International Journal of Neuropsychopharmacology (2004), 7, 299–309. Copyright © 2004 CINP
DOI: 10.1017/S1461145704004304

ARTICLE

Influence of immobilization stress on the levels of CaMKII and phospho-CaMKII in the rat hippocampus

Takami Suenaga, Shigeru Morinobu, Ki-ichiro Kawano, Takuya Sawada and Shigeto Yamawaki

Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University

Abstract

The phosphorylation of calcium/calmodulin-dependent protein kinase (CaMK) II, induced by an increase in the intracellular Ca^{2+} concentration, is involved in the alteration of brain functions such as memory formation. In the present study, we examined the influence of various immobilization stress paradigms on the phosphorylation of CaMKII (phospho-CaMKII) and CaMKII levels in the rat hippocampus. Immunoblot and immunohistochemical analyses were performed to examine the levels of CaMKII and phospho-CaMKII. Real-time quantitative polymerase chain reaction (PCR) was performed to analyse the mRNA levels of *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor subtypes. Acute (single) and repeated (4 d), but not chronic (14 d), stress exposure of 45 min or longer duration significantly increased phospho-CaMKII levels without affecting the levels of CaMKII. Pre-treatment with NBQX, a selective AMPA receptor antagonist, significantly prevented this stress-induced increase. In contrast, two NMDA receptor antagonists, LY235959 and MK-801, showed no inhibitory effect on phospho-CaMKII levels during acute stress. Neither acute nor chronic stress changed mRNA levels of NMDA and AMPA receptors. These results demonstrate that immobilization stress promotes the phosphorylation of CaMKII. The increase in the intracellular Ca^{2+} concentration by the activation of AMPA receptors may play a role in the stress-induced phospho-CaMKII in the rat hippocampus.

Received 21 May 2003; Reviewed 3 September 2003; Revised 19 November 2003; Accepted 23 November 2003

Key words: AMPA receptor, calcium/calmodulin-dependent protein kinase II, immobilization stress, NMDA receptor, phosphorylation.

Introduction

Various stresses can alter brain function through the phosphorylation of neurotransmitter receptors as well as the expression of neuronal genes. It has been revealed that stress can regulate the levels of receptors coupled with second messengers, such as cAMP or calcium (Ca^{2+}), which are closely involved in the phosphorylation of protein kinases (Duman, 1995). Numerous studies have demonstrated that immobilization stress increases the synaptic level of glutamate and the activation of its receptors (Bartanusz et al., 1995; Duman, 1995; Lowy et al., 1993, 1995; Schwendt and Jezova, 2000; Tocco et al., 1991). Thus, immobilization stress may alter Ca^{2+}-dependent signal transduction in the brain.

It is well known that one of the protein kinases, calcium/calmodulin-dependent protein kinase (CaMK) II, also plays an important role in synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Bejar et al., 2002; Giese et al., 1998; Lisman et al., 2002; Lledo et al., 1995; Malenka, 1995; Malenka et al., 1989) involved in memory formation (Izquierdo and Medina, 1995; Silva et al., 1992). Recently several studies have reported links between stress and phosphorylation of CaMKII (phospho-CaMKII) in regional changes in the hippocampus (Blank et al., 2002, 2003; Gerges et al., 2003). However, influence of immobilization stress on CaMKII under

Address for correspondence: Dr S. Yamawaki, Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.
Tel.: +81-82-257-5205 *Fax*: +81-82-257-5209
E-mail: yamawaki@hiroshima-u.ac.jp

various time paradigms has not been fully studied in the entire hippocampus. In addition, the mechanism by which stress affects the phosphorylation of CaMKII remains to be determined.

In the present study, we first examined whether various stress paradigms using immobilization affected the levels of CaMKII and phospho-CaMKII in the rat hippocampus. Secondly, we identified the signal transduction pathway involved in the up-regulation of phospho-CaMKII in response to a single immobilization stress.

Method

Animals

Male Sprague–Dawley rats weighing 250 g (Japan Charles River, Yokohama, Japan) were used in our studies. They were housed in groups of three per cage with food and water available ad libitum and kept on a 12 h:12 h light–dark cycle (lights on at 08:00 hours). All experimental procedures were approved by the Hiroshima University Medical Science Animal Care Committee.

Immobilization stress

In the acute study, rats were subjected to a single immobilization stress for either 45 or 90 min and sacrificed immediately after the end of the stress session. In order to investigate whether rapid reversal of the stress-induced phosphorylation of CaMKII was observed, some rats under 90-min immobilization were returned to their home cages for 60 min after the stress session, and then decapitated. In the 4-d repeated immobilization stress, rats were subjected to 45-min immobilization stress per day for 4 consecutive days. They were sacrificed immediately after the end of the stress session on day 4. In the chronic study, rats were subjected to repeated immobilization stress exposure of 90 min duration per day for 14 consecutive days and sacrificed immediately after the end of the stress session on day 14. To investigate the influence of repeated stress on the basal level of phospho-CaMKII in the hippocampus, some rats under chronic stress were returned to their home cages for 24 h after immobilization on day 13, and then sacrificed on day 14. Each rat was immobilized by placing it inside a disposable clear polyethylene rodent restraint cone (Harvard Apparatus, South Natick, MA, USA) with only the tail protruding. The large end of the cone was closed with tape at the base of the tail. The bag size was adjusted according to the size of the rat in order to achieve complete immobilization. A hole in the small end of the cone allowed the rats to breathe freely.

Another group of rats (sham) was kept under non-stress conditions prior to sacrifice.

Drug treatment

Among α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonists, NBQX, a selective and competitive AMPA receptor antagonist, has two advantages compared with CNQX. NBQX does not act as an *N*-methyl-D-aspartic acid (NMDA) antagonist (via the glycine site) and it shows greater central nervous system (CNS) activity when administered systemically (Smith et al., 1991). CNQX does not effectively cross the blood–brain barrier (BBB) (Birder and de Groat, 1992; Fink-Jensen et al., 1992; Kakizaki et al., 1996; Yoshiyama et al., 1995). In contrast, an anticonvulsant effect of NBQX occurred 15–60 min after administration in rats at the same dose as that used in the present study (Smith et al., 1991). NBQX disodium salt (Tocris, Ellisville, MO, USA) was dissolved in distilled water (DW) and 11.95 mg/kg was intraperitoneally injected 20 min prior to immobilization.

LY235959 (Tocris), a competitive NMDA receptor antagonist, was dissolved in DW and 2.5 or 5.0 mg/kg was intraperitoneally injected 20 min before commencement of immobilization stress. Prior studies have shown that LY235959 can pass through the BBB and has a plasma half life of 1.6 h (Chartoff et al., 1999; Dahlem and Eckstein, 1992).

(+)-MK-801 maleate (Tocris), a potent, selective and non-competitive NMDA receptor antagonist, was dissolved in DW and 0.5 mg/kg was intraperitoneally injected 20 min prior to immobilization. MK-801 can penetrate the BBB, and its peak plasma concentration and plasma half-life are 10–15 min and 1.6 h respectively (Hatfield et al., 1992). Sham rats for NBQX, LY235959 and MK-801 treatment were injected with the same amount of DW and were kept under non-stress conditions.

Nimodipine (Tocris), a central acting L-type Ca^{2+}-channel blocker, was suspended in 1% Tween-80 in saline and 2.5 or 5.0 mg/kg was intraperitoneally injected 45 min prior to immobilization, based on its duration of action (Taya et al., 2000). Sham rats for nimodipine treatment were injected with the same amount of 1% Tween-80 in saline and were kept under non-stress conditions.

Western blot

Following decapitation, the intact brain was quickly removed and transferred to an ice-cold artificial

cerebrospinal fluid (aCSF) solution of the following composition: 5 mM KCl, 126 mM NaCl, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, and 2 mM $MgSO_4$, containing 10 mM sodium fluoride (NaF). We removed the whole hippocampus from the brain in aCSF containing NaF. The tissues were then quickly frozen at −80 °C. The entire hippocampus was homogenized on ice with a Polytron homogenizer at top speed (30 000 rpm) in homogenization buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, 10 mM sodium phosphate, 25 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM DTT, 25 mM benzamidine, 25 mM NaF, 1 mM orthovanadate and 1 mM phenylmethylsulphonyl fluoride (in 100% ethanol). The insoluble material was removed by centrifugation at 350 *g* at 4 °C for 5 min. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (8.7–17.7 μg for the measurement of phospho-CaMKII, 0.87–1.78 μg for the measurement of α-CaMKII) for each group were separated on a 10% SDS gel (ATTO, Tokyo, Japan) and transferred to a PVDF membrane (ATTO) using a semi-dry blotting apparatus (Bio-Rad). The blots were blocked overnight at 4 °C with PVDF buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% I-Block™ and 0.1% Tween-20]. Then, the blots were incubated with anti-ACTIVE CaMKII pAb, (pT^{286}) (1:1000) (Promega, Madison, WI, USA) in PVDF buffer at room temperature for 2 h. After washing three times (15 min each) with 75 ml PVDF buffer, the membranes were incubated for 1 h with donkey anti-rabbit IgG (H+L) secondary antibody conjugate (Promega) diluted 1:8000 in PVDF buffer. After washing three additional times with PVDF buffer and twice with TBS [20 mM Tris–HCl (pH 7.5), 150 mM NaCl], the phospho-CaMKII band was detected by a colorimetric method (PerkinElmer, Boston, MA, USA).

The α-CaMKII level was determined using anti-CaMKII, α-subunit (Upstate Biotechnology, New York, NY, USA) and anti-mouse IgG, HRP-linked antibody (Cell Signaling, Beverly, MA, USA). After transferring, the membranes were blocked in TBS containing 5% non-fat dry milk and 0.05% Tween-20 (TBST-MLK) at 20–25 °C for 20 min with constant agitation, and then incubated overnight with anti-CaMKII (1:1000) in TBST-MLK at 4 °C. After washing twice with DW, the membranes were incubated with anti-mouse IgG, HRP-linked antibody (1:3000) in TBST-MLK at room temperature with agitation for 1.5 h. They were washed with DW twice and then incubated in TBS 0.1% Tween-20 for 4 min. After rinsing in 4–5 changes of DW, the CaMKII bands were detected by a colorimetric method (PerkinElmer). The density of the immunoreactive bands was quantified with a Macintosh-based ATTO Image analysis program (version 4.0; ATTO). We carefully selected the immunoreactive bands with densities within the linear range of film sensitivity.

Immunohistochemistry

Following decapitation, the intact brain was removed from the skull and quickly frozen using powdered dry ice and stored at −80 °C. Freshly frozen coronal brain sections (15 μm) through the hippocampus were cut using a cryostat, thaw-mounted onto slides and fixed with 4% paraformaldehyde for 5 min. Sections were washed three times with TBS (PBS 0.1% Triton X-100) and pre-treated with 10% H_2O_2 in methanol to neutralize the endogenous peroxidase activity. The sections were then washed twice in TBS for 10 min and autoclaved in TBS at 95 °C for 15 min. After being blocked in 10% sheep serum in TBS for 60 min, the sections were incubated overnight at 4 °C with anti-ACTIVE CaMKII pAb (pT^{286}) (Promega) diluted 1:500 in 10% sheep serum in TBS. After washing four times in TBS for 10 min each, the sections were incubated at room temperature for 180 min with goat anti-rabbit IgG (H+L) HRP conjugate (Zymed, San Francisco, CA, USA) diluted 1:500 in 10% sheep serum in TBS. They were then washed four times in TBS for 10 min each and exposed with Dako liquid DAB+substrate chromogen solution (Dako, Carpinteria, CA, USA). The immunohistochemical signal for phospho-CaMKII was detected using a digital video-image analyser (Nikon ACT-1, version 2.00, Tokyo, Japan).

Real-time quantitative polymerase chain reaction (RT–PCR)

Total RNA was extracted using the RNAqueous™ Total RNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. After treatment with RNase-free DNase I (Takara, Shiga, Japan), a single-stranded cDNA was synthesized using reverse transcriptase (Toyobo, Osaka, Japan). RT–PCR was performed with an ABI7700 sequence detection system [PerkinElmer (PE) Applied Biosystems, Foster City, CA, USA] to quantitate relative mRNA levels in samples. RT–PCR was performed to amplify the mRNA of NR1, NR2A, NR2B and GluR2. The primers and TaqMan hybridization probes were designed using Primer Express software (PE Applied Biosystems). Table 1 shows the sequences and fluorescent dyes of the PCR primers and TaqMan probes

Table 1. Sequences and fluorescent dye of PCR primers and TaqMan probes

NR1	
Forward primer	5′-GTTCTTCCGCTCAGGCTTTG-3′
Reverse primer	5′-AGGGAAACGTTCTGCTTCCA-3′
TaqMan probe	5′-FAM-CGGCATGCGCAAGGACAGCC-TAMRA-3′
NR2A	
Forward primer	5′-AGCCCCCTTCGTCATCGTA-3′
Reverse primer	5′-GACAGGGCACCGTGTTCCT-3′
TaqMan probe	5′-FAM-AGGACATAGACCCCCTGACTGAGACCTGTG-TAMRA-3′
NR2B	
Forward primer	5′-CCCCCAAGTTCTGGTTGGT-3′
Reverse primer	5′-TTTTGGGAACGAGCTTTGCT-3′
TaqMan probe	5′-FAM-TTGGCCGTCTTGGCCGTATCAGGZ-TAMRA-3′
GluR2	
Forward primer	5′-CGGGTAGGGATGGTTCAGTTT-3′
Reverse primer	5′-TGGCTACCTCCAAATTGTCGAT-3′
TaqMan probe	5′-FAM-CACTTCGGAGTTCAGACTGACACCCCA-TAMRA-3′

for each molecule. The TaqMan probe, which was designed to hybridize to the PCR products, was labelled with a fluorescent reporter dye at the 5′-end and a quenching dye at the 3′-end. PCR was carried out with TaqMan Universal PCR Master Mix (PE Applied Biosystems). All standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial denaturation at 50 °C for 2 min and 95 °C for 10 min. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95 °C for 15 s for melting and 60 °C for 1 min for annealing and extention. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the TaqMan Rodent GAPDH Control Reagents kit (PE Applied Biosystems).

The mRNA levels of NR1, NR2A, NR2B and GluR2 were detected by RT–PCR (ABI PRISM 7700 sequence detection system) and the ratio of the concentration of the target molecule to that of GAPDH (target molecule/GAPDH) in unknown samples was calculated.

Statistical analysis

Immunoreactive bands were quantified with a Macintosh-based ATTO Image analysis program, version 4.0. The mRNA levels by RT–PCR were calculated with an ABI PRISM 7700 sequence detection system. The data were expressed as mean ± S.E.M.

Data from the three groups in the acute and chronic immobilization stress studies were analysed by one-way ANOVA (Tukeys test for post-hoc comparison). Two-way ANOVA (drug × stress) was performed on the drug treatment and stress exposure data, and post-hoc comparisons were performed using Tukey's test. Experiments containing two groups were analysed by unpaired t test. Significance was determined at the level of $p < 0.05$.

Results

Influence of acute immobilization stress on the levels of phospho-CaMKII and CaMKII

The influence of different paradigms of acute immobilization stress on the levels of phospho-CaMKII and CaMKII in the hippocampus was examined by Western blot analysis. In these experiments rats were subjected to a single immobilization stress of either 45- or 90-min duration and sacrificed immediately after the end of the stress session. The phospho-CaMKII levels in the hippocampus were significantly increased in response to acute immobilization stress for 45 or 90 min (Figure 1a).

In rats which received 90-min immobilization and were then allowed to remain in their home cages for 60 min, there was no significant difference in the phospho-CaMKII levels in comparison with those of the sham group (results not shown). None of the single-immobilization stress paradigms changed CaMKII levels in the hippocampus (Figure 1b).

To identify the hippocampal cell layers where phospho-CaMKII was expressed and influenced by

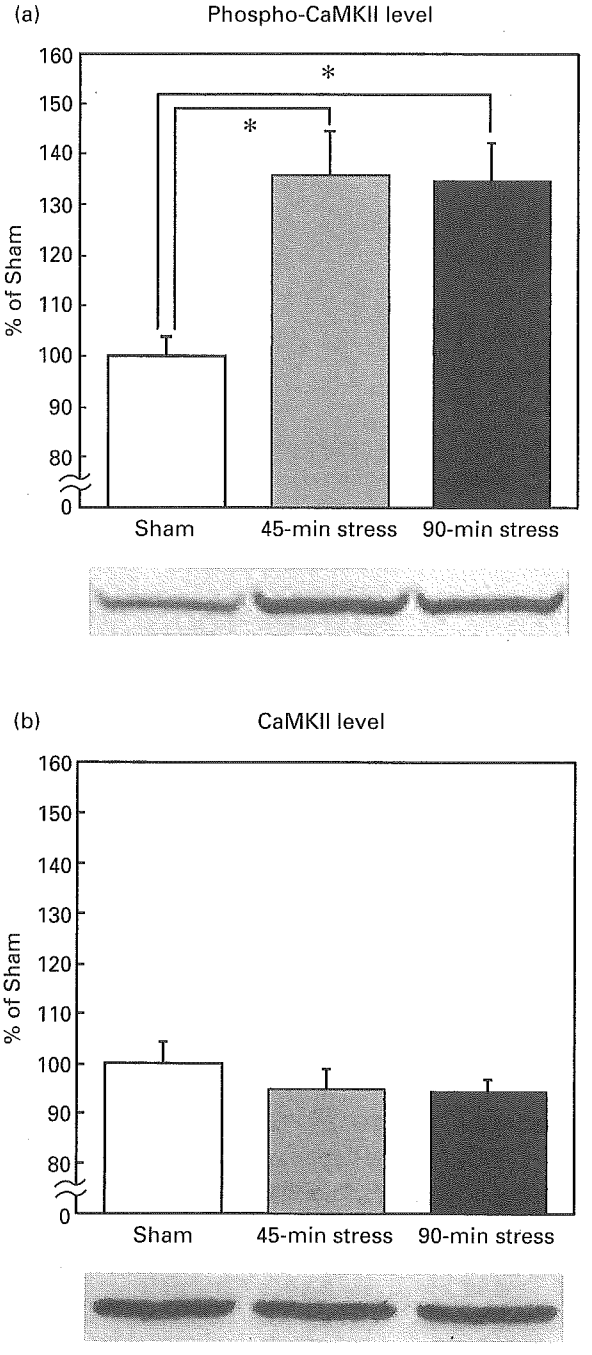

Figure 1. Influence of a single immobilization stress on the phospho-CaMKII and CaMKII immunoreactivity in the hippocampus, as determined by Western blot analysis. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to a single immobilization of either 45- or 90-min duration and sacrificed immediately after the end of the stress session. (a) Influence of a single immobilization on phospho-CaMKII levels. (b) Influence of a single immobilization on CaMKII levels. Sham, sham-treatment; 45-min stress, stress exposure of 45-min duration; 90-min stress, stress exposure of 90-min duration.

stress exposure, we examined the influence of immobilization stress on phospho-CaMKII expression by immunohistochemical analysis. In the sham-treated rat, immunohistochemical analysis demonstrated phospho-CaMKII immunoreactivity in the CA1, CA2 and CA3 pyramidal cell layers and dentate gyrus granular cell layers (Figure 2a). Acute immobilization stress for 45 min markedly increased the levels of phospho-CaMKII immunoreactivity in the CA1, CA2 and CA3 pyramidal cell layers (Figure 2b).

An increase in cellular Ca^{2+} level is initially required to induce the phosphorylation of CaMKII. It has been reported that the NMDA receptor, AMPA receptor and L-type voltage-gated Ca^{2+} channels play an important role in the regulation of Ca^{2+} influx from extracellular sources (Dolmetsch et al., 2001; Leonard et al., 2002; Pellegrini-Giampietro et al., 1997). To determine the main pathway involved in the increase in phospho-CaMKII levels induced by acute immobilization stress, we examined the effect of pre-treatment with LY235959, MK-801, NBQX and nimodipine on the stress-induced phosphorylation of CaMKII in the hippocampus.

Two-way ANOVA showed no significant drug effect for LY235959 [$F(1,44)=0.002$, $p=0.968$], a significant effect of stress [$F(1,44)=8.599$, $p=0.005$], and no interaction between LY235959 and stress [$F(1,44)=0.426$, $p=0.517$], indicating that pre-treatment with LY235959 (5.0 mg/kg) did not affect the increase of phospho-CaMKII levels in response to acute immobilization (Figure 3a). LY235959 (2.5 mg/kg) pre-treatment also had no effect on the stress-induced up-regulation of phospho-CaMKII (results not shown). For MK-801, significant effects of drug [$F(1,20)=16.259$, $p<0.001$], and stress [$F(1,20)=7.559$, $p=0.012$] were found, while the interaction between MK-801 and stress [$F(1,20)=0.365$, $p=0.553$] was not significant ($n=6$; sham: 100 ± 9.1; DW+stress: 126.4 ± 5.5; MK-801+stress: 153.3 ± 10.0; MK-801 alone: 136.5 ± 5.9).

Two-way ANOVA showed a significant drug effect for NBQX [$F(1,44)=8.177$, $p=0.007$], effect of stress [$F(1,44)=13.561$, $p<0.001$] and interaction between NBQX and stress [$F(1,44)=23.648$, $p<0.001$]; phospho-CaMKII levels in the DW+stress group were significantly increased compared with the sham group ($p<0.05$, post hoc) and NBQX pre-treatment+stress

Data are expressed as the percentage of the sham group and represent the mean+S.E.M. of 12 rats per group.
*$p<0.05$ compared to the sham group (one-way ANOVA with Tukey's test).

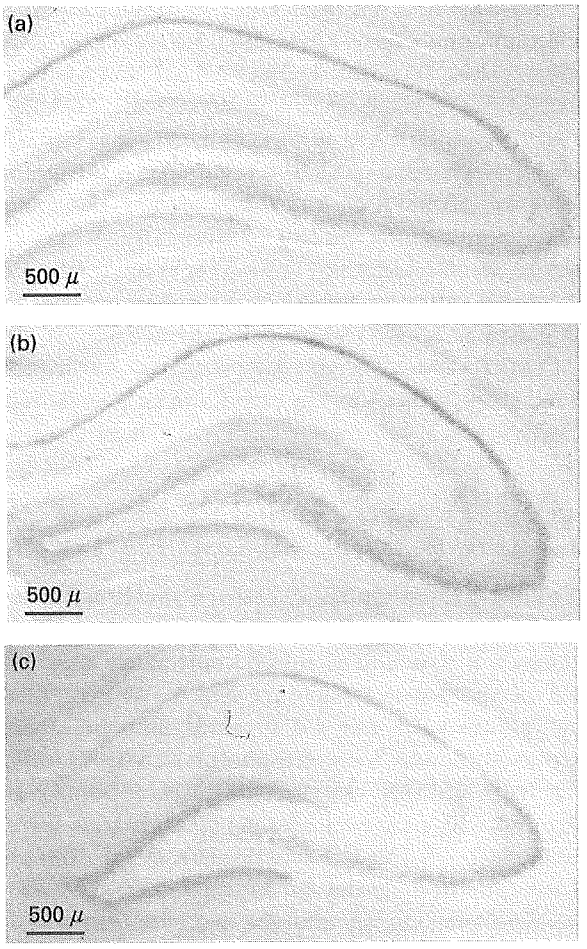

Figure 2. Influence of a single immobilization stress on the expression of phospho-CaMKII in the rat hippocampus as determined by immunohistochemical analysis. Representative immunohistochemical stains of phospho-CaMKII in the hippocampus are shown. Rats were intraperitoneally injected with either NBQX (11.95 mg/kg) or distilled water (DW), 20 min prior to the 45-min stress session, and sacrificed immediately after the stress session. (a) Sham: DW pre-treatment + non-stress condition. (b) DW + stress: DW pre-treatment + 45-min immobilization. (c) NBQX + stress, NBQX pre-treatment + 45-min immobilization.

group ($p < 0.05$, post hoc) (Figure 3b), demonstrating that NBQX significantly prevented the stress-induced up-regulation of phospho-CaMKII. Immunohistochemical analysis demonstrated that pre-treatment with NBQX attenuated the marked increase in the expression of phospho-CaMKII in response to acute immobilization stress in the CA1, CA2 and CA3 pyramidal cell layers (Figure 2c), compared with a stressed rat that had not been pre-treated with NBQX (Figure 2b).

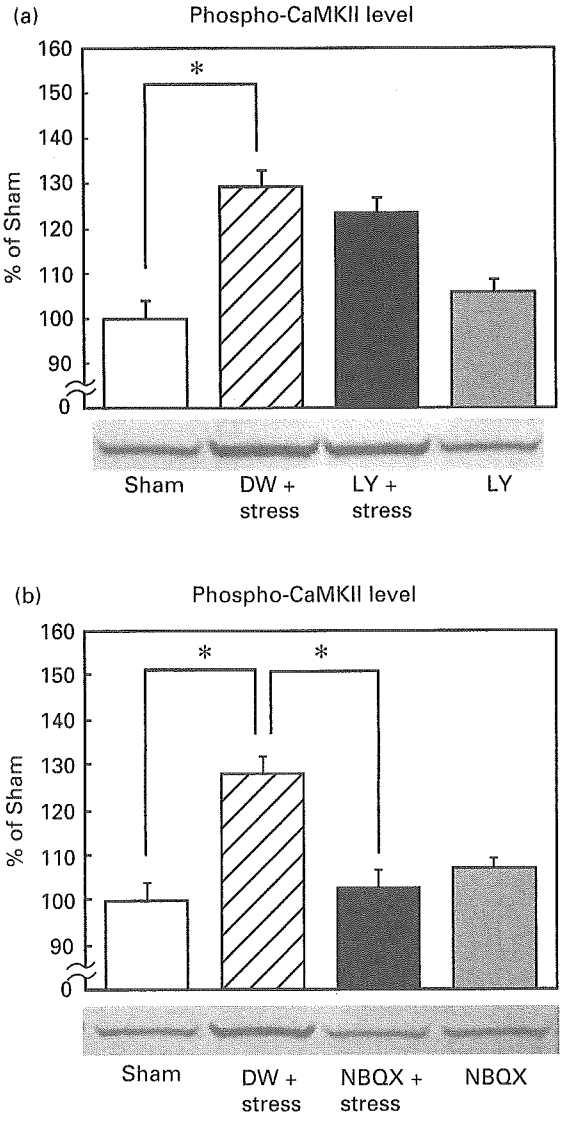

Figure 3. Effect of pre-treatment with LY235959 (5.0 mg/kg) and NBQX (11.95 mg/kg) on phospho-CaMKII levels following acute immobilization. Representative phospho-CaMKII Western blots are shown. (a) Rats were sacrificed immediately after the stress session. Sham, DW pre-treatment + non-stress condition; DW + stress, DW pre-treatment + 45 min immobilization; LY + stress, LY235959 pre-treatment + 45-min immobilization; LY, LY235959 pre-treatment + non-stress condition. Data are expressed as the percentage of the sham group and represent the mean ± S.E.M. of 12 rats per group. (b) Rats were sacrificed immediately after the stress session. Sham, DW pre-treatment + non-stress condition; DW + stress, DW pre-treatment + 45-min immobilization; NBQX + stress, NBQX pre-treatment + 45-min immobilization; NBQX, NBQX pre-treatment + non-stress condition. Data are expressed as the percentage of the sham group and represent the mean ± S.E.M. of 12 rats per group. Statistically significant differences, * $p < 0.05$. Two-way ANOVA followed by Tukey's test.

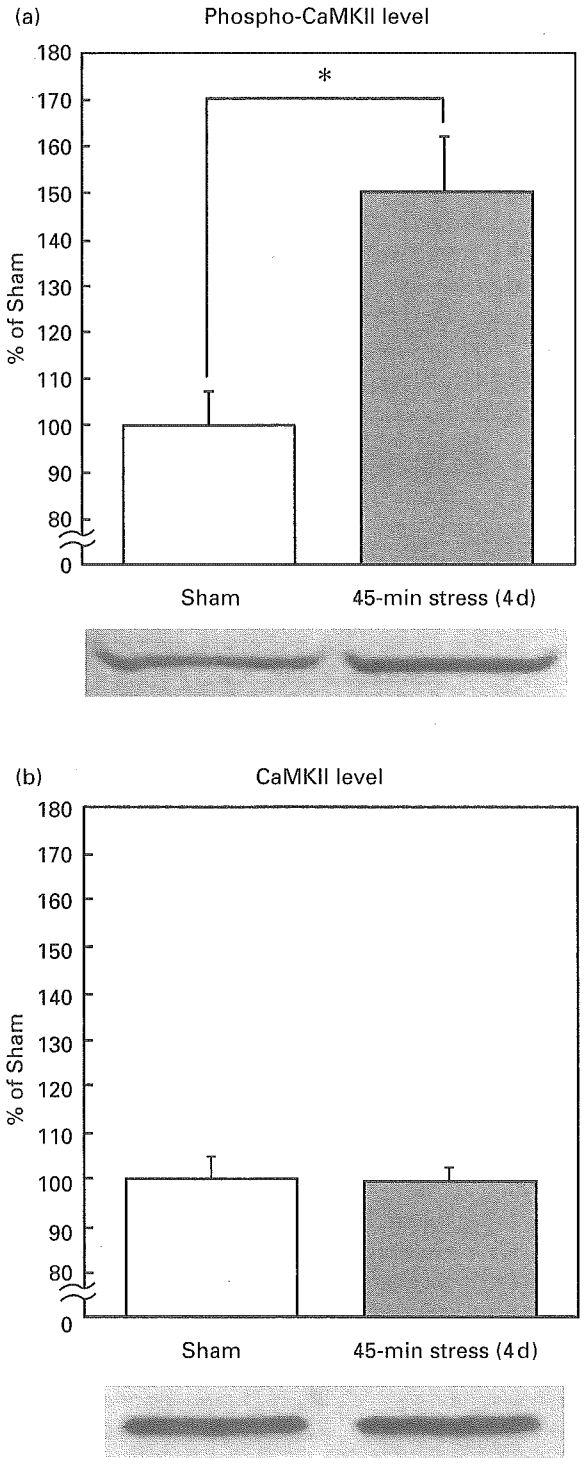

Figure 4. Effect of repeated immobilization stress for 4 days on phospho-CaMKII and CaMKII immunoreactivity as determined by Western blot analysis. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to 45- min immobilization stress per day for 4 consecutive days and sacrificed immediately after the end of the stress session on the last day. (a) Influence of repeated immobilization stress (4 d) on the phospho-CaMKII level. (b) Influence of repeated immobilization stress (4 d) on the CaMKII level. Sham, sham treatment; 45-min stress (4 d), repeated immobilization stress of 45-min duration for 4 d. Data are expressed as the percentage of the sham group and represent the mean ± S.E.M. of 6 rats per group. * $p<0.05$ compared to the sham group (unpaired t test).

Two-way ANOVA indicated no significant drug effect for nimodipine [$F(1,23)=0.867$, $p=0.362$], an effect of stress [$F(1,23)=24.256$, $p<0.001$] and no interaction between nimodipine and stress [$F(1,23)=0.001$, $p=0.975$], indicating that nimodipine (5.0 mg/kg) did not affect phospho-CaMKII levels under acute stress ($n=6$–7; sham: 100 ± 3.9; DW+stress: 128.1 ± 6.0; nimodipine+stress: 122.6 ± 6.7; nimodipine alone: 94.9 ± 5.5). Nimodipine (2.5 mg/kg) pre-treatment also had no effect on the stress-induced up-regulation of phospho-CaMKII (results not shown).

CaMKII levels were not changed by either drug, stress or both in the hippocampus (results not shown).

Influence of 4- and 14-d repeated immobilization stress on the levels of phospho-CaMKII and CaMKII

During 4-d repeated immobilization stress, rats were subjected to 45-min immobilization stress per day for 4 consecutive days and sacrificed immediately after the end of the stress session on day 4. Western blot analysis revealed that 4-d repeated immobilization stress significantly increased phospho-CaMKII levels, but not CaMKII levels, in the hippocampus (Figure 4a, b). In the chronic (14 d) study, rats were subjected to repeated immobilization stress (RIS) of 90 min duration per day for 14 consecutive days and sacrificed immediately after the end of the stress session on day 14 (RIS −14). Some rats exposed to repeated immobilization stress were returned to their home cages for 24 h after immobilization on day 13, and then sacrificed on day 14 (RIS −13+24 h). The levels of phospho-CaMKII or CaMKII in the hippocampus were not changed in rats exposed to 14-d chronic immobilization stress compared with the levels in either the sham group or the group sacrificed 24 h after repeated immobilization stress for 13 d (Figure 5a, b).

Immunohistochemical analysis demonstrated that chronic immobilization stress did not change the expression of phospho-CaMKII immunoreactivity in the CA1, CA2 and CA3 pyramidal cell layers in the hippocampus (results not shown).

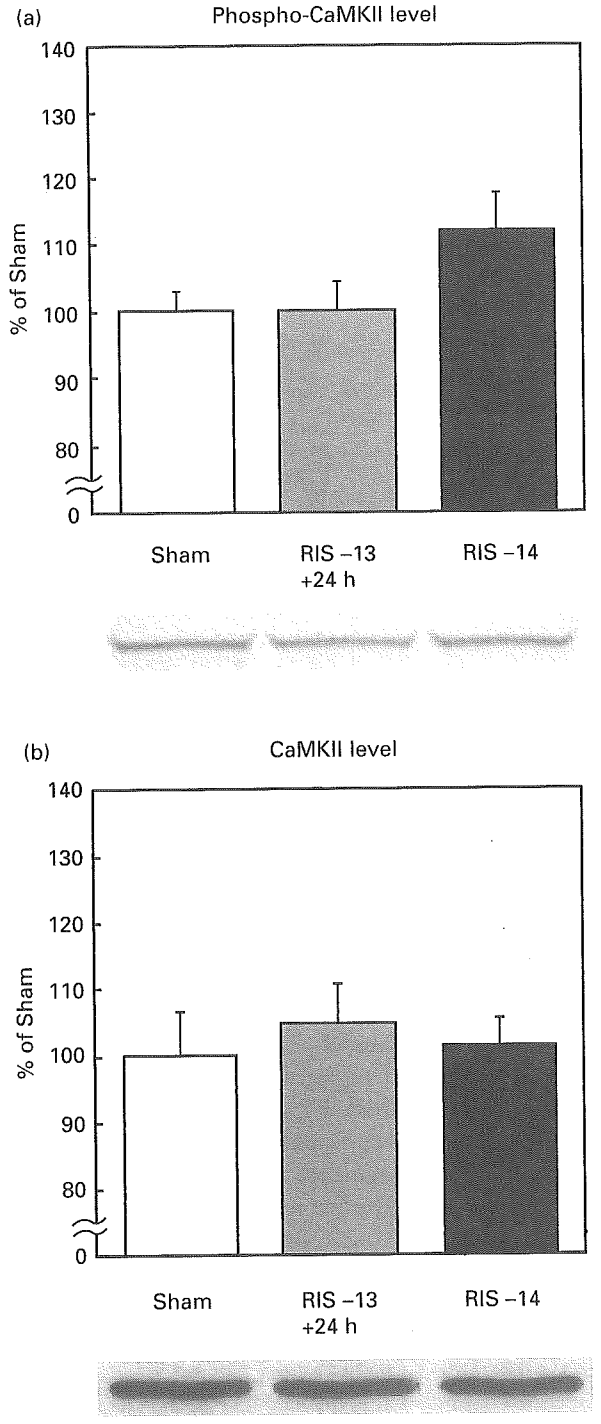

Figure 5. Influence of chronic immobilization stress on phospho-CaMKII and CaMKII immunoreactivity in the hippocampus. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to repeated immobilization stress (RIS) exposure of 90-min duration per day for 14 consecutive days and sacrificed immediately after the end of the stress session on the last day (RIS −14). Some rats under repeated immobilization stress were returned to their home cages for 24 h after immobilization on day 13, and then sacrificed on day 14 (RIS −13+24 h). (a) Influence of chronic stress on phospho-CaMKII levels. (b) Influence of chronic stress on CaMKII levels. Sham, sham-treatment. RIS −13+24 h, 24 h after the last session of repeated immobilization stress for 13 d. RIS −14, immediately after the last session of repeated immobilization stress for 14 d. Data are expressed as the percentage of the sham group and represent the mean ± S.E.M. of 11 rats per group.

Influence of acute and chronic immobilization stress on the mRNA levels of NMDA and AMPA receptors

Several different stress paradigms, including immobilization, were reported to alter the levels of NMDA and AMPA receptors (Bartanusz et al., 1995; Pellegrini-Giampietro et al., 1992; Schwendt and Jezova, 2000; Zelena et al., 1999). We therefore used RT–PCR analysis to determine whether acute or chronic immobilization stress changes the mRNA levels of these receptors in the rat hippocampus. In all experiments, rats were sacrificed immediately after the end of the final stress session. Neither acute nor chronic immobilization changed the mRNA levels of NR1, NR2A, NR2B or GluR2 in the hippocampus (Table 2).

Discussion

Phosphorylation of CaMKII in response to acute stress

The results of the present study demonstrated that phospho-CaMKII levels in homogenates derived from the entire rat hippocampus significantly increased immediately after the 45- and 90-min stress sessions and returned to basal levels 60 min after 90-min immobilization. Our findings differed from those of Blank et al. (2002, 2003) which had shown that the levels of phospho-CaMKII in the mouse CA1 region were decreased immediately after a single immobilization for 60 min and significantly increased 2 h after stress. Although the reason for this difference is uncertain, different experimental conditions such as the region of interest, the method of immobilization, and the effect of NaF in aCSF may account for the difference in the phosphorylation of CaMKII.

Several plausible mechanisms by which a single immobilization stress up-regulates the phosphorylation of CaMKII are considered. It is well known that elevation of intracellular Ca^{2+} levels induces Ca^{2+}-dependent phosphorylation of CaMKII and subsequently leads to autophosphorylation of $Thr^{286/287}$ in CaMKII, Ca^{2+}-independent activation (Fukunaga

Table 2. mRNA levels of NMDA receptors (NR1, NR2A, NR2B) and AMPA receptor (GluR2) in the hippocampus after immobilization stress

Treatment	NR1	NR2A	NR2B	GluR2
Acute stress				
Sham	100.0±9.2	100.0±10.9	100.0±4.1	100.0±6.6
Stress (45 min)	112.1±12.1	113.7±13.3	109.0±3.0	112.3±9.4
Stress (90 min)	103.7±4.3	99.7±7.7	113.4±7.3	110.6±6.9
Chronic stress				
Sham	100.0±2.2	100.0±2.6	100.0±2.6	100.0±1.9
Stress	96.0±2.5	108.1±3.1	107.8±3.9	102.8±4.0

Data are expressed as the percentage of the sham group and represent the mean± S.E.M. of 8–9 rats per group.

et al., 1989; Lou and Schulman, 1989). Since inescapable stress including acute immobilization increases the synaptic levels of glutamate (Duman, 1995; Lowy et al., 1993, 1995; McEwen and Magarinos, 1997; Schwendt and Jezova, 2000), it is conceivable that the activation of both NMDA and AMPA receptors are involved in the cellular Ca^{2+} influx in response to immobilization stress. Fukunaga et al. (1992) reported that treatment with glutamate can produce a long-lasting increase in the Ca^{2+}-independent CaMKII activity with cultured rat hippocampal neurons, mainly through the NMDA receptor. In contrast, the activation of AMPA receptors mediates fast excitatory synaptic transmission in the CNS, through depolarization of the post-synaptic cells and subsequent removal of the Mg^{2+} block in the NMDA receptors (Malenka, 1995; Malenka and Nicoll, 1999). There are also some reports that the AMPA receptor can respond to acute stress (Bartanusz et al., 1995; Tocco et al., 1991) and that Ca^{2+}-permeable AMPA receptors in the hippocampus play a role in Ca^{2+} influx (Gu et al., 1996; Pellegrini-Giampietro et al., 1997). Bartanusz and co-workers (1995) reported an increase of NR1 in the CA1 and CA3, an increase of NR2B only in the CA3 pyramidal layer, and decrease of GluR1 in both CA1 and CA3, 24 h after a single immobilization stress. We did not find any change in the mRNA levels of NR1, NR2A, NR2B or GluR2 in response to a single stress. The discrepancy is probably related to the different regions examined and times of decapitation. The present results show that pre-treatment with NBQX, but not LY235959 or MK-801, inhibited the phosphorylation of CaMKII, suggesting that the phosphorylation of CaMKII in response to acute immobilization may be mediated by AMPA, but not NMDA, receptors in the hippocampus. The increase in intracellular Ca^{2+} concentration by the activation of AMPA receptors may play a role in the induction of phospho-CaMKII by immobilization stress.

Influence of chronic immobilization stress on the phospho-CaMKII level

In contrast with both single and 4-d repeated immobilization stress, 14-d chronic immobilization did not affect the levels of CaMKII and phospho-CaMKII in the rat hippocampus. Whereas the results of the present study were consistent with previous findings that chronic psychosocial stress does not affect phospho-CaMKII levels in the rat dentate gyrus (Gerges et al., 2003), we did not find that chronic stress decreased total CaMKII levels. It is hypothesized that down-regulation of NMDA or AMPA receptors may suppress the induction of phospho-CaMKII by reducing Ca^{2+} influx in response to chronic stress. Our findings regarding NR1 mRNA levels were consistent with those of Schwendt and Jezova (2000). They reported the effect of repeated immobilization stress for 7 d on GluR1. In the present study, we examined GluR2, rather than GluR1, since the AMPA receptor is comprised of four subunits (GluR1-4) (Nakanishi, 1992) and the GluR2 subunit controls the Ca^{2+} permeability of the AMPA receptor (Pellegrini-Giampietro et al., 1997). In our study, we did not find any significant change in the mRNA levels of NR1, NR2A, NR2B nor GluR2 in the hippocampus in response to chronic stress. Therefore, it is unlikely that the down-regulation of NMDA or AMPA receptors in response to chronic immobilization stress leads to a decrease in the stress-induced up-regulation of phospho-CaMKII in the hippocampus.

Another tenable hypothesis is that the increase in the activity of protein phosphatases under chronic immobilization stress facilitates dephosphorylation of

phospho-CaMKII. Protein phosphatases such as PP1 and PP2A, can regulate Ca^{2+}-independent CaMKII activity through dephosphorylation of $Thr^{286/287}$ (Ishida et al., 1998; Strack et al., 1997a, b; Winder and Sweatt, 2001). Morinobu and colleagues (In Press) demonstrated that chronic immobilization significantly increased the activity of PP2A in the rat hippocampus. In this context, it is postulated that under repeated stress, the rate of dephosphorylation of phospho-CaMKII in the hippocampus is higher than the rate of phosphorylation of CaMKII.

Phospho-CaMKII in pathophysiology

CaMKII plays an important role in synaptic plasticity such as LTP and LTD related to memory formation including traumatic memories (Charney et al., 1993; Krystal, 1990). CaMKII phosphorylation under stress may be partly associated with the pathophysiology of stress-related disorders, such as post-traumatic stress disorder (PTSD). Further studies to elucidate the mechanism by which the activation of CaMKII and AMPA receptors leads to stress-induced memory alteration may promote our understanding of stress-related disorders.

Acknowledgements

This study was supported by a grant-in-aid for general scientific research from the Ministry of Education, Science, and Culture of Japan, a Health Science Research Grant for Research on Brain Science from the Ministry of Health and Welfare of Japan, and a grant from Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology (JST). The authors thank Dr T. Fukumoto and Ms M. Okamura for assisting with the Western blot analyses.

Statement of Interest

None.

References

Bartanusz V, Aubry JM, Pagliusi S, Jezova D, Baffi J, Kiss JZ (1995). Stress-induced changes in messenger RNA levels of N-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. *Neuroscience 66*, 247–252.

Bejar R, Yasuda R, Krugers H, Hood K, Mayford M (2002). Transgenic calmodulin-dependent protein kinase II activation: dose-dependent effects on synaptic plasticity, learning, and memory. *Journal of Neuroscience 22*, 5719–5726.

Birder LA, de Groat WC (1992). The effect of glutamate antagonists on c-fos expression induced in spinal neurons by irritation of the lower urinary tract. *Brain Research 580*, 115–120.

Blank T, Nijholt I, Eckart K, Spiess J (2002). Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. *Journal of Neuroscience 22*, 3788–3794.

Blank T, Nijholt I, Vollstaedt S, Spiess J (2003). The corticotropin-releasing factor receptor 1 antagonist CP-154, 526 reverses stress-induced learning deficits in mice. *Behavioural Brain Research 138*, 207–213.

Charney DS, Deutch AY, Krystal JH, Southwick SM, Davis M (1993). Psychobiologic mechanisms of posttraumatic stress disorder. *Archives of General Psychiatry 50*, 295–305.

Chartoff EH, Ward RP, Dorsa DM (1999). Role of adenosine and N-methyl-D-aspartate receptors in mediating haloperidol-induced gene expression and catalepsy. *Journal of Pharmacology and Experimental Therapeutics 291*, 531–537.

Dahlem AM, Eckstein JA (1992). Disposition of the novel NMDA antagonist, LY235959, in rats, dogs and monkeys. *Pharmaceutical Research 9*, S-278.

Dolmetsch RE, Pajvani U, Fife K, Spotts JM, Greenberg ME (2001). Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science 294*, 333–339.

Duman RS (1995). Regulation of intracellular signal transduction and gene expression by stress. In: Friedman MJ, Charney DS, Deutch AY (Eds.), *Neurobiology and Clinical Consequences of Stress* (pp. 27–43). Philadelphia: Lippincott-Raven.

Fink-Jensen A, Judge ME, Hansen JB, Jacobsen P, Turski L, Olney J, Honore T (1992). Inhibition of cisplatin-induced emesis in ferrets by the non-NMDA receptor antagonists NBQX and CNQX. *Neuroscience Letters 137*, 173–177.

Fukunaga K, Rich DP, Soderling TR (1989). Generation of the Ca^{2+}-independent form of Ca^{2+}/calmodulin-dependent protein kinase II in cerebellar granule cells. *Journal of Biological Chemistry 264*, 830–836.

Fukunaga K, Soderling TR, Miyamoto E (1992). Activation Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *Journal of Biological Chemistry 267*, 527–533.

Gerges NZ, Aleisa AM, Schwarz LA, Alkadhi KA (2003). Chronic psychosocial stress decreases calcineurin in the dentate gyrus: a possible mechanism for preservation of early LTP. *Neuroscience 117*, 869–874.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998). Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science 279*, 870–873.

Gu JG, Albuquerque C, Lee CJ, MacDermott AB (1996). Synaptic strengthening through activation of Ca^{2+}-permeable AMPA receptors. *Nature 381*, 793–796.

Hatfield RH, Gill R, Brazell C (1992). The dose–response relationship and therapeutic window for dizocilpine

(MK-801) in a rat focal ischaemia model. *European Journal of Pharmacology 216*, 1–7.

Ishida A, Kameshita I, Fujisawa H (1998). A novel protein phosphatase that dephosphorylates and regulates Ca^{2+}/calmodulin-dependent protein kinase II. *Journal of Biological Chemistry 273*, 1904–1910.

Izquierdo I, Medina JH (1995). Correlation between the pharmacology of long-term potentiation and the pharmacology of memory. *Neurobiology of Learning and Memory 63*, 19–32.

Kakizaki H, Yoshiyama M, de Groat WC (1996). Role of NMDA and AMPA glutamatergic transmission in spinal c-fos expression after urinary tract irritation. *American Journal of Physiology 270*, R990–996.

Krystal JH (1990). Animal models for posttraumatic stress disorder. In: Giller EL (Ed.), *Biological Assessment and Treatment of Posttraumatic Stress Disorder* (pp. 3–64). Washington, DC: American Psychiatric Press.

Leonard AS, Bayer KU, Merrill MA, Lim IA, Shea MA, Schulman H, Hell JW (2002). Regulation of calcium/calmodulin-dependent protein kinase II docking to N-methyl-D-aspartate receptors by calcium/calmodulin and alpha-actinin. *Journal of Biological Chemistry 277*, 48441–48448.

Lisman J, Schulman H, Cline H (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature Reviews Neuroscience 3*, 175–190.

Lledo PM, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC, Nicoll RA (1995). Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. *Proceedings of the National Academy of Sciences USA 92*, 11175–11179.

Lou LL, Schulman H (1989). Distinct autophosphorylation sites sequentially produce autonomy and inhibition of multifunctional Ca^{2+}/calmodulin-dependent protein kinase. *Journal of Neuroscience 9*, 2020–2032.

Lowy MT, Gault L, Yamamoto BK (1993). Adrenalectomy attenuates stress-induced elevations in extracellular glutamate concentrations in the hippocampus. *Journal of Neurochemistry 61*, 1957–1960.

Lowy MT, Wittenberg L, Yamamoto BK (1995). Effect of acute stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. *Journal of Neurochemistry 65*, 268–274.

Malenka RC (1995). LTP and LTD: dynamic and interactive processes of synaptic plasticity. *The Neuroscientist 1*, 35–42.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature 340*, 554–557.

Malenka RC, Nicoll RA (1999). Long-term potentiation – a decade of progress? *Science 285*, 1870–1874.

McEwen BS, Magarinos AM (1997). Stress effects on morphology and function of the hippocampus. *Annals of the New York Academy of Sciences 821*, 271–284.

Morinobu S, Fujimaki K, Kawano K, Tanaka K, Takahashi J, Ohkawa M, Yamawaki S, Kato N (In Press). Influence of immobilization stress on the expression and phosphatase activity of protein phosphatase 2A in the rat brain. *Biological Psychiatry*.

Nakanishi S (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science 258*, 597–603.

Pellegrini-Giampietro DE, Gorter JA, Bennet MV, Zukin RS (1997). The GluR2 (GluR-B) hypothesis: Ca^{2+}-permeable AMPA receptors in neurological disorders. *Trends in Neurosciences 20*, 464–470.

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992). Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proceedings of the National Academy of Sciences USA 89*, 10499–10503.

Schwendt M, Jezova D (2000). Gene expression of two glutamate receptor subunits in response to repeated stress exposure in rat hippocampus. *Cellular and Molecular Neurobiology 20*, 319–329.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science 257*, 206–211.

Smith SE, Durmuller N, Meldrum BS (1991). The non-N-methyl-D-aspartate receptor antagonists, GYKI 52466 and NBQX are anticonvulsant in two animal models of reflex epilepsy. *European Journal of Pharmacology 201*, 179–183.

Strack S, Barban MA, Wadzinski BE, Colbran RJ (1997b). Differential inactivation of postsynaptic density-associated and soluble Ca^{2+}/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. *Journal of Neurochemistry 68*, 2119–2128.

Strack S, Choi S, Lovinger DM, Colbran RJ (1997a). Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. *Journal of Biological Chemistry 272*, 13467–13470.

Taya K, Watanabe Y, Kobayashi H, Fujiwara M (2000). Nimodipine improves the disruption of spatial cognition induced by cerebral ischemia. *Physiology & Behavior 70*, 19–25.

Tocco G, Shors TJ, Baudry M, Thompson RF (1991). Selective increase of AMPA binding to the AMPA/quisqualate receptor in the hippocampus in response to acute stress. *Brain Research 559*, 168–171.

Winder DG, Sweatt JD (2001). Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. *Nature Review Neuroscience 2*, 461–474.

Yoshiyama M, Roppolo JR, de Groat WC (1995). Effects of GYKI 52466 and CNQX, AMPA/kainate receptor antagonists, on the micturition reflex in the rat. *Brain Research 691*, 185–194.

Zelena D, Makara GB, Jezova D (1999). Simultaneous blockade of two glutamate receptor subtypes (NMDA and AMPA) results in stressor-specific inhibition of prolactin and corticotropin release. *Neuroendocrinology 69*, 316–323.

Effect of Neonatal Isolation on the Noradrenergic Transduction System in the Rat Hippocampal Slice

KOUJI KUSAKA, SHIGERU MORINOBU, KI-ICHIRO KAWANO, AND SHIGETO YAMAWAKI*
Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

KEY WORDS fura-2; hippocampus; immunohistochemistry; intracellular calcium; noradrenaline

ABSTRACT Numerous studies suggest that early adverse experiences induce neurochemical, morphological, and functional changes in the hippocampus in adolescence and adulthood. The aim of this study was to identify the influence of neonatal isolation (NI) on noradrenaline (NA)-mediated intracellular calcium ($[Ca^{2+}]_i$) mobilization. To measure $[Ca^{2+}]_i$, we used the Ca^{2+}-sensitive dye fura-2 and analysis by fluorescence microscopy. First, we examined the contributions of adrenergic receptor subtypes to the NA-stimulated increase in $[Ca^{2+}]_i$ in the granule cell layers of the dentate gyrus (DG) and in the pyramidal cell layers of the CA3 in the hippocampus. Second, we found that the NA-stimulated $[Ca^{2+}]_i$ increment was significantly decreased in response to NI in these hippocampal regions. In addition, we examined the influence of environmental enrichment (EE) after weaning on the decrease in the NA-stimulated $[Ca^{2+}]_i$ increment induced by NI. The administration of EE reversed the influence of NI on the NA-stimulated $[Ca^{2+}]_i$ increment in the CA3 pyramidal cell layer but not in the DG granular cell layer in the hippocampus. These findings suggest that NI and EE after weaning may modulate hippocampal function by altering adrenergic receptor-mediated signal transduction during adolescence. **Synapse 54:223–232, 2004.**

INTRODUCTION

Numerous studies have shown that stress induces neurochemical, morphological, and functional changes in the hippocampus in humans (Bremner et al., 2003; Nakano et al., 2002; Vythilingam et al., 2002), nonhuman primates (Gould et al., 1997, 1998; Uno et al., 1989), and rodents (Huot et al., 2002; Watanabe et al., 1992). For example, the volume of right hippocampus measured by MRI in patients with posttraumatic stress disorders (PTSD) was significantly smaller and there was a significant correlation between the volume of the hippocampus and functional deficits in verbal memory (Bremner et al., 1995). An exposure to sustained social stress induced gastric ulcer formation as well as hippocampal degeneration in velvet monkeys (Uno et al., 1989). A series of studies by Sapolsky and colleagues (Sapolsky, 1996, 2000; Sapolsky et al., 1990) has indicated that an elevated secretion of glucocorticoids (GCs) in response to long-lasting stress was closely involved in the hippocampal abnormalities. In addition, several studies have demonstrated that early adverse experiences were associated with the enhanced response of the hypothalamic-pituitary-adrenal (HPA) axis to stress in later life (Anisman et al., 1998; Huot et al., 2002; Kalinichev et al., 2002; Meaney et al., 1996; Plotsky and Meaney, 1993).

On the other hand, it has been revealed that early adverse experiences play a major role in precipitating the onset of stress-related mental disorders, such as PTSD (Pine and Cohen, 2002) and major depression (Gilmer and McKinney, 2003). Brain imaging studies indicated that early adverse experiences may be associated with the smaller volume of the hippocampus in patients with severe depression (Vythilingam et al., 2002) and PTSD (Bremner et al., 2003). Furthermore, Huot et al. (2002) demonstrated that exposure to stress

Contract grant sponsors: Ministry of Education, Science, and Culture of Japan (grant-in-aid for general scientific research); Ministry of Health and Welfare of Japan (Health Science Research Grant for Research on Brain Science); Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology (JST).

*Correspondence to: Shigeto Yamawaki, M.D., Ph.D., Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.
E-mail: yamawaki@hiroshima-u.ac.jp

Received 5 November 2003; Accepted 20 August 2004

DOI 10.1002/syn.20086

Published online 13 October 2004 in Wiley InterScience (www.interscience.wiley.com).

or glucocorticoids early in life was associated with hippocampal atrophy and impairments in learning and memory in adult rats. Based on these findings, it is conceivable that the enhanced response of the HPA axis to stress induced by early adverse experiences may be crucial to the development of morphological and functional abnormalities of the hippocampus.

There are several studies which demonstrate the immediate (Kehoe et al., 1996a) as well as the long-lasting influence of neonatal isolation (NI) on central catecholamine functions (Kehoe et al., 1996b; Matthews et al., 2001; McCormick et al., 2002). For example, maternal separation increased the noradrenaline response to restraint stress in the paraventricular nucleus of the hypothalamus (Liu et al., 2000). However, in contrast to our knowledge of the HPA axis, little is known about how NI influences NA-mediated signal transduction in the hippocampus. In this context, we measured NA-induced intracellular calcium ($[Ca^{2+}]_i$) increments in the hippocampi of rats subjected to NI, using a fura-2 fluorescent technique, in order to elucidate the influence of NI on noradrenergic postsynaptic signal transduction. Environmental enrichment (EE) has been shown to improve performance in tests of spatial memory and induce neurogenesis in the hippocampus (Kempermann et al., 1997; Nilsson et al., 1999). Moreover, it has been reported that EE after weaning alleviates the adverse effects of maternal separation on both the HPA and the behavior in response to stress (Francis et al., 2002). Therefore, we also investigated whether the effect of NI was alleviated by EE in the present study.

MATERIALS AND METHODS

Animals

Female pregnant Sprague-Dawley rats were purchased from Charles River (Yokohama, Japan). These rats were housed individually in the breeding colony at constant room temperature (23 ± 2°C) and humidity (60%) with a 12/12 h light/dark cycle (lights on 0800–2000). Food and water were provided ad libitum. The litters were culled to 12 pups on postnatal day 1 (PN Day 1).

Preweaning housing conditions

The mothers and pups without NI were left entirely undisturbed until weaning. NI was undertaken according to Kehoe's method with a minor modification (Kehoe and Bronzino, 1999). Pups were isolated from the dam, nest, and siblings, and placed in individual round containers for a period of 1 h per day on PN Days 2–22. In all the experimental groups the dams and their 12 pups were housed in 38 × 23 × 20 cm clear plastic cages until weaning.

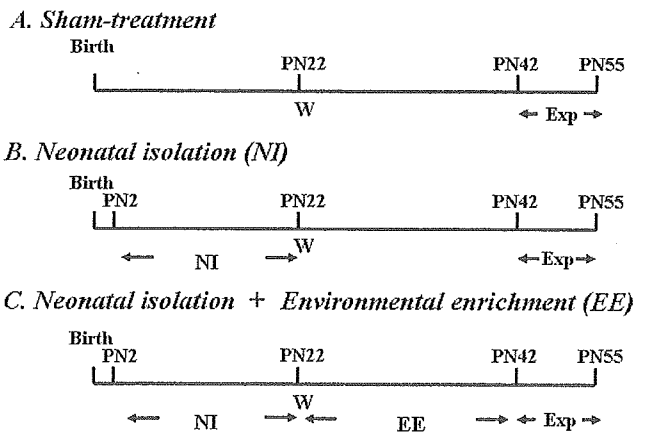

Fig. 1. Animal treatment paradigms. **A:** Sham-treated rats were left entirely undisturbed and decapitated on postnatal (PN) Days 42–55. **B:** Pups were isolated from the dam, nest, and siblings for a period of 1 h per day on PN Days 2–22 and decapitated on PN Days 42–55. **C:** Pups were isolated on PN Days 2–22 and received EE on PN Days 22–42 and decapitated on PN Days 42–55.

Postweaning housing conditions

After weaning (PN Day 22), we only used litters comprised of males and these were given ad libitum food and water. All animals were placed into groups of three and housed within 38 × 23 × 20 cm clear plastic cages. Sham-treated rats were left entirely undisturbed (Fig. 1A). Animals subjected to NI were randomly assigned to one of two groups. One group was left entirely undisturbed after weaning (Fig. 1B). The other group received EE on PN Day 22–42 (Fig. 1C). EE consisted of a few toys which were replaced regularly. The other housing conditions of EE, such as cage size and food, were identical to those of other groups. Animals were sacrificed by decapitation on PN 42–55. The mean date of decapitation day was PN 49 in all three groups. All animal procedures were carried out in strict accordance with the Hiroshima University School of Medicine Animal Care Committee.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} measurements were undertaken according to Kudo's method with a minor modification (Kudo et al., 1987). The hippocampus was isolated from rats and cooled by immersion for 10 min in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): NaCl, 124.0; KCl, 2.5; $CaCl_2$, 2.0; $MgCl_2$, 1.0; NaH_2PO_4, 1.25; $NaHCO_3$, 26.0; and glucose, 10.0; gassed with 95% O_2 / 5% CO_2. Transverse hippocampal slices about 300 μm thick were prepared by a rotary cutter (Rotorslicer DTY-7700, Dosaka EM, Kyoto, Japan). The slices were incubated in gassed ACSF for 60 min, then in 10 μM fura-2/AM (Dojindo Laboratory, Kumamoto, Japan) for 60 min at 37°C. A stable fura-2/AM solution was prepared by adding 1/10 volume of the detergent Cremophor EL (Sigma, St. Louis, MO) to a stock solution of fura-2/AM (1 mM in DMSO) and

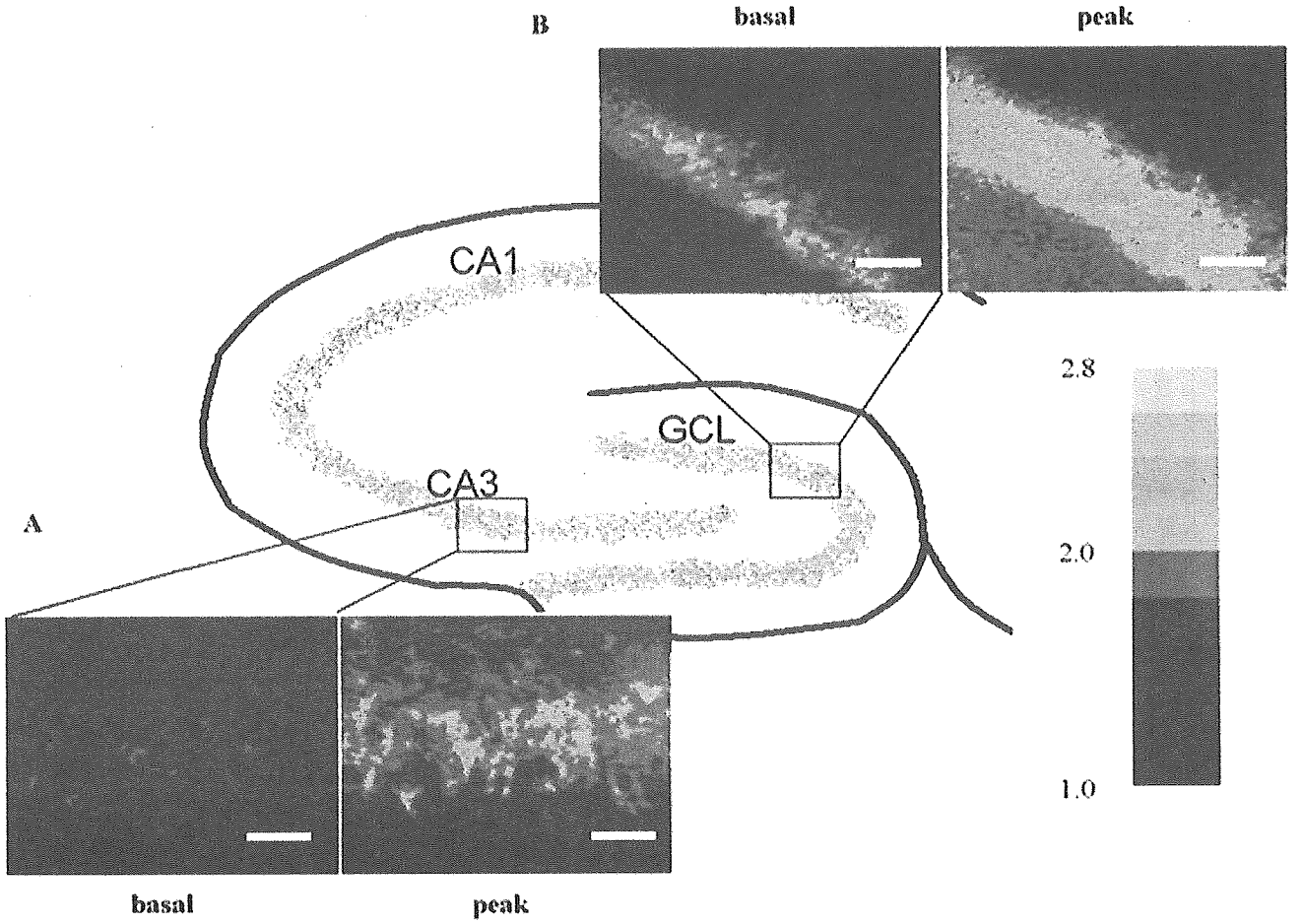

Fig. 2. A schematic drawing of a rat hippocampal slice. Changes in $[Ca^{2+}]_i$ in the stratum granulare in the dentate gyrus (DG) and the stratum pyramidale in the CA3 field were evaluated. The F_{340}/F_{380} ratio is represented by pseudocolor coding. **A:** Pseudocolor coding of the basal $[Ca^{2+}]_i$ value (left) and the peak value of $[Ca^{2+}]_i$ induced by application of 100 μM NA to the CA3 pyramidal cell layer (right). **B:** Similarly, basal $[Ca^{2+}]_i$ value (left) and peak value of $[Ca^{2+}]_i$ induced by application of 100 μM NA to the granule cell layer (GCL) in the dentate gyrus (right). Scale bars = 100 μm.

diluting it with buffer solution during sonication. The fura-2-loaded slices were incubated in warmed ACSF for another 30 min, then placed in a chamber made of a thin glass coverslip and a plastic wall. The chamber was continuously perfused with oxygenated ACSF (37°C; 3 ml/min). Noradrenaline (Nacalai Tesque, Kyoto, Japan) or phenylephrine (Sigma) was added to the ACSF with 500 μg/ml of the antioxidant sodium metabisulfite. Clonidine (Sigma), isoproterenol (Nacalai Tesque), prazosin (Sigma), yohimbine (Sigma), and propranolol (Sigma) were dissolved in the ACSF and administered by bath perfusion (3 ml/min). The emission intensity at 510 nm was imaged by means of an inverted microscope (Nikon, Japan) equipped with a CCD camera (Hamamatsu Photonics, Japan). Paired recordings of the 510 nm emission following 340 nm excitation (F_{340}) and 380 nm excitation (F_{380}) were made at 6-sec intervals. The F_{340}/F_{380} ratios were calculated using an Argus-50 digital fluorescence analyzer (Hamamatsu Photonics). The regions of interest were set in the stratum granulare and the stratum pyramidale, where the somas were stained most prominently. The peak values were used as the response data.

Data analysis

Statistical comparisons between two groups were performed using the Mann-Whitney U-test, with a significance level of $P < 0.05$.

RESULTS

Influence of NA on $[Ca^{2+}]_i$ in sham animals

Figure 2 shows a schematic drawing of a rat hippocampal slice. Pseudocolor coding of the basal and peak $[Ca^{2+}]_i$ values induced by the application of 100 μM of NA to the CA3 pyramidal cell layer and the granule cell layer in the dentate gyrus (DG) are shown in Figure 2A,B, respectively. Following exposure of the slices to NA, $[Ca^{2+}]_i$ increased rapidly, peaked after ~70 sec, then returned to resting levels (Fig. 3A). NA (10^{-6}-10^{-2} M) increased the peak level of $[Ca^{2+}]_i$ in the DG granule cell layer in a dose-dependent manner (Fig. 3B). The dose response ap-

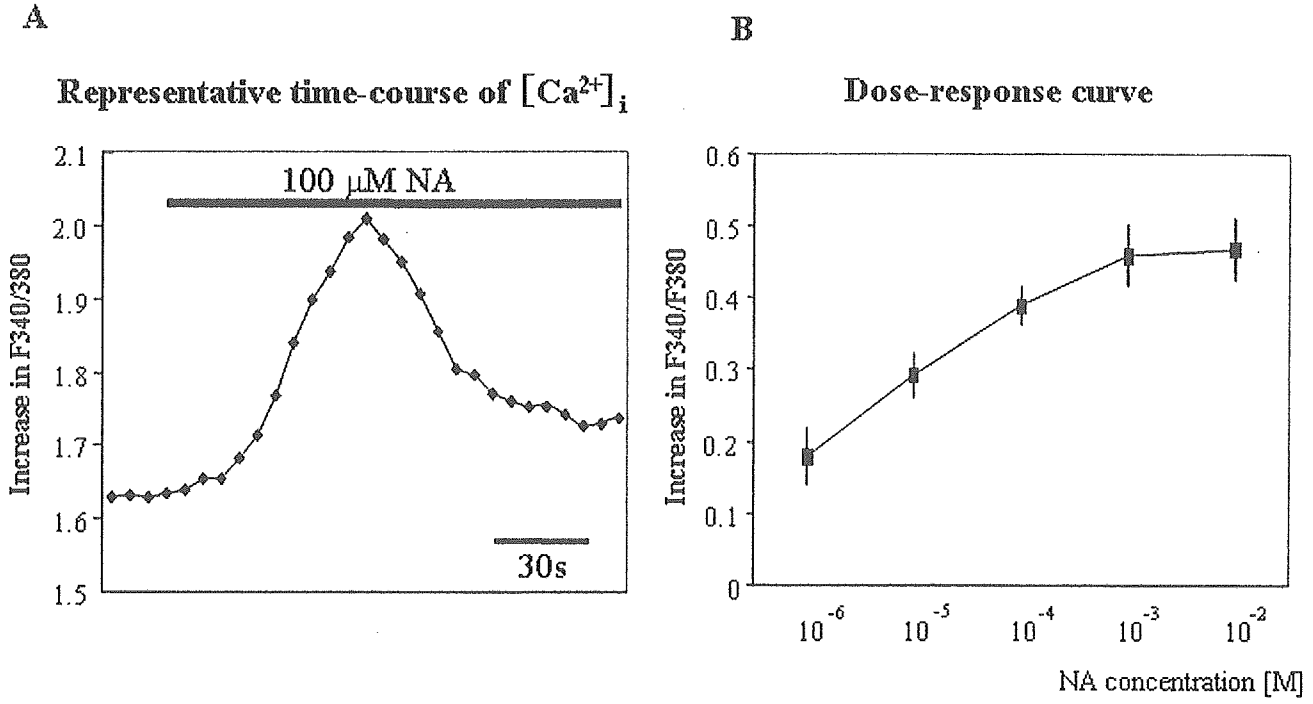

Fig. 3. **A:** The time-course of the $[Ca^{2+}]_i$ response to the application of 100 μM NA in the DG granule cell layer. **B:** The effects of various concentrations of NA on $[Ca^{2+}]_i$ in the DG granule cell layer. The $[Ca^{2+}]_i$ increases induced by bath application of NA were observed to change in a concentration-dependent manner. The results are the means ± SEMs (n = 6).

TABLE I. Several parameters of NA-induced $[Ca^{2+}]_i$ movement

	Sham	NI
DG granule cell layer		
The slope (increase/latency)	0.00546 ± 0.00099	0.00424 ± 0.00023
The latency of the peak(sec)	78.5 ± 5.7	69.3 ± 4.0
CA1 pyramidal cell layer		
The slope (increase/latency)	0.00401 ± 0.00024	0.00309 ± 0.00022
The latency of the peak(sec)	64.2 ± 4.1	61.0 ± 4.4
CA3 pyramidal cell layer		
The slope (increase/latency)	0.00415 ± 0.00033	0.00383 ± 0.00017
The latency of the peak(sec)	67.5 ± 3.8	55.0 ± 2.9[a]
	NI	**NI + EE**
DG granule cell layer		
The slope (increase/latency)	0.00430 ± 0.00021	0.00395 ± 0.00022
The latency of the peak(sec)	69.6 ± 4.2	72.7 ± 2.6
CA1 pyramidal cell layer		
The slope (increase/latency)	0.00388 ± 0.00024	0.00399 ± 0.00021
The latency of the peak(sec)	60.1 ± 3.8	62.9 ± 4.8
CA3 pyramidal cell layer		
The slope (increase/latency)	0.00392 ± 0.00019	0.00424 ± 0.00029
The latency of the peak(sec)	56.1 ± 3.2	64.8 ± 2.7[b]

Values are means ± SEM from six animals per group.
[a]$P < 0.05$, compared to sham.
[b]$P < 0.05$, compared to NI.

peared to plateau at 100 μM of NA, so this dose was used in subsequent experiments. We also analyzed other parameters such as the latency to the $[Ca^{2+}]_i$ peak and the slope (increase/latency) (Table I).

Characterization of the increase in $[Ca^{2+}]_i$

In order to clarify the AR subtypes involved in the stimulation of NA, we first examined the effects of adrenergic antagonists on the increase in $[Ca^{2+}]_i$ in hippocampal slices obtained from untreated rats (n = 5 in each group). As shown in Figure 4 and Table II, 100 μM of NA was applied in the presence of 100 μM of AR antagonist. Pretreatment with prazosin, an α_1-AR antagonist, markedly downregulated the NA-stimulated $[Ca^{2+}]_i$ increases in the DG granule cell ($P < 0.05$) and the CA3 pyramidal cell layers ($P < 0.05$). Pretreatment with yohimbine (an

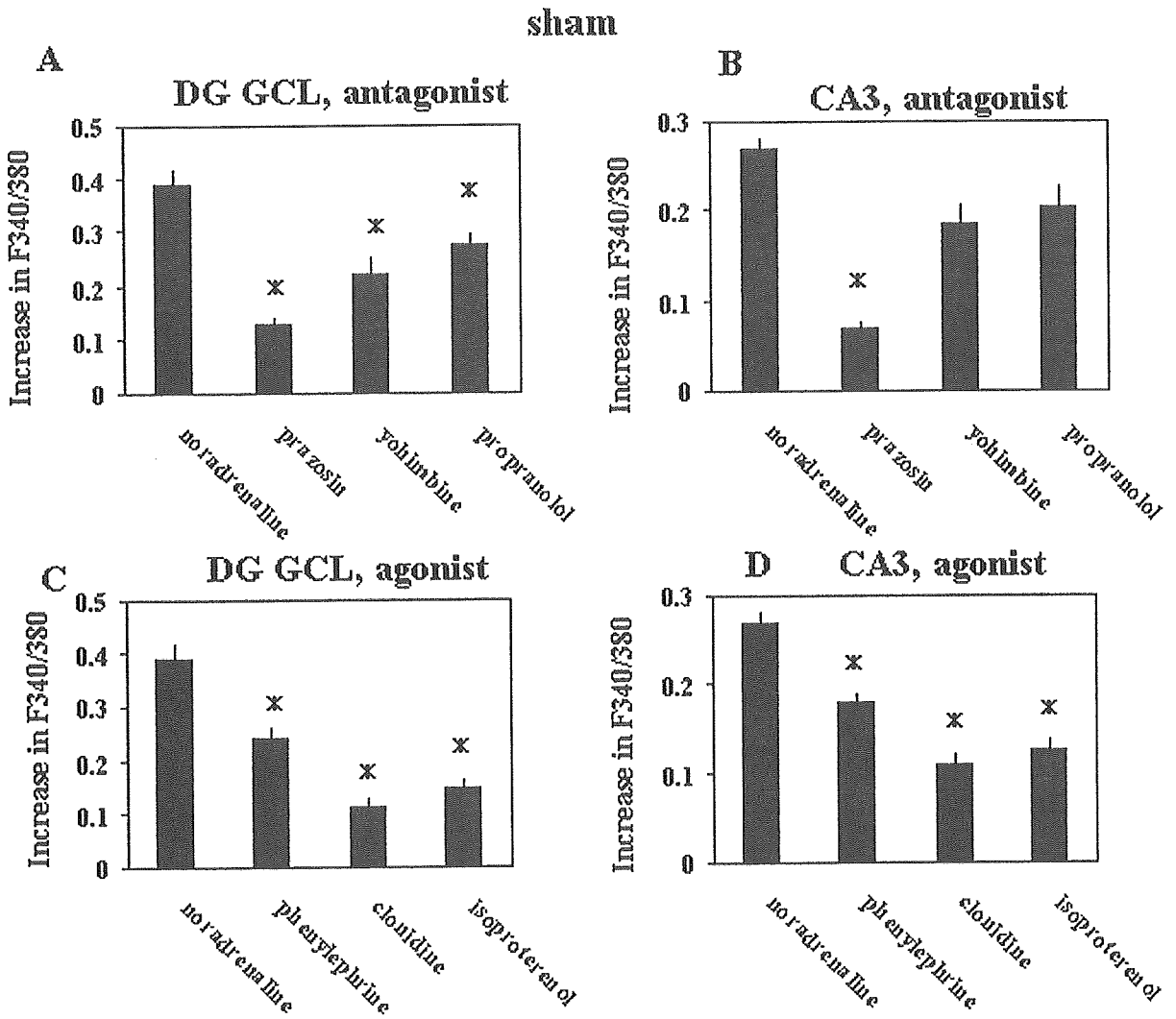

Fig. 4. Effects of AR antagonists and agonists on the $[Ca^{2+}]_i$ response in the DG granule cell (GCL) (**A,C**) and the CA3 pyramidal cell layers (CA3) (**B,D**) of sham-treated rats. The 100 μM NA-induced $[Ca^{2+}]_i$ increase was markedly reduced by 100 μM of prazosin, and partially reduced by 100 μM of yohimbine and 100 μM of propranolol in both the DG granule cell (**A**) and CA3 pyramidal cell layers (**B**). 100 μM of phenylephrine markedly increased $[Ca^{2+}]_i$ compared to 100 μM of clonidine and isoproterenol in both the DG granule cell (**C**) and CA3 pyramidal cell layers (**D**). The results are the means ± SEM (n = 5).

α_2-AR antagonist) and propranolol (a β-AR antagonist) suppressed the NA-stimulated $[Ca^{2+}]_i$ increases in both cell layers to a lesser extent (Fig. 4, Table II).

Second, prior to investigating the effects of adrenergic agonists on NA-induced $[Ca^{2+}]_i$, we examined the dose-dependent effects of α_1-, α_2-, and β-adrenergic agonists on $[Ca^{2+}]_i$ in the DG granule cell (Fig. 5A) and CA3 pyramidal cell layers (Fig. 5B). Phenylephrine, an α_1-AR agonist, markedly increased $[Ca^{2+}]_i$ in a concentration-dependent manner, reaching a plateau at 100 μM in both the DG granule cell (Fig. 5A) and the CA3 pyramidal cell layers (Fig. 5B). Clonidine, an α_2-AR agonist, at doses up to 1 mM, elicited modest increases in $[Ca^{2+}]_i$ in both the DG granule cell (Fig. 5A) and the CA3 pyramidal cell layers (Fig. 5B). Isoproterenol increased $[Ca^{2+}]_i$ in a concentration-dependent manner up to 1 mM in both the DG granule cell (Fig. 5A) and the CA3 pyramidal cell layers (Fig. 5B).

Third, based on the dose-dependent curve results, we compared the effects of 100 μM concentrations of the α_1-, α_2-, and β-adrenergic agonists on $[Ca^{2+}]_i$ in the DG granule cell and CA3 pyramidal cell layers (Fig. 4, Table II) (n = 5 in each experiment). Phenylephrine markedly increased $[Ca^{2+}]_i$ in both cell layers (Fig. 4C,D, Table II). Clonidine and isoproterenol also increased $[Ca^{2+}]_i$ in both cell layers, but to a lesser extent (Fig. 4C,D, Table II).

Influence of NI on NA-stimulated $[Ca^{2+}]_i$ increase

Effect of NI on $[Ca^{2+}]_i$

The peak value of NA-stimulated $[Ca^{2+}]_i$ in DG granule cell layer slices from NI-treated rats was significantly lower than that in the slices from sham-treated rats (F_{340}/F_{380} ratios were 0.278 ± 0.029 and 0.389 ±

TABLE II. Effect of antagonists and agonists on the NA-induced [Ca^{2+}] response in the dentate gyrus granule cell layer (DG GCL) and the CA3 pyramidal cell layer (CA3 PCL)

DG granule cell layer

		Noradrenaline	Prazosin	Yohimbine	Propranolol
Sham	F340/F380	0.389 ± 0.028	0.127 ± 0.010*	0.224 ± 0.028*	0.275 ± 0.018*
	% of NA	100.0 ± 7.1	32.7 ± 2.7*	57.6 ± 7.3*	70.8 ± 4.7*
NI	F340/F380	0.278 ± 0.029	0.099 ± 0.013*	0.156 ± 0.027*	0.179 ± 0.018*
	% of NA	100.0 ± 10.4	35.6 ± 4.6*	56.0 ± 9.7*	64.6 ± 6.4*
		Noradrenaline	Phenylephrine	Clonidine	Isoproterenol
Sham	F340/F380	0.389 ± 0.028	0.243 ± 0.021*	0.114 ± 0.015*	0.150 ± 0.013*
	% of NA	100.0 ± 7.1	62.4 ± 5.4*	29.2 ± 3.9*	38.4 ± 3.4*
NI	F340/F380	0.278 ± 0.029	0.170 ± 0.021*	0.101 ± 0.015*	0.121 ± 0.013*
	% of NA	100.0 ± 10.4	61.1 ± 7.7*	36.2 ± 5.4*	43.7 ± 4.8*

CA3 pyramidal cell layer

		Noradrenaline	Prazosin	Yohimbine	Propranolol
Sham	F340/F380	0.269 ± 0.012	0.070 ± 0.006*	0.187 ± 0.020	0.205 ± 0.020
	% of NA	100.0 ± 4.6	25.9 ± 2.4*	69.5 ± 7.3	76.2 ± 7.6
NI	F340/F380	0.209 ± 0.009	0.067 ± 0.017*	0.138 ± 0.021	0.172 ± 0.006
	% of NA	100.0 ± 4.3	32.0 ± 8.1*	66.1 ± 9.9	82.5 ± 2.8
		Noradrenaline	Phenylephrine	Clonidine	Isoproterenol
Sham	F340/F380	0.269 ± 0.012	0.178 ± 0.010*	0.108 ± 0.013*	0.125 ± 0.013*
	% of NA	100.0 ± 4.6	66.3 ± 3.8*	40.2 ± 4.8*	46.5 ± 4.8*
NI	F340/F380	0.209 ± 0.009	0.144 ± 0.011*	0.098 ± 0.009*	0.118 ± 0.007*
	% of NA	100.0 ± 4.3	68.9 ± 5.5*	46.9 ± 4.5*	56.7 ± 3.4*

Values are means ± SEM from five animals per group.
*$P < 0.05$, compared to noradrenaline.

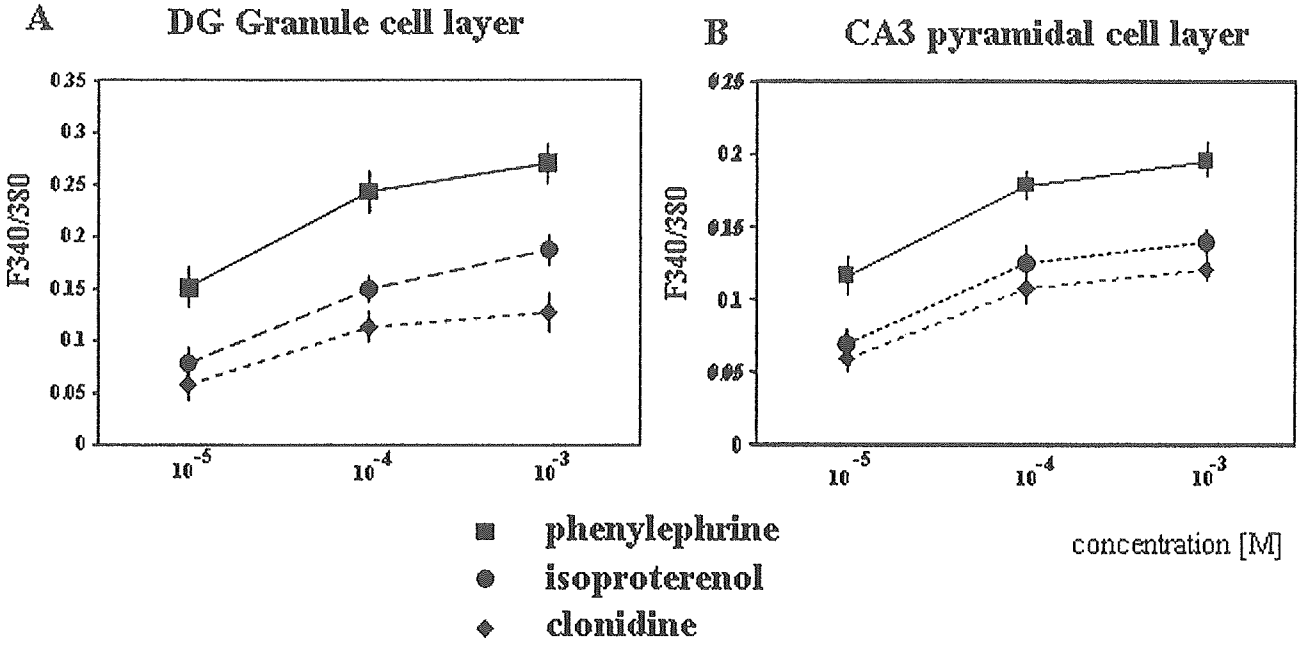

Fig. 5. Dose-dependent curves of the $[Ca^{2+}]_i$ responses to the application of various adrenergic agonists in the DG granule cell layer (**A**) and CA3 pyramidal cell layer (**B**). Phenylephrine, an α_1-AR agonist, markedly increased $[Ca^{2+}]_i$ in both the DG granule cell (**C**) and the CA3 pyramidal cell layers (**D**), reaching a plateau at 100 μM. Clonidine, an α_2-AR agonist, and isoproterenol, a β-AR agonist, also increased $[Ca^{2+}]_i$ in both cell layers but to a lesser extent (**C,D**). The results are the means ± SEM (n = 6).

0.028, respectively; $P < 0.05$) (Fig. 6A). Similarly, there was a significant difference in the peak values of NA-stimulated $[Ca^{2+}]_i$ in the CA3 pyramidal cell layer slices from NI and sham-treated rats (F_{340}/F_{380} ratios were 0.209 ± 0.009 and 0.269 ± 0.012, respectively; $P < 0.05$) (Fig. 6C). However, in the CA1 pyramidal cell layer no significant difference was detected (F_{340}/F_{380} ratios were 0.238 ± 0.019 and 0.233 ± 0.014, respectively; Fig. 6B). We also compared other parameters between the sham-treated and NI-treated rats, such as the latency to the $[Ca^{2+}]_i$ peak and the slope (increase/latency) (Table I). Data from two

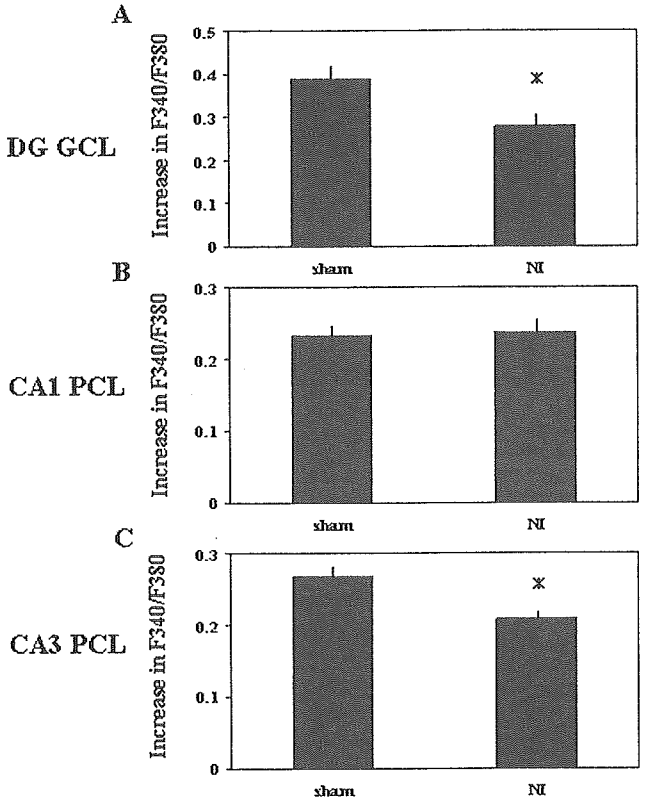

Fig. 6. Effect of NI on 100 μM NA-stimulated $[Ca^{2+}]_i$ increase. The 100 μM NA-mediated $[Ca^{2+}]_i$ increase was significantly downregulated by NI in both the DG granule cell (**A**) and the CA3 pyramidal cell layers (**C**) ($P < 0.05$), but not in the CA1 pyramidal cell layer (**B**). The results are the means ± SEM (n = 6).

slices per individual rat were averaged and six rats were used per group.

Characterization of the $[Ca^{2+}]_i$ increment in NI-treated rats

It is plausible that NI may regulate α_1-, α_2-, and β-adrenergic receptor pathways independently. Therefore, to examine this we measured the effects of 100 μM α_1-, α_2-, and β-adrenergic antagonists and agonists on $[Ca^{2+}]_i$ in hippocampal slices obtained from NI-treated rats (n = 5 in each experiment) (Fig. 7, Table II). Table II shows the F_{340}/F_{380} values as well as the ratio to NA. The suppression of NA-stimulated $[Ca^{2+}]_i$ increases by the adrenergic antagonists was similar in both NI-treated and sham-treated hippocampal slices (Fig. 7, Table II). Likewise, the effects of the three adrenergic agonists on the $[Ca^{2+}]_i$ increases in the hippocampal slices were similar in sham-treated and NI rats (Fig. 7, Table II).

Influence of EE on the NI-induced attenuation in $[Ca^{2+}]_i$ increment

We investigated whether the effect of NI was alleviated by EE in the present study. The NI-induced decrease in the 100 μM NA-stimulated $[Ca^{2+}]_i$ increase was alleviated by EE in the CA3 pyramidal cell layer (NI + EE, F_{340}/F_{380} ratio: 0.260 ± 0.022, NI, F_{340}/F_{380} ratio: 0.212 ± 0.011; Fig. 8C), but not in the DG granule cell layer (NI + EE, F_{340}/F_{380} ratio: 0.280 ± 0.015, NI, F_{340}/F_{380} ratio: 0.279 ± 0.022; Fig. 8A). The NA-stimulated increase in $[Ca^{2+}]_i$ in the CA1 pyramidal cell layer was not changed by NI or by EE (Fig. 8B). We also analyzed other parameters such as the latency to the $[Ca^{2+}]_i$ peak and the slope (increase/latency) (Table I). The results represent the mean values and SEMs obtained from six rats.

Influence of EE on NA-stimulated $[Ca^{2+}]_i$ increase

In rats with EE the peak increases in NA-stimulated $[Ca^{2+}]_i$ were 0.406 ± 0.030 in the DG granule cell layer and 0.280 ± 0.015 in the CA3 pyramidal cell layer (n = 6 in each group). No significant difference was detected between the sham-treated rats and EE-treated rats.

DISCUSSION

Mechanisms by which noradrenaline increases $[Ca^{2+}]_i$

The present study using AR antagonists and agonists indicates that the NA-stimulated increase in $[Ca^{2+}]_i$ was mediated predominantly by α_1-receptors, but α_2- and β-ARs contributed to the increase in $[Ca^{2+}]_i$ as well. It is reported that the α_1-AR-mediated increase in $[Ca^{2+}]_i$ is due to enhanced Ca^{2+} release from intracellular compartments, such as the endoplasmic reticulum, as well as to enhanced Ca^{2+} influx from extracellular sources (Minneman, 1988). It is well known that the stimulation of α_1-ARs induces the activation of phospholipase C (PLC) via G-proteins, and subsequently produces inositol 1,4,5-triphosphate (IP_3) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC (Minneman and Esbenshade, 1994). IP_3 can bind to its specific receptor on the endoplasmic reticulum and increase Ca^{2+} flux through the IP_3R channel (Berridge, 1993). In addition, the influx of extracellular Ca^{2+} may be through voltage-dependent and voltage-independent calcium channels (Minneman, 1988). Taken together, it is conceivable that two different signal transduction mechanisms through the α_1-ARs are involved in the increase in $[Ca^{2+}]_i$ mediated by NA.

It was also evident in this study that α_2- and β-ARs in the rat hippocampus were stimulated by NA application. Facilitation of the opening of VOCCs (voltage-operated Ca^{2+} channels) is suggested to be induced by activation of α_2-ARs in porcine uterine longitudinal muscle (Kitazawa et al., 2000) and by β-ARs in cultured rat astrocytes (MacVicar and Tse, 1988). In addition, it is reported that cyclic-AMP-dependent phosphorylation of IP_3 receptors increases $[Ca^{2+}]_i$ in response to the activation of α_1-ARs in lipid vesicles

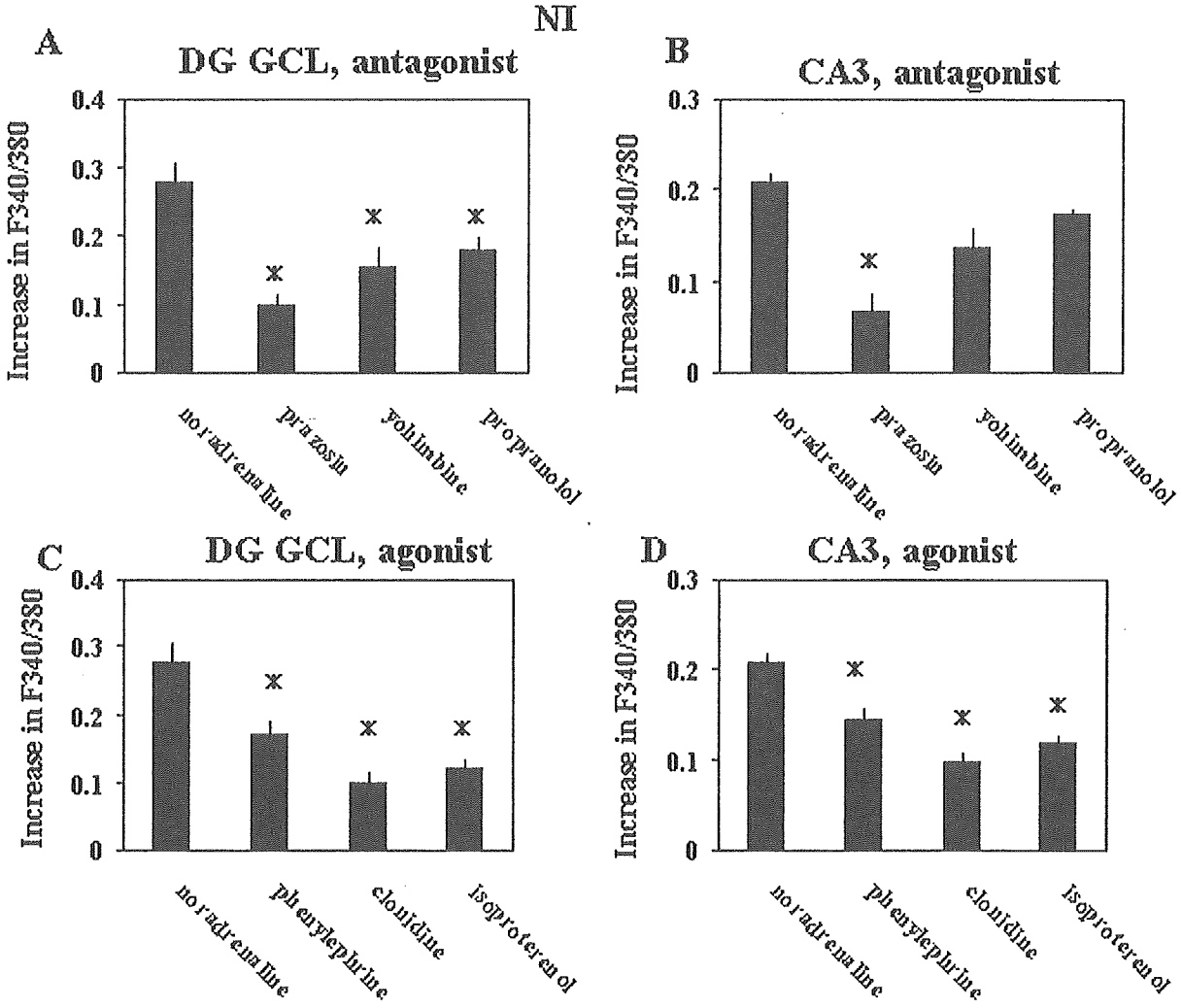

Fig. 7. Effects of adrenergic antagonists and agonists on the $[Ca^{2+}]_i$ responses in the DG granule cell (GCL) (**A,C**) and the CA3 pyramidal cell layers (CA3) (**B,D**) of NI-treated rats. The 100 μM NA-induced $[Ca^{2+}]_i$ increase was markedly reduced by 100 μM prazosin, and partially reduced by 100 μM yohimbine and 100 μM propranolol in each cell layer (**A,B**). 100 μM phenylephrine markedly increased $[Ca^{2+}]_i$ in both the DG granule cell (**C**) and CA3 pyramidal cell layers (**D**) while 100 μM clonidine and 100 μM isoproterenol had less of an effect (**C,D**). Note that the ratios of each agent to NA are similar to those shown in Figure 4. The results are the means ± SEM (n = 5).

from mouse cerebellum (Nakade et al., 1994). Thus, further study will be necessary to determine whether the $[Ca^{2+}]_i$ increase mediated by NA is derived from extracellular Ca^{2+} or from intracellular Ca^{2+} storage sites.

Effect of NI on $[Ca^{2+}]_i$

In the present study, we found that NA-stimulated $[Ca^{2+}]_i$ induction was downregulated by NI in both the DG granule cell and CA3 pyramidal cell layers. To clarify the difference between sham-treated and NI-treated rats in terms of NA-induced $[Ca^{2+}]_i$ movement, we characterized the NA-induced $[Ca^{2+}]_i$ increment in both rat groups. The α_1-, α_2-, and β-adrenergic receptor-mediated $[Ca^{2+}]_i$ increases were suppressed to a similar degree in both the NI- and sham-treated slices, so it is clear that NI did not suppress α_1-, α_2-, and β-adrenergic receptor pathways independently.

Possible mechanisms of $[Ca^{2+}]_i$ increment suppression via adrenaline receptor function

A couple of studies have shown that neonatal isolation did not alter the total number of cells in the dentate gyrus granule cell and the CA3 pyramidal cell layers (Huang et al., 2002; Huot et al., 2002), so the reduction in NA-stimulated $[Ca^{2+}]_i$ in the neonatal isolated hippocampi was not considered to be caused by a reduction in the total number of cells. It is possible that suppression of signal transduction pathways, such as Ca^{2+} influx through VOCCs or IP_3 receptors on the endoplasmic reticulum may be involved in the downregulation of NA-stimulated $[Ca^{2+}]_i$ induction in re-

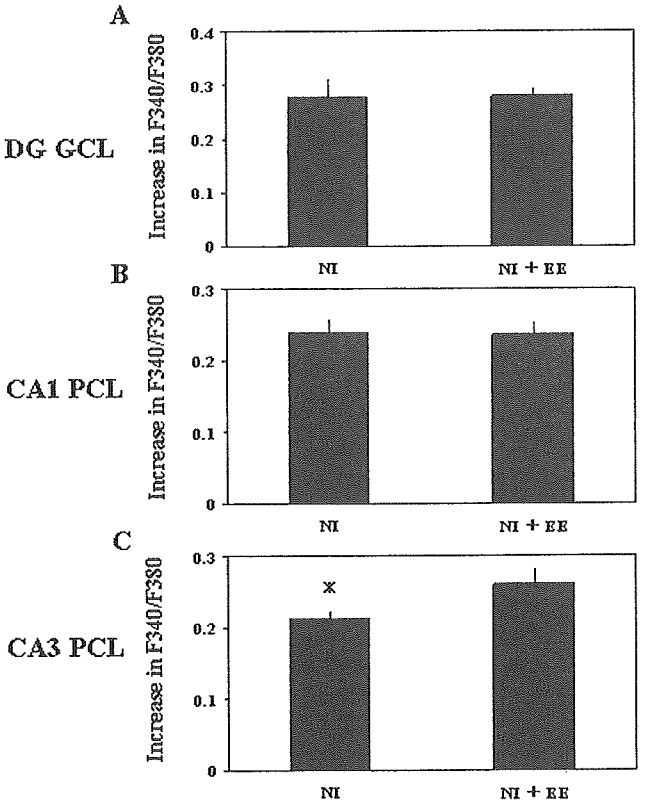

Fig. 8. Influence of EE on the downregulation of NA-stimulated $[Ca^{2+}]_i$ increment induced by NI. The downregulation in NA-stimulated elevation in $[Ca^{2+}]_i$ by NI was alleviated in response to EE in the CA3 pyramidal cell layer (**C**), but not in the DG granule cell layer (**A**). The NA-stimulated increase in $[Ca^{2+}]_i$ in the CA1 pyramidal cell layer was not affected by EE (**B**). The results represent the mean values and SEM (n = 6).

sponse to NI. Therefore, further studies examining the involvement of other pathways are required.

Significance of the decreased NA-induced $[Ca^{2+}]_i$ increment in NI-treated rat hippocampus

The decreased elevation in $[Ca^{2+}]_i$ might lead to a reduction in the phosphorylation of calcium/calmodulin-dependent protein kinase (CaMK) II and cAMP-responsive element binding protein (CREB), which are involved in the alteration of brain functions such as memory formation (Kida et al., 2002; Silva et al., 1992a,b). In addition, it is reported that NA promotes long-term potentiation in the rat hippocampus and plays an essential role in memory consolidation (Izumi and Zorumski, 1999; Kobayashi and Yasoshima, 2001). Taken together, it is plausible that the downregulation in NA-stimulated $[Ca^{2+}]_i$ induction in the hippocampus by NI may, at least in part, be involved in the impairment of memory function. In fact, Huot et al. (2002) reported that NI induced the impairment in spatial learning. On the other hand, it is well known that N-methyl-D-aspartate (NMDA) receptors regulate the levels of $[Ca^{2+}]_i$ essential for the induction of long-term potentiation (LTP). Therefore, it will be necessary to investigate whether NI alters $[Ca^{2+}]_i$ via NMDA receptors.

Effect of EE on the attenuated NA-induced increase in $[Ca^{2+}]_i$

Francis et al. (2002) demonstrated that EE alleviated the effects of maternal separation on both the HPA and the behavioral responses to stress. In this context, we also examined whether EE modulated the downregulation by NI of the NA-stimulated $[Ca^{2+}]_i$ increase in the CA3 pyramidal cell and DG granule cell layers. The downregulation in the NA-stimulated increase in $[Ca^{2+}]_i$ was alleviated by EE in the CA3 pyramidal cell layer, but not in the DG granule cell layer.

In published studies, the concentrations of antagonists used were typically less than 1 mM (Kobayashi et al., 1999; Okamoto et al., 1995). However, it is possible that the 100 μM antagonist concentrations used in the current study might have been insufficient to fully inhibit the ARs for the purpose of investigating the mechanisms of NA-induced $[Ca^{2+}]_i$ increases.

Effect of raising environment on the NA-induced $[Ca^{2+}]_i$ and the region-dependency of hippocampus

The NA-stimulated $[Ca^{2+}]_i$ increase was attenuated by NI in the DG granule cell and CA3 pyramidal cell layers. This downregulation was alleviated by EE in the CA3 pyramidal cell layer, but not in the DG granule cell layer. The influences of neonatal isolation and environmental enrichment are region-dependent. The mechanisms of the region-dependent influences might be associated with the different distributions of various types of cells, such as excitatory and inhibitory neurons and glial cells, since the activation of noradrenergic receptors also affects $[Ca^{2+}]_i$ mobilization in those cells. In order to elucidate a more precise mechanism, further studies are required.

In summary, whereas NA-stimulated $[Ca^{2+}]_i$ increment was downregulated by NI in both the DG granule cell and CA3 pyramidal cell layers, the administration of EE following NI alleviated the downregulation only in the CA3 pyramidal cell layer. These findings suggest that raising environments, such as NI and EE, may modulate hippocampal function by altering adrenergic receptor-mediated signal transduction during adolescence.

ACKNOWLEDGMENTS

The authors thank Drs. Y. Kudo and H. Miyakawa for technical support, and Drs. A. Kagaya, H. Jitsuiki, and K. Kurata for helpful advice.