Behavioral/Systems/Cognitive

Environmental Enrichment Enhances Neurogranin Expression and Hippocampal Learning and Memory But Fails to Rescue the Impairments of Neurogranin Null Mutant Mice

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Environmental enrichment is known to enhance hippocampal neurogenesis and cognitive functions. Neurogranin (Ng), a specific substrate of protein kinase C (PKC), is abundantly expressed in brain regions important for cognitive functions. Deletion of Ng in mice causes severe deficits in spatial learning and long-term potentiation (LTP) in the hippocampal CA1 region. These Ng−/− mice, as compared with Ng+/+, respond poorly after treatment of their hippocampal slices with agents that activate signaling molecules important for learning and memory, including Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), PKC, protein kinase A (PKA), extracellular signal-regulated kinase (ERK), and cAMP response element-binding protein (CREB). In the present study, adult mice were housed in either regular home cages (control group) or more spacious cages with an exercise wheel and change of toys twice per week (enriched group) for at least 3 weeks. Enriched Ng+/+ and Ng+/− mice showed enhanced LTP in the hippocampal CA1 after high-frequency stimulation, but Ng−/− mice were affected only minimally. Behaviorally, the enriched Ng+/+ and Ng+/−, but not Ng−/− mice, performed significantly better than their respective control cohorts in Morris water maze and in step-down fear conditioning. Enriched Ng+/− mice also showed improvement in the radial arm maze. Quantitative immunoblot analyses showed that the enriched groups of all three genotypes exhibited elevated hippocampal levels of αCaMKII and CREB, but not ERK. Interestingly, enrichment caused a significant increase in hippocampal Ng levels both in Ng+/+ and Ng+/− mice that seemed to contribute to their improved LTP and behavioral performances. These results suggest that Ng gates the neuronal signaling reactions involved in learning and memory. During environmental enrichment, these Ng-regulated reactions are also critical for the enhancement of synaptic plasticity and cognitive functions.

Key words: **neurogranin; environmental enrichment; Ca^{2+}/calmodulin; learning and memory; synaptic plasticity; hippocampus**

Introduction

Environmental enrichment has been shown to enhance hippocampal neurogenesis (Kempermann et al., 1997; van Praag et al., 2000; Bruel-Jungerman et al., 2005) and mitigate deficits in cognitive functions in several mutant animal models (Need et al., 2003; Spires et al., 2004; Jankowsky et al., 2005; Li and Tang, 2005). It is believed that these complex environmental stimuli induce structural changes in the neuronal network to enhance synaptic efficacy and improve learning and memory. Mechanistically, modulation of transcriptional, translational, and post-translational events are essential for neuronal structural modification leading to increasing neurotransmitter-mediated signal transduction efficiency. An increase in the network connectivity and/or the efficacy of signal amplification within a connection are the likely consequences of exposing an animal to an enriched environment. Rodents housed in enriched environments with toys and exercise wheels and increased social interaction with cage mates exhibit significant changes in synaptic morphology and neuronal function compared with those housed in standard cages (van Praag et al., 2000). The enriched environment rectifies cognitive deficits in mutant mice resulting from deletion of NMDA receptor 1 subunit (Rampon et al., 2000) and K^+ channel regulatory subunit Kvβ1.1 (Need et al., 2003).

Previously, we have shown that neurogranin knock-out mice (Ng−/−) exhibited severe deficits in performing the hippocampus-dependent tasks (Pak et al., 2000; Miyakawa et al., 2001). In these tasks, heterozygous mice (Ng+/−) although not as impaired as Ng−/− mice, did not perform as well as wild-type mice (Ng+/+). Their cognitive deficits were likely caused by their attenuated Ca^{2+}- and Ca^{2+}/calmodulin (CaM)-mediated signalings. *In vitro* treatment of acute hippocampal slices with

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pharmacological agents that stimulate protein kinase C (PKC), protein kinase A (PKA), and their down-stream targets only evoke meager responses in Ng−/− mice (Wu et al., 2002; Wu et al., 2003). In addition, high-frequency stimulation (HFS)-induced long-term potentiation (LTP) in the hippocampal CA1 region of Ng−/− mice is also greatly attenuated as compared with their Ng+/+ littermates, in part because of reduced neuronal Ca^{2+} transients (Huang et al., 2004). Interestingly, the hippocampal levels of Ng in Ng+/− mice seemed to vary broadly among individual mice and their performances in Morris water maze were positively correlated to their hippocampal Ng content (Huang et al., 2004). These findings led us to hypothesize that Ng modulates neuronal signaling by a "mass-action mechanism;" namely, the higher the Ng level in neurons, the greater the efficiency of signal amplification.

In this study, we sought to investigate whether environmental enrichment could improve the performances of mice and, more specifically, to rescue the cognitive impairments of Ng +/− and Ng−/− mice and affect the expression of the various signaling molecules in the hippocampus. Several behavioral testing paradigms were used to measure the effect of enrichment on mouse performance. We also tested the HFS-induced LTP in the acute hippocampal slices of these mice. Our results showed that environmental enrichment enhanced LTP and behavioral performances in both Ng+/+ and Ng+/− mice but only minimally affected the Ng−/− mice. Enrichment also enhanced hippocampal expressions of signaling molecules important for learning and memory like Ng, Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), and cAMP response element-binding protein (CREB).

Materials and Methods

Environmental enrichment. Animal protocols were approved by the National Institute of Child Health and Human Development Animal Care and Use Committee. Wild-type and Ng mutant mice were bred in-house and were colonies derived from >10 back-crossings from the original 129/Sv by C57BL/6 mixed background. Animals were housed in groups and maintained on a 12 h light/dark cycle, and the behavioral tests were performed during the light phase of the cycle. For environmental enrichment, mice (3–5 months of age) of different genotypes (Ng+/+, Ng+/−, and Ng−/−) were mixed and randomly divided into control and enriched groups. Control mice were housed in regular cages (30 cm length × 19 cm width × 13 cm height) whereas the enriched mice were in more spacious ones (48 cm length × 26 cm width × 16 cm height) with rodent toys of various shapes and colors and an exercise running wheel. Toys were changed twice a week during bedding changes and mice were kept under such enriched environment for at least 3 weeks before behavioral testing or electrophysiological recording. At the conclusion of behavioral testing, the animals were killed, the brains were removed, and both the right and left hippocampi were dissected and kept frozen for later immunoblot analyses. Animals used for water maze tasks were not tested for the radial arm maze and vise versa. Step-down fear conditioning was always performed with previously tested animals. Also, animals used for the behavioral testing would not be used for electrophysiological recordings. Thus, not the same sample size was used across all behavioral testing, and the *n* numbers of each test were indicated in the figure.

Electrophysiology. Electrophysiological recordings were performed in the CA1 region of the transverse hippocampal slices (400 μm), which had been kept in oxygenated artificial CSF (ACSF) [containing the following (in mM): 124 NaCl, 4.9 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25.6 $NaHCO_3$, and 10 D-glucose, pH 7.4] at room temperature for 1–2 h for recovery after slicing. The slices were placed in a submerged-type chamber superfused with oxygenated ACSF at a flow rate of ~2 ml/min. Glass electrodes (1–4 MΩ) filled with ACSF were used for stimulation of Schaffer collateral/commissural fibers as well as for recordings of field EPSPs (fEPSPs) from the stratum radiatum of the CA1 region. The slope of the fEPSP was used to measure the potentiation. After establishing a stable baseline, the slices were subjected to high-frequency stimulation (100 Hz for 1 s) with a current that evoked ~30% of the maximal response. Potentials were amplified using an Axoclamp-2B (Molecular Devices, Union City, CA), digitized by CED Power 1401 (Cambridge Electronic Design, Cambridge, UK), and analyzed using Signal 2 software (Cambridge Electronic Design). Values obtained after HFS were expressed as percentage of their respective baseline recordings. For comparison of the responses between groups, the last 10 min blocks of recordings were analyzed

Morris water maze. To learn the hidden platform version of the water maze, mice received three blocks of three training trials per day (interblock interval, ~2 h) for 4 consecutive days. In all trials, mice were allowed to swim until they landed on the platform or 60 s had elapsed. At the end of the fourth day of training, a probe test without the platform was conducted. The swim path, time, and distance were recorded in all trials (Videomex; Columbus Instruments, Columbus, OH). After the completion of hidden platform tasks, mice were given the visible platform version of the test.

Step-down fear conditioning. Mice, one at a time, were placed on a plastic platform (10 × 9 × 2.5 cm high) which was sitting on a metal grid and located inside a stainless steel box (23 × 23 × 23 cm high). When they stepped down onto the grid and had all four of their paws on it, they received a 2 s, 0.5 mA foot shock and were immediately returned to their home cages. Twenty-four hours after conditioning, mice were returned to the platform in the box and were tested for their retention from stepping down from the platform or when 10 min had elapsed. The foot shock was omitted during testing.

Radial arm maze. The equipment consisted of eight equal radial arms, 11.4 × 41.9 × 10.1 cm high, with a central octagonal arena, 27.4 cm wide, all with transparent lids. There was a sliding door at the entrance of each arm, and three of them had distinctive drawings of shapes serving as visual cues in addition to those posted on the nearby wall. During the duration of training, food was available for mice only from 4:00 P.M. to 8:00 P.M., whereas water was unlimitedly provided. Body weight of the animal was monitored every other day to avoid >20% reduction. Mice were first group-habituated (3–4 mice at a time) in the maze, where baits (sugar pellet of ~10 mg and of assorted color) were scattered throughout the maze, and then they were habituated again while food was present only in four of the arms, which would be designated as the baited arms in the later trials. Only one pellet was placed at the end of each designated arm during actual training. Training consisted of two daily trials; the mouse was placed in the central area for 5 s before opening the doors to the arms. Each trial lasted 3 min or until all four baits were consumed, during which all entries into baited or unbaited arms, with consumption or no consumption of bait, were recorded. In tabulating the performances, the percentage of working memory error was defined as any repeated entries among total entries, and the percentage of reference memory error (long-term memory) as the percentage of entries into an unbaited arm and those without consumption of bait when entering baited arms out of total entries.

Immunoblot analysis. Each hippocampus was homogenized with 250 μl of homogenization buffer containing the following: 50 mM Tris/Cl, pH 7.8, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 50 mM KF, 5 mM Na-pyrophosphate, 50 nM okadaic acid, 50 μM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 5 μg/ml each of leupeptin, aprotinin, and pepstatin, and 1% SDS. Protein concentrations were determined by BCA (Pierce, Rockford, IL). Normally, 30 μg of protein was loaded per lane onto 8–16% gradient gels containing 0.1% SDS. After electrophoresis and transfer to nitrocellulose membrane, the lower portion of the membrane was blotted for Ng (antibody 270 or 2641), and the upper portion for CREB, αCaMKII, and extracellular signal-regulated kinase (ERK) consecutively. Control and enriched samples of the same genotype were run on the same gel, so were some control samples of all genotypes for direct comparisons. The bands of each immunoblot were scanned and quantified [using Kodak (Rochester, NY) Imaging Station and its analysis software] from films developed with ECL

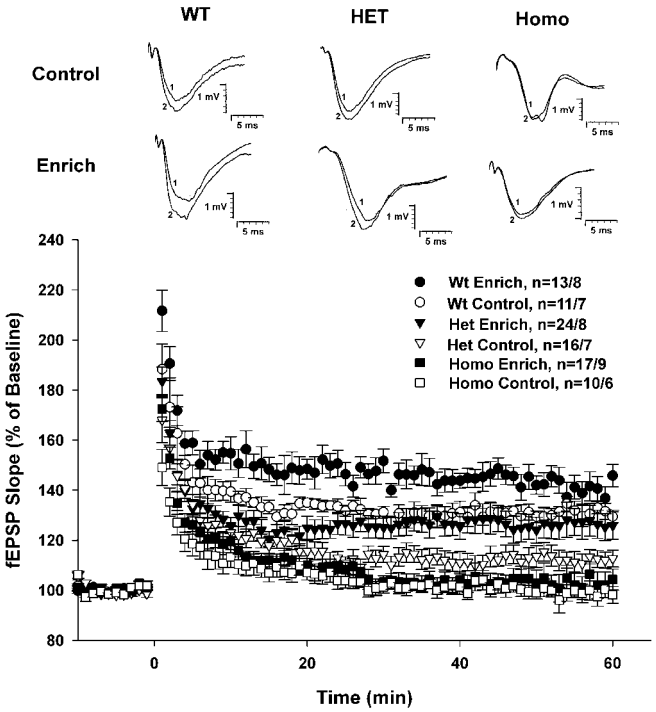

Figure 1. LTP in the hippocampal CA1 region as affected by environmental enrichment. LTP was induced by HFS (100 Hz for 1 s), and the fEPSP at the CA1 region was recorded for 60 min. The slope of the fEPSP was determined and the steady-state levels of responses among Ng+/+ (Wt), Ng+/− (Het), and Ng−/− (Homo) during the last 10 min blocks were as follows (percentage of mean ± SEM of baseline): Wt enrich, 141.4 ± 1.0%; Wt control, 131.1 ± 0.5%, Het enrich, 126.6 ± 0.3%; Het control, 112.6 ± 0.4%; Homo enrich, 103.0 ± 0.6%; Homo control, 99.1 ± 0.5%. One-way ANOVA by pairwise comparisons using the Holm–Sidak method were performed: Wt control versus Het control and Wt and Het control versus Homo control were all significantly different with $p < 0.001$; Wt enrich and Het enrich versus their respective controls are also all significantly different with $p < 0.001$. This HFS protocol does not evoke significant LTP for either control or enriched Ng−/− mice. Representative traces of the fEPSP before (1) and 60 min after (2) HFS are shown. Calibration: 1 mV, 5 ms.

and exposed in the linear range for each antibody. Data were expressed as percentage of control or percentage of Ng+/+ control.

Statistical analysis. Results are expressed as mean ± SEM. Statistical comparisons were made by one-way ANOVA using the Holm–Sidak method for pairwise comparisons to identify significant differences. In all cases, $p < 0.05$ was considered significant.

Results

Environmental enrichment on LTP in hippocampal CA1 area

LTP, the activity-dependent change in synaptic strength, has been proposed as a cellular mechanism underlying learning and memory. Environmental enrichment is known to modify neurogenesis and synaptic strength in the hippocampal neurons (Foster et al., 1996; Kempermann et al., 1997). We examined whether environmental enrichment could modify hippocampal LTP of Ng+/+, Ng+/−, and Ng−/− mice (Fig. 1). Treatment of hippocampal slices with a single 1 s burst of 100 Hz stimulation evoked a strong potentiation in Ng+/+ control mice (131.1 ± 0.5%; $n = 11/7$); the potentiation was much reduced in Ng+/− control mice (112.6 ± 0.4%; $n = 16/7$) and neared nil in Ng−/− mice (99.1 ± 0.5%; $n = 10/6$). Enriched experiences significantly enhanced LTP in both Ng +/+ (141.4 ± 1.0%; $n = 13/8$) and Ng+/− (126.6 ± 0.3; $n = 24/8$) mice. The enriched Ng+/− mice showed a potentiation level nearly approaching that of the control Ng+/+ mice. But this enrichment regimen only minimally affected LTP for the enriched over control Ng−/− mice (enriched Ng−/−: 103 ± 0.6%, $n = 17/9$). It seems that missing Ng led to a reduced response of the activity-dependent synaptic plasticity enhancement.

Enrichment on animal behaviors

Previously (Pak et al., 2000; Huang et al., 2004), we showed that Ng−/− mice exhibited severe deficits in both the acquisition and retention of spatial memory in Morris water maze as compared with their Ng+/+ littermates, whereas Ng+/− mice performed less well than those of Ng+/+ mice. In the present experiments, we sought to determine whether environmental enrichment and voluntary exercise could improve their performances in the water maze, especially among Ng−/−mice. We have adopted a less intense training protocol for the hidden platform test by giving three trials per block and three blocks per day for 4 consecutive days. Under this regimen, again, the Ng+/+ performed significantly better than Ng+/− and Ng−/− mice, and Ng+/− mice also performed better than Ng−/− mice (Fig. 2*A*). As for the effect of environmental enrichment, both enriched Ng+/+ and Ng+/− mice performed significantly better than their nonenriched counterparts, but it did not rescue the deficits exhibited among Ng−/− mice. During the probe test, selectivity of mice swimming in the target quadrant was analyzed both in terms of time spent in the quadrant and number of entries to the target quadrant. As shown in Figure 2*B*, enriched Ng+/+ and Ng+/− mice spent significantly more time than their controls swimming in the target quadrant. Counting the number of entries into target quadrant gave similar results (data not shown). Their preferences for the target quadrant over other quadrants (percent of time spent in target quadrant/averaged percent of time spent in other quadrants) for Ng+/+, Ng+/−, and Ng−/− mice were as follows: control/enriched 1.37 ± 0.06/2.07 ± 0.10, 1.17 ± 0.03/1.53 ± 0.03, and 0.91 ± 0.06/1.14 ± 0.09, respectively (a value of one or less means no preference). Apparently, environmental enrichment also positively affected Ng+/+ and Ng+/− mice in the probe test, however, the effect on Ng−/− mice was minimal. In the visible platform test, there were no significant differences among genotypes or between enriched and control groups (data not shown).

Morris water maze learning is a hippocampus-dependent spatial task, and we sought to use other more complex behavioral training paradigms consisting of spatial, emotional, and food motivations, such as step-down fear avoidance and the radial arm maze, to test the effect of enrichment. In the step-down fear avoidance test, a fear-motivated learning task, animals associated a platform present in a given context (spatial recognition) with a shock given to the foot (fear) when they step down from the platform. In the radial arm maze, using food as motivation, animals use visual spatial cues for place learning to execute their spatial working and reference memory. As shown in Figure 3, during step-down fear conditioning trials, all groups exhibited similar short retention times before stepping down the platform and receiving a shock. During testing for their memory of the aversive experiences 24 h later, there were significant differences in retention times among genotypes (Ng+/+, 416 ± 41.0 s; Ng+/−, 319.1 ± 36.7 s; Ng−/−, 151.9 ± 39.3 s; Ng+/+ vs Ng+/−, $p < 0.01$; Ng+/+ vs Ng−/−; and Ng+/− vs Ng−/−, $p < 0.001$). Enrichment caused a significant improvement of both Ng+/+ and Ng+/− mice. Enriched Ng+/+ and Ng+/− mice (544.4 ± 25.8 and 443.4 ± 37.8 s, respectively) compared with their corresponding controls were significantly different with $p < 0.001$ and $p = 0.001$, respectively. Although there was a

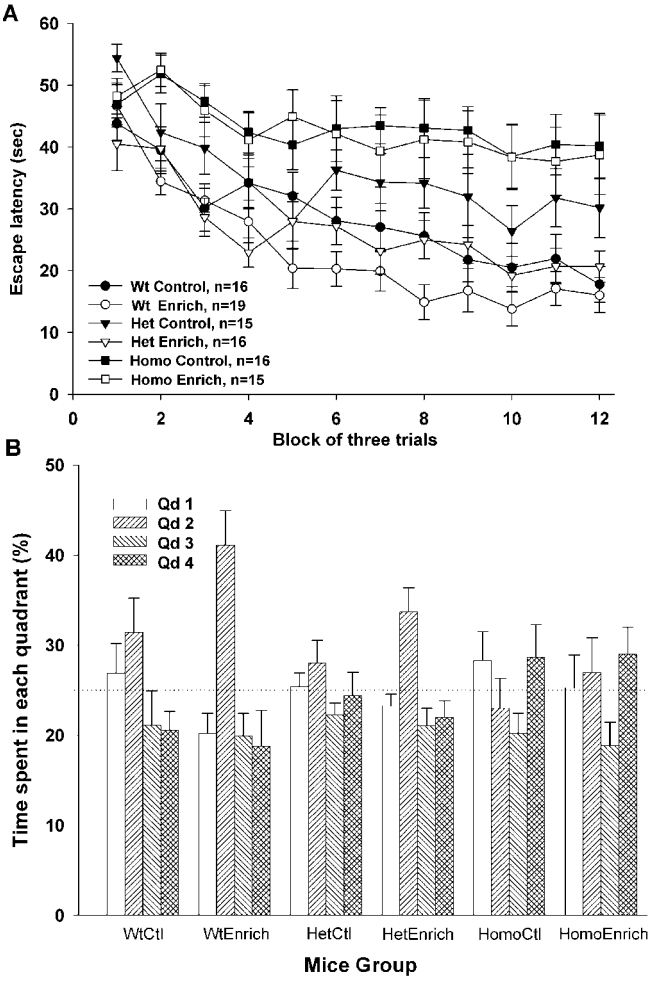

Figure 2. Performances in Morris water maze as affected by environmental enrichment. ***A***, In the first part of the experiment, mice received 12 blocks of training (3 blocks/d and 3 trials/block for 4 consecutive days) in the hidden-platform version of the water maze. The graph shows the escape latency to find the hidden platform for Ng+/+ (Wt), Ng+/− (Het), and Ng−/− (Homo) mice over successive blocks of trials. The averaged escape latencies for the last three blocks of trials among the groups of mice were as follows (mean ± SEM): Wt control, 20.1 ± 1.2 s; Wt enrich, 15.7 ± 1.0 s; Het control, 29.5 ± 1.6 s; Het enrich, 20.2 ± 0.5 s; Homo control, 39.7 ± 0.6 s; Homo enrich, 38.3 ± 0.3 s. One-way ANOVA pairwise comparisons using the Holm–Sidak method were performed: Wt control versus Het control, and Wt and Het control versus Homo control were significantly different with $p < 0.001$; Wt and Het enrich versus their corresponding controls were also significantly different with $p < 0.01$ and $p < 0.001$, respectively. Homo enrich were not significantly different from their control with $p = 0.331$. ***B***, The same number of animals was used in the probe trial. During the 1 min duration of the probe trial, the percentage of times spent in the previously trained quadrant (second Qd) among the groups of animals were (mean ± SEM): Wt control, 31.3 ± 3.8; Wt enrich, 41.1 ± 3.8; Het control, 28.0 ± 2.5; Het enrich, 33.7 ± 2.7; Homo control, 23.0 ± 3.4; Homo enrich, 25.5 ± 3.4. Analyzing their selectivity for the target quadrant over other quadrants for control/enriched mice showed 1.37 ± 0.06/2.07 ± 0.10, 1.17 ± 0.03/1.53 ± 0.03, and 0.91 ± 0.06/1.14 ± 0.09, for Ng+/+, Ng+/−, and Ng−/− mice, respectively (a value of 1 denotes no selectivity). Enriched Ng+/+ and Ng+/− mice showed significant preferences over their controls for the target quadrant, whereas Ng−/− mice, either control or enriched, showed no such preference.

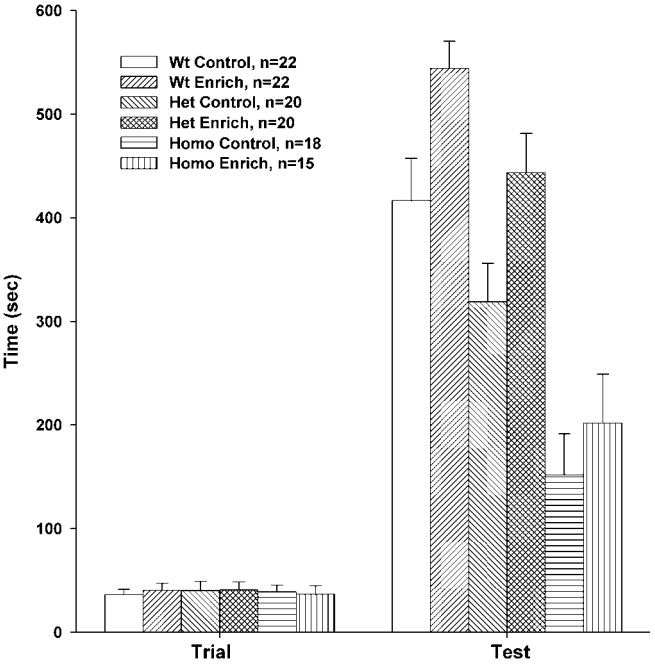

Figure 3. Performances in the step-down fear conditioning as affected by environmental enrichment. Retention times during the conditioning trial among Ng+/+ (Wt), Ng+/− (Het), and Ng−/− (Homo) mice were all very short and not different from each other. Retention times 24 h after conditioning were as follows (mean ± SEM): Wt control, 416.6 ± 41.0 s; Wt enrich, 544.4 ± 25.8 s; Het control, 319.1 ± 36.7 s; Het enrich, 443.4 ± 37.8 s; Homo control, 151.9 ± 39.3 s; Homo enrich, 201.8 ± 47.2 s. Pairwise comparisons by the Holm–Sidak method showed the Wt control versus Het control, Wt control versus Homo control, and Het control versus Homo control were all significantly different with $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively. Wt and Het enrich versus their respective controls were also significantly different with $p < 0.001$, but Homo enrich versus Homo control was not significantly different with $p = 0.231$.

tendency for the enriched Ng−/− mice (201.8 ± 47.2 s) to perform better than their respective controls, the improvement was not significant among them (Ng−/− enrichment vs control, $p = 0.231$).

Learning performance in the eight-arm radial maze showed that mice of all three genotypes, even those housed in regular home cages, improved their time required to retrieve all baits after training (Fig. 4*A*). Among the control groups, the rate of learning of Ng+/+ mice was faster than those of Ng+/− and Ng−/− mice; these latter two groups progressed at similar rate (Fig. 4*Aa*). The enrichment didn't further improve the rate of learning of Ng+/+ (Fig. 4*Ab*) or Ng −/− mice (*Ad*), although enriched Ng+/− mice improved in time needed to consume all four baits (*Ac*). For reference memories (remembering the arms where baits were located so not to enter the unbaited arm), although all groups showed clear learning curves (Fig. 4*Ba*), Ng+/+ mice were significantly better than Ng+/− mice, and Ng+/− better than Ng−/− mice in not making errors entering unbaited arms. The enriched Ng+/+ mice didn't improved their performance over the control mice (Fig. 4*Bb*). Enriched Ng−/− mice seemed to do well initially but didn't continue improving and were not significantly different from their control group (Fig. 4*Bd*) at the end of the study. Enriched Ng+/− mice (Fig. 4*Bc*), however, made the most significant improvement over their control group in remembering the baited arm and performed as well as Ng+/+ mice, if not better. As for the working memory error (Fig. 4*C*), all control groups showed progress across the training (Fig. 4*Ca*); Ng+/+ and Ng+/− mice obviously performed much better than the Ng−/− mice in not re-entering the previously visited arms, but the progress of Ng+/+ mice was not distinguishable from that of Ng+/− mice. Most interestingly, environmental enrichment didn't seem to affect working memory

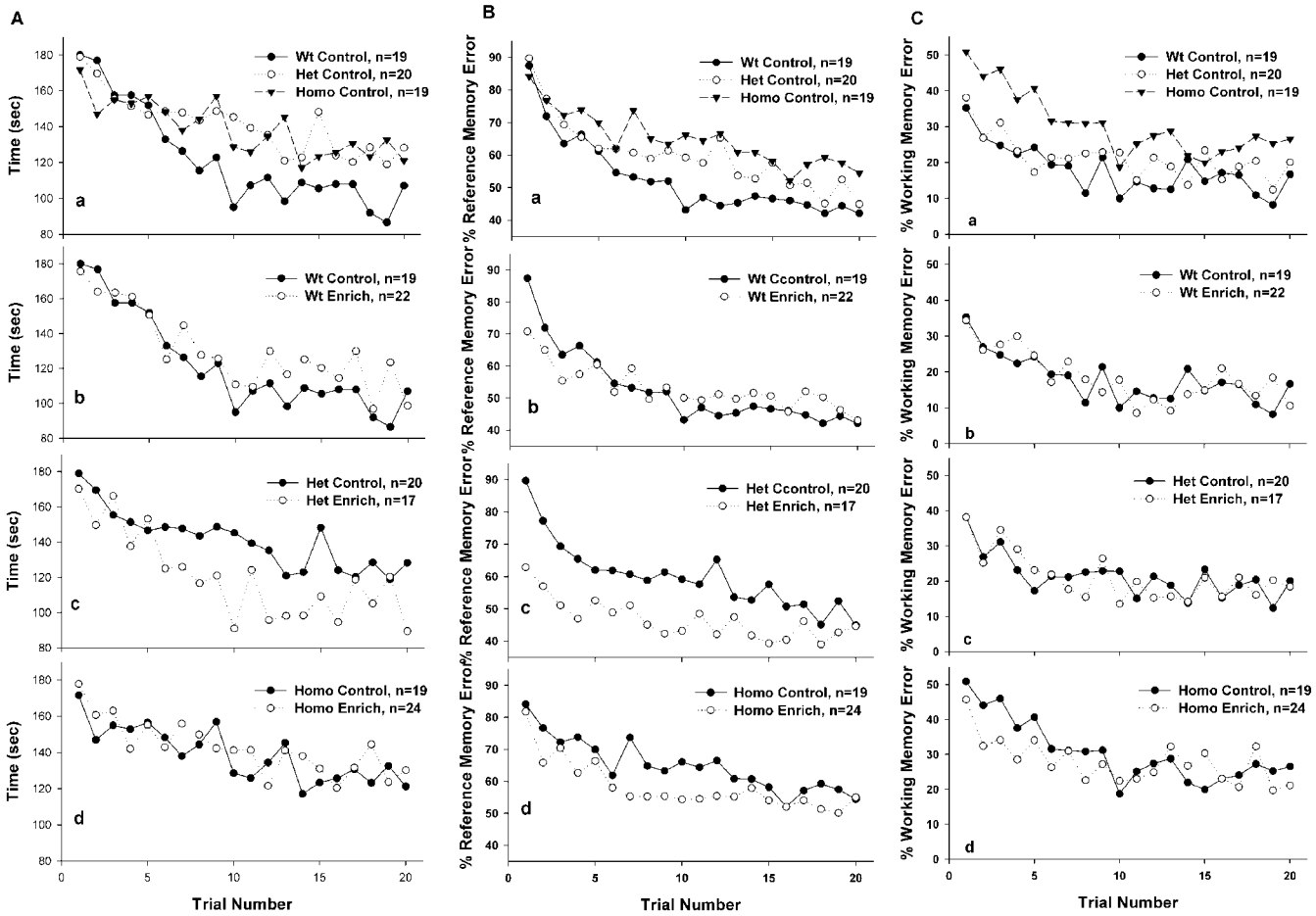

Figure 4. Performances in radial-arm maze as affected by environmental enrichment. After initial habituation, there were always four baits at arm 1, 2, 4, and 7 to start the training. There were two trials per day, one in the morning and the other one in the afternoon. ***A***, Progression of time required to retrieve all four baits or if 3 min had elapsed throughout 20 blocks of trial in 10 d. Averaged times for the last six trials among Ng+/+ (Wt), Ng+/− (Het), and Ng−/− (Homo) mice were as follows (mean ± SEM): Wt control, 101.2 ± 3.9 s; Wt enrich, 113.9 ± 5.5 s (***Ab***); Het control, 128.1 ± 4.3 s; Het enrich, 106.3 ± 5.1 s (***Ac***); Homo control, 126.1 ± 1.9 s; Homo enrich, 130.24 ± 3.4 s (***Ad***). The Wt control was significantly different from either Het or Homo control with $p < 0.001$, but Het control and Homo control exhibited no significant differences (***Aa***). Het enrich was significantly different from Het control with $p < 0.001$ (***Ac***), but Wt enrich or Homo enrich was not significantly different from its respective control (***Ab***, ***Ad***). ***B***, Percentage reference memory errors averaged from the last six trials were as follows (percent error mean ± SEM): Wt control, 44.3 ± 0.8; Wt enrich, 48.0 ± 1.4; Het control, 50.4 ± 2.0; Het enrich, 42.0 ± 1.2; Homo control, 56.4 ± 1.1; Homo enrich, 52.8 ± 0.8. Wt control compared with Het and Homo control, and Het control versus Homo control were all significantly different with $p = 0.002$, $p < 0.001$, and $p = 0.002$, respectively (***Ba***). Het enrich was significantly different from Het control ($p < 0.001$) (***Bc***), but Wt and Homo enrich were not different from their respective controls (***Bb***, ***Bd***). ***C***, Percentage working memory errors averaged from the last six trials were as follows (percent error mean ± SEM): Wt control, 14.0 ± 1.5; Wt enrich, 15.8 ± 1.5 (***Cb***); Het control, 18.4 ± 1.6; Het enrich, 18.7 ± 1.0 (***Cc***); Homo control, 24.3 ± 1.1; Homo enrich, 24.5 ± 2.2 (***Cd***). Wt and Het controls were significantly different from Homo control with $p < 0.001$ and $p = 0.01$, respectively, but Wt control was not different from Het control (***Ca***). As for the enrichment, none of the enriched groups were significantly different from their respective controls.

on any of the genotypes. These findings showed the severe deficits of Ng−/− mice in the learning and memory of several behavioral testing paradigms, and significant improvement was not attained through environmental enrichment, but evidently achieved among Ng+/+ and Ng+/− mice.

Enrichment-induced changes in hippocampal protein expression

Environmental enrichment has been shown to increase immunoreactivities of CREB (Williams et al., 2001), NR2A, NR2B, and GluR1 (glutamate receptor 1) (Tang et al., 2001), mRNAs of NR1, and BDNF (Guilarte et al., 2003) in the hippocampus. Figure 5, *A* and *B*, shows the representative immunoblot analyses depicting levels of hippocampal protein expression in control and enriched groups from experiments comparing Ng+/+ and Ng+/−, and Ng+/+ and Ng−/− mice, respectively. Figure 5*C* shows comparative levels of expression in control mice of all three genotypes. All comparisons were made with samples of control and enriched groups analyzed on the same gel and were expressed as percent of control as shown in Figure 5*D*. Enrichment has boosted Ng expression in both Ng+/+ and Ng+/− mice significantly over their respective controls (Ng+/+, 121.4 ± 2.6%, $p < 0.001$; Ng+/−, 131.0 ± 4.6%, $p < 0.001$). Because Ng+/− controls had 49.8 ± 3.4% of the Ng of Ng+/+ control mice, enriched Ng+/− mice increased their Ng to 65.2% of that of Ng+/+ control mice. Immunoreactivities of αCaMKII in all genotypes were increased among the enriched animals versus their respective controls (Ng+/+, 119.3 ± 2.9%, $p < 0.001$; Ng+/−, 117.9 ± 2.3%, $p < 0.001$; Ng−/−, 114.1 ± 4.2%, $p < 0.001$). Immunoreactivities of CREB in all genotypes were also increased among the enriched groups versus their respective control animals (Ng+/+, 117.2 ± 2.4%, $p < 0.001$; Ng+/−, 113 ± 2.9%, $p < 0.001$; Ng−/−, 110 ± 2.8%, $p < 0.001$). Although ERK has been implicated in long-term synaptic changes and behavior (Sweatt, 2004), environmental enrichment did not have a significant effect on the expression of ERK in all of these animals (data not shown).

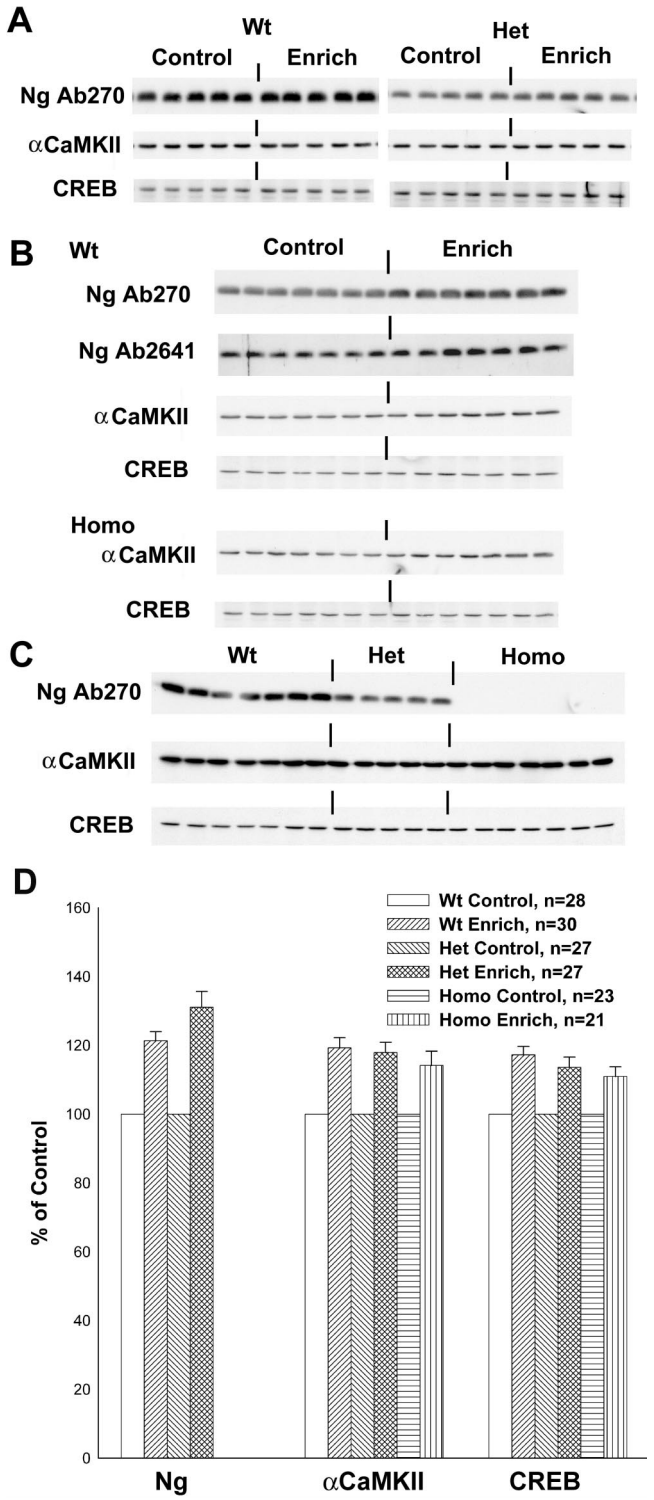

Figure 5. Immunoblot analyses for hippocampal Ng, αCaMKII, and CREB expression of control and enriched animals. Shown are representative immunoblots of hippocampal samples from experiments comparing Ng+/+ (Wt) and Ng+/− (Het) mice (***A***), Ng+/+ (Wt) and Ng−/− (Homo) (***B***), and control levels of expression of all three genotypes (***C***). ***D***, Number of animals used in the analyses was indicated in the graph. For some animals, both their right and left hippocampi were analyzed; thus, the actual number of samples included for statistical analysis was greater than the number of animals. Densitometric measurements were performed for all of the blots. Percentage changes in enriched samples were compared with those control samples that were blotted on the same membrane. Calculated from ***C***, Het control expressed Ng at 49.8 ± 3.4% ($p < 0.001$; $n = 13$) of that of Wt mice; thus, enriched Het expressed at 65.5% Ng of that of Wt. Expressions of αCaMKII and CREB were compatible among control animals of all genotypes (***C***).

Discussion

In the present study, we showed that environmental enrichment exerted many positive behavioral responses on Ng+/+ and Ng+/− mice, however, it did not rescue the cognitive deficits of Ng−/− mice. In addition, environmental enrichment enhanced LTP in the hippocampal CA1 area of Ng+/+ and Ng+/− mice, but had limited effect on that of Ng−/− mice. These observations suggested that the environmental enrichment protocol used in this study did not provide sufficient stimulation on Ng−/− mice to induce neurogenesis and modification of neuronal networks to be evident at behavioral and electrophysiological levels. Under our casual observation, we found that an enriched environment did increase the in-cage activity of the wild-type as well as the mutant mice as compared with those controls housed in a regular home cage, and that the enriched mice did make use of the running wheel for exercise. Recently, it was noted that mice with a point mutation that prevented autophosphorylation at threonine-286 of the αCaMKII were also nonresponsive to environmental enrichment to rescue their cognitive impairments (Need and Giese, 2003). In certain respects, Ng−/− mice resemble αCaMKII T286A mutant mice in their impairments of hippocampus-dependent spatial learning and memory and deficits in hippocampal LTP (Giese et al., 1998). It has been shown that autophosphorylation of αCaMKII at threonine-286 is necessary and sufficient to induce LTP (Giese et al., 1998; Bejar et al., 2002). The deficits of Ng−/− mice in the autophosphorylation of αCaMKII (Pak et al., 2000) provide a link in the similarities in phenotypes between these two mutant mice.

Ng has been proposed as a CaM buffer in neurons to modulate $[Ca^{2+}]_i$ by a "mass-action" mechanism (Huang et al., 2004), which predicts the greater the Ng level, the higher the $[Ca^{2+}]$-transients under any given stimulus-induced Ca^{2+} influx. Because postsynaptic influx of Ca^{2+} at the synapses in the hippocampal CA1 region is an essential step for the induction of LTP, in the absence of CaM-buffering Ng, the influxed Ca^{2+} will be sequestered by CaM (estimated cellular concentration ~20 μM) resulting in a small rise of free Ca^{2+}, too low to trigger the kinase-mediated phosphorylation cascades. It seems that Ng plays a pivotal role in gating neuronal Ca^{2+} signals for the activation of αCaMKII and other Ca^{2+}- and Ca^{2+}/CaM-dependent reactions for the enhancement of synaptic plasticity. Without Ng, the activation threshold of a neuron becomes too high to be responsive to environmental enrichment as examined behaviorally and electrophysiologically among Ng−/− mice.

Although the present enrichment protocol did not rescue Ng−/− mice from their impairments in cognitive behaviors and LTP, it enhanced the expression of signaling components αCaMKII and CREB; both are important for learning and memory. The extent of increases of these two proteins by enrichment were comparable among Ng+/+, Ng+/−, and Ng−/− mice; however, significant improvement in LTP and performances of certain behavior tests were seen among Ng+/+ and Ng+/− mice, but not Ng−/− mice. These findings indicate that a certain element of enrichment is responsible for the increased expression of these proteins but not sufficient for improving the behavioral performances. Recently, it was shown that different types of environmental enrichment, such as cognitive stimulation, voluntary exercise, and acrobatic training, had distinctive effects on spatial memory and expression of a presynaptic protein, synaptophysin (Lambert et al., 2005). For example, wheel running exercise, but not cognitive stimulation or acrobatic training, improves spatial

working memory relative to controls, despite the fact that both exercise and cognitive stimulation increase synaptophysin levels in the neocortex and hippocampus. It seems that our enrichment paradigms, which include rodent toys for cognitive stimulation and a running wheel for exercise, are effective to stimulate the expression of αCaMKII and CREB among the experimental cohorts. However, the enrichment-induced increase in these proteins is not sufficient to enhance LTP and improve cognitive behaviors of Ng−/− mice. Notably, enrichment also increases the expression of Ng among Ng+/+ and Ng+/− over their respective controls, and the enriched groups of these two genotypes exhibit enhanced LTP and improvement in certain behavioral tests. These results are indicative of the potential contribution of increasing Ng concentration in the enhancement of synaptic plasticity and learning and memory. Although, most interestingly, the enriched Ng+/− mice expressed 31% more Ng than their control, which reached merely 65% of the Ng level of the wild-type control, and yet their behavioral performance and LTP were improved to nearly as good as the wild-type control.

Among several behavioral-testing paradigms used in this study, we noticed that environmental enrichment improved the performances of Ng+/+ and Ng+/− mice in most of the behavioral tests. In the hidden platform version of the Morris water maze, enrichment induced a noticeable improvement of Ng+/+ and Ng+/− mice during the acquisition phase of the trial, as well as an increase in the acuity of seeking the location of the removed platform during the probe test. In the current study, we have reduced the number of trial/block to three from the previously used four-trial/block to slow down the rate of learning for monitoring the effects of environmental enrichment. Indeed, under this less-intensive training protocol, the performances of the controls of Ng+/+ and Ng+/− were not as good as their respective ones under the more vigorous training regimen (Huang et al., 2004). This was especially evident during the probe test, under which both Ng+/+ and Ng+/− controls searched for the missing platform over a much broader area as compared with the enriched mice, which focused more on the target quadrant.

In the step-down inhibitory avoidance task, enrichment enhanced the platform-retention time of Ng+/+ and Ng+/− but not significantly for Ng−/− mice. This fear-motivated learning is a hippocampus- and amygdala-dependent task. A single trial training profoundly enhanced the memory of Ng+/+, Ng+/−, and Ng−/− mice in an Ng dose-dependent manner (Fig. 3). These findings agree with our previous observations of the Ng dose-dependent improvement in performance in Morris water maze (Huang et al., 2004). It should be noted that even without Ng, as among Ng−/− mice, aversive foot-shock experience still can be registered as memory. Enrichment significantly enhanced the performance of Ng+/+ and Ng+/− mice with concomitant increase in their hippocampal Ng content; we have not yet determined whether the Ng level in the amygdala of the enriched group was also elevated. For Ng−/−, enrichment also seemed to exhibit a trend to improve performance, albeit not significantly, suggesting that Ng plays a pivotal role in transmitting enrichment-mediated synaptic responses.

In the food-motivated eight-arm maze, training improved the performances of Ng+/+ better than Ng+/− mice, and Ng−/− exhibited much less progress. Enrichment exhibited significant improvement for Ng+/− mice, especially their reference memory. Enrichment seemed to also improve the reference memory of Ng−/− mice during the early trials, but the improvement did not persist. Enrichment did not seem to have any effect on the working memory of all three genotypes of mice. It is interesting to point out that the present enrichment protocol has hardly improved the performances of Ng+/+ mice in the eight-arm maze. It is possible that this maze task is not that challenging and that the control Ng+/+ mice were already performing at their optimal levels.

In conclusion, environmental enrichment enhances the expression of hippocampal Ng, which positively correlates with the improvement of LTP and performances of spatial and emotional learning and memory for Ng+/+ and Ng+/− mice. Without Ng, like Ng−/− mice, an animal performs poorly on cognitive and emotional memory tasks and the enrichment affects these mice only minimally. The signaling mechanisms using Ca^{2+}, Ca^{2+}/CaM, and cAMP are significantly attenuated in Ng−/− mice (Wu et al., 2002). This provides a logical explanation for the poor performance of Ng−/− mice in a variety of cognitive tasks. Although enrichment also increases the expression of other important signaling molecules, such as αCaMKII and CREB in Ng−/− mice, these proteins cannot facilitate the learning without Ng to serve as an upstream regulator to enhance the neurotransmitter-mediated Ca^{2+}-transients. These results provide additional evidence for Ng playing a pivotal role in modulating the signaling pathways for learning and memory in mammals.

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