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# Src is Implicated in Hepatic Ischemia Reperfusion-Induced Hippocampus Injury and Long-Term Cognitive Impairment in Young Mice via NMDA Receptor Subunit 2A Activation

Xiangyang Yu,[a] Lili Jia,[b] Kuoqi Yin,[a] Jingshu Lv,[a] Wenli Yu[b]* and Hongyin Du[b]

[a] Tianjin Medical University First Center Clinical College, Tianjin, China

[b] Department of Anesthesiology, Tianjin First Center Hospital, Tianjin, China

**Abstract—Hepatic ischemia reperfusion (HIR) has been found to induce hippocampus injury and cognitive dysfunction. The N-methyl-D-aspartate (NMDA) receptor subunit 2A (NR2A) is an important factor mediating excitotoxicity and neurons injury, and autophosphorylation of Src can up-regulate tyrosine phosphorylation of NR2A to improve its activity. However, the role of Src and NR2A in HIR-induced hippocampus injury in young mice remains unknown. In this study, we found that serum biomarkers of brain injury (S100β and NSE) increased significantly and reached highest after reperfusion of 3 days which had the same trend with the levels of p-Src and p-NR2A. Interactions between Src and NR2A or PSD95 were increased after HIR. Hippocampal neuron apoptosis was increased, and long-term cognitive impairment was found after reperfusion of 1 month. Inhibition of Src and NR2A with PP2 and NVP-AAM077 respectively not only down-regulated the levels of p-Src and p-NR2A, but also ameliorated hippocampal neurons apoptosis and long-term cognitive impairment after HIR. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ) and interleukin (IL)-6 were increased after reperfusion of 3 days, while PP2 and NVP-AAM077 treatment didn't attenuate the changes. And no difference was found in serum TNF-α, IFN-γ, IL-6 concentrations as well as the levels of Src, p-Src, NR2A, p-NR2A, PSD95 among the four groups after reperfusion of 1 month. In summary, HIR can lead to hippocampus injury and long-term cognitive dysfunction, and Src-PSD95-NR2A pathway plays an important role in the process.** 

**Key words: hepatic ischemia reperfusion, hippocampus, Src, NR2A, cognitive function, tyrosine phosphorylation.**

## INTRODUCTION

Pediatric liver transplantation is the most effective way to treat end-stage liver disease in children (Rand and Olthoff, 2003; Kasahara et al., 2017). However, The incidence of neurological complications after pediatric liver transplantation can reach as high as 46% which has higher mortality compared with adults (Erol et al., 2007; Fernandez et al., 2010). What's more, recent researches indicated that pediatric liver transplantation could induce long-term cognitive dysfunction (Bucuvalas, 2013; Kaller et al., 2013). Hepatic ischemia reperfusion (HIR) is a common pathophysiological process during liver transplantation which involves the release of reactive oxygen species and mediators of inflammation, and activates the neutrophil-mediated inflammatory responses as well (Tu et al., 2016; Karimi et al., 2017; Ma et al., 2017). Recent evidence revealed that HIR could damage the central nervous system (CNS), leading to neurons apoptosis and spatial cognitive impairment in adult rodent, and the effect may be related with the N-methyl-D-aspartate (NMDA) receptors (Wang et al., 2014; Tu et al., 2016; Jia et al., 2017; Ma et al., 2017). Whether HIR can induce brain injury and long-term cognitive function in children is still unknown. Hippocampus is one of the most important parts of the brain which is associated with the abilities of learning and memory (Karimi et al., 2017). Therefore we detected the effect of HIR on hippocampus in young mice in the present study.

Normal brain functions are dependent on the dynamic balance between excitatory and inhibitory inputs. NMDA

*Corresponding author. Fax: +86-022-23627130.
E-mail address: yzxwenliyu@163.com (W. Yu).

*Abbreviations:* AI, apoptosis index; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BDNF, brain-derived neurotrophic factor; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid); ELISA, enzyme linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HIR, hepatic ischemia reperfusion; IFN-γ, interferon-γ; IL-6, interleukin-6; MWM, Morris water maze; NMDA, N-methyl-D-aspartate; NR2A, N-methyl-D-aspartate receptor 2A subunit; NSE, neuron-specific enolase of enzyme; PMSF, phenylmethyl sulfonylfluoride; PSD95, postsynaptic density protein 95; SrcPTKs, Src family of non-receptor protein tyrosine kinases; TNF-α, tumor necrosis factor α.

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receptors are the primary excitatory glutamatergic receptors expressed in brain, the functions of which are closely associated with hippocampal learning and memory functions. (Morris, 1989; Nakanishi, 1992; Jung et al., 2010). In physiological conditions, NMDA receptors are involved in the synaptic transmission and synaptic plasticity in CNS (Liu et al., 2004; Zhang et al., 2016). However, some pathological conditions such as brain ischemia, traumatic brain injury, epilepsy, depression and certain neurodegenerative disorders have been linked with excessive activation of NMDA receptors which can induce a large influx of $Ca^{2+}$ leading to excitotoxicity of neurons(Raza et al., 2004; Hou et al., 2007; Wu and Hou, 2010; Sonmez et al., 2015; Reus et al., 2016; Zhang et al., 2016; Yang et al., 2017). NMDA receptors function as multi-protein complexes which are centered on a core heterotetramer containing ligand-binding sites and an ion channel pore (Scanlon et al., 2017). The heterotetramers are composed of two obligate NMDA receptor 1 (NR1) subunits and two NR2 subunits, NR2A-D (Laube et al., 1998; Cull-Candy et al., 2001). The intracellular segments of these subunits contain multiple serine and tyrosine phosphorylation sites, and phosphorylation modification can affect the channel characteristics of NMDA receptors (Yang and Leonard, 2001). Src family of non-receptor protein tyrosine kinases (SrcPTKs) are members of non-receptor protein tyrosine kinases and involved in many cellular functions (Salter and Kalia, 2004). In the developing CNS, an important function of SrcPTKs is to regulate NMDA receptors activity and NMDA receptor-dependent synaptic plasticity (Salter and Kalia, 2004; Wu and Hou, 2010; Qiao et al., 2017). Src, a member of SrcPTKs, has been reported to up-regulate the tyrosine phosphorylation of NMDA receptor subunit 2A (NR2A) and increase the activity of NMDA receptors (Kohr and Seeburg, 1996; Rong et al., 2001; Thornton et al., 2003; Wu and Hou, 2010). Src plays a primary role in regulating the activity of NMDA receptors through several tyrosine phosphorylation sites of NR2A such as Y1325, Y1387 and Y1292, among which Y1325 is the principal phosphorylation site associated with Src (Yang and Leonard, 2001; Taniguchi et al., 2009). Intermolecular autophosphorylation of Src at Y416 can displace Y416 from the substrate binding site and allow the kinase access to substrates, which can up-regulate the activity of Src (Salter and Kalia, 2004). Postsynaptic density protein 95 (PSD95) has been found to assemble NR2A subunits and Src through its N-terminal PDZ domains which acts as a bridge linking Src kinases with its substrate—NR2A subunits, and mediates NR2A tyrosine phosphorylation by Src (Tomita et al., 2001; Wu and Hou, 2010).

With reference to the above studies, we proposed a hypothesis that HIR induced autophosphorylation of Src as well as tyrosine phosphorylation of NR2A via enhanced interaction of Src-PSD95-NR2A signaling modules, as a result, the function of NMDA receptors was enhanced excessively which resulted in the neuronal apoptosis in the hippocampus, and finally led to long-term cognitive dysfunction in young mice. To confirm the hypothesis, we chose 2-week mice to make 70% HIR model. Blood samples were used to detect the biomarkers of brain injury, aminotransferase and inflammatory cytokines. The phosphorylation of Src and NR2A was observed through western blot. Hippocampal neurons apoptosis was determined by TUNEL staining. Interactions among Src, PSD95 and NR2A were detected by Immunoprecipitation. Finally, we analyzed the long-term cognitive function after HIR and again detected the inflammatory cytokines and the activity of Src and NR2A after reperfusion of 1 month. PP2 (a inhibitor of SrcPTKs) and NVP-AAM077 (a inhibitor of NR2A) were used to further confirm the role of Src and NR2A in the model.

## EXPERIMENTAL PROCEDURES

### Animals

C57BL/6 mice (2-weeks) bought from Experimental animal center of the Chinese people's Liberation Army Military Medical Science Academy (Beijing, China), weighing 4–6 g, were housed under standard conditions with lights from 8:00 to 20:00. The temperature was controlled at 23–24 °C. Water and food were available. All experimental protocols on animals complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Tianjin First Center Hospital (Tianjin, China).

### Animal groups

Twenty-five mice were randomly divided into five groups ($n$ = 5/group) for enzyme linked immunosorbent assay (ELISA) and western blot: normal control group (NC group), HIR6h group, HIR1d group, HIR3d group and HIR7d group. To detect the effect of PP2 or NVP-AAM077 treatment on the tyrosine phosphorylation of Src or NR2A as well as the neurons apoptosis in sham-operated animals, 15 mice were randomly divided into three groups ($n$ = 5/group): vehicle pretreatment + sham-operated group (sham group), PP2 pretreatment + sham-operated group (sham(PP2) group) and NVP-AAM077 pretreatment + sham-operated group (sham(NVP) group). Another 72 mice were randomly divided into four groups ($n$ = 18/group): vehicle pretreatment + sham-operated group (sham group), vehicle pretreatment + HIR group (HIR group), PP2 pretreatment + HIR group (PP2 group) and NVP-AAM077 pretreatment + HIR group (NVP group). Five of the mice from each group were killed 3d after the surgery, their brains were taken for TUNEL staining and blood samples were collected for detection of alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ) and interleukin (IL)-6, another five mice from each group were killed 3d after the surgery and their hippocampi were taken for western blot and immunoprecipitation, the rest of the mice were used for detecting the long-term cognitive function 1 month after HIR, then the mice were killed for detection of



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