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Involvement of Brain-Derived Neurotrophic Factor in Spatial Memory Formation and Maintenance in a Radial Arm Maze Test in Rats

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Brain-derived neurotrophic factor (BDNF) regulates both short-term synaptic functions and activity-dependent synaptic plasticity such as long-term potentiation. In the present study, we investigated the role of BDNF in the spatial reference and working memory in a radial arm maze test. The radial arm maze training resulted in a significant increase in the BDNF mRNA expression in the hippocampus, although the expression in the frontal cortex did not change. When spatial learning was inhibited by treatment with 7-nitroindazole, an inhibitor of brain nitric oxide synthase, the increase in the hippocampal BDNF mRNA did not occur. To clarify the causal relation between BDNF mRNA expression and spatial memory formation, we examined the effects of antisense BDNF treatment on spatial learning and memory. A continuous intracerebroventricular infusion of antisense BDNF oligonucleotide resulted in an impairment of spatial learning, although the sense oligonucleotide had no effect. Treatment with antisense, but not sense, BDNF oligonucleotide was associated with a significant reduction of BDNF mRNA and protein levels in the hippocampus. Furthermore, treatment with antisense BDNF oligonucleotide in rats, which had previously acquired spatial memory by an extensive training, impaired both reference and working memory. There were no differences in locomotor activity, food consumption, and body weight between the antisense and sense oligonucleotide-treated rats. These results suggest that BDNF plays an important role not only in the formation, but also in the retention and/or recall, of spatial memory.

Key words: BDNF; working memory; reference memory; radial arm maze; antisense oligonucleotide; LTP; hippocampus

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, which plays important roles in the survival, maintenance, and growth of neurons (Barde et al., 1982; Leibrock et al., 1989). BDNF supports the survival of many types of neurons, including mesencephalic dopaminergic neurons (Knüsel et al., 1991), septal cholinergic neurons (Nonner et al., 1996), and striatal GABAergic neurons (Ventimiglia et al., 1995). This neurotrophin influences the development of patterned connections (Cabelli et al., 1995) and growth and complexity of dendrites in the cerebral cortex (McAllister et al., 1995).

BDNF is also implicated in the modulation of synaptic transmission. Regarding the short-term synaptic function, BDNF enhances synaptic transmission (Lohof et al., 1993; Lessmann et al., 1994; Levine et al., 1995; Kang and Schuman, 1995, 1996), increases phosphorylation of subunits of NMDA receptors (Suen et al., 1997; Lin et al., 1998), and facilitates acetylcholine release (Knipper et al., 1994). In activity-dependent synaptic plasticity, BDNF enhances long-term potentiation (LTP) in the hippocampus (Figurov et al., 1996) but blocks the induction of long-term depression (LTD) in the visual cortex (Kinoshita et al., 1999). High-frequency stimulation, which induces LTP, increases BDNF mRNA in hippocampal slices (Patterson et al., 1992). In BDNF mutant mice, LTP is impaired, but the changes were restored by either adenovirus-mediated re-expression (Korte et al., 1995) or by the bath application of BDNF (Patterson et al., 1996).

Because LTP is considered a potential cellular mechanism underlying learning and memory, BDNF may be involved in memory processes The water maze learning was impaired in BDNF mutant mice (Linnarsson et al., 1997) or in rats that had received an intracerebroventricular infusion of anti-BDNF antibody (Mu et al., 1999). A relation between BDNF mRNA expression and memory was shown in the passive avoidance test (Ma et al., 1998) and in the water maze test (Kesslak et al., 1998). It should be noted, however, that the behavioral tests used so far to investigate the role of BDNF in learning and memory are negatively motivated stressful paradigms and that various stresses and sensory stimulation affect BDNF expression (Ernfors et al., 1991; Nibuya et al., 1995; Smith et al., 1995).

Nitric oxide (NO) is a free radical gas with a role in synaptic plasticity, including learning and memory (Garthwaite and Boulton, 1995; Yamada and Nabeshima, 1998). A brain NO synthase (NOS) inhibitor 7-nitroindazole (7-NI) inhibited the spatial learning of rats (Zou et al., 1998a). Notably, Xiong et al. (1999) have demonstrated a mutual regulation of NO and BDNF synthesis *in vitro* and *in vivo*.

In the present study, we investigated the role of BDNF in learning and memory using a less stressful eight-arm radial arm maze test. We examined whether spatial memory formation is associated with changes in the level of BDNF mRNA in the brain. We also studied the changes in BDNF mRNA level when spatial learning is inhibited by treatment with 7-NI. Furthermore, to clarify the causal relation between BDNF expression and memory formation, we investigated the effects of BDNF antisense treatment on spatial learning and memory.

MATERIALS AND METHODS

Materials. Male Wistar rats (7-weeks-old; Charles River Japan, Yokohama, Japan) weighing 230 ± 10 gm at the beginning of experiments were used in the study. They were housed three per cage with *ad libitum* access to food and water under controlled laboratory conditions (a 12 hr light/dark cycle with lights on at 9:00 A.M., 23 ± 0.5°C, 50 ± 0.5% humidity). 7-NI was purchased from Lancaster Synthesis (Lancashire, UK). All experi-

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ments were performed in accordance with the *Guidelines for Animal Experiments of the Nagoya University School of Medicine*, the *Guiding Principles for the Care and Use of Laboratory Animals* approved by the Japanese Pharmacological Society, 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 from 1 to 8 (48 × 12 cm), extending radially from a central area (32 cm in diameter), with a 5 cm edge around the apparatus. The training procedure was essentially the same as described previously (Zou et al., 1998a, 1999). Briefly, each animal was placed individually in the center of the maze and subjected to a reference and working memory task for 28 d, where the same four arms (numbers 1, 2, 4, and 7) were baited for each daily training trial. The other four arms (numbers 3, 5, 6, and 8) 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). 7-NI (50 mg/kg) was suspended in peanut oil at a dose of 50 mg/kg and administered p.o. 60 min before each training trial of the radial arm maze test. Control animals were administered with peanut oil in a volume of 1 ml/kg and subjected to the maze training. The third group of animals was prepared as nontrained control rats, which were kept on a restricted diet as were the other groups of animals, put in the radial arm maze everyday without maze training, and given four food pellets.

Antisense oligonucleotide treatment. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. The infusion cannula was connected to a miniosmotic pump (total capacity, 200 μl; Alza, Palo Alto, CA), which was filled with BDNF antisense or sense oligonucleotides, and was implanted into the right ventricle (anterior −0.3, lateral 1.2, ventral 4.5), according to the atlas of Paxinos and Watson (1982). The pump was placed subcutaneously in the neck of the rat. The BDNF antisense and sense oligonucleotides were continuously infused into the cerebral ventricle at a dose of 3.6 nmol/d. The infusion of the oligonucleotides was maintained for 2 weeks (flow rate, 0.5 μl/hr; Alzet 2002) or 4 weeks (flow rate, 0.25 μl/hr; Alzet 2004). Phosphothioate oligonucleotides were custom-synthesized at SAWADY technology (Tokyo, Japan) and dissolved in sterile pyrogen-free 0.9% saline. The sequences of antisense and sense oligonucleotides were 5′-TCTTC-CCCTTTTAATGGT-3′ and 5′-ACCATTAAAAGGGGAAGA-3′, respectively, which correspond to amino acids 114–119 of BDNF (Acheson et al., 1995). The rats were allowed a 3 d recovery period after the surgery for implantation of the infusion cannula. On day 4 after the start of oligonucleotide infusion, the rats were subjected to the reference and working memory task, and the test was continued for the indicated time period.

Measurement of locomotor activity and food consumption. Locomotor activity, food consumption, and body weight in rats treated with antisense or sense oligonucleotides of BDNF were measured to see whether motor function and/or motivation were affected by the treatment. Each rat was placed in a locomotor cage (25 × 42 × 20 cm), with photobeams placed 2 cm above the floor at 1 inch intervals along two sides of the cage (Columbus Instruments). Locomotor activity was measured for 10 min (Zou et al. et al., 1998b). 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 cutoff time of 180 sec (Zou et al. et al., 1998b).

RT-PCR. Total RNA was extracted from brain tissues by a method previously described (Chamberlain and Burgoyne, 1996) (Qiagen, Tokyo, Japan). The levels of BDNF mRNA in brain tissues were determined by RT-PCR. The mRNA for β-actin was used as an internal control, to be coamplified with BDNF mRNA. Total RNA (1 μg) was converted into cDNA using oligo(dT) 12–18 primer (Life Technologies, Tokyo, Japan) and Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a total reaction volume of 25 μl (RT-reaction mixture). PCR was performed using one twenty-fifth of the RT-reaction mixture, 0.5 μM of each (forward and reverse) primers, and ReadyToGo PCR Beads (Amersham Pharmacia Biotech, Tokyo, Japan) in a total reaction volume of 25 μl. The primers used were as follows: BDNF: 5′-CGTGATCGAG-GAGCTGTTGG-3′ (forward) and 5′-CTGCTTCAGTTGGCCTT-TCG-3′ (reverse), and β-actin: 5′-TGCTCGACAACGGCTCCG-GCATGT-3′ (forward) and 5′CCAGCCAGGTCCAGACGCAGGAT-3′ (reverse) (Bova et al., 1998). In a preliminary experiment, the number of PCR cycles and denaturation temperature were tested to ascertain a linear working range for all PCR products. The experimental amplification protocol consisted of a first round at 94°C for 5 min and then 27 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 2 min at 72°C on a programmable thermal cycler (PCR Thermal Cycler; Takara, Shiga, Japan). The PCR products were visualized by ethidium bromide staining under UV light after electrophoresis on a 1.5% agarose gel.

BDNF enzyme immunoassay. The BDNF levels were measured by enzyme immunoassay (EIA) as described (Nawa et al., 1995). Briefly, anti-BDNF antisera were prepared as described previously and were used as the primary antibody in BDNF detection to coat polystyrene 96-well microtiter plates for EIA. The high-affinity anti-BDNF antibodies were biotinylated with NHS-LC-Biotin and used as secondary antibody (Nawa et al., 1995). The EIA detected trace amounts of BDNF (>1 pg/assay) and they did not cross-react with 1000-fold excess amounts of other neurotrophins.

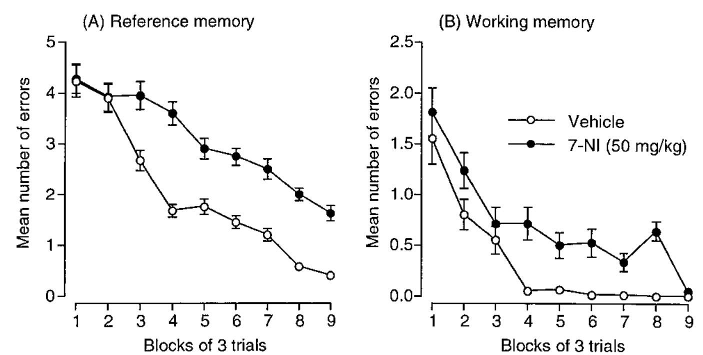

Figure 1. Spatial reference (*A*) and working (*B*) memory formation in rats treated with vehicle ($n = 20$) and 7-NI ($n = 14$). 7-NI (50 mg/kg) was administered p.o. every day 60 min before each training trial. Each value represents the mean ± SE. 7-NI significantly impaired reference ($p < 0.001$) and working memory ($p < 0.001$) formation.

Statistical analysis. Results were expressed as the mean ± SE. The significance of differences was determined by a one-way or two-way ANOVA, followed by Bonferroni's test or the Student–Newman–Keuls test for multigroup comparisons. Student's *t* test was used for two-group comparisons. An ANOVA with repeated measures was conducted for analyzing data of the radial arm maze. A p value <0.05 was regarded as statistically significant.

RESULTS

To examine whether radial arm maze training affects BDNF mRNA expression in the brain, one group of animals ($n = 20$) was trained for the reference and working memory task for 28 d. The second group ($n = 14$) was treated with a brain NOS inhibitor 7-NI (Babbedge et al., 1993; Moore et al., 1993) every day before the training, which has been previously demonstrated to inhibit the spatial learning in this test (Yamada et al., 1995; Zou et al., 1998a) and to decrease BDNF protein level in the hippocampus (Han et al., 2000). The third group of animals ($n = 3$) was prepared as nontrained rats that were kept on a restricted diet like the other groups of animals, put on the radial arm maze everyday without maze training, and given four food pellets on the maze.

Figure 1 shows the alterations of performance in rats produced by repeated daily training in the reference and working memory task. An ANOVA with repeated measures revealed significant effects of group ($F_{(1,32)} = 72.336$; $p < 0.0001$), trial ($F_{(8,256)} = 68.412$; $p < 0.0001$), and group by trial interaction ($F_{(8,256)} = 4.761$; $p < 0.0001$) in reference memory (Fig. 1*A*). In working memory, there were significant effects on group ($F_{(1,32)} = 19.987$; $p < 0.0001$) and trial ($F_{(8,256)} = 33.196$; $p < 0.0001$), but not group by trial interaction ($F_{(8,256)} = 1.343$; $p = 0.2225$) (Fig. 1*B*). These results indicate that 7-NI inhibits both spatial reference memory and working memory formation.

Three groups of rats were killed either immediately, 15, or 30 min after the maze training on day 28. Because the prefrontal cortex and hippocampus are involved in reference and working memory (Olton et al., 1979; Kesner et al., 1987; Chiba et al., 1997; Porter et al., 2000), BDNF mRNA levels in the frontal cortex, including anterior cingulate cortex and motor area of frontoparietal cortex, and the whole hippocampus were measured by RT-PCR (Fig. 2*A,C*). There was no difference in the BDNF mRNA level before and after the taking of the four food pellets in the nontrained rats, suggesting that food consumption per se has no effect on BDNF mRNA level (data not shown).

A two-way ANOVA of the data in the hippocampus of the trained rats with or without 7-NI treatment revealed a significant effect of 7-NI treatment ($F_{(1,28)} = 49.618$; $p < 0.0001$), time ($F_{(2,28)} = 5.054$; $p = 0.0134$), and treatment by time interaction ($F_{(2,28)} = 10.436$; $p = 0.004$) (Fig. 2*B*). A one-way ANOVA of the vehicle-treated trained rats including the control animals revealed a signif-

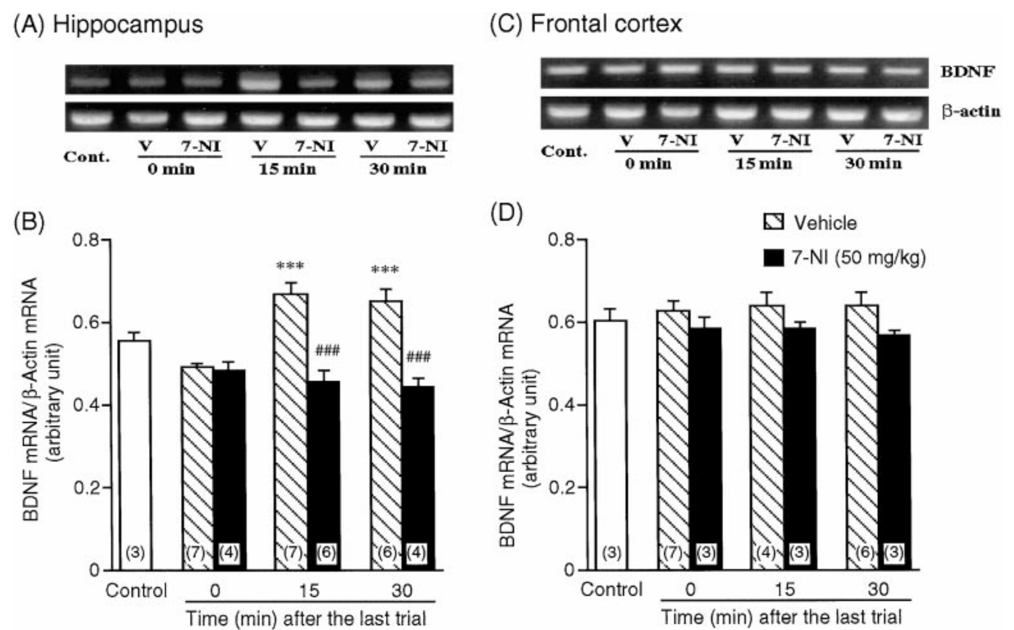

Figure 2. Changes in BDNF mRNA expression in the hippocampus and frontal cortex of rats associated with spatial memory formation. Rats were trained for the reference and working memory task for 28 d. 7-NI was administered every day 60 min before each training trial. Animals were killed immediately, 15, or 30 min after the last training. The levels of BDNF and β-actin were measured by RT-PCR. Representative gel patterns showing BDNF and β-action cDNA bands at different times after training in the hippocampus (*A*) and frontal cortex (*C*). The quantitative results of RT-PCR in the hippocampus (*B*) and frontal cortex (*D*). The number in parentheses shows the number of animals. The BDNF mRNA level was normalized with the β-action mRNA level. Each value represents the mean ± SE. $^{***}p < 0.001$ versus immediately after the training (0 min). ###$p < 0.001$ versus corresponding vehicle-treated rats.

icant group effect ($F_{(3,19)} = 13.736$; $p < 0.0001$). *Post hoc* analysis with Bonferroni's test indicated that the level of BDNF mRNA in the hippocampus was significantly increased 15 and 30 min after the training trial compared with the level immediately after the training trial ($p < 0.001$) (Fig. 2*B*). When spatial learning was inhibited by treatment with 7-NI, the changes in BDNF mRNA expression were not observed ($F_{(3,13)} = 2.8555$; $p = 0.0780$). There was a significant difference (Student's *t* test, $p < 0.001$) in BDNF mRNA levels between vehicle- and 7-NI-treated rats 15 and 30 min, but not immediately after the last training trial (Fig. 2*B*). A significant correlation was found between the number of reference memory errors on day 28 and BDNF mRNA levels in the hippocampus at 15 ($r = -0.695$; $p = 0.0067$) and 30 min ($r = -0.693$; $p = 0.024$), but not immediately ($r = 0.035$; $p = 0.921$), after the training trial. There was no correlation between the number of working memory errors on day 28 and BDNF mRNA levels in the hippocampus at any time points examined. In the frontal cortex, a two-way ANOVA revealed a small but significant effect of 7-NI treatment ($F_{(1,20)} = 4.804$; $p = 0.0404$), although no significant effects were observed in time ($F_{(2,20)} = 0.038$; $p = 0.9626$) and treatment by time interaction ($F_{(2,20)} = 0.102$; $p = 0.9034$) (Fig. 2*D*).

These results suggest that spatial memory formation is associated with an increase in BDNF mRNA in the hippocampus, a brain structure being involved in spatial learning and memory in the radial arm maze test (Olton et al., 1979). However, it is still unclear whether the alteration is the cause or the effect of spatial learning. To clarify the causal relation between the changes in BDNF levels and spatial learning, rats first received surgery for continuous intracerebroventricular infusion of antisense BDNF oligonucleotide, and after a 4 d recovery period they were subjected to the spatial learning test.

Figure 3 illustrates the maze performance in nonoperated control rats and the rats that received a continuous intracerebroventricular infusion of antisense or sense BDNF oligonucleotide. An ANOVA with repeated measures of the data from the three groups revealed significant effects of group ($F_{(2,16)} = 193.410$; $p < 0.0001$), trial ($F_{(6,96)} = 50.681$; $p < 0.0001$), and group by trial interaction ($F_{(12,96)} = 6.172$; $p < 0.0001$) on the reference memory formation (Fig. 3*A*). *Post hoc* analysis with Bonferroni's test indicated that antisense BDNF oligonucleotide treatment significantly inhibited spatial reference memory formation ($p < 0.001$) (Fig. 3*A*).

In the working memory formation, an ANOVA with repeated measures indicated significant effects of group ($F_{(2,16)} = 38.368$; $p < 0.0001$) and trial ($F_{(6,96)} = 15.435$; $p < 0.001$), but not group by trial interaction ($F_{(12,96)} = 1.227$; $p = 0.2761$) (Fig. 3*B*). *Post hoc* analysis with Bonferroni's test revealed that antisense BDNF oligonucleotide treatment significantly inhibited spatial working memory formation ($p < 0.001$), although the sense oligonucleotide treatment had no effect (Fig. 3*B*).

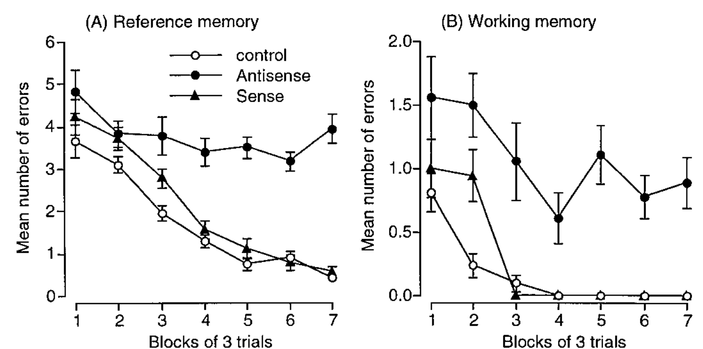

Figure 3. Spatial reference (*A*) and working (*B*) memory formation in nonoperated control rats ($n = 7$) and rats that were continuously infused with BDNF antisense ($n = 6$) and sense oligonucleotides ($n = 6$) into the cerebral ventricle. The maze training was started 4 d after the start of continuous intracerebroventricular infusion of the oligonucleotide. Each value represents the mean ± SE. Antisense BDNF treatment significantly impaired reference ($p < 0.001$) and working memory formation ($p < 0.001$).

We also examined the effects of BDNF antisense oligonucleotide on locomotor function and appetite, which affect performance in the radial arm maze test. There were no differences in locomotor activity, food consumption, and body weight between the antisense and sense oligonucleotide-treated rats ($p > 0.05$, Student's *t* test) (Fig. 4*A–C*). Thus, it is unlikely that the impairment of the maze performance in rats treated with antisense BDNF oligonucleotide is a secondary effect of the motor dysfunction or an altered motivational state.

To confirm the effect of continuous intracerebroventricular infusion of antisense BDNF oligonucleotide on BDNF synthesis, rats were killed on day 22, and BDNF mRNA and protein levels were determined (Fig. 5). A one-way ANOVA revealed a significant group effect in BDNF protein ($F_{(2,13)} = 5.6356$; $p < 0.05$) and mRNA levels ($F_{(2,9)} = 26.218$; $p < 0.001$). *Post hoc* analyses revealed that both BDNF protein (Fig. 5*A*) and mRNA (Fig. 5*B*) levels in the hippocampus of the antisense oligonucleotide-treated rats were significantly lower than those in nonoperated control ($p < 0.05$). Neither BDNF protein nor mRNA levels were affected by treatment with the sense oligonucleotide.

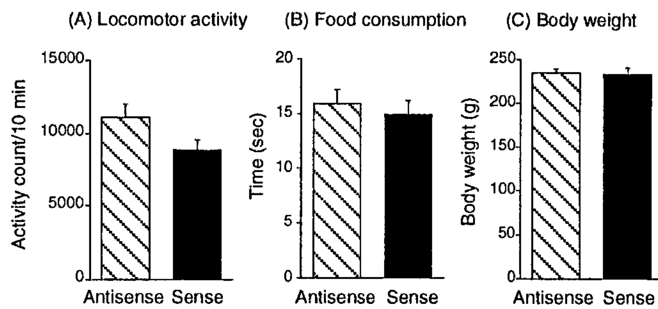

Figure 4. Effects of continuous infusion of BDNF antisense (n = 6) and sense oligonucleotides (n = 6) into the cerebral ventricle on locomotor activity, food consumption, and body weight. Rats were continuously infused with BDNF antisense or sense oligonucleotide into the cerebral ventricle and were subjected to the radial maze test for 21 d. Locomotor activity (*A*), food consumption (*B*), and body weight (*C*) were measured on the day after the last training. Each value represents the mean ± SE.

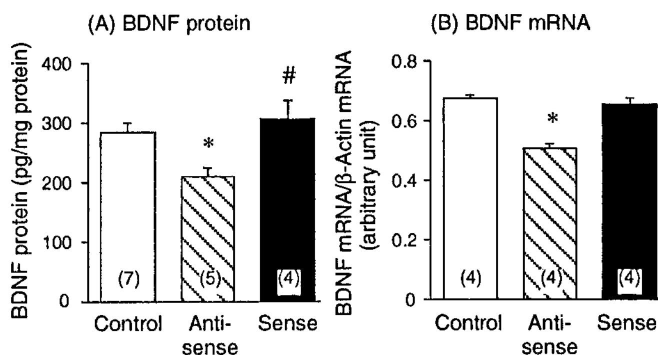

Figure 5. Effects of continuous infusion of BDNF antisense and sense oligonucleotides into the cerebral ventricle on BDNF protein (*A*) and mRNA (*B*) levels in the hippocampus. Rats were continuously infused with BDNF antisense or sense oligonucleotide into the cerebral ventricle and were subjected to the radial maze test for 21 d. The rats treated with antisense or sense BDNF oligonucleotide and nontrained control rats were killed on the day after the last training for the measurement of BDNF protein (*A*) and mRNA (*B*) levels. The number in parentheses shows the number of animals. Each value represents the mean ± SE. *$p < 0.05$ versus control. #$p < 0.05$ versus antisense-treated group.

Finally, to investigate the role of BDNF in memory retention and/or recall, antisense BDNF oligonucleotide was infused in rats that had been trained for the reference and working memory task for 28 d. The rats underwent surgery for continuous intracerebroventricular infusion of either antisense or sense BDNF oligonucleotide on day 29. After a 4 d recovery period, they were subjected to the same reference and working memory task.

Figure 6 shows the effects of antisense BDNF treatment on previously acquired spatial memory. An ANOVA with repeated measures of the data after the surgery revealed a significant effect of group ($F_{(1,19)}$ = 61.035; $p < 0.0001$), but not trial ($F_{(5,95)}$ = 0.289; p = 0.9178) and group by trial interaction ($F_{(5,95)}$ = 0.303; p = 0.9100) on reference memory (Fig. 6*A*). There were also significant effects of group ($F_{(1,19)}$ = 34.410; $p < 0.0001$), but not trial ($F_{(5,95)}$ = 0.127; p = 0.9859) and group by trial interaction ($F_{(5,95)}$ = 0.127; p = 0.9859) on working memory (Fig. 6*B*). These results indicated that the number of both reference and working memory errors was significantly increased by treatment with antisense BDNF oligonucleotide compared with the sense oligonucleotide.

DISCUSSION

Memory formation is considered to involve both short-term changes in electrical properties and long-term structural alterations in synapses. Short-term changes may include LTP (Barnes, 1995) and LTD (Ito, 1986), whereas long-term morphological alterations may involve synaptogenesis and neuropil growth (Burns and Augustine, 1995; Edwards, 1995). Because BDNF significantly modulates both forms of synaptic changes, it has been suggested to play a role in neuronal plasticity such as learning and memory (Lo, 1995; Thoenen, 1995).

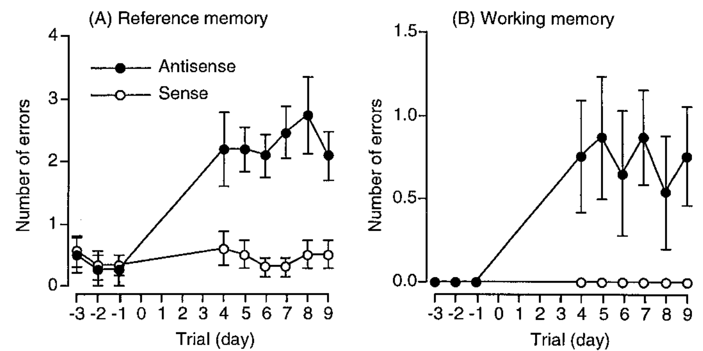

Figure 6. Effects of continuous infusion of BDNF antisense and sense oligonucleotide into the cerebral ventricle on spatial reference (*A*) and working (*B*) memory retention and/or recall in rats which had previously acquired the spatial reference and working memory. Rats were first trained for the reference and working memory task for 28 d and then received a surgical operation for the continuous intracerebroventricular infusion of BDNF antisense (n = 11) or sense oligonucleotide (n = 10). The radial maze test was started 4 d after the start of oligonucleotide treatment. Each value represents the mean ± SE. Antisense BDNF treatment significantly increased the number of reference ($p < 0.0001$) and working memory errors ($p < 0.0001$).

We demonstrated in the present study that spatial memory formation is associated with an increase in BDNF mRNA level in the hippocampus in the radial arm maze test. The increase in BDNF mRNA was not observed when learning was inhibited by treatment with a brain NOS inhibitor, 7-NI (Babbedge et al., 1993; Moore et al., 1993). The continuous intracerebroventricular infusion of antisense BDNF oligonucleotide resulted in a severe impairment in reference and working memory formation, which was accompanied by a reduction of BDNF mRNA and protein levels in the hippocampus. Furthermore, the antisense BDNF treatment disrupted previously formed spatial memory. Our findings suggest an essential role for BDNF in the acquisition, as well as retention and/or recall, of spatial memory.

With regard to a role for BDNF in memory formation, it has been demonstrated, by using nuclease protection assay and *in situ* hybridization, that the level of BDNF mRNA was elevated in the hippocampus but not cerebellum, striatum, frontal, and middle or caudal neocortex in the learning group compared with yoked and sedentary controls after 3 and 6 d of training in the water maze (Kesslak et al., 1998). It is also reported, using a RT-PCR method, that the BDNF mRNA level in the dentate gyrus of the hippocampus was significantly increased in rats showing good retention compared with the poor retention control animals (Ma et al., 1998). All these studies, including our own, suggest that learning is associated with an alteration of BDNF mRNA in the hippocampus in spite of differences in levels of stress and the types of motivation involved in the behavioral tasks used. It should be noted, however, that there was no apparent increase in BDNF mRNA levels immediately after the training trial on day 28. The results suggest that previous training for 27 d has no residual effect on BDNF mRNA levels, and the levels may get turned over between training trial days. Therefore, it remains to be determined how such alterations of BDNF mRNA expression after training trial contribute to spatial reference and working memory formation.

We have used RT-PCR instead of Northern blotting or nuclease protection assay to quantify BDNF mRNA, under conditions that PCR cycles were within a linear working range for all PCR products, and the levels of β-actin mRNA were determined as internal controls. Further experiments should be undertaken with Northern blot analysis to determine the levels and *in situ* hybridization histochemistry to map the distribution of BDNF mRNA expression.

The paucity of agents that can selectively and potently block the synthesis of BDNF or its receptor trkB has led to investigations into the role of BDNF in learning and memory using BDNF and trkB mutant mice. Because most mice deficient in BDNF (Ernfors et al., 1994) and trkB (Klein et al., 1993) die after birth, behavioral experiments had so far been limited to the heterozygous mutant mice. The results are controversial. One study has shown that heterozygous BDNF mutant mice show a moderate but significant impairment of water maze learning without any effect on memory retention (Linnarsson et al., 1997), whereas another study has failed to detect deficits of performance in the water maze test (Montkowski and Holsboer, 1997). Recently, Minichiello et al. (1999) generated conditionally gene targeted mice in which the knock-out of the trkB gene is restricted to the forebrain and occurs only during postnatal development (trkB-CRE mutant mice), and they analyzed their ability to learn and memorize. The trkB-CRE mutant mice completely failed the more stressful water maze test, partly failed the eight-arm maze test, but succeeded in simple passive avoidance learning, suggesting a role for trkB receptor signaling in complex learning. The results also implied that procedural long-term memory is relatively spared, whereas short-term plasticity within the hippocampus is impaired in the trkB-CRE mutant mice (Minichiello et al., 1999).

Our results clearly indicate that BDNF plays an essential role in both reference and working memory. Reference memory refers to memory for information that remains constant over repeated trials and is, therefore, trial-independent. Working memory refers to memory in which the information to be remembered changes in repeated trials, and thereby, is trial-dependent (Olton et al., 1979). Both hippocampus and cerebral cortex have been suggested to contribute to reference and working memory (Kesner et al., 1987; Jarrard, 1993; Durkin, 1994), and these brain areas contain relatively high levels of BDNF proteins (Nawa et al., 1995; Katoh-Semba et al., 1997). Although learning-associated change in the BDNF mRNA level was observed only in the hippocampus, not in the frontal cortex, it remains to be determined which brain structure or structures are the site or sites of action of BDNF in spatial reference and working memory. A direct focal infusion of antisense BDNF oligonucleotide into the hippocampus or the frontal cortex may allow a more detailed dissection of the role for BDNF in reference and working memory.

The impairment of BDNF synthesis induced by antisense BDNF treatment resulted in an impairment of memory retention and/or recall, as well as memory formation, in the radial arm maze test. There was no difference, however, in memory retention between the BDNF mutant and wild-type mice, as assessed by performance in a spatial probe test immediately and 4 weeks after training for the water maze (Linnarsson et al., 1997). The reasons for the discrepancy between the two studies are unclear. BDNF is involved in the modulation of synaptic transmission such as cholinergic and glutamatergic neurotransmission, and produces a fast physiological response, similar to those observed in classical neurotransmitters (Berninger and Poo, 1996). These effects may explain the impairment of memory retention induced by antisense BDNF treatment because inhibition of cholinergic and glutamatergic neurotransmission impairs memory retention in rats (Nabeshima, 1993; Yamada et al., 1996a; Zou et al., 1998b, 1999). The BDNF mutant mice, on the other hand, failed to show such impairment because of possible compensations by neural systems for the gene mutation.

It is important to establish the earliest time point at which BDNF antisense treatment is effective in downregulating BDNF mRNA and protein. Although we did not perform such experiments, the last experiment indicated that the antisense infusion for 4 d was sufficient to disrupt spatial memory (Fig. 6). Therefore, we consider that a relatively short-term intracerebroventricular infusion (at least 4 d) of antisense BDNF oligonucleotide may be effective in downregulating BDNF mRNA and protein under our experimental condition.

There is evidence that NO is involved in the mechanisms of synaptic plasticity, including LTP (Bliss and Collingridge, 1993; Garthwaite and Boulton, 1995; Son et al., 1996), LTD (Shibuki and Okada, 1991), and learning and memory *in vivo* (Yamada et al., 1995, 1996a,b; Zou et al., 1998a). Thus, it appears that BDNF and NO have similar effects on synaptic activities and learning and memory processes. Mutual regulation of these two molecules in their synthesis has been demonstrated *in vitro* and *in vivo* (Xiong et al., 1999). In the present study, 7-NI inhibited spatial memory formation, which was associated with a failure of alteration of BDNF mRNA induced by the maze training. These effects of 7-NI are consistent with our previous findings that 7-NI inhibits pentylenetetrazole-induced kindling, a form of synaptic plasticity that was accompanied by prevention of the kindling-induced increase in BDNF protein levels in the hippocampus (Han et al., 2000). It should be elucidated why 7-NI suppressed BDNF mRNA expression at longer intervals (15 and 30 min), but not immediately, after learning.

In summary, this study, in which BDNF synthesis has been diminished by the continuous intracerebroventricular infusion of BDNF antisense oligonucleotide, suggests an essential role for BDNF in spatial memory formation as well as memory retention and/or recall. Further work, in which infusion of antisense oligonucleotide will be restricted to certain brain structures such as the hippocampus, will shed light on the mechanism and on neuronal pathways that require BDNF/trkB signaling in learning and memory processes.

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