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Research report

Spatial learning alters hippocampal calcium/calmodulin-dependent protein kinase II activity in rats

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Abstract

This study investigated the role of hippocampal CaM-kinase II (calcium/calmodulin-dependent protein kinase II) in spatial learning. In Experiment I, three groups of rats received 1, 2 or 5 days of training on a spatial task in the Morris water maze with a hidden platform, while a control group was trained on a nonspatial task with a visible platform. The acquisition rate in the spatial task was slower than that in the nonspatial task. However, rats receiving 5 days of spatial training had the highest Ca^{2+}-independent activity of CaM-kinase II compared with the controls receiving nonspatial training and rats having 1 or 2 days of spatial training. Furthermore, the level of hippocampal Ca^{2+}-independent CaM-kinase II activity was correlated with the final performance on the spatial task. In Experiment II, rats received intra-hippocampal injections of a specific CaM-kinase II inhibitor – KN-62 – before each training session. In comparison with the vehicle-injected controls, pretraining injection of KN-62 retarded acquisition in the spatial task but had no effect on the nonspatial task. These results, taken together, indicated that the activation of CaM-kinase II in the hippocampus is not only correlated to the degree of spatial training on the Morris water maze, but may also underlie the neural mechanism subserving spatial memory.

Keywords: Hippocampus; Morris water maze; Excitatory synapse; Calcium/calmodulin-dependent protein kinase II; KN-62

1. Introduction

The hippocampus has been implicated in learning and memory [9]. Studies have shown that this brain structure is particularly involved in acquisition and storage of spatial information [23,24,26,28,33]. Animals with lesions of the hippocampus show impairments in the acquisition of various learning tasks relying on spatial cues, such as: the radial eight-arm maze [28], the circular platform task [23] and the Morris water maze [26]. Furthermore, long-term potentiation (LTP), a prominent feature of hippocampal physiology [4,5], is a prevailing neurobiological model for learning and memory [4,36]. LTP can be induced by behavioral learning engaging the hippocampus, and many treatments affecting LTP also influence the performance in learning tasks including the Morris water maze [27,32,39].

It is well established that LTP induction in the hippocampal CA1 region is dependent upon activation of *N*-methyl-D-aspartate (NMDA) receptors [7,8]. The NMDA receptor is one of the three ionotropic glutamatergic receptors, and opening of this ionophore requires sufficient postsynaptic depolarization in addition to ligand binding. Once opened, the NMDA channel allows transient influx of calcium ions, which in turn activate postsynaptic cellular mechanisms ultimately responsible for the induction of LTP [1,18–20,25]. Pharmacological studies have consistently shown that 2-amino-5-phosphonovaleric acid (AP5), a specific NMDA antagonist, not only blocks LTP induction but also interferes with spatial learning [27]. Conversely, administration of D-cycloserine to stimulate the glycine site of NMDA receptors facilitates LTP induction [3] as well as enhances behavioral learning in aged rats [2].

The influx of calcium ions into post-synaptic neurons results in the activation of various protein kinases [12]. Studies employing pharmacological interventions suggest that activation of protein kinases is necessary for the induction of LTP [18–20,30]. Among the variety of protein kinases present in the excitatory synapses, calcium/calmodulin-dependent protein kinase II (CaM-kinase II) is of particular interest because it alone consti-

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tutes 30 to 50% of total postsynaptic density protein [14]. Interestingly, studies have shown that activation of the CaM-kinase II causes phosphorylation of non-NMDA glutamate receptors, which could be blocked by either AP5 or a specific inhibitor – KN-62 [35]. Further, injection of activated CaM-kinase II enhanced kainate responses in hippocampal neurons [22]. Thus, CaM-kinase II may serve as a link between the induction and expression of LTP.

A role of CaM-kinase II in learning and memory receives support from the finding that mutant mice lacking the CaM-kinase II gene not only showed impaired LTP but also had much poorer performance than controls in learning the Morris water maze. Administration of KN-62 into the hippocampus or amygdala of rats has been shown to cause a memory deficit in an inhibitory avoidance task [39]. However, there is no evidence showing that hippocampal CaM-kinase II is indeed activated by spatial training, a point that is essential to hypotheses positing links between the enzyme and the encoding/utilization of spatial information. The present study addressed this issue by measuring the CaM-kinase II activity after various amounts of spatial and nonspatial learning, and examining the effect of suppressing hippocampal CaM-kinase II on acquisition of spatial versus nonspatial task.

2. Materials and methods

2.1. Subjects

110 male Sprague-Dawley rats (200–250 g) were used. They were housed in the animal center and maintained on 12:12 h light/dark cycle with light on at 7:00 A.M. Eighty rats were assigned by random into four groups in Experiment I ($n = 20$ for each group) to receive various degrees of training in the Morris water maze. Another 30 rats were divided into two groups in Experiment II ($n = 15$ for each group), which received intra-hippocampal injection of KN-62 or vehicle when trained in the Morris water maze.

2.2. Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Atropine sulfate (0.4 mg/kg) was given 30 min before anesthesia to prevent congestion. The anesthetized rat was mounted on a stereotaxic apparatus and two cannulae made of 23 gauge stainless steel tubing implanted bilaterally into the hippocampi. The coordinates were AP −4.3 mm, ML +2.0 mm, DV −3.0 mm according to Paxinos and Watson [29]. Three jewelry screws were implanted on the skull serving as anchors and the whole assembly was affixed on the skull with dental cement. The rats were monitored and handled daily. A week of recuperation was allowed before the behavioral training.

2.3. Behavioral tasks

2.3.1. Morris water maze

The water maze training was conducted in a circular pool of 2 m diam., with 30 cm deep of water. A circular platform (8 cm diam.) made of Plexiglas was placed 1 cm beneath the water level at a specific location for the entire session. Water was made cloudy by addition of milk. Distinctive visual cues were set up on the wall surrounding the pool. The behavioral training procedure was modified from that of earlier studies [26,27]. Briefly, the rat was accustomed to the water by swimming freely in the pool for 1 min and then removed from water and placed in a holding cage for 30 s before the learning trial began. In each training trial, the rat was placed into the water at random positions. The time for the rat to get onto the platform was recorded. The time limit for each trial was 90 s. After reaching the platform, the rat was allowed to stay on it for 30 s in each trial. Each daily session consisted 5 trials. Three groups of rats were trained on the maze for 1, 2 or 5 days, which represented the initial, early and asymptotic levels of acquisition, respectively. An additional group of rats serving as the non-spatial learning control was trained in the water maze under condition in which the platform was marked and elevated 2 cm above the water level.

2.3.2. Locomotor activity

To assess the effect of intra-hippocampal injection of KN-62 on motor activity, injected rats were subjected to an open-field test. An open-field platform (76 × 76 cm) was divided by lines into equal squares (4 × 4 cm) and elevated 65 cm above ground. The rat was placed at one corner and the number of lines it crossed within 5 min period was recorded.

2.4. Protein kinase assay

After receiving a predetermined amount of training, rats were sacrificed by decapitation. The hippocampi were quickly dissected out and frozen in liquid nitrogen. The samples were transferred to a −70°C freezer for storage until the biochemical assay [35].

Hippocampi were homogenized in 200 μl homogenization buffer: 50 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM EGTA, 5 mM EDTA; 1 mM DTT; 0.1% Triton X-100; 25 mM NaF, 100 mM β-glycerophosphate, 15 mM $NaPP_i$, 100 mM sodium vanadate, 0.4 mM microcystin; 5 μM leupeptin, μM trypsin inhibitor, 5 μM aprotinin, 1 mM PMSF. Samples were centrifuged at 14,000 × g for 5 min, and the supernatants were collected. The protein kinase assay was carried out at 30°C for 2 min, Syntide-2 was used as the CaM-kinase II substrate [13]. The reaction mixture was: (final conc.) 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, 1 mg/ml BSA, 5 μM PKA inhibitor, 2 μM PKC inhibitor, 40 μM [γ-^{32}P]ATP (specific activ-

ity 3000 cpm/pmol) and 40 μM syntide-2; in a final volume of 25 μl. In the total activity condition 1 mM $CaCl_2$ and 3 μM calmodulin were added; for the calcium-independent activity condition 1 mM EGTA was added instead. Each sample was assayed in duplicate. The reaction was stopped by spotting onto a P81 filter paper. The filter papers were washed several times with 75 mM phosphoric acids, and were counted by liquid scintillation counter.

2.5. Drug administration

Intra-hippocampal injection was delivered to a conscious rat. The rat was placed in a small container to restrain it from drastic movement. Drug was administered through a 30-gauge injection needle connected to a 10 μl Hamilton syringe by a polyethylene tubing (PE-100). The injection was administered at the rate of approximately 1 μl per min; a total of 1 μl KN-62 (i.e. 7.0 μg per site) was injected into each site. The rat was injected 15 min before each behavioral training session. KN-62, 1-(*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl)-4-phenylpiperazine, a specific CaM-kinase II inhibitor [37] was purchased from Seikagaku Inc., and was dissolved in 0.1% dimethyl sulfoxide (DMSO) diluted with saline. DMSO at this concentration was used for control injection.

2.6. Histological verification

Animals were sacrificed with an excess dose of sodium pentobarbital (i.p.). The brain was perfused through the heart with saline followed by 10% formalin. Brain sections (40 μm) were collected and stained with cresyl violet. Cannulae placements were identified as tracks on the stained brain sections.

2.7. Statistical analysis

The escape latency in the Morris water maze and the number of lines crossing in open-field activity, as well as counts of the syntide-2 phosphorylation were analyzed by analysis of variance and Student's *t*-tests. The Pearson product-moment correlation coefficient between behavioral performance at Day 5 and the level of activated CaM-kinase II activity of the rats was calculated and statistically tested for significance.

3. Results

3.1. Experiment I

In general, rats showed improvement in the escape latency over time in the Morris water maze learning (Fig. 1). However, the improvement rate of the rats in the spatial learning groups was significantly lower ($F_{1,4} = 89.106$, $P < 0.001$) than that in the non-spatial learning control group. The rats in the non-spatial learning group could learn to escape by visualizing the salient platform. Separate *t*-tests indicated that the escape latency (mean ± S.E.M.) of the rats in the spatial learning groups was significantly less on Day 5 (3.850 ± 0.988) than on Day 1 (45.360 ± 3.000) and Day 2 (36.880 ± 3.320) ($P < 0.001$). There were no statistical differences in the escape latency between Day 1 and Day 2 for the rats in the spatial learning groups. However, the escape latency of the rats in the visually guided group was significantly reduced on Day 2 (16.870 ± 2.044) compared to that at Day 1 (34.750 ± 3.333) ($P < 0.001$). This result suggested that substantial learning had occurred in one session, if the escaping response could rely on direct visualization of the marked platform. Rats in the spatial learning groups, who had to rely on spatial cues to locate the submerged platform position, showed very little learning for the first two days of training.

The biochemical assays showed no differences in the total CaM-kinase II activity across the groups (Fig. 2). However, the 5-day training rats in the spatial learning group on day 5 had a significantly higher percentage of Ca^{2+}-independent activity of hippocampal CaM-kinase II than did the rats in the visually guided group (13.334 ± 1.870 vs. 6.505 ± 1.342; $t_{38} = 5.861$, $P < 0.001$) (Fig. 2). There were no differences in the percentage of Ca^{2+}-independent activity of hippocampal CaM-kinase II between the visually guided controls and the spatial learning group on Day 1 or Day 2. Furthermore, the percentage of Ca^{2+}-independent activity of hippocampal CaM-kinase II was inversely correlated with the behavioral performance of rats on Day 5 ($r = -0.884$, $P < 0.001$) (Fig. 3).

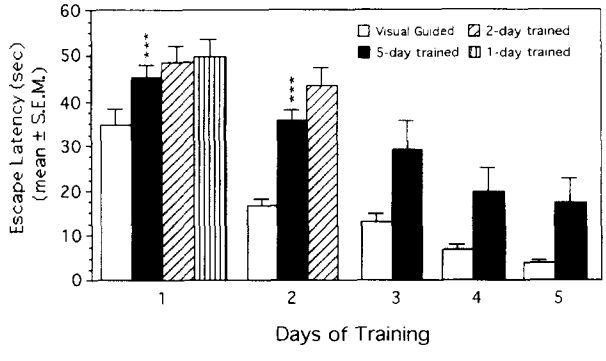

Fig. 1. Acquisition performance of rats in the Morris water maze. The rats in the visually guided control group were trained on the maze with a visible platform; other groups of rats were trained with a submerged platform for 1, 2, or 5 days. There was a significant difference in the escape latencies between the control and the 5-day groups ($F_{1,4} = 89.106$, $P < 0.001$). There were statistically significant differences in the performance between Day 1 and Day 5 ($P < 0.001$)as well as between Day 2 and Day 5 ($P < 0.001$) for the 5-day training group.

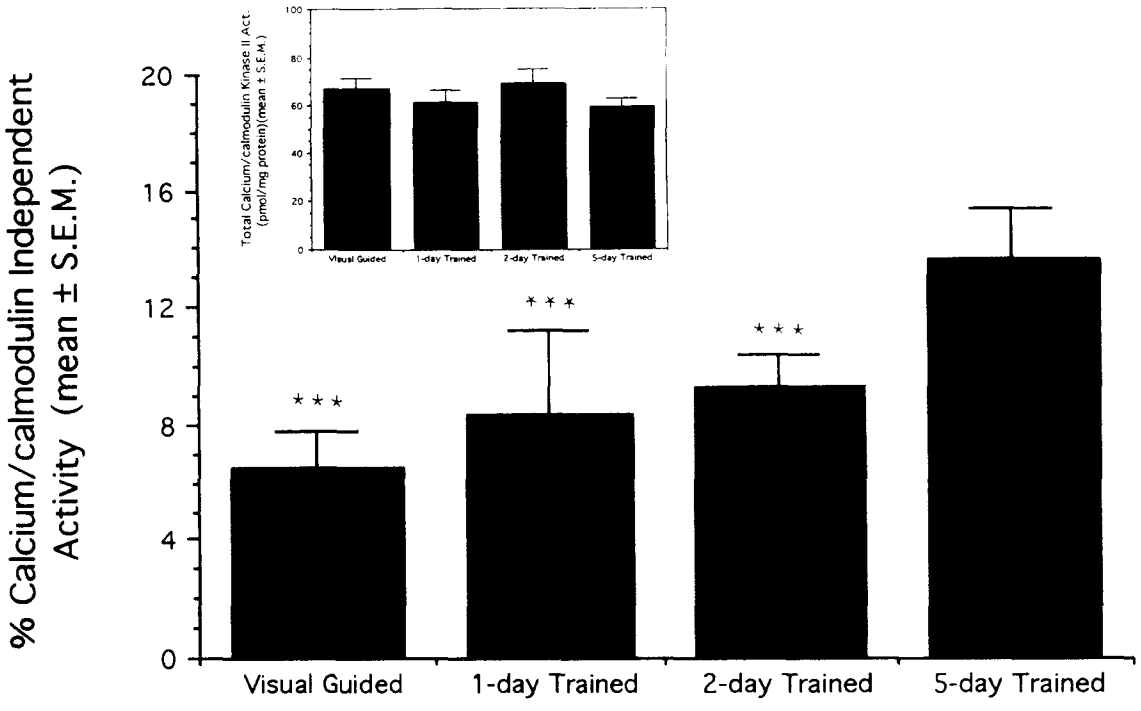

Fig. 2. Percentages of Ca^{2+}/calmodulin-independent CaM-kinase II activity in the rat hippocampi after Morris water maze training. There was a significant elevation of the Ca^{2+}/calmodulin-independent activity in the hippocampi after 5 days of training on the spatial water maze relative to the other three groups ($P < 0.001$). No differences were found among 1-day, 2-day spatial training groups and visually guided groups. The inset shows total CaM-kinase II activity, no difference in the total CaM-kinase II activity among the four groups.

3.2. Experiment II

Rats showed deficits in the Morris water maze learning after intra-hippocampal injection of KN-62. The KN-62 injected rats showed no improvements in the escape latency throughout five days of training, in contrast to the normal acquisition rate of the vehicle (0.1% DMSO) injected controls (Fig. 4). There was a statistically significant difference in the escape latency between the KN-62 and vehicle injected groups on Day 5 ($t_{28} = 30.469$, $P < 0.001$). As a supplementary control, four intra-hippocampal KN-62 injected rats were tested in the Morris water maze under a visible platform setting. These rats showed no deficit in the acquisition, indicating that the KN-62 effect was specific to spatial learning. Locomotor activity did not differ between the KN-62 or vehicle injected groups. There were an average of 138.50 ± 8.30 squares crossed by rats from both groups in 5 min. The histological verification confirmed the positions of the cannula tips were indeed in the hippocampal region.

The CaM-kinase II activity of intra-hippocampal injected rats were assayed on Day 5. There were no differ-

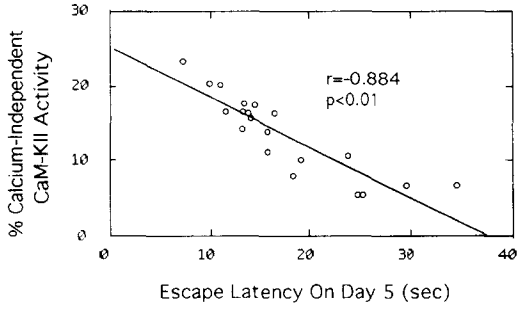

Fig. 3. The correlation between the escape latency and the hippocampal kinase activity of the spatial training rats on day 5. $r = -0.884$; $P < 0.001$.

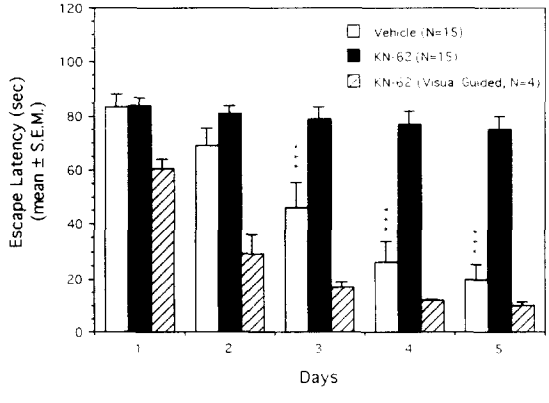

Fig. 4. Performance of rats in the Morris water maze after receiving KN-62 (shaded bar) or vehicle (open bar) during acquisition. Intra-hippocampal injection of KN-62 impaired acquisition of the spatial task ($F_{1,4} = 126.414$, $P < 0.001$). The striped bar represents the rats injected with KN-62 and were trained on the maze with a visible platform. There was a statistically significant difference between the KN-62 and vehicle groups in the escape latency on Day 3, 4 and 5 ($P < 0.001$). The rats injected with KN-62 were able to learn the task if the platform was visible.

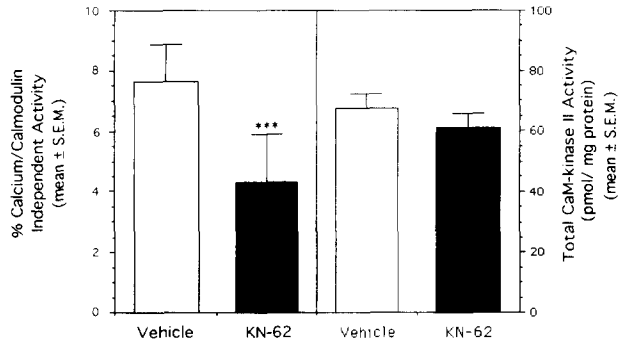

Fig. 5. The CaM-kinase II assay of the rat hippocampi after injection of KN-62 (shaded bar) or vehicle (open bar). There were no differences in the total kinase activity between the groups. However, the KN-62 injected group had a significantly lower percentage of Ca^{2+}/calmodulin-independent CaM-kinase II activity ($t_{26} = 5.768$, $P < 0.001$).

ences in the total CaM-kinase II activity between the KN-62 and vehicle injected groups (Fig. 5). However, the percentage of Ca^{2+}-independent activity of hippocampal CaM-kinase II was significantly lower in the KN-62 injected group (including the four KN-62 injected rats trained on the visible platform setting) than in the vehicle injected group (4.302 ± 1.667 vs. 7.505 ± 1.342) ($t_{26} = 5.768$, $P < 0.001$).

4. Discussion

Results of Experiment I indicated that activity of CaM-kinase II in the rat hippocampus was significantly altered by training on the Morris water maze, a task widely used for assessing spatial learning and memory in behavioral studies [26,27,31]. Rats in the spatial learning group learned the task gradually and their performance did not reach the asymptote until Day 4 or Day 5. This improvement rate was slower than that in non-spatial learning. The biochemical assay of the hippocampal CaM-kinase II demonstrated that in comparison with the non-spatial learning controls, the percentage of Ca^{2+}-independent activity was significantly increased only by spatial training.

The elevation of Ca^{2+}-independent activity appeared only after 5 days of spatial training but not after 1 or 2 days of training. Therefore, this elevation of Ca^{2+}-independent activity is not likely due to stress or emotional arousal involved in water maze learning, otherwise the change would have been most prominent on Day 1 of spatial training, at which time the rat presumably was most highly stressed [17]. The 2-day trained group was designed to represent an early stage of learning, during which time the memory trace for performing the Morris water maze learning had not been well established. Thus, the relative elevation of Ca^{2+}-independent activity of CaM-kinase II becomes apparent in the hippocampus of a rat learning spatial information only when it approached asymptotic performance level.

It is interesting to note that the percentage of Ca^{2+}-independent CaM-kinase II activity assayed immediately after the behavioral performance was highly correlated with the escape latency on Day 5 of spatial training. This correlation could not be interpreted by performance factors such as less motor efforts invested in escaping because the non-spatial learning group which escaped even faster showed no such activity elevation. This finding suggests that the activated level hippocampal CaM-kinase II may be involved in fine tuning of performance in a spatial memory task once a certain level of mastering has been reached. Thus, the strength of a formed memory trace for a spatial task may be related, at least in part, by the level of activated CaM-kinase II in a hippocampal circuit.

Results of the second experiment establish a causal relationship between the hippocampal CaM-kinase II activation and spatial learning. The rats receiving intra-hippocampal injection of a specific inhibitor of CaM-kinase II – KN-62 [37] – showed a marked acquisition deficit in spatial learning. These results are consistent with and extend previous findings that intra-hippocampal or intra-amygdala injection of KN-62 impairs retention in an inhibitory avoidance task [39]. Because the drug was given prior to training in each session, the observed effect may be due to influences of the drug on sensory or motor abilities rather than learning spatial information per se. However, the lack of effects of KN-62 on the visually guided non-spatial learning task and the open field activity renders this interpretation unlikely. The vehicle injected control rats were able to acquire the task at a rate comparable to that in Experiment I in which no injection was given, this rules out the possibility that any toxicity of 0.1% DMSO would have contribute to the effect of KN-62 on spatial learning. These findings thus suggest that KN-62, a drug which specifically blocks the calmodulin binding site of CaM-kinase II and results in failure of CaM-kinase II activation [37], specifically retards the acquisition of spatial learning.

It is tempting to speculate the mechanism of how Ca^{2+}-independent CaM-kinase II in the hippocampus is involved in the memory processing of spatial information [15,16]. The abundance of CaM-kinase II in the hippocampus, particularly in the postsynaptic density [14] invites the prediction that the enzyme may play a significant role in the regulation of synaptic functions, such as excitatory transmission in the glutamatergic system [12]. A model has been proposed suggesting that CaM-kinase II may mediate the interaction of NMDA and non-NMDA receptors in LTP induction and expression [34]. Thus, CaM-kinase II may mediate the potentiated neural activity in the circuitry underlying spatial learning.

The elevation of Ca^{2+}-independent CaM-kinase II suggests that this enzyme may still effectively catalyze whatever the changes underlying the neuronal and behavioral plasticity even after neural activation or behavioral training. However, the persistence of the hippocampal Ca^{2+}-in-

dependent CaM-kinase II is not congruent with the duration of spatial memory [6,12]. Given that continuous kinase activity does not appear to be required for LTP maintenance [21], and that the phosphorylated substrate protein has a relatively short half-life, it can be assumed that the plasticity underlying behavioral changes may involve modifications in biochemical cascades beyond phosphorylation of existing proteins. The involvement of CaM-kinase II in stimulation of gene expression [10] and regulation of gene transcriptional processes [38] have been reported. Therefore, the phosphorylation activity of CaM-kinase II may act on both the receptor (or other cytosolic) proteins and the nuclear phosphoproteins. Both processes are the crucial characteristics of neuronal plasticity. The significance of these processes in acquisition and storage of spatial information should be explored in the future.

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