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ORIGINAL RESEARCH ARTICLE

Phosphatidylinositol 3-kinase: a molecule mediating BDNF-dependent spatial memory formation

M Mizuno[1]*, K Yamada[1]*, N Takei[2], MH Tran[1], J He[1], A Nakajima[1], H Nawa[2] and T Nabeshima[1]

[1]*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [2]*Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan*

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Brain-derived neurotrophic factor (BDNF) plays a critical role in synaptic plasticity such as long-term potentiation (LTP), a form of synaptic correlate of learning and memory. BDNF is also implicated in learning and memory. We have demonstrated that radial arm maze training in rats for spatial learning and memory results in a significant increase in the BDNF mRNA expression in the hippocampus. Moreover, antisense BDNF oligonucleotide treatment impaired not only acquisition, but also maintenance and/or recall of spatial memory in the maze. Although these results suggest a role of BDNF for spatial memory processes, the signal transduction mechanisms that mediate the actions of BDNF remain unknown. Here we show that phosphorylation of BDNF receptor tyrosine kinase B (TrkB), phosphatidylinositol 3-kinase (PI3-K) and Akt, a target of PI3-K, in the hippocampus increased in parallel with spatial memory formation. Moreover, an activation of translational processes was suggested in the hippocampus after the maze training. When spatial learning was inhibited by antisense BDNF oligodeoxynucleotide, the activation was diminished. Chronic treatment with PI3-K inhibitor wortmannin impaired spatial learning. Our findings suggested that activation of TrkB/PI3-K and protein synthesis signaling pathway by BDNF in the hippocampus is important for spatial memory.
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The binding of Brain-derived neurotrophic factor (BDNF)[1–4] to its receptor tyrosine kinase B (TrkB) leads to autophosphorylation of tyrosine residues in the intracellular domain of the receptor and subsequent activation of cytoplasmic signaling pathways. Tyrosine phosphorylated TrkB serves as a scaffold for the recruitment of adaptor proteins and enzymes, including mitogen-associated protein kinase (MAPK), phospholipase C-γ (PLC-γ) and PI3-K that further transduce the BDNF signal.[5–7] To examine whether spatial learning and memory are associated with activation of BDNF/TrkB signaling, phosphorylated TrkB was measured in the hippocampus of rats trained for the spatial reference and working memory task in a radial arm maze.[4,8] Figure 1a shows the changes in performance produced by daily training (three trials per day). An ANOVA with repeated measures revealed a significant effect of training on reference ($F(7,84) = 60.890$, $P < 0.0001$) and working memory ($F(7,84) = 40.209$, $P < 0.0001$), indicating spatial memory formation. The animals were killed immediately after the last training trial on days 1, 4 and 8, or 30 min after the training on day 8, to analyze the phosphorylation of TrkB. The level of phosphorylated TrkB was extremely low in the nontrained control animals. In trained rats, the level was increased on days 1 and 4, and peaked on day 8 immediately after the training. It then decreased 30 min after the training on day 8 (Figure 1b). These results suggest that TrkB phosphorylation after the maze training increases in parallel with spatial memory formation. In contrast, there was no significant difference in the phosphorylated TrkA or C levels between control and trained animals on day 8 (Figure 1c and d).

We next analyzed whether TrkB phosphorylation after maze training is associated with an activation of PI3-K, MAPK and/or PLC-γ. Levels of phosphorylated PI3-K were significantly increased in the trained rats, whereas the phosphorylation of MAPK and of PLC-γ was not changed (Figure 2a–c). Phosphorylated Akt, a target of PI3-K,[9] levels after maze training on day 8 were also significantly increased in the trained rats (Figure 2d). *src* homology 2 (SH2) domains are utilized in the intracellular signaling of various growth factors. TrkB and PI3-K possess SH2 domains and PI3-K binds with TrkB via SH2 domains.[10] To test for increased binding of PI3-K with Trk, TrkB was immunoprecipitated with anti-TrkB antibodies, and then blotted with anti-PI3-K antibodies. Figure 2e shows that levels of PI3-K proteins co-immunoprecipitated with TrkB were significantly increased in the trained rats.

Protein synthesis is required for BDNF-dependent LTP in the hippocampus.[11] A recent study showed that BDNF increases protein synthesis by activating initiation and elongation steps in mRNA translation and the TrkB/PI3-K signaling pathway is involved in this activation.[12] We, therefore, hypothesized that BDNF-regulated translational activation is involved in the spatial memory processes. Translation of mRNA into protein is generally divided into initiation and elongation. During initiation and elongation, eukaryotic initiation factors (eIFs) and elongation factors (eEFs) are involved.[13] Eukaryotic initiation

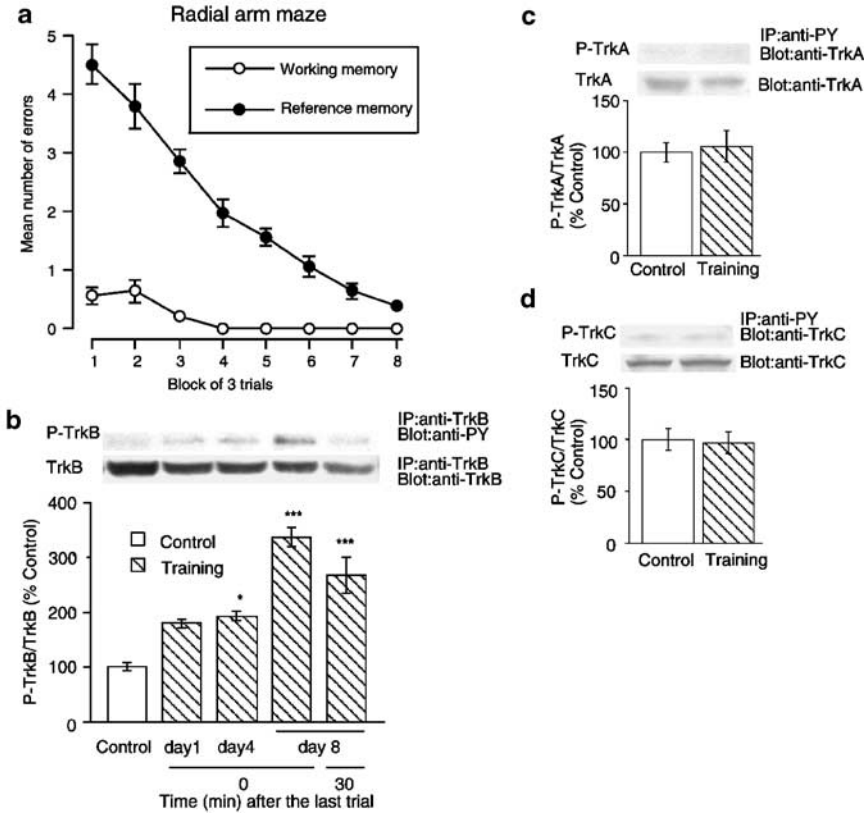

Figure 1 Spatial learning-associated activation of the TrkB/PI3-K pathway in the hippocampus. (a) Changes in performance of rats on repeated daily training (8 days) for the reference and working memory task. (b) Learning-associated TrkB phosphorylation in the hippocampus from day 1 to day 8. Learning-associated activation of TrkA (c) and TrkC (d) in the hippocampus. Data are expressed as a percentage of control. Each value represents the mean ± s.e.m. (a: $n=13$; b–d: $n=5$). $^{*}P<0.05$, $^{***}P<0.001$ *vs* control.

factor 4E binding protein 1 (4E-BP1) is a negative regulator for eukaryotic initiation factor 4E (eIF4E). 4E-BP1 is inactivated by mammalian target of rapamycin (mTOR)-dependent phosphorylation.[14,15] Phosphorylation on 4E-BP1 by mTOR erases the suppressive effect on eIF4E. Eukaryotic elongation factor-2 (eEF-2) kinase is a negative regulator of eukaryotic mRNA translation. It phosphorylates and inactivates eEF-2, thereby reducing the rate of peptide chain elongation.[16,17] It is suggested that the PI3-K/Akt/mTOR signaling pathway plays an important role in the regulation of mRNA translation.[18,19] As shown in Figure 2f and g, phosphorylated 4E-BP1 levels were significantly increased, whereas phosphorylated eEF2 levels were decreased in the hippocampus of rats immediately after the training on day 8, suggesting that the radial arm maze training results in an activation of translational processes in the hippocampus.

We also examined whether treatment with antisense BDNF oligodeoxynucleotide inhibits memory formation and signal transduction. Our previous study demonstrated that a continuous infusion of antisense, but not sense, BDNF oligodeoxynucleotide into the cerebral ventricle markedly reduced BDNF protein and mRNA levels in the hippocampus.[4] The mean numbers of reference and working memory errors during the last block of three trials in rats which received 21 consecutive daily training trials (one trial/day) with concurrent infusion of antisense or sense BDNF oligonucleotide are shown in Figure 3a. Antisense BDNF treatment significantly impaired both spatial reference and working memory formation. Of note, spatial learning-associated activation of TrkB (Figure 3b) and PI3-K (Figure 3c) was completely diminished by treatment with antisense BDNF oligodeoxynucleotide, while the sense oligodeoxynucleotide had no effect. Phosphorylated MAPK (Figure 3d) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) levels (Figure 3f), which were not influenced by the maze training itself, were reduced by antisense BDNF treatment. Neither spatial learning nor antisense BDNF treatment affected PLC-γ phosphorylation (Figure 3e). These findings imply that although various signaling molecules may be regulated by BDNF, the TrkB/PI3-K signaling pathway is associated with BDNF-dependent spatial learning.

Finally, we examined the effects of the PI3-K inhibitor wortmannin on spatial learning. Continuous intracerebroventricular infusion of wortmannin (2.5–25 μM) impaired spatial learning in a dose-dependent manner: A one-way ANOVA with repeated measures

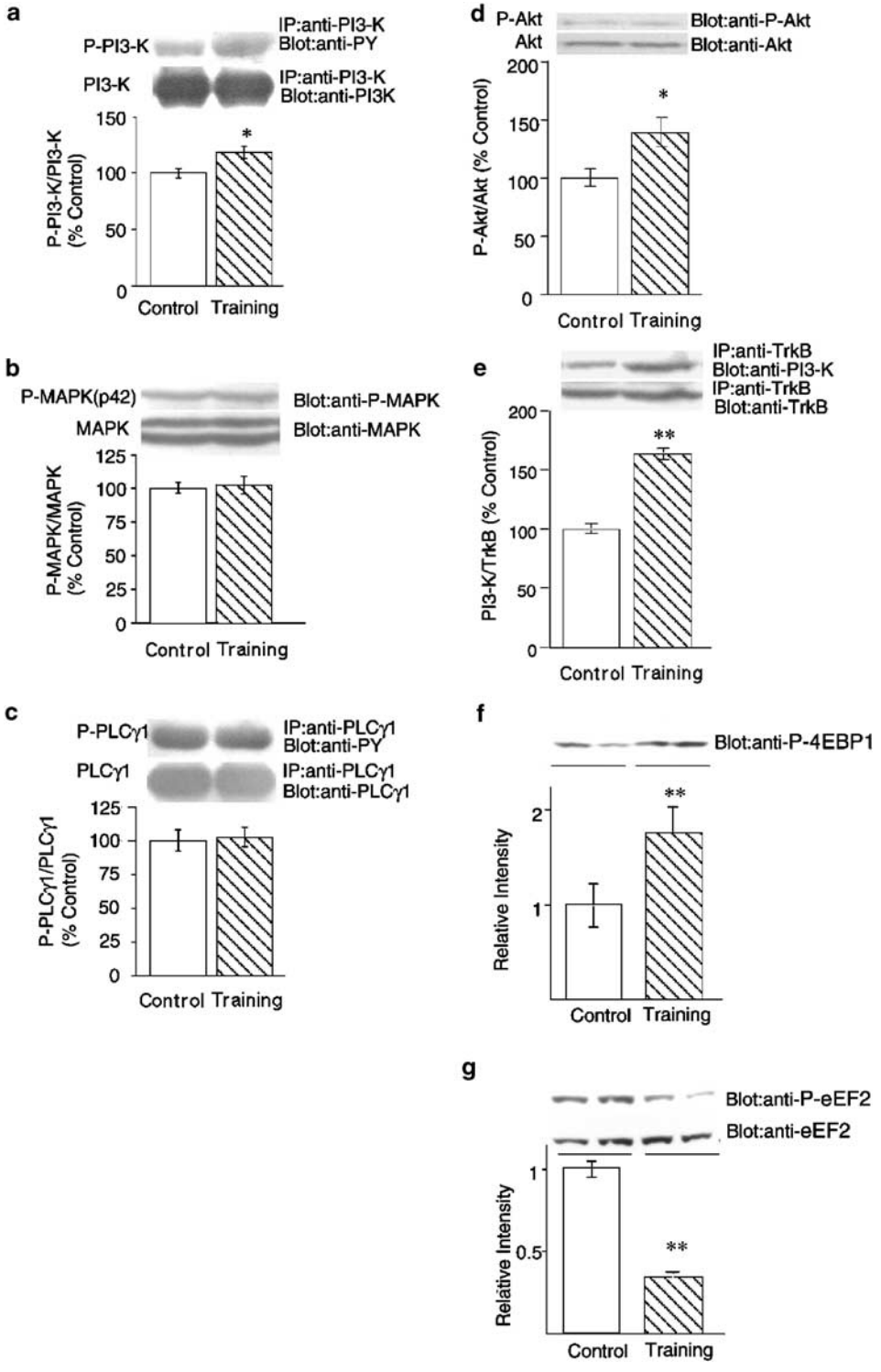

Figure 2 Spatial learning-associated activation of the PI3-K pathway in the hippocampus. Learning-associated activation of PI3-K (a), MAPK (b), PLC-γ-1 (c), Akt (d) and PI3-K coimmunoprecipitation with TrkB (e) in the hippocampus. ($n=5$). Learning-associated changes in phosphorylated 4E-BP1(f) and eEF2 (g) levels in the hippocampus. Data are expressed as a percentage of control. Each value represents the mean $\pm$ s.e.m. ($n=4$). $^{*}P<0.05$, $^{**}P<0.01$ *vs* control.

of the data revealed significant effects of group ($F(2,21)=12.466$, $P=0.0003$) and blocks of trials ($F(4,84)=211.913$, $P<0.0001$), but not the interaction ($F(8,84)=1.885$, $P=0.0730$), on reference memory (Figure 4a). There were also significant effects of group ($F(2,21)=49.258$, $P<0.0001$) and blocks of trials ($F(4,84)=19.205$, $P<0.0001$), but not the interaction ($F(8,84)=1.148$, $P=0.3405$), on working mem-

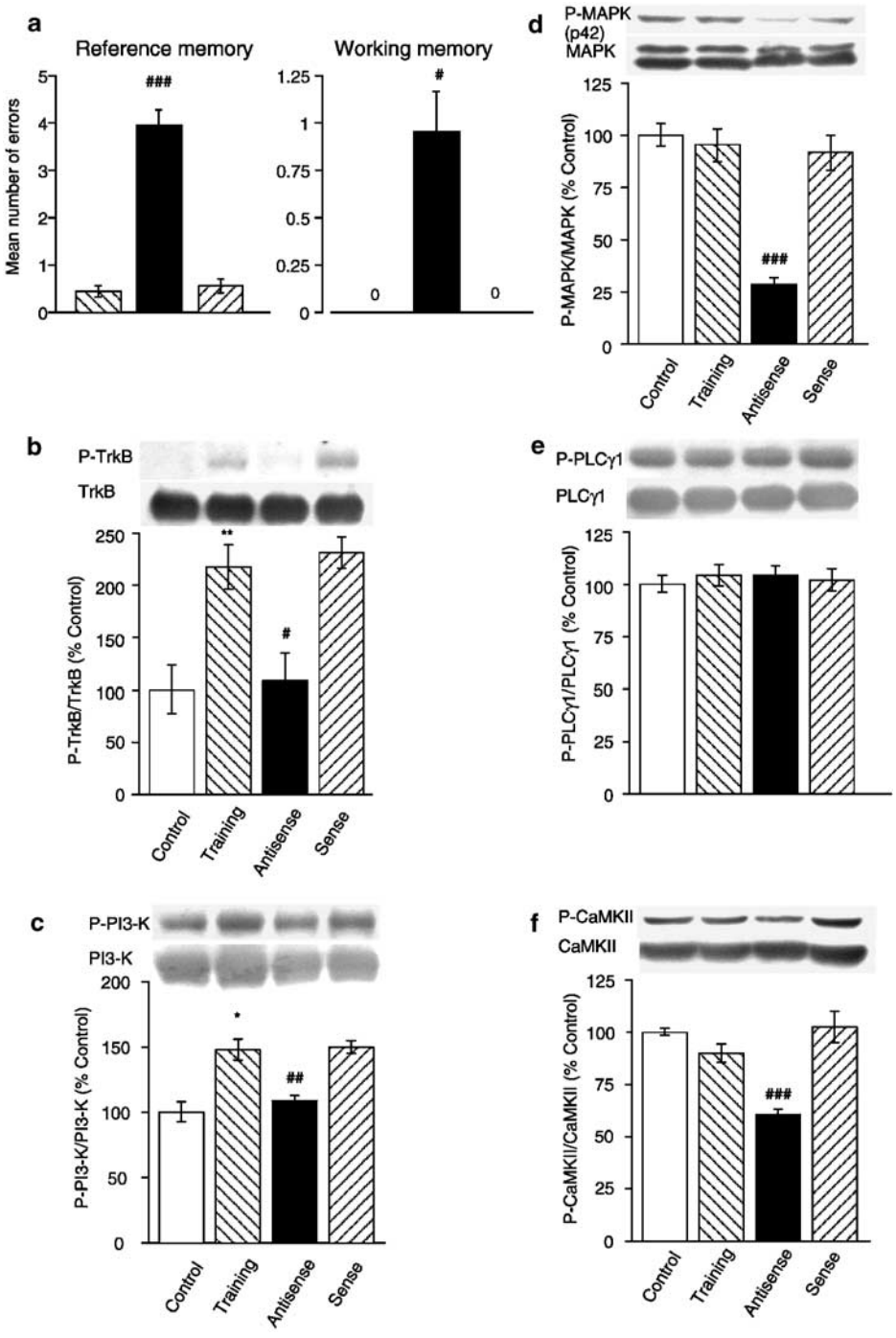

Figure 3 Effect of antisense BDNF oligonucleotide treatment on spatial learning (a), and the learning-associated activation of TrkB (b), PI3-K (c), MAPK (d), PLC-γ-1 (e) and CaMKII (f) in the hippocampus. Data are expressed as a percentage of control. Each value represents the mean $\pm$ s.e.m. ($n=5$). $^{*}P<0.05$, $^{**}P<0.01$ *vs* control, $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ *vs* trained rats.

ory (Figure 4b). *Post hoc* analysis with Scheffe's test indicated that the number of both reference (control *vs* 25 μM wortmannin, $P<0.001$) and working memory errors (control *vs* 2.5 and 25 μM wortmannin, $P<0.0001$, respectively) was significantly increased by treatment with wortmannin. There was no apparent difference in locomotor activity ($F(2,21)=0.9114$, $P=0.4173$) and food consumption ($F(2,21)=0.2036$, $P=0.8174$) among the animals during the trials (data not shown). To confirm the effect of wortmannin on PI3-K signaling events, rats were killed after the behavioral test and phosphorylated PI3-K and Akt protein levels were determined. The increase in phosphorylated PI3-K (Figure 4c) and Akt (Figure

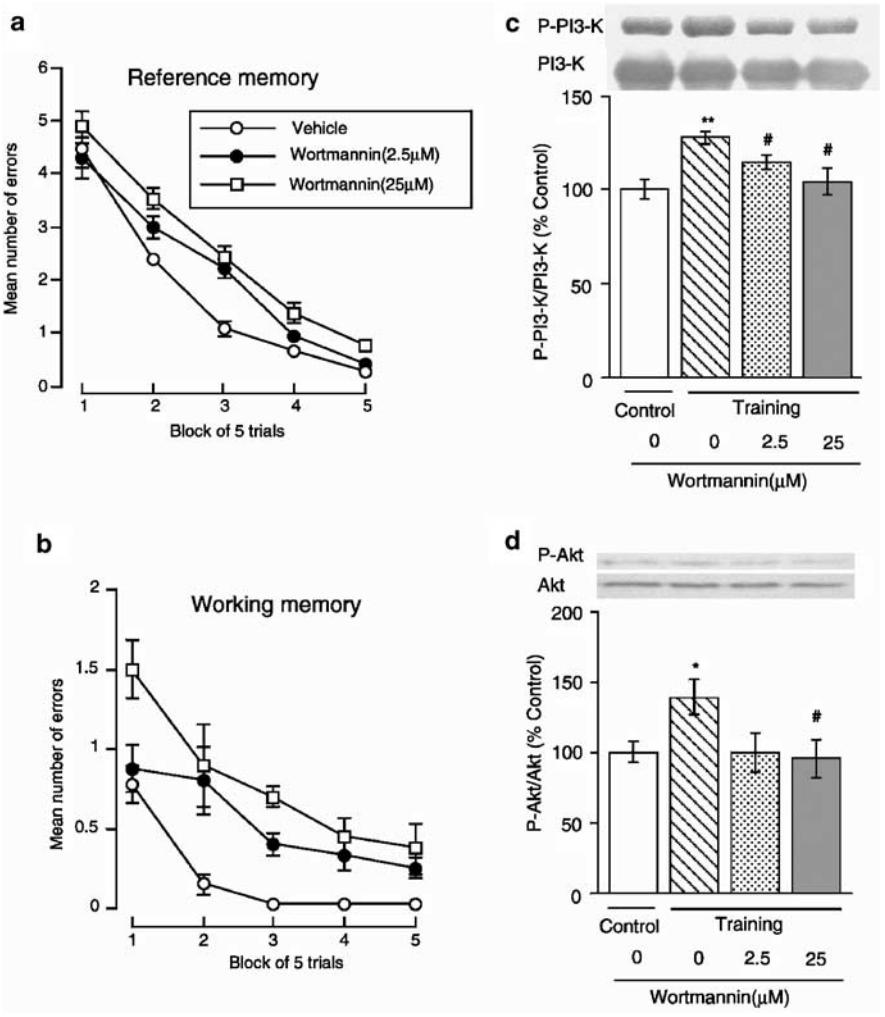

Figure 4 Effect of continuous intracerebroventricular infusion of wortmannin on spatial learning. Changes in the number of reference (a) and working memory errors (b) PI3-K (c) and Akt activation (d) in the hippocampus. Data are expressed as a percentage of control. Each value represents the mean ± s.e.m. (a and b: $n=8$, c and d: $n=5$). $^{*}P<0.05$, $^{**}P<0.01$ *vs* control, $\#P<0.05$ *vs* trained rats.

4d) levels induced by spatial learning was reduced in the wortmannin-treated rats compared to the vehicle-treated rats.

We demonstrated in the present study that activation of the TrkB/PI3-K signaling pathway induced by BDNF is critical for spatial learning in the radial arm maze. The involvement of the BDNF/TrkB system in spatial learning is specific because neither NGF or NT-3 mRNA levels (data not shown) nor TrkA or C protein phosphorylation levels changed in the trained animals. Our findings support the hypothesis that neuronal plasticity is the primary function of BDNF.[20]

Spatial-learning-induced activation of PI3-K/Akt was evidenced as an increase in the level of phosphorylated proteins in the hippocampus, while no changes in MAPK or PLC-γ phosphorylation were detected. Furthermore, treatment with antisense BDNF oligonucleotide, which resulted in an impairment of spatial memory formation, completely abolished the activation. These results suggest a role for BDNF-dependent activation of the TrkB/PI3-K/Akt signaling pathway in spatial memory formation. Several studies have shown that MAPK activity is critical for memory formation.[21–24] For instance, activation of MAPK was shown by immunohistochemistry in the dorsal hippocampus of rats that had acquired spatial memory in the Morris water maze, although the activation was not detected by Western blotting.[25] To check the sensitivity of the antibodies used in the present study, we examined the effect of BDNF treatment (100 ng/ml, for 15–30 min) of adult rat hippocampal slices *in vitro*. BDNF treatment led to an increase in the level of phosphorylated Trk, MAPK, PLC-γ and Akt. Thus, it is possible that the activation of MAPK induced by spatial learning is restricted to a limited population of cells, which we failed to detect by immunoblotting.

The reactivation of spatial memory by radial arm maze training in well-trained rats resulted in an increase in phosphorylated 4E-BP1, and a decrease

in phosphorylated eEF-2, indicating an increase in activity to translate mRNA into protein, although we did not measure protein synthesis by itself. Protein synthesis is required for BDNF-dependent LTP in the hippocampus,[11] and BDNF increases protein synthesis by enhancing translation initiation via multiple signaling pathway including PI3-K and Akt.[12] Collectively, one of the mechanisms by which BDNF facilitates spatial learning and memory may be the activation of translation processes via the TrkB/PI3-K/Akt signaling pathway. Further studies are required to test this hypothesis. It also remains to be determined whether activation of the TrkB/PI3-K signaling pathway is a point of convergence of multiple receptor-mediated processes for spatial memory formation because the blockade of neurotransmitter receptors such as *N*-methyl-D-aspartate (NMDA) receptors is known to inhibit spatial learning and memory.[26]

Finally, we demonstrated that pharmacological blockade of PI3-K activity with wortmannin impaired spatial learning. In this regard, it was reported that intracerebroventricular injection of wortmannin inhibited the expression of LTP in the hippocampus in rats,[27] and that activation of PI3-K was necessary for BDNF to modulate high-frequency transmission in the neonatal hippocampus.[5] On the other hand, a recent study showed that BDNF induces hipppocampal LTP *in vivo* through MAPK and selective induction of the activity-regulated cytoskeleton-associated protein (Arc).[28] It is plausible that not only PI3-K but also other molecules may be involved in BDNF-dependent spatial memory formation.

Methods

Subjects

Male Wistar rats (7 weeks old, Charles River Japan, Yokohama, Japan) weighing 230 ± 10 g at the beginning of experiments were used in the present study. They were housed 3 per cage under controlled laboratory conditions (a 12 h light/dark cycle with lights on at 09:00, $23 \pm 0.5^{\circ}$C, 50 ± 0.5% humidity). Prior to the radial maze experiments, the animals were kept on a restricted diet (10 g per day) and body weight was maintained at 85% of their free-feeding weight over a 1-week period, with water being available *ad lib*. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Radial arm maze task

The radial arm maze used in the present study consisted of eight arms, numbered 1–8 (48×12 cm), extending radially from a central area, with a 5-cm edge around the apparatus (Neuroscience, Inc., Osaka, Japan). The training procedure was as described previously.[4,8] Before the actual training began, the animals were shaped for 4 days to run to end of the arms and consume the bait, in groups of four. The bait was initially available throughout the maze, but gradually was restricted to the food cup. Following this shaping period, each animal was placed individually in the center of the maze and trained for a reference and working task for three trials per day over 8 days, where the same four arms were baited (a single 50 mg food pellet) for each daily training trial. The other four arms were never baited. The training trial continued until all four baits in the food cups had been consumed or until 5 min had elapsed. Measures were made of the number of reference memory errors (entering an arm that was not baited) and working memory errors (entering an arm containing food but previously entered). Re-visits to unbaited arms were counted as reference memory errors. The control rats were kept on a restricted diet as trained animals, put on the radial arm maze everyday without maze training, and given four food pellets. They were killed on day 8.

Intracerebroventricular infusion of antisense oligonucleotide and wortmannin

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. An infusion cannula connected to a mini-osmotic pump (Alza, Palo Alto, CA, USA) placed subcutaneously in the neck of the rat was implanted into the right ventricle (*A*, −0.3; *L*, 1.2; *V*, 4.5; according to the rat brain atlas[29]). BDNF antisense and sense oligonucleotides were continuously infused into the cerebral ventricle at a dose of 3.6 nmol/day for 25 days (flow rate, 0.25 μl/h). Phosphothioate oligonucleotides were custom synthesized at SAWADY technology, and dissolved in sterile pyrogen-free 0.9% saline. The sequences of antisense and sense oligonucleotides were 5′-TCTTCCCCTTTTAATGGT-3′ and 5′-ACCATTAAAAGGGGAAGA-3′, respectively, which correspond to amino acid 114–119 of BDNF.[30] Four days after the operation, the rats were subjected to the reference and working memory test (one trial per day for 21 days[4]).

Wortmannin was dissolved in 1% DMSO/saline at concentrations of 2.5 and 25 μM, and infused for 1 week (flow rate, 0.5 μl/h). The rats were allowed a 1-day recovery period following the surgery for implantation of the infusion cannula. The maze training was conducted for 5 days with five trials per day because of instability of the reagent. Immediately after the last training trial, the animals were killed for biochemical analysis.

Locomotor activity and food consumption in rats treated with wortmannin were measured to see if motor function and/or food consumption were affected by the treatment. After the training trials on day 3 of the maze training, each rat was placed in a locomotor cage ($25 \times 42 \times 20$ cm), with photobeams placed 2 cm above the floor at 1-in intervals along two sides of the cage (Columbus Instruments, USA), and the locomotor activity was measured for 10 min. After measurement of locomotor activity, rats were individually placed in a home cage, and then 10 baits,

which were the same as those used in the radial arm maze test, were provided. The time taken to consume all 10 baits was recorded, with a cut-off time of 180 s min.[4]

Immunoprecipitation and Western blotting

The hippocampus from each rat was lysed at 4°C in a lysis buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 10 mM sodium diphosphate decahydrate, 0.5 mM DTT, 0.2 mM PMSF, 4 μg/ml pepstatin, 4 μg/ml aprotinin and 4 μg/ml leupeptin, pH 7.4. The lysate was then centrifuged at 10 000 *g* for 10 min. The protein concentration of the supernatant was determined with a Protein Assay Rapid kit (Wako, Osaka, Japan). Protein A-Sepharose (Amersham Pharmacia) was incubated with either monoclonal anti-TrkB (Santa Cruz Biotechnology), anti-PLC-γ-1 (Upstate Biotechnology), anti-PI3-K p85 (Upstate Biotechnology) or antiphosphotyrosine (anti-PY) antibodies (Upstate Biotechnology) for 6 h and then incubated with each lysate (0.5 mg of protein) overnight. The immunoprecipitate was boiled in Laemmli sample buffer, separated on a 7.5% polyacrylamide gel and subsequently transferred to a PVDF membrane (Millipore). The membranes were blocked with Detector Block Kit (KPL) for 2 h at room temperature, and probed with either anti-PY, anti-TrkA or anti-TrkC antibodies overnight at 4°C. Other antibodies used for Western blotting were antiphospho-ERK (Santa Cruz Biotechnology), antiphosphoAkt (Sell Signaling Technology) and antiphospho αsubunit CaMKII antibodies (Affinity Bioreagents, Inc.). The membranes were washed with TBST (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl, 0.1% Tween 20) and probed with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The immune complexes were detected by ECL (Amersham Pharmacia) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry. To calculate the value phosphorylated form of each protein *vs* nonphosphorylated form, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 10 min, incubated with either anti-TrkB, anti-PLC γ-1, anti-PI3-K p85, anti-TrkA, anti-TrkC, anti-ERK1 (K-23) (Santa Cruz Biotechnology), anti-α subunit CaMKII (Affinity Bioreagents, Inc.) or anti-Akt antibodies (Cell Signaling Technology), and detected as described above. Immunoprecipitation and Western blotting for phospho-4EBP1, phospho-eEF2 and eEF2 were performed as described previously.[12]

Statistical analysis

Results were expressed as means $\pm$ s.e.m. The significance of differences was determined by a one-way or two-way ANOVA, followed by Bonferroni's test for multi-group comparisons. Student's *t*-test was used for two-group comparisons. An ANOVA with repeated measures, followed by Scheffe's test, was used for analyzing data of the radial arm maze. A *P* value less than 0.05 was regarded as statistically significant.

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Correspondence: T Nabeshima, PhD, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8560, Japan.
E-mail: tnabeshi@med.nagoya-u.ac.jp.
*These authors contributed equally to this work.
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