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·Original Article·

Synaptic non-GluN2B-containing NMDA receptors regulate tyrosine phosphorylation of GluN2B 1472 tyrosine site in rat brain slices

Heng Ai[2,*], Wen Lu[1,*], Mao Ye[1], Wei Yang[1]

[1]*Key Laboratory of Medical Neurobiology of Ministry of Health of China, Zhejiang Province Key Laboratory of Neurobiology, Department of Neurobiology, Zhejiang University School of Medicine, Hangzhou 310058, China*

[2]*Department of Physiology, Zhejiang Medical College, Hangzhou 310053, China*

[*]These authors contributed equally to this work.

Corresponding author: Wei Yang. E-mail: yangwei@zju.edu.cn

ABSTRACT

Activation of N-methyl-*D*-aspartate receptors (NMDARs) mediates changes in the phosphorylation status of the glutamate receptors themselves. Previous studies have indicated that during synaptic activity, tyrosine kinases (Src and Fyn) or phosphatases (PTPα and STEP) are involved in regulating the phosphorylation of NMDARs. In this study, we used immunoblotting to investigate the role of an NMDAR subpopulation on the phosphorylation level of the GluN2B subunit at the Y1336 and Y1472 sites in rat brain slices after NMDA treatment. We found that NMDA stimulation dramatically decreased the phosphorylation level of GluN2B at Y1472 in a dose- and time-dependent manner, but not at Y1336. Extrasynaptic NMDAR activation did not reduce the phosphorylation of GluN2B at Y1472. In addition, ifenprodil, a selective antagonist of GluN2B-containing NMDARs, did not abolish the decreased phosphorylation of GluN2B at Y1472 triggered by NMDA. These results suggest that the activation of synaptic GluN2A-containing NMDARs is required for the decreased phosphorylation of GluN2B at Y1472 that is induced by NMDA treatment in rat brain slices.

Keywords: N-methyl-*D*-aspartate receptors; tyrosine phosphorylation; Y1472; synapse; extrasynaptic receptors; GluN2B-containing NMDA receptors

INTRODUCTION

Glutamate, the major excitatory neurotransmitter in the brain, plays a vital role in many functions in the nervous system by activating ionotropic as well as metabotropic glutamate receptors. Ionotropic receptors include those activated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-*D*-aspartate (NMDA) and kainate. The NMDAR is a heteromeric molecule containing four subunits, two fundamental GluN1 subunits that are essential, and two GluN2 subunits (GluN2A and GluN2B). Recent evidence further revealed GluN3 subunits (GluN3A and GluN3B) that also modulate the properties of the NMDAR[1-3]. Dysfunction of NMDARs occurs in many neurological diseases, including stroke, epilepsy, Alzheimer's disease, and Parkinson's disease[4,5].

Phosphorylation of NMDARs is one of the most important mechanisms of their regulation[5]. Previous electrophysiological studies have shown that NMDAR currents in neurons are governed by a balance between tyrosine phosphorylation and dephosphorylation[6,7]. Accumulating evidence also shows that tyrosine phosphorylation of GluN2B plays a critical role in modulating NMDAR functions[5]. Three major tyrosine residues have been identified in the GluN2B C-terminus, designated Y1252, Y1336 and Y1472[5]. In particular, Y1472 is thought to be the major tyrosine phosphorylation site enzymatically catalyzed by Fyn and Src kinases[8,9], and is directly involved in synaptic plasticity. For instance, phosphorylation

at Y1472 is increased during long-term potentiation (LTP) and decreased during long-term depression (LTD)[10], as well as preventing the endocytosis of GluN2B by disrupting AP-2 binding to GluN2B[8]. In addition, the fear learning and memory of Y1472F knock-in mice (a knock-in mutation of the Tyr1472 site to phenylalanine) is impaired[11], providing the first evidence for the physiological function of Y1472 *in vivo*. Very recently, Harrington *et al.* found that phosphorylation of GluN2B at Y1472 is necessary for the formation of taste memory in rats[12]. In addition, some studies have also shown that phosphorylation at Y1336 is involved in calpain-mediated cleavage of GluN2B[13]. All this evidence strongly suggests that tyrosine phosphorylation of GluN2B is vital for the processes that underlie physiological and pathological plasticity in the brain.

Although previous studies have revealed the phosphorylation regulation of AMPARs by NMDA treatment[14], little is known about tyrosine phosphorylation changes in GluN2B under NMDA stimulation. Furthermore, which NMDAR subtype is involved remains unknown. Thus, the present study was designed to investigate the phosphorylation changes of GluN2B at both Y1472 and Y1336 in acute rat brain slices after NMDA stimulation.

MATERIALS AND METHODS

Antibodies and Reagents

The secondary antibodies, goat anti-mouse IgG Dylight^{TM}680 and goat anti-rabbit IgG Dylight^{TM}800, were from Thermo Electron Corp. (San Jose, CA). GluN2B mouse monoclonal antibody was made in our lab as described previously[15,16]. Phospho-GluN2B antibodies (pY1472 and pY1336) were from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Preparation of Acute Hippocampal Slice

Male Sprague-Dawley rats (4–6 weeks) were used in this study. All animal experiments were performed in accordance with the ethical guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Zhejiang University Animal Experimentation Committee. Preparation of transverse hippocampal slices was performed as described previously[17]. The rats were deeply anesthetized with ether. The brain was immediately removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (in mmol/L): 124 NaCl, 4 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 1 KH_2PO_4, and 25.7 $NaHCO_3$. The hippocampus was carefully dissected out on a plate soaked with ice-cold oxygenated ACSF and unrolled on an agar block. Subsequently, 400-μm transverse slices were cut on a Vibratome 1000 Plus tissue-chopper (St. Louis, MO). The slices were transferred to a chamber perfused with 26°C ACSF bubbled with 95% O_2/5% CO_2 and allowed to recover for at least 1 h before use.

Treatment of Acute Hippocampal Slices

The hippocampal slices were transferred to 26°C oxygenated ACSF with (in μmol/L) 1 TTX, 40 CNQX, 100 APV, and 5 nimodipine for 3 h[18] prior to NMDA treatment as below.

The slices were incubated in different concentrations of NMDA (10–300 μmol/L) in 26°C oxygenated Mg^{2+}-free ACSF supplemented with (in μmol/L) 1 TTX, 40 CNQX, and 5 nimodipine for 3 min to assess dose-related effects, or incubated in 20 μmol/L NMDA for different times.

Extrasynaptic NMDARs were selectively activated according to the procedures described previously[14,18]. Briefly, after blockade in ACSF with (in μmol/L) 1 TTX, 40 CNQX, and 5 nimodipine for 3 h, the slices were transferred to 50 μmol/L MK801 in 26°C oxygenated ACSF containing 10 μmol/L bicuculline and 5 μmol/L nimodipine for 15 min. The MK801 was removed by rinsing in ACSF for at least 15 min. Then the slices were incubated in 20 μmol/L NMDA in ACSF (lacking Mg^{2+}) containing (in μmol/L) 1 TTX, 40 CNQX, and 5 nimodipine for 3 min.

Selective inhibition of GluN2B-containing NMDARs was accomplished by applying ACSF with 10 μmol/L ifenprodil for 20 min. Then the slices were treated with 20 μmol/L NMDA in ACSF (lacking Mg^{2+}) containing (in μmol/L) 1 TTX, 40 CNQX, 5 nimodipine, and 10 ifenprodil for 3 min.

SDS-PAGE and Semi-Quantitative Western Blotting Analysis

Sample preparation was performed as described previously[19]. Briefly, slices were homogenized in ice-cold homogenization buffer (10 mmol/L Tris-HCl buffer, pH 7.4, containing in mmol/L 320 sucrose, 1 Na_3VO_4, 5 NaF, 1

EDTA and 1 EGTA). The homogenate was subjected to a first centrifugation at 700 g for 10 min at 4°C to remove nuclei and debris, followed by a second centrifugation at 37 000 g at 4°C for 40 min to obtain a pellet. This pellet, which is considered to be a crude membrane fraction, was resuspended in 10 mmol/L Tris-HCl (pH 7.4). Finally, the same volume of 2× loading buffer (125 mmol/L Tris-HCl, pH 6.8, 4% SDS, 10% dithiothreitol, 15% glycerol) was added to generate samples for SDS-PAGE. Equal amounts of the samples were subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in TBST for 1 h at room temperature, then incubated with specific primary antibody (anti-GluN2B, anti-phospho-GluN2B pY1336, or anti-phospho-GluN2B pY1472) at 4°C overnight, followed by incubation with goat anti-mouse IgG Dylight^{TM}680 or goat anti-rabbit IgG Dylight^{TM}800 for 2 h at room temperature. The signal intensity in each blot was quantified using Li-Cor/Odyssey (Li-Cor Biosciences, Lincoln, NE). Phospho-GluN2B signals were normalized to the total GluN2B protein signals.

Data Analysis

Data are presented as mean ± SEM from at least three independent experiments. Statistical analysis was performed with Graphpad Prism 5.0 software. Statistical differences were determined by one-way ANOVA. P <0.05 was considered to be statistically significant.

RESULTS

NMDAR Activation Regulates Phosphorylation of GluN2B at Y1472 but not Y1336

To evaluate the effects of NMDA activation on the tyrosine phosphorylation of GluN2B subunit residues Y1336 and Y1472, a series of concentrations of NMDA (from 10 to 300 μmol/L) was applied to the slices. Stimulation of slices with 10 μmol/L NMDA induced minor phosphorylation changes at Y1472 (Fig. 1A). However, 20 to 300 μmol/L NMDA significantly decreased the phosphorylation at Y1472 (Fig. 1A). Conversely, NMDAR activation resulted in no significant changes of GluN2B phosphorylation at Y1336 at all NMDA concentrations used (Fig. 1B). Thus, our results suggested that bath application of NMDA that selectively activates NMDARs, reduces the phosphorylation level of GluN2B at Y1472, but not at Y1336.

NMDA Stimulation Induces a Persistent Decrease of GluN2B Phosphorylation at Y1472

To characterize the time-window of phosphorylation changes

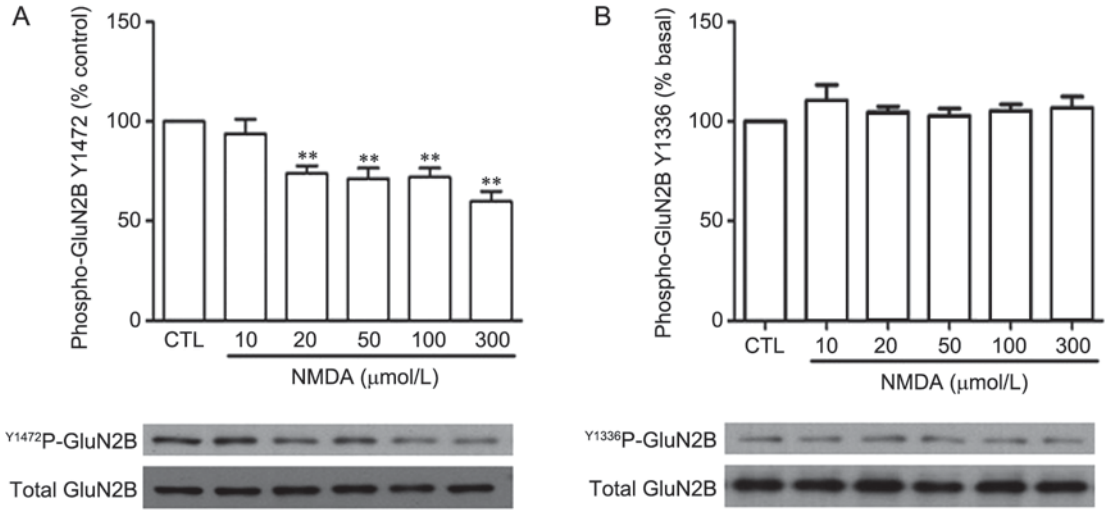

Fig. 1. Phosphorylation of GluN2B at Y1472 decreased during NMDAR activation. NMDA (10–300 μmol/L) was applied to acute rat brain slices and the tyrosine phosphorylation level of GluN2B was assessed by Western blot. A: Quantification of phospho-GluN2B at Y1472, normalized to total GluN2B. Mean ± SEM (n = 5–6). **P <0.01 compared with control (CTL). B: Quantification of phospho-GluN2B at Y1336, normalized to total GluN2B. Mean ± SEM (n = 5–6).

at Y1472, we examined its phosphorylation level after different durations of NMDA application. NMDA at 20 μmol/L was chosen to stimulate brain slices since 20–300 μmol/L elicited responses similar to those described above. A brief application (3 min) of NMDA resulted in a significant reduction in the phosphorylation level at Y1472 (Fig. 2). Even with a long period of stimulation (30 min), the phosphorylation level remained comparable to that at 15 min, much lower than the untreated control group. Taken together, these data suggest that the phosphorylation changes at Y1472 induced by NMDA last for at least 30 min.

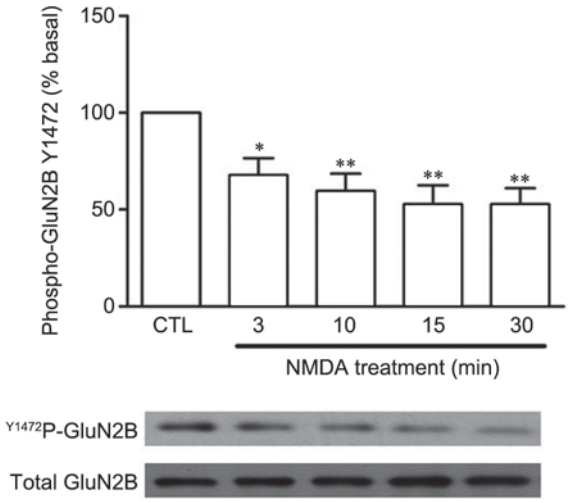

Fig. 2. The decrease of phosphorylation of GluN2B at Y1472 during NMDAR activation is persistent, lasting for at least 30 min. There were significant differences in phosphorylation between the untreated control and NMDA (20 μmol/L)-treated groups at different durations (*n* = 6). Data are presented as mean ± SEM. **P* <0.05; ***P* <0.01 compared with control (CTL).

Extrasynaptic Activation of NMDARs Fails to Elicit Detectable Changes of GluN2B Phosphorylation at Y1472

It is of interest to determine whether the changes in GluN2B phosphorylation at Y1472 are due to activation of synaptic or extrasynaptic NMDARs. A growing body of evidence shows that activation of synaptic or extrasynaptic NMDARs leads to distinct or even opposite consequences *via* activation of different downstream signaling pathways[20]. In order to verify the contribution of one subpopulation of NMDARs, we used the extrasynaptic activation protocol as described previously[14]. Our data clearly revealed that the selective extrasynaptic activation of NMDARs failed to induce any detectable phosphorylation changes compared to that of the untreated control group (Fig. 3), suggesting that synaptic NMDAR activation is responsible for the changes of GluN2B phosphorylation at Y1472.

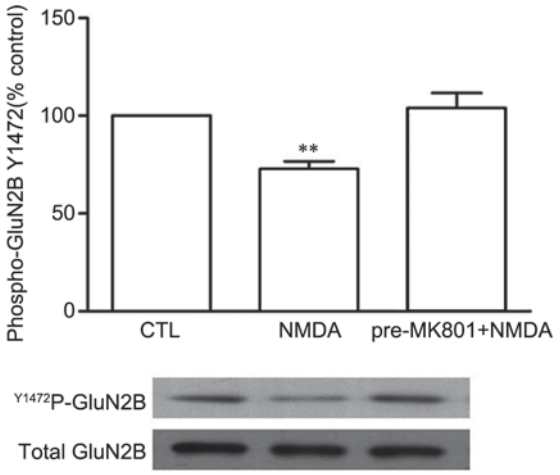

Fig. 3. Extrasynaptic activation of NMDARs fails to induce changes in GluN2B phosphorylation at Y1472. NMDA treatment (20 μmol/L, 3 min) decreased GluN2B phosphorylation at Y1472, while extrasynaptic activation failed to induce such changes (*n* = 6). Data are presented as mean ± SEM. ***P* <0.01 compared with control (CTL).

GluN2A Subunit-containing NMDARs Predominate in the Down-regulation of GluN2B Phosphorylation at Y1472

Considerable evidence shows that, in developmentally mature synapses, GluN2A-containing NMDARs occur in synaptic locations, while those containing GluN2B mainly occupy extrasynaptic sites[21,22]. Based on the results shown in Fig. 3, we speculated that GluN2A-containing NMDARs play a dominant role in mediating the decreased GluN2B phosphorylation at Y1472. Indeed, selective blockade of GluN2B-containing NMDARs by 10 μmol/L ifenprodil failed to inhibit the phosphorylation changes induced by NMDA (Fig. 4). Conversely, the NMDA-induced reduction of phosphorylation at Y1472 was abolished by the NMDAR antagonist MK801 that blocked the open NMDARs that

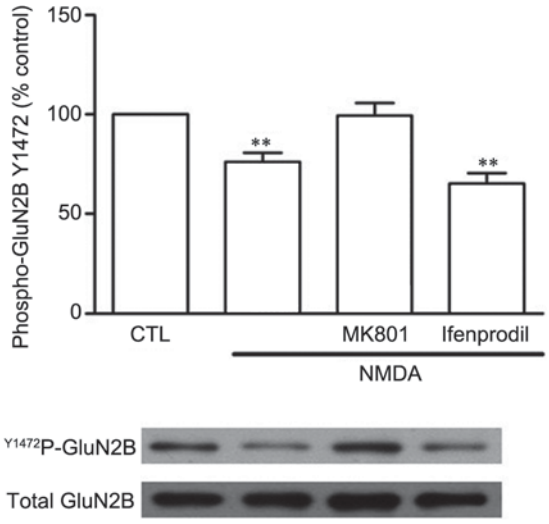

Fig. 4. Blockade of GluN2B-containing NMDARs had no effect on the changes in GluN2B phosphorylation at Y1472 during NMDA treatment. NMDA treatment (20 μmol/L, 3 min) still decreased the phosphorylation of GluN2B at Y1472. Phosphorylation of GluN2B at Y1472 was similar to the control group when MK-801 was used to block total NMDARs (*n* = 5). Ifenprodil and NMDA treatment gave the same results as NMDA treatment alone (*n* = 5). Data are presented as mean ± SEM. ***P* <0.01 compared with control (CTL).

were activated during enhanced synaptic activity by bath incubation with bicuculline. This suggested that the synaptic NMDARs which do not contain GluN2B are responsible for the phosphorylation changes.

Taken together, the results shown in Figures 3 and 4 strongly suggest that synaptic GluN2A, but not extrasynaptic GluN2B-containing NMDARs, mediate the NMDA-induced reduction of GluN2B phosphorylation at the Y1472 site.

DISCUSSION

Many studies have focused on the regulation of phosphorylation of AMPARs by NMDAR activation. The GluN2 subunits of NMDAR are differentially tyrosine-phosphorylated, which is important for regulating NMDAR function[23]. Previous studies have indicated that many factors such as brain-derived neurotrophic factor, dopamine receptors, and Src family kinases[24-26] regulate the tyrosine phosphorylation of GluN2A and GluN2B subunits. However, the self-contribution of NMDAR activation to GluN2 tyrosine phosphorylation is still unclear. To address this issue, we blocked neuronal excitability by using a cocktail of inhibitors (TTX, CNQX and nimodipine) during NMDA treatment in brain slices. In this system, NMDA treatment only activated the NMDARs but no other downstream effectors. The present results demonstrated that (1) NMDAR activation only resulted in decreased GluN2B phosphorylation at Y1472, but not at Y1336; (2) NMDA at 20 to 300 μmol/L consistently reduced the phosphorylation level of GluN2B at Y1472; and (3) synaptic GluN2A-containing NMDARs play a critical role in modulating GluN2B phosphorylation at Y1472.

In the present study, our data first showed that NMDAR activation decreased tyrosine phosphorylation of GluN2B at Y1472, but not at Y1336, suggesting that NMDAR activation differentially regulates different tyrosine sites of the GluN2B subunit.

Previous studies have indicated that 20 μmol/L NMDA treatment induces chemical LTD[27], but a very high concentration of NMDA, such as 100 or 300 μmol/L, induces neuron death[28]. In our study, 10 μmol/L NMDA did not induce any detectable changes in GluN2B Y1472, but concentrations from 20 to 300 μmol/L decreased it in a time-dependent pattern. These data implied that both chemical LTD induction and NMDAR-mediated excitotoxicity result in a decrease in NMDARs on the cell surface, possibly by promoting the endocytosis of GluN2B-containing NMDARs.

NMDARs are located in neuronal cell membranes at synaptic and extrasynaptic sites, where they are believed to mediate distinct physiological and pathological processes[29,30]. To further identify whether synaptic or extrasynaptic NMDARs are responsible for regulating the tyrosine phosphorylation of GluN2B at Y1472, we selectively blocked the synaptic NMDARs in brain slices. Our data showed that synaptic NMDAR inhibition abolished the reduction in GluN2B phosphorylation at Y1472 induced by NMDA. Interestingly, a recent study indicated that GluN2B phosphorylation at Y1472 is associated with an enrichment of synaptic NMDARs[31], suggesting that these receptors control the synaptic pool of NMDARs by regulating the tyrosine phosphorylation of GluN2B at Y1472.

It is also well known that GluN2A and GluN2B are the prevalent subunits in NMDARs in the forebrain[32] and they

occur at both synaptic and extrasynaptic sites. In adulthood, GluN2A is dominant in the synapse, while the extrasynaptic region contains more GluN2B[21,22]. Here, by applying the GluN2B-specific inhibitor ifenprodil, we demonstrated that GluN2A-containing NMDARs were responsible for the reduction of phosphorylation of GluN2B at Y1472 induced by NMDA in brain slices. It is known that GluN2B is dominant in early development, while GluN2A expression gradually increases during brain development. Until now, accumulating evidence suggests that expression of both GluN2A and GluN2B subunits in rats is stable four weeks after birth[33,34]. Therefore, the expression of the GluN2A and GluN2B subunits was stable in our model, which implied that our results represent a general mechanism in adult rats.

Previous studies indicated that GluN2A-containing NMDARs are associated with neuroprotection, while GluN2B-containing NMDARs are involved in cell death[35]. In other words, synaptic NMDARs play a neuroprotective role[36], while extrasynaptic NMDARs are conducive to neuronal excitotoxicity[36]. Our results showed that a high concentration of NMDA (100 or 300 μmol/L) decreased tyrosine phosphorylation of GluN2B at Y1472. According to these data, it is possible that synaptic GluN2A-containing NMDARs protect neurons from NMDA-induced excitotoxicity by reducing the number of GluN2B-containing NMDARs on the cell surface. It will be interesting to uncover how synaptic GluN2A-containing NMDAR activation regulates the tyrosine phosphorylation of GluN2B at Y1472.

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