

Modulation of NMDA Receptor Subunits Expression by Concanavalin A

Soyong Jang¹ · Ji-Yeon Yu¹ · Jung-Hyuck Ahn² · Seikwan Oh¹

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Abstract The processes of N-methyl-D-aspartate (NMDA) receptor subunits expression were examined in cortical neurons and rat brain in order to investigate how the concanavalin A (Con A) modulates neuronal cells. Con A modulated the expression of NMDA receptor subunits in cultured cortical cells. Con A augmented the level of intracellular Ca^{2+} by α -amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA). We determined whether activation of AMPA receptors was involved in the regulation of NMDA receptor expression with Con A by blocking the desensitization of AMPA receptors. The results showed that AMPA receptor antagonists suppressed NMDA receptor subunits expression in Con A-treated cortical neuronal cells. PMA elevated the expression of NMDA receptor subunits, while PKC inhibitor and tyrosine kinases inhibitor suppressed the expression of NMDA receptor subunits. Furthermore, it was shown that NMDA receptor subunits expression was modulated in a region-specific manner after the sustained microinfusion of Con A into the cerebroventricle of the rat brain. Collectively, it could be presumed that the AMPA receptor activation was involved in Con A-induced modulation of NMDA receptor subunits expression.

Keywords Concanavalin A · NMDA · AMPA · PKC · In situ hybridization

Introduction

Many plant lectins are capable of binding to the carbohydrate moieties of complex glycoconjugates, without altering the covalent structure of the recognized glycosyl ligands, and cross-linking glycosylated membrane receptors on cells [1]. Thus, activation of the receptors and transmembrane signal transduction pathways leads to a wide variety of cellular responses, including recognition, adhesion, signal transduction, mitogenic stimulation, cytotoxicity, and apoptotic mechanisms [2].

Concanavalin A (Con A) acts as a mitogen for monocytes, T cells, and cancer cells [3–5]. Tyrosine phosphorylation induced by mitogens plays a pivotal role in cell proliferation, differentiation, and transformation [6]. Tyrosine phosphorylation is the initial step in cell signaling induced by extracellular stimulants, including growth factors, cytokines, antigens, and extracellular matrix [7]. The activated receptors phosphorylate a series of cytoplasmic proteins, which stimulate a cascade of events leading to activation of transcription factors in the nucleus [8]. These transcription factors lead to an increase in the levels of transcription and translation of certain genes, finally leading to growth, proliferation or differentiation [6, 7].

Ionotropic glutamate receptors rapidly undergo desensitization upon prolonged exposure to the excitatory transmitter L-glutamate, as well as to a variety of structurally related agonists. Several studies have suggested that desensitization of glutamate receptors may modulate the magnitude and duration of postsynaptic excitatory currents; therefore, desensitization apparently can play a role in limiting excitation mediated by this transmitter [9].

The effects of Con A on excitatory amino acid receptors are of particular interest. Many of the characterized neurotransmitter receptors, or their subunits, consist of

✉ Seikwan Oh
skoh@ewha.ac.kr

¹ Department of Molecular Medicine, Tissue Injury Defense Research Center, School of Medicine, Ewha Womans University, Seoul 158-710, South Korea

² Department of Biochemistry, School of Medicine, Ewha Womans University, Seoul 158-710, South Korea

glycoproteins [10]. In locust muscle, in vertebrate neurons and mammalian neurons, desensitization of glutamate receptors can be blocked by lectins such as Con A or wheat-germ agglutinin. Compounds such as cyclothiazide, diazoxide and aniracetam, which are structurally distinct from the lectins, also inhibit glutamate receptor desensitization [9]. The binding of lectins such as Con A to surface carbohydrate groups can lead to the cross-linking and/or aggregation of membrane proteins. Similarly, Con A binds to the asparagines-linked oligosaccharides on nicotinic acetylcholine and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [9]. Therefore, Con A appears to inhibit the conformational change leading from the active, open state to the desensitized state, and thereby increases the steady-state current of the receptor. The lectin may act to lock, i.e., immobilize, the activatable, nondesensitized receptor conformation [9, 11].

Synaptic plasticity refers to neural connections that change in strength in response to development, experience, and pathology. These changes of synaptic gain affect neural communication and may underlie the behavioral changes exhibited in learning and memory [12]. Functional NMDA receptor (NMDAR) complexes are composed of NR1 and NR2 subunits as well as of adaptor, signaling, cytoskeletal, and cell-adhesion proteins. It is known that the NR2A and NR2B subunits predominate in the adult forebrain, although many brain regions contain predominantly NR2A subunits (e.g., the hippocampus) [13].

Several studies have reported that recognition of complex carbohydrates by lectins plays an important role in the regulation of cell metabolism [5]. Although Con A acts as a mitogen in various cells, such as T cells, monocytes, and cancer cells, the precise mechanisms underlying the cellular responses to Con A have yet to be fully elucidated in neuronal systems. In the present study, the effect of Con A was determined by investigating whether Con A modulates protein phosphorylation and NMDA receptor expression in neuronal cells and rat brains.

Experimental Procedures

Chemicals and Animals

Con A (type IV salt free), L-glutamine, glucose, poly-D-lysine, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Horse serum, fetal bovine serum (FBS), and Eagle's minimal essential media (EMEM) were purchased from Hyclone (Logan, Utah, USA). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzof[quinoxaline-2,3-dione (NBQX), and 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-

2,3-Benzodiazepine (GYKI-52466) were purchased from Calbiochem (La Jolla, CA, USA).

Sprague–Dawley rats (male, 260–280 g) were obtained from Daehan Biolink (Eumsung, Korea), and maintained under standard conditions (24 ± 3 °C) under a 12 h light/dark cycle (light on at 07:00 a.m.) with free access to food and water. All procedures involving rats were performed in accordance with the guidelines issued by the Animal Care and Use Committee of the School of Medicine, Ewha Womans University in Korea.

Cell Culture

Cerebral cortices were removed from the brains of 16-day-old ICR fetal mice. The neocortices were triturated and plated at a density of approximately 4×10^5 cells/culture well on 24-well plates precoated with 100 μ g/ml poly-D-lysine and 4 μ g/ml laminin, containing EMEM supplemented with horse serum (5 %), FBS (5 %), 2 mM glutamine, and 20 mM glucose. Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO₂. After 6 days in vitro (DIV), the cultures were transferred to the plating media containing 10 μ M cytosine arabinoside without FBS. The cultures were then fed twice per week. To identify the condition of the cell, mixed cortical cell cultures containing neurons and glia (9:1, DIV 12–14) were exposed to the excitatory amino acid glutamate in EMEM supplemented without 10 % horse serum. The morphology of the degenerating neurons was observed under a phase contrast microscope over the next 24 h.

Measurement of Cell Viability

Cell number and viability were assessed using the WST-1 reagent (Roche, Indianapolis, IN, USA). This colorimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 substrate 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulphonate] into formazan by mitochondrial dehydrogenase in live cells. This was followed by incubation with the WST-1 reagent at a dilution of 1:10 in the original conditioned media at 37 °C for 2 h. After thorough shaking, the amount of formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm, while the reference wavelength is 650 nm. Absorbance readings were normalized against control wells containing only medium.

Intracellular Ca²⁺ Imaging on Confocal Microscopy

Cultured neuronal cells were incubated at room temperature for 30 min with a Fura2-AM loading buffer (10 μ M, Molecular Probes, Eugene, Oregon, USA). Experimental

solution contained (mmol/l): NaCl 136, KCl 4, NaH₂PO₄ 0.33, NaHCO₃ 4, CaCl₂ 2, MgCl₂ 1.6, HEPES 10, glucose 10. Cells were continuously superfused during experiments. For complete de-esterification of Fura2-AM, wash out the loading buffer and remove any extracellular dye, and cells were superfused with experimental solution for with/without Con A (0.25 µg/ml) for 30 min before experiments were started. Ca²⁺ measurements were performed with a laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) using a 40× oil-immersion objective. Fura2-Ca²⁺ was excited by laser (380 nm) and emitted fluorescence was collected through a 510 nm long-pass emission filter. Fluorescence images were recorded for 5 min.

Changes of Intracellular Ca²⁺ Level in Fluorimeter

Cultured neuronal cells (24-well plate) were incubated with a Fura2-AM loading buffer (10 µM, Molecular Probes, Eugene, Oregon, USA) for 30 min. Neuronal cells were washed with experimental solution [as described in confocal microscopy section] for 5 min and exposed with/without Con A (0.25 µg/ml) for 30 min before measurements. Measurements were performed with a fluorescence plate reader (Molecular Devices, Palo Alto, CA, USA) at room temperature. Cells were excited at 340 and 380 nm and the emitted fluorescence was collected at 510 nm at every 7.9 s. The intracellular Ca²⁺ level was measured as the ratio of fluorescence at 340 and 380 nm (F340/F380 nm in ratio units). Two seconds after stopping the stimulation during steady state conditions, AMPA (10 µM) was applied directly onto the cells.

Immunoblot Analysis

Cultured cells were washed twice with cold PBS and then lysed in ice-cold modified lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 0.01 % Triton X-100, and protease inhibitor mixture pH 8.0). Cellular debris was cleared by centrifugation. The supernatants were aliquoted and stored at -70 °C for further use. Samples were assayed for protein concentration using bicinchoninic acid reagents (Rockford, IL, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5 % non-fat dry milk in TBST solution. The blots were incubated with the NR1, NR2A, NR2B (BD Biosciences, CA, USA), and anti-phosphotyrosine, 4G10 (Upstate, CA, USA) antibodies. After washing with TBST, HRP-conjugated secondary antibodies (Cell signaling technology, MA, USA) were applied and the blots were developed using the enhanced

chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Prolonged Infusion of Con A into Cerebroventricle with Osmotic Minipump

Rats were implanted with guide cannulae for Con A infusion. Briefly, rats were anesthetized with ketamine (50 mg/kg) and xylazine (1 mg/kg) s.c., and standard stereotaxic surgery was performed using a Kopf stereotaxic frame. A 21-gauge stainless steel cannula was implanted in the right lateral ventricle (L: 1.3 mm; A-P: -0.5 mm; D-V: -4.5 mm) using the bregma as the stereotaxic reference point [14]. The cannula was held in place with rapid-setting dental acrylic and anchored to the skull with an aluminum protective cap and steel screws. Rats were allowed 1 week to recover before osmotic minipump implantation. Minipumps were implanted (s.c.) as previously described [15]. Briefly, under ether anesthesia, a small incision was made behind the ears and the subcutaneous space was expanded with hemostatic forceps. Saline and Con A were filtered through a 0.2 µm sterile syringe filter and then used to fill an osmotic minipump (Alzet 2 ML 1, Alza, Palo Alto, CA, USA). The minipump was then implanted and connected directly to the cannula via 6 cm long PE-60 tubing. Con A was infused into the rat cerebroventricle using an osmotic minipump at a flow rate of 1 µg/10 µl/h for 7 days. The incision made on the back was closed with cyanoacrylate glue, and dental acrylic was layered on top of the polyethylene tubing.

In situ Hybridization

The purified oligodeoxynucleotide probes complementary to rat NMDA subunit cDNA of NR1 (5'-CGT GCT AAG GAA ACT CAG GTG GAT ACT CTT GTC AGA GTA GAT GGA-3'; residues 375–420), NR2A (5'-AGA AGG CCC GTG GGA GCT TTC CCT TTG GCT AAG TTT C-3'; residues 567–579), NR2B (5'-GGG CCT CCT GGC TCT CTG CCA TCG GCT AGG CAC CTG TTG TAA CCC-3'; residues 557–572) and NR2C (5'-TGG TCC ACC TTT CTT GCC CTT GGT GAG GTT CTG GTT GTA GCT-3'; residues 562–576) were obtained from New England Nuclear (Boston, MA, USA) [13, 16]. The probe was labeled at its 3' end using terminal deoxynucleotidyl transferase and [α -³⁵S]dATP (New England Nuclear). Ten picomoles of probe in tailing buffer (0.1 M potassium cacodylate, 25 mM Tris base, 1.0 mM cobalt chloride, and 0.2 mM dithiothreitol, pH 7.0) and 50 pmol of [α -³⁵S]dATP were incubated with 36 units of terminal deoxynucleotidyl transferase at 37 °C for 30 min. The reaction was terminated by the addition of 400 µl of Nensorb-20 solution on ice. The

labeled probe was eluted in 50 % ethanol using the Nensorb-20 column chromatography.

In situ hybridization was performed according to the method described by Tseng et al. [17]. The probe solution (5×10^5 dpm/ μ l) was diluted with hybridization buffer [50 % formamide, 10 % dextran sulfate, 1 % Denhardt's solution, 100 mM DTT, 0.025 % tRNA from *Escherichia coli* and 0.05 % DNA from salmon tests in $4 \times$ standard sodium citrate buffer [(SSC, $1 \times$ SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0)]. The final radioactivity of the hybridization solution was about 1×10^4 dpm/ μ l. Frozen brain sections were dried at room temperature for 10 min and fixed in 4 % paraformaldehyde in PBS, pH 7.4, for 10 min. The slides were rinsed three times in PBS for 3 min, rinsed once in $2 \times$ SSC for 3 min, and dipped once in deionized water. Each brain slice was hybridized with 35 μ l of hybridization buffer under a coverslip to prevent tissue drying and then incubated overnight at 40 °C in a high humidity environment. After hybridization, the coverslips were carefully removed in $1 \times$ SSC, and the slides were rinsed in $1 \times$ SSC three times for 3 min each to remove excess hybridization buffer. Slides were then washed twice, for 15 min each, in $2 \times$ SSC + 50 % formamide at 55 °C, followed by washing twice for 15 min in $1 \times$ SSC at room temperature. Finally, slides were briefly dipped in deionized water and air-dried. Competition hybridization was carried out in the presence of an excess amount (70-fold) of unlabeled probe, which showed negligible non-specific hybridization in the final image.

After hybridization, dried tissue sections were juxtaposed to Hyperfilm- β max (Amersham, Arlington Heights, IL, USA) together with a standard slide (ARC-146, ARC St. Louis, MO). Following a 2-week exposure period, the film was developed in Kodak D19 at room temperature for 5 min and fixed for 10 min. Autoradiograms were analyzed by a digital scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) operating on the image acquisition and analysis program ImageQuant 3.3 (Molecular Dynamics, Sunnyvale, CA, USA). The density of the brain regions was recorded by marking 3–6 areas on the bilateral slides of the brain.

Statistical Analysis

All values were expressed as the mean \pm S.E.M., and comparisons between groups were made using analysis of variance followed by *t* test, with $*p < 0.05$, $**p < 0.01$ considered as significant. The mean values of in situ hybridization were determined from 5 to 6 rats for each group and expressed as the mean \pm S.E.M., in nCi/g wet brain tissue. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by Newman–Keuls

multiple comparison test. Differences with $*p < 0.05$, $**p < 0.01$ were defined as statistically significant.

Results

Con A Induced Cytotoxicity in a Dose-Dependent Manner in Cultured Cortical Cells

A previous study showed that Con A induced neuronal cell death in cultured cortical cells [18]. In our study, to determine the cytotoxic effects of Con A, cell viability was measured following Con A (0.1–100 μ g/ml) treatment for 24 h in cortical cells. Con A did not induce cytotoxicity at low concentrations (lower than 1 μ g/ml) although induce cytotoxicity at high concentrations (over than 10 μ g/ml) (Fig. 1). Native lectins showed equal effect as mitogens at concentrations below 6 μ g/ml in mouse spleen cells [19]. Based on these results, a low concentration of Con A which did not induce neurotoxicity was used to investigate the effect of Con A in neuronal cells in our study.

Con A Increased the Expression of NMDA Receptor Subunits

This study investigated whether Con A modulates the expression of receptors such as NMDA receptor subunits in cortical cells. Con A was applied for 24 h to cultured cortical cells, and immunoblot analysis was performed

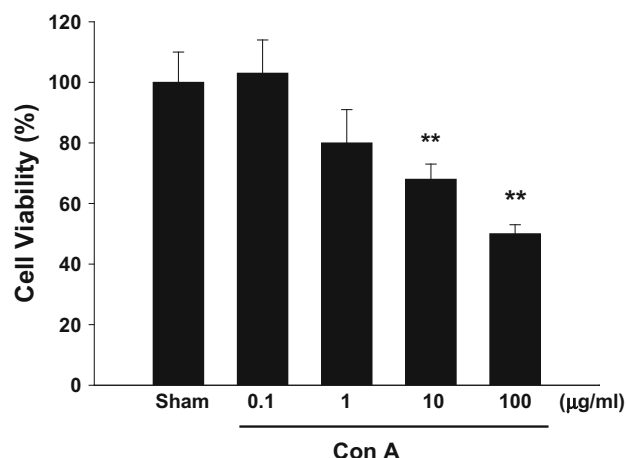


Fig. 1 The effect of Con A on the viability of cultured cortical cells. Cortical cells (4×10^5 cells per well) were treated with Con A at various concentrations (0.1–100 μ g/ml) for 24 h, and cell viability was measured using WST-1. High concentrations of Con A induced a considerable amount of cytotoxicity, whereas low concentrations of Con A did not affect cell viability. All values are expressed as the mean \pm S.E.M., from three independent experiments. $**p < 0.01$ indicated a significant difference between the sham group and Con A-treated group, respectively

using anti-NMDA receptor subunit (NR1, NR2A, and NR2B) antibodies. Interestingly, Con A (0.05–0.25 $\mu\text{g/ml}$) treatment increased the expression of NMDA receptor subunits NR1, 2A and 2B (Fig. 2) and this result showed that Con A modulated the expression of NMDA receptor subunits.

Con A Augmented the AMPA-Induced $[\text{Ca}^{2+}]_i$ in Cultured Cortical Cells

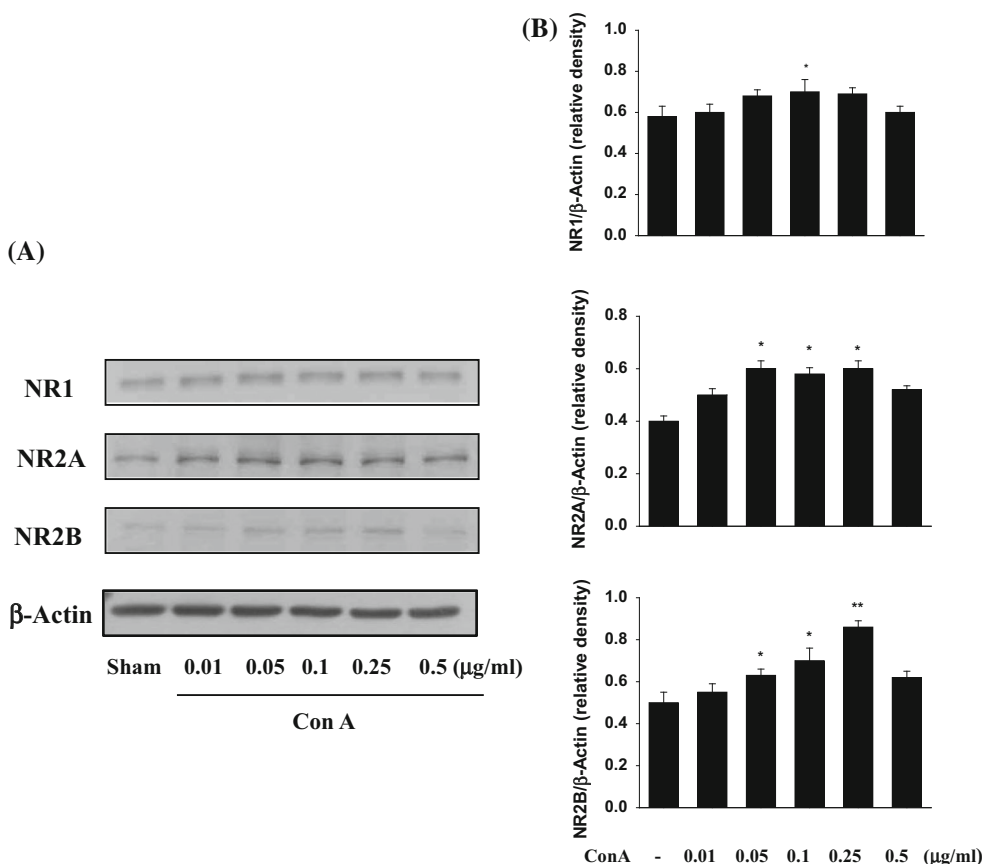
Calcium-permeable AMPA receptors are expressed in cultured cerebellar granule cells and hippocampal neurons as well as in cortical neurons [20]. Elevation of intracellular calcium ($[\text{Ca}^{2+}]_i$) through AMPA receptors may or may not be required for its neurotoxicity but appears to be required for synaptic plasticity effects [21, 22]. The augmentative effect of Con A on the action of AMPA receptors was investigated in cultured cortical cells by measuring elevation of $[\text{Ca}^{2+}]_i$ with Fura2 imaging. Stimulation of cultured cortical neurons with AMPA (10 μM) induced elevation of $[\text{Ca}^{2+}]_i$ while ConA alone did not induce $[\text{Ca}^{2+}]_i$ elevation. However, the treatment with Con A (0.25 $\mu\text{g/ml}$) for 30 min before AMPA application resulted in the augmentation of AMPA-induced

elevation of $[\text{Ca}^{2+}]_i$ in cultured neuron (Fig. 3). However, Con A did not augment the NMDA-induced elevation of $[\text{Ca}^{2+}]_i$ in cultured neuronal cells (data not shown).

AMPA Receptor Antagonists Suppressed the Con A-induced Elevation of NMDA Receptor Subunits Expression in Cultured Cortical Cells

We determined whether the AMPA receptors affect the Con A-induced NMDA receptor subunits expression. As Con A is known to block the desensitization of AMPA receptors [9, 20], AMPA receptor antagonists were used in this study: NBQX, a potent and selective competitive AMPA receptor antagonist; and GYKI52466, a non-competitive AMPA receptor antagonist. Cortical cells were pretreated with the AMPA receptor antagonists NBQX, or GYKI52466 for 30 min and then cells were treated with Con A for 24 h for immunoblot analysis. Con A increased the expression of NR1, NR2A and NR2B level, and the expression of NR2B was reduced by NBQX (10 and 20 μM). Particularly, expression of subunits 2A and 2B was inhibited by GYKI52466 (10 and 20 μM) (Fig. 4). The inhibition of Con A-induced expression of NMDA receptor subunits by the AMPA receptor antagonist indicated that

Fig. 2 Expression of NMDA receptor subunits in Con A-treated cultured cortical cells. Cell extracts were prepared from Con A-treated (24 h) cortical cells, and immunoblot analysis was performed using anti-NMDA receptor subunit antibodies NR1, NR2A, and NR2B. Con A greatly increased the expression of NR2A and NR2B in comparison to NR1



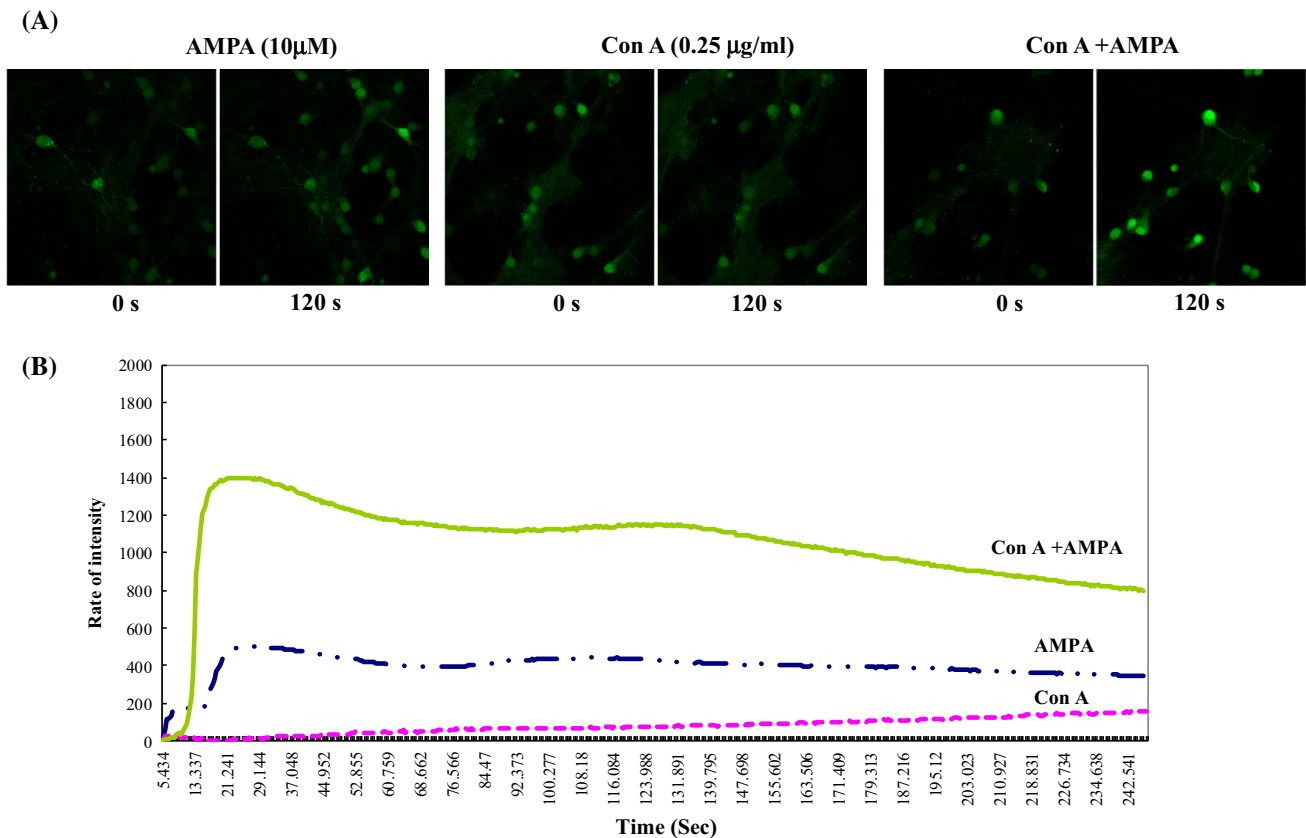


Fig. 3 Enhancement of intracellular Ca^{2+} by Con A on AMPA-induced Ca^{2+} elevation. **a** Cultured cortical neurons were loaded with the Fura2 fluorescence in the presence or in the absence of 0.25 $\mu\text{g/ml}$ Con A for 30 min. Ca^{2+} measurements were performed with a laser scanning confocal microscope using a $\times 40$ oil-immersion objective. Fura2- Ca^{2+} was excited by laser (380 nm) and emitted fluorescence was collected through a 510 nm long-pass emission filter. Fluorescence images were recorded for 5 min. Each experimental condition was performed in five repeated experiment, and about 6–13 cells were analyzed in each field of the microscope. **b** Cultured neuronal cells

(24-well plate) were incubated with a Fura2-AM loading buffer (10 μM) for 30 min. Neuronal cells were washed with experimental solution for 5 min and exposed with/without Con A (0.25 $\mu\text{g/ml}$) for 30 min before measurements. Measurements were performed with a fluorescence plate reader at room temperature. Cells were excited at 340 and 380 nm and the emitted fluorescence was collected at 510 nm at every 7.9 s. The intracellular Ca^{2+} level was measured as the ratio of fluorescence at 340 and 380 nm. Two seconds after stopping the stimulation during steady state conditions, AMPA (10 μM) was applied directly onto the cells

Con A-induced NMDA receptor expression might occur through activation (or blocking the desensitization) of the AMPA receptor.

PMA Induced Tyrosine Phosphorylation and Increased the Expression of NMDA Receptor Subunits in Cultured Cortical Cells

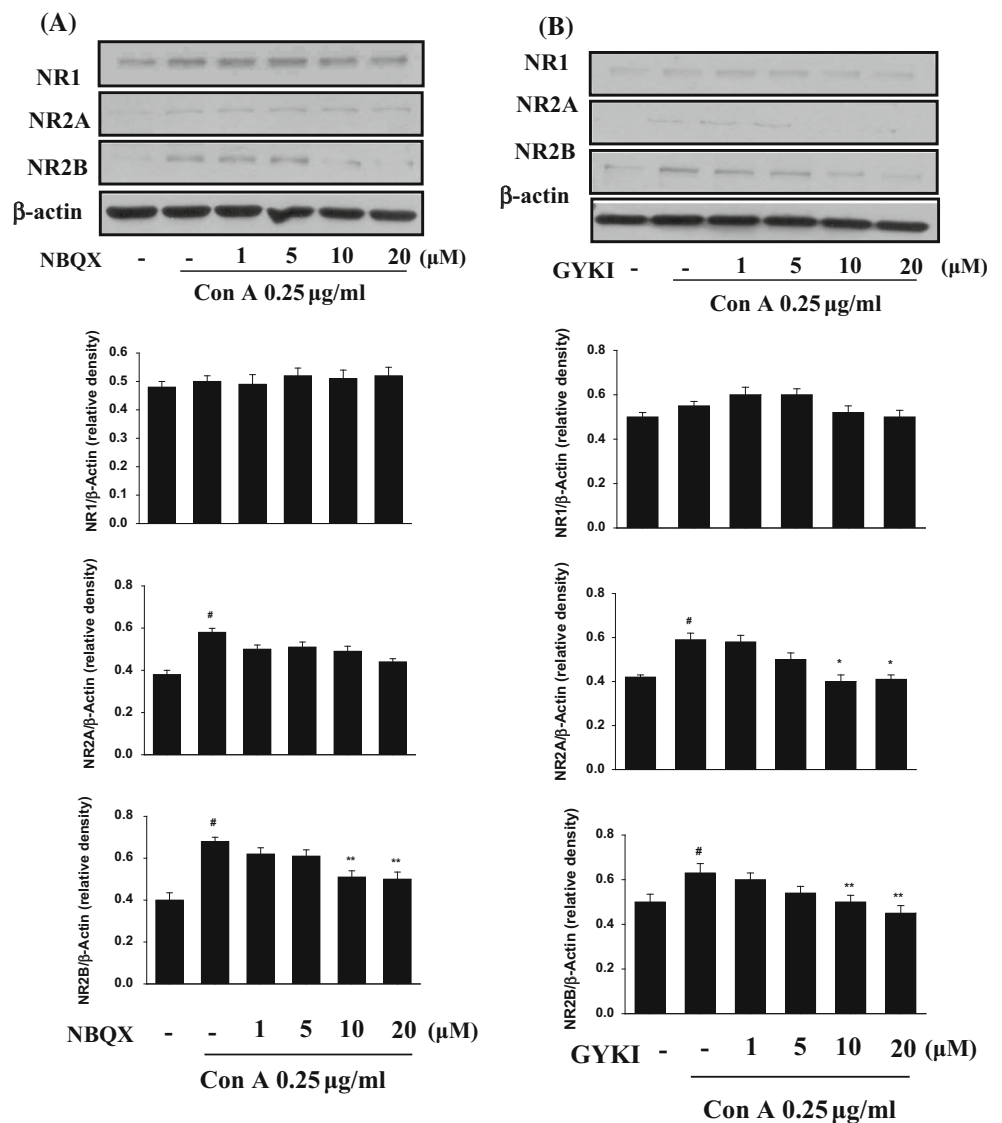
The effect of phorbol 12-myristate 13-acetate (PMA), known as a PKC activator, was ascertained through the induction of tyrosine phosphorylation and NMDA receptor expression in cortical cells. The tyrosine phosphorylation (Fig. 5a) and expression of NMDA receptor subunits NR1, NR2A, and NR2B (Fig. 5b, c) was increased in PMA-treated (24 h) cortical neurons as Con A did. These results indicated that the activity of Con A resembles that of PMA

in terms of tyrosine phosphorylation and augmentation of NMDA receptor subunit expression.

The Expression of NMDA Receptor Subunits was Suppressed by PKC Inhibitor or TRK Inhibitor

To determine whether the effect of Con A on NMDA receptor expression was mediated by activation of PKC using an inhibitor of PKC, chelerythrine chloride was used. Interestingly, pretreatment of cells with chelerythrine chloride 30 min before application of ConA inhibited the Con A-induced upregulation of NMDA receptor subunits expression (Fig. 6a). The specificity of tyrosine phosphorylation by Con A was evaluated by using inhibitor of tyrosine kinases inhibitor, hypericin. Hypericin strongly suppressed the Con A-induced expression of NR1, NR2A

Fig. 4 Expression of NMDA receptor subunits via activation of the AMPA receptor in Con A-treated cortical cells. Cells were pretreated with the AMPA/KA receptor antagonists NBQX, or GYKI52466 for 30 min and then exposed to Con A (0.25 µg/ml) for 24 h. The effects of the AMPA/KA receptor antagonists on Con A-induced NMDA receptor subunit expression in cortical cells were determined by immunoblot analysis. NBQX reduced the expression of NR2B by Con A, while GYKI52466 significantly inhibited the induction of Con A-induced NR2B expression. β-Actin was also measured as a control. The bands were quantified by densitometry in order to obtain the relative levels of NMDA receptor subunits. All values are expressed as the mean ± S.E.M., from three independent experiments. #*p* < 0.01 indicate significant difference between the sham group and Con A-treated group, respectively. **p* < 0.05 and ***p* < 0.01 indicate significant difference between the Con A-treated group and AMPA/KA receptor antagonists, respectively



and NR2B (Fig. 6b). These results suggested that the activation of tyrosine kinases was involved in the Con A-induced modulation of NMDA receptor subunits expression, and that the effect of Con A was mediated through the activation of PKC and/or tyrosine kinase for the tyrosine phosphorylation.

Expression of NMDA Receptor Subunits was Elevated in a Region-Specific Manner in the Con A-infused Rat Brain

The modulating effect of Con A on the expression of NMDA receptor subunits in cultured cortical neurons was also observed in in vivo experiment. Con A was continuously infused into rat cerebroventricle through an osmotic minipump at a flow rate of 1 µg/10 µl/h for 7 days, and

brain tissues were subsequently sectioned. After the infusion of Con A, the expression of the NR1 subunit was elevated (110–120 %) in the frontal cortex, caudate-putamen, thalamus, hippocampus, inferior colliculus of brainstem, and granule layer of cerebellum as compared to the saline-infused group, respectively (Fig. 7; Table 1). The expression of the NR2A subunit was increased (110–130 %) in the dentate gyrus of hippocampus and granule layer of cerebellum, while the expression of the NR2B subunit was elevated in the frontal cortex, caudate-putamen, thalamus, and hippocampus as compared to the saline-infused group, respectively. The expression of NR2C subunit was increased (120 %) in the granule layer of cerebellum, as compared to the saline-infused group (Fig. 7; Table 2). These results indicated that Con A elevated the expression of NMDA receptor subunits in a region-specific manner in the brain.

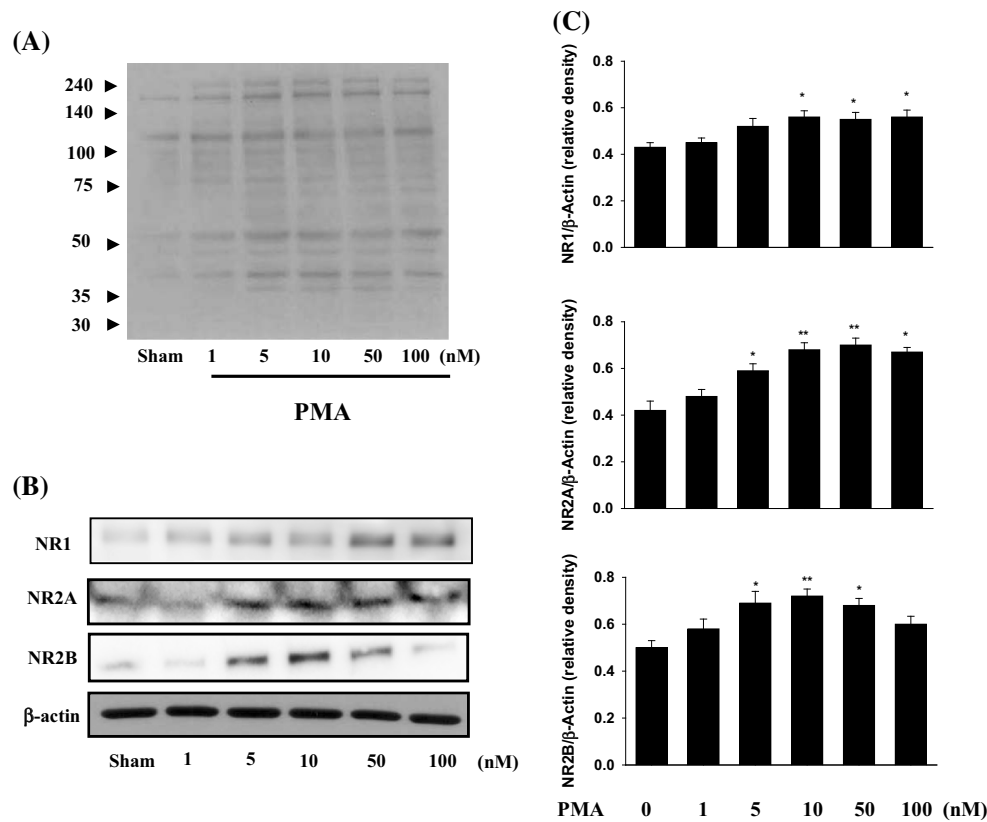


Fig. 5 Induction of tyrosine phosphorylation and expression of NMDA receptor subunits in PMA-treated cortical neurons. **a** Cells were treated with PMA for 24 h in order to determine whether tyrosine phosphorylation was induced via PKC activation. Phosphotyrosine-containing proteins were detected by immunoblot analysis using anti-phosphotyrosine antibody, 4G10. PMA induced the phosphorylation of tyrosine. **b** Cell extracts were prepared from PMA-treated cortical cells for immunoblot analysis using anti-NMDA

receptor subunit antibodies NR1, NR2A, and NR2B. PMA induced the expression of NR1, NR2A, and NR2B like as pattern of NMDA receptor subunits modulated by Con A. β -Actin was also measured as a control. **c** The bands were quantified by densitometry in order to obtain the relative levels of NMDA receptor subunit expression. All values are expressed as the mean \pm S.E.M., from three independent experiments. * $p < 0.05$ and ** $p < 0.01$ indicate significance between the sham group and PMA-treated group, respectively

Discussion

The plant lectin, concanavalin A (Con A), exerts a myriad of biological effects. Con A exhibits a tetrameric conformation at physiological pH, and it may play an important role in cell adhesion, signal transduction, mitogenic stimulation, cytotoxicity, and apoptotic mechanisms [2, 9]. The mechanisms by which Con A exerts these biological effects have been proposed as its ability to bind and crosslink membrane glycoproteins. Among these actions of Con A, the mitogenic effect of Con A has been established in several different cells. However, the biological effects of Con A on neuronal cells have not been clearly defined. This study was focused on the effect of Con A on the NMDA receptor expression in neuronal cells.

The Con A-induced modulation of NMDA receptor subunit expression was determined. The NMDA receptors, the most abundant glutamate receptor in the CNS, are

universally distributed throughout the brain [13]. The NMDA receptor is primarily implicated in excitatory neurotransmission, and it is thought to play a vital role in normal CNS function [23]. Activation of the NMDA receptor contributes to diverse physiological responses including synapse formation and plasticity that underlie learning and memory [24, 25]. In addition, NMDA receptors have been implicated in schizophrenia, epilepsy, ischemic brain damage, and neurodegenerative disorders. Thus, the expression of NMDA receptor subunits has been the focus of numerous efforts to improve the function of the brain, and they are major targets in the development of anti-neurodegenerative agents [23].

Based on facts that influx of calcium through AMPA appears to be required for synaptic plasticity effects [21]. It was determined whether Con A affects the AMPA-induced $[Ca^{2+}]_i$ level in cultured cortical cells. This result showed that Con A augmented the elevation of $[Ca^{2+}]_i$ in AMPA-treated cortical cells. Recent study showed that Ca^{2+}

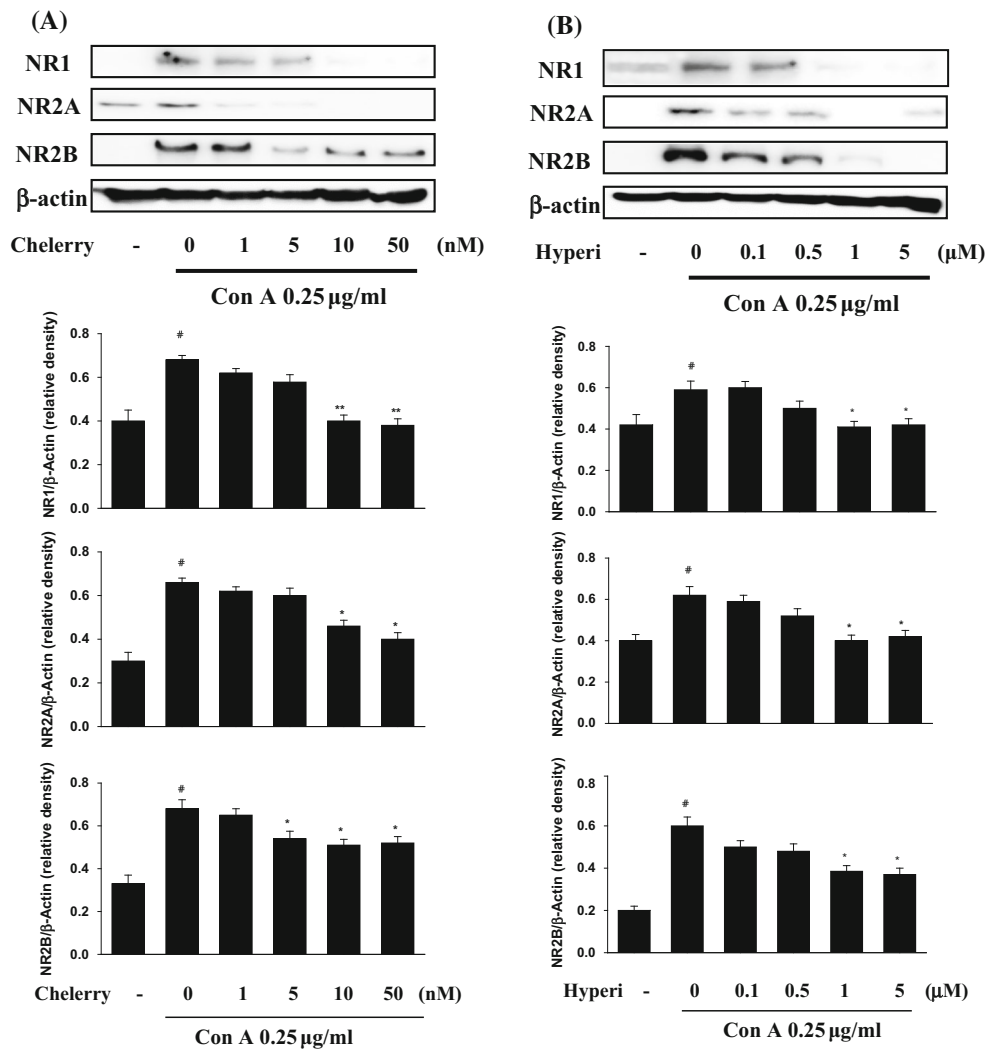


Fig. 6 The effect of PKC and tyrosine kinase inhibitors on the Con A-induced expression of NMDA receptor subunits. **a** The effects of PKC inhibitor on Con A-induced NMDA receptor subunits expression in cortical cells were determined by immunoblot analysis. Cells were pretreated with PKC inhibitor, chelerythrine chloride (1, 5, 10, 50 nM) for 30 min and then exposed to Con A for 24 h. PKC inhibitor partly inhibited the induction of Con A-induced NMDA receptor subunits expression. **b** Cells were pretreated with tyrosine kinases inhibitor, hypericin (0.1, 0.5, 1, 5 μM) for 30 min and then

exposed to Con A for 24 h. The effects of tyrosine kinases inhibitor on Con A-induced NMDA receptor subunit expression were determined by immunoblot analysis. Tyrosine kinases inhibitor inhibited the induction of Con A-induced NR2A and NR2B subunits expression. β-Actin was also measured as a control. [#]*p* < 0.01 indicate significant difference between the sham group and Con A-treated group, respectively. **p* < 0.05 and ***p* < 0.01 indicate significant difference between the Con A-treated group and inhibitor-treated group, respectively

increase in the cytosol can be due to activation of NMDA receptors, and/or of voltage-dependent Ca²⁺ channels. NMDA receptors appeared to be implicated in the effects of CX614, which is AMPA receptor modulator, on mTOR phosphorylation. It is possible that NMDA receptors could also be implicated in the effects of CX614 on BDNF release. Voltage-dependent Ca²⁺ channels were also required for BDNF release as evidenced by TrkB phosphorylation and for activation of downstream regulators of translation, such as phosphorylation of mTOR. Therefore, it was indicated that both intracellular and extracellular

Ca²⁺ is involved in CX614-dependent phosphorylation of TrkB [22].

Con A exhibited the mitogenic effects similar to those of the PKC activator, PMA, through the induction of tyrosine phosphorylation as well as expression of NMDA receptor subunits NR1, NR2A, and NR2B. The specificity of PKC activation and tyrosine kinases activation by Con A was furtherly evaluated by using inhibitors of PKC (chelerythrine chloride) and inhibitor of tyrosine kinases (hypericin). Chelerythrine and hypericin suppressed the Con A-induced upregulation of NMDA receptor subunits

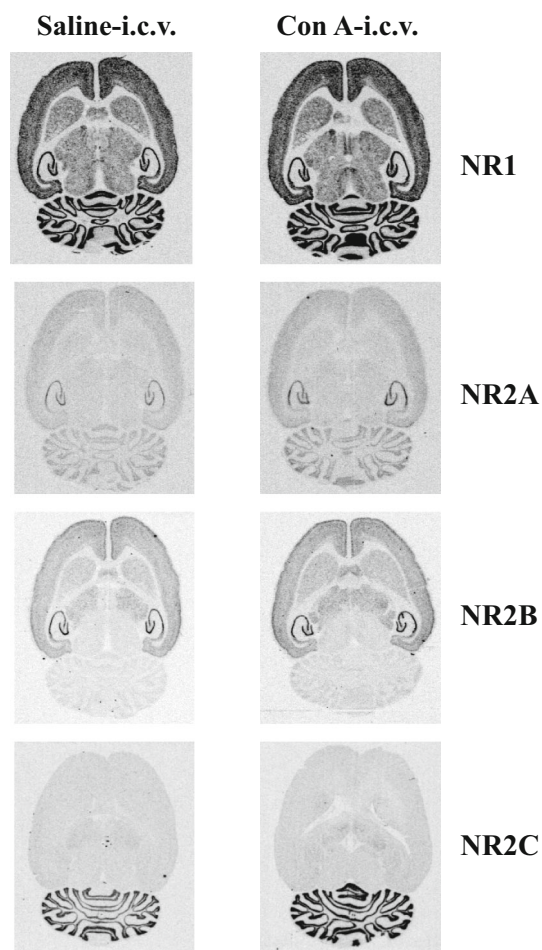


Fig. 7 Representative in situ images of NMDA receptor subunits in horizontal sections of the rat brain after Con A infusion. Con A was continuously infused (flow rate, 1 $\mu\text{g}/10 \mu\text{l}/1 \text{ h}$) into the cerebroventricle using an osmotic minipump for 7 days. The expression of NMDA receptor subunits was modulated in a region-specific manner after microinfusion of Con A into the cerebroventricle

expression. These results suggested that the mitogenic effect of Con A was secondary to the activation of tyrosine kinases and PKC on the induction of tyrosine phosphorylation and the expression of NMDA receptor subunits. In fact, activation of Trk receptors promotes PLC γ activity, giving rise to diacylglycerol which activates PKC [26].

In this experimental results, it was shown that Con A treatment increased the expression of NMDA receptor subunits, and this activity was significantly inhibited by AMPA receptor antagonist, NBQX or GYKI52466. These results suggest that Con A modulated the expression of the NMDA receptor directly and/or through AMPA receptor activation in cortical neuronal cells. Modulation of the NMDA receptor by the infusion of Con A into the cerebroventricle was confirmed in the rat brain. The results showed that the expression of NMDA receptor subunits

Table 1 Changes of NMDA receptor NR1 subunit mRNA level in Con A-infused rats

Regions	mRNA levels (nCi/g tissue)	
	Saline	Con A [#]
Cortex		
Frontal cortex	37.60 \pm 0.42	41.34 \pm 0.72**
Temporal cortex	40.54 \pm 0.89	44.33 \pm 1.06*
Entorhinal cortex	42.53 \pm 1.87	45.93 \pm 1.23
Cingulate	41.28 \pm 1.01	45.37 \pm 0.81**
Caudate-putamen	27.55 \pm 0.66	31.16 \pm 0.35**
Septum	29.4 \pm 0.90	30.95 \pm 1.64
Thalamus	25.60 \pm 0.88	29.19 \pm 0.62**
Hippocampus		
CA1	60.172 \pm 0.76	65.11 \pm 2.03
CA3	86.11 \pm 2.61	89.82 \pm 4.01
Dentate gyrus	63.62 \pm 2.19	76.62 \pm 2.96**
Brainstem		
Inferior colliculus	23.66 \pm 0.81	26.12 \pm 0.72*
Central gray	29.38 \pm 1.06	30.17 \pm 1.49
Cerebellum		
Granule layer	92.09 \pm 3.96	103.30 \pm 1.84*

Con A # was continuously infused (flow rate, 1 $\mu\text{g}/10 \mu\text{l}/1 \text{ h}$) into rat brain by osmotic minipump for 7 days

All values are expressed as mean \pm S.E.M., from 5 to 6 rats

* $p < 0.05$; ** $p < 0.01$ for difference from respective control groups

was elevated in a region-specific manner after prolonged Con A infusion.

It has been known that the ligand binding affinities of recombinant NMDA receptors depend on subunit combination, and regional differences in subunit composition of NMDA receptors may underlie their functional and pharmacological heterogeneity [27]. When the heteromeric receptors were constructed from the NR1 subunit and a subunit of the NR2 subfamily, NR1–NR2B receptors display the highest affinity for [^3H]glutamate, whereas NR1–NR2A receptors show the highest affinity for antagonists, [^3H]CGP39653 and [^3H]MK-801, with high affinity. NR1–NR2C receptor exhibits lower affinities for glutamate and NMDA and display considerably lower affinities for antagonist as compared to NR1–NR2A and NR1–NR2B receptors [27]. NR2 expression may be the rate-limiting step in functional NMDA receptor expression [28]. Therefore, changes of subunit NR2A or NR2B could modulate overall NMDA receptor activity [29] and contribute to the formation of new NMDA receptors with distinct functional characteristics [30].

Recent study showed that mice overexpressing NR2B subunit in forebrain display enhancement in LTP and

Table 2 Changes of NMDA receptor NR2 subunits mRNA level in Con A-infused rats

Regions	mRNA levels (nCi/g tissue)	
	Saline	Con A [#]
NR2A		
Frontal cortex	10.32 ± 0.34	11.07 ± 0.30
Hippocampus		
CA1	24.95 ± 0.98	26.39 ± 0.57
CA3	18.54 ± 0.85	18.37 ± 0.95
Dentate gyrus	18.09 ± 0.46	24.04 ± 1.03**
Cerebellum		
Granule layer	13.88 ± 0.24	16.41 ± 0.37**
NR2B		
Cortex		
Frontal cortex	8.63 ± 0.25	10.31 ± 0.37**
Temporal cortex	10.79 ± 0.52	13.4 ± 0.36**
Entorhinal cortex	10.57 ± 0.53	11.38 ± 0.41
Cingulate	12.22 ± 0.34	13.71 ± 0.28**
Caudate putamen	5.93 ± 3.96	7.31 ± 0.19**
Thalamus	7.67 ± 0.27	9.52 ± 0.32**
Hippocampus		
CA1	28.15 ± 1.59	29.11 ± 0.92
CA3	16.94 ± 0.88	22.19 ± 1.01**
Dentate gyrus	25.03 ± 1.37	28.94 ± 1.14*
NR2C		
Cerebellum		
Granule layer	42.44 ± 1.60	51.13 ± 2.05**

Con A # was continuously infused (flow rate, 1 µg/10 µl/1 h) into rat brain by osmotic minipump for 7 days

All values are expressed as mean ± S.E.M., from 5 to 6 rats

* $p < 0.05$; ** $p < 0.01$ for difference from respective control groups

learning with respect to control mice. Thus, the increase in expression and/or activity of NR2B subunits could improve cognitive capabilities in mice. These data suggest that procedures that enhance the NR2B-containing NMDA receptor pathways could provide potential therapeutic strategies to diminish the cognitive impairment that occurs during normal aging or in disease [31]. Therefore, these results may suggest that the functions of NMDA and AMPA receptors in the regulation of gene expression are closely related to each other.

Collectively, Con A augmented the AMPA receptor functions and NMDA receptor subunits (NR1, NR2A, NR2B) expression via the activation of PKC/tyrosine kinases in neuronal cells. In addition, prolonged infusion of Con A into cerebroventricle induced the elevation of NMDA receptor subunits expression in a region-specific manner in rat brain.

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