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Research report

# 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☆

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**Abstract**

Transient ischemia increases tyrosine phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor. Several tyrosine kinases are involved in this process. In this study, effect of ischemia and reperfusion (I/R) on tyrosine phosphorylation of NMDA receptor subunit 2A (NR2A) and the interaction of two tyrosine kinases, Src and Pyk2, with NR2A was investigated. Four-vessel occlusion was used to produce transient (15 min) cerebral ischemia in SD rats. Tyrosine phosphorylation of NR2A in hippocampus was enhanced after 15 min of reperfusion and reached its peak level at 6 h of reperfusion. The increase sustained for at least 24 h. Src and Pyk2 co-immunoprecipitated with NR2A and the binding increased after I/R, which also reached a peak at 6 h of reperfusion. Besides, Src and Pyk2 were activated after I/R. These increases were prevented by ketamine, a selective NMDA receptor antagonist, which was administered to the SD rats 20 min before ischemia. Moreover, Src and Pyk2 coprecipitated with each other. These data show that NR2A, Src and Pyk2 might form a protein complex in vivo and the interaction suggests a possible mechanism of signal transduction in the postischemic hippocampus.


*Theme:* Disorders of the nervous system

*Topic:* Ischemia

*Keywords:* NMDA receptor; Cerebral ischemia; Tyrosine phosphorylation; Src; Pyk2; Interaction

## 1. Introduction

The hippocampus is one of the most vulnerable brain regions to ischemic damage. Transient cerebral ischemia causes release of glutamate acid, which may induce the influx of $Ca^{2+}$ via *N*-methyl-D-aspartate (NMDA) receptor [1]. Protein tyrosine phosphorylation is thought to be an important way of regulating activity of NMDA receptor. Though the effect of ischemia on tyrosine phosphorylation of NMDA receptor has been well documented [15,24], intracellular signaling pathways induced by ischemia and reperfusion (I/R) which lead to altered tyrosine phosphorylation of NR are still not sure. Recently, several molecules including Src and proline-rich tyrosine kinase 2 (Pyk2) have been shown to be involved in the process [21,28].

Src is a non-receptor protein tyrosine kinase(PTK) of 60 kDa known as c-Src and v-Src, the former is widely distributed in many tissues. The family of Src-related PTKs now includes nine members (Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk) [2]. Src may be a regulator of the NR ion channel and may bind to NR through its Src homology 2 (SH2) domain [23,28], which can bind to phospho-tyrosine (pY). Another tyrosine kinase abundant in central neuron system, Pyk2, was recently thought to be upstream of Src-MAPK (mitogene-activated protein kinase) cascade [7,18]. It may play an important role in transferring $Ca^{2+}$ signals to Src. Both these kinases are activated by autophosphorylation of tyrosine residues (Src–Tyr416, Pyk2–Tyr402) [5,27]

In this report, the effect of I/R and ketamine on the tyrosine phosphorylation of NMDA receptor subunit 2A

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(NR2A) and the interaction of Src and Pyk2 with NR2A were investigated. The interaction suggests a possible mechanism of modulating the function of the NMDA receptor after transient cerebral ischemia.

## 2. Materials and methods

### 2.1. Animal surgical procedures

Adult male Sprague–Dawley (SD) rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 200–250 g were used. Transient cerebral ischemia was induced by four-vessel occlusion as described before [16]. Briefly, rats were anesthetized and both vertebral arteries were occluded permanently by electrocautery. On the next day, both carotid arteries were occluded with aneurysm clips for 15 min. During ischemia, animals were required to meet the following criteria: (1) completely flat EEG during occlusion, (2) maintenance of dilated pupils and absence of a cornea reflex due to strong light stimulation, and (3) rectal temperature was maintained at 37–37.5°C. Animals not meeting these criteria were excluded. Sham control animals received the same surgical procedures except those carotid arteries were not occluded. When necessary, ketamine (Sigma, St. Louis, USA) or dissolvent was administered to the rats by abdominal injection 20 min before occlusion.

### 2.2. Tissue preparation

At various times of reperfusion, animals were killed by decapitation and the hippocampus were removed and quickly frozen in liquid nitrogen. Before homogenization, the hippocampus were warmed to −5–0°C and homogenized in ice-cold 0.32 mol/l sucrose containing 50 mmol/l MOPS (Sigma), pH 7.4, 100 mmol/l KCl, 1 mmol/l $Na_3VO_4$ (Sigma), 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml each of leupeptin, pepstatin A and aprotinin (Sigma). The homogenizates were centrifuged at 800 *g* for 10 min at 4°C. Supernatants were collected and stored at −80°C for use. Protein concentration was determined by the method of Lowry et al. [8].

### 2.3. Immunoprecipitation and immunoblot

Immunoprecipitation of NR2A, Src or Pyk2 was performed as in Pei et al. [15]. Tissue homogenizates (400 μg of protein) were diluted 4-fold with 0.05 mol/l Hepes buffer, pH 7.4, containing 10% glycerol, 0.15 mol/l NaCl, 1% Triton X-100, 0.5% Nonidet P-40, and 1 mmol/l each of EDTA, EGTA, PMSF and $Na_3VO_4$. Samples were preincubated for 1 h with 25 μl protein A/G-sepharose CL-4B (Amersham Pharmacia, Buckinghamshire, UK) and then centrifuged to remove any protein adhered nonspecifically to the protein A/G. The supernatants were incubated with 1–2 μg proper antibodies for 4 h or overnight at 4°C. Protein A/G-sepharose (25 μl) was added and the incubation continued for 2 h. Samples were centrifuged at 10,000 *g* and the pellets were washed three times with HEPES buffer. Bound proteins were eluted by adding 2×SDS–PAGE sample buffer (20 μl) and boiled at 100°C for 5 min. Samples were then centrifuged and supernatants were used for immunoblot (IB).

For immunoblot, the supernatants were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were electrotransferred onto nitrocellulose membrane (Amersham Pharmacia) by the method of Sambrook et al. [20]. The membrane was probed with the indicated antibodies at 4°C overnight. Detection was carried out by alkaline phophatase conjugated sheep anti-mouse IgG or sheep anti-rabbit IgG (Sigma, 1:10,000) and developed using NBT/BCIP color substrate (Promega, Madison, USA). After immunoblot, the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP upland, CA).

### 2.4. Antibodies

Rabbit polyclonal anti-NR2A was purchased from Chemicon (Temecula, CA). Mouse monoclonal anti-Pyk2 and anti-phosphotyrosine (PY20) were obtained from Pharmingen (Lexington, KY). Mouse monoclonal anti-Src was purchased from Calbiochem (Cambridge, MA).

### 2.5. Statistics

Values were expressed as mean±S.D. from three independent animals. Statistical analysis of the results was carried out by one-way analysis of variance (ANOVA) followed by the Duncan's new multiple range method or Newman–Keuls test.

## 3. Results

### 3.1. Effects of ketamine on altered tyrosine phosphorylation of NR2A induced by I/R in hippocampus

Time course of I/R induced tyrosine phosphorylation of NR2A was determined (Fig. 1A and B). Tyrosine phosphorylation of NR2A was decreased slightly after transient cerebral ischemia (15 min) and increased after reperfusion, which reached its peak lever at 6 h of reperfusion, then decreased again. The increase sustained for at least 24 h. The protein amount of NR2A decreased after ischemia, and there were significant decreases after 24 h of reperfusion. I/R induced increase of tyrosine phosphorylation was dose-dependent inhibited by ketamine, a selective NMDA receptor antagonist administered to the rats by abdominal

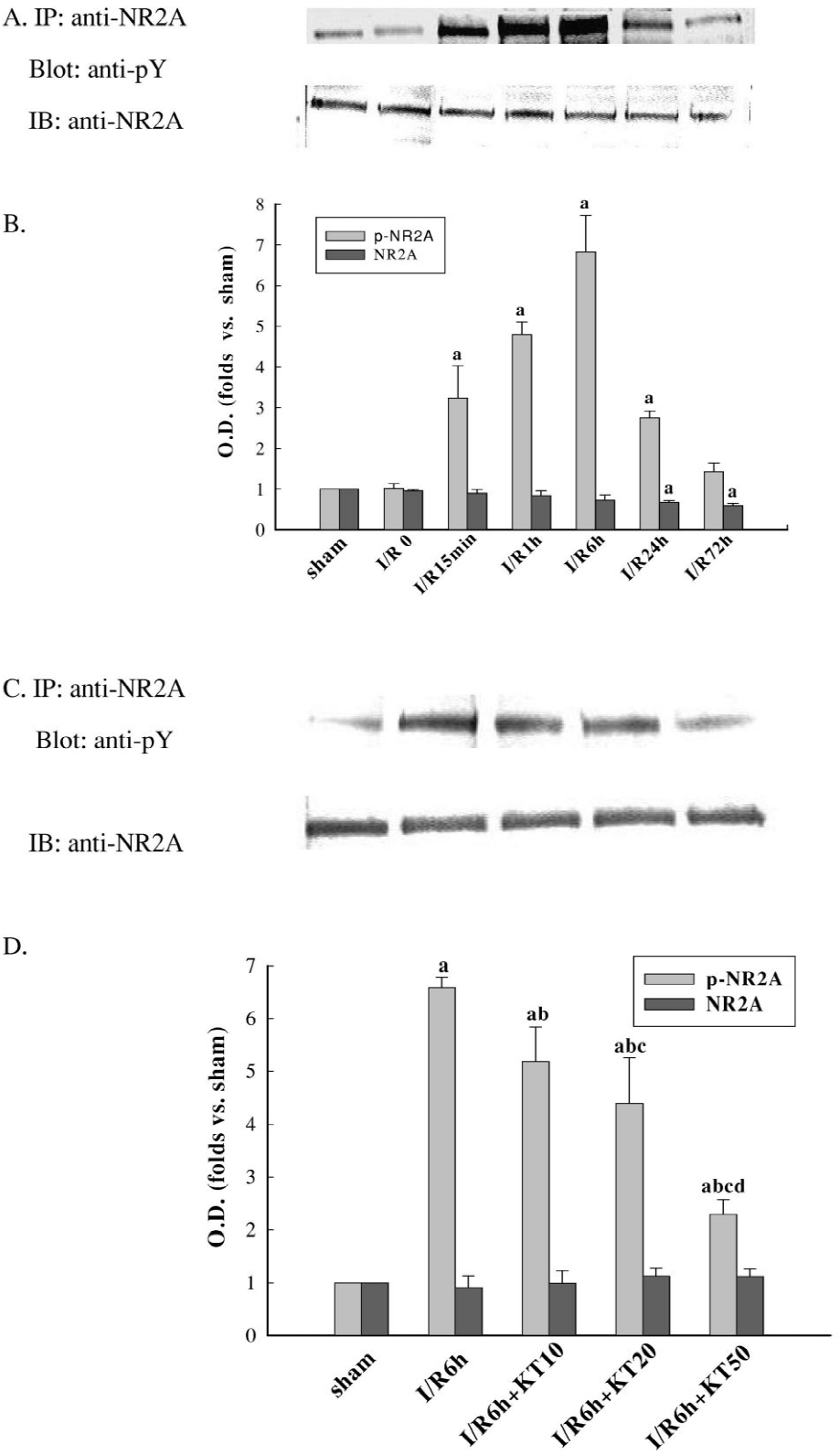


Fig. 1. Effect of ketamine on I/R induced tyrosine phosphorylation of NR2A. A, B: Time course of I/R induced altered tyrosine phosphorylation of NR2A from sham and ischemic animals that had 0, 15 min, 1, 6 or 24 h of reperfusion. C, D: Effect of ketamine on I/R induced tyrosine phosphorylation of NR2A. Homogenates of rat hippocampus from animals were immunoprecipitated (IP) with anti-NR2A specific antibody and blotted (blot) with anti-pY antibody. Bands corresponding to NR2A were scanned and the intensities were represented as folds vs. sham control. Similar amounts of NR2A were detected in sham and drug administered groups by immunoblotting (IB) with anti-NR2A antibody. Data were expressed as mean±S.D. from three independent animals ($n=3$). [a]$P<0.05$ vs. sham control, [b]$P<0.05$ vs. I/R6 h, [c]$P<0.05$ vs. KT10, [d]$P<0.05$ vs. KT20. KT10, KT20, KT50: ketamine of 10, 20 or 50 mg/kg was administered to the rats 20 min before ischemia and hippocampus were removed at 6 h of reperfusion.

injection 20 min before ischemia (top panel of Fig. 1C and D, determined at I/R 6 h). Dissolvent (water) had no effect on the phosphorylation (date not shown). A total of 50 mg/kg ketamine partially inhibited the phosphorylation of NR2A (from 6.8 to 2.1 folds vs. Sham control). The protein level of NR2A, as indicated by NR2A immunoreactivities, was unaffected by ketamine (bottom panel of Fig. 1C and D).

### *3.2. Time course of interaction of Src and Pyk2 with NR2A after I/R*

The interaction of Src and Pyk2 with NR2A was determined at various times of reperfusion from immunoprecipitation followed by western blot. NR2A could co-immunoprecipitate with both Src and Pyk2, respectively, and the bindings increased rapidly and reached their peaks (2.8 and 3.0 folds vs. sham control, respectively) at 6 h of reperfusion (Fig. 2).

### *3.3. Effect of ketamine on the interactions of Src and Pyk2 with NR2A*

Ketamine was administered 20 min before 15 min of ischemia. The associations of NR2A with Src and Pyk2 were determined at 6 h of reperfusion. Both of the bindings were inhibited by 50 mg/kg ketamine (from 2.8 and 3.0 folds to 1.2 and 1.1 folds, respectively) (Fig. 3A,B). Ketamine had no effect on the amounts of Src or Pyk2. Besides, tyrosine phosphorylation of Src and Pyk2 increased after I/R, and the increase was inhibited by 50 mg/kg ketamine (Fig. 3C,D)

### *3.4. Src, Pyk2 and NR2A co-immunoprecipitated with each other*

NR2A could co-immunoprecipitate with Src and Pyk2 and the bindings increased after I/R vs. sham control. Moreover, Src and Pyk2 co-immunoprecipitated with each other and the binding also increased after I/R. The protein

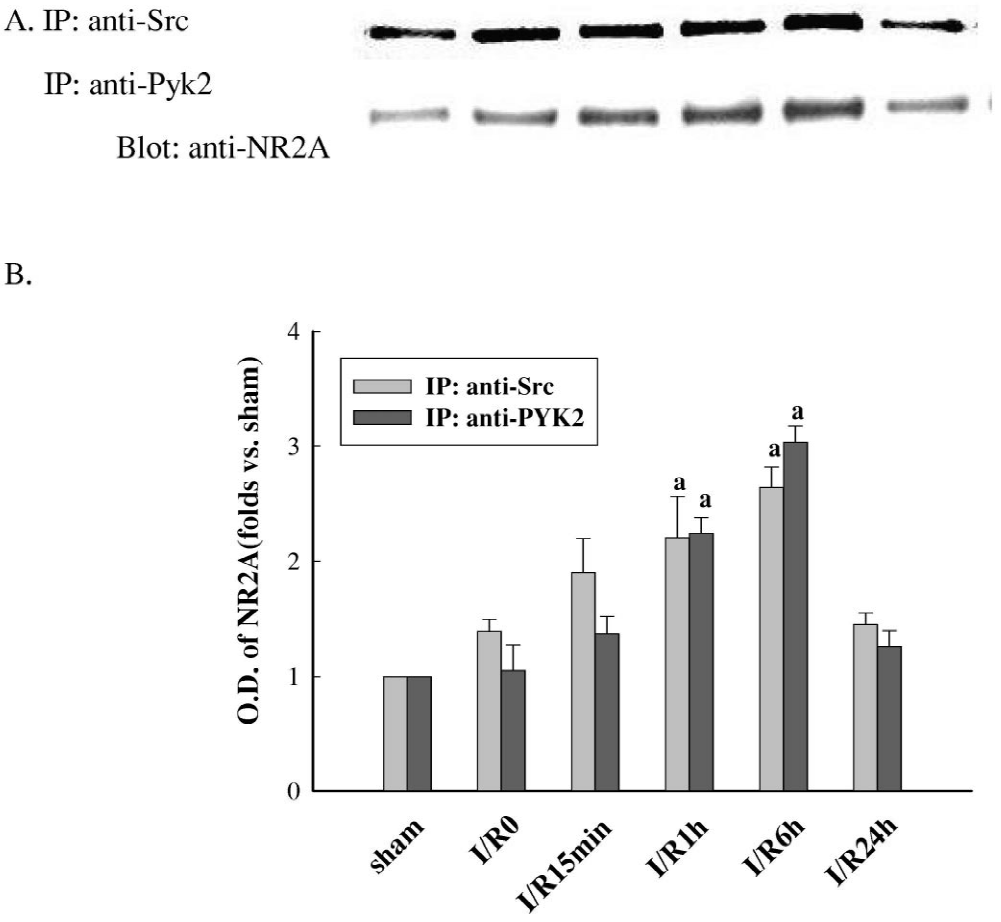


Fig. 2. Time course of interaction of Src and Pyk2 with NR2A. Homogenates of rat hippocampus from sham control or ischemic animals that had 0, 15 min, 1, 6 or 24 h of reperfusion were immunoprecipitated with anti-Src or anti-Pyk2 specific antibodies and blotted with anti-NR2A antibody (A). Bands corresponding to NR2A were scanned and the intensities were represented as folds vs. sham control (B). $^{a}P<0.05$ vs. sham control, $n=3$.

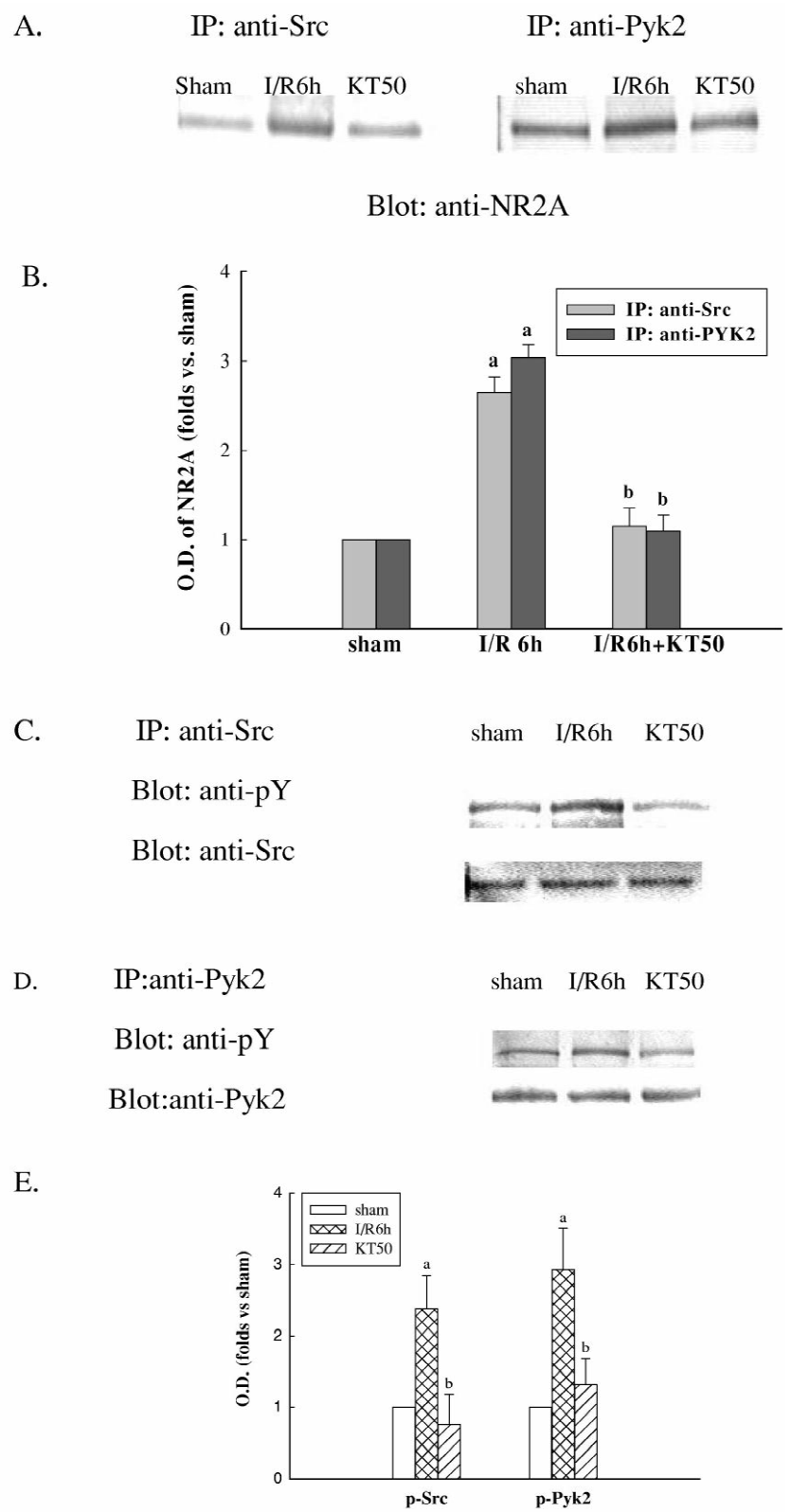


Fig. 3. Effect of ketamine on I/R induced altered interaction of Src and Pyk2 with NR2A. Homogenates of rat hippocampus from sham control or ischemic animals that had been administered ketamine of 50 mg/kg·w 20 min before ischemia and recovered for 6 h were immunoprecipitated with anti-Src or anti-Pyk2 specific antibody, and blotted with anti-NR2A antibody (A). Bands corresponding to NR2A were scanned and the intensities were represented as folds vs. sham control (B). Tyrosine phosphorylation of Src and Pyk2 increased after I/R, and was inhibited by ketamine. Ketamine had no effect on the expression of Src or Pyk2 at I/R6 h (C,D). $^{a}P<0.05$ vs. sham control, $^{b}P<0.05$ vs. I/R6 h, $n=3$.

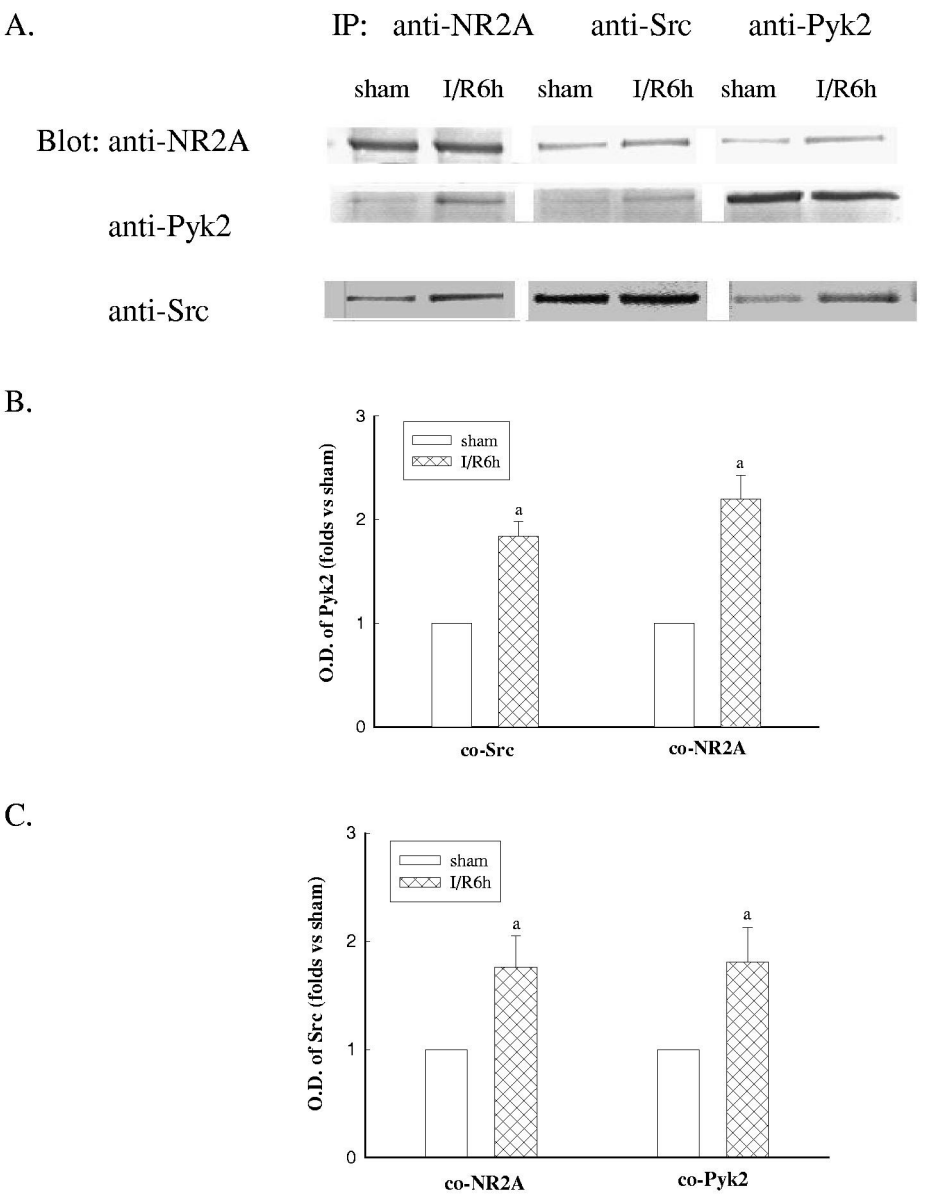


Fig. 4. Co-immunoprecipitation of Src, Pyk2 and NR2A homogenates of hippocampus from sham control and ischemic animals at 6 h of reperfusion (I/R6 h) were immunoprecipitated with anti-NR2A, anti-Src or anti-Pyk2 specific antibodies respectively, then blotted with anti-NR2A, anti-Src or anti-Pyk2 antibodies (A). Bands corresponding to Pyk2 were scanned and the intensities were expressed as folds vs. sham control (B). 'co-Src' and 'co-NR2A' represented the Pyk2 co-immunoprecipitated with Src and NR2A respectively. Bands corresponding to Src were also quantified (C). 'co-NR2A' and 'co-Pyk2' represented the Src co-immunoprecipitated with NR2A and Pyk2 respectively. [a] $P<0.05$ vs. sham control, $n=3$.

levels of NR2A, Src and Pyk2 were not affected by I/R (Fig. 4).

## 4. Discussion

Modulation of NMDA receptors in the brain by protein phosphorylation may play a central role in the regulation of synaptic plasticity, neuronal development, and neurological disorders [11,12,22]. There are many serine, threonine and tyrosine residues in C terminuses of NR1 and NR2 subunits. Biochemical studies have demonstrated that NR are directly phosphorylated at these sites by PKA, PKC and protein tyrosine kinases (PTKs) [6,25], whereas protein phosphorylation of NR2 subunits is an important way of regulating NMDA receptor function [3,6].

In this report, we studied the effect of I/R on tyrosine phosphorylation of NR2A. I/R increased tyrosine phosphorylation of NR2A, which reached a peak of 6.8 folds of sham control at 6 h of reperfusion. The increase was prevented by ketamine, a selective NMDA receptor antagonist. These results showed that I/R could lead to

enhanced and lasting openness of NMDA receptor ion channel, and the openness was self-regulated by NMDA receptor.

It had been demonstrated that Src family PTKs modulated openness of NR ion channel and Src bound to NR1 and NR2B [15,28]. The binding was due to interaction of SH2 domain and phospho-tyrosine (pY) [23]. Proteins of the Src family PTKs have a common domain organization with each segment designated as a Src-homology (SH) region such as SH1 (catalytic), SH2 and SH3 domains [27]. SH2 and SH3 domains mediate protein–protein interactions in cellular signaling cascades and are found in many proteins outside the Src family kinases [14]. Here we first demonstrated that Src bound to NR2A in vivo. The time course of the binding was similar to that of I/R induced tyrosine phosphorylation of NR2A, and the binding reached its peak level at I/R 6 h. The binding was prevented by ketamine. These data showed that Src was closely related to the function of NR.

We demonstrated that tyrosine phosphorylation of Src and Pyk2 increased after I/R and the increases were prevented by ketamine, whereas activation of Src or Pyk2 depended on their autophosphorylation of tyrosine residues [9,13,26]. These data showed that Src and Pyk2 were activated via NR after I/R. In this report, Pyk2 co-immunoprecipitated with Src and the binding increased after I/R. Pyk2 also co-immunoprecipitated with NR2A and the binding changed of the same process as that of Src. But there's no proof that Pyk2 binds to NR2A directly. Pyk2 is a member of the Fak family of non-receptor protein tyrosine kinases. It has two proline-rich domains (29 and 23.3% proline, respectively), which could bind to the SH3 domain [7,17,27]. Therefore, NR2A, Src and Pyk2 might form a ternary complex and the ternary complex may be a formation of NR2A–(SH2)Src(SH3)–Pyk2.

Our data showed that Src and Pyk2 bound to NR2A earlier than the increase in tyrosine phosphorylation of NR2A after I/R. Evidence obtained in PC12 cells and HEK-293 cells indicate that Pyk2 is involved in the $Ca^{2+}$-induced activation of tyrosine phosphorylation and downstream Src-MAPK signaling cascades [7,18]. Cheung et al. [4] found that Pyk2 was activated earlier than Src after I/R. Thus, we think that there may be a mechanism of postischemic events (Fig. 5). Stimulation of NR and other ion channels (such as L-type voltage dependent calcium channel, L-VDCC) induced by cerebral ischemia leads to an influx of $Ca^{2+}$, which activates Pyk2 via $Ca^{2+}$/CaM or PKC [10,18,21]. Activated Pyk2 may bind to Src and activates it. Src binds to NR subunits and phosphorylates them on tyrosine residues. Phosphorylated NR subunits may bind to more Src, which causes enhanced NMDA receptor function and further boost the entry of $Ca^{2+}$. Src also promotes the opening of L-VDCC [19]. Therefore the positive feedback promotes the I/R induced influx of $Ca^{2+}$ and kinase cascades, and damages the ischemic cells more seriously.

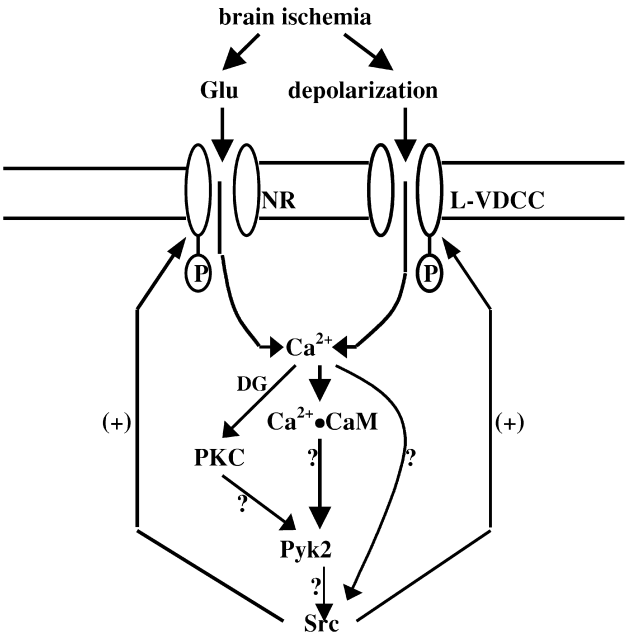


Fig. 5. Model of I/R induced tyrosine phosphorylation of NR2A. Stimulation of NMDA receptor after I/R leads to influx of $Ca^{2+}$. The activation of Pyk2 by $Ca^{2+}$ via CaM or PKC causes activation of Src, which also phosphorylates NMDA receptor and L-VGCC and boost influx of $Ca^{2+}$. The feedback leads to more activation of protein tyrosine kinases.

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