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Enrichment enhances the expression of *sgk*, a glucocorticoid-induced gene, and facilitates spatial learning through glutamate AMPA receptor mediation

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Abstract

We have previously demonstrated that the serum and glucocorticoid-inducible kinase (*sgk*) gene plays a causal role in facilitating memory performance in rats. Environment enrichment is known to facilitate spatial learning. We therefore examined the effect of enrichment on *sgk* expression. We also examined the role of *sgk* in spatial and nonspatial learning and the regulation of *sgk* expression by activation of different glutamate receptors. Both real-time polymerase chain reaction and Western blot analyses revealed that enrichment training preferentially increased *sgk* mRNA and protein levels in the hippocampus. Transfection of *sgk* mutant DNA to the hippocampal CA1 area markedly impaired spatial learning, fear-conditioning learning and novel object-recognition learning in rats, but enrichment training effectively reversed these learning deficits. Meanwhile, S422A mutant DNA transfection prevented enrichment-induced spatial learning facilitation. In studying glutamate receptor regulation of *sgk* expression, we found that blockade of *N*-methyl-D-aspartate (NMDA) receptors in general, and the NR2B subunit in particular both effectively blocked enrichment-induced spatial learning facilitation, but they did not block enrichment-induced *sgk* expression. Upon various glutamate agonist infusions, only α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) increased *sgk* mRNA levels significantly in the hippocampus. Furthermore, blockade of AMPA receptors effectively blocked both enrichment-induced spatial learning facilitation and *sgk* expression. These results indicate that there is a dissociation between NMDA receptor activation and *sgk* expression. Enrichment enhanced spatial learning through both NMDA and AMPA receptor activation, whereas enrichment-induced *sgk* expression is specifically mediated through AMPA receptors. These results suggest that *sgk* could serve as a novel molecular mechanism, in addition to the NMDA receptor NR2B, underlying enrichment-induced learning facilitation.

Introduction

The development of an animal's behaviour is regulated by both genetic and environmental factors. Several studies have revealed that animals reared in an enriched environment show improved learning and memory performance in a variety of behavioural tasks, including spatial learning and memory (Kempermann *et al*., 1997; Nilsson *et al*., 1999), recognition memory (Rampon *et al*., 2000a) and memory for fear-conditioning learning (Rampon *et al*., 2000a; Duffy *et al*., 2001). In parallel with these findings, enriched experience is shown to facilitate long-term potentiation (LTP), a synaptic model for long-term memory, in the hippocampus (van Praag *et al*., 1999; Duffy *et al*., 2001). In addition, enrichment also increases synaptic strength (Foster *et al*., 1996), synaptic transmission (Green & Greenough, 1986) and the synaptic density (Rampon *et al*., 2000a) of hippocampal neurons. These results together suggest that environmental enrichment enhances synaptic plasticity in the hippocampus.

Some neurochemical and molecular changes associated with environmental enrichment have been reported. For example, the expression of nerve growth factors (Torasdotter *et al*., 1998) and neurotrophins (Young *et al*., 1999; Ickes *et al*., 2000) were increased in animals exposed to an enriched environment. Enrichment also increases glucocorticoid receptor (GR) mRNA expression (Olsson *et al*., 1994) and cAMP-responsive element binding protein (CREB) phosphorylation (Bevilaqua *et al*., 1999; Young *et al*., 1999) in the hippocampus. Although glucocorticoids, CREB, and some neurotrophins, such as brain-derived neurotrophic factor, play important roles in memory consolidation (Ma *et al*., 1998; Silva *et al*., 1998; Roozendaal, 2000), it is not known whether they also serve as the molecular mechanisms of enrichment-induced cognitive changes. By contrast, transgenic mice with *N*-methyl-D-aspartate receptor (NMDAR) NR2B subunit overexpression show better learning and memory performance (Tang *et al*., 1999). Enrichment also increases NR2B protein levels in mouse forebrain, and it prevents further improvement in certain learning tasks in NR2B transgenic mice (Tang *et al*., 2001). These results suggest that the NMDAR NR2B subunit could serve as a molecular mechanism of enrichment-induced learning and memory facilitation.

Glucocorticoids are known to facilitate memory performance in a variety of learning tasks in different animals (Sandi, 1998; Roozendaal, 2000). This effect is suggested to be mediated through a glucocorticoid receptor (GR)-signalling pathway because impairment in spatial learning and memory was observed in GR-deficient mice (Oitzl *et al*., 1997). In addition, transgenic mice bearing the GR antisense construct show impairment in both water maze and radial arm maze learning tasks (Rousse *et al*., 1997). In a recent study, we have further demonstrated that the serum and glucocorticoid-inducible kinase (*sgk*) gene plays an important role in memory consolidation of spatial learning in rats. Transfection of the *sgk* wild-type DNA

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enhances, whereas transfection of the *sgk* mutant DNA impairs, water maze performance in rats (Tsai *et al*., 2002). In the present study, we examined whether *sgk* serves as another molecular mechanism, in addition to NR2B, underlying enrichment-induced learning facilitation. We also examined the role of *sgk* involved in spatial and nonspatial learning and the regulation of *sgk* expression by different glutamate receptor activation.

Materials and methods

Animals

Adult (2–3 months old) and aged (12 months old) male Sprague–Dawley rats bred at the Animal Facility of the Institute of Biomedical Sciences (IBMS), Academia Sinica, Taiwan, were used. Animals were housed in a room maintained on a 12 : 12 h light : darkness cycle (light on at 06.00 h) with food and water available continuously. Experimental procedures followed the Guidelines of Animal Use and Care of IBMS, Academia Sinica. For plasmid DNA and drug infusions, animals were anaesthetized with pentobarbital (40 mg/kg, i.p.) and subjected to stereotaxic surgery. Two 23-gauge, stainless-steel, thin-wall cannulae were implanted bilaterally to hippocampal CA1 area at the following coordinates: 3.5 mm posterior to the bregma; 2.5 mm lateral to the midline; and 3.4 mm ventral to the skull surface. DNA and drugs were infused to the CA1 at a rate of 0.5 μL/min (0.8 μL each side) after rats recovered from the surgery. All drugs were purchased from Tocris, UK.

Environmental enrichment

Animals were placed in an enriched chamber (50 cm × 40 cm × 40 cm) for 1 h a day, for a period of 2 weeks. Small houses, running wheel, stairways, tunnels and various toys were provided in the chamber. Holes of irregular shape were drilled on the wall of the houses for the rat to explore. Cheese, crackers and fruit were provided. Animals in the control group were also brought to the experimental room every day for 1 h; they were also handled by the same experimenter every day for 10 min. In some cases, glutamate agonists/antagonists were infused to the CA1 area 30 min before the rat was placed in the chamber every day. Animals were subjected to the spatial learning task the next day after the last enrichment training. For *sgk* DNA transfection, the plasmid DNA was infused to the CA1 area at the end of 14 days enrichment training or administered every other day starting 2 days before enrichment training. Spatial learning tasks were performed 48 h after DNA transfection or at the end of enrichment training.

Water maze learning

The water maze used was a plastic, circular pool (2.0 m diameter and 0.6 m height) filled with water (25 ± 2 °C) to a depth of 20 cm. A circular platform (8 cm diameter) was placed at a specific location away from the edge of the pool. The top of the platform was submerged 1.5 cm below the water surface. Water was made cloudy by the addition of bleaching powder. Distinctive visual cues were set on the wall.

For spatial learning, animals were subjected to four trials a session, two sessions a day, with one given in the morning and the other in the afternoon. A total of four sessions were given. In each of the four trials, animals were placed at four different starting positions spaced equally around the perimeter of the pool in a random order. Animals were given 120 s to find the platform. If an animal could not find the platform in this time, it was guided to the platform. After mounting the platform, animals were allowed to stay there for 20 s. The time that each animal took to reach the platform was recorded as the escape latency.

In the visible platform experiment, a flag was mounted on the platform and the platform was raised 2.5 cm above the water surface. In addition, bleaching powder was not added so that the animals could see the location of the platform from the water.

Novel object recognition learning

The procedure used for novel object recognition was similar to that of Tang *et al*. (1999) with slight modifications. A different batch of animals was used for this experiment. Animals were habituated to an open field box (45 × 45 × 35 cm) individually for 30 min one day before the experiment. During training, two different novel objects were placed in the open field box and the rats were allowed to explore the objects for 5 min. The criteria used for exploration were a distance less than 1.5 cm between the rat and the object or a direct contact of the rat to the object. The time each rat spent to explore each object was recorded. During the retention test given 8 h later, the animals were placed back into the same box but one of the familiar objects was replaced with a novel object of approximately the same size. The time each rat spent exploring the two objects during 5 min was also recorded. Exploratory preference was calculated as the ratio of time spent exploring each object to that of exploring both objects during the training and retention tests, respectively.

Fear-conditioning learning

The next day after the object recognition task, the same animals were subjected to the fear-conditioning task. Animals were placed in a fear-conditioning chamber (45 × 30 × 45 cm). The conditioned stimulus (CS) used was an 85-dB sound (2800 Hz), and the unconditioned stimulus (UCS) was a foot shock of 0.75 mA for 1 s. Three seconds after presentation of CS, scrambled shocks were delivered to the rats randomly five times. The intershock-interval ranged from 3 s to 10 s. At the end of the CS/UCS pairing, animals remained in the chamber for 30 s for immediate freezing measurements. Eight hours later, the animals were placed back into the same chamber and the time spent for the freezing response (contextual freezing) was recorded for 3 min as the retention measure.

Real-time polymerase chain reaction

Animals were killed by decapitation immediately after the fear-conditioning vetention test. Their brains were removed and the left-side hippocampal tissue was subjected to real-time polymerase chain reaction (PCR) analysis and the right-side tissue subjected to Western blot analysis. A pilot study was first carried out to demonstrate that the hippocampal *sgk* mRNA level is not lateralized in each individual animal (our unpublished observations). Total RNA was isolated using the Ultraspec II RNA isolation system (Biotecx Inc., TX, USA) and followed by RNase-free RQ1 DNase (Promega Inc., WI, USA) treatment to remove the potential contamination of genomic DNA. The cDNA was synthesized from 0.5 μg total RNA with 0.5 μg oligo-dT in a 20-μL reaction volume by Superscript II reverse transcriptase (Invitrogen Inc., CA, USA).

Real-time PCR analysis of *sgk* mRNA was performed by using the PRISM 7700 sequence detection system with TaqMan method. The house-keeping enzyme hypoxanthine phosphoribosyl transferase (HPRT) was used as an internal standard (Murphy *et al*., 1993) that was coamplified with the *sgk* mRNA because its range of detection is similar to that of SGK (Fig. 1A). The primers and fluorogenic probes were designed by Primer Express software (Applied Biosystem Inc., CA, USA). The primers and the probe used for *sgk* were: forward primer, 5′-GGT GCT AGC TCT AAA GGA GCT TGA-3′; reverse primer, 5′-CCT GCA TCT TCC TTC TCA CTG A-3′; and probe, 5′-6FAM TGC CGC CTG AGA CGC ACC TTG TAMRA-3′, which amplified a 73-bp region of the *sgk* cDNA (nucleotides 1608–1680; GeneBank accession number L01624). The primers and probe used for

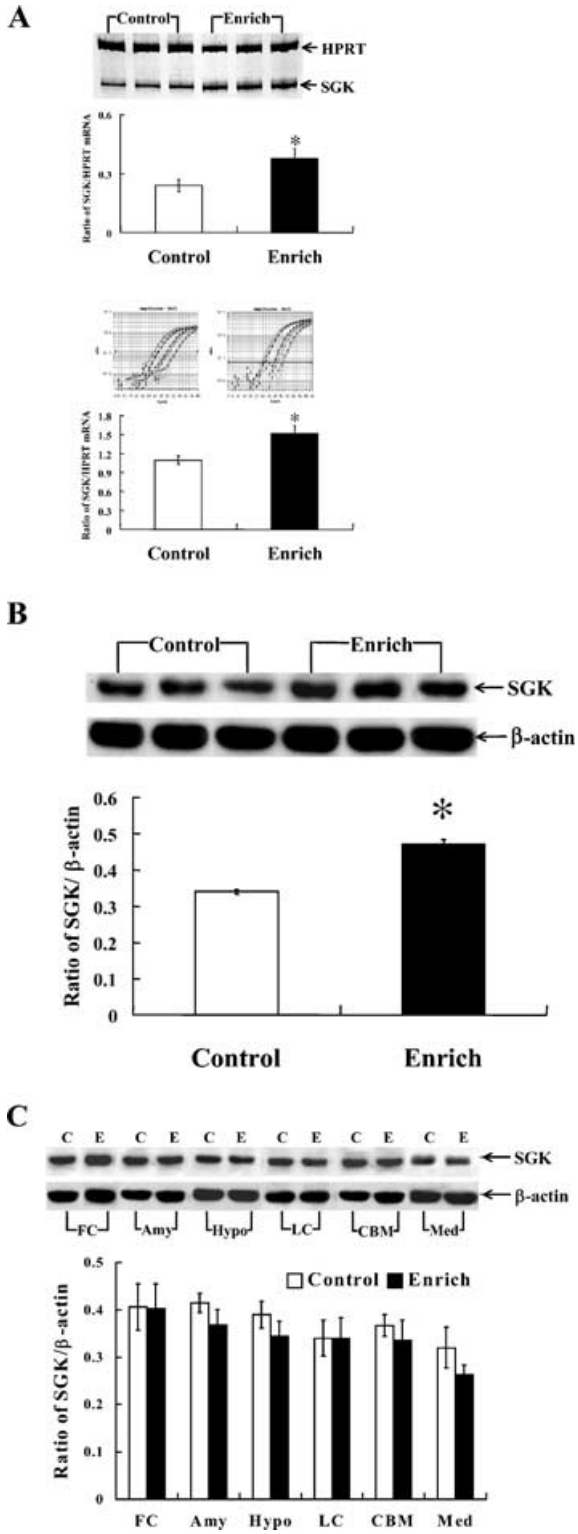

FIG. 1. Effect of environmental enrichment on *sgk* mRNA expression and protein levels. (A) Reverse transcription-PCR (upper) and real-time PCR (lower) analyses both revealed a significant increase in *sgk* mRNA level in the hippocampus of adult rats upon enrichment training (both $P < 0.05$). Inserts are standard curves for *sgk* and HPRT. (B) The same enrichment training also increased SGK protein levels in the hippocampus ($P < 0.05$). (C) *sgk* mRNA is similarly distributed in various brain regions and that enrichment training did not affect *sgk* mRNA expression in areas other than the hippocampus (all $P > 0.05$). FC, frontal cortex; Amy, amygdala; Hypo, hypothalamus; LC, locus coeruleus; CBM, cerebellum; Med., medulla. Data are expressed as mean ± SEM, $n = 7$ each group; $^{*}P < 0.05$.

HPRT were: forward primer, 5′-GCC GAC CGG TTC TGT CAT-3′; reverse primer, 5′-TCA TAA CCT GGT TCA TCA TCA CTA ATC-3′; and probe, 5′-VIC TCG ACC CTC AGT CCC AGC GTC G TAMRA-3′, which amplified a 69-bp region of the HPRT cDNA (nucleotides 11–79; GeneBank accession number X62084). The quantities of *sgk* and HPRT from the same sample were analysed simultaneously but in separate tubes. Each assay was carried out in duplicate. The amplification mixture contained 1 μL reverse transcription product, 200 nM of each primer, 250 nM probe, and TaqMan universal PCR master mix (P/N 4304437) in a total of 25-μL reaction volume. The thermal conditions were: 2 min at 55 ° and 10 min at 95 °; followed by 40 cycles at 95 ° for 15 s and 60 ° for 1 min.

The mRNA for *sgk* and HPRT in each sample was estimated from a threshold cycle number (C_T), which is correlated inversely with the abundance of its initial mRNA level. The C_T was then converted to relative quantity of mRNA by using a standard curve calibrated according to the manufacturer's instructions. The standard curve for *sgk* and HPRT was created by serial dilution of the RT products from control samples (Fig. 1A). Semiquantitative analysis of *sgk* gene expression was normalized in relation to HPRT gene expression.

Western blot

The hippocampal tissue was sonicated in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, pepstatin A (20 μg/mL), leupeptin (20 μg/mL) and aprotinin (20 μg/mL). The lysate was centrifuged at 14 000 ***g*** for 10 min at 4 °C and the supernatant was assayed for protein concentration.

Equal amounts of protein extract (20 μg) were subjected to 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, MA, USA) by a semi-dry transfer method. The membrane was blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature (RT), and then incubated with SGK1 antibody (1 : 500; UBI, NY, USA) for 2 h at RT. After washing (0.1% Tween 20 in TBS buffer), three times for 10 min each, the membrane was incubated with horseradish peroxidase (HRP)-conjugated IgG (1 : 2500, UBI) in 0.05% TBST containing 2% BSA for 1 h at RT. After extensive washes, the peroxidase signals were detected by chemiluminescence (ECL plus; Amersham, NJ, USA). To intensify the signal of SGK, the membrane containing the SGK protein was removed for the second anti-SGK antibody incubation. Before incubation, the membrane was stripped in 0.2 M glycine (pH 2.5) and 0.05% Tween at 80 ° for 20 min, then rinsed twice with 0.09 M boric acid (pH 7.4) containing 0.9% NaCl and 0.05% Tween 20. The membrane was reblocked and incubated with SGK1 antibody (1 : 1000; UBI) at RT overnight. After washing, the membrane was incubated with HRP-conjugated IgG (1 : 2500; UBI) for 1 h at RT. The protein bands were visualized through chemiluminescence.

To prepare for β-actin reprobing, the blot was stripped again and reblocked with 5% skim milk in 0.05% TBST. The membrane was then incubated with anti-β-actin monoclonal antibody (1 : 1000; Chemicon) in 0.05% TBST containing 5% skim milk for 1 h at RT. After washing, the blot was incubated with HRP-conjugated IgG (UBI) for 1 h at RT in 0.05% TBST containing 5% skim milk and visualized through chemiluminescence. The protein band was quantified with a computing densitometer and the IMAGE Software (NIH, USA).

Plasmid DNA preparation and intrahippocampal gene transfection

Haemagglutinin (HA) from the influenza virus is commonly used as an epitope protein for detecting specific DNA transfection because antibodies raised against HA do not react with over 20 chemically

synthesized peptides (Green *et al*., 1982). For construction of the HA-tagged DNA plasmid (HA-SGK), full-length *sgk* was cloned by amplifying the rat hippocampal *sgk* cDNA with the primers 5′-CGG AAT TCA CCG TCA AAA CCG AGG CTC G-3′ and 5′-GCT CTA GAT CAG AGG AAG GAG TCC ATA GG-3′. The PCR product was subcloned between the *EcoR*I and *Xba*I sites of the mammalian expression vector pcDNA3-HA. The Ser422 mutant (kinase-deficient HA-SGK S422A) was generated by PCR using the primers 5′-CGG AAT TCA CCG TCA AAA CCG AGG CTC G-3′ and 5′-GCT CTA GAT CAG AGG AAG GAG TCC ATA GGA GGG GCA TAG GCG AAG-3′ with HA-SGK as a template and inserted into the pcDNA3-HA expression vector. The efficiency of the *sgk* mutant DNA transfection was confirmed by a decreased SGK activity in HEK 293 cells (Park *et al*., 1999). Presumably, decreased SGK activity would also occur in the hippocampus upon *in vivo* S422A mutant DNA transfection. Before injection, plasmid DNA was diluted in 5% glucose to a concentration of 5 μg/μL. Branched polyethylenimine (PEI; 25 kDa; Sigma) was diluted to a concentration of 0.1 M in 5% glucose and added to the DNA solution. The mixture was vortexed for 30 s and allowed to equilibrate at RT for 10 min before injection.

For *in vivo* gene transfection, animals were anaesthetized with pentobarbital (40 mg/kg, i.p.) and subjected to stereotaxic surgery. Two 23-gauge stainless steel thin-wall cannulae were implanted bilaterally to the CA1 area at the coordinates described above. After recovery from the surgery, 0.8 μL of a 5% glucose solution containing 2 μg plasmid DNA complexes with 10 PEI equivalents were injected into the CA1 area (0.5 μL/min). The injection needle was left in place for 5 min to limit the diffusion of the injected DNA.

Immunohistochemistry

Two days after transfection, rats were anaesthetized with pentobarbital (100 mg/kg, i.p.) and perfused with ice-cold phosphate-buffered saline (PBS; 100 mM, pH 7.4), followed by 4% paraformaldehyde. Brains were removed and postfixed in 20% sucrose−4% paraformaldehyde solution for 20–48 h. After postfixation, brains were frozen, cut into 20-μm sections on a cryostat and mounted on poly-L-lysine-coated slides. Brain sections were rinsed with 1 × PBS for 10 min at RT and permeablized with precold EtOH/CH_3COOH (95% : 5%) for 10 min, followed by three times with 1 × PBS for 10 min. The sections were preincubated in a blocking solution containing 3% normal goat serum, 3% BSA and 0.2% Triton X-100 in 1 × PBS for 2 h at RT, followed three times by washes in 1 × PBS for 10 min. For immunofluorescence analysis, tissue sections were incubated with a mouse monoclonal anti-HA antibody (Boehringer Mannheim; 1 : 100) in blocking buffer at 4 °C overnight. Sections were washed three times in 1 × PBS and incubated in the secondary antibody (goat anti-mouse FITC-conjugated IgG antibody; Sigma; 1 : 100) in 1 × PBS for 1 h at RT. Digital photomicrographs were taken with an Olympus digital C-3030 camera mounted on a Zeiss microscope (Oberkochen, Germany).

Statistics

All behavioural data were analysed by two-way analysis of variance (ANOVA) with repeated measure. All biochemical data were analysed by Student's *t*-test or one-way ANOVA. Specific comparisons between groups were made by Dunnett's *t*-test or the Newman–Keuls method.

Results

Effect of enriched environmental training on SGK expression

Because enrichment enhances spatial learning and that *sgk* mRNA levels are increased in animals showing better performance in the spatial learning task, we predicted that enriched environmental training should increase *sgk* expression. The present study examined this hypothesis. Animals were divided into two groups and subjected to regular housing and enriched environmental training, respectively ($n = 7$ each group). We conducted reverse transcription-PCR and real-time PCR analyses and both results revealed that enriched environmental training significantly increased *sgk* mRNA levels in the hippocampus ($t_{1,12} = 2.63$ and $t_{1,12} = 2.31$, both $P < 0.05$; Fig. 1A). To examine whether this effect was also evident at the protein level, we performed Western blot analyses. Results revealed that enrichment also markedly increased SGK protein levels in the hippocampus ($t_{1,12} = 2.47$, $P < 0.05$; Fig. 1B). We further examined whether the effect of enrichment is also observed in other brain areas. Results indicated that enrichment training did not significantly alter SGK protein levels in any other brain areas examined (all $P > 0.05$; Fig. 1C). In addition, SGK was expressed similarly in the various brain regions examined (Fig. 1C).

Enrichment-induced recovery of spatial and nonspatial learning deficits

We have previously demonstrated that transfection of S422A mutant DNA to the hippocampus significantly impaired water maze performance in rats (Tsai *et al*., 2002). We have also shown that enrichment increased *sgk* expression in the hippocampus. In the present experiment we further examined whether the increased *sgk* expression plays a functional role in enhancing learning and memory. For the spatial learning task, animals were divided into three groups ($n = 8$ each group). Rats in Group I and Group II received pcDNA3 vector and S422A mutant DNA transfection, respectively. Rats in Group III were placed in the enriched chamber every day for 2 weeks before S422A DNA transfection. Results revealed an overall significant effect of these treatments on spatial learning ($F_{2,21} = 6.97$, $P < 0.01$; Fig. 2A). Further analyses indicated that S422A mutant DNA transfection markedly impaired water maze performance ($q = 4.75$, $P < 0.05$). However, enriched environmental training significantly reversed this spatial learning deficit ($q = 0.48$, $P > 0.05$ when comparing the S422A + enrichment group with the control group). The first trial performance was not significantly different among these three groups ($F_{2,21} = 1.57$, $P > 0.05$). To further examine whether *sgk* serves as an underlying mechanism of enrichment-induced spatial learning facilitation, separate animals were divided into three groups ($n = 8$ each group). Rats in Group I received pcDNA3 vector transfection only; rats in Group II received pcDNA3 vector transfection but were subjected to enrichment training for 2 weeks; rats in Group III received S422A DNA transfection and were subjected to enrichment training for 2 weeks. The first pcDNA3 or S422A mutant DNA infusion was given 2 days before enrichment training and it was given repeatedly every other day thereafter because transient transfection shows an optimal expression 48–72 h after transfection (Abdallah *et al*., 1996). Results from Fig. 2B revealed a significant treatment effect ($F_{2,21} = 7.13$, $P < 0.01$). Further analyses indicated that enrichment training markedly improved water maze performance in rats ($q = 5.29$, $P < 0.05$), but this effect was blocked by prior S422A mutant DNA transfection ($q = 0.57$, $P > 0.05$ when comparing the S422A + enrichment group with the control group). Further analyses revealed that these animals do not differ significantly in their water maze performance when tested under visible platform learning (all $P > 0.05$).

We next evaluated whether S422A mutant DNA transfection impaired retention performance of other hippocampal-dependent learning tasks in addition to spatial learning. A different batch of animals was used. Animals were similarly divided into three groups as described above ($n = 9$ each group). Two days after pcDNA3 vector or S422A mutant DNA transfection, these animals were subjected to

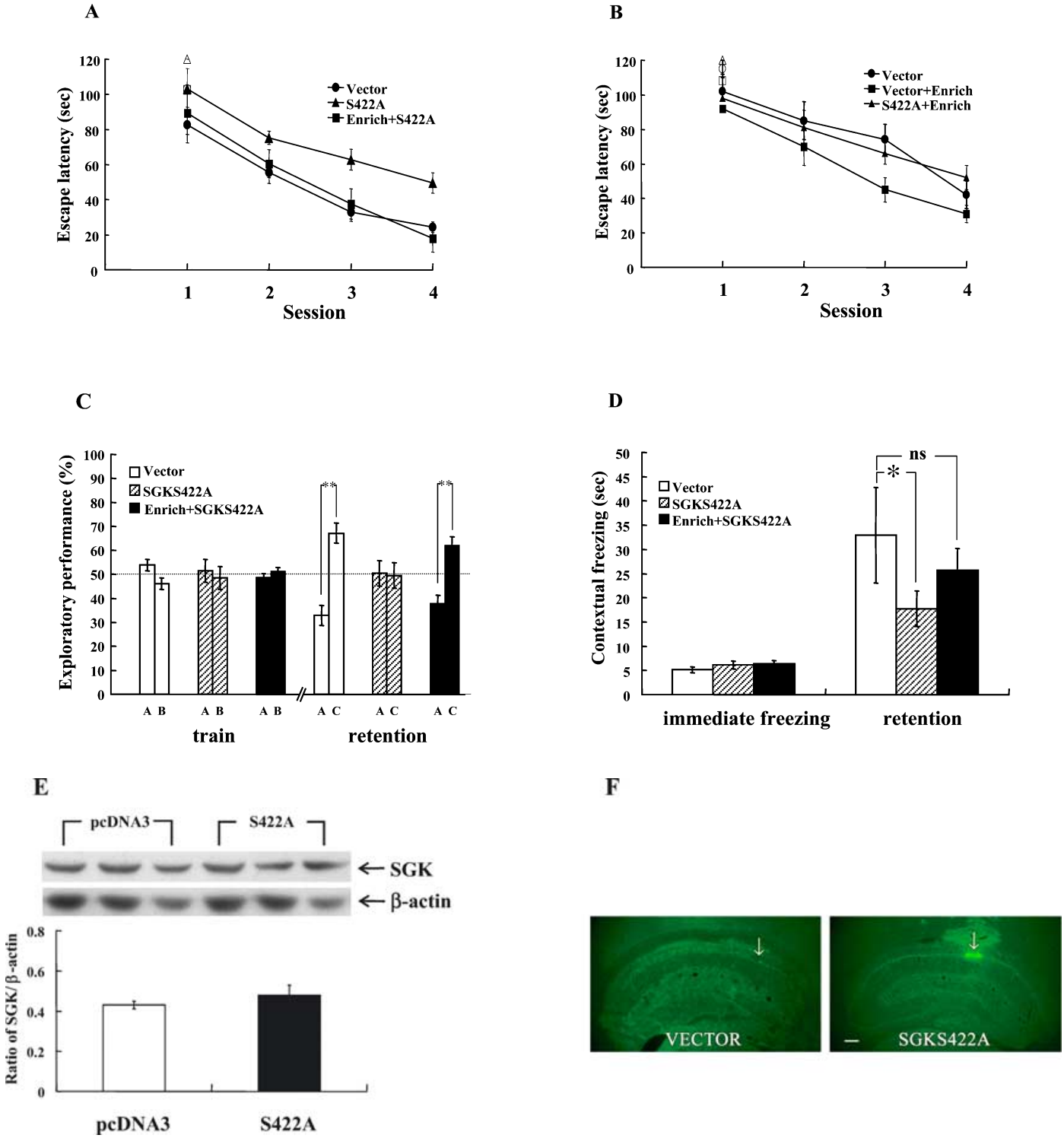

FIG. 2. Effects of enrichment training and *sgk* mutant (S422A) DNA transfection on various learning performance in rats. (A) S422A DNA transfection to hippocampal CA1 area significantly impaired water maze performance in rats and this effect is blocked by prior enrichment training ($P < 0.01$, $n = 8$ each group). (B) Enrichment markedly improved water maze performance and this effect is blocked by prior S422A mutant DNA transfection ($P < 0.01$, $n = 8$ each group). Open symbols represent the first trial performance of each corresponding group. (C) S422A DNA transfection to the CA1 area markedly impaired memory retention for novel object recognition learning and this effect is reversed by prior enrichment training ($P < 0.01$, $n = 9$ each group). (D) S422A DNA transfection also significantly impaired retention for fear-conditioning learning and this effect was partially, but significantly, reversed by the same enrichment training ($P < 0.05$, $n = 9$ each group). Data are mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$. (E) Western blot analysis showing that S422A mutant DNA transfection did not alter SGK protein level in the hippocampus ($P > 0.05$, $n = 6$ each group). (F) Representative immunohistochemical stainings showing transfection of pcDNA3 vector (left) and of HA-tagged S422A mutant DNA (right) in the hippocampal CA1 area. The FITC-conjugated IgG secondary antibody and anti-HA primary antibodies were used. Arrows indicate the sites of transfection. Scale bar, 30 μm.

object recognition learning and fear-conditioning learning. As shown in Fig. 2C, these animals did not differ markedly in their preference to object A and B during recognition training (all $P > 0.05$). During the retention test, the pcDNA3 vector-transfected animals showed a significant preference to the novel object C (replacing object B) over object A ($t_{1,16} = 6.35$, $P < 0.01$). This novel object recognition was not observed in S422A mutant DNA-transfected animals ($t_{1,16} = 0.53$, $P > 0.05$). However, enriched environmental training significantly reversed this memory deficit ($t_{1,16} = 5.43$, $P < 0.01$).

We then assessed the effect of S422A mutant DNA transfection and enrichment training on fear-conditioning learning in the same animals. As shown in Fig. 2D, these animals did not differ in their immediate freezing responses to fear conditioning ($P > 0.05$). The freezing response was in general higher during the retention test, but there was not an overall significant treatment effect ($F_{2,24} = 1.59$, $P > 0.05$). Further analyses indicated that S422A mutant DNA transfection significantly decreased the freezing response ($t_D = 2.51$, $P < 0.05$ when compared with the vector group). Enriched environmental training partially, but significantly, reversed this retention deficit ($t_D = 1.17$, $P > 0.05$ when compared with the control group).

To assess whether S422A mutant DNA transfection decreased SGK protein level, we conducted Western blot analysis. Results from Fig. 2E revealed that S422A transfection did not alter SGK protein levels in the hippocampus ($t_{1,10} = 0.52$, $P > 0.05$). Immunohistochemistry against the HA-tagged protein was carried out to confirm the efficiency of *sgk* DNA transfection. As shown in Fig. 2F, no specific labelling was observed in the hippocampus upon non-HA vector transfection (left), but fluorescence labelling was observed in the CA1 area in HA-SGKS422A mutant DNA-transfected animals (right).

Effects of enrichment on spatial learning and SGK expression in aged rats

Enriched environment is known to facilitate spatial learning in adult rat and we have demonstrated that enrichment training enhanced SGK expression in the hippocampus. As behavioural plasticity and synaptic plasticity can decline with age (Nakamura *et al*., 1999), in the present experiment we examined whether enrichment also enhances spatial learning and SGK expression in aged rats. Animals (12 months old) were divided into two groups as described above (control group and enriched group, $n = 8$ each group). Results revealed that enriched environmental training markedly improved water maze performance in aged rats ($F_{1,14} = 8.31$, $P < 0.01$, Fig. 3A). The first trial performance was also significantly different ($t_{1,14} = 2.54$, $P < 0.05$). We further examined whether enrichment enhanced sensory and motor coordination in aged rats might therefore improve their spatial learning performance. Results from Fig. 3B revealed that enrichment training tended to improve water maze performance under visible platform learning ($F_{1,14} = 3.86$, $P = 0.07$). The first trial performance was significantly different ($t_{1,14} = 3.82$, $P < 0.05$).

After the behavioural experiments, we then analysed hippocampal SGK protein level in these animals. Results from Fig. 3C revealed that enrichment training significantly increased the SGK level by more than twofold in the hippocampus ($t_{1,14} = 5.04$, $P < 0.01$).

Role of NMDA receptor in enrichment-induced spatial learning facilitation and SGK expression

From the above experiments, we have demonstrated that rats exposed to an enriched environment showed better spatial learning performance and increased SGK expression in the hippocampus. Because the NMDA receptor plays an important role in spatial learning (Morris *et al*., 1986), in the present experiment, we examined whether the above effects were mediated through the NMDA receptor. All animals were cannulated bilaterally and were divided into three groups: the control group; enriched group; and DL-2-amino-5-phosphonopentanoic acid (AP5) + enrichment group ($n = 8$ each group). In the last group, 0.5 μg AP5 was infused to the CA1 area 30 min before enrichment training every day for 2 weeks continuously. The control and enriched groups received saline infusions. Results from Fig. 4A indicated that there was an overall significant treatment effect ($F_{2,21} = 3.53$, $P < 0.05$). Further analyses revealed that enrichment training consistently and significantly improved water maze performance ($q = 2.85$, $P < 0.05$), but this effect was completely blocked by pretreatment with AP5 ($q = 0.77$, $P > 0.05$ when comparing the AP5 + enrichment group with the control group). The first trial performance was not markedly different ($F_{2,21} = 0.52$, $P > 0.05$). Results from Fig. 4B revealed that there was not a significant difference in their performance under visible platform learning ($F_{2,21} = 1.81$, $P > 0.05$).

We then analysed the SGK protein level from these animals. Results from Fig. 4C revealed that enrichment training consistently and markedly increased SGK expression in the hippocampus ($t_D = 2.27$, $P < 0.05$, Dunnett's *t*-test). However, prior AP5 treatment did not block enrichment-induced SGK expression ($q = 0.98$, $P > 0.05$ when comparing the AP5 + enrichment group with the enriched group).

Role of NMDAR NR2B in enrichment-induced spatial learning facilitation and SGK expression

Recent studies have demonstrated that transgenic mice with NMDAR NR2B overexpression show better learning and memory performance (Tang *et al*., 1999). Moreover, enrichment also increases the NR2B protein level (Tang *et al*., 2001). In the present experiment, we examined whether the NR2B subunit could also mediate the effect of enrichment on spatial learning and SGK expression. Animals were divided into three groups: the control group; enriched group; and ifenprodil + enrichment group ($n = 8$ each group). In the last group, 6 μg ifenprodil was infused to the CA1 area 30 min before enrichment training every day for 2 weeks. The other two groups received saline infusions. Results from Fig. 5A revealed an overall significant treatment effect ($F_{2,21} = 11.04$, $P < 0.01$). Further analyses indicated that enrichment training markedly improved spatial learning performance ($q = 3.57$, $P < 0.05$). Prior treatment with the NR2B antagonist ifenprodil completely prevented the effect of enrichment on spatial learning ($q = 6.64$, $P < 0.01$ when comparing the ifenprodil + enrichment group with the enrichment group). The first trial performance was not markedly different ($F_{2,21} = 1.07$, $P > 0.05$). In analysing the sensory and motor functions of these animals, we found that ifenprodil infusion markedly impaired water maze performance under visible platform learning ($q = 2.96$, $P < 0.05$ when compared with the control group).

In analysing the SGK protein level of these animals, we found that enrichment training consistently and markedly increased SGK protein expression in the hippocampus ($t_D = 2.25$, $P < 0.05$, Dunnett's *t*-test; Fig. 5C). However, prior ifenprodil treatment did not block the effect of enrichment on SGK expression ($q = 0.82$, $P > 0.05$ when comparing the ifenprodil + enrichment group with the enrichment group).

Regulation of SGK expression by different glutamate receptors

The above results demonstrate that although the NMDA receptor plays an important role in enrichment-induced spatial memory facilitation, it did not mediate enrichment-induced SGK expression. In this experiment, we examined the effects of different glutamate receptor activation on *sgk* expression in the hippocampus (see Fig. 6). Animals

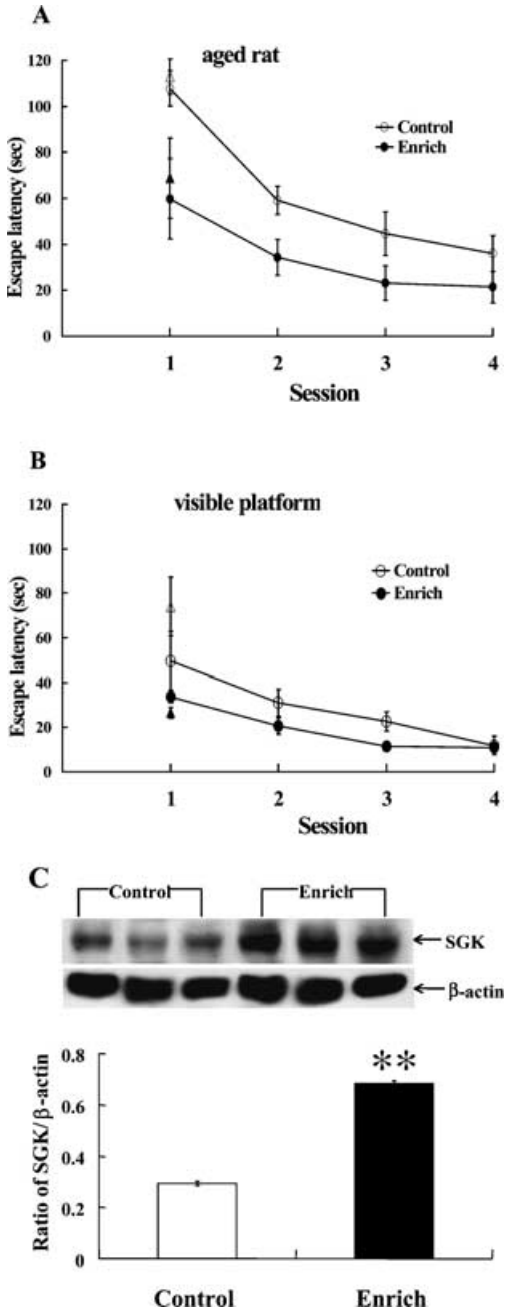

FIG. 3. Effect of environmental enrichment on spatial learning and SGK expression in the hippocampus of aged rats (A) Enrichment training significantly improved water maze performance in aged rats ($P < 0.01$). (B) Enrichment training tended to improve water maze performance under visible platform learning ($P = 0.07$). The first trial latency was significantly reduced in aged rats upon enrichment training under both conditions (both $P < 0.05$). △ and ▲, first trial performance of control and enriched group, respectively. (C) Enrichment training markedly increased SGK protein level in the hippocampus ($P < 0.01$). Data are mean ± SEM, $n = 8$ each group. $^{**}P < 0.01$.

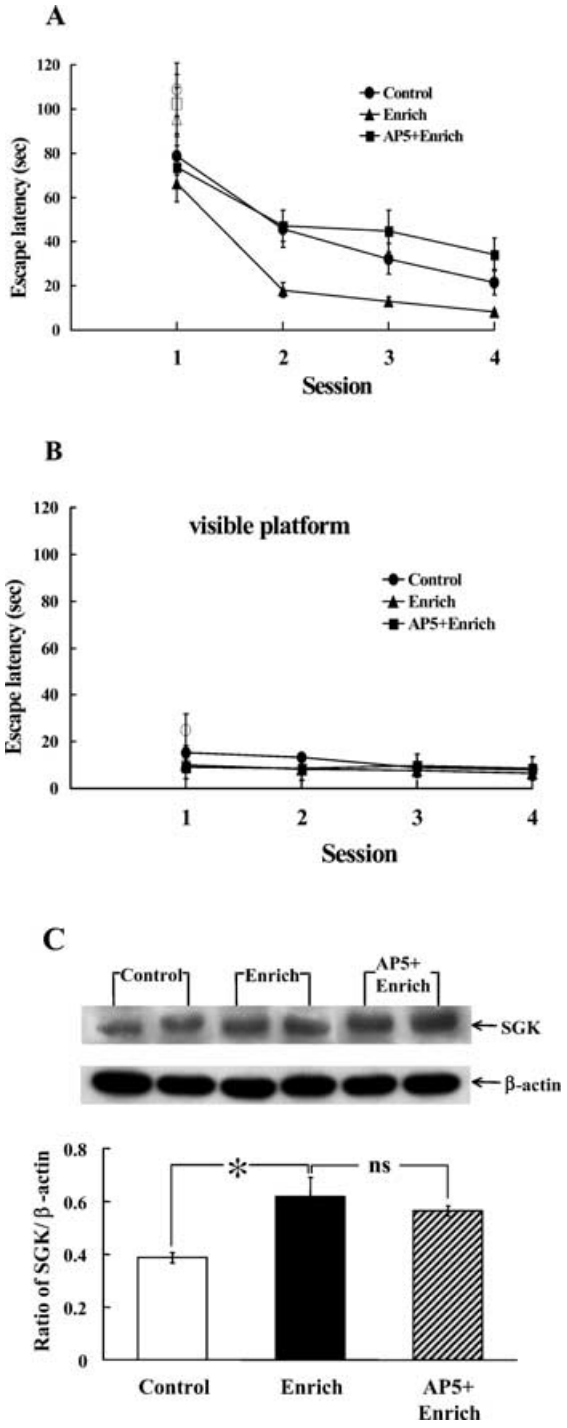

FIG. 4. Role of NMDA receptor in enrichment-induced spatial learning facilitation and SGK expression. (A) Enrichment training significantly improved water maze performance in rats, and this effect was completely blocked by AP5 (0.5 μg) pretreatment ($P < 0.05$). (B) Neither AP5 pretreatment nor enrichment training affected water maze performance under visible platform learning ($P > 0.05$). (C) Enrichment training markedly increased SGK protein level in the hippocampus ($P < 0.05$), but this effect is not prevented by prior AP5 infusion ($P > 0.05$). Data and open symbols are expressed as in Fig. 2. $n = 8$ each group; $^{*}P < 0.05$.

were divided into four groups to receive: saline; NMDA (0.05 μg); (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD; 2 μg); and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; 2 μg) infusions to the CA1 area, respectively (0.8 μL each side, $n = 5$ each group). Concentrations of NMDA, ACPD and AMPA were determined from other studies that showed these to have a significant behavioural or biochemical changes when injected into the hippocampus (Bianchin *et al*., 1994; Quillfeldt *et al*., 1994; Huang *et al*., 1998). Animals were killed 30 min after infusion and the hippocampus was subjected to real-time PCR analysis of *sgk* mRNA expression. Statistical analysis revealed an overall significant treatment effect ($F_{3,16} = 8.06$, $P < 0.01$; Fig. 6). Further analyses indicated that only

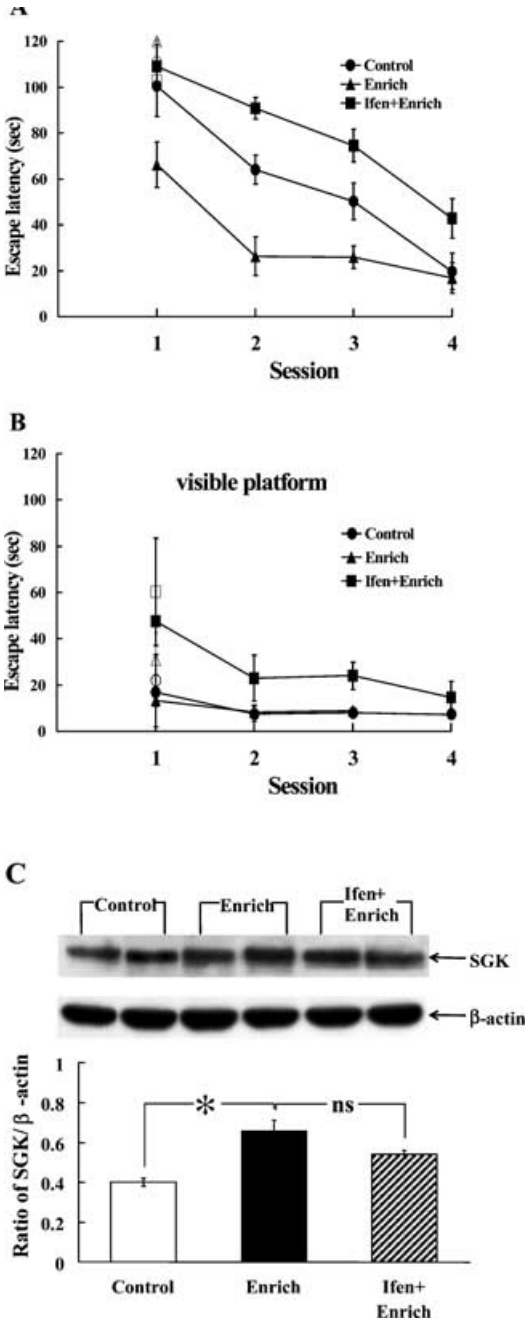

FIG. 5. Role of NMDAR NR2B in enrichment-induced spatial learning facilitation and SGK expression. (A) Enrichment significantly improved water maze performance in rats, and this effect was completely blocked by pretreatment with the NR2B antagonist ifenprodil (6 μg) ($P < 0.01$). (B) Ifenprodil pretreatment also markedly impaired water maze performance under visible platform learning ($P < 0.05$). (C) Enrichment significantly increased SGK protein level in the hippocampus ($P < 0.05$), but this effect was not blocked by prior ifenprodil infusion ($P > 0.05$). Data and open symbols are shown as in Fig. 2. $n = 8$ each group. Ifen, ifenprodil. $^{*}P < 0.05$.

AMPA infusion markedly increased *sgk* mRNA level in the hippocampus ($t_D = 3.05$, $P < 0.01$, Dunnett's *t*-test).

Role of AMPA receptor in enrichment-induced spatial learning facilitation and SGK expression

The above results demonstrate that the glutamate AMPA receptor regulates *sgk* mRNA expression in the hippocampus, but it is not known whether the AMPA receptor also mediates the effect of

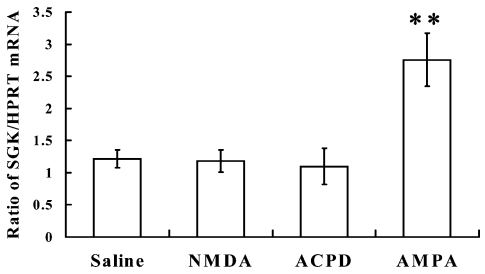

FIG. 6. Role of different glutamate receptors in regulation of *sgk* expression. Animals received bilateral CA1 infusion of different glutamate receptor agonists including NMDA (0.05 μg), ACPD (2 μg), and AMPA (2 μg) and *sgk* expression was examined 30 min later. Real-time PCR analysis showed that only AMPA infusion significantly increased *sgk* mRNA levels in the hippocampus ($P < 0.01$). Data are mean ± SEM, $n = 5$ each group. $^{**}P < 0.01$.

enrichment on spatial learning. The present experiment examined this hypothesis. Animals were divided into three groups: control; enriched; and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(F)quinoxaline-7-sulphonamide disodium (NBQX) + enrichment group ($n = 6$–8 each group). In the last group, NBQX (6 μg) was infused to the CA1 area bilaterally 30 min before enrichment training every day for 2 weeks. The other groups received saline infusions. As shown in Fig. 7A, enrichment training markedly enhanced spatial learning performance in rats ($q = 3.16$, $P < 0.05$). Prior infusion of the AMPA receptor antagonist NBQX completely blocked the effect of enrichment on spatial learning ($q = 6.85$, $P < 0.01$ when comparing the NBQX + enrichment group with the enrichment group). We further analysed whether any difference in their sensory and motor coordination may affect their spatial learning performance. Statistical analysis indicated that NBQX treatment markedly impaired the first trial performance under visible platform learning ($t_D = 2.92$, $P < 0.01$ when compared with the control group, Dunnett's *t*-test).

We finally analysed the SGK protein levels of these animals. Results from Fig. 7C revealed an overall significant treatment effect ($F_{2,19} = 13.19$, $P < 0.01$). Further analyses indicated that enrichment markedly increased SGK protein levels in the hippocampus ($t_D = 4.76$, $P < 0.01$, Dunnett's *t*-test), and this effect was completely blocked by NBQX pretreatment ($q = 6.04$, $P < 0.01$ when comparing the NBQX + enrichment group with the enrichment group).

Discussion

Various studies have demonstrated that enrichment training improved spatial learning performance in rats (Kempermann *et al*., 1997; Nilsson *et al*., 1999), but its underlying mechanism is relatively unknown. In studying the molecular mechanism of enrichment-induced cognitive changes, NMDAR NR2B has been suggested to play an important role, because enrichment increases NR2B expression and it prevents further improvement in certain learning tasks in NR2B transgenic mice (Tang *et al*., 2001). In a recent study, we have demonstrated that the *sgk* gene is causally involved in spatial learning performance in rats (Tsai *et al*., 2002). We hypothesized that *sgk* might serve as another molecular mechanism of enrichment-induced learning facilitation. This speculation is supported by our finding that enrichment training significantly increased *sgk* mRNA and protein levels in the hippocampus. These results are consistent with the reports that enrichment increases the level of GR mRNA (Olsson *et al*., 1994) and enrichment reverses decreased GR mRNA expression induced by stroke (Dahlqvist *et al*., 1999). Furthermore, *sgk* is widely and evenly distributed in the various brain regions examined and enrichment training preferentially increased *sgk* expression in the

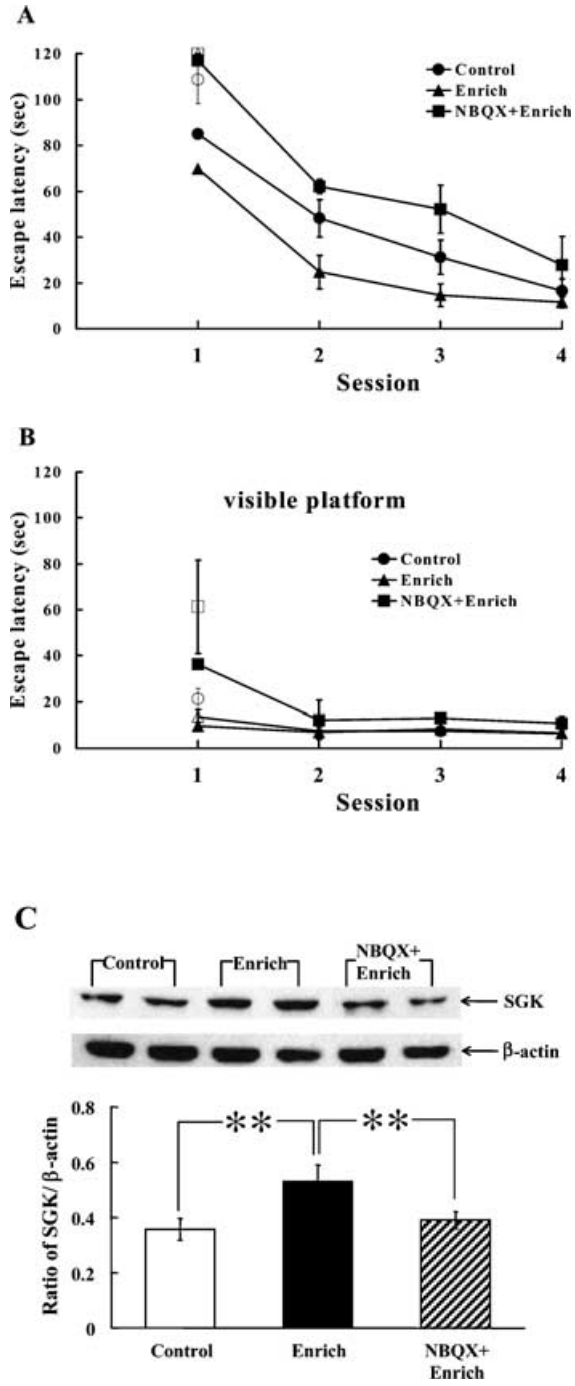

FIG. 7. Role of AMPA glutamate receptor in enrichment-induced spatial learning facilitation and SGK expression. (A) Enrichment significantly facilitated water maze performance in rats, and this effect was completely blocked by pretreatment with the AMPA receptor antagonist NBQX (6 μg; $P < 0.01$). (B) NBQX did not markedly affect water maze performance under visible platform learning except at the first trial ($P < 0.05$). (C) Enrichment significantly increased SGK protein levels in the hippocampus ($P < 0.01$), and this effect was completely blocked by prior NBQX treatment ($P < 0.01$). Data and open symbols are shown as in Fig. 2. $n = 6$–8 each group; $^{**}P < 0.01$.

hippocampus. There are several possible explanations for the preferential selectivity of enrichment on *sgk* expression in the hippocampus. First, the hippocampus is rich in AMPA receptors (Monaghan *et al*., 1984) and GRs (van Steensel *et al*., 1996) and both receptors mediate the effect of enrichment and regulate *sgk* expression. Therefore, it is much easier to observe an effect of enrichment on *sgk* expression in this area. Alternatively, there could be subgroups of neurons in these other regions examined and SGK is inhomogeneously distributed in these neurons. Further investigation of the cellular distribution of SGK should help to resolve this issue. In the present study, although enrichment increased *sgk* expression in the hippocampus, we can not exclude the possibility of arousal-induced *sgk* expression that is confounded in the effect of enrichment. However, the effect of handling stress should have been eliminated because control animals were also handled every day.

To assess the functional significance of enrichment-induced *sgk* expression, we examined the effect of enrichment on *sgk* mutant DNA-induced spatial learning deficit. We also examined the role of *sgk* involved in enrichment-induced spatial learning facilitation. In addition, we investigated the role of *sgk* in two types of associative learning that also require the integrity of hippocampal neurons; the fear-conditioning learning (Phillips & LeDoux, 1992) and novel object recognition learning (Mumby *et al*., 1996). Our results revealed that *sgk* mutant DNA transfection impaired spatial learning, fear-conditioning learning and recognition learning. It also prevented the facilitating effect of enrichment on spatial learning. These results suggest that *sgk* participates in memory formation of both spatial and non-spatial learnings involving the hippocampus, and it possibly serves as an underlying mechanism of enrichment training. By contrast, S422A transfection did not alter SGK protein levels in the hippocampus. These results suggest that S422A mutant DNA transfection probably decreases SGK activity (Park *et al*., 1999) and blocks the normal SGK signalling to impair memory formation rather than affecting the SGK protein content. In another study, we have similarly demonstrated that transfection of the focal adhesion kinase (FAK) mutant DNA markedly impaired long-term potentiation induction without altering FAK protein level in the hippocampus (Yang *et al*., 2003). Studies are currently underway to examine this hypothesis. Moreover, enrichment training effectively reversed the learning deficits caused by S422A mutant DNA transfection. This is probably a result of enhanced SGK expression and signalling upon enrichment training because prior S422A DNA transfection blocked the effect of enrichment on water maze learning. However, we can not exclude the possibility that enrichment training also enhances other gene expressions to compensate for the impairing effect of S422A mutant DNA transfection. The lack of an effect of *sgk* mutant DNA transfection and enrichment on water maze performance under visible platform learning suggests that they do not alter the motivation and sensory/motor functions of these animals to affect their spatial learning performance.

The hippocampus-associated functions could decline with age. These include decreases in synaptic plasticity (Nakamura *et al*., 1999), cognitive function (Winocur, 1998) and neurogenesis (Kuhn *et al*., 1996). It is possible that the effect of enrichment is also attenuated in aged rats. In examination of this hypothesis, we found that enrichment produced a consistent and significant effect in enhancing both spatial learning performance and *sgk* expression in the hippocampus of aged rats. These results are consistent with the report that experience induces neurogenesis in the senescent hippocampus (Kempermann *et al*., 1998). Furthermore, enhancement in spatial learning in aged rats may be partly a result of improvement in their sensory/motor functions because the first trial performance was also significantly improved in enriched rats under both invisible and visible platform learning ($P < 0.05$). These results are consistent with the finding that environmental enrichment enhances visual acuity in mice (Prusky *et al*., 2000). These results together suggest that *sgk* could play an important role in neuroplasticity during ageing. Evidence supporting this notion comes from the observation that GR protein level and DNA binding are decreased in the hippocampus of

aged rats (Murphy *et al*., 2002). Future studies examining the alteration of *sgk* expression during ageing and the relationship between *sgk* and diseases associated with ageing should help further clarify the role of *sgk* in neuroplasticity. It will also be interesting to examine the effect of enrichment on *sgk* expression in other brain areas in aged rat.

In examining the role of NMDA receptors involved in enrichment-induced spatial learning facilitation and *sgk* expression, we found that blockade of NMDA receptors in general, and the NR2B subunit in particular, both effectively blocked the facilitating effect of enrichment on spatial learning. However, these treatments did not block enrichment-induced *sgk* expression. Although these results are consistent with the report that the NMDA receptor plays an important role in spatial learning (Morris *et al*., 1986), they also suggest that the NMDA receptor does not regulate *sgk* expression. Further gene regulation studies indicated that *sgk* expression is upregulated selectively by AMPA receptor activation. Together with the report that *sgk* expression facilitates spatial learning (Tsai *et al*., 2002), the present result is consistent with the finding that the AMPA receptor is involved in memory consolidation of avoidance learning in rats (Liang *et al*., 1996). It is also consistent with the report that a specific increase in AMPA receptor synthesis is associated with the late-phase of LTP in rat hippocampus (Nayak *et al*., 1998). It will be interesting to examine whether *sgk* expression is associated with the late-phase of LTP in future studies. By contrast, blockade of AMPA receptors effectively blocked both enrichment-induced spatial learning facilitation and *sgk* expression. These results are congruent with the finding that enrichment selectively increases ^{3}H-AMPA binding and augments AMPA receptor-mediated synaptic responses in the hippocampus (Foster *et al*., 1996). Furthermore, both AMPA-induced CREB phosphorylation and *sgk* activation depend on phosphatidylinositol 3-kinase signalling (Park *et al*., 1999; Perkinton *et al*., 1999). These results together suggest that AMPA receptor activation and *sgk* activation may share some common signalling pathways. However, although ifenprodil completely blocked the effect of enrichment on spatial learning, it also impaired water maze performance under visible platform learning. This is probably because at the concentration of ifenprodil used (6 μg), it also impaired the animal's sensory/motor coordination to a certain extent. However, a significant effect of ifenprodil on spatial learning was still observed even the latter effect is taken into consideration ($P < 0.01$ vs. $P < 0.05$). The same situation also occurred during NBQX treatment for the first trial performance.

In the present study, transfection was carried out in a limited area in the CA1 region, but significant behavioural changes were observed. One possible explanation for this is that the defective neurons transfected with S422A could convey the wrong signalling information to neighbouring neurons through cross-talk, and consequently amplify the effect of *sgk* mutant DNA; however, the exact mechanism remains to be elucidated.

In a recent study that used the oligonucleotide microarray technique, Rampon *et al*. (2000b) have reported many gene expression changes in the mouse brain associated with environmental enrichment. Yet, *sgk* is not one of these genes. There are several possible explanations. First, in that study the cortex tissue was examined, whereas in the present study the hippocampus was the target of interest. Enrichment training might induce different gene expressions in different brain regions. This explanation is supported by our finding that *sgk* expression is not significantly altered in rat frontal cortex upon enrichment training (Fig. 1C). Second, oligonucleotide microarray might not be sensitive enough to detect relatively small changes in gene expression (about 40–50% in the present study), because in their study, most gene expression changes were higher than twofold. Third, the *sgk* gene might not be included in the original oligonucleotide array. Enrichment was also shown to induce various immediate early gene expression changes (Bisler *et al*., 2002), and some of these genes were shown to be associated with synaptic plasticity. Whether *sgk* is associated with the expression of these immediate early genes is currently under investigation. In addition, we have demonstrated in the present study that *sgk* plays an important role in various hippocampal-dependent learning tasks. Whether *sgk* also participates in nonhippocampal-dependent learning requires further investigation.

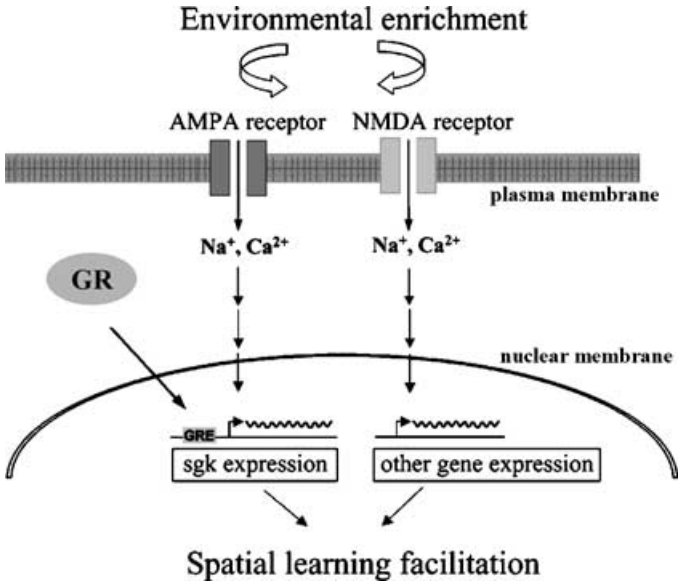

FIG. 8. A schematic diagram showing the relationship between environmental enrichment, glutamate receptor activation, *sgk* expression and spatial learning facilitation. Enrichment training activates both glutamate NMDA and AMPA receptors to facilitate spatial learning performance. However, *sgk* expression-mediated learning facilitation is mediated specifically through AMPA receptor activation. Glucocorticoid receptor (GR) also targets the GRE promoter sequence to enhance *sgk* expression.

In conclusion, environmental enrichment was shown to enhance synaptic plasticity and improve various learning performances associated with the hippocampus, and the NMDAR NR2B subunit is suggested to be one of the molecular mechanisms responsible for these changes. Here we reported that enrichment training enhanced spatial learning through activation of both NMDA and AMPA receptors, but enrichment-induced *sgk* expression is mediated specifically through AMPA receptor activation (Fig. 8). Thus, *sgk* could serve as another molecular mechanism, which is independent of the NMDA receptor, underlying enrichment-induced learning facilitation.

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ANOVA, analysis of variance; BSA, bovine serum albumin; CREB, cAMP-responsive element binding protein; CS, conditioned stimulus; AP5, DL-2-amino-5-phosphonopentanoic acid; GR, glucocorticoid receptor; HA, haemagglutinin; HPRT, hypoxanthine phosphoribosyl transferase; HRP, horseradish peroxidase; LTP, long-term potentiation; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(F)-quinoxaline-7-sulphonamide disodium; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, room temperature; SGK, serum and glucocorticoid-inducible kinase.

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