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

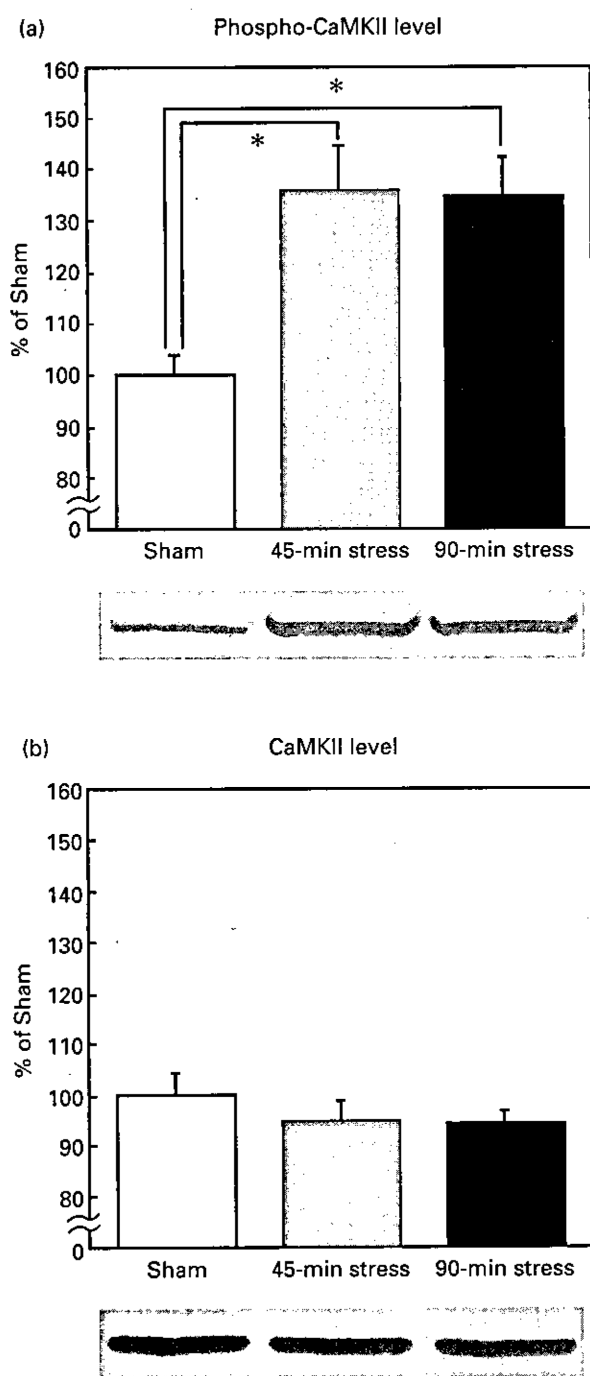

Figure 1. Influence of a single immobilization stress on the phospho-CaMKII and CaMKII immunoreactivity in the hippocampus, as determined by Western blot analysis. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to a single immobilization of either 45- or 90-min 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.

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).

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

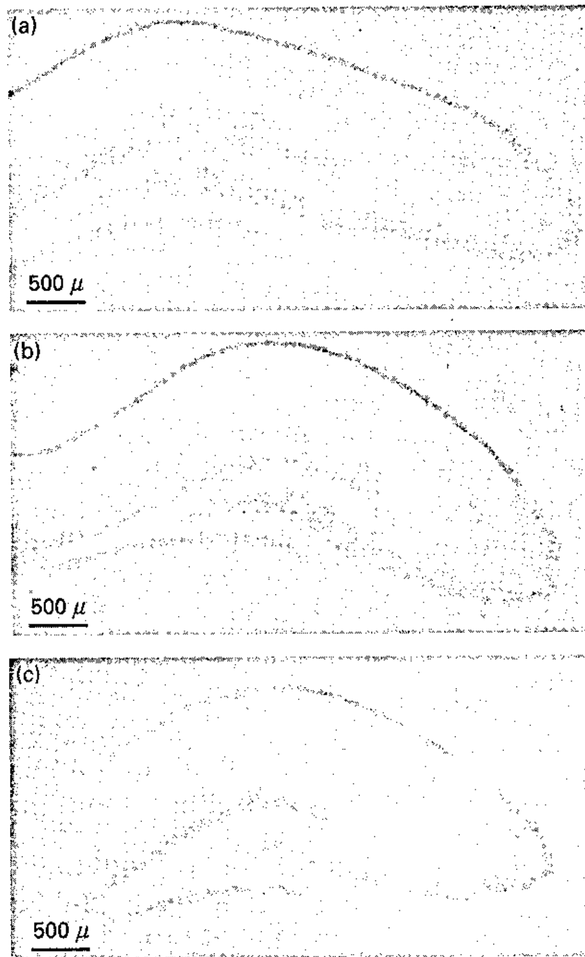

Figure 2. Influence of a single immobilization stress on the expression of phospho-CaMKII in the rat hippocampus as determined by immunohistochemical analysis. Representative immunohistochemical stains of phospho-CaMKII in the hippocampus are shown. Rats were intraperitoneally injected with either NBQX (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).

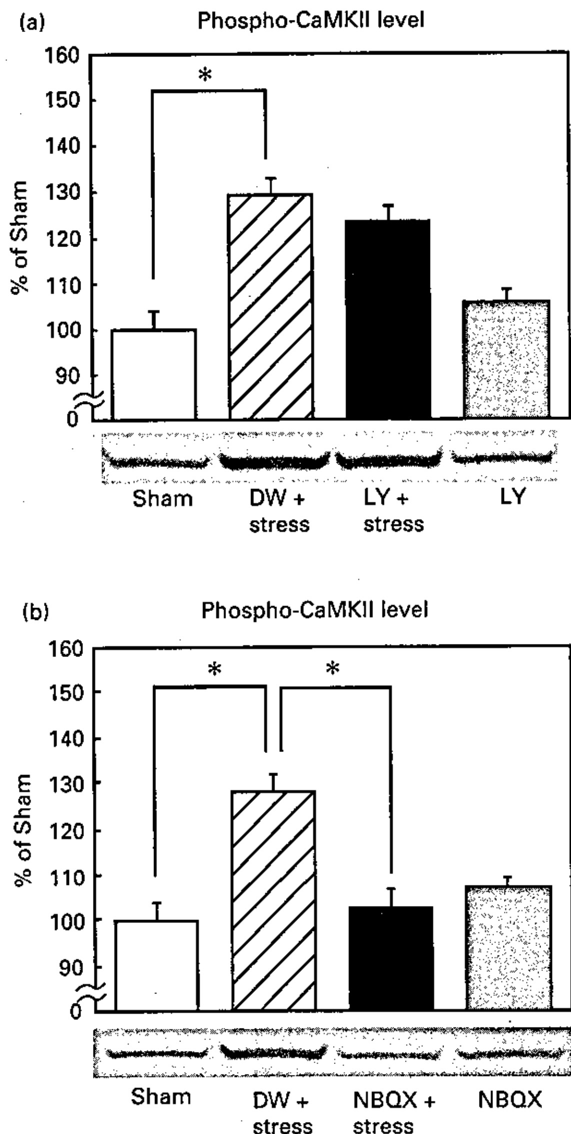

Figure 3. Effect of pre-treatment with LY235959 (5.0 mg/kg) and NBQX (11.95 mg/kg) on phospho-CaMKII levels following acute immobilization. Representative phospho-CaMKII Western blots are shown. (a) Rats were sacrificed immediately after the stress session. Sham, DW pre-treatment + non-stress condition; 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.

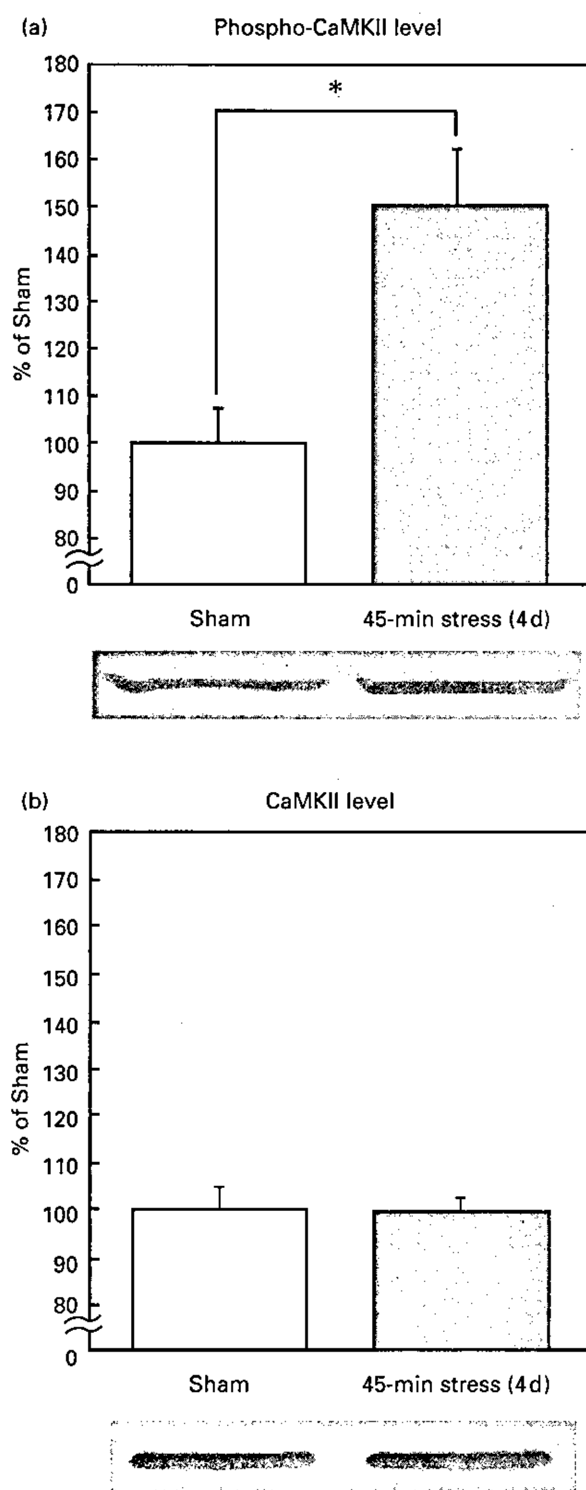

Figure 4. Effect of repeated immobilization stress for 4 days on phospho-CaMKII and CaMKII immunoreactivity as determined by Western blot analysis. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to 45- min immobilization stress per day for 4 consecutive days and 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 $\pm$ 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).

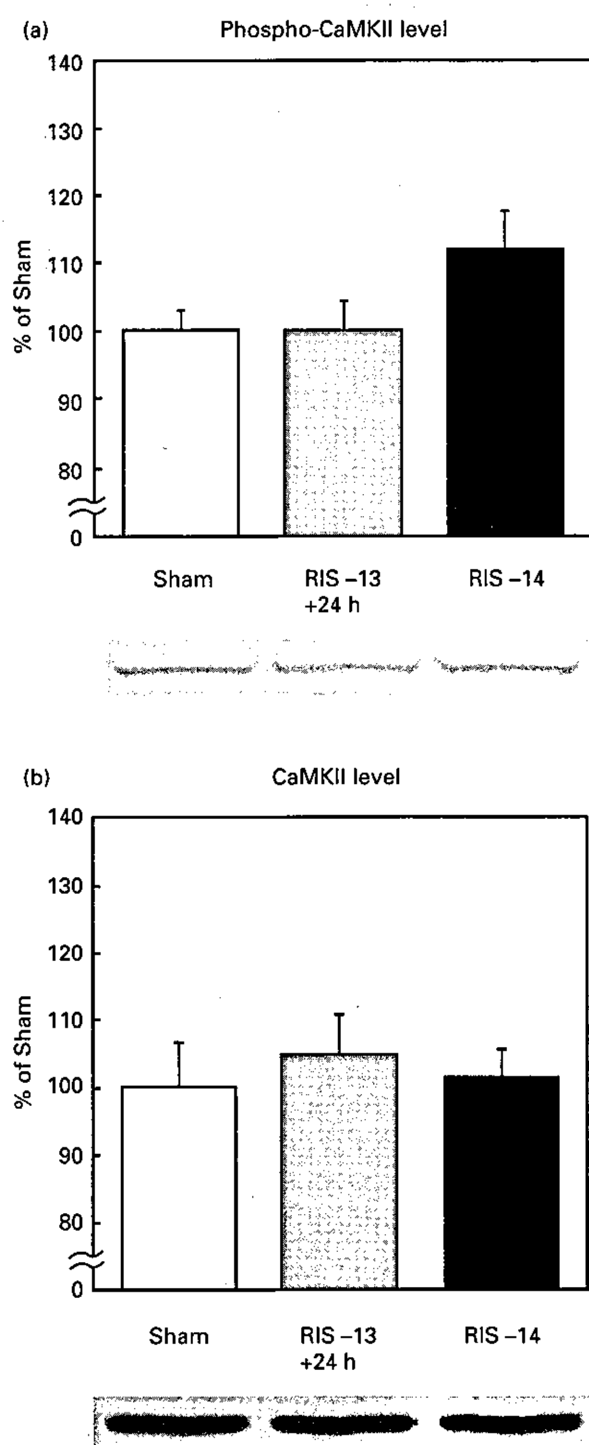

Figure 5. Influence of chronic immobilization stress on phospho-CaMKII and CaMKII immunoreactivity in the hippocampus. Representative immunoblots of phospho-CaMKII and CaMKII are shown. Rats were subjected to repeated immobilization stress (RIS) exposure of 90-min duration per day for 14 consecutive 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.

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Statement of Interest

None.

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REDUCTION IN LEVELS OF AMPHIPHYSIN 1 mRNA IN THE HIPPOCAMPUS OF AGED RATS SUBJECTED TO REPEATED VARIABLE STRESS

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Abstract—Various neurobiological studies of aging indicate that elevated levels of circulating glucocorticoids lead to hippocampal vulnerability to stress, though little is known about the molecular mechanism underlying stress vulnerability in the elderly. We have compared the gene expression profiles in the hippocampus of aged (20 months) and adult (3 months) rats in response to repeated variable stress (RVS) for 4 days, using a cDNA array technique and real-time quantitative PCR, to identify putative genes involved in the mechanism of stress vulnerability in the elderly. We found a significant decrease in the levels of amphiphysin 1 mRNA in aged rats subjected to RVS compared with treated and untreated adult rats or to untreated aged rats. Similarly, we found a significant decrease in hippocampal levels of amphiphysin 1 mRNA in aged rats subjected to RVS for 8 days, but not in those subjected to a single VS. These findings suggest that the decrease in the hippocampal levels of amphiphysin 1 mRNA in response to repeated stress may be involved in the stress vulnerability in the elderly, and may lead to the disturbance of learning and memory under stressful conditions in the elderly.
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Key words: G1/S-specific cyclin E, amphiphysin 2, stress vulnerability, aging, learning and memory, cDNA array.

The molecular mechanisms of the vulnerability to stress in later life are being elucidated by growing evidence that neurochemical, morphological, and functional changes are induced in the aged brain (Smith, 1996; McEwen, 2002). An age-related increase in circulating glucocorticoids, induced by enhanced activity of the hypothalamo–pituitary–adrenacortical (HPA) axis, has been suggested to be closely associated with hippocampal vulnerability to stress in the elderly (Siegel et al., 1989; Issa et al., 1990; Akil H et al., 1993; Lupien et al., 1994; Strokes, 1995; Herman et al., 2001). For example, Landfield and colleagues (1981) demonstrated that the number of hippocampal neurons in aged rats was significantly decreased compared with the number in young rats, and that elevated levels of circulating glucocorticoids in aged rats might be involved in this decrease. In addition, decreased neurogenesis was observed in the dentate gyrus of aged rats compared with adult rats (Kuhn et al., 1996), and increased activity of the HPA axis has been shown to lead to decreased neurogenesis in aged rats (Cameron and McKay, 1999).

Furthermore, it has been postulated that stress might accelerate age-related changes in the hippocampus. An increased loss of hippocampal neurons in response to sustained exposure to elevated glucocorticoids has been closely linked to memory impairments (Landfield et al., 1981). In healthy elderly people, stressful conditions have been reported to decrease declarative memory performance significantly (Lupien et al., 1997).

Although it is well known that the increased activity of the HPA axis plays an important role in hippocampal dysfunction in the elderly (Lupien et al., 1999), there are likely additional mechanisms by which stress accelerates hippocampal damage in the aged. For instance, increased activity of the mitogen-activated protein kinases, p38 and c-Jun N-terminal kinase, due to an increase in the level of interleukin-1β in the aged brain, has been linked to both apoptotic changes and age-dependent impairment in long-term potentiation in the hippocampus (McGahon et al., 1999; Martin et al., 2002). Taken together, these data suggest that a number of age-related changes in the brain, and the hippocampus in particular, may be associated with vulnerability to stress in later life and may underlie the specific symptoms of stress in the elderly, such as memory deficit. Therefore, examination of stress-induced changes in gene expression in the aged rat brain may help elucidate the molecular mechanism of stress vulnerability in the elderly.

In this context, we first examined changes in the profile of hippocampal gene expression in response to repeated variable stress in adult and aged rats using a cDNA array technique. We subsequently performed real-time quantitative PCR to identify genes specifically regulated by stress in aged rats.

EXPERIMENTAL PROCEDURES

Animals

Eight-week old male Sprague–Dawley rats were purchased from Japan Charles River (Yokohama, Japan). Rats were housed

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Abbreviations: ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPA, hypothalamo–pituitary–adrenacortical; LTD, long-term depression; RVS, repeated variable stress; SVS, single variable stress.

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(three rats per cage) at constant room temperature (23±2 °C) and humidity (60%) with a 12-h light/dark cycle (lights on 8:00 a.m.–8:00 p.m.). Food and water were provided *ad libitum*. Adult and aged rats were housed until the ages of 3 or 20 months, respectively.

Stress paradigm

Both aged (20 M) and adult (3 M) rats were subjected to single or repeated episodes of the variable stress paradigm. The variable stress paradigm applied in this study consisted of immobilization for 1 h, shaking at high speed for 1 h, and forced swimming for 30 min, each applied once (single variable stress; SVS), or once on each of 4 or 8 successive days (repeated variable stress; RVS). This variable stress paradigm was designed to reduce habituation of corticosterone in response to stress and to produce a larger effect on adrenal weight (Gannon and McEwen, 1990; Magarinos and McEwen, 1995). In each experiment, rats were killed immediately after the variable stress, and sections of hippocampus were quickly removed and stored at −70 °C. All animal procedures were performed in strict accordance with the Hiroshima University School of Medicine Animal Care Committee. Our institutional guidelines are in compliance with national and international laws and policies, and all efforts were made to minimize the number of animals used and their suffering.

cDNA expression array

Because it was likely that acute stress did not induce marked differences in hippocampal gene expression, we used a 4-day exposure to RVS when comparing the gene expression profiles of aged and adult rats. Total RNA from the hippocampus of five rats in each group subjected to RVS was isolated with the RNAqueous Phenol-free Total RNA Isolation Kit (Ambion, Austin, TX, USA) and pooled. After treatment with RNase-free DNase I (Takara, Kusatsu, Japan), 1 μg of the pooled total RNA was used as a template for the synthesis of ^{32}P-labeled cDNA probes. The cDNA probes were synthesized by reverse transcriptase in the presence of [α-^{32}P] dATP. The cDNA probes (2×10^6 cpm/μl) were denatured and then hybridized to an Atlas Rat Stress cDNA Expression Array (Clontech, Palo Alto, CA, USA), which contains 207 genes. Then, the arrays were exposed to a BAS-MS imaging plate (Fuji Film, Tokyo, Japan) for 2 h, and the levels of gene expression were quantified using a MacBAS2000 imaging analyzer (version 2.3; Fuji Film). We calculated the change in gene expression as a percentage of the expression observed in the adult RVS group, using three internal reference controls (tubulin α1, ornithine decarboxylase and cytoplasmic β-actin) recommended by the manufacturer for normalization, to ensure the comparability of the aged RVS and adult RVS samples.

Real-time quantitative PCR

Total RNA was isolated from the hippocampus with the RNAqueous Phenol-free Total RNA Isolation Kit (Ambion). After treatment with RNase-free DNase I (Takara), real-time quantitative PCR was performed with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) to quantify the relative levels of mRNA in the samples. Real-time quantitative PCR was performed to amplify amphiphysin 1 and G1/S-specific cyclin E. The following primers and TaqMan hybridization probes were designed using Primer Express software (PE Applied Biosystems): amphiphysin 1 forward primer, 5′-CTGGGCCAGTTT CCTGACAT-3′; amphiphysin 1 reverse primer, 5′-TGTCGTAAT CCACGAGCTTCC-3′; amphiphysin 1 probe, 5′-AAGAATCGC ATCGCTAAGCGCAGC-3′; G1/S-specific cyclin E forward primer, 5′-TTGCTGGCCTCTGCTGG-3′; G1/S-specific cyclin E reverse primer, 5′-CTCACGACCACTCGCCGT-3′; G1/S-specific cyclin E probe, 5′-TGTCGCAGGGTTGCTGTTGATAGGG-3′. The TaqMan probes, which were designed to hybridize to the PCR products, were labeled 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. Each plate contained the same standard. 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 (heating at 95 °C for 15 s and 60 °C for 1 min) were performed. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the TaqMan Rodent GAPDH Control Reagents kit (PE Applied Biosystems). The PCR assay of unknown samples was performed simultaneously with standard samples (rat brain tissue) to construct a standard curve. The relative concentrations of GAPDH and amphiphysin 1 or G1/S-specific cyclin E in unknown samples were calculated from this standard curve, and we calculated the ratio of the relative concentrations of amphiphysin 1 or G1/S-specific cyclin E (already normalized by GAPDH expression) to the relative concentration of GAPDH.

Statistical analysis

Results were expressed as mean±S.E.M. and subjected to statistical analysis. The results of experiments containing two groups of rats were subjected to unpaired Student's *t*-test. Statistical analysis between four groups was determined by two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Significance was set at $P<0.05$.

RESULTS

Analysis of gene expression profiles in the hippocampus of aged rats subjected to RVS

To elucidate the influence of aging on changes in hippocampal gene expression induced by 4 days of RVS, we used the Atlas Rat Stress cDNA Expression Array (Clontech; Fig. 1). Examination of the gene expression profiles following episodes of RVS in aged and adult rats revealed genes that were up-regulated >3.0-fold or down-regulated <0.3-fold in aged rats relative to adult rats. Based on this analysis, we found 25 genes that were up-regulated by at least 3.0-fold and 15 genes that were down-regulated by at least 0.3-fold in aged rats subjected to RVS (Table 1). However, since the signals observed for many of the mRNAs detected by the arrays were close to the background signal, we focused on genes whose expression was markedly higher than the background signal. We identified two genes (encoding amphiphysin1 and G1/S-specific cyclin E) whose expression significantly differed between these two groups. In contrast, the ratio of the levels of amphiphysin 2, another isoform of amphiphysin, between aged and adult rats subjected to RVS was 1.13 (aged/adult). Because of the low sensitivity of these cDNA expression arrays, we performed real-time quantitative PCR analysis to confirm these differences in expression.

The influence of RVS on the levels of amphiphysin1 and G1/S-specific cyclin E mRNA in the hippocampus of aged and adult rats

We measured the levels of amphiphysin 1 mRNA in the hippocampus of four groups of rats (adult rats without stress, aged rats without stress, adult rats subjected to RVS, and aged rats subjected to RVS) using real-time

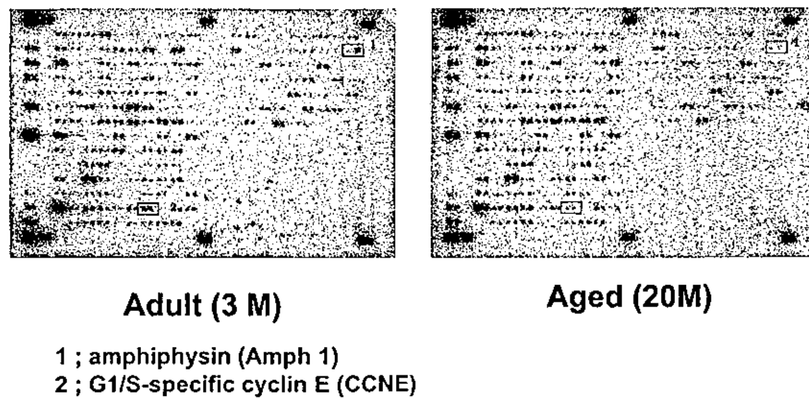

Fig. 1. Hippocampal gene expression profiles determined by Atlas Rat Stress cDNA Expression Array. Total RNA from the hippocampus of five rats in each group subjected to RVS for 4 days was isolated with the RNAqueous Phenol-free Total RNA Isolation Kit (Ambion), and pooled. The cDNA probes synthesized from the pooled total RNA by reverse transcriptase, were hybridized to the Atlas Rat Stress cDNA Expression Array (Clontech), and the arrays were exposed to Kodak films as described in the Materials and Methods. Left: Gene expression profile in adult rats subjected to RVS (n=5). Right: Gene expression profile in aged rats subjected to RVS (n=5). 1, Amphiphysin 1; 2, G1/S-specific cyclin E.

quantitative PCR. Statistical analysis revealed a significant interaction of age and RVS (F=3.7; df=3,26; P=0.02; Fig. 2). Post hoc analysis revealed that there was a significant decrease in the levels of hippocampal amphiphysin 1 mRNA in aged rats subjected to RVS when compared with any of the other test groups (Fig. 2). In addition, the hippocampal levels of G1/S-specific cyclin E mRNA were determined in adult and aged rats subjected to RVS. Statistical analysis revealed no significant difference in the levels of G1/S-specific cyclin E mRNA between these two groups (Fig. 3).

The influence of a single cycle of variable stress on the hippocampal levels of amphiphysin1 mRNA in aged and adult rats

We next examined whether a single exposure to variable stress could also produce differences in the levels of amphiphysin 1 mRNA between aged and adult rats. There was no significant difference in the levels of amphiphysin 1 mRNA between these two groups (Fig. 4).

The influence of long-term variable stress on the levels of amphiphysin1 mRNA in the hippocampus of aged and adult rats

We also examined whether long-term exposure to variable stress could produce differences in the levels of amphiphysin 1 mRNA between aged and adult rats. Both groups of rats were subjected to variable stress for 8 consecutive days. The hippocampal levels of amphiphysin 1 mRNA in aged rats subjected to CVS for 8 days were significantly lower than those in adult rats subjected to CVS for the same period (Fig. 5).

DISCUSSION

In this study, we examined the effects of aging on RVS-induced gene expression in the rat hippocampus, with the aim of elucidating the molecular mechanism underlying stress vulnerability in the elderly. We found that the levels of amphiphysin 1, but not G1/S-specific cyclin E, were significantly decreased in aged rats exposed to RVS for 4 days compared with similarly treated adult rats. There was no significant difference in the levels of amphiphysin 1 mRNA between aged and adult rats in the absence of stress treatment.

The amphiphysin protein is known to be associated with synaptic vesicles (Lichte et al., 1992). Two isoforms of amphiphysin (amphiphysin 1 and 2) are expressed in mammals and amphiphysin 1 is primarily expressed in the brain (De Camilli et al., 1993; Butler et al., 1997). Recent studies have demonstrated that amphiphysin is linked to synaptic vesicle endocytosis and recycling through interactions with dynamine, AP-2, and clathrin (Di Paolo et al., 2002; Shupliakov et al., 1997; Wigge and McMahon, 1998).

The release of neurotransmitters by synaptic vesicle exocytosis, as well as the recycling of synaptic vesicles from the plasma membrane, plays an important role in the regulation of higher brain function. In this context, it is likely that the significant decrease in the levels of amphiphysin 1 in the hippocampus of aged rats subjected to RVS may lead to some impairment in cognitive functions controlled by the hippocampus, such as learning and memory. Interestingly, Di Paolo and his associates (2002) have recently demonstrated that amphiphysin 1-knockout mice exhibit decreased efficiency of synaptic vesicle recycling and impairment in spatial learning. Based on these findings, it is plausible that aged rats subjected to RVS might have some dysfunction of learning and memory. However, since the marked decrease in locomotor activity in response to RVS (4 d) was found in adult rats [locomotor activity was measured for 30 min immediately after the last VS by SCANET MV-10 (Toyo Industry, Toyama, Japan); n=6; sham: 3502±606 count/30 min; RVS: 2723±253 count/30 min; unpublished observations], it is conceivable that an administration of RVS may prevent a precise evaluation of cognitive dysfunction in aged rats.

Table 1. Hippocampal genes whose mRNA expression was markedly changed by RVS for 4 days in aged rats in comparison with those in adult rats as determined by cDNA expression assay[a]

Gene	Adult (PSL/mm^2)	Aged (PSL/mm^2)	Adult/aged
Genes upregulated in aged rats			
CYP7	0.07	4.59	65.53
PAP3	0.03	1.05	35.03
MAO-A	0.21	4.55	21.69
Adrenodoxin precursor	0.19	4.05	21.33
Thromboxane-A synthase	0.33	5.10	15.47
FMO4	0.3	4.22	14.07
Nuclear factor IV	0.32	4.44	13.87
HMG2	0.87	6.87	7.90
GSTM2	0.77	5.71	7.41
GST YA	0.81	5.04	6.22
Cu-Zn SOD3	0.5	3.00	6.00
RPA	0.57	3.20	5.62
p53 Nuclear oncoprotein	0.78	4.09	5.24
Epoxide hydratase	1.22	6.39	5.23
RecA-like protein HsRad51	1.14	5.77	5.06
MRP1	0.71	3.19	4.49
FMO1	1.26	5.59	4.44
Multidrug resistance protein	1.5	6.36	3.54
MGST1	1.79	6.34	4.24
Rhodanase	1.69	3.46	3.42
LCAD	2.48	7.97	3.22
Selenoprotein	0.91	2.92	3.21
GST5-5	2.2	7.01	3.18
Genes downregulated in aged rats			
CCNE	30.7	6.81	4.51
Amphiphysin 1	26.47	7.49	3.53

[a] Total RNA was isolated from the hippocampus of five aged and five adult rats subjected to RVS, and subjected to the Atlas Rat Stress cDNA Expression Array (Clontech) followed by analyses with the MacBAS imaging analyzer (Fuji Film). Three internal controls (tubulin α1, ornithine decarboxylase, cytoplasmic β-actin) recommended by the manufacturer were used. The results were obtained from a single array determination. Since the PSL values of three adult hippocampal genes in the group of genes upregulated in aged rats as well as 12 aged hippocampal genes in the group of genes downregulated in aged rats were below zero, these genes were eliminated in Table 1. CCNE, G1/S-specific cyclin E; Cu-Zn SOD3, copper–zinc-containing superoxide dismutase 3 precusor; CYP7, cytochrome P450 VII; FMO1, hepatic flavin-containing monooxygenase 1; FMO4, hepatic flavin-containing monooxygenase 4; GST5-5, glutathione-S-transferase subunit 5 θ; GSTM2, glutathione-S-transferase Yb subunit; GST YA, glutathione-S-transferase Ya subunit; HMG2, high mobility group protein 2; LCAD, long chain-specific acyl-CoA dehydrogenase precusor; MAOA, flavin-containing monoamine A; MGST1, microsomal glutathione S-transferase; MRP1, mismatch repair protein 1; PAP3, pancreatitis-associated protein 3 precusor; PSL, photo-stimulated luminescence; RPA, replication protein A 32-kDa subunit.

In addition, Wang and Linden (2000) have demonstrated that the induction of cerebellar long-term depression (LTD), considered a model of learning and memory, is closely associated with postsynaptic clathrin-mediated endocytosis, at least in part, mediated by amphiphysin. Amphiphysin 2 has also been reported to play a role in the recycling of synaptic vesicles in the context of a stable heterodimer with amphiphysin 1 (Wigge et al., 1997). The lack of amphiphysin 1 expression by knockout mice markedly decreases the stability of amphiphysin 2 without affecting the expression of its mRNA (Di Paolo et al., 2002). Using the technique of differential mRNA display, Napolitano and colleagues (1999) demonstrated that increased levels of amphiphysin two in the rat striatum were involved in the induction of striatal LTD. These findings indirectly support the hypothesis that a reduction in amphiphysin 1 might impair learning and memory.

It has been postulated that stress can accelerate disorders of cognitive dysfunction, such as the memory impairment, in the elderly. Long-term administration of low-intensity foot shock significantly damaged hippocampal neurons in aged compared with adult or young rats (Kerr et al., 1991). Age-related gonadal hypofunction accelerated the damage to CA3 and CA4 neurons in response to the stress of immobilization or swim in rats (Mizoguchi et al.,

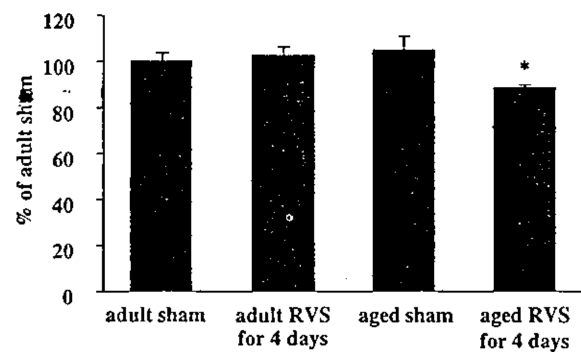

Fig. 2. Expression of amphiphysin 1 mRNA in the hippocampus of adult rats with sham treatment (adult sham), adult rats subjected to RVS for 4 days (adult RVS), aged rats with sham treatment (aged sham), and aged rats subjected to RVS for 4 days (aged RVS). The level of amphiphysin 1 mRNA was determined by real-time quantitative PCR as described in the Experimental Procedures. Results are expressed as the percentage of adult sham. The mean±S.E.M. (n=6–9 per group) is shown. Post hoc analysis revealed that there was a significant decrease in the hippocampal levels of amphiphysin 1 mRNA in aged rats subjected to RVS in comparison with any of the remaining groups (* $P<0.05$, two-way ANOVA followed by the Student-Newman-Keuls test).

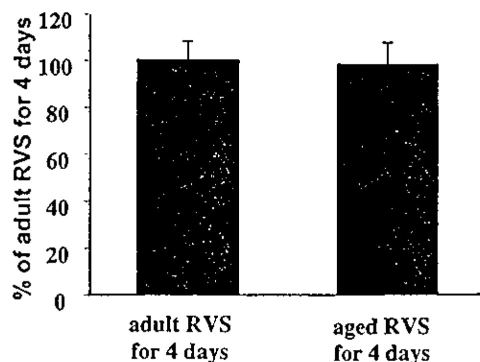

Fig. 3. Expression of G1/S-specific cyclin E mRNA in the hippocampus of adult and aged rats subjected to RVS for 4 days. The level of G1/S-specifc cyclin E mRNA was determined by real-time quantitative PCR as described in the Experimental Procedures. Results are expressed as the percentage of adult rats subjected to RVS. The group of aged RVS and adult RVS contained six and eight rats, respectively.

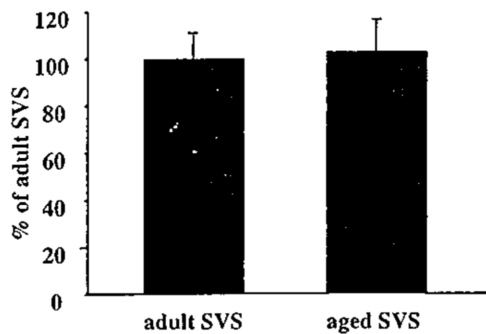

Fig. 4. Expression of amphiphysin 1 mRNA in the hippocampus of adult and aged rats subjected to SVS. The level of amphiphysin 1 mRNA was determined by real-time quantitative PCR as described in the Experimental Procedures. Results are expressed as the percentage of adult rats subjected to SVS. Each group contained eight rats.

1992). A brief exposure to stress (public speaking task) has been reported to decrease declarative memory performance significantly in healthy elderly people, and it has been suggested that chronic exposure to stress might be more predictive of declarative memory deficits (Lupien et al., 1997). We have found a significant decrease in levels of amphiphysin 1 in the hippocampus of aged rats subjected to RVS, suggesting that the down-regulation of amphiphysin 1 in response to stress in the elderly might be involved in the pathogenesis of stress-induced memory impairments. However, since the decrease in amphiphysin 1 mRNA in the hippocampus of aged rats is relatively small, immunoblot analysis will be necessary to determine whether these changes lead to a reduction in levels of the amphiphysin protein. In addition, further experiments are required to determine whether such a relatively small reduction in amphiphysin 1 can influence learning and memory.

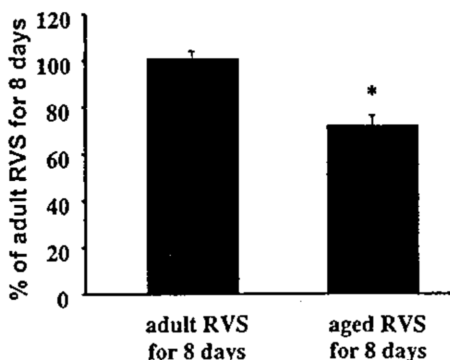

Fig. 5. Expression of amphiphysin 1 mRNA in the hippocampus of adult and aged rats subjected to RVS for 8 days. The level of amphiphysin 1 mRNA was determined by real-time quantitative PCR as described in the Experimental Procedures. The levels of the hippocampal mRNA in the aged rats subjected to RVS (8 day) were significantly lower than those in the adult rats subjected to RVS (8 d; * $P<0.05$, unpaired Student's t-test). Results are expressed as the percentage of adult rats subjected to RVS (8 d). Each group contained six rats.

CONCLUSION

We found a significant decrease in the levels of amphiphysin 1 mRNA in the hippocampus of aged rats subjected to RVS compared with adult rats subjected to RVS. This finding suggests that the RVS-induced decrease in the hippocampal levels of amphiphysin 1 in aged rats may be, at least in part, associated with stress-related memory impairment in the elderly. These results may promote our understanding of the neurobiological mechanism of stress vulnerability in the elderly.

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

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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.** © 2004 Wiley-Liss, Inc.

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

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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

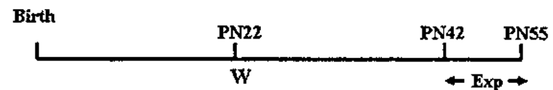
Animals

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).

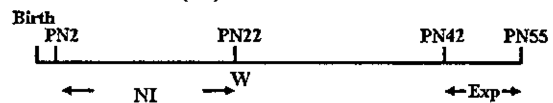
Preweaning housing conditions

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.

A. Sham-treatment



B. Neonatal isolation (NI)



C. Neonatal isolation + Environmental enrichment (EE)

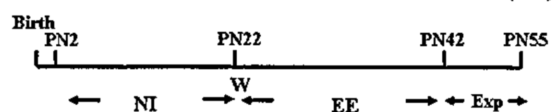


Fig. 1. Animal treatment paradigms. A: Sham-treated rats were left entirely undisturbed and decapitated on postnatal (PN) Days 42–55. B: Pups were isolated from the dam, nest, and siblings for a period of 1 h per day on PN Days 2–22 and decapitated on PN Days 42–55. C: Pups were isolated on PN Days 2–22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

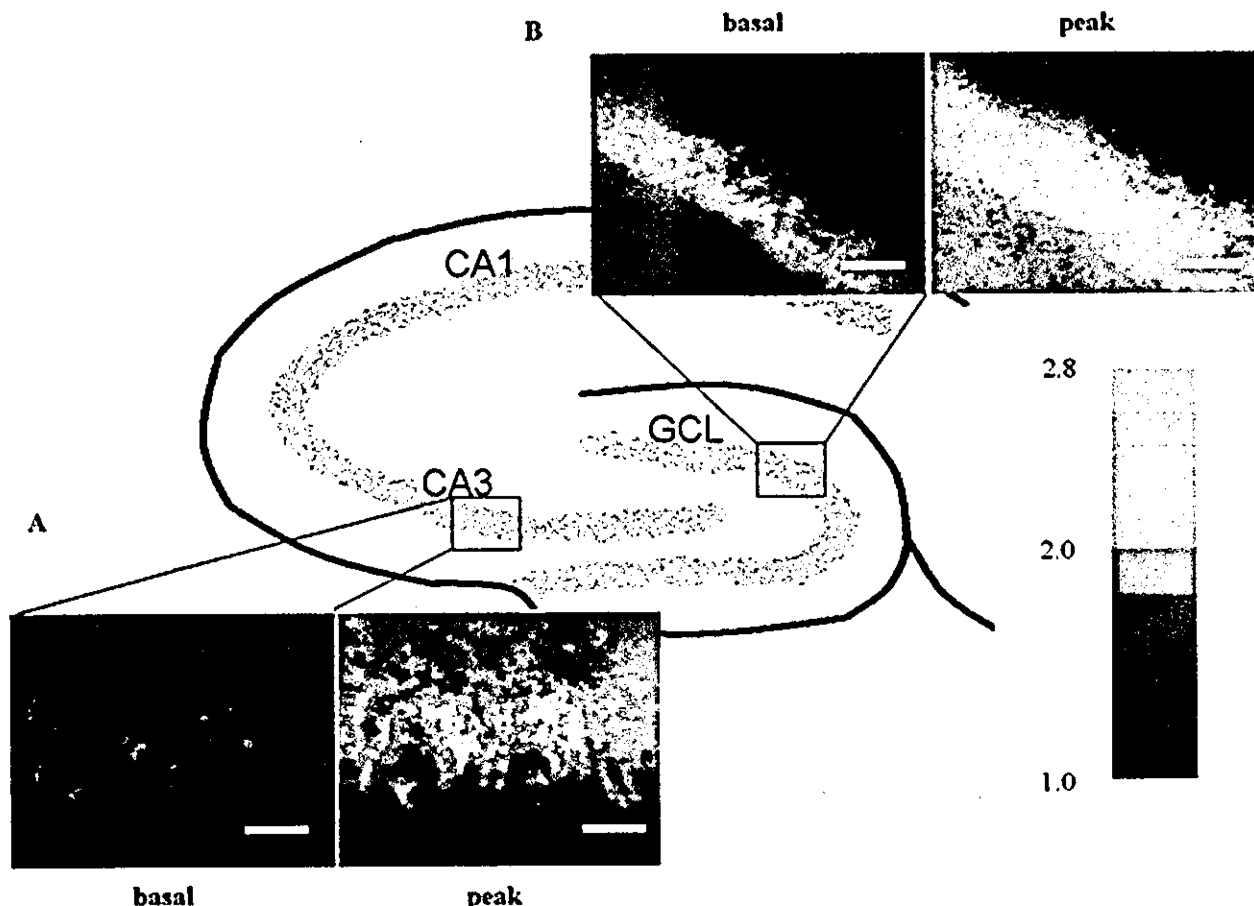


Fig. 2. A schematic drawing of a rat hippocampal slice. Changes in $[Ca^{2+}]_i$ in the stratum granulare in the dentate gyrus (DG) and the stratum pyramidale in the CA3 field were evaluated. The F_{340}/F_{380} ratio is represented by pseudocolor coding. **A:** Pseudocolor coding of the basal $[Ca^{2+}]_i$ 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-

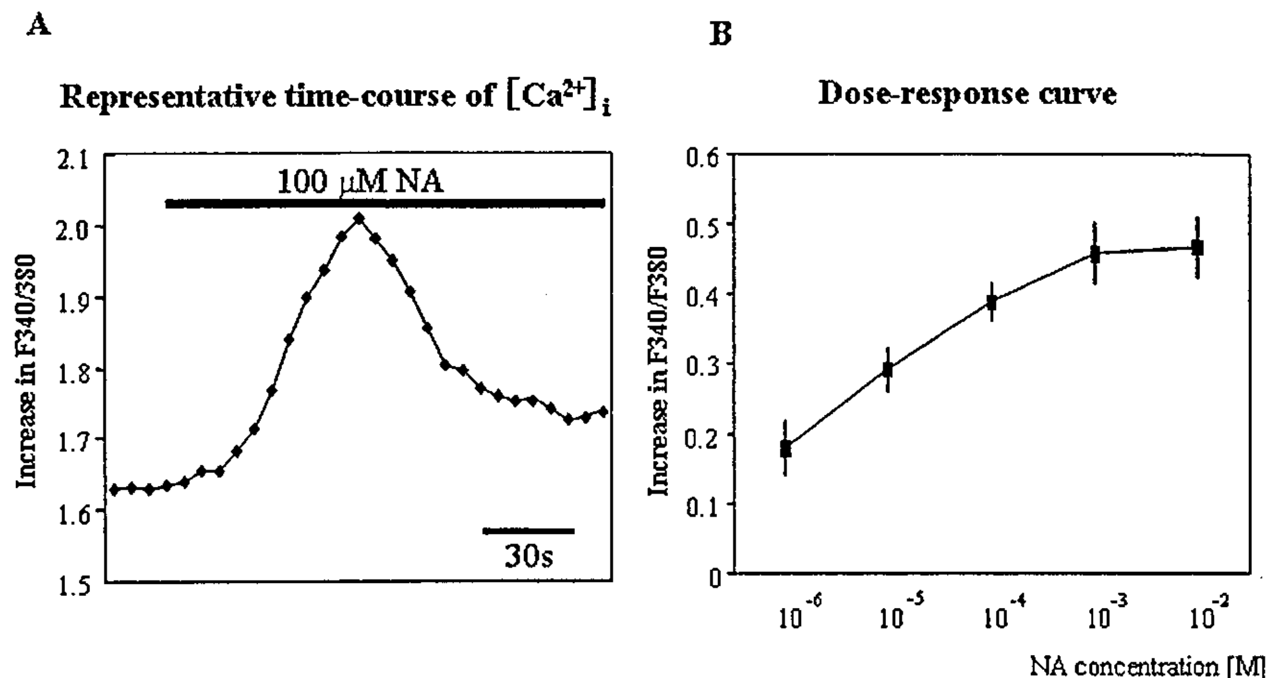


Fig. 3. A: The time-course of the $[Ca^{2+}]_i$ response to the application of 100 μM NA in the DG granule cell layer. B: The effects of various concentrations of NA on $[Ca^{2+}]_i$ in the DG granule cell layer. The $[Ca^{2+}]_i$ increases induced by bath application of NA were observed to change in 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

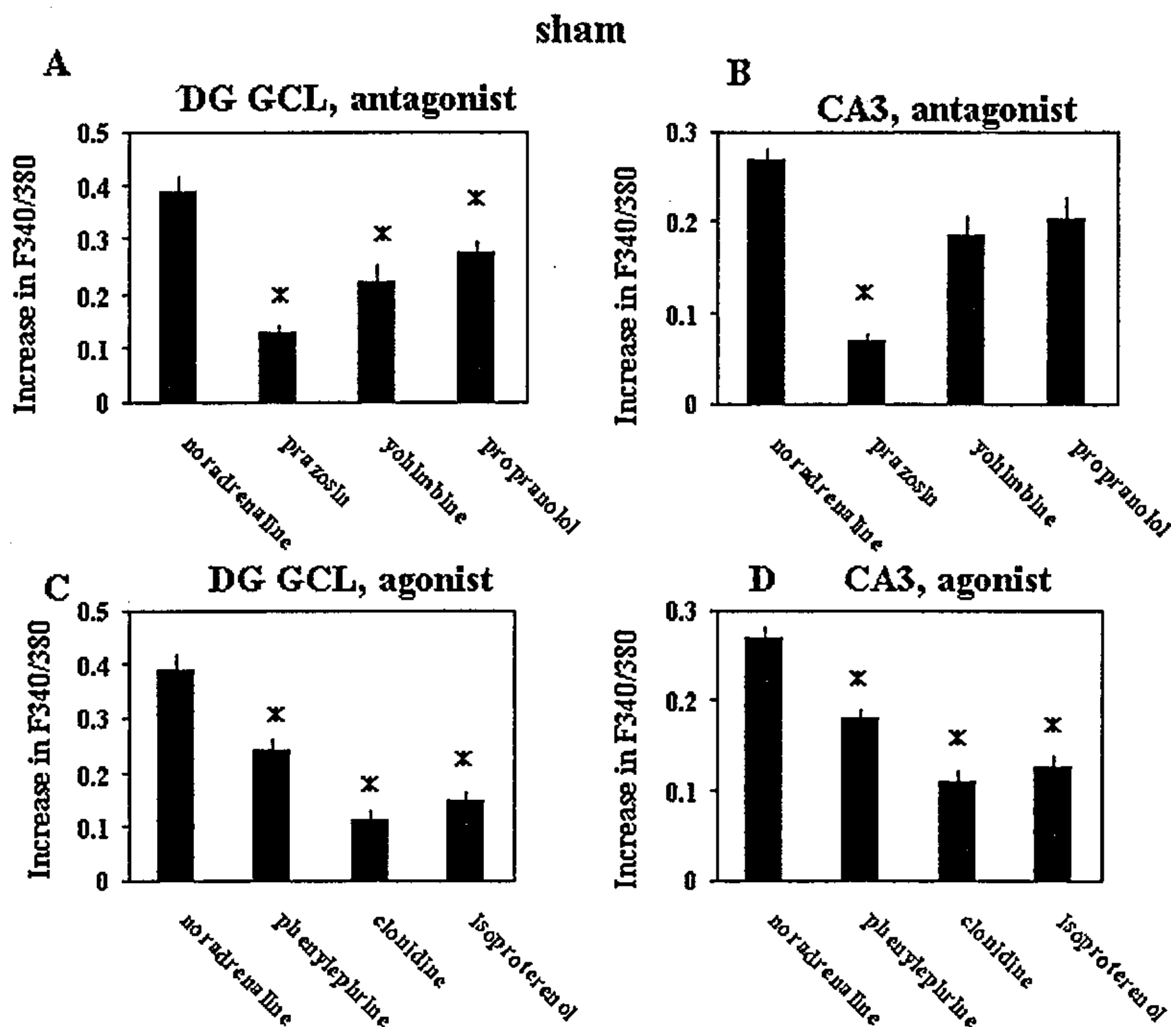


Fig. 4. Effects of AR antagonists and agonists on the $[Ca^{2+}]_i$ response in the DG granule cell (GCL) (**A**,**C**) and the CA3 pyramidal cell layers (CA3) (**B**,**D**) of sham-treated rats. The 100 μM NA-induced $[Ca^{2+}]_i$ increase was markedly reduced by 100 μM of prazosin, and partially reduced 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.

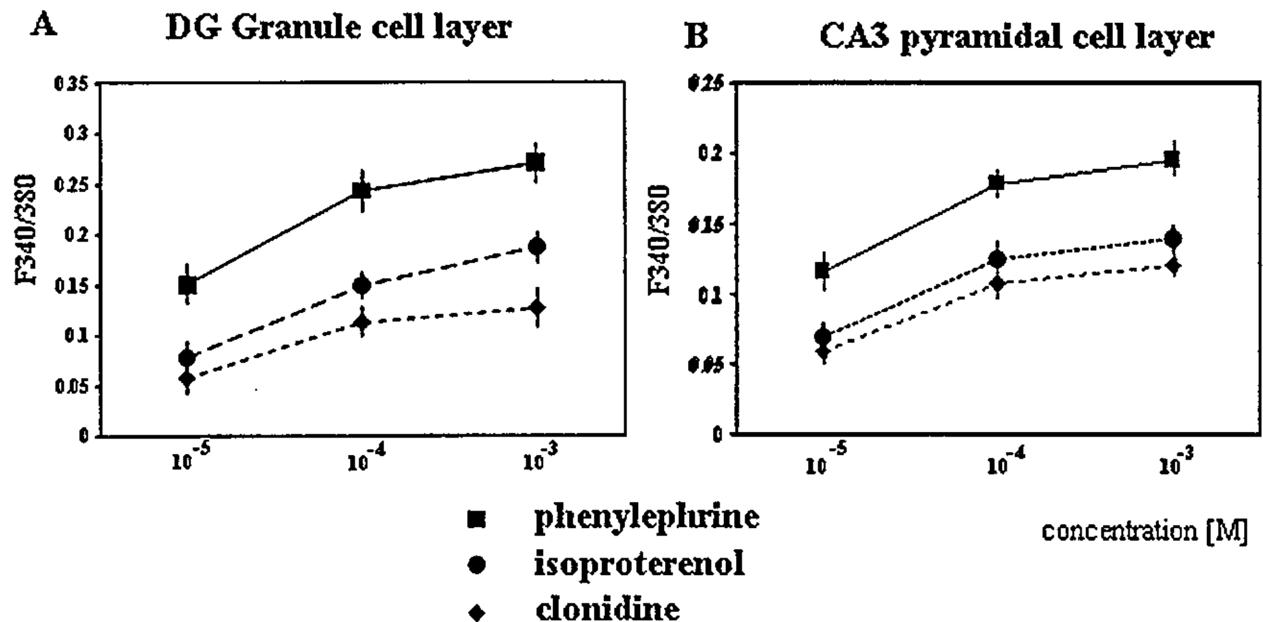


Fig. 5. Dose-dependent curves of the [Ca^{2+}]$_i$ responses to the application of various adrenergic agonists in the DG granule cell layer (A) and CA3 pyramidal cell layer (B). Phenylephrine, an α_1-AR agonist, markedly increased [Ca^{2+}]$_i$ in both the DG granule cell (C) and the CA3 pyramidal cell 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