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# Involvement of Glial Cell Line-Derived Neurotrophic Factor in Activation Processes of Rodent Macrophages

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The physiological roles of glial cell line-derived neurotrophic factor (GDNF) expressed in the microglia/macrophages of the injured spinal cord have not yet been clarified. mRNA expression of chemokines, including monocyte chemoattractant protein (MCP)-1, was evoked within 1 hr after transection of the spinal cord, and GDNF mRNA expression was similarly up-regulated. Immunohistochemical analysis showed that GDNF was coexpressed with MCP-1 in the CD11b-positive cells. Therefore, we examined further the effects of GDNF on cultured rat peritoneal macrophages. GDNF enhanced the phagocytic activity of the macrophages via GFR $\alpha$ -1, glycosylphosphatidylinositol-anchored specific binding site of GDNF, in a c-Ret-independent manner. The influence of autocrine and/or paracrine GDNF synthesis was evaluated by performing activation experiments using macrophages cultured from heterozygous (+/-) GDNF gene-deficient mice or wild-type (+/+) mice. There were no morphological differences dependent on genetic types or stimulators. However, the GDNF mRNA level, but not the MCP-1 or GFR $\alpha$ -1 mRNA level, was substantially lower in the mutant macrophages than in the +/+ cells irrespective of stimulation with MCP-1 or lipopolysaccharide (LPS). The phagocytic activity enhanced by MCP-1 or LPS was significantly lower in the mutant cells (+/-) than in the +/+ ones, demonstrating the involvement of endogenous GDNF in the activation processes of macrophages *in vitro* and suggesting that not only neuroprotective function but also activation of macrophages is effected by the GDNF produced after a spinal cord injury. © 2005 Wiley-Liss, Inc.

**Key words:** macrophages; phagocytic activity; spinal cord; glial cell line-derived neurotrophic factor; GDNF; monocyte chemoattractant protein-1; MCP-1

Several experimental strategies have been employed to minimize tissue damage and to enhance axonal growth and regeneration after injury to the central nervous system (CNS). The failure of axonal regeneration after CNS

injury is thought to result in part from the expression of molecules inhibitory for axonal growth (Fawcett and Asher, 1999), the lack of neurotrophic factors (Widenfalk et al., 2001), and/or inflammatory reactions (Franzen et al., 1998).

Inflammation is one of the responses occurring immediately after spinal cord injury and is likely to cause secondary injury that magnifies the primary injury and facilitates neuronal dysfunction. This inflammatory response is characterized by the invasion of leukocytes into the injury site and the activation of resident glial cells, which is regulated by chemokines in the case of various injuries and diseases of the CNS (Glabinski et al., 1995; Adams and Lloyd, 1997; Rollins, 1997). Several studies have shown that accumulation of macrophages around the lesion site is correlated with the secondary injury that appears after a spinal cord injury (Blight, 1985, 1992; Popovich et al., 1997; Zhang et al., 1997). On the other hand, inflammation is also thought to contribute to the induction of neurotrophic factors beneficial to damaged neurons. It has been shown that glial cell line-derived neurotrophic factor (GDNF), one of the most potent neurotrophic factors for motoneurons (Lin et al., 1993; Henderson et al., 1994), is expressed in leukocytes and especially in activated microglia/macrophages of the injured brain (Batchelor et al., 1999; Wei et al., 2000). There is also a report showing that the activated microglia/macrophages in the injured spinal cord express GDNF (Satake et al., 2000; Widenfalk et al., 2001). How-

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ever, the interaction between neurotrophic factors and chemokines in the injured spinal cord and the physiological significance of GDNF expression in microglia/macrophages have not been studied.

In this study, we found that 1) microglia/macrophages recruited into the transected spinal cord coexpressed both GDNF and one of the chemokines, monocyte chemoattractant protein (MCP)-1; 2) cultured peritoneal macrophages responded to exogenous GDNF by enhancing their phagocytic activity and expression of MCP-1 mRNA; and 3) macrophages cultured from the heterozygous (+/-) GDNF gene-deficient mice were activated by MCP-1 or lipopolysaccharide (LPS) to a much lesser extent than those cultured from the +/+ mice. These results suggest that GDNF induced in activated macrophages behaves as a cytokine for macrophages and participates in the development of inflammation in the injured CNS in addition to functioning in its role as a neurotrophic factor.

## MATERIALS AND METHODS

### Animals and Surgery

Animals were cared for according to the guidelines for experimental animal care issued from the Office of the Prime Minister of Japan. Adult male Wistar rats (7–8 weeks old, 150–200 g; Nippon SLC, Shizuoka, Japan) were anesthetized by an intraperitoneal injection with pentobarbital (30 mg/kg). After laminectomy at the level of the ninth thoracic vertebra, the exposed spinal cords were completely transected with a razor blade. Afterward, the incision sites were sutured. The generation of the GDNF gene-deficient mice has been described elsewhere (Picher et al., 1996).

### Reverse Transcription-Polymerase Chain Reaction

The spinal cords were dissected out from the anesthetized animals 0, 1, 3, 6, 12, 24, or 48 hr after the spinal cord transection, and segments (5 mm in length) of each spinal cord just rostral or caudal to the transected site were collected and pooled. RNA was prepared from the collected tissues by using Isogen (Nippon Gene, Tokyo, Japan), which is basically composed of guanidine isothiocyanate. The reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the instructions. In brief, 500 ng of RNA was denatured for 2 min at 70°C and reverse transcribed with MMLV reverse transcriptase for 1 hr at 42°C in the presence of 1  $\mu$ M CDS primer. Afterward, the transcriptase was inactivated by heating for 7 min at 72°C. The synthesized cDNA was amplified with each pair of primers by PCR. The amplification was carried out with the thermal cycler at 94°C for 3 min, followed by 20–36 cycles consisting of 94°C for 30 sec, 52–60°C for 30 sec, and 72°C for 30 sec. The products were electrophoresed on 2% agarose gels. The predicted sizes of amplified cDNA fragments, the numbers of PCR cycles, and the annealing temperatures are indicated in Table I.  $\beta$ -Actin mRNA was used as an internal control. The intensity of the bands was analyzed by use of image-analysis software (Mac BAS;

Fuji Film, Tokyo, Japan). RNA from rat peritoneal macrophages cultured in the presence of GDNF, MCP-1 (Prepro-Tech, Rocky Hill, NJ), or LPS (Sigma, St. Louis, MO) was prepared and analyzed by RT-PCR as described above.

### Immunohistochemical Study

Two days after the spinal cord transection, animals were anesthetized with pentobarbital (30 mg/kg) and then cardioperfused with cold 4% (w/v) paraformaldehyde solution prepared in 0.1 M phosphate buffer, pH 7.3 (the fixative). The spinal cord was postfixed with the same fixative for 2 hr, soaked in 20% (w/v) sucrose for 1 day, and frozen in embedding compound (Tissue-Tec; Miles, Elkhart, IN). Coronal sections of 30  $\mu$ m thickness were cut with a cryostat (model CM 1800; Leica, Deerfield, IL) and thawed on coverslips bearing covalently linked amino groups (Sumitomo Bakelite, Tokyo, Japan). The tissue sections were next dried and subsequently soaked in the fixative for 5 min to cross-link covalently the tissue sections to the coverslips. Then, they were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.3% (v/v) Triton X-100 (TT buffer) at 37°C for 30 min to make the cell membrane permeable to antibodies. Next, the sections were incubated with TT buffer containing anti-GDNF rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution or anti-MCP-1 rabbit antibody (Biogenesis, Poole, United Kingdom) at a 1:1,000 dilution or antigial fibrillary acidic protein mouse antibody (GFAP; Boehringer Mannheim Biochemical, Indianapolis, IN), anti-CD11b mouse antibody (Chemicon, Temecula, CA), and anti-NeuN mouse antibody (Chemicon) at a 1:1,000 dilution at 4°C overnight, and subsequently incubated at room temperature for 2 hr with TT buffer containing both fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG and rhodamine-labeled anti-mouse IgG antibodies (Chemicon). After having been washed with TT buffer, the sections were observed for fluorescence with a confocal laser microscope (Zeiss model L SM510).

### Cell Cultures

Macrophages were obtained from the peritoneal cavity of adult male Wistar rats or adult GDNF gene-deficient mice [wild-type mice (+/+) or heterozygous mice (+/-)] 2 days after an intraperitoneal injection of 200  $\mu$ g of concanavalin A (Sigma) in 5 ml phosphate-buffered saline (PBS). The cells were washed with PBS and cultured in RPMI 1640 medium (Sigma) containing 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Cansera Int., Rexdale, Canada); 1 hr later, the medium was aspirated to remove nonadherent cells and replaced with fresh medium.

### Phagocytosis Assay

The FITC-labeled latex beads (1.0  $\mu$ m in diameter; Polysciences, Warrington, PA) were opsonized by incubation with human IgG (Sigma) at 4°C overnight, washed, and suspended in PBS. Cells pretreated or not with phosphatidylinositol-specific phospholipase C (PI-PLC, 0.5 U/ml; Sigma) for 1 hr were cultured in the presence of GDNF, MCP-1, or LPS and then incubated in the medium containing 0.01% FITC-labeled latex beads for 30 min at 37°C. Excess beads were washed away with PBS, and incorporated beads were visualized with a fluorescence

TABLE I. Sequences of Oligonucleotide Primers Used in the RT-PCR

| Gene            | Upstream primer                      | Downstream primer                    | Product size (bases) | Annealing temperature (°C) | Cycle number |
|-----------------|--------------------------------------|--------------------------------------|----------------------|----------------------------|--------------|
| GDNF            | GAGAGGAATCGGCAG<br>GCTGCAGCTG        | CAGATACATCCACAT<br>CGTTTAGCGG        | 337                  | 60                         | 36           |
| NGF             | GGGGATCCTCCACCC<br>ACCCAGTCTCCAC     | GGCAAGTCAGCCTCT<br>TCTTGTAGCCTTCC    | 376                  | 60                         | 30           |
| BDNF            | GGAATTCGAGTGATG<br>ACCATCCTTTTCCTTAC | CGGATCCCTATCTTC<br>CCCTTTAATGGTCAGTG | 771                  | 60                         | 31           |
| NT-3            | TATGCAGAACATAAG<br>AGTCAC            | CTACGAGTTTGTGT<br>TTTCTG             | 294                  | 55                         | 36           |
| MCP-1           | TAGCATCCACGTGCT<br>GTCTC             | CATTCAAAGGTGCTG<br>AAGTCC            | 299                  | 55                         | 25           |
| MIP-1 $\alpha$  | TGCCCTTGCTGTTCT<br>TCTCT             | AGATCTGCCGGTTTC<br>TCTTG             | 200                  | 55                         | 30           |
| MIP-1 $\beta$   | CTCTCTCCTCCTGCT<br>TGTGG             | CACAGATTTGCCTGC<br>CTTTT             | 200                  | 55                         | 30           |
| MIP-2           | AGGGTACAGGGGTTG<br>TTGTG             | TTTGGACGATCCTCT<br>GAACC             | 204                  | 55                         | 30           |
| RANTES          | ATATGGCTGGACAC<br>CACTC              | CGCACTTCTTCTCTG<br>GGTTG             | 167                  | 55                         | 30           |
| CCR2            | CTGCCCTACTTGTG<br>ATGGT              | AACGCAGCACTGTGT<br>CATTC             | 264                  | 55                         | 30           |
| GFR $\alpha$ -1 | TCCAGCCACATAACC<br>ACAAA             | CTTCAACAGAAGCCC<br>CTGAG             | 297                  | 55                         | 30           |
| c-Ret           | CGGCACACCTCTGCT<br>CTATG             | CTGGAGGAAGACGGT<br>GAGCA             | 237                  | 55                         | 35           |
| $\beta$ -Actin  | GTGGGCGGCCCTAGG<br>CACCAG            | CTCTTTAATGTACAG<br>CAGCAT            | 542                  | 55                         | 32           |

microscope. Phagocytic activity was quantified as the number of FITC-positive cells (phagocytosing macrophages) per total cells.

## RESULTS

### Injury-Induced mRNA Expression of Neurotrophic Factors

Time-dependent mRNA expression of GDNF or neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), was examined in the segment adjacent to the transection site of the rat spinal cord (Fig. 1). NGF, BDNF, and GDNF mRNA levels were significantly elevated after the injury, but the NT-3 mRNA level remained constant throughout the experimental period. The increase was greatest for NGF mRNA, followed by that for GDNF mRNA. The NGF mRNA level started to increase 1 hr after the transection, peaked at 6 or 12 hr, and gradually declined until 48 hr. The GDNF mRNA started to rise slightly later, at 3 hr after the injury, attained its maximal level 12 hr postoperation, and gradually declined until 48 hr. However, the change in the mRNA level of BDNF was relatively small, though significant; i.e., an increase was detected first at 6 hr and was sustained until 24 hr after the injury. These results for NGF and GDNF gene expression are in agreement with previous findings (Widenfalk et al., 2001; Murakami et al., 2002). We were particularly interested in GDNF with respect to

spinal cord regeneration, because GDNF has potent neurotrophic activity on motoneurons and CNS neurons that send axons into the spinal cord.

### Injury-Induced mRNA Expression of Chemokines

Various chemokines are up-regulated in the injured CNS, including the spinal cord (McTigue et al., 1998; Huang et al., 2000; Lee et al., 2000). They are thought to act as chemoattractants to recruit immune cells, such as leukocytes and microglia/macrophages, to the injury site, thus triggering inflammatory reactions. To compare the gene expression profile of GDNF with that of various chemokines, we monitored by RT-PCR the time-dependent mRNA expression of MCP-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, and regulated upon activation normal T-cell expressed and secreted (RANTES) in the segment adjacent to the transection site of the spinal cord. The expression profiles of these mRNAs were similar to each other. Especially, the pattern of MIP-1 $\beta$  quite resembled that of MIP-1 $\alpha$ , so the results for MIP-1 $\beta$  are not shown. The expression of all chemokines tested was evoked 1 hr after the injury, peaked at 6 or 12 hr, and was sustained at a significantly high level until 48 hr (Fig. 2). These patterns resembled the expression profile of GDNF mRNA, suggesting shared mechanisms for expression between chemokines and GDNF in the injured spinal cord.

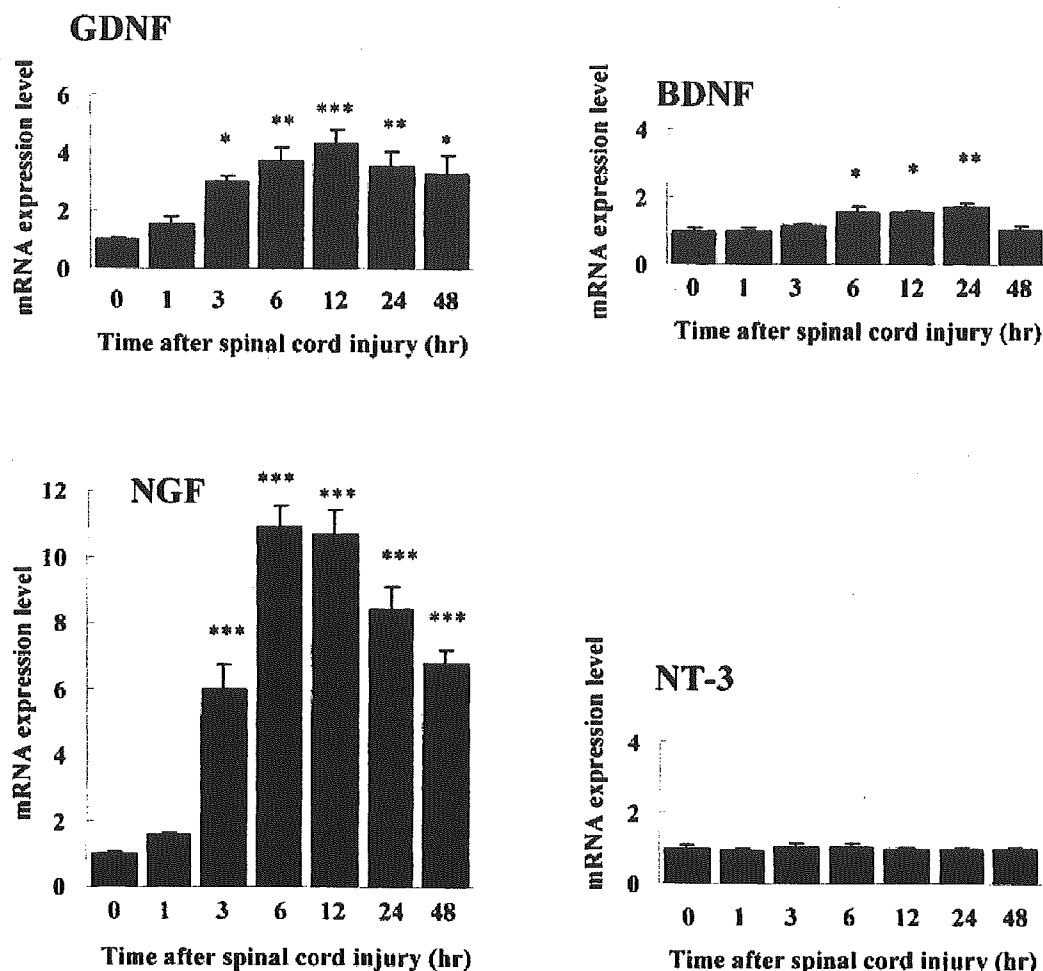


Fig. 1. Expression of neurotrophic factor mRNAs in the sites adjacent rostrally or caudally to the transection site of the rat spinal cord. The time-dependent mRNA expression of neurotrophic factors, including GDNF, NGF, BDNF, and NT-3, after spinal cord transection was monitored by RT-PCR. Total RNA of the tissue was subjected to RT-PCR using primers specific for the respective target genes (see Table I), and PCR products were applied to 2% agarose gels. The bands corresponding to neurotrophic factor mRNA- or  $\beta$ -actin mRNA-

derived cDNA were densitometrically quantified, and the ratio of the intensity of each neurotrophic factor band to that of the  $\beta$ -actin band (ratio of neurotrophic factor/ $\beta$ -actin) was calculated. The values are expressed as the means  $\pm$  SE ( $n = 3$ ) of the -fold increase in the ratio of GDNF/ $\beta$ -actin, regarding the value of the mice of control group (0 hr) as 1.0. Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### Expression of GDNF and MCP-1 in the Injured Spinal Cord

Mac1/CD11b-positive cells, implying activated microglia/macrophages (Lu and Pitha, 2001; Babcock et al., 2003), appeared in a limited fashion in both rostral and caudal areas just adjacent to the injury site (data not shown). GDNF or MCP-1 immunoreactivity (-ir) was observed predominantly around the injury site and was colocalized with most of the Mac1/CD11b-positive cells (Fig. 3A-F). These results demonstrate that activated microglia/macrophages synthesized both GDNF and MCP-1 in the transected spinal cord. MCP-1-ir (Fig. 3G-I), but not GDNF-ir (data not shown),

was colocalized with GFAP-ir in fibrous structures in the area over 5 mm distant from the injury site, implying the expression of MCP-1 in the processes of astrocytes, as reported previously (Che et al., 2001; Babcock et al., 2003). This finding may suggest that the physiological roles of GDNF are at least partially different from those of MCP-1, because glial production of MCP-1 was shown to be important for leukocyte responses to CNS axonal injury (Babcock et al., 2003). The MCP-1-ir-positive and GFAP-ir-negative cells were sparse in the same area distant from the injury site (Fig. 3I). They might resemble neurons morphologically, but they were likely to be activated microglia/

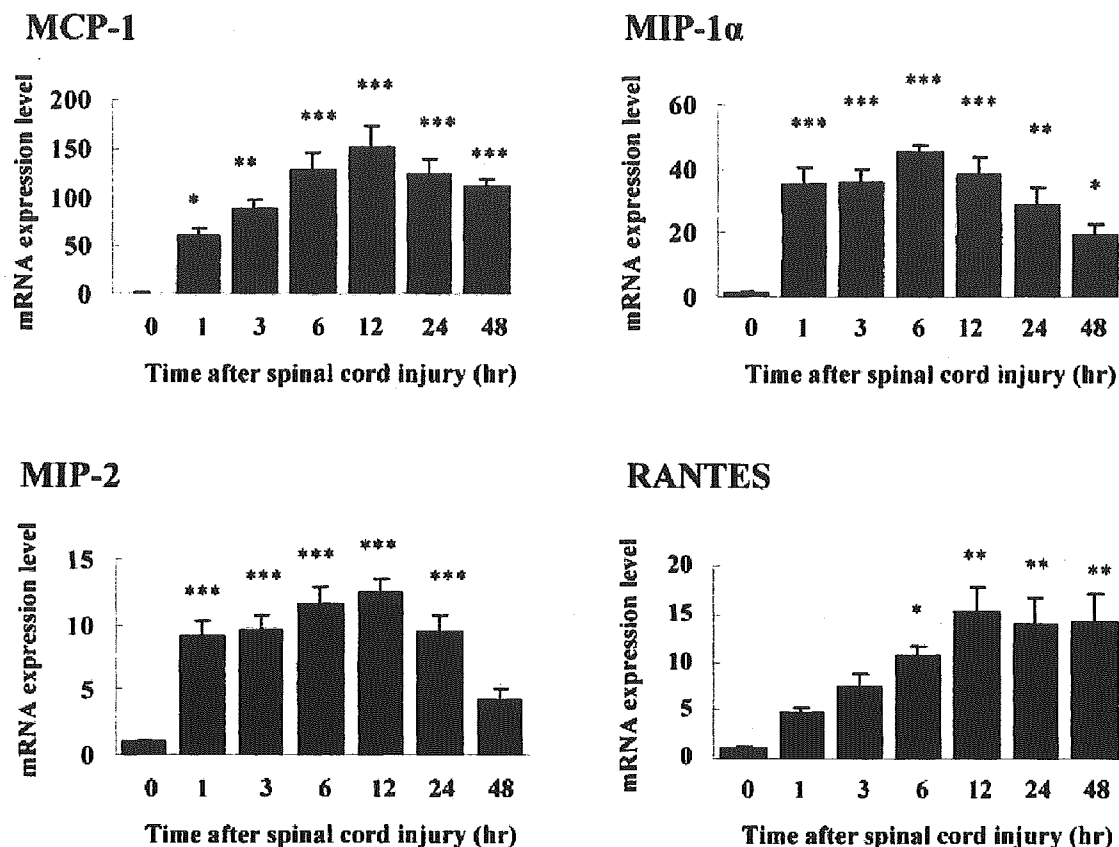


Fig. 2. Expression of chemokine mRNAs in the sites adjacent rostrally or caudally to the transected site of the rat spinal cord. The time-dependent mRNA expression of chemokines, including MCP-1, MIP-1 $\alpha$ , MIP-2, and RANTES, after spinal cord transection was monitored by RT-PCR. The ratio of the intensity of chemokine cDNA products

to that of  $\beta$ -actin cDNA products was calculated. The values are expressed as the means  $\pm$  SE ( $n = 3$ ) of -fold increase over the control group (0 hr). Significant differences from the value of the corresponding control group were determined by one-way ANOVA and Tukey test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

macrophages because of intensive MCP-1 expression, which is poor in neurons. Because MCP-1 expression has been shown to occur on injured neurons (Flugel et al., 2001), combined expression of MCP-1 and NeuN, an antigen specific for differentiated neurons, was examined in the areas just adjacent to the injury site. As demonstrated in Figure 3J–L, NeuN and MCP-1 were separately expressed, demonstrating that neurons in the injured spinal cord did not always express MCP-1.

#### Expression of Receptors for GDNF or MCP-1

To examine whether the activated microglia/macrophages can respond to GDNF, we analyzed by RT-PCR the mRNA expression of receptors for GDNF in macrophages cultured from the rat peritoneal cavity. In addition to the mRNA of MCP-1 receptor CCR2, that of GFR $\alpha$ -1, a glycosyl-phosphatidylinositol (GPI)-anchored binding site of GDNF, was detected, but not that of the tyrosine kinase c-Ret, which transduces the GDNF signal intracellularly (Fig. 4). Recent experiments showed that GDNF actions could be mediated by GFR $\alpha$ -1 in the absence of c-Ret

(Paratcha et al., 2003). Therefore, the cultured macrophages used in this work hereinafter seem to respond to GDNF in an autocrine and/or local paracrine manner through GFR $\alpha$ -1 alone.

#### Effects of GDNF and MCP-1 on Phagocytosis Activity of Cultured Macrophages

Next, dose- and time-dependent effects of GDNF on the phagocytic activity of cultured rat peritoneal macrophages were examined (Fig. 5). Although relatively higher concentrations (100–1,000 ng/ml) and longer exposure times (over 12 hr) were necessary, GDNF significantly facilitated the phagocytosis of latex beads. On the other hand, MCP-1 was effective at a concentration as low as 10 ng/ml and showed potent activity as early as 1 hr after exposure. LPS caused a high level of phagocytic activity at 100 ng/ml. The GDNF activity was completely lost when the macrophages were pretreated with PI-PLC, which liberates GFR $\alpha$ -1, the GPI-anchored specific binding site of GDNF, demonstrating a specific and direct action of GDNF on macrophage phagocytosis via this

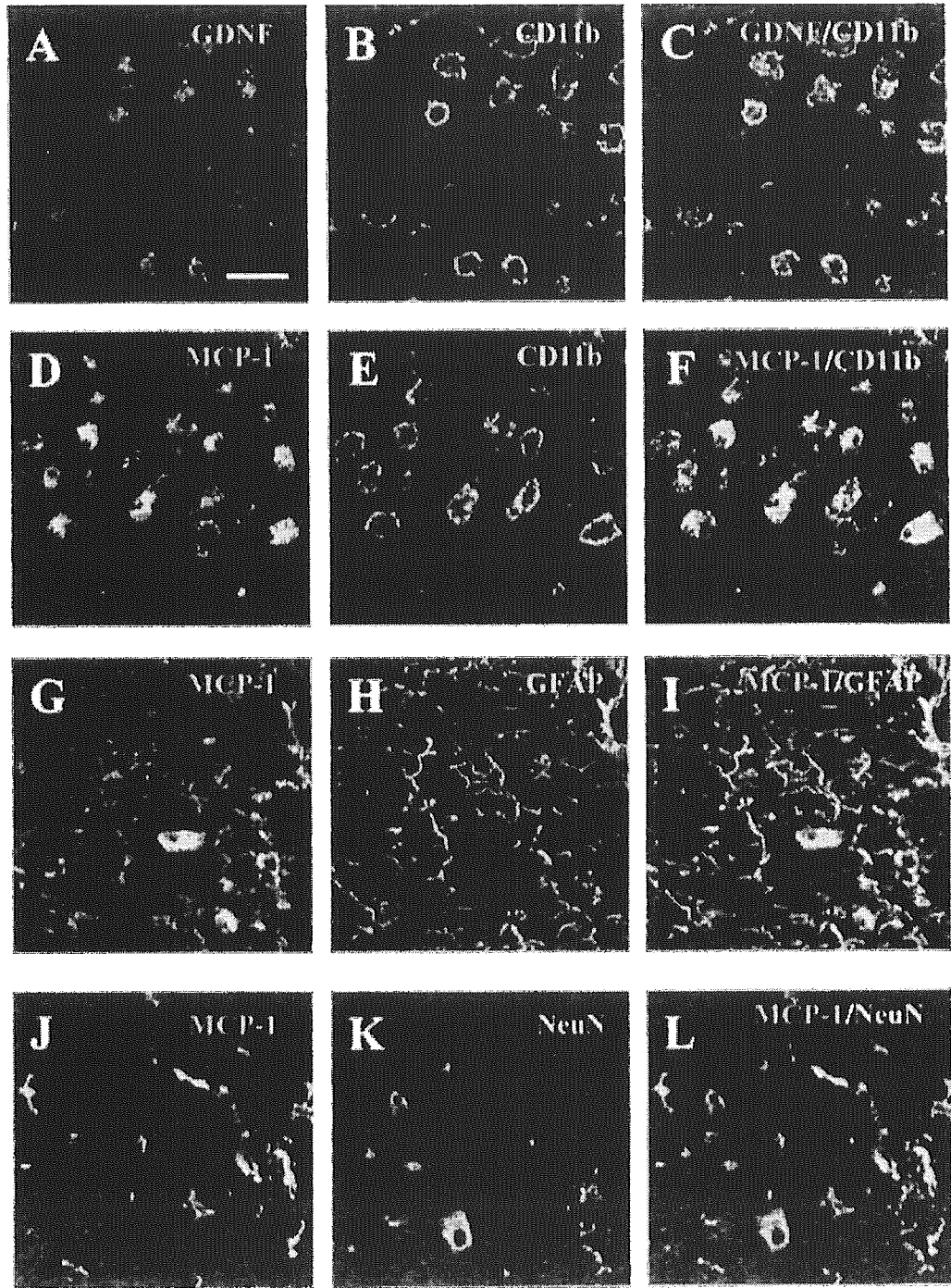


Fig. 3. Localization of GDNF- and MCP-1-ir in the injured rat spinal cord. Rats were anesthetized and cardioperfused 2 days after spinal cord transection. The spinal cord was dissected out, postfixed, and frozen in embedding compound. Coronal sections of 30  $\mu\text{m}$  thickness were cut from the area 2 mm (A-F) or 5 mm (G-L) distant from the transection site of the spinal cord and used for immunostaining analysis. The sections were reacted with specific antibody against GDNF (A), MCP-1 (D,G,J), GFAP (H), NeuN(K) or CD11b (B,E) and visualized with FITC-conjugated anti-rabbit IgG (A,D,G,J) or rhodamine-conjugated anti-mouse IgG (B,E,H,K). A and B are merged in C, D and E in F,G and H in I, and J and K in L. Scale bar = 20  $\mu\text{m}$ .

GPI-linked receptor component. On the other hand, MCP-1 was fully active, but LPS showed only half of its original activity toward the PI-PLC-treated macrophages. A part of the phagocytic activity elicited by LPS was shown to be mediated through GPI-anchored CD14 (Schiff et al., 1997). Therefore, the PI-PLC treatment liberated not only GFR $\alpha$ -1 but also CD-14 from the

surface of the macrophages, resulting in the reduction of GDNF or LPS activity. Neither GDNF nor MCP-1, nor their combination, showed any mitotic activity on macrophages, as judged from the lack of increase in cell number (data not shown). These results demonstrate that GDNF has phagocytosis-stimulating activity, like MCP-1 and LPS, and suggest that GDNF behaves as a cytokine

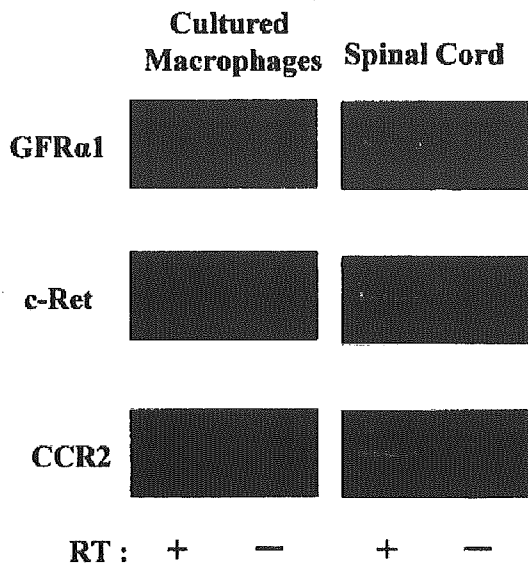


Fig. 4. Expression of GDNF and MCP-1 receptors in rat peritoneal macrophages. RT-PCR analysis detected mRNAs of GFR $\alpha$ -1 and CCR2 in total RNA prepared from cultured rat peritoneal macrophages but no c-Ret mRNA (RT<sup>+</sup>). No signal was obtained when the RT reaction was conducted without reverse transcriptase (RT<sup>-</sup>).

that acts on microglia/macrophages in the injured spinal cord.

**Effects of GDNF on mRNA Expression of MCP-1 in Cultured Macrophages**

Next, the effects of GDNF on the mRNA expression of MCP-1 and its own mRNA were investigated. As shown in Figure 6, GDNF stimulated significantly the expression of MCP-1 mRNA at 100 and 1,000 ng/ml but did not induce any significant elevation of GDNF mRNA. MCP-1 markedly stimulated the expression of MCP-1 mRNA as low as 10 ng/ml, but that of GDNF mRNA only at higher concentrations 100 and 1,000 ng/ml. LPS stimulated both mRNAs at a concentration of 100 ng/ml. These results suggest that the phagocytosis-stimulating activity of GDNF is mediated by GDNF-induced MCP-1.

**Activation of the Macrophages Cultured From GDNF Gene Mutants**

To clarify the involvement of GDNF in the activation processes of macrophages, we tested the effects of agents that activate macrophages on the macrophages cultured from GDNF gene-deficient +/- or +/+ mice. Macrophages from homozygous mice (-/-) could not be used for this experiment, because the mice died immediately after birth. First, mRNA expression of GDNF, GFR $\alpha$ -1, and c-Ret gene in the cerebral cortex and cultured macrophages was analyzed by RT-PCR (Fig. 7A). GDNF, GFR $\alpha$ -1, and c-Ret mRNAs were expressed in the cerebral cortex of both mutant (+/-) and

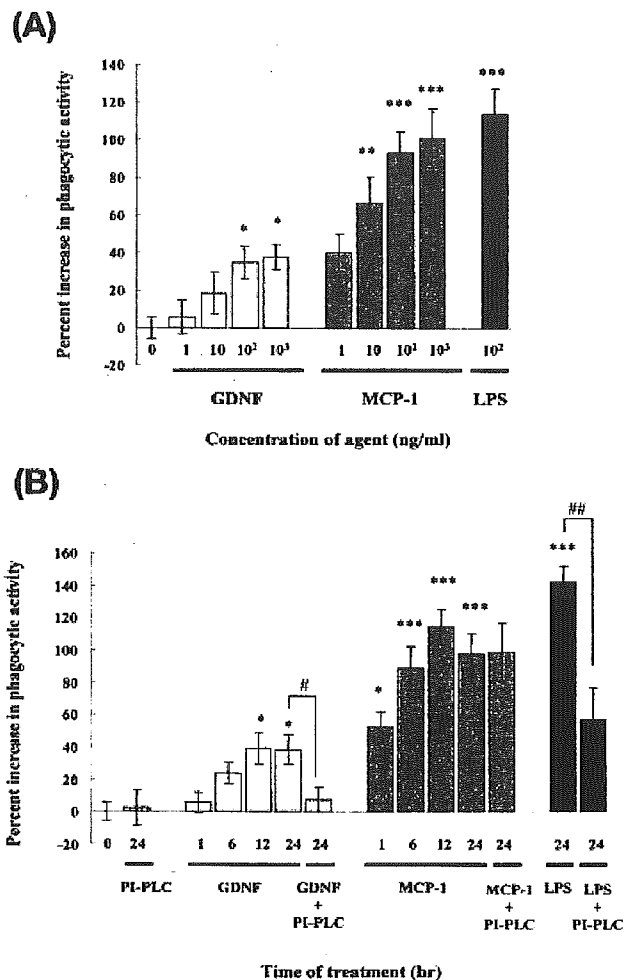


Fig. 5. Dose-dependent (A) and time-dependent (B) increase in phagocytic activity of cultured macrophages after exposure to GDNF, MCP-1, or LPS. Peritoneal macrophages were cultured in the presence of FITC-labeled latex beads after incubation with or without GDNF, MCP-1, or LPS at the indicated concentrations for 24 hr (A) or at 100 ng/ml for the indicated times (B). Some of the cells were pre-treated with PI-PLC (0.5 U/ml) for 1 hr to liberate GPI-anchored binding sites, such as GFR $\alpha$ -1 for GDNF and CD14 for LPS. Phagocytic activity is expressed as percentage increase in the number of macrophages bearing FITC-labeled beads/total number of macrophages (mean  $\pm$  SE, n = 5). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. the value of the control group (0 hr, without any reagents; one-way ANOVA and Tukey test); ##*P* < 0.05, ###*P* < 0.01 vs. the value of the group treated with PI-PLC (Student's *t*-test).

+/+ littermates. However, c-Ret mRNA was not expressed in the macrophages of either genetic type, consistent with the data on cultured rat macrophages shown in Figure 4. The expression level of GDNF mRNA was always lower in the +/- than in the +/+ mice before and after the stimulation with MCP-1 or LPS (Fig. 7A,Ba). The level of GFR $\alpha$ -1 mRNA tended to be lower in the +/- than in the +/+ mice in both cerebral cortex and



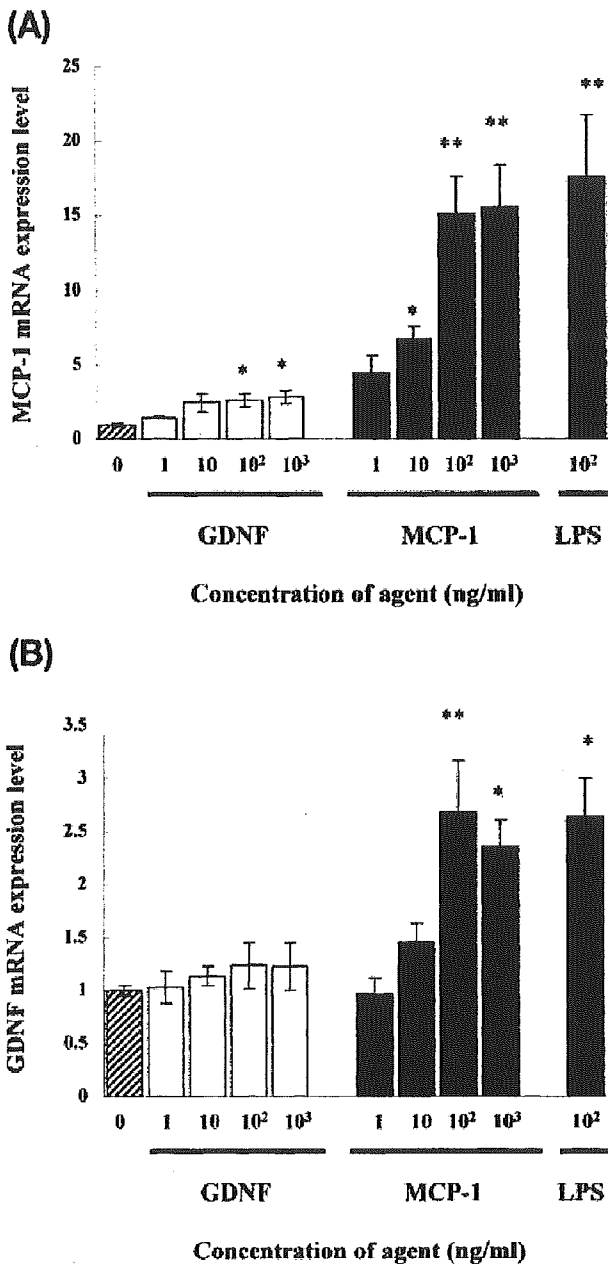


Fig. 6. Dose-dependent effects of GDNF, MCP-1, and LPS on the mRNA expression of MCP-1 (A) or GDNF (B) of cultured rat peritoneal macrophages. Macrophages were cultured with or without MCP-1, GDNF, or LPS at various concentrations for 24 hr, and then total RNA of the macrophages was prepared and subjected to RT-PCR with primers specific for MCP-1 (A) or GDNF (B). The ratio of the intensity of cDNA products to that of  $\beta$ -actin cDNA products was calculated. The values are expressed as mean  $\pm$  SE (n = 3) of -fold increase over the control group (0 ng/ml). Significant differences from the value of the control group (0 ng/ml) were determined by one-way ANOVA and Tukey post hoc test. \**P* < 0.05, \*\**P* < 0.01.

macrophages (Fig. 7A), but the difference was not statistically significant (Fig. 7Bb). MCP-1 mRNA expression was markedly facilitated by the stimulation with MCP-1 or LPS but was the same for both genetic types (Fig. 7Bc).

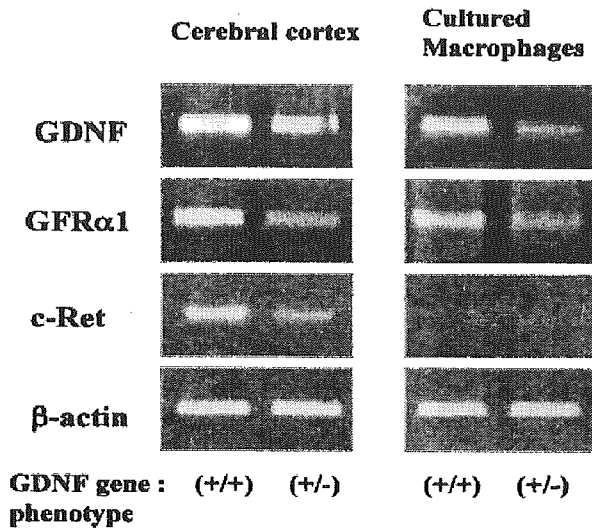
There were no morphological differences in the macrophages with respect to the genetic type or stimulant (Fig. 8A). The phagocytosis activity of the +/+ macrophages was significantly enhanced by the treatment with GDNF, MCP-1, or LPS or with MCP-1 or LPS but not with GDNF, in the +/- cells (Fig. 8B). However, the value of the phagocytosis activity induced was significantly lower in the +/- than in the +/+ macrophages when the cells were treated with GDNF, MCP-1, or LPS. The data thus indicate that the GDNF signal is important and is involved in phagocytosis-stimulating processes, i.e., activation processes of macrophages.

### DISCUSSION

Our present study was aimed at addressing the physiological significance of GDNF produced by macrophages in the injured CNS, and the results suggest that GDNF plays a role as a cytokine to activate microglia/macrophages in an autocrine/paracrine manner. Our observation that mRNAs of GDNF, NGF, BDNF, and all chemokines examined were up-regulated around the injury site of the rat spinal cord is essentially consistent with previous findings (McTigue et al., 1998; Lee et al., 2000; Satake et al., 2000; Widenfalk et al., 2001). However, detailed expression profiles of these factors had not been studied before. The mRNA expression profile of GDNF resembled very much that of NGF or various chemokines, including MCP-1 (Figs. 1, 2). Indeed, NGF is a well-known cytokine that stimulates phagocytosis and chemotaxis of macrophages (Susaki et al., 1996; Kobayashi and Mizisin, 2001). These findings prompted us to examine whether GDNF plays an inflammatory role following spinal cord injury. We focused on GDNF and compared its biological actions particularly on cultured macrophages with those of MCP-1, because GDNF and MCP-1 were coexpressed in microglia/macrophages around the transected site of the spinal cord (Fig. 3). Furthermore, Batchelor et al. (1999) reported that the synthesis of GDNF was increased in macrophages around the site of striatal injury, demonstrating a possibility that GDNF participates as a cytokine or chemokine to induce inflammatory reactions following spinal cord injury.

We initially examined the expression of the receptors of GDNF or MCP-1 in rat peritoneal macrophages, and we detected GFR $\alpha$ -1 mRNA, but not c-Ret mRNA (Figs. 4, 7). Generally, GDNF first binds to GFR $\alpha$ -1, and this complex then activates Ras/ERK and phosphatidylinositol 3-kinase/Akt pathways through the tyrosine kinase receptor c-Ret (Jing et al., 1996; Trupp et al., 1999). However, GDNF can also signal through activation of Src family kinase via GFR $\alpha$ -1 in the absence of c-Ret (Trupp et al., 1999; Pezeshki et al., 2001). In fact, exogenous GDNF enhanced phagocytic activity of the cultured macrophages via GFR $\alpha$ -1, a GPI-anchored GDNF binding protein (Fig. 5), demonstrating that GDNF could directly

**A**



**B**

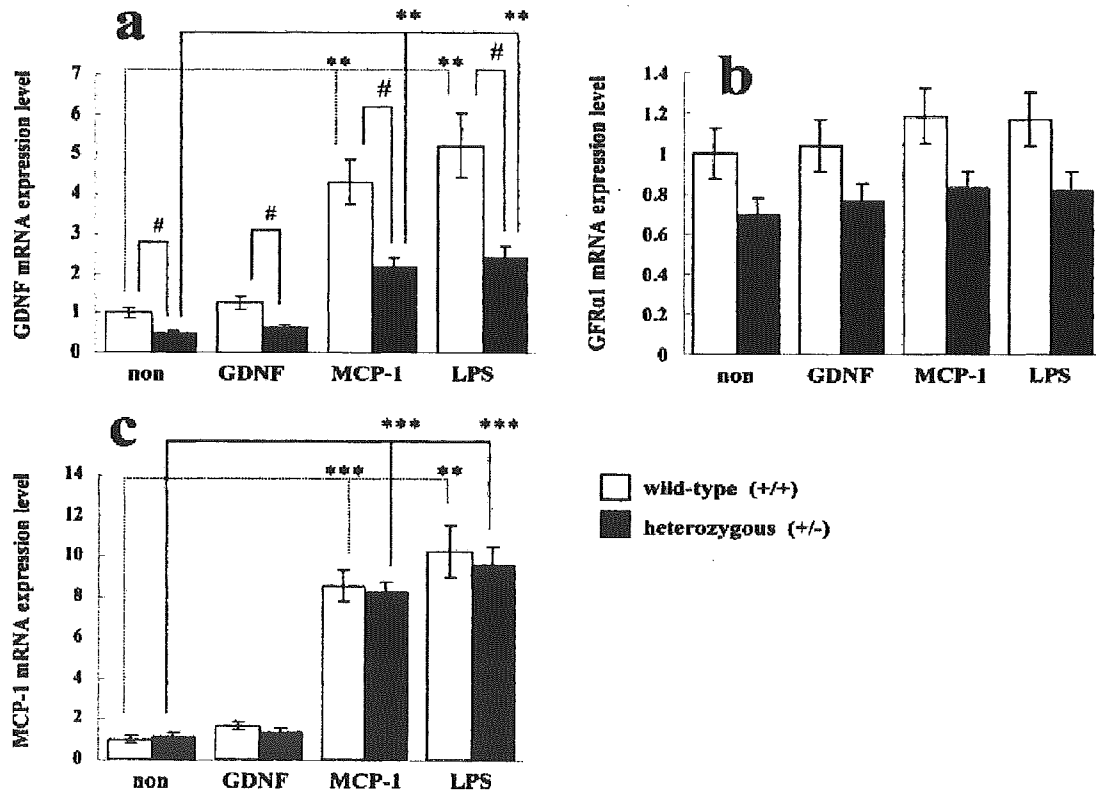
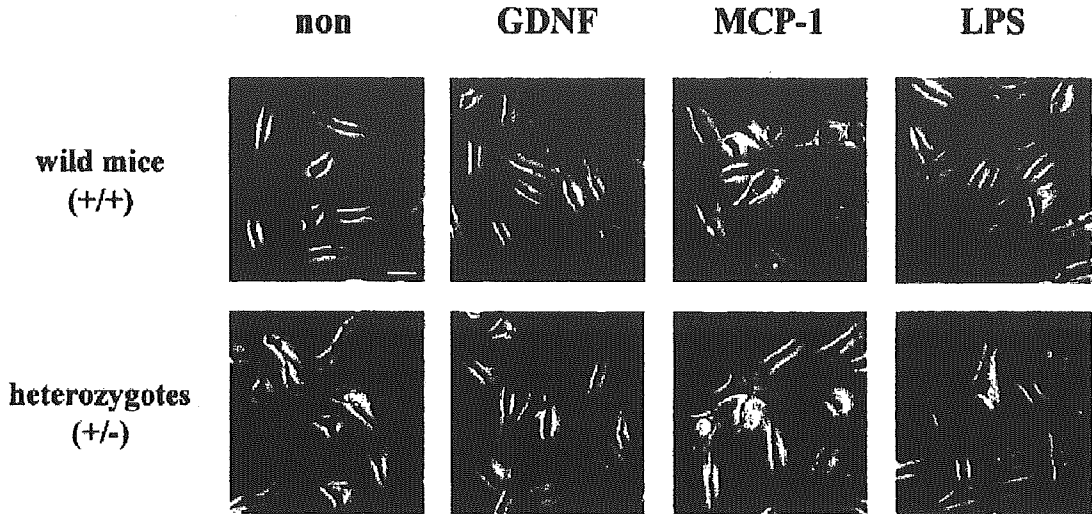


Fig. 7. Effects of GDNF, MCP-1, and LPS on mRNA expression of GDNF and its receptors, GFRα-1 and c-Ret, in the macrophages cultured from wild-type mice or GDNF gene mutants. **A**: mRNA expression of GDNF and of its receptors, GFRα-1 and c-Ret, in the cerebral cortex and cultured macrophages of GDNF gene-deficient heterozygous mice (+/-) and wild-type mice (+/+) was examined by RT-PCR. The macrophages were cultured with or without GDNF, MCP-1, or LPS (100 ng/ml) for 24 hr, after which total RNA of the

cells was prepared and subjected to RT-PCR with primers specific for GDNF, GFRα-1, or MCP-1. **B**: The ratio of the intensity of cDNA products to that of the β-actin cDNA product was calculated. The values are expressed as mean ± SE (n = 3) of -fold increase over the nontreatment group of +/+ macrophages. Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: \*\**P* < 0.01, \*\*\**P* < 0.001, #*P* < 0.05.

**A**



**B**

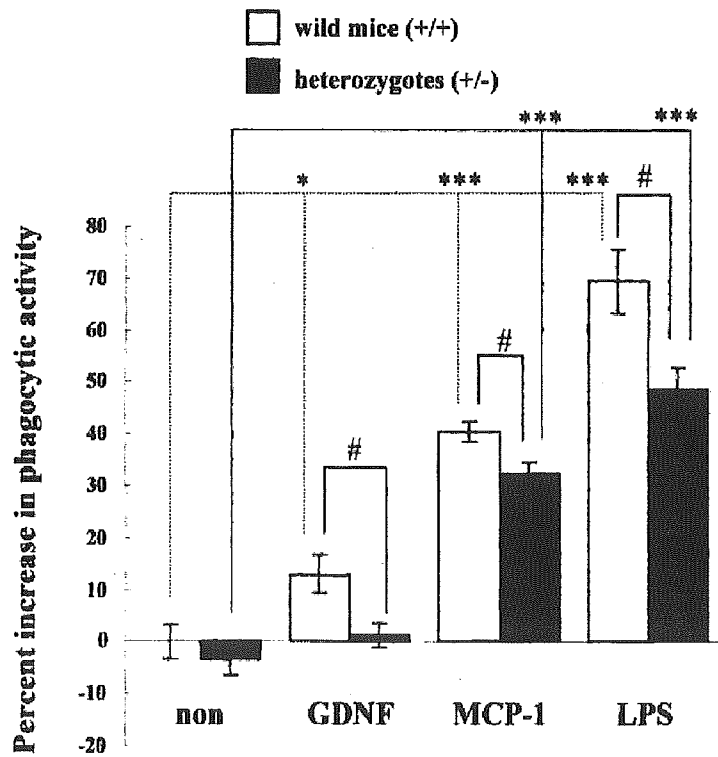


Fig. 8. Effects of GDNF, MCP-1, and LPS on the morphology (A) and phagocytic activity (B) of the macrophages cultured from wild-type mice or GDNF gene mutants. A: Macrophages from wild-type mice (+/+) or GDNF gene-deficient heterozygous mice (+/-) were cultured for 24 hr in medium supplemented or not with GDNF, MCP-1, or LPS (100 ng/ml), and then photographs of them were taken under a phase-contrast microscope. B: The +/+ or +/- macrophages were cultured for 30 min in medium containing FITC-labeled latex beads after incubation with or

without 100 ng/ml of GDNF, MCP-1, or LPS for 24 hr. FITC-positive cells (phagocytosing macrophages) were counted under a fluorescence microscope, and the percentage of positive cells per total cells was calculated. The values are expressed as mean  $\pm$  SE (n = 4) of the percentage increase over the control group (non). Statistical significance was assessed using one-way ANOVA followed by Tukey post hoc test. Significance of differences as indicated by the brackets: \*P < 0.05, \*\*\*P < 0.001, #P < 0.05. Scale bar = 20  $\mu$ m.

influence macrophage function through activation of Src family kinase in a c-Ret-independent manner. This implies an autocrine/paracrine action of GDNF on macrophages.

It is likely that GDNF stimulated phagocytosis of macrophages by the mediation of GDNF-induced chemokines, such as MCP-1. However, MCP-1 mRNA was expressed at a similar level in both +/+ and mutant +/- macrophages before or after stimulation (Fig. 7Bc) in spite of the lower expression of GDNF mRNA in the mutant macrophages (Fig. 7Ba). Therefore, GDNF mRNA expression is correlated better than MCP-1 mRNA expression with induction of the phagocytic activity, which may be supported by our preliminary result showing that the mutant macrophages secreted about half the GDNF protein secreted by the wild-type mice. It is possible that GDNF directly participates in the activation processes of the macrophages.

Exogenously infused GDNF can exert behavioral and anatomic neuroprotection against spinal cord injury (Cheng et al., 2002). Therefore, GDNF produced after a spinal cord injury is likely to exert predominantly neurotrophic effects on injured neurons. It is also conceivable that GDNF produced from microglia/macrophages in the injured spinal cord induces more activation of macrophages and more expression of GDNF and MCP-1 from these activated macrophage and that the increased GDNF acts as a neurotrophic factor for injured neurons. However, successful regeneration after a spinal cord injury is difficult, because the inflammatory response, such as activation of macrophages, is delayed and limited. In fact, GDNF might not have been produced so much because of the limited number of activated macrophages. Zeev-Brann et al. (1998) showed that activation of microglia/macrophages was enhanced when the cells were exposed to sciatic nerve segments but was inhibited in the presence of optic nerve segments, suggesting the existence of substances in the CNS inhibitory toward the activation of macrophages. Such inhibition may reduce the neurotrophic influence of GDNF in the injured site and result in failed regeneration. Macrophages are also known to have not only beneficial but also harmful effects, the latter caused by the production of nitric oxide. Indeed, infusion of a chemokine antagonist attenuated macrophage infiltration and induced axonal sprouting in the injured spinal cord (Ghirnikar et al., 2001). This is contradictory to the beneficial effects of macrophages. We propose that a delayed inflammatory response may have a harmful influence on a spinal cord injury but that an early one may be beneficial for neuronal regeneration because of an increased production of neurotrophic factors such as GDNF (Fig. 1).

In summary, we demonstrate here that GDNF was up-regulated in microglia/macrophages after spinal cord transection and enhanced phagocytic activity and expression of GDNF or chemokine mRNA of cultured rodent peritoneal macrophages. GDNF produced after a spinal cord injury might not only have a beneficial influence on

the injury but might also participate in the inflammatory response.

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# Long-Lasting Impairment of Associative Learning Is Correlated with a Dysfunction of *N*-Methyl-D-aspartate–Extracellular Signaling-Regulated Kinase Signaling in Mice after Withdrawal from Repeated Administration of Phencyclidine

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## ABSTRACT

In humans, the administration of phencyclidine causes schizophrenic-like symptoms that persist for several weeks after withdrawal from phencyclidine use. We demonstrated here that mice pretreated with phencyclidine (10 mg/kg/day s.c. for 14 days) showed an enduring impairment of associative in a Pavlovian fear conditioning 8 days after cessation of phencyclidine treatment. Extracellular signaling-regulated kinase (ERK) was transiently activated in the amygdalae and hippocampi of saline-treated mice after conditioning. In the phencyclidine-treated mice, the basal level of ERK activation was elevated in the hippocampus, whereas the activation was impaired in the amygdala and hippocampus after conditioning. Exogenous *N*-methyl-D-aspartate (NMDA), glycine, and spermidine-induced

ERK activation was not observed in slices of hippocampus and amygdala prepared from phencyclidine-treated mice. Repeated olanzapine (3 mg/kg/day p.o. for 7 days), but not haloperidol (1 mg/kg/day p.o. for 7 days), treatment reversed the impairment of associative learning and of fear conditioning-induced ERK activation in repeated phencyclidine-treated mice. Our findings suggest an involvement of abnormal ERK signaling via NMDA receptors in repeated phencyclidine treatment-induced cognitive dysfunction. Furthermore, our phencyclidine-treated mice would be a useful model for studying the effect of antipsychotics on cognitive dysfunction in schizophrenia.

In humans, phencyclidine, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, has been shown to induce schizophrenia-like psychosis representing positive

(e.g., hallucination and paranoia) and negative symptoms (e.g., emotional withdrawal and motor retardation) and cognitive dysfunction (Javitt and Zukin, 1991). It is interesting that such schizophrenia-like symptoms persisted for several weeks after withdrawal from long-term phencyclidine use (Rainey and Crowder, 1975; Allen and Young, 1978; Jentsch and Roth, 1999). Phencyclidine psychosis has been demonstrated to represent a drug-induced model of schizophrenia (Javitt and Zukin, 1991). We have reported previously that repeated phencyclidine treatment induces persistent enhancement of immobility in a forced swimming test (Noda et al., 1995, 2000) and social behavior deficit in mice (Qiao et al., 2001), which would be an animal model of negative symp-

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; ERK, extracellular signaling-regulated kinase; NR1 subunit; *N*-methyl-D-aspartate receptor  $\zeta$  subunit; MK-801, dizocilpine maleate; VEH, vehicle; OLZ, olanzapine; HAL, haloperidol; PCP, phencyclidine; ANOVA, analysis of variance; TBS, Tris-buffered saline; BDNF, brain-derived neurotrophic factor; PCP, phencyclidine.

toms. In cognitive function, it is well known that one-time administration of phencyclidine induces a transient cognitive dysfunction (Nabeshima et al., 1986; Noda et al., 2001). Jentsch and colleagues (1997a,b) have reported that repeated phencyclidine treatment induces an enduring working memory impairment in the T-maze of rats and memory impairment in an "object retrieval with a detour" task of monkeys. However, there were contradictory results about the enduring impairment of working memory in rats (Stefani and Moghaddam, 2002; Li et al., 2003). Although it has reported recently that spatial learning is impaired 1 day after repeated phencyclidine treatment in object recognition test of mice (Mandillo et al., 2003), it is not clear whether long-lasting cognitive dysfunction occurred in other types of learning and memory tasks.

Pavlovian fear conditioning is a useful tool for investigating associative learning, because the neuronal circuits underlying this task have been mapped (Maren, 2001). The cued fear conditioning depends on the amygdala, whereas the contextual fear conditioning depends on both the hippocampus and amygdala. Recent studies have identified the molecular basis of learning in this task. Activation of extracellular signaling-regulated kinase (ERK) via NMDA receptors in the hippocampus and amygdala is required for associative learning in fear conditioning (Atkins et al., 1998; Schafe et al., 2000; Athos et al., 2002).

The present study was designed to test the hypothesis that phencyclidine-pretreated mice develop an impairment of associative learning in Pavlovian fear conditioning after withdrawal from phencyclidine, and such impairment is mediated via a dysfunction of NMDA-ERK signaling. We attempted to investigate whether an impairment of associative learning is produced after repeated administration of phencyclidine and the changes of ERK activation in phencyclidine-treated mice after fear conditioning and NMDA receptor stimulation. Finally, for determining whether our model could be used as a model of the cognitive dysfunction in schizophrenia, the effects of haloperidol (a typical antipsychotic) and olanzapine (an atypical antipsychotic) on the associative learning in phencyclidine-treated mice were investigated.

## Materials and Methods

**Animals.** Male mice of the ddY strain (Japan SLC Inc., Shizuoka, Japan), weighing 25 to 27 g at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum, and were maintained on a 12/12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

**Cued and Contextual Fear Conditioning.** Cued and contextual fear conditioning was performed according to a previous report (Nagai et al., 2003) with a minor modification. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (17 × 27 × 12.5 cm) for 1 min and then in the conditioning cage (25 × 31 × 11 cm) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co. Ltd., Osaka, Japan). This procedure

was repeated four times with 15-s intervals. Cued and contextual tests were carried out 1 to 2 h or 1 day after fear conditioning. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical with the conditioned stimulus. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 min in the absence of the conditioned stimulus.

**Drugs.** Phencyclidine HCl [1-(1-phenylcyclohexyl) piperidine hydrochloride] was synthesized by the authors according to the method of Maddox and colleagues (1965) and was checked for purity. Haloperidol (Sigma Chemical, St. Louis, MO) was purchased commercially. Olanzapine was supplied by Eli Lilly & Co. (Indianapolis, IN). Phencyclidine was dissolved in saline. Haloperidol and olanzapine were suspended in saline containing 0.1% (w/v) carboxymethyl cellulose sodium salt.

**Drug Treatment.** Phencyclidine (10 mg/kg/day s.c.) or saline was administered once a day for 14 consecutive days. Fear conditioning was performed 8 days after the withdrawal of repeated phencyclidine treatment. For investigating the effects of repeated antipsychotic treatment, mice were administered haloperidol (1 mg/kg/day p.o.), olanzapine (3 mg/kg/day p.o.), or vehicle once a day for 7 consecutive days from 1 day after the final phencyclidine treatment. On the day after the final haloperidol or olanzapine treatment, fear conditioning was performed. On the basis of our previous studies in a forced swimming test of mice (Noda et al., 1995, 2000), the doses of phencyclidine, olanzapine, and haloperidol were chosen. All compounds were administered in a volume of 0.1 ml/10 g body weight.

**Western Blotting Analysis.** Western blotting analysis was performed as described previously (Mizuno et al., 2002) with a minor modification. Before (basal) and immediately, 1 and 24 h after fear conditioning, the mice were killed by decapitation, and the brain was immediately removed. Hippocampi and amygdalae were rapidly dissected out, frozen, and stored at -80°C until used. The brain samples were homogenized in ice-cold buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin, 4 μg/ml aprotinin, and 4 μg/ml leupeptin for measuring ERK; or 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin for measuring the NMDA receptor ζ subunit (NR1) and actin]. The lysate was centrifuged at 8000g for 10 min at 4°C. The protein concentration of the supernatant was determined by a Bradford assay (Bio-Rad, Hercules, CA). Sample buffer was added to the supernatant, and the mixture was boiled at 95°C for 5 min. Equivalent amounts of protein (50 μg) were electrophoresed on SDS-polyacrylamide gel, transferred to PVDF membranes (Millipore Corporation, Billerica, MA), and blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The membranes were incubated with anti-phospho-ERK antibody (1:1000; New England Biolabs, Beverly, MA), anti-NR1, CT antibody (1:1000; Upstate Biotechnology, Lake Placid, NY), or anti-actin antibody (C-11) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membranes were washed with the washing buffer (50 mM Tris-HCl, pH 7.4, 0.05% Tween 20, and 150 mM NaCl) three times for 10 min each. After incubation with secondary antibodies conjugated to horseradish peroxidase, the membranes were washed with the washing buffer three times for 10 min each. The immune complex was detected by an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray film. Images on X-ray film were captured using charge-coupled device camera (Atto Bioscience, Tokyo, Japan). The band intensities were quantitatively analyzed using the Atto Densitograph Software Library Lane Analyzer. To measure total (phospho- and nonphospho-) ERK, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 10 min and incubated with anti-ERK antibody (1:1000; New

England Biolabs) at 4°C overnight. The immune complex was detected as described above. To evaluate the ERK activation, the phospho-ERK levels were normalized to the total ERK levels in the same membranes, and then each phospho-ERK/total ERK level was normalized to basal phospho-ERK/total ERK level in saline-treated mice. To evaluate the NR1 levels, the NR1 levels were normalized to the actin levels in the same membranes, and then each NR1/actin level was normalized to NR1/actin level in saline-treated mice.

**Immunohistochemical Analysis.** Immunohistochemical analysis was performed as described previously (Schafe et al., 2000; Kim et al., 2003) with a minor modification. Before and immediately after fear conditioning, mice were anesthetized with chloral hydrate (200 mg/kg i.p.) and then transcardially perfused with ice-cold 20 mM Tris-buffered saline (TBS) followed by 4% paraformaldehyde in 0.1 M Tris-HCl buffer, pH 7.4. Perfused brains were fixed at 4°C for 24 h in the same fixative and then immersed in 30% sucrose in TBS until the brain sank to the bottom. Immersed brains were cut into 30- $\mu$ m transverse free-floating sections using a horizontal sliding microtome. For immunohistochemistry, sections were blocked in 0.3% hydrogen peroxide in TBS for 30 min and then followed by 30-min incubation in 0.4% Triton X-100, 4% normal goat serum, and 0.25% bovine serum albumin in TBS. After incubation in primary antibody against phospho-ERK (1:200) at 4°C for 48 h, sections were incubated in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at room temperature for 2 h, and then followed by 1 h incubation in Avidin-Biotin complex (Vector Laboratories) at room temperature. 3-3'-Diaminobenzidine was used as a chromogen. According to a previous article (Sananbenesi et al., 2002), the density of phospho-ERK-stained cells were analyzed from the standardized area of 0.72 mm<sup>2</sup> in lateral and central amygdala, 1.58 to 1.70 mm posterior to bregma, using computer-based image analysis system (C. Imaging System; Compig, Mars, PA) attached to a light microscope (Olympus BX60-FLB-3; Olympus, Tokyo, Japan). To evaluate the ERK activation, the phospho-ERK levels after fear conditioning were normalized to the mean basal phospho-ERK level in each treatment.

**Slice Stimulation.** Slice stimulation was essentially performed as described previously (Mamiya et al., 2003) with a minor modification. Eight days after the final phencyclidine treatment, the mice were killed by decapitation, and the brain was immediately removed. Amygdalae and hippocampi were dissected. Hippocampi were sliced at a thickness of 300  $\mu$ m in a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK). Amygdalae was sliced manually with a blade. After preincubation at 37°C in Ringer's buffer (10 mM HEPES-NaOH, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 10 mM glucose; gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) for 5 min, each slice was stimulated with NMDA (100  $\mu$ M), glycine (10  $\mu$ M), and spermidine (1 mM) for 5 min. After NMDA receptor stimulation, the slices were washed with ice-cold Ringer's buffer two times. The slices were homogenized as described above for Western blotting analysis.

**Statistical Analysis.** Statistical analysis was performed by one-way or two-way analysis of variance (ANOVA) using Bonferroni's test. The unpaired *t* test was used to compare two sets of data. A value of *p* < 0.05 was considered statistically significant. Data were expressed as the mean  $\pm$  S.E.M.

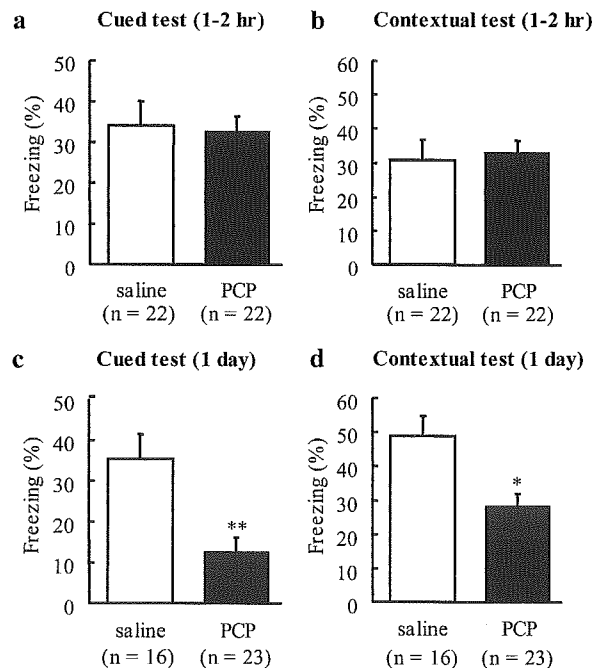
## Results

**Effect of Repeated Phencyclidine Treatment on Fear Conditioning in Mice.** In the preconditioning phase, both saline- and phencyclidine-treated mice hardly showed the freezing response. There were no differences in basal levels of freezing response between the two groups (data not shown). In cued (amygdala-dependent but hippocampus-independent) and contextual (amygdala- and hippocampus-dependent) fear conditioning, animals learned to fear tone and

context associated with the room for foot shock. Both saline- and phencyclidine-treated mice showed marked cued and contextual freezing response 1 to 2 h after fear conditioning, and there was no significant difference between the two groups (Fig. 1, a and b). However, when cued and contextual freezing response was measured 1 day later, the phencyclidine-treated mice exhibited less freezing response (cued test: *p* < 0.01; contextual test: *p* < 0.05; Fig. 1, c and d). No alterations of nociceptive response were found in the phencyclidine-treated mice: the minimal current required to elicit flinching/running, jumping, or vocalization in the phencyclidine-treated mice was the same as that in the saline-treated mice (data not shown).

**ERK Activation in the Amygdala and Hippocampus after Fear Conditioning.** Phospho-ERK levels in the amygdala and hippocampus of the saline-treated mice were significantly increased immediately after fear conditioning, compared with basal levels (before conditioning) (*p* < 0.01, Fig. 2, a and b). The increase in phospho-ERK levels was transient and returned to basal levels within 1 h in both regions. In the amygdalae of the phencyclidine-treated mice, fear conditioning did not significantly increase the phospho-ERK levels compared with the basal level (Fig. 2a), and the phospho-ERK level immediately after conditioning in the phencyclidine-treated mice was significantly lower than that in the saline-treated mice (*p* < 0.05, Fig. 2a).

The basal phospho-ERK level in the hippocampi of the phencyclidine-treated unconditioned mice was significantly elevated compared with that in the saline-treated unconditioned mice (*p* < 0.05, Fig. 2b). However, fear conditioning did not cause a further increase compared with the basal level (Fig. 2b). Fear conditioning did not cause any changes in



**Fig. 1.** Effect of repeated phencyclidine treatment on performance of fear conditioning in mice. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Cued and contextual tests were performed 1 to 2 h (a, cued test; b, contextual test) and 1 day after fear conditioning (c, cued test; d, contextual test). Values correspond to mean  $\pm$  S.E.M. \*, *p* < 0.05 and \*\*, *p* < 0.01 versus saline-treated mice (unpaired *t* test).



total ERK levels in the amygdalae (Fig. 2c) and hippocampi (Fig. 2d) of the saline- or phencyclidine-treated mice.

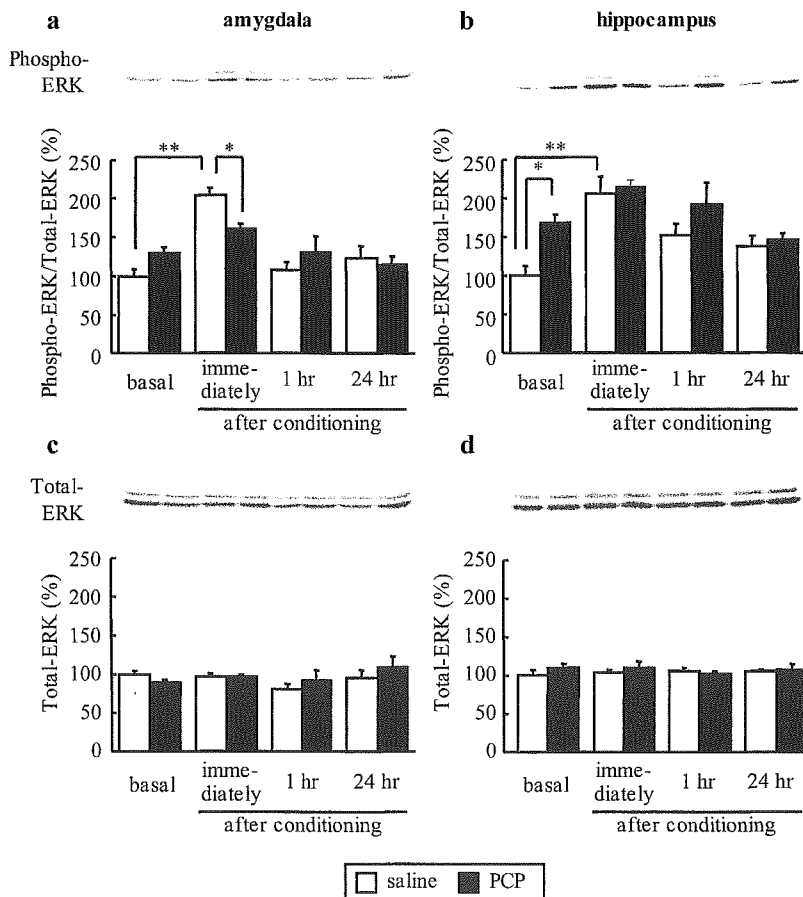
Because amygdala consists of heterogeneous nuclei, we measured phospho-ERK levels in the lateral amygdala, which is an essential region for fear conditioning, and the central amygdala using immunohistochemical methods (Fig. 3, a–h). Repeated treatment with phencyclidine induced an increase in the basal phospho-ERK levels in the lateral and central amygdalae compared with those in saline-treated mice, but not significantly (Fig. 3a). Although the phospho-ERK levels in the lateral amygdalae of both saline- and phencyclidine-treated mice were significantly increased immediately after fear conditioning compared with the basal levels ( $p < 0.01$ , Fig. 3a), these changes were more pronounced in saline-treated mice than in phencyclidine-treated mice ( $p < 0.01$ , Fig. 3c). There were no significant changes in the central amygdala, but fear conditioning tended to increase the phospho-ERK levels in the central amygdalae of saline- and phencyclidine-treated mice (Fig. 3b). ERK activation in the central amygdalae of saline-treated mice tended to be intense compared with that of phencyclidine-treated mice (Fig. 3d).

**ERK Activation in the Slices of Amygdala and Hippocampus by NMDA Receptor Stimulation.** To confirm that ERK activation is facilitated after NMDA receptor stimulation, we measured the phospho-ERK levels in slices of amygdala or hippocampus stimulated with NMDA (100  $\mu$ M), glycine (10  $\mu$ M), and spermidine (1 mM). Under our experimental conditions, the increase in phospho-ERK levels was detected 5 min after stimulation with NMDA, glycine, and

spermidine compared with the basal level (without stimulation) in the amygdalae ( $p < 0.01$ , Fig. 4a) and hippocampi ( $p < 0.05$ , Fig. 4b) prepared from saline-treated mice. The basal levels of phospho-ERK in the amygdalae (Fig. 4a) and hippocampi (Fig. 4b) of phencyclidine-treated mice tended to be increased compared with those of saline-treated mice. However, stimulation with NMDA, glycine, and spermidine did not cause a further increase in phospho-ERK levels of the amygdalae (Fig. 4a) or hippocampi (Fig. 4b) prepared from phencyclidine-treated mice. After stimulation, phospho-ERK level in the amygdalae of phencyclidine-treated mice was significantly lower than that of saline-treated mice ( $p < 0.05$ , Fig. 4a). There was no significant difference in total ERK levels in amygdala (Fig. 4c) and hippocampus (Fig. 4d) between saline- and phencyclidine-treated mice.

**Effect of Repeated Phencyclidine Treatment on the Protein Levels of NR1 in the Amygdala and Hippocampus.** We measured the protein levels of NR1, which is the obligatory subunit of NMDA receptors, in the amygdala and hippocampus using the same schedule as fear conditioning. However, there was no significant difference in NR1 levels of the amygdala (Fig. 4e) and hippocampus (Fig. 4f) between saline- and phencyclidine-treated mice.

**Effects of Antipsychotics on the Impairment of Associative Learning Induced by Repeated Phencyclidine Treatment.** We investigated the effects of repeated treatment with olanzapine and haloperidol on associative learning in saline- or phencyclidine-treated mice. Fear conditioning was performed 1 day after repeated olanzapine (3 mg/kg/day p.o. for 7 days) or haloperidol (1 mg/kg/day p.o.



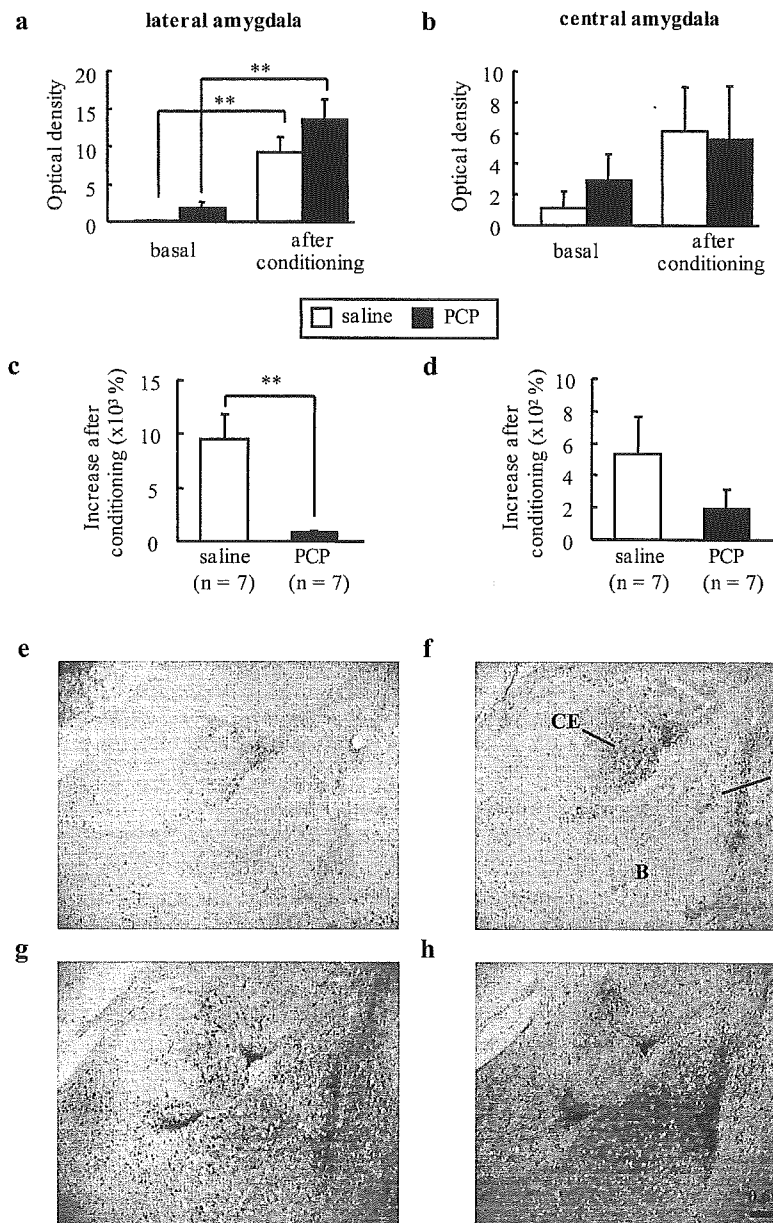
**Fig. 2.** ERK activation in the amygdala and hippocampus after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) ( $n = 10$  in each group before or immediately after fear conditioning) and hippocampi (b) ( $n = 7$  in each group) of saline- and phencyclidine-treated mice after fear conditioning. Representative Western blots and total ERK immunoreactivity in the amygdalae (c) and hippocampi (d) of saline- and phencyclidine-treated mice after fear conditioning. Values correspond to the mean  $\pm$  S.E.M. Results with two-way ANOVA were: a, treatment,  $F_{1,64} = 0.000007$  ( $p = 0.998$ ); time,  $F_{3,64} = 17.09$  ( $p < 0.01$ ); treatment-by-time interaction,  $F_{3,64} = 4.56$  ( $p < 0.01$ ); b, treatment,  $F_{1,48} = 7.32$  ( $p < 0.01$ ); time,  $F_{3,48} = 9.29$  ( $p < 0.01$ ); treatment-by-time interaction  $F_{3,48} = 1.51$  ( $p = 0.22$ ); c, treatment,  $F_{1,64} = 0.47$  ( $p = 0.50$ ); time,  $F_{3,64} = 1.45$  ( $p = 0.24$ ); treatment-by-time interaction,  $F_{3,64} = 0.84$  ( $p = 0.48$ ); d, treatment,  $F_{1,48} = 1.01$  ( $p = 0.32$ ); time,  $F_{3,48} = 0.14$  ( $p = 0.94$ ); treatment-by-time interaction,  $F_{3,48} = 0.59$  ( $p = 0.62$ ). Basal, before conditioning; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Bonferroni's test).

for 7 days) treatment. Repeated olanzapine and haloperidol treatment did not affect the cued (Fig. 5a) and contextual (Fig. 5c) freezing response in saline-treated mice. On the other hand, repeated olanzapine treatment reversed the impairment of associative learning produced by repeated phencyclidine treatment in both cued ( $p < 0.05$ , Fig. 5b) and contextual tests ( $p < 0.01$ , Fig. 5d). Repeated haloperidol treatment did not reverse the phencyclidine-induced impairment of associative learning in either test (Fig. 5, b and d).

**Effects of Repeated Antipsychotic Treatments on the Impairment of ERK Activation Induced by Repeated Phencyclidine Treatment.** Repeated treatment with olanzapine or haloperidol did not affect the phospho-ERK levels in the amygdalae of saline-treated mice (Fig. 6a). When olanzapine was repeatedly administered to phencyclidine-treated mice, fear conditioning significantly increased the phospho-ERK level in the amygdala ( $p < 0.01$ , Fig. 6b). Fear conditioning did not increase the phospho-ERK levels in

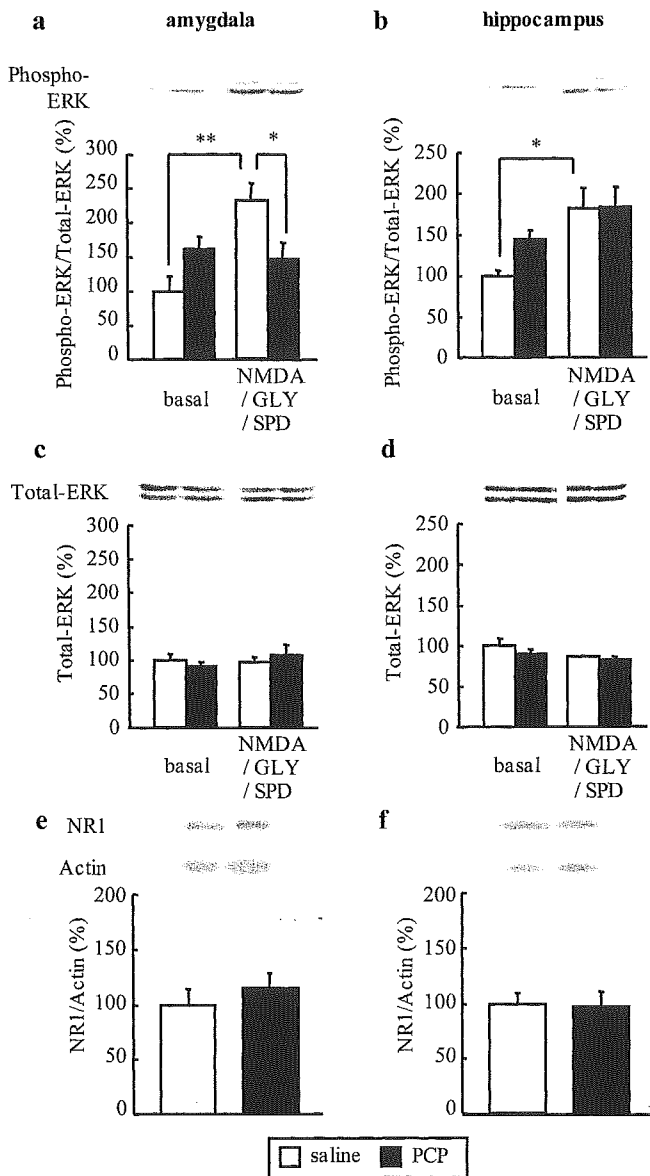
the amygdalae of phencyclidine/haloperidol-treated mice (Fig. 6c). Total ERK levels in the amygdalae of saline- and phencyclidine-treated mice were not changed by repeated olanzapine (Fig. 6, d and e) and haloperidol (Fig. 6, d and f) treatment.

Olanzapine treatment significantly increased the basal phospho-ERK level in the hippocampi of saline-treated mice ( $p < 0.05$ , Fig. 7a) and tended to increase the basal phospho-ERK level in the hippocampi of phencyclidine-treated mice (Fig. 7b). Haloperidol treatment significantly increased the basal phospho-ERK levels in the hippocampi of both saline- ( $p < 0.05$ , Fig. 7a) and phencyclidine-treated mice ( $p < 0.05$ , Fig. 7c). A further increase in phospho-ERK levels was caused by fear conditioning in saline/vehicle- ( $p < 0.01$ , Fig. 7a), saline/olanzapine- ( $p < 0.05$ , Fig. 7a), saline/haloperidol- ( $p < 0.05$ , Fig. 7a), and phencyclidine/olanzapine-treated mice ( $p < 0.05$ , Fig. 7b), but not in phencyclidine/vehicle- (Fig. 7, b and c) and phencyclidine/haloperidol-treated mice (Fig. 7c). Total-ERK levels in the hippocampi of saline- and



**Fig. 3.** ERK activation in the lateral amygdala and central amygdala after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Phospho-ERK levels in the lateral (a) and central amygdalae (b) of saline- and phencyclidine-treated mice before (basal) and immediately after fear conditioning ( $n = 6$  in saline-treated mice before fear conditioning,  $n = 8$  in phencyclidine-treated mice before fear conditioning,  $n = 7$  in each group immediately after fear conditioning). The increasing percentage of phospho-ERK expression immediately after fear conditioning in the lateral (c) and central amygdalae (d) of saline-treated and phencyclidine-treated mice. Values correspond to the mean  $\pm$  S.E.M. of 7 mice. Representative photomicrographs of phospho-ERK expression in the amygdalae of saline-treated (e and g) and phencyclidine-treated (f and h) mice. Phospho-ERK was markedly increased in the amygdala immediately after fear conditioning (g and h), compared with basal levels (e and f). Results with two-way ANOVA were the following: a: treatment,  $F_{1,24} = 2.72$  ( $p = 0.11$ ); time,  $F_{1,24} = 32.38$  ( $p < 0.01$ ); treatment-by-time interaction,  $F_{1,24} = 0.59$  ( $p = 0.45$ ); b: treatment  $F_{1,24} = 0.06$  ( $p = 0.81$ ); time,  $F_{1,24} = 2.31$  ( $p = 0.14$ ); treatment-by-time interaction,  $F_{1,24} = 0.20$  ( $p = 0.66$ ). \*\*,  $p < 0.01$  [Bonferroni's test (a) or unpaired  $t$  test (c)]. B, basolateral amygdala; CE, central amygdala; LA, lateral amygdala.

phencyclidine-treated mice were not changed by repeated olanzapine (Fig. 7, d and e) or haloperidol (Fig. 7, d and f) treatment.



**Fig. 4.** ERK activation by NMDA receptor stimulation and the protein levels of NR1 in the amygdala and hippocampus. Slices of amygdala or hippocampus were stimulated with NMDA (100  $\mu$ M), glycine (GLY; 10  $\mu$ M), and spermidine (SPD; 1 mM) 8 days after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) and hippocampi (b) of saline- and phencyclidine-treated mice after the stimulation ( $n = 6$ ). Representative Western blots and total ERK immunoreactivity in the amygdalae (c) and hippocampi (d) of saline- and phencyclidine-treated mice after the stimulation ( $n = 6$ ). The NR1 levels were measured 8 days after the cessation of phencyclidine treatment. Representative Western blots and NR1/actin immunoreactivity in the amygdala (e) ( $n = 5$ ) and hippocampus (f) ( $n = 8$ ) of saline- and phencyclidine-treated mice. Values correspond to the mean  $\pm$  S.E.M. Results with two-way ANOVA were: a, treatment,  $F_{1,20} = 0.25$  ( $p = 0.62$ ); stimulation,  $F_{1,20} = 6.34$  ( $p < 0.05$ ); treatment-by-stimulation interaction,  $F_{1,20} = 10.36$  ( $p < 0.01$ ); b, treatment,  $F_{1,20} = 1.40$  ( $p = 0.25$ ); stimulation,  $F_{1,20} = 9.80$  ( $p < 0.01$ ); treatment-by-stimulation interaction,  $F_{1,20} = 1.19$  ( $p = 0.29$ ); c, treatment,  $F_{1,20} = 0.05$  ( $p = 0.82$ ); stimulation,  $F_{1,20} = 0.74$  ( $p = 0.40$ ); treatment-by-stimulation interaction,  $F_{1,20} = 0.04$  ( $p = 0.84$ ); d, treatment,  $F_{1,20} = 1.44$  ( $p = 0.24$ ); stimulation,  $F_{1,20} = 3.42$  ( $p = 0.08$ ); treatment-by-stimulation interaction,  $F_{1,20} = 0.33$  ( $p = 0.57$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Bonferroni's test).

## Discussion

There are many reports that a single administration of phencyclidine or ketamine alters cognition in healthy volunteers or patients with schizophrenia via glutamatergic hypofunction caused by blockade of NMDA receptors (Javitt and Zukin, 1991; Adler et al., 1998; Tamminga, 1998). Because this cognitive impairment recovers quickly, the ability of a single phencyclidine administration to impair memory in human is caused, at least in part, by phencyclidine-induced transient confusion (Javitt and Zukin, 1991; Ellison, 1995). The study of short-term phencyclidine administration to rodents may be relevant to some of the short-lasting cognitive effects of phencyclidine in humans (e.g., disorientation and dissociative states), whereas long-term phencyclidine would be more relevant to cognitive deficits in schizophrenia because cognitive deficits in schizophrenia is enduring and is not usually accompanied by impairment of orientation to time, place, and person. In the present study, the impairment of associative learning, which depends on the amygdala and hippocampus, was observed 8 days after withdrawal from repeated phencyclidine treatment. This impairment of associative learning was detected 24 h, but not 1 to 2 h, after fear conditioning, suggesting a failure of memory consolidation. As far as we know, this is the first report of long-lasting impairment of associative learning after withdrawal from repeated phencyclidine treatment. This persistent impairment of learning was consistent with reports of enduring working memory impairment in a T-maze of rats and memory impairment in an "object retrieval with a detour" task of monkeys (Jentsch et al., 1997a,b). It was also consistent with the clinical observation that schizophrenia-like psychosis persisted for several weeks after withdrawal from long-term phencyclidine use in humans (Rainey and Crowder, 1975; Allen and Young, 1978; Jentsch and Roth, 1999). Furthermore, associative learning is disrupted in patients with schizophrenia (Rushe et al., 1999), and dysfunction of the amygdala and hippocampus contributes to the pathophysiology of schizophrenia (Heckers et al., 1998; Edwards et al., 2001). A single dose of phencyclidine may produce a reversible neurotoxic effect related to the cognitive deficits, whereas repeated phencyclidine treatment could produce long-lasting structural changes in cerebral cortex and hippocampus (Ellison, 1995; Olney and Farber, 1995; Olney et al., 1999). These results suggest that repeated phencyclidine treatment causes the long-lasting impairment of associative learning and the neuronal and/or signal circuit needed to perform associative learning task.

Recent studies have demonstrated a critical role of ERK activation in associative learning in cued and/or contextual fear conditioning (Atkins et al., 1998; Schafe et al., 2000; Athos et al., 2002). Our finding, that phencyclidine-treated mice failed to activate ERK, was consistent with the impairment of associative learning in fear conditioning. In the amygdalae and hippocampi of saline-treated mice, phospho-ERK levels were transiently increased immediately after fear conditioning and returned to basal levels within 1 h. The time course of ERK activation in our experiments was consistent with that in long-term potentiation experiments (Davis et al., 2000), whereas it was different from reports that showed ERK activation 1 h after fear conditioning in rats (Atkins et al., 1998; Schafe et al., 2000). The reason for

this discrepancy is unknown. One possibility is the different duration of unconditioned stimulation (foot shock) between our condition (total duration, 20 s) and the previously reported condition (1 or 5 s) (Atkins et al., 1998; Schafe et al., 2000).

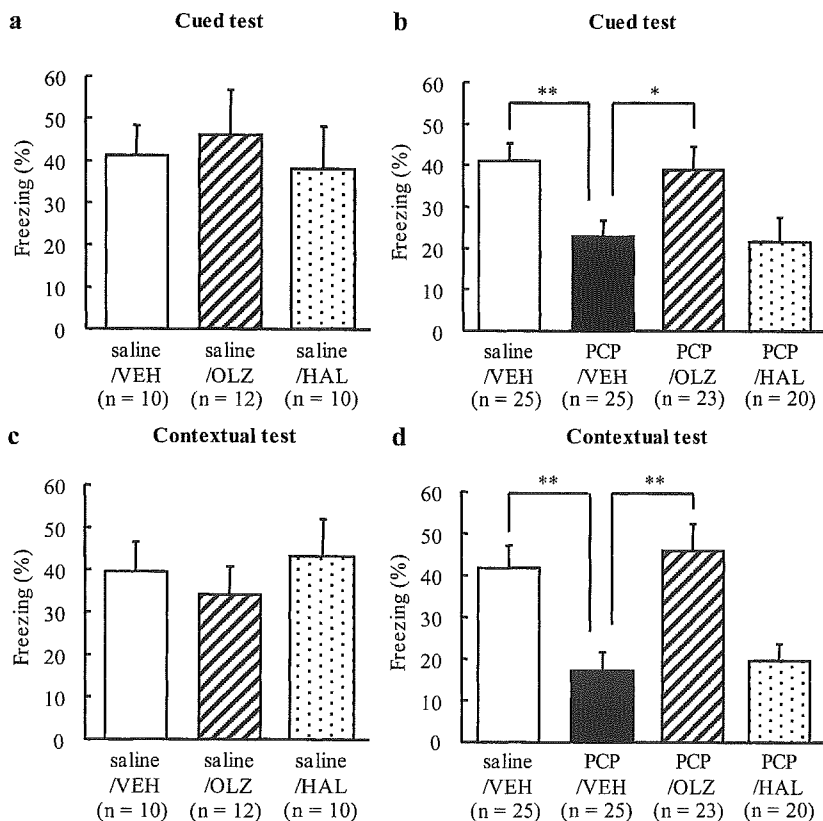
Although fear conditioning-induced ERK activation in the amygdalae of phencyclidine-treated mice was completely abolished in Western blotting, ERK activation in the lateral amygdalae of phencyclidine-treated mice was less pronounced but observed in immunohistochemical analysis. Although the reason for the difference is unknown, it might be caused by the difference of sensitivity in two methods. As it has been reported (Schafe et al., 2000), fear conditioning induced ERK activation most intensely in the lateral amygdala, whereas in the other nuclei of amygdala, ERK activation is weak and variable among individual mice. Therefore, immunohistochemical analysis would be more sensitive compared with Western blotting. However, an important finding in the present study is that fear conditioning-induced changes in the lateral amygdala were more pronounced in saline-treated mice than in phencyclidine-treated mice.

A previous report demonstrated that the activation of ERK is mediated via NMDA receptors in fear conditioning, because a single treatment with MK-801, a noncompetitive NMDA receptor antagonist, blocks both the activation of ERK and associative learning (Atkins et al., 1998). We investigated NMDA-ERK signaling after stimulation with exogenous NMDA, glycine, and spermidine in slices of the amygdala and hippocampus. In the amygdalae and hippocampi from saline-treated mice, phospho-ERK levels were increased after the stimulation. However, stimulation with NMDA, glycine, and spermidine failed to increase phospho-ERK levels in the amygdalae and hippocampi from phencyclidine-

treated mice. Our results clearly suggest that repeated phencyclidine treatment disrupts the activation of ERK mediated via NMDA receptors. The dysfunction of NMDA-ERK signaling is not accompanied by changes in the NR1 levels in the amygdala and hippocampus. It might be caused by the functional alterations to the NMDA receptor itself or alterations to the intracellular signaling via NMDA receptors.

Kyosseva and colleagues (2001) have reported that continuous phencyclidine infusion increases basal phospho-ERK levels in the cerebellum, but not in the hippocampus, brain stem, and frontal cortex of rats. It contrasts with our results that repeated phencyclidine treatment increased the basal level of phospho-ERK in the hippocampus. Although the reason for discrepancy as to the affected region is unknown, it might be caused by some differences of experimental protocol about species (mice versus rats), phencyclidine administration schedule (pulsatile injection for 14 days versus continuous infusion for 10 days), and duration of withdrawal from phencyclidine (8 days versus 1 day). The increase in basal ERK activation might be caused by adapted response to alterations of neurotransmitters or intracellular signaling as a result of repeated phencyclidine treatment. However, the adapted responses might be insufficient to mediate further ERK activation in associative learning or NMDA receptor stimulation. The most important finding in our study is the disruption of NMDA-ERK signaling during learning *in vivo* as well as on stimulation with exogenous NMDA, glycine, and/or spermidine *ex vivo*. The dysfunction of ERK signaling via NMDA receptors in phencyclidine-treated mice is consistent with the hypothesis of dysfunction of the glutamatergic system in schizophrenia (Carlsson et al., 1997; Olney et al., 1999).

We measured phospho-ERK levels in the mice that were



**Fig. 5.** Effects of repeated antipsychotic treatment on the impairment of associative learning induced by repeated phencyclidine treatment. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed 1 day after the final antipsychotic treatment. Cued (a and b) and contextual tests (c and d) were performed 1 day after fear conditioning. Values correspond to the mean  $\pm$  S.E.M. Results with one-way ANOVA were: a,  $F_{2,29} = 0.38$  ( $p = 0.69$ ); b,  $F_{3,89} = 4.72$  ( $p < 0.01$ ); c,  $F_{2,29} = 0.18$  ( $p = 0.84$ ); d,  $F_{3,89} = 8.14$  ( $p < 0.01$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (Bonferroni's test).