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Molecules and Cells

Changes in Vascular Endothelial Growth Factor (VEGF) Induced by the Morris Water Maze Task

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The present study was undertaken to evaluate the effects on hippocampal vascular endothelial growth factor (VEGF) levels in rats when they experience hippocampal-dependent spatial learning via the Morris water maze (MWM) task. Rats underwent one of two different versions of the MWM: weak or intensive. After one day of intensive training, a highly sensitive enzyme-linked immunosorbent assay (ELISA) was used to measure VEGF protein levels in the hippocampus, cortex, and serum, and higher levels were found in the trained group compared to a naive control group. VEGF levels also increased in rats that swam only for durations equal to the intensive training periods. In contrast, rats trained under the weaker MWM paradigm for five days showed a decrease in hippocampal VEGF protein level. Mimicking increases in neuronal VEGF in the hippocampus by direct infusion of VEGF into CA1 resulted in up-regulation of the phosphorylation of the cAMP response element-binding (CREB) protein and the Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII). These results suggest that VEGF may be a physiological parameter involved in learning procedures that include physical activity.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen that plays a critical role in angiogenesis under physiological and pathological conditions (Leung et al., 1989; Takahashi and Shibuya, 2005). In recent years research on the functions of VEGF and VEGFR has expanded to include effects on neurons, with both neuroprotective and neurotrophic effects being reported (Nowacka and Obuchowicz, 2011). For example, enhancing the expression of VEGF increases neurogenesis in the subgranular zone of the dentate gyrus in the hippocampus (Jin et al., 2002). In addition, enhancing the expression of VEGF in the rodent hippocampus by gene delivery not only enhances neurogenesis, but also results in improved behavioral performance in learning and memory (Cao et al., 2004). While these studies demonstrate that chronic manipulations of VEGF signaling can influence hippocampal functions and behavioral processes, the influence of behavioral tasks on the expression of VEGF has not been thoroughly examined.

The Morris water maze (MWM) (Morris, 1984) task has been the most extensively used and accepted means by which behavioral physiologists and pharmacologists measure spatial learning, especially in rodents. The MWM is a challenging task for rodents that employs a variety of mnemonic processes, such as acquisition and spatial localization of relevant visual cues. It requires animals to swim and successfully navigate to escape a water maze (McNamara and Skelton, 1993; Morris, 1984). It is likely that the swimming contributes considerably to vasocirculation processes in rats since several lines of evidence confirm that blood circulation is relevant to the neurotrophic factor levels in the brain.

We have previously shown that membrane depolarization and glutamatergic receptor activation induces VEGF164 expression in hippocampal neuronal cultures (Kim et al., 2008). We have also demonstrated that VEGF is released from neurons in an activity-dependent manner, and it enhances neuronal plasticity in the hippocampus. Having obtained evidence that VEGF activates hippocampal neuronal functions, we wondered whether VEGF is involved in hippocampal dependent learning.

In the current study, we aimed to investigate the effects of the MWM task on the hippocampal protein level of the VEGF. We considered two versions of the MWM task, intensive and weak, and our findings indicate that the VEGF protein level in the hippocampus is increased by the intensive, but not by the weak MWM task.

MATERIALS AND METHODS

Hidden platform water maze task Experiment 1 ("Weak" learning paradigm)

The training and testing procedures were adapted from those described in previous studies and have been shown to be hippocampal-based (Packard et al., 1994). Animals were subjected to one session consisting of three training trials (with a 15-min intertrial interval) to find a fixed submerged platform (6" diameter) and escape from a circular water maze (72" in diameter, 24.5" high, 22-24°C water temperature). The starting points were distributed randomly across the four quadrants (two start-

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ing points per quadrant; the animal always faced the wall when placed in the water). If escape did not occur within 60 s, the animal was manually guided to the platform. After climbing the platform, the animal stayed on it for 60 s and then was placed in a holding cage until the next trial. After the last trial, the animals were returned to their home cages. Animals were subjected to the same session for five days at 24-h intersession intervals. Animals were sacrificed two hours after completing the spatial learning task, under pentobarbital anesthesia. Blood samples were collected retro-orbitally. The frontal cortex and two hippocampi were then rapidly isolated and frozen for ELISA. Animals' movements and the time taken to reach the platform were monitored automatically using a computerized tracking system (SmarTrack; Smartech, USA).

Experiment 2 ("Intensive" learning paradigm)
Independent groups of animals were subjected to four sessions of training at 30-min intersession intervals during one day. Each session consisted of three training trials (with a 15 min inter-trial interval) to find a fixed submerged platform (6" in diameter) and escape from a circular water maze (72" in diameter, 24.5" high, 22-24°C water temperature). The starting points were distributed randomly across the four quadrants (two starting points per quadrant; the animal always faced the wall when placed in the water). Animals were divided into two groups and sacrificed at 2 h or 24 h after completing the spatial learning task, under pentobarbital anesthesia. Blood samples were collected retro-orbitally. The frontal cortex and two hippocampi were then rapidly isolated and frozen for ELISA. Animal care and experiments were conducted in accordance with the 2004 Guide for the Care and Use of Laboratory Animals (Korea National Institute of Health) and the Hanyang University Veterinary committee. All values included in the figure legends represent mean ± SEM. Statistical analysis of the biochemical experiments was performed using ANOVA test (SPSS 16.0).

Drugs and surgical procedures
Stereotaxic surgeries were performed under ketamine (80 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.) anesthesia. Bilateral viral injections were performed with coordinates -4.1 mm (anterior/posterior), ± 2.4 mm (lateral), and -4.1 mm (dorsal/ventral) relative to the Bregma (Paxinos et al., 1980). Rats received bilateral microinjection of human recombinant VEGF165 (50 ng/side, R&D systems, USA) or saline (0.9%) into the CA1 of the hippocampus. A total volume of 3.0 μl was infused into each side over 15 min, and the injection syringe was left in place for an additional 5 min to allow diffusion. Needles were removed and the scalp incision was closed with wound clips. The drugs were prepared as stock solutions, stored at -70°C, and diluted to their final concentrations in the perfusion solution immediately before use.

VEGF production detection
The presence of VEGF protein was assessed by the Quantikine VEGF ELISA kit according to the manufacturer's instructions (R&D Systems, USA). Total protein concentration was determined by BCA (Pierce Chemical, USA).

Immunohistochemistry
Rats were anesthetized deeply with pentobarbital and perfused via the ascending aorta with saline until the outflow became clear. They were then perfused with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 20 min. The brains were removed rapidly, frozen immediately, and stored at -70°C. Serial sections (40 μm/section) were made coronally throughout the entire anteroposterior extension of the hippocampus. Sections were incubated with rabbit anti-p-CREB (Cell Signal.ing Technology) or rabbit anti-p-CaMKII (Affinity Bioreagents, Inc) for p-CREB or p-CaMKII labeling. To measure the intensity of immunoreactivity in the hippocampal CA1, images were acquired with a digital camera (Nikon E800) and analyzed using an image analysis program (AnalySIS version 3.0, Soft Image Analysis System GmBH). In each image, a ROI (region of interest), which represented the CA1, was determined by free hand drawing, and mean optical values in each ROI were measured. Results represent the ratio of the intensity, which was computed by dividing the mean optical value of total protein by the corresponding value of phosphorylated protein.

Semiquantitative PCR and quantitative real time RT-PCR
Total RNA was prepared using Trizol reagent (Life Technologies Inc, USA). The following PCR primers were used: rat VEGF-A 164 sense, 5′-GTACCTCCACCATGCCAAGT-3′ and antisense, 5′-CAAGGCTCACAGTGATTTTCTGG-3′; Hif1-α sense, 5′-GTCTCGAGATGCAGCCAGATCTCG-3′ and antisense, 5′-GGTCAGATGATCAGAGTCCAAAGC-3′: rat BDNF sense, 5′-GTGACAGTATTAGCGAGTGGG-3′ and antisense, 5′-GGGTAGTTCGGCATTGC-3′; and GAPDH sense, 5′-CTC GTCTCATAGACAAGATGGTGAAG-3′ and antisense, 5′-AGA CTCCACGACATACTCATCACC-3′. Transcript levels of each gene were normalized to the amount of the GAPDH housekeeping gene in the individual samples, and mean optical value changes were compared using the ImageJ program (provided for free on the Wright Cell Imaging Facility's website). Statistical significance was calculated using Student's *t*-test.

RESULTS

Membrane depolarization activates VEGF transcription
Figure 1A shows that animals that received training had significantly shorter latencies to escape onto the hidden platform on the second day (*latency:* first day: 37.9 ± 3.3 s; second day: 26.9 ± 2.3 s; third day: 27.5 ± 2.4 s; fourth day: 23.5 ± 2.4 sec, repeated-measure ANOVA, $p < 0.05$). The latency was further shortened on the fifth day of training (Fig. 1A; fifth day: 18.3 ± 2.8 s, repeated-measure ANOVA, $p < 0.01$). These results indicate that the animals could be evaluated for biochemical changes caused by hippocampal dependent learning.

For the analysis of VEGF produced from the hippocampus following hippocampal-dependent learning, the hippocampi were isolated and processed for ELISA. After five days of MWM training, the plasma VEGF in the swimming group was significantly lower than that in the naive control group (Fig. 1B; Ctl vs. swimming, one-way ANOVA, $p < 0.01$). The MWM group showed plasma VEGF levels similar to those of the swimming rats (Fig. 1B; one-way ANOVA, Ctl vs. MWM, $p < 0.05$; swimming vs. MWM, $p > 0.1$). In the hippocampus, there was a significantly greater decrease detected in the VEGF levels after five days of spatial learning compared to swimming (Fig. 1B; swimming vs. MWM, one-way ANOVA, $p < 0.05$). The VEGF level in the cortex was significantly decreased in the spatial learning group compared to the control, but not to swimming groups (Ctl vs. MWM, $p < 0.01$; swimming vs. MWM, $p > 0.05$). These results indicate that the hippocampal-dependent learning paradigm of the MWM task reduces VEGF in the brain including the hippocampus and also in the blood.

We therefore trained animals under an intensive learning paradigm. In order to avoid creating a stressful condition, the training was executed for only one day. Figure 2A shows that animals that received intensive training had significantly shorter

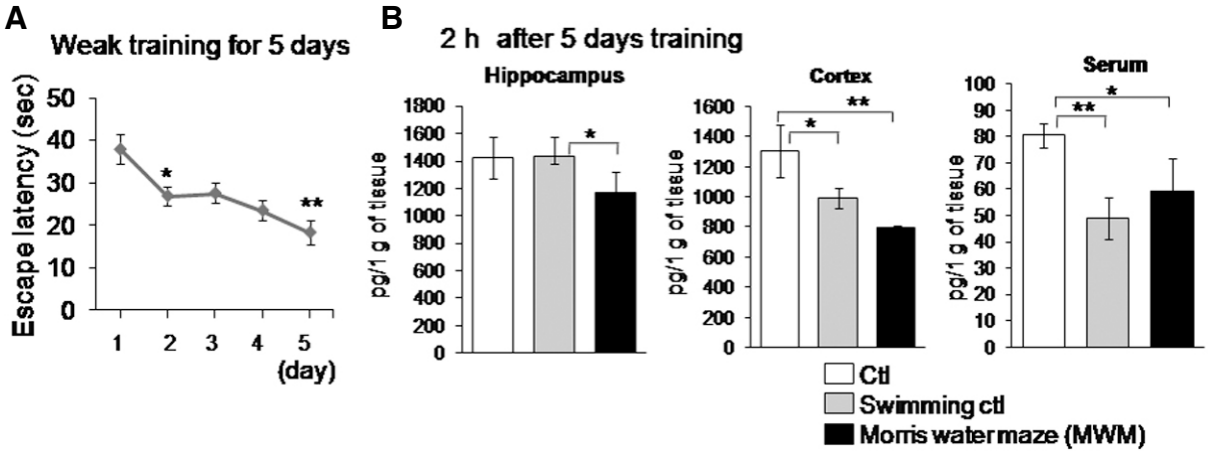

Fig. 1. Effects of MWM on VEGF protein levels for a one-session-per-day procedure conducted for five consecutive days. (A) Acquisition curves for the weak version of the MWM hidden platform test. The latency in seconds is presented for each day. Each point represents the mean latency in sec ± SEM. (B) VEGF protein level in the hippocampus, cortex and serum detected by ELISA. Each point represents the mean ± SEM; n = 5-8 rats per group, *$P < 0.05$, **$P < 0.01$.

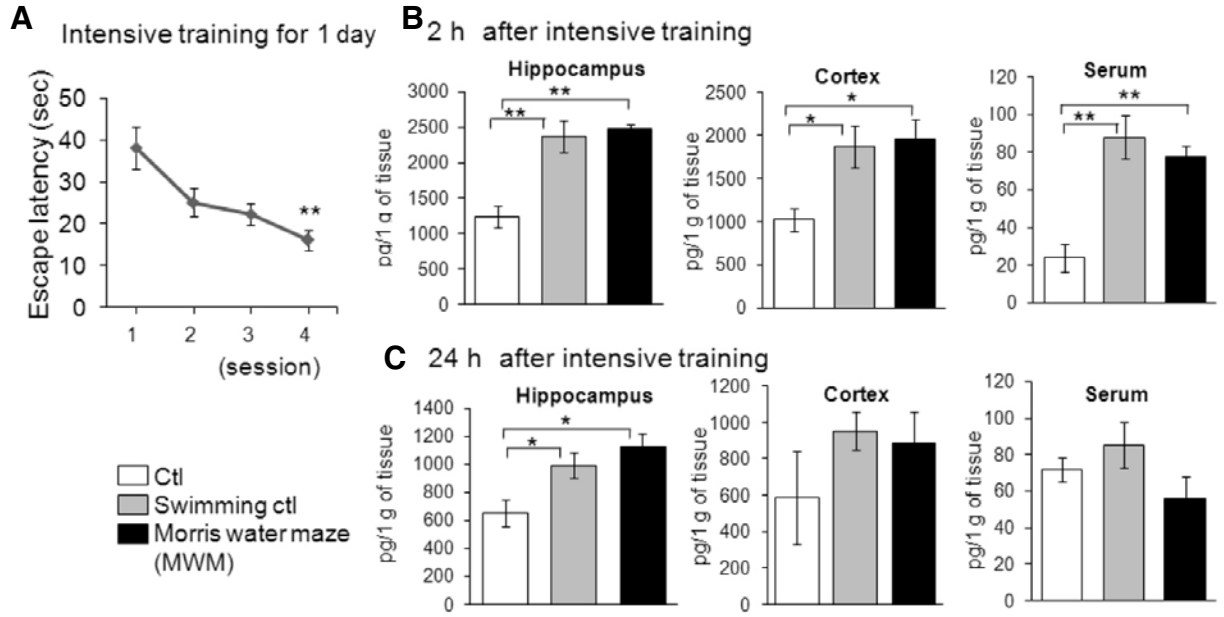

Fig. 2. Effects of MWM on VEGF protein levels for a three-session-per-day procedure conducted for one day. (A) Acquisition curves for the intensive version of the MWM hidden platform test. The latency in seconds is presented for each day. Each point represents the mean latency in sec ± SEM. (B, C) VEGF protein level in the hippocampus, cortex, and serum detected by ELISA 2 h (B) and 24 h (C) after training. Each point represents the mean ± SEM; n = 5-8 rats per group, *$P < 0.05$, **$P < 0.01$.

latencies to escape onto the hidden platform by the fourth session (*latency:* first session: 38.2 ± 5.1 s; fourth session: 16.2 ± 2.5 s, repeated-measure ANOVA, $p < 0.01$). Two hours after the intensive training, the VEGF protein level was significantly higher in the hippocampus of the trained animals compared to the controls (Fig. 2B; Ctl vs. MWM, one-way ANOVA, $p < 0.01$). In addition, the VEGF protein levels were significantly increased in rats who swam only (Fig. 2B; Ctl vs. swimming, one-way ANOVA, $p < 0.01$). There was no difference in the hippocampal VEGF protein levels between the rats in the swimming group and the rats treated with the intensive MWM. The VEGF level in the cortex also increased in both the swimming and MWM groups compared to controls (Fig. 2B; Ctl vs. MWM, Ctl vs. swimming, respectively, one-way ANOVA, $p < 0.05$). There was no significant difference between the swimming and MWM groups. The plasma VEGF was significantly higher in the swimming and MWM groups compared to the naive control group (Fig. 2B; Ctl vs. swimming, Ctl vs. MWM, one-way ANOVA, $p < 0.01$).

In another experimental group, 24 h after experiencing the same intensive MWM, the VEGF levels in the hippocampus and cortex were substantially lower compared to those examined two hours after the MWM (Fig. 2C), indicating that increases in the VEGF protein were transient. There was a sig-

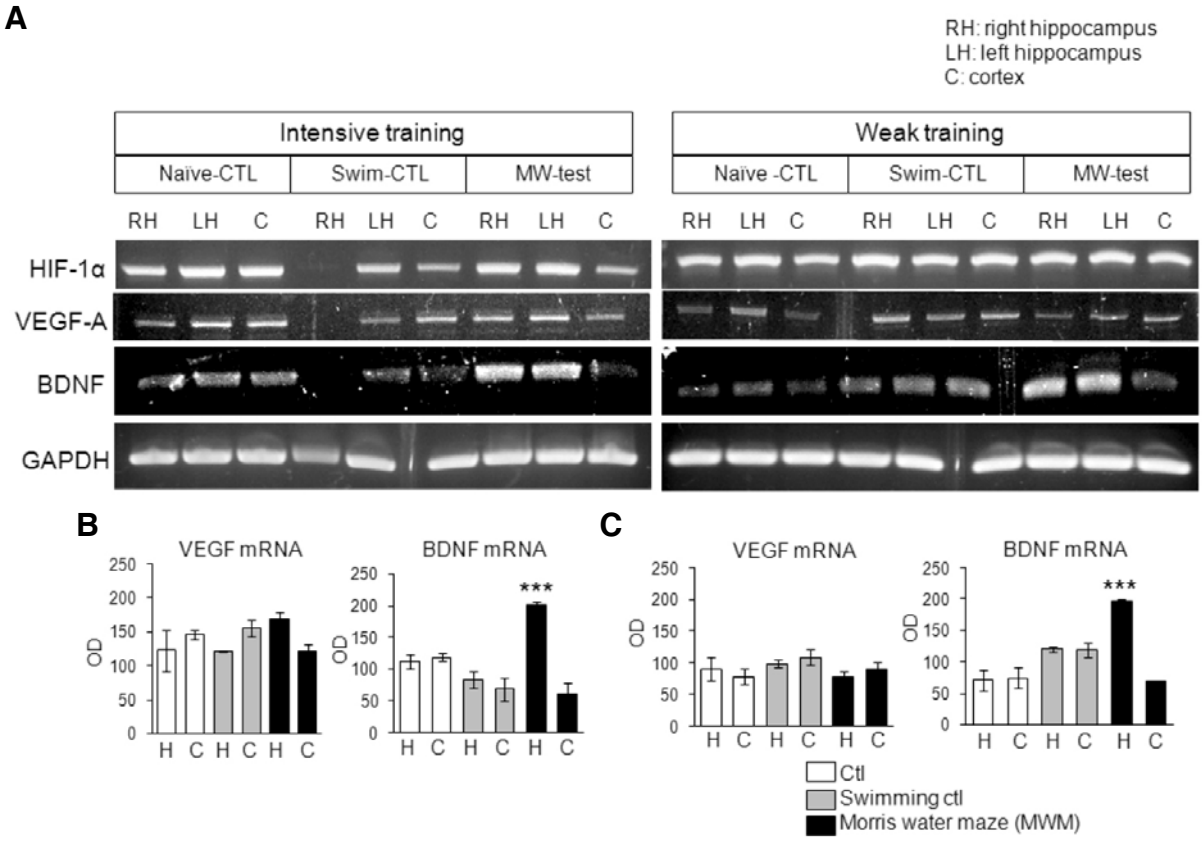

Fig. 3. VEGF mRNA expression in rats subjected to two MWM paradigms. (A) Representative RT-PCR. (B, C) Expression of VEGF and BDNF in MWM-trained rats ($n = 4$ naïve, $n = 4$ swim, $n = 5$ intensive MWM, $n = 4$ weak MWM; ***$P < 0.001$ intensive MWM compared with swim). H, hippocampus; C, cortex.

nificant increase in hippocampal VEGF in the swimming and MMW groups compared to naive controls (Fig. 2C; Ctl vs. swimming, Ctl vs. MWM, one-way ANOVA, $p < 0.05$). However, there were no significant differences between the swimming and MWM groups in either the hippocampus or the cortex (Fig. 2C). The plasma VEGF levels were similar between the groups. These results indicate that the VEGF protein is induced in the hippocampus, cortex, and blood in the course of performing the intensive MWM task. Since the increase in VEGF protein in the MWM group was similar to that seen in the swimming group, these results further suggest that the increase in VEGF protein might not be associated with hippocampal-dependent spatial learning.

To more closely evaluate the effects of learning on VEGF protein levels within the hippocampus, total RNA was isolated from rapidly dissected hippocampal formations from animals that were subjected to weak five-day learning or intensive learning. In spite of the robust increase in VEGF protein, semi-quantitative RT-PCR analysis showed little learning-induced change in the abundance of VEGF mRNA (Gomez-Pinilla et al., 1997) in both weak and intensive MWM paradigms (Figs. 3A, 3B and 3C). VEGF is expressed in neurons (Ogunshola et al., 2002; Oosthuyse et al., 2001) and is induced by specific stressors, including hypoxia in neurons. The expression of HIF-1α was not changed compared to control levels (Fig. 3A).

We examined the expression of brain-derived neurotrophic factor (BDNF), which plays a central role in synaptic plasticity, learning, and memory (Tang et al., 2002), in order to compare the effects of MWM on BDNF with those on VEGF. The level of BDNF mRNA was increased in the rats that performed either weak or intensive MWM tasks (Fig. 3B; swimming vs. MWM, $p < 0.001$, respectively). Together with the results of ELISA, these findings indicate that VEGF might be induced at a translational, but not transcriptional, level in response to MWM.

Given the evidence that VEGF is released in the hippocampus during the learning procedure, we wondered whether VEGF regulates neuronal functions. Therefore, we investigated the intracellular signaling molecules potentially activated by VEGF in an attempt to characterize the possible contribution of VEGF to hippocampal neuronal functions. A link between VEGF and the CREB pathways in neurons has been demonstrated by numerous groups (Lee et al., 2009; 2010). Therefore we investigated the potential link between VEGF and signaling pathways related to synaptic plasticity, including the CREB downstream pathway, using a variety of antibodies. To mimic VEGF release in hippocampal neurons, rats received stereotaxic injections of human recombinant VEGF165 or vehicle. Injection of VEGF165 (50 ng/ml) induced an increase in the expression of p-CREB 2 h after infusion.

Calcium/calmodulin protein kinase II (CaMKII) is activated in response to neuronal activity and is required for synaptic plasticity (Schmitt et al., 2005). Therefore, we assessed whether CaMKII was responsive to VEGF infusion. In fact, we found that VEGF had a significant effect on the phosphorylation of CaMKII,

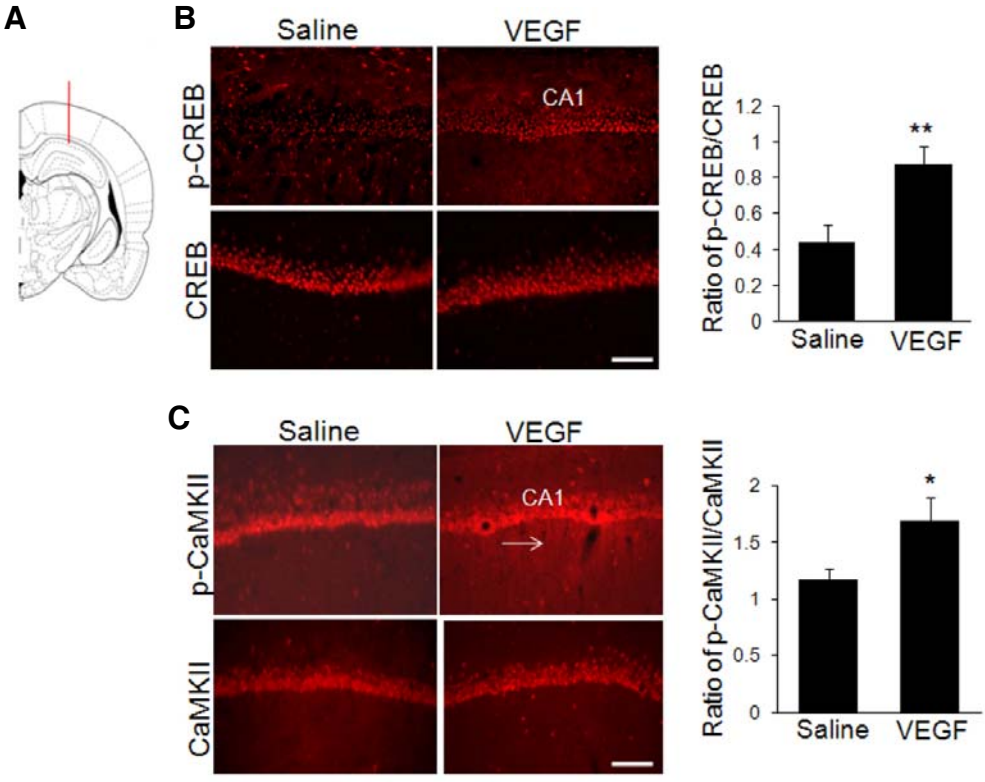

Fig. 4. Effects of stereotaxic VEGF treatment on protein levels in the CA1. (A) VEGF (50 ng/side) into was bilaterally injected into the hippocampal CA1 subregion in rats and immunohistochemistry was performed as described in the "Materials and Methods." (B) VEGF increased p-CREB immunoreactivity in the pyramidal cells. (C) VEGF increased p-CaMKII immunoreactivity in the dendrites (arrow). Scale bar, 50 μm. (Graphs) Quantification of relative p-CREB and p-CaMKII levels in the hippocampal CA1 2 h after the injection of VEGF. $^{*}P < 0.05$, $^{**}P < 0.01$.

implying that activation of the CaMKII signaling pathways may play a significant role in the VEGF-mediated responses. Phosphorylation of CREB and CaMKII also appears to be greatly increased within a similarly rapid time period. Together, these results indicate that the CREB and CaMKII pathways are activated following VEGF stimulation, consistent with previous reports for other types of cells (Rodgers and Theibert, 2002). Overall, our results in conjunction with those from previous studies demonstrate that VEGF is produced in the hippocampus in an activity-dependent manner and plays a role in the activation of signaling pathways, which appear to be the CREB and CaMKII pathways.

DISCUSSION

Our results demonstrate that VEGF is oppositely influenced by the two spatial learning paradigms that we tested: weak and intensive MWM tasks. The weak MWM training was even weaker than that tested previously (van Praag et al., 2005). The acquisition curves for rats given one session per day for five consecutive days (weak training) and three sessions per day for one day (intensive training) are illustrated. We have used these learning protocols in our laboratory in previous studies (Jo et al., 2007). For both approaches, the rats learned to locate the hidden platform with progressively shorter latencies over the course of the study. We found that weak-MWM-trained subjects given only one session per day were somewhat less efficient at producing VEGF than intensive-MWM-trained subjects given multiple sessions for one day. In fact, VEGF levels were significantly decreased in the hippocampus and cortex after weak MWM training, whereas intensive MWM training increased the VEGF protein levels in the hippocampus and cortex. Further, we found the weak and intensive MWM to be useful for learning-induced BDNF studies (Beversdorf et al., 1999; Hernandez and Terry, 2005).

The MWM task includes muscle exercise that can lead to increased expression of HIF1. HIF-1α regulates VEGF by binding to an HRE (Wu et al., 2007), and HIF-1α mRNA increases transiently in the mouse brain in response to short-term environmental enrichment (Rampon et al., 2000). These data imply that changes in exercising muscle that lead to increased expression and stability of HIF-1α and VEGF (Gustafsson et al., 1999) may be paralleled in the brain. However, our results show that HIF-1α mRNA is not increased by either weak or intensive MWM training, consistent with previous results.

The current results support previous studies showing an exercise-induced increase in BDNF mRNA levels, while no significant change was detected in HIF-1α mRNA expression. A number of previous studies have established that the activation of the CaMKII cascade is a necessary step in long-term spatial memory (Bach et al., 1995; Yasuda and Mayford, 2006). Our immunohistochemistry results using hippocampal sections indicated that increased VEGF results in a marked increase in dendritic CaMKII phosphorylation. Activation of CaMKII in dendrites may influence memory formation by enhancing neuron signaling. Future studies will need to be performed to determine the effects of VEGF-induced CaMKII activation responsible for synaptic plasticity, learning, and memory.

In conclusion, the present study is the first to clearly demonstrate that levels of VEGF protein production in the brain after MWM learning may differ depending on the intensity of the training trials. These findings suggest that the neurotrophic effects of VEGF account for the learning-induced cellular modifications caused by learning paradigms involving physical activity. Further, these results are consistent with the suggestion that VEGF is one of the factors involved in hippocampal-dependent learning.

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