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Memory-improving effect of formulation-MSS by activation of hippocampal MAPK/ERK signaling pathway in rats

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MSS, a comprising mixture of maesil (*Prunus mume Sieb. et Zucc*) concentrate, disodium succinate and Span80 (3.6 : 4.6 : 1 ratio) showed a significant improvement of memory when daily administered (460 mg/kg day, p.o.) into the normal rats for 3 weeks. During the spatial learning of 4 days in Morris water maze test, both working memory and short-term working memory index were significantly increased when compared to untreated controls. We investigated a molecular signal transduction mechanism of MSS on the behaviors of spatial learning and memory. MSS treatment increased hippocampal mRNA levels of NR2B and TrkB without changes of NR1, NR2A, ERK1, ERK2 and CREB. However, the protein levels of pERK/ERK and pCREB/CREB were all significantly increased to 1.5 ± 0.17 times. These results suggest that the improving effect of spatial memory for MSS is linked to MAPK/ERK signaling pathway that ends up in the phosphorylation of CREB through TrkB and/or NR2B of NMDA receptor. [BMB reports 2008; 41(3): 242-247]

INTRODUCTION

Learning capabilities, including memory, are central processes that affect on the daily life and activity of modern persons. These include a series of mental processes, for example, attention, short term memory, long term memory, reasoning, coordination of movement, planning of tasks and so on. Also, learning capabilities usually influence directly the mental activity of modern persons, and ultimately, have an effect on the quality of life. There is now a consensus that the human hippocampus is involved in episodic memory (memory of events or episodes that one experienced personally at a particular time and place). Likewise, it is out of the question that the hippocampus is associated with spatial or positional memory in animals other than human beings (1). A change in the hippocampus has a close connection with learning, memory, emotional control or the like. The hippocampus shows synaptic plasticity in response to various paradigms including long term potentiation (LTP) (2) of which spatial training in the Morris water maze (MWM) has been the most actively investigated (3, 4).

Several intracellular signal transduction pathways are related to transmission of information that propagated the initial signal from interaction of membrane-receptor to the nucleus. CREB (cyclic AMP-response-element-binding protein) located within the nucleus is transcription factor interacting importantly in transmission process of stimulus-transcription coupling that extra-cellular stimuli to cell membrane elicit changes in gene expression. Change of gene expression affects ultimately the function of individual neuron and whole neuronal circuit by regulation of expression of several neuronal proteins (5). Adenylyl cyclase (AC), cAMP, Ca^{2+} and mitogen-activated protein kinase (MAPK) are associated with CREB-regulated gene expression as well as CREB. This signal transduction pathway has been researched severely since the pathway was implicated being involved in synaptic plasticity (6) and induction of antidepressant effect (7). Pretraining infusions of antisense oligodeoxynucleotides directed against CREB mRNA significantly impaired memory for MWM test (8). In CA1 of hippocampus, phosphorylation of CREB increased during forms of LTP (9, 10). In addition, expression of NMDA receptor (NMDAR) and tyrosine receptor kinase B (TrkB) has been shown to be related to signal transduction mechanism for memory formation (11). TrkB, a receptor of brain-derived neurotrophic factor (BDNF), is also necessary for the synaptic plasticity in the CA1 region of the rat hippocampus and for the retention of memory (12) as well as NMDAR (13).

Succinic acid (SA) is an intermediate metabolite of tricarboxylate cycle (TCA cycle) and is typical compound playing a very important role in the energy metabolism of brain mitochondria. Succinic semialdehyde (SSA) and succinic acid disodium salt (SS) were administered to normal subjects in order to observe the higher nerve activity (14). As a result, it was suggested that SSA enhances the excitability of the cerebral cortex to increase verbal system activity, whereas SS has the possibility of psychoenergizer that stabilizes the excitability of the cerebral cortex. However, there is still no study on the effect of SA on the improvement of memory and learning capabilities related to normal hippocampal conditions. The dicar-

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boxylic acids of the TCA cycle are known to be very limited with respect to passage through the blood-brain barrier (BBB), thus, the way by which dicarboxylic acids influence the brain can also be limited (15, 16). However, stress conditions influence the state of the brain hippocampus according to the regulation of CRF (corticotrophin releasing factor) by HPA-axis rather than BBB. Anti-depressant drugs can inhibit steroid transporters in BBB and neuron in patients with depression, animal and cell models. Corticosterone is associated with the inhibitory mechanism of the steroid transporters via HPA-axis, which is increased due to endogenous glucocorticoids (17, 18). Accordingly, it is expected that, when anti-depressant drugs are administered in combination with the dicarboxylic acid SA in a normal condition for a given period of time, they may have an effect on the plasticity-memory ability of the brain hippocampus. Authors found the putative antidepressant effect of maesil (*Prunus mume Sieb. et Zucc*) concentrate (MS) and then examined the various combining effects on the spatial memory. We finally obtained a comparative improved formulation on the basis of the spatial memory test from the combination of MS, SA or its disodium salt (SS), and sorbitan-monooleate (Sp, span 80). MSS, a composition comprising a mixture of MS with SS and SP (MS : SS : SP = 3.6 : 4.6 : 1 ratio), showed a significant improvement of memory in normal rats. We provide an evidence that the MSS may act as an activator of MAPK/ERK (The mitogen-activated protein kinase/extracellular-signal regulated kinase) signaling pathway which mediates a memory formation on the basis of behaviors in this paper.

English abbreviations used to indicate the test components: In each of animal tests, the name of each group was defined using the English abbreviations of administered components. English abbreviations used to indicate the components are as follows, SA: succinic acid; SS: succinic acid disodium salt; MS: maesil concentrate; Sp: Span80; and DW: distilled water. When various components are administered at the same time, the compositions will be listed using the abbreviations thereof. The amount (mg) of each component administered per kg bodyweight of each test animal was described as a subscript on the name of each composition. For example, a test animal group administered with mixture of 180 mg of MS, 230 mg of SS with 50 mg of Span80 per kg of bodyweight was described as $MS_{180}SS_{230}Sp_{50}$ (MSS).

RESULTS

In MWM, Co-administration of SA with Span80 enhanced the learning ability as compared to SA-administered group in normal rats, although the working memory in correct quadrant of $SA_{100}Sp_{100}$-administered group was not so significant (Supplementary-Fig. 1, Supplementary-Table 1). The administration of MS_{180} did not show any significant differences as compared to control during the 4 sessions of learning, and in the short term working memory index. However, the sole administration of MS for 3 weeks showed dose-dependent antidepressant effect at MS_{90}, MS_{180} and then maintained the level to some extent at MS_{360}, MS_{720} in TST (Supplementary Fig. 2). Thus, MS_{180} was

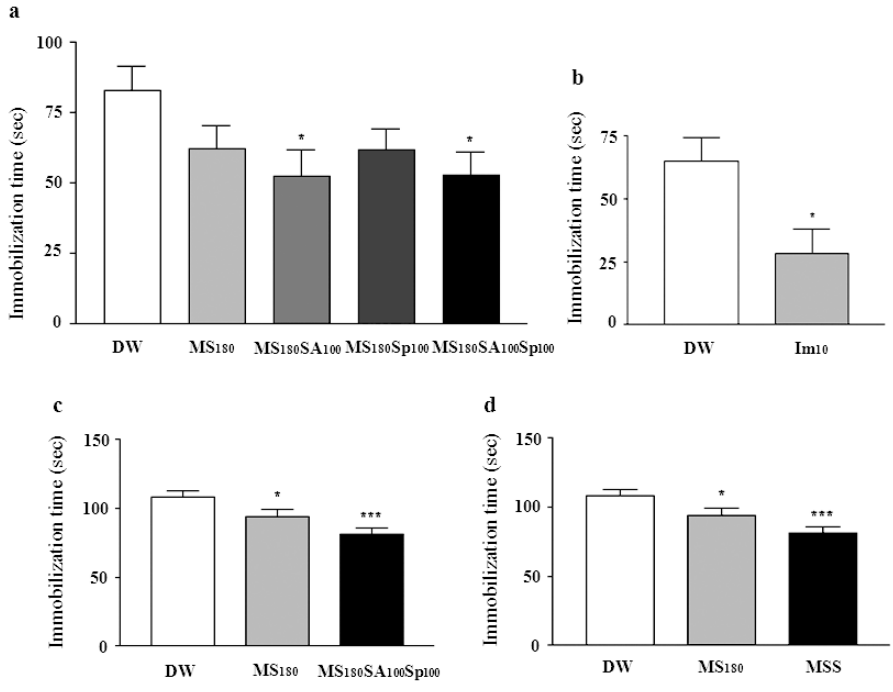

Fig. 1. Anti-depressive effects on the treatment of MSS in tail suspension test. Values are expressed as the mean values ± S.E.M. *, $P < 0.05$; ***, $P < 0.001$ vs control in Dunnett's Multiple Comparison Test. ICR mice were observed for 5 min in tail suspension test (TST) after oral administration of drug or formulations for a week (n = 18-20/group). Both $MS_{180}SA_{100}$ and $MS_{180}SA_{100}SP_{100}$ were significantly showed anti-depressive effect in TST compared to their control values. Imipramine (Im, 10 mg/kg b.w.) as a positive control was intraperitoneally injected once a day for a week. Formulation $MS_{180}SA_{100}Sp_{50}$ (lower left) and $MS_{180}SS_{230}Sp_{50}$ (lower right) also exerted a significant difference in TST as well as working memory test (See text, Fig. 2). Im10: imipramine 10 mg/kg b.w.

selected as a suitable dosage by considering further experiments. Interestingly, the mixtures containing MS_{180} as combined with SA_{100} and/or Sp_{100} showed more potent antidepressive effects than MS_{180} alone when orally administered for one or three weeks in TST (Fig. 1). The combining effect with MS_{180} in working memory test suggested that MS sould be related with sensory motor activity. Unfortunately, $MS_{180}SA_{100}$ mixture without Span 80 did not clearly show significant improving effect on the escape latency in learning, and retention time in working memory test, and then index in the short-term working memory test (supplementary Fig. 3). However, $MS_{180}SA_{100}SP_{100}$ administration for 3 weeks exerted a significant increase of swimming velocity in working memory test caused by the increase of distance moved in correct quadrant (Supplementary Table 1). Therefore, further investigation was needed to know whether the combined treatment of MS and SA, with Span80 could actually increase the moving distance (or velocity) in both test of working memory and its short term working memory or not. After trying to vary the Sp concentration, in order to get the best formulation under our experimental conditions, we found that the concentration of Sp could give similar effects in 50, 75, 100 mg/kg b.w (data not shown). Among these dosages we fixed arbitrarily the concentration of Sp as 50 mg/kg. Fig. 2 has shown the comparative results of $MS_{180}SA_{100}$ with Sp_{50} on learning and memory in MWM test. Formulation-$MS_{180}SA_{100}Sp_{50}$ exerted not so significant effect in learning (two-way ANOVA F: 3.476, P = 0.0675 vs control) (Fig. 2) but significant in antidepressant effect (lower left graph of Fig. 1). Unlike formulation- $MS_{180}SA_{100}Sp_{50}$, MSS showed significant effects in learning (P = 0.035, f = 9.304) and memory (Fig. 2) as well as antidepressant effect (lower right graph of Fig. 1). In MSS-administered rats, the working memory increased significantly about 1.7 times as compared to control in moved distance within 30 cm diameter circle where platform had located at the center of the quadrant, and then the short term working memory index was increased about 1.3 times (Fig. 2). For a reference, the mean frequency passed through the area of platform in this test increased 1.6 times in MSS administered group as compared to control data.

In order to explain the behavior-analysis data of the MWM test as a molecular signal transduction mechanism, following the 3-week administration of MSS, the brain hippocampus of the test animals was isolated after the final behavior test. RNA was isolated from the brain hippocampus, and then cDNA was constructed from the isolated RNA. Using the cDNA as a template, real-time PCR was performed using primers of NR1, NR2A, NR2B, ERK1, ERK2, TrkB, CREB and BDNF, and the mRNA copy level relative to GAPDH was calculated (Fig. 3A). As results, the mRNA levels of TrkB and NR2B were significantly increased compared to those of the control group, but there were no differences in other mRNA levels between the two groups. Also, when the pERK1/2 and pCREB were analyzed, the amounts of the ERK1/2 and CREB proteins were not changed, however, the phosphorylation of the proteins was significantly increased as compared to control group (see Fig. 3B, $P < 0.01$, vs. control). It suggests that, when MSS was orally and daily administered for 3 weeks, a memory-related signal transduction process should be significantly activated to enhance synaptic plasticity. Also, this shows that the change in the signal transduction-related molecules of the test animals may correlate with the improvement of memory and learning functions.

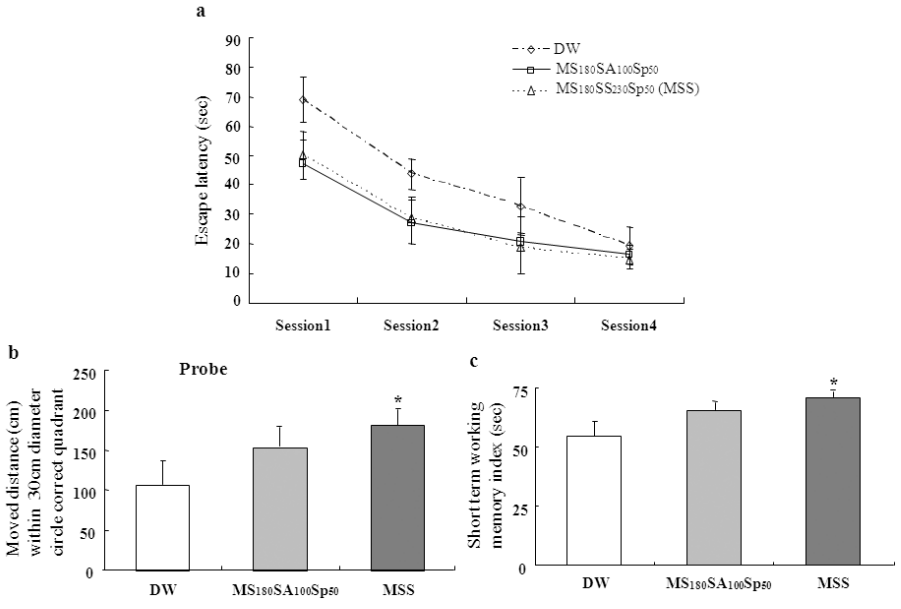

Fig. 2. Effect of spatial memory and learning on 3-week-treatment of MSS in Morris water maze test. Values are expressed as the mean escape latencies ± S.E.M. of 4 trials/session/day for 4 days of training in Morris water maze test. *, $P < 0.05$ vs control in both probe trial test (lower left) and the short term working memory index test (lower right). The results suggest that formulation-MSS, including sodium salt (SS) instead of SA, is rather effective enhancing the both working memory and short term working memory index. The short-term working memory index was measured by 4 trials per session at the same starting position after putting on the hidden escape platform located in opposite site. The index values were obtained by the subtraction of escape latencies between the first and the average of subsequent trials. Morris water maze test began at the 1st day of the 3rd week of administration. MSS: $MS_{180}SS_{100}Sp_{50}$.

DISCUSSION

A dicarboxylic acids in TCA cycle intermediates such as SA has a possibility to by pass BBB to some extent by the sodium dependent dicarboxylate transporters NaDC-3 (19), though it is not concretely known whether NaDC-3 exists in BBB and takes charge of the transport them into the brain or not. The BBB transport ratio of these TCA cycle dicarboxylic acids was much lowered as results of the experiment on dicarboxylic acid transport and metabolism examined after injection of isotope labeled fumaric acid, malic acid into mice (15). There were no data about SA transport in BBB. However, the composition comprised of MS, SA and Sp improved "the short-term working memory" when tested just after probe trial, compared to the results of the control.

Evidences from the studies of human and animal indicate that stress inhibits a series of various hippocampal memory (20). It has been characterized that memory and concentration ability significantly decrease in most case of the depression (21). This is the reason why prescribes psychotropic drugs in the clinical treatment (22). With regard to the effects of maesil, an effect for the facilitation of intestinal absorption (23) and a relaxing effect for the stress (24) were already reported. However, no evident data about the anti-depression effect was provided thus far.

Our study did not directly show how MSS roles in the supply of substrate "SA" in passing through BBB. However, our data suggested that MSS fortified hippocampal memory and/or improved the memory and learning signal transduction pathway. Among various compositions tested, MSS showed most significant effect on overall test process such as "escape latency" and "working memory" test. MSS also subsequently improved the short-term working memory. The minimal effective interval to improve memory and learning was thought to need at least 3 weeks of consecutive daily treatment for the administration of MSS under our experimental conditions (data

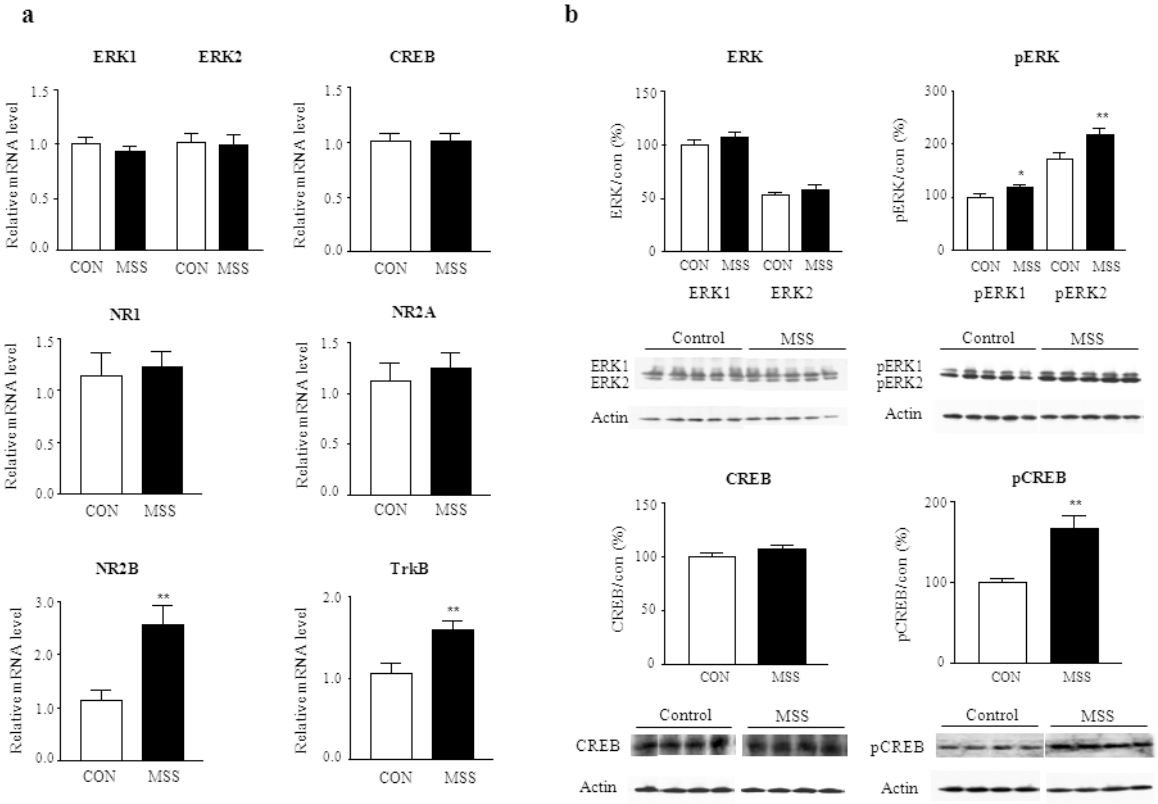

Fig. 3. Analysis of memory-related signal molecules in the hippocampus of MSS-administered rats. Each data represents the mean ± SEM (n = 7). *: $P < 0.05$ and **: $P < 0.01$ versus control values in Student's t test. Formulation-MSS ($MS_{180}SS_{230}SP_{50}$) was administered daily for 3 weeks. (a) mRNA expression of memory related genes. After testing the short term working memory, the expression level of mRNA on ERK1, ERK2, CREB, TrkB, NR1, NR2A, NR2B was evaluated in hippocampus of rats. The mRNA levels of TrkB and NR2B increased significantly as compared with control (**$P < 0.01$), respectively. MSS administration increased TrkB 1.5 times of control, and NR2B 2.3 times of control, but there were no differences in other mRNA levels between the two groups. (b) Western blot analysis and their relative densitometries of ERK1/2, pERK1/2, CREB and pCREB expression. The protein levels pERK/ERK and pCREB/CREB were all increased to 1.27 and 1.68 times, respectively.

not shown). It was suggested that the treatment of composition containing Sp was necessary to get synergic effect. Sp (Span80) is structurally correspondent to tween80. Though tween80 was also effective on memory when used with SA, we rather investigated with span80 in this paper to select more simple structure than tween80, which has no polyoxyethylene moiety in its molecular structure. Considering the roles of surfactant (25), Sp in MSS composition was thought to act on the absorption, metabolism or distribution of SS rather than that of MS. However, this case has not yet been properly investigated.

The minimal effective interval to improve memory and learning was thought to need at least 3 weeks of consecutive daily treatment for the administration of MSS under our experimental conditions (data not shown). To compare the formulation MSS-administered group with control, we analyzed the changes of the signal-transduction molecules after finishing overall behavioral tests. Hippocampus separated from each rat brain was employed to study improving effects of learning and memory (Fig. 3).

As results, MSS treatment increased hippocampal mRNA levels of NR2B and TrkB to 2.3, 1.5 times, respectively ($P < 0.01$) (Fig. 3A). The mRNA levels of NR1, NR2A and protein ERK1, ERK2 and CREB were not changed but the pERK and pCREB were all increased to 1.27 and 1.68 times, respectively ($P < 0.01$) (Fig. 3B). Activation of ERK in hippocampus fortifies the memory through a variety of learning in mammals and various kinases involved in the phosphorylation of CREB, ERK is a regulator of CREB phosphorylation, which performs an important role in memory and synaptic plasticity (26).

In conclusion, we presented behavioral evidences that MSS-formulation improved memory and learning ability and enhanced anti-depressant effect. When accompanied with the anti-depressant effect, the formulation improved spatial memory behavior at the molecular signal transduction level. Our results suggest that the improving effect of spatial memory for MSS is linked to MAPK/ERK signaling pathway that ends up in the phosphorylation of CREB through TrkB and/or NR2B of NMDA receptor.

MATERIALS AND METHODS

MSS and animals

MSS was prepared from the three materials mixture comprising maesil concentrate (MS) with disodium succinate (SS) and Span80 (Sp) (3.6 : 4.6 : 1 ratio). Disodium succinate (SS, sodium succinate dibasic hexahydrate) and Span80 (Sorbitan monooleate, Sp) were purchased from Sigma and Aldrich, respectively. Adult Sprague Dawley rats (male, 6 weeks old) for Morris water maze (MWM) test and ICR mice (male, 4 weeks old) for Tail Suspension test (TST) purchased from Core tech., Central Animal Research Facility, Korea, were used after a week adjustment in the new environment.

Behavioral tests

MWM test began at the 1st day of the 3rd week of administration. After 4 or 5 days of the final learning training, a working memory test called as probe trial was performed without platform at 24 hr later. In the short-term working memory test which subsequently performed after probe trial, data analysis was focused upon the savings in escape performance between the first and subsequent trials (27, 28). Authors expressed the short-term spatial memory as a 'short-term working memory index'. These values, more detailed, were obtained by the subtraction of the escape latencies between the first and the average of subsequent 2nd-4th trials (See the supplementary information). Anti-depressant effect was tested using a tail suspension test (TST), which is most generally used for the screening of anti-depressant drugs (29).

Analysis for the changes of signal transduction molecules in brain

In case of test animals, which showed a significant difference in a probe test, the brain hippocampus was isolated and then, the mRNA expression levels of memory and learning-related signaling substances e.g., CREB, NMDAR (NR1, NR2A, NR2B), ERK1/2 and TrkB were examined comparatively with the control group in order to analyze the improvement of learning and memory function of the composition-administered group with regard to molecular signaling mechanisms.

Analysis for phosphorylation of ERK1/2 and CREB in brain

The brain hippocampal tissues from each rat were rapidly removed and homogenized in lysis buffer. The supernatant of the 13,000 rpm of homogenate was used as a test sample after protein assay. Equivalent amounts of protein for each sample were resolved in 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinilidene difluoride (PVDF) membranes (Amersham Bioscience). The primary antibodies used were pERK1/2 (phosphorylated ERK1/2), ERK1/2 (1 : 1,000, Santa Cruz); pCREB (phosphorylated CREB), CREB (1 : 500, Upstate). Quantitation was performed with reference to the invariant cytoskeletal protein, β-actin and expressed additionally as a percentage of control.; β-actin (1 : 1,000, Sigma).

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