation detected by the fluorescent DNA binding dye 4,6-diamidino-2-phenylindole (DAPI, Sigma) was used as the indication of depolarization-induced apoptosis-like cell death. Briefly, cells grown on each coverslip restored at different time points were incubated with 10 μg/ml DAPI at 37°C for 30 min, then washed with phosphate buffered saline (PBS) and excited with vertical fluorescent at 400 nm. With fluorescence collected at 455 nm, apoptosis-like cells were characterized by the presence of condensed and fragmented nuclei, as opposed to the diffuse staining observed in non-apoptotic cells. Each sample was pooled from at least four coverslips. The proportion of apoptosis-like cells was calculated as a percentage of total cells counted in 10 microscopic fields (400×).

Preparation of cell extracts

To detect NR2A tyrosine phosphorylation or interactions of Src with NR2A, cultured neurons at different time of restoration were rinsed with PBS, and then were scraped off with 80 μl homogenization buffer containing 50 mM MOPS (3-(N-morpholino) propane-sulfonic acid; pH 7.4), 20 mM sodium pyrophosphate, 20 mM ß-phosphogrycerol, 320 mM sucrose, 0.2 mM dithiothreitol, 100 mM KCl, 50 mM NaF, 0.5 mM $MgCl_2$, 1 mM each of EDTA, EGTA, Na_3VO_4, p-nitrophenyl phosphate (PNPP), 1% Triton X-100, 0.05% NP40 and protease inhibitors: 1 mM phenyl-methylsulfonyl fluoride, 5 μg/ml each of aprotinin, leupeptin, pepstatin A and benzamidine. Homogenates were collected and quickly frozen in liquid nitrogen, then were sonicated three times and protein concentrations were determined by the Lowry's method. Samples were stored at -80°C until assay and thawed only once.

Immunoprecipitation and immunoblot(ting)

Cell samples (400 μg of total proteins of each sample) were diluted four fold with immunoprecipitation (IP) buffer containing 50 mM 2-[4-(2-Hydroxyethyl)-1piperazine]ethane-sulfonic acid (HEPES) (pH 7.1), 150 mM NaCl, 1 mM $ZnCl_2$, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM each of EDTA, EGTA, Na_3VO_4, PNPP and protease inhibitors as indicated above, then pre-incubated with 20μl protein A sepharose CL-4B (Amersham, Buckinghamshire, UK) for 1 h at 4°C to remove proteins adhered nonspecifically to protein A. After centrifugation, supernatants were incubated with 1-2μg indicated antibodies for 4 h or overnight at 4°C. Protein A (20μl) was added and the incubation continued for 2 h at 4°C. Immune complexes were centrifuged at 4°C and the pellets were washed three times with immunoprecipitation buffer. Bound proteins were eluted by boiling for 5 min in 2×sample buffer (0.12M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 20% glycerol and 0.003% bromophenol blue), then centrifuged and the supernatants were collected for protein analysis by immuoblotting (IB).

For IB, the supernatants were separated on 7.5% acrylamide SDS-PAGE (poly-acrylamide gel electrophoresis), then electro-transferred onto nitrocellulose membrane (pore size: 0.45 μm; Amersham). After blocking with 3% bovine albumin, the membrane was probed with the indicated primary antibodies at 4°C overnight. Bound antibodies were detected by alkaline phosphatase conjugate goat anti-mouse immunoglobulin (Ig) G or goat anti-rabbit IgG (Sigma). Immunoreactivity was detected by NBT/BCIP assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software; UVP Inc., Up-land, CA, USA) to detect relative optical densities.

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Antibodies

Rabbit anti-NR2A antibody was obtained from Chemicon. Phospho-tyrosine antibody used in IB and all of the secondary antibodies were purchased from Sigma. Phospho-tyrosine antibody used in IP was purified in our laboratory[12]. Anti-Src antibody and anti-actin antibody were sourced from Sant Cruz.

Statistics

Data were presented as means ± S.D. from five independent cultures. Statistical analysis of the results was performed by one-way analysis of variance followed by the Duncan's new multiple range method. $P < 0.05$ was considered significant.

Results

Time course of apoptosis-like cell death induced by depolarization

Cultured cortical neurons DIV15-16 were exposed to 75 mM KCl in l-DMEM for 20 min to induce depolarization, then restored in original feeding medium for 6, 12, 18, 24, 36 or 48 h. Apoptosis-like cell death was detected by DAPI staining, which increased from 17% of sham group to 55%-81% after 12-48 h restoration (Figure 1).

Effects of EGTA, Cd^{2+}, nimodipine, genistein, MK-801 and PP2 on depolarization-induced apoptosis-like cell death

To investigate possible signal transduction pathways involved in the depolarization-induced cell death, cultured cortical neurons were pre-incubated with EGTA, Cd^{2+}, nimodipine, genistein, MK-801, PP3 and PP2, and then exposed to 75 mM KCl. Depolarization-induced apoptosis-like cell death was detected with DAPI staining after 24 h restoration in original feeding medium without the above drugs. Results showed that the increased apoptosis-like cell death was inhibited by EGTA, Cd2+, nimodipine, genistein, MK-801, coapplication of nimodipine and MK-801, and PP2 from 81% to 35%, 45%, 43%, 40%, 50%, 32%, and 39%, respectively (Figure 2A). Vehicle (0.2% DMSO) and PP3 had no significant effect on the depolarization-induced apoptosis-like cell death. In addition, L-VGCC agonist BayK-8644 incubation could also induce the neuronal apoptosis-like cell death. Typical photographs with DAPI staining are presented to show depolarization-induced apoptosis-like cell death and the inhibitory effects of the drugs listed above (Figure 2B).

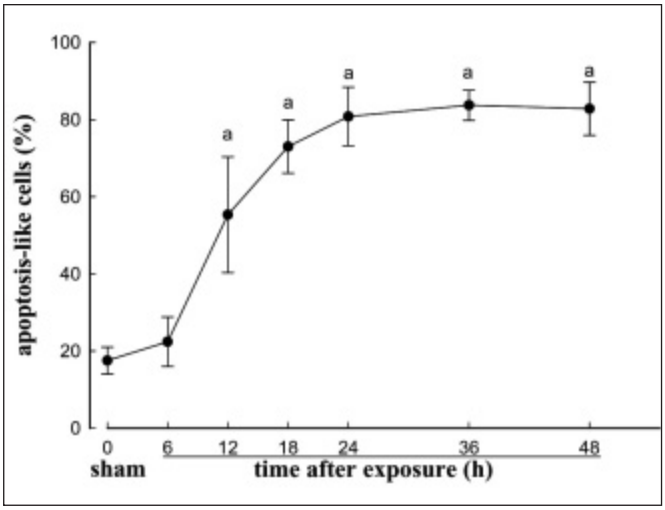

Figure 1: *Time course of apoptosis-like cell death in cultured rat cortical neurons induced by KCl depolarization. DIV15-16 cultured rat cortical neurons were exposed to 75 mM KCl, 20 min for depolarization. Cells were restored in l-DMEM and DAPI staining was carried out at 6, 12, 18, 24, 36 and 48 h after restoration using 10 μg/ml DAPI. Cells of group of sham control were restored for 24 h. Apoptotic-like cells were expressed as percent of total cells counted in 10 microscopic fields (×400) for DAPI staining.* [a]*P<0.05 vs. group of sham.*

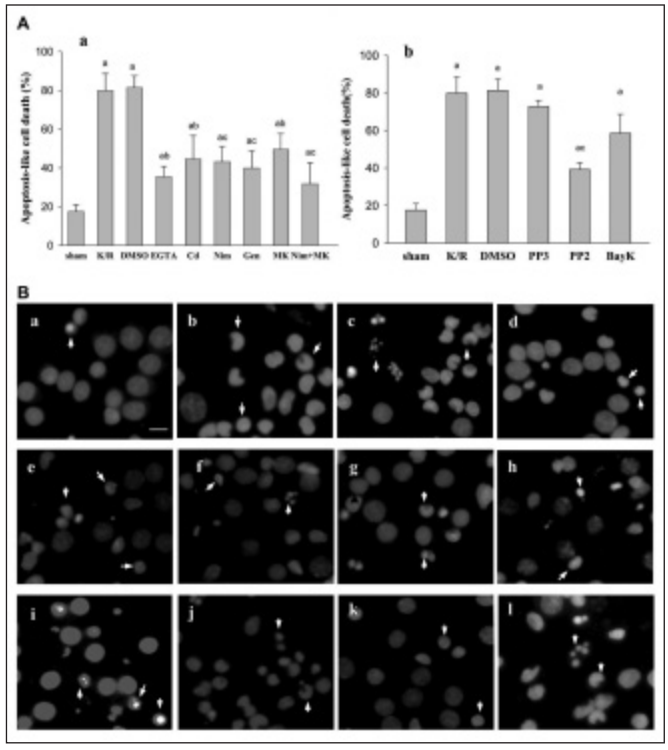

Figure 2: *Effects of some drugs on KCl depolarization (K/R) induced apoptosis-like cell death in cultured rat cortical neurons. (A) Quantitative representations expressed as percentage of total cells counted in 10 microscopic fields (×400) for DAPI staining.* [a]*P<0.05 vs. group of sham,* [b]*P<0.05 vs. group of K/R,* [c]*P<0.05 vs. group of DMSO. (B) Typical photographs showing DAPI staining of inhibitory effects of EGTA, Cd^{2+}, nimodipine (Nim), genistein (Gen), MK801 (MK) and PP2 on KCl depolarization induced apoptosis-like cell death. (a) Cells were not exposed to high concentration KCl, (b) cells exposed to 75 mM KCl for 20 min, (c-i) 0.2% DMSO, 5 mM EGTA, 100 μM Cd^{2+}, 10 μM Nim, 10 μM Gen, 20 μM MK-801, and combination of Nim and MK-801 (Nim+MK) were added 20 min before and during depolarization, (j, k) cells were exposed to 2 μM PP3 and PP2 from 20 min before to the end of restoration, (l) cells were exposed to 1 mM BayK 8644 for 20 min followed restoration, respectively. DAPI staining was carried out at 24 h after restoration. Typical nuclear condensations and fragmentation of apoptosis were marked by arrows. Scale bar=10 μm.*

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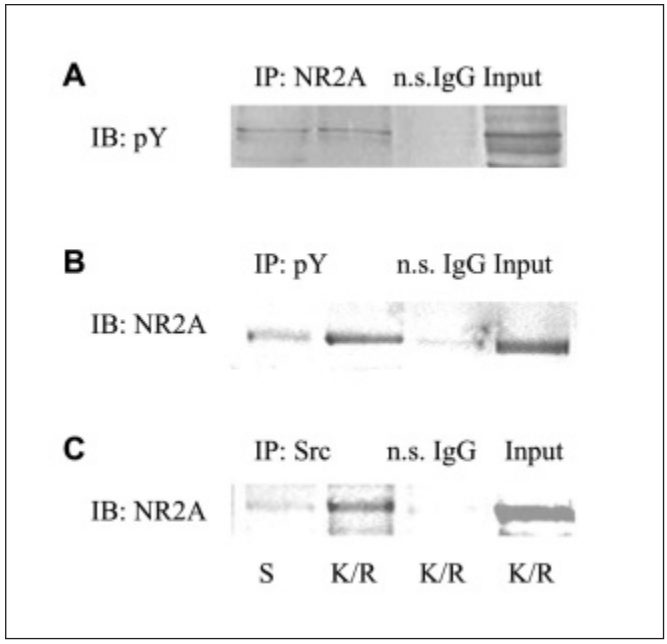

Figure 3: *Increased tyrosine phosphorylation of NR2A and interaction of Src with NR2A induced by KCl depolarization. Homogenates (400 μg of total protein) from sham-operated controls (Sham) and post-depolarization samples at 12 h of restoration (K/R) were immunoprecipitated (IP) with anti-NR2A antibody (anti-NR2A), anti-phosphotyrosine antibody (anti-pY), anti-Src antibody or nonspecific IgG (n.s. IgG), and the precipitates were analyzed by immunoblotting (IB) with anti-pY (A) or anti-NR2A (B, C). In the lane marked input, 100 μg of protein without immunoprecipitation were loaded. At least three independent experiments were performed and one typical experiment is presented.*

Increased tyrosine phosphorylation of NR2A and interaction of Src with NR2A induced by KCl depolarization

To study the relationship between NMDA receptors and L-VGCCs, tyrosine phosphorylation of NR2A was detected after depolarization. Either immunoprecipitated with anti-NR2A antibody (anti-NR2A, Chemicon) followed by immunoblot with anti-phosphotyrosine antibody (anti-pY, Sigma) or immuno-precipitated with anti-pY followed by Blot with anti-NR2A, a band of 170 kDa was detected (Figure 3A and B), and a band of the same molecular weight in the lane marked input was also detected, which indicated that NR2A was phosphorylated at tyrosine residues. Interactions of tyrosine kinase Src with NR2A were also detected by immunoprecipitation with anti-Src antibody (Sant Cruz, USA) followed by immunoblot with anti-NR2A antibody. The results showed that interactions of Src with NR2A increased after depolarization (Figure 3C). While immunoprecipitated with nonspecific mouse or rabbit IgG no significant band corresponding to NR2A was detected.

Time course of tyrosine phosphorylarion of NR2Aand interactions of Src with NR2A induced by depolarization

Cortical cultures were depolarized and then restored in previous medium for 0, 1, 6, 12, 18 or 24 h, repectively. Tyrosine phosphorylation of NR2A and interaction of Src with NR2A were detected by immunoprecipitation followed by immunoblot. Tyrosine phosphorylation of NR2A increased after restoration and reached a peak level (3.4 fold relative to sham control) at 12 h of restoration, then decreased (top strip in Figure 4A, B), whereas protein express of NR2A and actin, an inner control, had no significant change (middle strip in Figure 4A, B). In addition, interactions of Src with NR2A also increased significantly from 1h to 18 h after restoration, which reached a peak level at 12 h (2.5 fold relative to sham control, bottom strip in Figure 4A, B).

Effects of EGTA, Cd^{2+}, nimodipine, genistein and MK-801 on tyrosine phosphorylation of NR2A and interaction of Src with NR2A

Effects of EGTA, nimodipine, genistein and MK-801 on depolarization-induced tyrosine phosphorylation of NR2A and

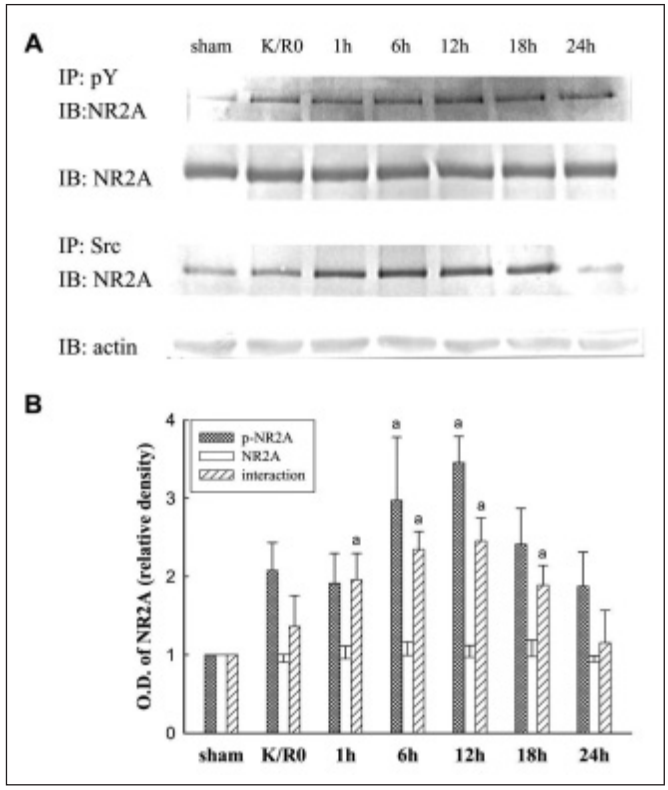

Figure 4: *Time course of tyrosine phosphorylation of NR2A and interaction of Src with NR2A induced by KCl depolarization. Cultured rat cortical neurons were exposed to 75 mM KCl 20 min for depolarization. Cells were harvested at 0, 1, 6, 12, 18 and 24 h after restoration. Tyrosine phosphorylation of NR2A was detected using immunoprecipitation (IP) with anti phosphotyrosine antibody (anti-pY) followed by immunoblotting with anti-NR2A antibody (anti-NR2A) (top strip in A), protein expression of NR2A was detected using IB with anti-NR2A antibodies, respectively (second strip in A). Interaction of Src with NR2A was detected using IP with anti-Src followed by IB with anti-NR2A (third strip in A). Inner control of actin was detected with IB (bottom strip). Bands corresponding to NR2A were scanned and the intensities were quantified (B). $^{a}P<0.05$ vs. group of sham (n=5).*

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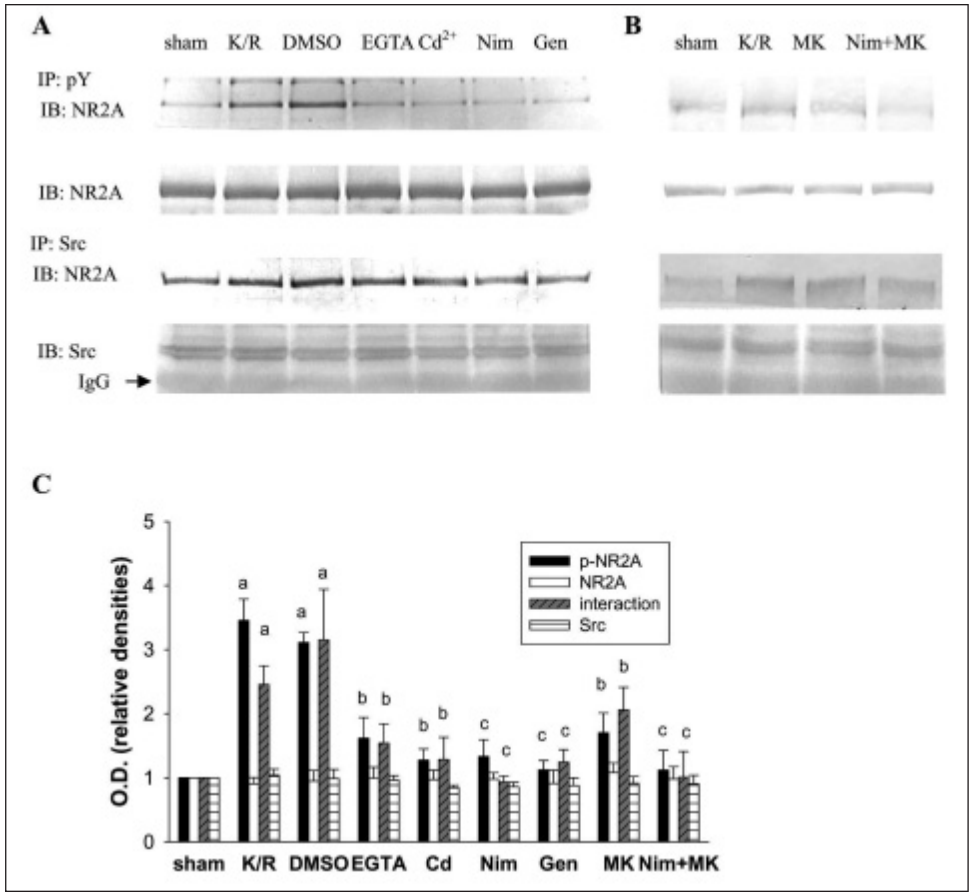

Figure 5: *Effects of EGTA, Cd^{2+}, nimodipine, genistein and MK-801 on KCl depolarization induced tyrosine phosphorylation of NR2A and interaction of Src with NR2A. Cultured rat cortical neurons were exposed to 75 mM KCl 20 min for depolarization. For drug administration, 0.2% DMSO, 5 mM EGTA, 100 μM Cd^{2+}, 10 μM nimodipine, and 100 μM genistein were added 20 min before and during depolarization, respectively. Cells were harvested at 12h after restoration. Tyrosine phosphorylation (top strip in A), protein expression of NR2A (second strip and Src (bottom strip in A) and interaction of Src with NR2A (third strip in A) were detected. Bands were scanned and the intensities were quantified (B). $^{a}P<0.05$ vs. group of sham, $^{b}P<0.05$ vs. group of K/R, $^{c}P<0.05$ vs. group of vehicle (DMSO) (n=5).*

interactions of Src with NR2A were studied. Cortical cultures were depolarized and restored in previous medium for 12 h, and then cells were harvested. Increased tyrosine phosphorylation of NR2A was inhibited by EGTA, Cd^{2+}, nimodipine, genistein, MK-801 and combined treatment of nimodipine and MK-801 incubated 20 min before and during the depolarization from 3.4 fold to 1.6, 1.3, 1.3, 1.1, 1.7 and 1.1 fold relative to sham control, respectively (top strip in Figure 5A, B). The increased interactions of Src with NR2A were also inhibited by EGTA, Cd^{2+}, nimodipine, genistein, MK-801 and combined treatment of nimodipine and MK-801 from 2.5 fold to 1.5, 1.3, 0.9, 1.2, 2.0 and 1.0 fold relative to sham control, respectively (third strip in Figure 5A, B), whereas protein expression of NR2A (second strip) or Src (bottom strip in Figure 5A, B) had no significant change after drug administration. Vehicle control (DMSO) had no statistically significant effect on the NR2A tyrosine phosphorylation or the interactions of Src with NR2A.

Effects of PP2 on tyrosine phosphorylation of NR2A and interaction of Src with NR2A

To further study mechanisms of depolarization-induced NR2A tyrosine phosphorylation, Src family kinase inhibitor PP2 and its non-effect analog PP3 were administered into culture medium 20 min before and during depolarization and restoration. Cortical cultures were depolarized and restored in previous medium for 12 h, and then cells were harvested. Increased tyrosine phosphorylation of NR2A was inhibited by 2 μM PP2 from 3.11 to 0.85 fold relative to sham control (top strip in Figure 6A, B). The increased interactions of Src with NR2A were also inhibited by PP2 from 3.15 to 0.84 fold (the third strip in Figure 6A, B), whereas PP3 had no significant effect on the NR2A tyrosine phosphorylation or the interactions of Src with NR2A after depolarization (Figure 6). Protein expression of NR2A (the second strip in Figure 6A) and Src (the bottom strip in Figure 6A) had no significant change.

Discussion

The VGCCs (including L-, N-, P-, Q- and T- types) expressed in cerebral neurons change with age, indicating special functions of different types[5]. Membrane depolarization may activate VGCCs directly and lead to influx of extracellular calcium. In this paper, we found that 20 min incubation in 75 mM KCl induced depolarization and led to about 80% apoptosis-like cell death after 24 h restoration, which showed that excessive activation of VGCCs could do great harm to cortical cultures. As EGTA (a Ca^{2+} chelator), Cd^{2+} (a blocker of VGCC), nimodipine (a specific inhibitor of L-VGCC) and MK-801 (a blocker of NMDA receptor) partly inhibited depolarization-induced

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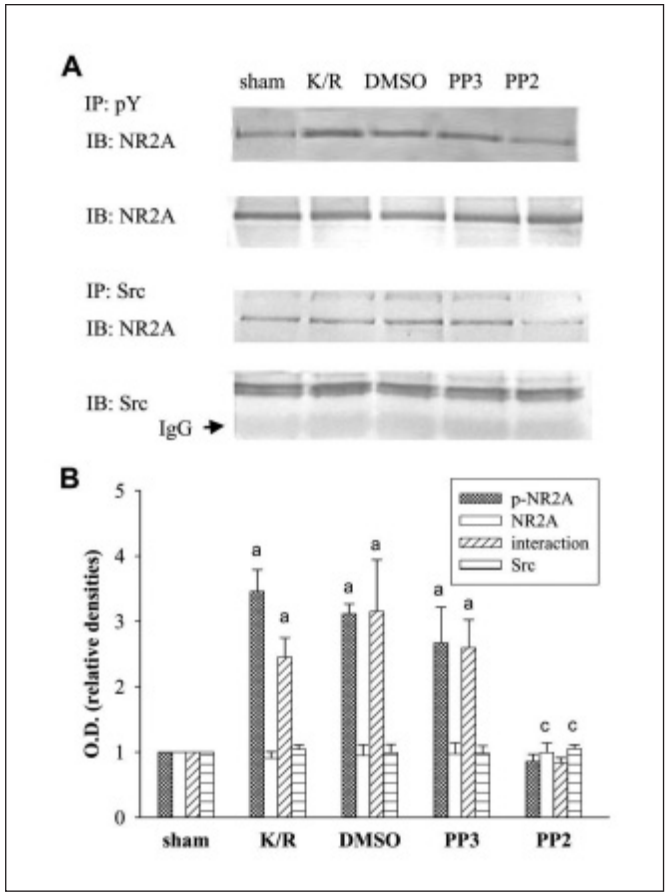

Figure 6: *Effect of PP2 on KCl depolarization induced tyrosine phosphorylation of NR2A and interaction of Src with NR2A. Cultured rat cortical neurons were exposed to 75 mM KCl 20 min for depolarization. For drug administration, 0.02% DMSO, 2 μM PP3 and 2 μM PP2 were added from 20 min before to the end of restoration, respectively. Cells were harvested at 12 h after restoration. Tyrosine phosphorylation (top strip in A), protein expression (second strip in A), interaction of Src with NR2A (third strip in A) and expression of Src (bottom strip in A) were detected. Bands were scanned and the intensities were quantified (B).* $^{a}P<0.05$ *vs. group of sham,* $^{c}P<0.05$ *vs. group of vehicle (DMSO) (n=5).*

apoptosis, the results indicated that excessive extracellular Ca^{2+} influx through VGCCs, especially L-VGCCs, and NMDA receptors contributed to the process.

Functional NMDA receptors are composed of NR1, NR2(A-D) and NR3(A/B) subunits. Both NR1 and NR2 contribute, as a heterotetramer, to NMDA receptor ion channels[13-15]. Tyrosine phosphorylation of NR2 subunit C-terminals is considered an important mechanism to up-regulating activity of NMDA receptor ion channels[16-18]. Previous studies showed that tyrosine phosphorylation of NR2 subunits increased after brain ischemia and the NR2 phosphorylation was auto-regulated by activation of NMDA receptors[19]. Function of NMDA receptors was also mediated by openness of L-VGCCs after brain ischemia, which indicated cross-talk between NMDA receptors and L-VGCCs through tyrosine phosphorylation *in vivo*[9]. Here we found that tyrosine phosphorylation of NR2A increased after depolarization, and the increase was inhibited by EGTA, Cd^{2+} and nimodipine. The results indicate that activation of NMDA receptors might be involved in depolarization-induced cortical culture apoptosis-like cell death. Genistein could significantly inhibit KCl depolarization induced cell death and tyrosine phosphorylation of NR2A, which showed that protein tyrosine kinase played a vital role in the process.

It has been demonstrated that Src family tyrosine kinases (SFKs, including Src, Fyn, Lyn, Yes and Lck) expressed in the central nervous system modulate the electrophysiological activity of NMDA receptor ion channel and tyrosine phosphorylation of NR2 subunits[16-18]. Src and Fyn may bind with NR2 subunits through tyrosine residues of NR2A and SH2 domain directly or through PDZ domains of PSD-95 indirectly, and the binding was involved in brain ischemia induced tyrosine phosphorylation of NR2 subunits[8,20-22]. As depolarization induced apoptosis-like cell death and NR2A tyrosine phosphorylation were both inhibited by genistein, we detected the protein interaction of Src with NR2A. Binding of Src with NR2A increased after depolarization, and the increase was inhibited by EGTA, Cd^{2+}, nimodipine and genistein. Moreover, although the NMDA receptors were blocked during depolarization by MK-801, the depolarization could still promote the tyrosine phosphorylation of NR2A and interaction of Src with NR2A. The data further proved that the process was largely triggered by the influx of Ca^{2+} from L-VGCCs. We also found that Src family kinase inhibitor PP2, but not its non-effect analog PP3, could partly prevent the cell death and inhibit the increased tyrosine phosphorylation of NR2A and interaction of Src with NR2A. The results indicate that tyrosine kinase Src might be activated by the depolarization-induced influx of Ca^{2+} and involved in the tyrosine phosphorylation of NR2A.

Electrophysiological studies showed that NMDAR currents in neurons are governed by a balance between tyrosine phosphorylation and dephosphorylation: inhibiting endogenous phosphotyrosine phosphatase (PTP) activity or introducing exogenous Src enhance NMDAR currents, whereas inhibiting endogenous PTK activity or introducing exogenous PTP suppresses NMDAR currents[17,23]. The addition of exogenous Src or Fyn potentiates recombinant NMDAR-mediated currents in HEK293 cells[24-25]. Moreover, SFKs were implicated as endogenous upregulators of NMDAR activity through the use of a phosphopeptide (pYEEI peptide) SFK activator, which is a ligand for SFK SH2 domains. The activating peptide increased synaptic NMDAR-mediated currents in cultured neurons, whereas the SFK inhibitor PP2 could decrease the NMDAR currents in neurons from colitis[26-27]. Our previous work reported that brain ischemia/reperfusion might increase the interaction of Src/Fyn, PSD-95 and L-VGCC α1C subunit and tyrosine phosphorylation of L-VGCC α1C subunit[10]. Ross[28] reported that L-VGCC-mediated whole-cell currents could be up-regulated by Src kinase. All these findings suggested that SFK might phosphorylate NMDAR subunits and L-VGCC α-subunits and lead to increased NMDAR and L-VGCC-mediated synaptic currents, which contribute to Ca^{2+}-dependent synaptic plasticity or pathological events such as brain ischemia[21].

However, the administration of nimodipine, MK-801, or the combination of nimodipine and MK-801 could not absolutely prevent the depolarization-induced apoptosis, whereas NR2A

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tyrosine phosphorylation was inhibited by a combination of nimodipine and MK-801. As brain ischemia and depolarization could induce the pre-synaptic glutamate release, and subsequent activation of other glutamate receptors, such as GluR6-KA receptors, could lead to neuronal injury[29], the results suggest that L-VGCC and NMDAR cross-talk might just be one of the possible signal pathways involved in brain ischemia and depolarization-induced neuronal death.

Taken together, the above results *in vitro* further prove our previous proposed hypothesis of cross-talk of L-VGCCs with NMDA receptors *in vivo*[8,9]. Briefly, high concentration KCl induced depolarization led to influx of Ca^{2+} through L-VGCCs. Increased intracellular Ca^{2+} activated Src kinase, the latter bound with NR2A and promoted tyrosine phosphorylation of NR2A and L-VGCC α-subunits, which caused the up-regulation and the persistent openness of NMDA receptors and led to overload of intracellular Ca^{2+}-induced apoptosis-like cell death.

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References

1. Bano D, Nicotera P. Glutamate-independent calcium toxicity: introduction: Ca^{2+} Signals and neuronal death in brain ischemia. Stroke. 2007;38 (2 suppl):674-6.
2. Kuriyama H, Nakagawa M, Tsuda M. Intracellular Ca^{2+} changes induced by in vitro ischemia in rat retinal slices. Exp Eye Res. 2001;73(3):365-74.
3. Altier C, Spaetgens RL, Nargeot J, Bourinet E, Zamponi GW. Multiple structural elements contribute to voltage-dependent facilitation of neuronal alpha 1C (CaV1.2) L-type calcium channels. Neuropharmacology. 2001;40(8):1050-7.
4. Wankerl K, Weise D, Gentner R, Rumpf JJ, Classen J. L-type voltage-gated Ca^{2+} channels: a single molecular switch for long-term potentiation/long-term depression-like plasticity and activity-dependent metaplasticity in humans. J Neurosci. 2010; 30(18): 6197-204.
5. Timmermann DB, Westenbroek RE, Schousboe A, Catterall WA. Distribution of high-voltage-activated calcium channels in cultured gamma-aminobutyric acidergic neurons from mouse cerebral cortex. J Neurosci Res. 2002;67(1):48-61.
6. Lukyanetz EA, Stanika RI, Koval LM, Kostyuk PG. Intracellular mechanisms of hypoxia-induced calcium increase in rat sensory neurons. Arch Biochem Biophys. 2003;410(2):212-21.
7. Robert F, Bert L, Stoppini L. Blockade of NMDA-receptors or calcium-channels attenuates the ischaemia-evoked efflux of glutamate and phosphoethanolamine and depression of neuronal activity in rat organotypic hippocampal slice cultures. C R Biol. 2002;325(4):495-504.
8. Hou XY, Zhang GY, Yan JZ, Chen M, Liu Y. Activation of NMDA receptors and L-type voltage-gated calcium channels mediates enhanced formation of Fyn–PSD95–NR2A complex after transient brain ischemia. Brain Res. 2002;955(1-2):123-32.
9. Liu Y, Hou XY, Zhang GY, Xu TL. L -type voltage-gated calcium channel attends regulation of tyrosine phosphorylation of NMDA receptor subunit 2A induced by transient brain ischemia. Brain Res. 2003;972(1-2):142-8.
10. Hou XY, Zhang GY, Yan JZ, Liu Y. Increased tyrosine phosphorylation of a1C subunits of L-type voltage-gated calcium channels and interactions among Src/Fyn, PSD-95 and a1C in rat hippocampus after transient brain ischemia. Brain Res. 2003;979(1):43-50.
11. Jiang Q, Gu Z, Zhang G. Activation, involvement and nuclear translocation of c-Jun N-terminal protein kinase 1 and 2 in glutamate-induced apoptosis in cultured rat cortical neurons. Brain Res. 2002; 956(2):194-201.
12. Pei L, Li Y, Wang DG, Zhang GY, Cui ZC, Zhu ZM. Preparation, purification and application of O-phosphotyrosine protein specific antibody. Acta Acad Med Xuzhou. 2000;20(2):87-91.
13. Schotanus SM, Chergui K. NR2A-containing NMDA receptors depress glutamatergic synaptic transmission and evoked-dopamine release in the mouse striatum. J Neurochem. 2008; 106(4):1758-65.
14. Das S, Sasaki YF, Rothe T, et al. Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. Nature. 1998;393(6683):377-81.
15. Petrenko AB, Yamakura T, Baba H, Shimoji K. The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. Anesth Analg. 2003;97(4):1108-16.
16. Hayashi T, Thomas GM, Huganir RL. Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. Neuron. 2009;64 (2):213-26.
17. Lu WY, Xiong ZG, Lei S, et al. G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. Nat Neurosci. 1999;2(4):331-8.
18. Lei G, Xue S, Chery N, et al. Gain control of N-methyl-D-aspartate receptor activity by receptor-like protein tyrosine phosphatase alpha. EMBO J. 2002;21(12):2977-89.
19. Liu Y, Zhang G, Gao C, Hou X. NMDA receptor activation results in tyrosine phosphorylation of NMDA receptor subunit 2A (NR2A) and interaction of Pyk2 and Src with NR2A after transient cerebral ischemia and reperfusion. Brain Res. 2001;909 (1-2):51-8.
20. Cui H, Hayashi A, Sun HS, et al. PDZ Protein Interactions Underlying NMDA Receptor-Mediated Excitotoxicity and Neuroprotection by PSD-95 Inhibitors. J Neurosci. 2007;27(37): 9901-15.
21. Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. Nat Rev Neurosci. 2004;5:317-28.
22. Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T. PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. Proc Natl Acad Sci. 1999;96(2):435-40.
23. Wang YT, Salter MW. Regulation of NMDA receptors by tyrosine kinases and phosphatases. Nature. 1994;369(6477):233-5.
24. Xu J, Weerapura M, Ali MK, et al. Control of excitatory synaptic transmission by C-terminal Src kinase. J Biol Chem. 2008;283 (25):17503-14.
25. Kohr G, Seeburg PH. Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. J Physiol. (Lond.) 1996;492(Pt 2):445-52.
26. Yu XM, Askalan R, Keil GJ, Salter MW. NMDA channel regulation by channel-associated protein tyrosine kinase Src. Science. 1997;275(5300):674-8.
27. Li J, McRoberts JA, Ennes HS, et al. Experimental colitis modulates the functional properties of NMDA receptors in dorsal root ganglia neurons. Am J Physiol Gastrointest Liver Physiol. 2006;291(2):G219-28.
28. Ross GR, Kang M, Akbarali HI. Colonic inflammation alters Src kinase-dependent gating properties of single Ca^{2+} channels via tyrosine nitration. Am J Physiol Gastrointest Liver Physiol. 2010;298(6):G976-84.
29. Li C, Xu B, Wang WW, et al. Coactivation of GABA receptors inhibits the JNK3 apoptotic pathway via disassembly of GluR6-PSD-95-MLK3 signaling module in KA-induced seizure. Epilepsia. 2010;51(3):391-403.