with causal mechanisms underlying the development of hyperexcitability, or as inhibitory processes.

Neurotrophic factors appear to play a key role in these changes, since their expression changes during the pathophysiology of the seizures [9]. However, it remains uncertain whether they act as promoting factors of epileptogenesis or act as endogenous anti-epileptogenic substances. The expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) is rapidly up-regulated following seizures [9]. In addition, their expression is enhanced in the hippocampus of temporal lobe epilepsy patients [14], suggesting that these neurotrophic factors play pivotal roles in the epileptogenesis or anti-epileptogenesis [9]. Indeed, several studies indicate that NGF promotes kindling epileptogenesis [1,29]. In addition, antibodies to NGF attenuate cholinergic sprouting and increase the size of basal forebrain cholinergic neurons that are associated with seizure-induced injury caused by pilocarpine [7]. In contrast, the role of BDNF remains controversial. A chronic infusion of BDNF in the hippocampus delays [20] and accelerates [21] epileptogenesis. Decreased BDNF signaling in transgenic mice reduces epileptogenesis [10], and expression of BDNF increased in pilocarpine seizures [22]. In contrast to these neurotrophic factors, NT-3 mRNA undergoes a delayed down-regulation, suggesting that the neurotrophic factor has a different function in epileptogenesis. NT-3 inhibits seizure development and related synaptic reorganization [31]. Deletion of the NT-3 gene retards the development of kindling epileptogenesis [3]. Up-regulation of basic fibroblast growth factor (b-FGF, FGF-2) follows seizure induced by chemical [6,9] and electrical kindling [9,25]. Long-term, low-dose infusion of bFGF prevents kainate-induced hippocampal cell loss, although it has no effect on seizure latency or duration [12]. Thus, chronic elevation of bFGF levels after seizures may prevent hippocampal cell damage. There is a significant elevation of heparin-binding epidermal growth factor-like growth factor (HB-EGF) mRNA and its protein [17] after kainate injection. The present study was designed to examine the role of these neurotrophic factors in the cellular changes that occur following the seizure-induced damage. The roles of these neurotrophic factors were evaluated by estimating the changes in their expression in a pilocarpine model of seizures. For comparison, the effects of repeated seizures were studied on the pathophysiological changes which occurred in the epilepsy-prone EL mice [16] after repeated vestibular stimulation. The results obtained suggest that NGF, BDNF, NT-3, and HB-EGF are involved in the cellular changes that occur following seizure-induced damage.

2. Materials and methods

2.1. Animals

The epilepsy-prone EL mice were propagated in our laboratory. The C57BL/6 (B6) mice and ddY mice, parent strain of EL mice, were purchased from Shizuoka Experimental Animals Co., Hamamatsu, Japan. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Nagoya University. The mice were housed in a polystyrene cage in a temperature-regulated (22 ± 2 °C), light-controlled (lighted from 0700 to 1900 h) room and were fed ad libitum with a commercial stock diet (CE-2, Japan CLEA Co., Ltd., Urawa). Both sexes of the mice were used when they are 5 weeks old.

2.2. Vestibular stimulation of EL mice

Some EL mice were subjected to 30 'tosses', 15 cm high, once a week, starting at 5 weeks old, for 3, 7, and 15 weeks, respectively. The mice were killed 4 h after the final vestibular stimulation.

2.3. Chemical convulsant treatment

Pilocarpine hydrochloride (Sigma-Aldrich, P6503) was dissolved in 0.85% NaCl (saline, 15 mg/ml) and filtered through a 0.45-μm membrane filter (Advantec Co., Ltd.) and injected i.p. to some groups of B6 mice at a dose of 300 mg/kg body weight. Fifteen minutes before the injection of pilocarpine, the mice were injected i.p. with atropine methyl bromide dissolved in saline (0.25 mg/ml) at a dose of 5 mg/kg. Two hours thereafter, the mice were injected i.p. with diazepam (Cercine, Takeda Pharmaceutical Co., Ltd., 0.5 mg/ml) at a dose of 10 mg/kg. The control mice were injected with saline instead of pilocarpine. For assay of neurotrophic factor expression in the brain, the mice were killed 4 h after the pilocarpine injection. Four hours and 1, 2, 28, and 90 days after the pilocarpine injection, the mice were anesthetized and underwent intracardiac infusion of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS for histochemical analysis. None of the pilocarpine-treated EL mice were subjected to vestibular stimulations.

2.4. BrdU-labeling

Division of hippocampal cells was evaluated using the bromodeoxyuridine (BrdU, Sigma, B5002) labeling method. They were killed 4 h and 1 and 2 days after pilocarpine treatment and received i.p. injection of 50 mg/kg of BrdU 2 h before sacrifice. Some other B6 mice were injected with BrdU twice daily on the 2nd and 3rd day to examine the fate of BrdU-labeled cells and killed 28 and 90 days after the pilocarpine treatment.

2.5. Northern blot analysis

For assays of the expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF, FGF-2), epidermal growth

factor (EGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), and neurotrophin 3 (NT-3), the mice were killed 4 h after the vestibular stimulation and pilocarpine injection, and the brains were quickly removed, placed in 1 ml of ISOGENE (Nippon Gene Co., Ltd., Tokyo) and homogenized (HG30, Hitachi, Tokyo). The total RNA was extracted and Northern blot analyses were performed as previously described [16] using the following radiolabeled cDNA fragments as probes, which were prepared by RT-PCR screening of the mouse library using the following oligonucleotides as primers: [NGF] forward: 5′-TCCTAGT-GAAGATGCTGTGC-3′, reverse: 5′-ACTCTCAACAGGAT-TGGAGG-3′; [HB-EGF] forward: 5′-ATGCTGAAGCTCTT TCTGGC-3′, reverse: 5′-ACGCCCAACTTCACTTTCTC-3′; [FGF-2] forward: 5′-AAGCGGCTCTACTGCAAGAA-3′, reverse: 5′-AACAGTATGGCCTTCTGTCC-3′; [NT-3] forward: 5′-ATCAAGCTGATCCAGGCGGA-3′, reverse: 5′-GTCAGTGCTCGGACATAGGT-3′. 1.1 kb *Eco*RI and *Bam*HI fragment of BDNF cDNA and 1.2 kb *Pst*I fragment of β-actin cDNA were used, respectively, as probes for Northern blot analysis of these proteins. The expression of each mRNA was measured using a BAS 2000 bioimaging analyzer (Fuji Film, Tokyo) and normalized to the levels of β-actin mRNA.

2.6. Histochemistry

Mice were anesthetized before undergoing intracardiac perfusion of sodium sulfide followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The mice were killed 4 h and 1, 2, 3, 7, 28, and 90 days after the pilocarpine treatment. For BrdU analysis, the drug was injected i.p. at a dose of 50 mg/kg 2 h before intracardiac perfusion of sodium sulfoxide and 4% paraformaldehyde. The brains were isolated and immersed overnight in 4% paraformaldehyde at 4 °C and then dehydrated by treating with 20% sucrose. The tissues were embedded in O.C.T. compound (Tissue-Tek; Mikes, Elkhart, IN) and frozen. Frozen sections (10 μm) were cut using a cryostat microtome (Leica), then transferred to MAS-coated slides (MATSUNAMI, S-9441) and air-dried. Coronal cryosections of 10 μm thickness, including hippocampus, were processed for histological staining.

2.7. BrdU analysis

Counting of BrdU-labeled cells was performed on 3–6 sections per animal. The sections were sampled at an interval of 100 μm beginning at a random point close to the front of the hippocampus in order to avoid counting the same cell in 2 sections. BrdU- and NeuN-double positive cells were confirmed with Apo Tome microscope (Carl Zeiss).

2.8. Antibodies

To avoid nonspecific staining, M.O.M. (mouse on mouse) Immunodetection Kit (VECTOR, BMK-2202) was used throughout. The following antibodies were used: a monoclonal anti-BrdU [mouse immunoglobulin G (IgG); COSMOBIO, BU1/75] at a dilution of 1:100, a monoclonal anti-NeuN [mouse IgG; CHEMICON,] at a dilution of 1:400, a monoclonal anti-glial fibrillary acidic protein (GFAP, an astrocytric marker; rabbit IgG; IMMUNON) at a dilution of 1:100, and a monoclonal anti-CD11 (rat IgG, DSHB, M1/70) at a dilution of 1:1. To detect neuronal damage after seizures, Fluoro-Jade B (FJB, Histo-Chem., Inc.) staining method was used. FJB is an anionic fluorescein and has a specific affinity for degenerating neurons [23].

2.9. Statistical analysis

Welch's test or Student's t test after two-way ANOVA was used to determine the significance of differences of the data in each experiment [26].

3. Results

All EL mice, which were subjected to the vestibular stimulation once a week, manifested repeated severe seizures within 3 weeks. However, they did not exhibit status epilepticus throughout the whole experimental period of 20 weeks. Upon pilocarpine injection, some (23/54) B6 mice exhibited severe tonic–clonic convulsions within 30 min and were grouped as 'pilocarpine 2'. Some other mice (21/54), which did not manifest seizures were grouped as 'pilocarpine 1'. In contrast, none of the pilocarpine-treated EL mice exhibited tonic–clonic seizure although they were given a same dose of the drug. None of the EL mice exhibited seizures even when they were injected with pilocarpine at its dose which caused the response in some B6 mice nor by repeated vestibular stimulations.

3.1. Neurotrophin expression

Figs. 1A–F illustrate the expression of various neurotrophic factors in the brain of ddY, EL, and B6 mice, which were subjected to vestibular stimulation and treated with the chemical convulsant, pilocarpine, respectively. As shown, expression of NGF, BDNF, and HB-EGF was markedly increased only in the brain of the pilocarpine-treated mice, which manifested tonic–clonic convulsions (Figs. 1A–C, E, F). In contrast, expression of NT-3 decreased significantly in both ddY mice and B6 mice (Fig. 1D). It is noteworthy that there was no meaningful change in expression of these neurotrophic factors in the brains of B6 mice which showed no sign of convulsions, even though they were treated with the same dose of pilocarpine (Fig. 1, pilocarpine 1). It should be noted also that expression of these neurotrophic factors did not change significantly in the EL mice, although they manifested repeatedly severe convulsions after periodical vestibular stimulations.

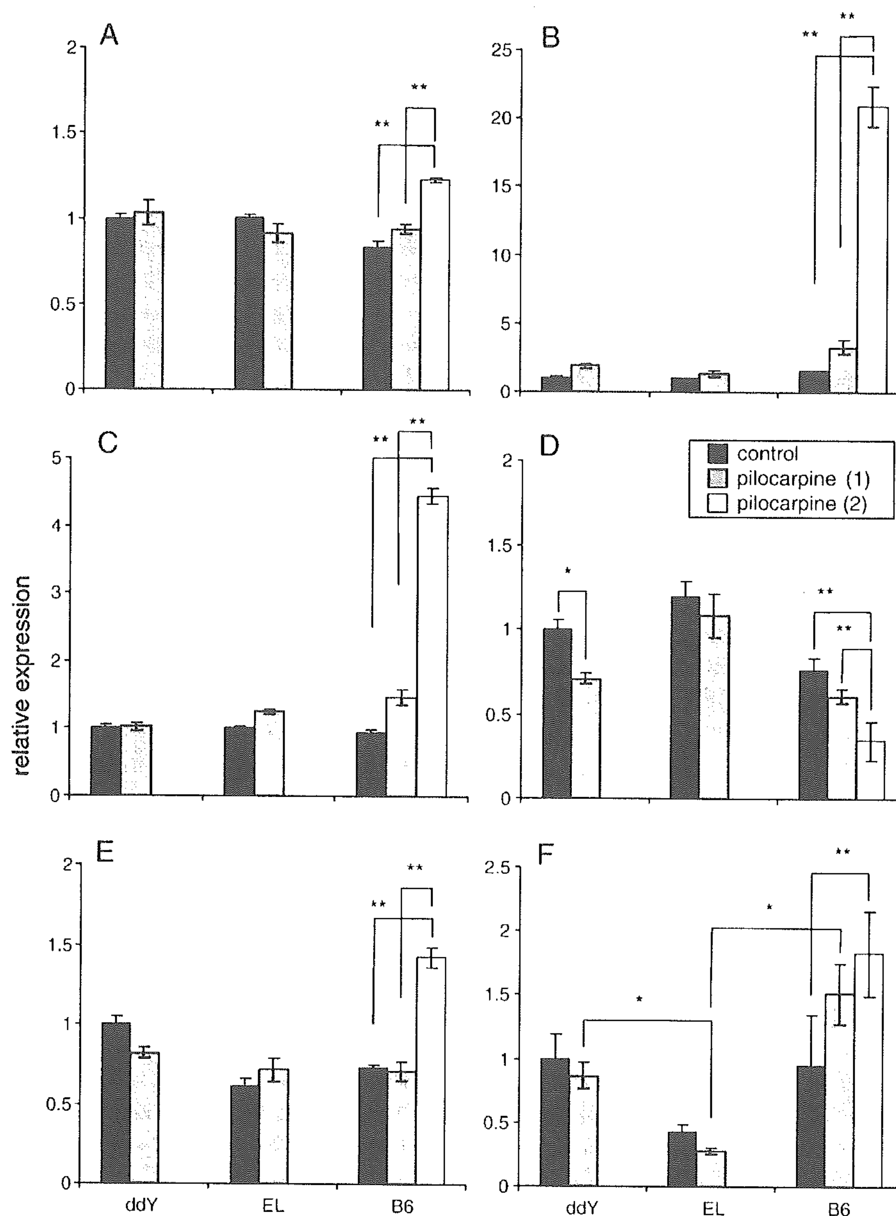


Fig. 1. The relative expression of NGF (A), 4.2 kb BDNF (B 1.5 kb BDNF (C), NT-3 (D), HB-EGF (E), and FGF-2 (F), respectively, in the brains of the ddY mice, EL mice, and C57BL/6 (B6) mice. Some of these mice were injected i.p. with pilocarpine hydrochloride (Sigma, P6503, 300 mg/kg, hatched bars). Fifteen minutes before the injection of pilocarpine, the mice were injected i.p. with atropine methyl bromide (5 mg/kg). Two hours after the injection of pilocarpine, the mice were injected i.p. with diazepam (10 mg/kg). The mice exhibiting tonic–clonic seizures after injection of pilocarpine were grouped as pilicarpine 2 and the remaining mice, which did not exhibit seizures, were grouped as pilocarpine 1. Control mice were injected with 0.85% NaCl instead of pilocarpine. Expression of the neurotrophic factors were measured 4 h after pilocarpine injection. None of the mice were subjected to vestibular stimulation in the study. For details, see Materials and methods. Mean ± SEM of 4–5 animals per group. Statistically different from the control mice: *$P < 0.05$, **$P < 0.01$.

3.2. Histochemistry

Fig. 2 illustrates the histochemical analysis of BrdU-positive cells in the dentate gyrus of control and pilocarpine-treated B6 mice. As shown, the BrdU-positive cells in the control mice were confined to the subgranular zone. The B6 mice which manifested tonic–clonic seizures had about 9.7 and 15.1 BrdU-positive cells, in average, per 10-μm cryosection 2 days and 28 days, respectively, after treatment of pilocarpine (Fig. 2C). In contrast, there were 1.5 and 2.1 BrdU-positive cells on 2 days and 28 days, respectively, in the control mice (Fig. 2B). The double-staining analysis for BrdU and NeuN, a marker of neuronal cells, showed enhanced neurogenesis in the B6 mice which manifested tonic–clonic seizures (Fig. 2). The number of NeuN- and BrdU-double positive cells increased from 0.03, on average, on 2 days to 1.2 on 28 days in the control mouse, and from 1.2 on 2 days to 1.9 cells per cryosection on 28 days after pilocarpine injection. In contrast, there was no appreciable change in the number of BrdU-positive cells in

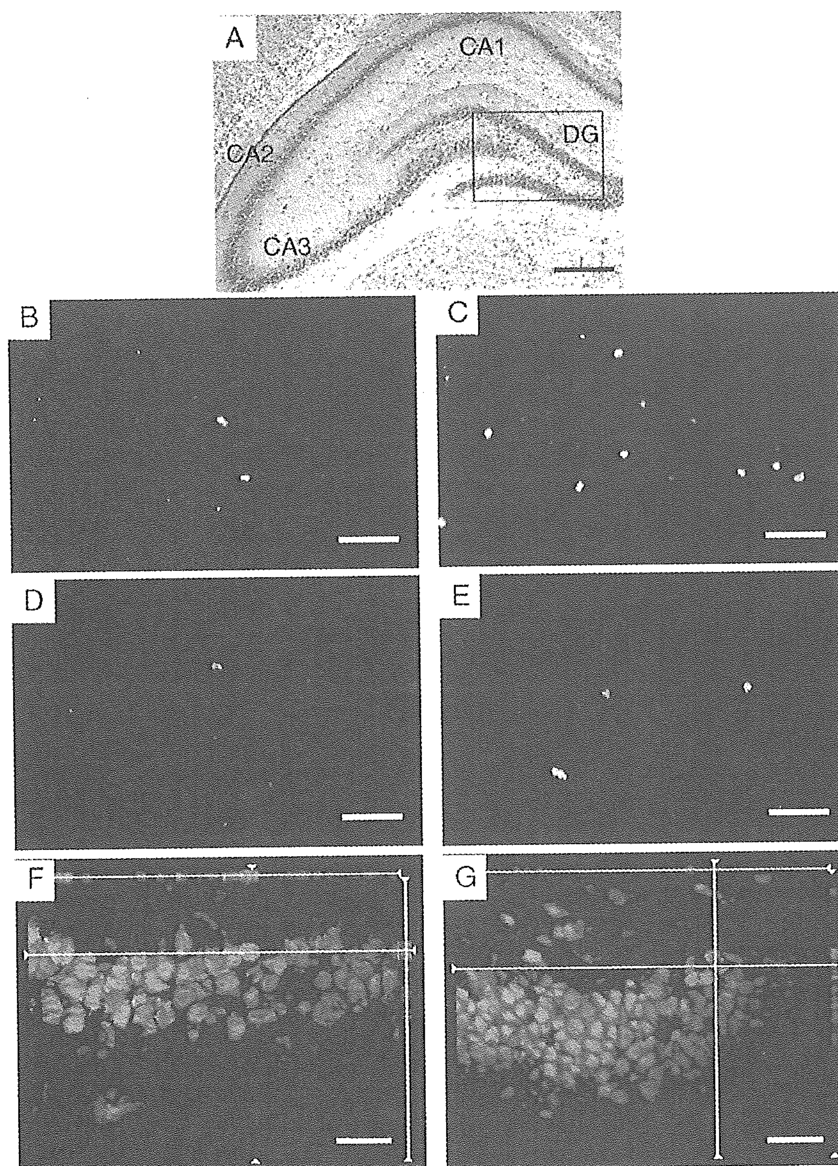


Fig. 2. Structure of mouse hippocampus showed by Nissl staining (A) and BrdU-positive cells in the dentate gyrus of C57BL/6 (B6) mice (B–D) and EL mouse (E). (B) Baseline division of hippocampal cells of a saline-treated control mouse. (C) Increased BrdU–positive cells observed after 2 days in the hippocampus of a B6 mouse treated with pilocarpine and manifested tonic–clonic seizures. Note the clustering of BrdU–positive nuclei in the subgranular proliferative zone at the border of the hilus and granule cell layer. (D) The B6 mouse which manifested no seizures in spite of pilocarpine injection showed BrdU incorporating as same level as saline-treated control. (E) EL mouse experienced repeatedly seizures also did not show increased BrdU-positive cells. (F, G) BrdU (green)- and NeuN (red)-double labeling for control (F) and pilocarpine-seized B6 mouse observed after 2 days (G). Note that the BrdU (green)- and NeuN (red)-double positive cells were noted in the pilocarpine-seized B6 mouse (G), whereas these cells were rarely observed in the saline-treated mice (F). CA1-3, pyramidal cell layer CA1-3 region; DG, dentate gyrus. Scale bar: panel A, 300 μm; panels B–G, 50 μm.

the mice, which exhibited no sign of tonic–clonic convulsions (Fig. 2D). Furthermore, there was no change in the number of BrdU-positive cells in the brain of EL mice, which experienced repeatedly severe seizures after consecutive vestibular stimulations (Fig. 2E). BrdU (green)- and NeuN (red)-double positive cells were noted after 2 days in the pilocarpine-seized B6 mouse (Fig. 2G), whereas these cells were rarely observed in the saline-treated mice (Fig. 2F).

Fig. 3 shows the cells stained with Fluoro-Jade B, a marker of damaged neurons, in the brain of mice, which manifested tonic–clonic convulsions after pilocarpine injection. As shown, a number of Fluoro-Jade B-positive cells were observed after 2 days in the hilus and CA1 pyramidal cell layer of the pilocarpine-treated B6 mice which exhibited tonic–clonic convulsions (Figs. 3C, D). No Fluoro-Jade B-positive cells were noted in the corresponding region of the saline-injected B6 mice (Fig. 3A) and in the B6 mice which manifested no seizures, although they were injected with pilocarpine (data not shown). A marked neuronal cell loss was observed after 90 days in the CA1 and CA3 region of the B6 mice which exhibited tonic–

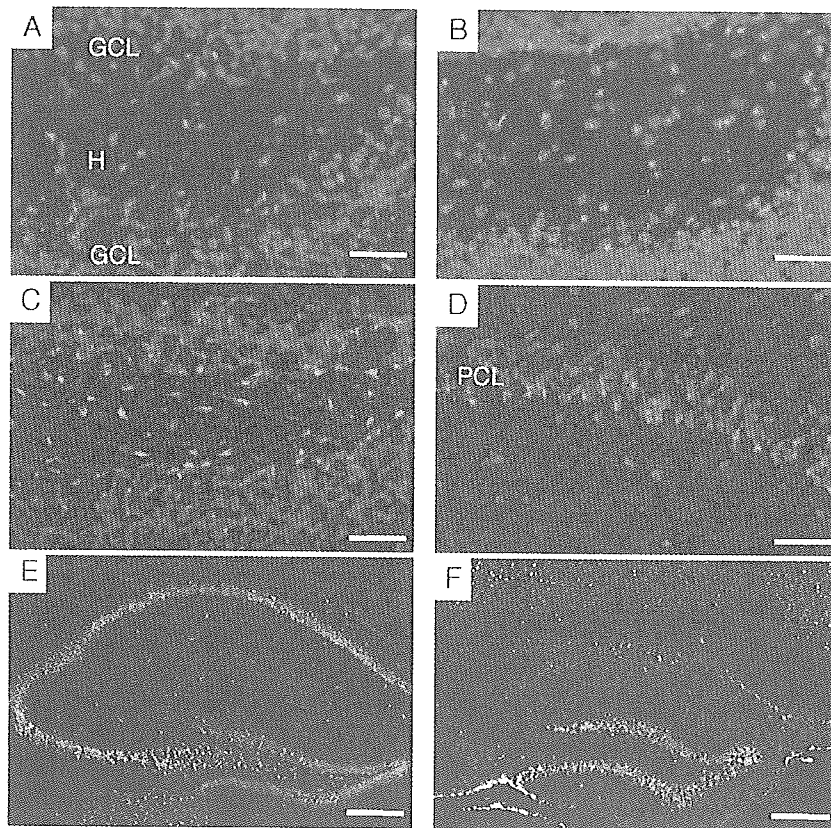
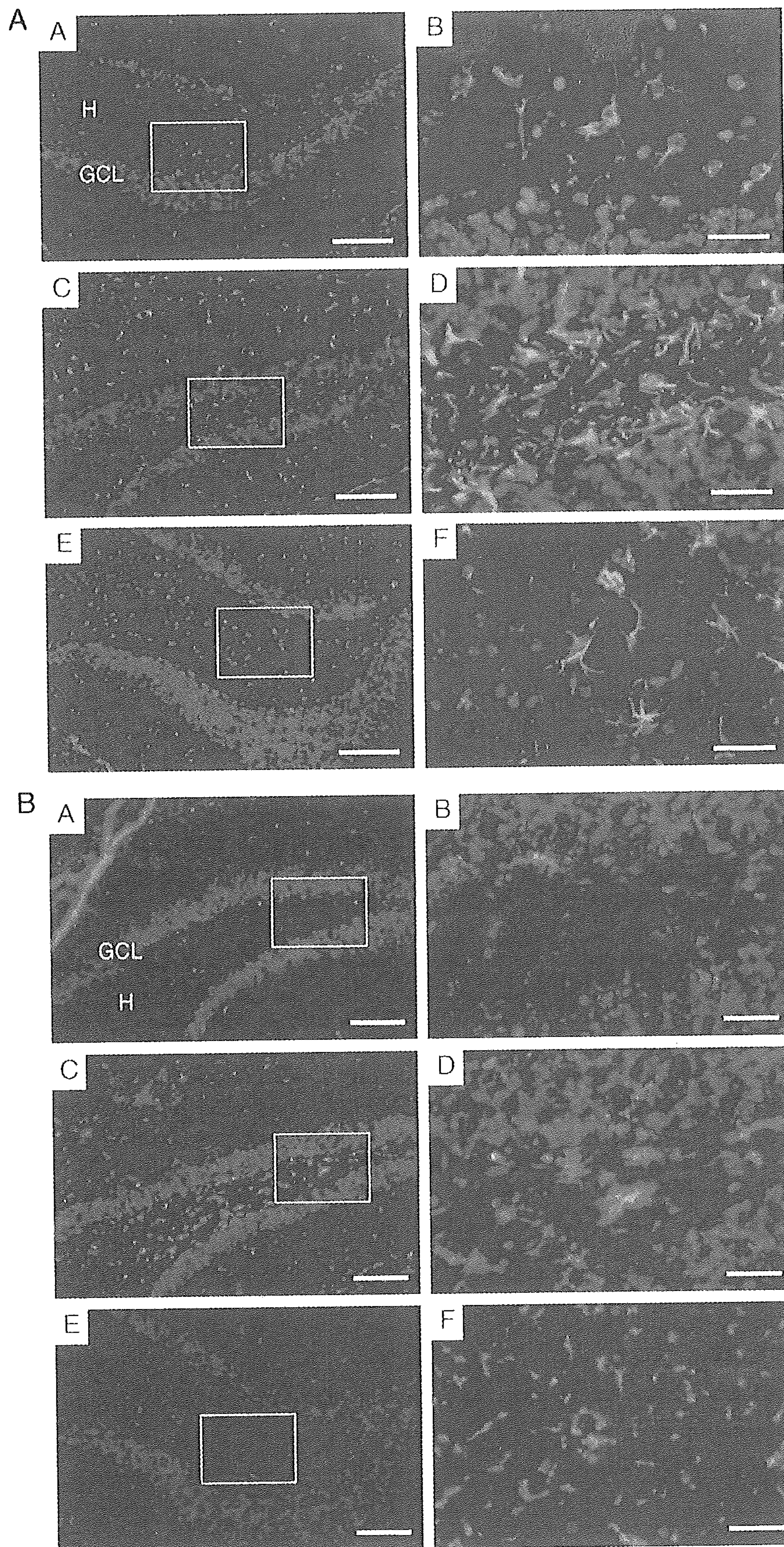


Fig. 3. FJB-positive damaged neurons and loss of NeuN-positive neurons in the hippocampus. FJB-positive cells of the saline-treated C57BL/6j (B6) mouse (A), and the EL mouse which experienced convulsions repeatedly by vestibular stimulations (B). (C) Increased FJB-positive cells observed after 2 days in the B6 mouse which exhibited tonic–clonic seizures after pilocarpine treatment. (D) FJB-positive cells are also detected in the CA1 region of the B6 mouse, which manifested tonic–clonic seizures. (E) Image of NeuN staining in the control B6 mouse. (F) Loss of pyramidal NeuN-positive cells observed after 90 days in the B6 mouse, which manifested tonic–clonic seizures after pilocarpine treatment. GCL, granule cell layer; H, hilus; PCL, pyramidal cell layer (CA1 region). Scale bars: panels A–D, 50 μm; panels E and F, 200 μm.

clonic seizures (Figs. 3E, F). There were no Fluoro-Jade B-positive cells in the brains of the EL mice, which experienced severe convulsions repeatedly as shown in Fig. 3B. Figs. 4A and B illustrate changes in the number of astrocytes, and microglia as evidenced by staining with anti-GFAP and anti-CD11b antibodies, respectively. As shown, the number of these cells was apparently increased after 2 days in the various regions of the hippocampus of the mice, which exhibited tonic–clonic seizures (Figs. 4AC, D and BC, D). Here again, there was no change in the number of these cells in the brain of the B6 mice, which did not manifest convulsions after pilocarpine injection (data not shown), nor in the stimulated EL mice, even though they experienced severe seizures repeatedly (Figs. 4AE, F and BE, F).

4. Discussion

The results obtained here suggest that the neurotrophic factors such as NGF, BDNF, HB-FGF, and NT-3 may be involved in the cellular changes that occur following seizure-induced damage in the mice manifesting tonic–clonic seizures induced by pilocarpine injection. It was evidenced by the findings that their expression changes during seizures, although we show here only the changes in mRNA levels and not of proteins of the neurotrophic factors. First, it was shown here that NGF and BDNF are up-regulated in the mice manifesting tonic–clonic seizures after pilocarpine injection (Figs. 1A, B), confirming those of previous animal experiments [9,13] and in the hippocampus of temporal lobe epilepsy patients [14]. Several studies indicate that NGF promotes epileptogenesis [1,7,29]. In contrast, the roles of BDNF and b-FGF remain controversial [10,20,21]. It was also shown here that HB-EGF was up-regulated in the mice exhibiting tonic–clonic seizures caused by pilocarpine injection (Fig. 1E). These results are consistent with those reported previously showing that HB-EGF expression is significantly up-regulated in kainate-induced seizures [17]. To our knowledge, there is no previous report on the effects of HB-EGF on seizure severity. NT-3 mRNA undergoes a down-regulation in the mice manifesting tonic–clonic seizures after injection of pilocarpine (Fig. 1D), confirming the previous reports, which demonstrated its decreased expression in the kainate-induced [11] and kindling-dependent [4]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seizure models, suggesting that down-regulation of NT-3 was performed in order to prevent further development of pathological events. These results are consistent with the previous reports showing that NT-3 has a facilitatory effect on kindling and sprouting [3,31]. Expression of b-FGF (FGF-2) was enhanced in the pilocarpine-injected mice which exhibited tonic–clonic seizures (Fig. 1F). These results are consistent with those of the previous reports, showing that its expression follows seizure induced by chemical [6,9] and electrical kindling [9,25]. Long-term, low-dose infusion of bFGF prevents kainate-induced hippocampal cell loss, although it has no effect on seizure latency or duration [12]. It should be noted that these changes in neurotrophic factor expression were measured, in the present study, in the entire brain, and not in isolated regions such as hippocampus. Thus, it is possible that we could have missed changes in expression in more limited areas of the brain.

It should be noted that all histological and biochemical changes examined here were observed only in the mice, which elicited tonic–clonic seizures. First, the cells stained with Fluoro-Jade B, a marker of damaged neurons, were noted only in the mice that experienced tonic–clonic seizures after injection of pilocarpine. In contrast, Fluoro-Jade B-positive cells were not observed in the mice that showed no sign of seizures even though they were treated with the same dose of pilocarpine. In addition, there was no cell stained with Fluoro-Jade B in the activated EL mice although they exhibited severe seizures repeatedly. Second, the number of BrdU-positive cells, a marker of division of dividing cells, increased significantly only in the mice, which exhibited tonic–clonic seizures after injection of pilocarpine, whereas no appreciable change in the number of BrdU-stained cells was observed in the mice that showed no seizures, although they were treated with pilocarpine. Furthermore, there was no significant change in the number of BrdU-positive cells in the activated EL mice, which experienced severe seizures repeatedly. It is noteworthy that an increase in the number of BrdU-positive cells was evident as late as 2 days after the pilocarpine injection and not after 1 day (data not shown). Third, activation of astrocytes and microglia were noted mainly in the mice that showed tonic–clonic seizures after pilocarpine treatment. Fourth, the changes of expression of NGF, BDNF, HB-FGF, and NT-3 occurred, here again, only in the mice that exhibited tonic–clonic convulsions after pilocarpine injection. Furthermore, no significant change was observed in expression of these neurotrophic factors in the activated EL mice, which manifested severe seizures. There may simply be strain differences, and therefore severe seizures in B6 mice may result in severe damage, whereas severe seizures in EL mice may not elicit damage, regardless of whether the seizures are tonic–clonic or not. This is clearly correlated with whether neurogenesis and glial activation occur as well, although the correlation cannot be made with neurotrophic factors for the reasons given above. These results together suggest that neuronal damage occurring in the brain of the mice manifesting tonic–clonic seizures is accompanied by neurogenesis.

It remains uncertain whether seizure-induced neuronal degeneration is responsible for enhancement of progenitor cell division. Nakagawa et al. have shown that pyramidal neuronal degeneration is associated with enhancement of progenitor cell division in kainic acid-induced seizures but not in a kindling model [15]. As shown in Figs. 2 and 3, the number of Fluoro-Jade B-stained cells and BrdU-positive cells was increased only in the mice which exhibited tonic–clonic seizures after pilocarpine injection. Hence, it is possible that neurogenesis occurs when the degree of seizures was severe enough to cause neuronal damage.

It is noteworthy that none of the EL mice manifested tonic–clonic seizure even when they were treated with the pilocarpine. In addition, any of the histological changes examined was not observed in these mice, nor in the EL mice which experienced repeatedly seizure episodes after periodical vestibular stimulations. This may indicate that the EL mice are resistant to various epileptogenic stimuli. None of the pilocarpine-treated EL mice and their control animals was subjected to vestibular stimulations. Hence, one might not expect up-regulation of neurotrophic factors, nor in labeling of BrdU, FJB, GFAP, or CD11b already in the control animals. In fact, there was no difference in expression of these factors between the control EL mice and the control B6 mice.

Mounting evidence indicates that neurotrophic factors such as NGF, BDNF, and NT-3 have functional roles in the regulation of pathophysiological changes that associated with seizure-induced brain injury. For example, NGF is known to have important roles in the remodeling of networks that follow repetitive seizures [7]. Granule cell mRNA levels for NGF, BDNF, and NT-3 correlate with neuronal loss and mossy fiber sprouting in the epileptic human hippocampus [14]. Together, these growth factors may regulate the downstream events that follow severe seizure induced by pilocarpine injection.

Fig. 4. (A) A, B: GFAP, a marker of astrocyte-positive cells in the hippocampus of the saline-treated control C57BL/6j (B6) mouse. C, D: Increased GFAP-positive cells after 2 days in the mouse exhibited tonic–clonic seizures caused by pilocarpine injection. E, F: GFAP-positive cells in the EL mouse, which experienced seizures repeatedly after vestibular stimulation. GCL, granule cell layer; H, hilus. Scale bars: panels A, C, and E, 100 μm; panels B, D, and F, 25 μm. (B) A, B: CD11b, a marker of activated microglia-positive cells in the hippocampus of the saline-treated B6 mouse. C, D: Increased activated microglial cells observed after 2 days in the B6 mouse, which exhibited tonic–clonic seizures after pilocarpine treatment. E, F: CD11b-positive cells in the EL mouse, which experienced seizures repeatedly by periodical vestibular stimulations. H, hilus; GCL, granule cell layer. Scale bars: panels A, C, and E, 100 μm; panels B, D, and F, 25 μm.

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REGULAR PAPER

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Cytokine production of activated microglia and decrease in neurotrophic factors of neurons in the hippocampus of Lewy body disease brains

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Abstract Dementia is a frequent complication of Parkinson's disease (PD) and usually occurs late in the protracted course of the illness. We have already reported numerous MHC class II-positive microglia in the hippocampus in PD patients, and that this phenomenon may be responsible for functional changes in the neurons and the cognitive decline in PD patients. In this study, we have investigated the distribution of activated microglia and the immunohistochemical and the mRNA expression of several cytokines and neurotrophic factors of the hippocampus in PD and dementia with Lewy bodies (DLB). The brains from five cases of PD and five cases of DLB that were clinically and neuropathologically diagnosed, and those from four normal controls (NC) were evaluated by immunohistochemistry using anti-HLA-DP, -DQ, -DR (CR3/43), anti-α-synuclein, anti-brain-derived neurotrophic factor (BDNF), and anti-glial fibrillary acidic protein antibodies. In addition, the mRNA expressions of cytokines (IL-1α, IL-1β, TNF-α, IL-6, TGF-β) and neurotrophic factors (BDNF, GDNF, NGF, NT-3) of these brains were evaluated by the reverse transcription-PCR method. MHC class II-positive microglia were distributed diffusely in the hippocampus of PD and DLB brains. Although the cytoplasm of pyramidal and granular cells of the hippocampus in NC brains was strongly stained by anti-BDNF antibodies, it was only weakly stained in PD and DLB brains. The mRNA expression of IL-6 was significantly increased in the hippocampus of PD and DLB brains, and that of BDNF was significantly decreased in the hippocampus of DLB brains. The increased number of activated microglia and the production of neurotrophic cytokines such as IL-6, together with the decreased expression of the neurotrophic factors of neurons in the hippocampus of PD and DLB brains, may be related to functional cellular changes associated with dementia.

Keywords Lewy body disease · Microglia · Major histocompatibility complex class II antigen · Interleukin-6 · Brain-derived neurotrophic factor

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Introduction

Microglia, which are now widely accepted as being of a mononuclear, phagocyte lineage, qualify as immunocompetent cells in the central nervous system (CNS) by virtue of their ability to express major histocompatibility complex (MHC) class II antigens [24, 29, 57]. Microglia with resting or ramified morphology seldom express those antigens, and the up-regulation of MHC class II antigen is an early consequence of activation,

the threshold of detection being reached prior to the onset of visible morphological changes. MHC class II expression on microglia is also up-regulated in pathological situations where neurons degenerate [38, 45, 46]. Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are two such conditions [30, 41, 44]. The salient pathological features of PD are selective neuronal loss, presumably by apoptosis, and the presence of Lewy bodies (LB) in the affected regions [32, 54]. The presence of activated microglia and the absence of reactive astrocytosis in the substantia nigra (SN) of patients with PD suggest microglial involvement in the pathological process of dopaminergic neurons [49]. DLB is a recently recognized cause of neurodegenerative dementia and is clinically characterized by fluctuating but progressive cognitive impairment, parkinsonism, and psychosis with recurrent hallucinations. The neuropathological hallmarks are widely distributed LB throughout the paralimbic and neocortical regions as well as SN [48]. We have already demonstrated that MHC class II-positive activated microglia were widely distributed in the affected regions, including the hippocampus, frequently in association with α-synuclein-positive Lewy neurites or monoaminergic neurites in PD brains [30]. These activation of microglia may be related to reduction of neurotransmitters or neurotrophic factors of adjacent neurons. Nowadays glial cells are known to possess neurotrophic properties that are essential to the survival of dopaminergic and cholinergic neurons [52]. In the present study, we report an increased number of activated microglia, the production of neurotrophic cytokines such as interleukin (IL)-6, and a decreased expression of the neurotrophic factors of neurons in the hippocampus of PD and DLB brains.

Materials and methods

Subjects

Autopsy brains from five clinically and neuropathologically confirmed cases of PD (ages at death 71–81 years, mean 74.8 years), five cases of DLB (ages at death 65–79 years, mean 74.6 years), and four age-matched individuals (ages at death 73–81 years, mean 75.8 years) were examined in this study. All PD patients had presented clinically with resting tremor, rigidity and akinesia, and had responded to levodopa during the course of their disease. Neuropathologically, the brain specimens showed neuronal loss in the SN, locus ceruleus and dorsal vagal nuclei, and Lewy bodies appeared in the SN, locus ceruleus, dorsal vagal nuclei and neocortex [17]. The diagnosis of DLB was made according to the consensus criteria for its pathological diagnosis [48]. Neuropathological findings of PD and DLB are summarized in Table 1. PD-related pathological staging was evaluated according to the Braak stages [7].

Conventional histopathology

All brains were removed within 12 h of death and immersed in 20% neutral-buffered formalin and fixation periods of all brains were within 3 weeks and there was no significant difference between PD, DLB and controls. Each brain region was sliced into 5-mm-thick sections along various planes: cerebrum in the frontal plane, brainstem and spinal cord in the horizontal plane, and cerebellum in the sagittal plane. The tissues were embedded in paraffin and sectioned at a 10-μm thick-

Table 1 Neuropathological findings of PD, DLB and control cases. For NFT, the stage (I–V) is shown; for Aβ, stages of cortical Aβ deposition (A–C) are shown, PD stages are given as 1–6 (*0* , no PD lesions). The table lists the predisposed induction sites of the PD-related pathology. The degree of pathology is assessed semiquantitatively and indicated by: –, absent; +, slight; + +, moderate; + + +, severe (*PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *NFT* neurofibrillary tangle, *aβ* β-amyloid, *ol* olfactory bulb,*dm* dorsal IX/X motor nucleus, *rm* nucleus raphes magnus and/or gigantocellular reticular nucleus. *co* coeruleus-subcoeruleus complex, *sn* posterior portion of substantia nigra , *db* interstitial nucleus of the diagonal band and/or basal nucleus. of Meynert, *CA2*. second sector of the Ammon's horn, *mc* transentorhinal region and/or ectorhinal region, *hc* high-order sensory assosiation areas and prefrontal areas of the neocortex, *fc* first-order sensory assosiation areas and premotor areas and/or primary sensory and motor fields of the neocortex)

	Age	Sex	Dulation (years)	NFT	aβ	PD	ol	dm	rm	co	sn	db	CA2	mc	hc	fc
1	81	M	13	II	A	3	+ +	+ + +	+ +	+ +	+	+	-	-	-	-
2	74	F	13	II	A	3	+ + +	+ +	+ +	+ +	+ +	+	-	-	-	-
3	74	M	11	II	A	4	+ + +	+ + +	+ +	+ + +	+ +	+	+	+	-	-
4	74	M	11	II	A	4	+ + +	+ + +	+ + +	+ +	+ +	+	+	+	-	-
5	71	M	8	II	A	4	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	-	-
6	77	M	9	II	A	6	+ + +	+ + +	+ +	+ +	+ + +	+ + +	+ +	+ + +	+ +	+
7	65	M	5	II	A	6	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
8	75	M	4	II	A	6	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+
9	79	M	13	II	A	6	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
10	77	M	11	II	A	6	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
11	73	F	-	I		0	-	-	-	-	-	-	-	-	-	-
12	74	M	-			0	-	-	-	-	-	-	-	-	-	-
13	75	M	-			0	-	-	-	-	-	-	-	-	-	-
14	81	M	-			0	-	-	-	-	-	-	-	-	-	-

ness. For routine histological examinations, each section was stained with hematoxylin and eosin (H-E), and the Klüver-Barrera (K-B) method was used.

Immunohistochemical staining

The 10-µm-thick sections were deparaffinized and rehydrated according to the standard procedures for immunohistochemistry. They were then subjected to microwave treatment for 30 min in 0.01 M citrate buffer at pH 6.0, removed from the buffer to cool down to room temperature, and treated for 20 min with 0.3% H_2O_2 solution in 0.01 M phosphate-buffered saline at pH 7.4. After blocking, they were incubated for 74 h at 4°C with primary antibodies, treated with biotinylated second antibodies (DAKO, Carpinteria, CA) for 1 h at room temperature, and then incubated with avidin-labeled horseradish peroxidase (DAKO) for 1 h at room temperature. Peroxidase labeling was visualized by brief incubation in 0.01% 3,3'-diaminobenzidine and 0.1% H_2O_2 in 0.05 M TRIS-HCl buffer at pH 7.6. Nuclei were counterstained with hematoxylin. Double immunostaining was also performed. The first cycle was carried out as mentioned above, and stained sections were again subjected to a microwave treatment for 30 min in 0.01 M citrate buffer at pH 6.0. The second immunohistochemical cycle was carried out similarly to the first one, except that it was incubated with avidin-labeled alkaline phosphatase (DAKO), and immunolabeling was visualized by incubation in fast red. The primary antibodies used in this study were monoclonal antibody to human HLA-DP, DQ, DR (clone CR3/43) at a dilution of 1:100 (DAKO A/S, Glostrup, Denmark), monoclonal antibody to human GFAP (clone 6F2) at a dilution of 1:400 (DAKO A/S), monoclonal antibody to human brain-derived neurotrophic factor (BDNF; clone 35928.11) at a dilution of 1:100 (R&B Systems Inc., USA), and polyclonal goat antibody to human α-synuclein (N-19) at a dilution of 1:100 (Santa Cruz, CA).

Reverse transcription-PCR method

Ribonucleic acid (average 21.86 mg/punched tissue) extracted from punched samples using a modified acid phenol-guanidine method was used as a template for first-strand cDNA synthesis as follows. A random primer (0.1 mg) was incubated at 95°C for 10 min with the RNA (1 µg) at a volume of 30 µl, and then placed on ice for 5 min. Next, the mixture was incubated at 37°C for 90 min with a mixture of 100 U M-MLV reverse transcriptase (GIBCO BML), 1× reverse transcription (RT) buffer, 10 mM dithiothreitol, 40 U RNasin, and 0.56 mM each of dATP, dGTP, dCTP and dTTP in a volume of 50 µl, then repeated at 95°C for 10 min. The cDNA was amplified with Taq DNA polymerase (Takara, Tokyo) using primer pairs specific to NGFβ (sense primer: AGTTTTACCAAGGGAGCA, antisense primer: GGCAGTGTCAAGGGAATG), BDNF (sense primer: AAGAAAGCCCTAACCAGT, antisense primer: CGAAAGTGTCAGCCAATG), NT-3 (sense primer: GCTTATCTCCGTGGCATC, antisense primer: TGTTGTCGCAGCAGTTCG), glial cell line-derived neurotrophic factor (GDNF; sense primer: GCCAGAGGATTATCCTGA, antisense primer: CCCAGACCCAAGTCAGTG), IL-6 (sense primer: TCAATGAGG AGACTTGCC, antisense primer: TGAGTTGTCATGTCCTGC), TGFβ (sense primer: AGCTGTACATTGACTTCC, antisense primer: GGACAGCTGCTC CACCTT), TNFα (sense primer: CCCAGGCAGTCAGATCAT, antisense primer: GGCAGAGAGGTTGAC), IL-1α (sense primer: AGGAAGAAATCATC AAGC, antisense primer: TGGGCAGTCACATACAAT) or IL-1β (sense primer: TGGCTTATTACAG TGGCA, antisense primer: AAGAAGGTGCTCAG GTCA) for 40 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min), and GAPDH (sense primer: GAAGGTGAAGGTCGGAGTC, antisense primer: GAAGATGGTGATGGGATTTC) for 30 cycles. The 195-bp (NGFβ), 260-bp (BDNF), 257-bp (NT-3), 240-bp (GDNF), 259-bp (IL-6), 251-bp (TGFβ), 296-bp (TNFα), 274-bp (IL-1α), 247-bp (IL-1β) and 228-bp (GAPDH) PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed.

Quantification

Average numbers of HLA-DP, DQ, DR (CR3/43)-positive cell counts in the hippocampus, amygdala and transentorhinal cortex were calculated as the sum of reactive microglia in five ×200 fields of five different sections. Stat View (Abacus Co., Cary, NC) was used for statistical analysis. Differences were analyzed by the Mann-Whitney test. Statistical significance was confirmed using backward elimination at a probability value of 0.05.

Results

Immunohistochemical study

An immunohistochemical study using anti-α-synuclein showed that a few α-synuclein-positive presynaptic terminals were stained in the hippocampus CA2/3 region and dentate gyrus of NC brains (Fig. 1A, Fig. 2A). However, there were also some α-synuclein-positive Lewy neurites in the hippocampus CA2/3 region (Fig. 1B) and mossy fibers in the dentate gyrus (Fig. 2B) of PD brains. In DLB brains, Lewy neurites (Fig. 1C) and mossy fibers (Fig. 2C) were more numerous.

An immunohistochemical study using anti-HLA-DP, DQ, DR (CR3/43) showed that CR3/43-positive activated microglia were not seen in the hippocampus CA2/3 region and dentate gyrus of NC brains (Fig. 1D,

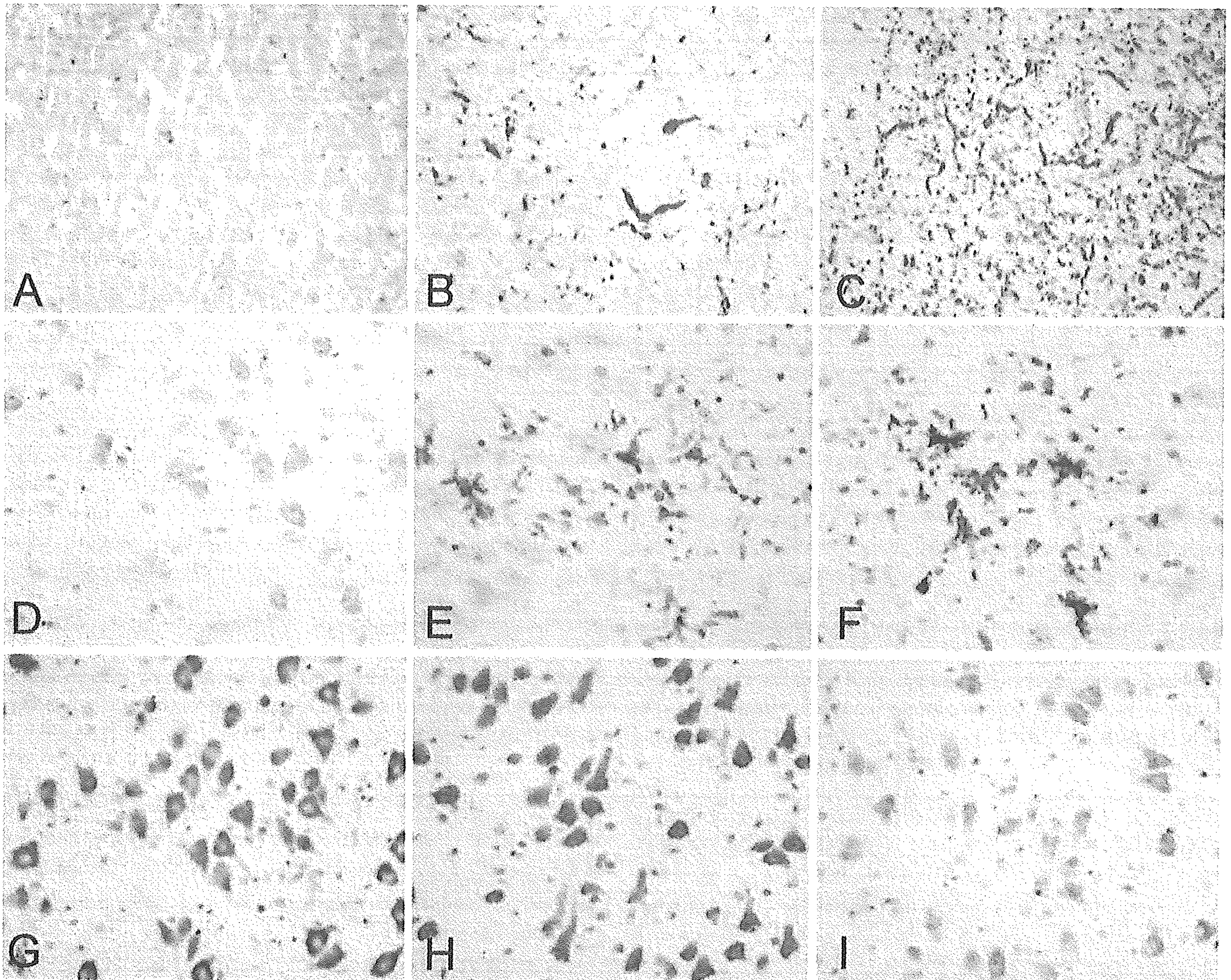


Fig. 1 Immunohistochemical studies of the hippocampus CA2/3 region in NC (**A**, **D**, **G**), PD (**B**, **E**, **H**) and DLB (**C**, **F**, **I**). Immunohistochemical study was carried out with anti-α-synuclein (**A**–**C**). **A** A few α-synuclein-positive presynaptic terminals are seen in NC. **B** There are some α-synuclein-positive Lewy neurites in PD. **C** In DLB, Lewy neurites are more numerous. Immunohistochemical study was also carried out with anti-HLA-DP, DQ, DR (CR3/43) (**D**–**F**). **D** CR3/43-positive microglia are not seen in NC. **E**, **F** In PD and DLB, many CR3/43-positive ramified-shaped microglia are seen. Another immunohistochemical study was carried out with anti-BDNF (**G**–**I**). **G** Almost all neuronal cytoplasm is stained in NC. **H** Almost all neuronal cytoplasm is weakly stained, and nuclei are strongly stained in PD. **I** In DLB, almost all neuronal cytoplasm and nuclei are weakly stained (*NC* normal control, *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *BDNF* brain-derived neurotrophic factor). ×380

Fig. 2D). In PD and DLB brains, many CR3/43-positive ramified-shaped microglia were seen in the hippocampus CA2/3 region (Fig. 1E, F) and dentate gyrus (Fig. 2E, F).

An immunohistochemical study using anti-BDNF showed that almost all neurons were strongly stained in the cytoplasm of NC brains (Fig. 1G, Fig. 2G). In PD brains, almost all neuronal cytoplasm was weakly stained, and their nuclei were strongly stained (Fig. 1H, Fig. 2H). In DLB, almost all neuronal cytoplasm and nuclei were weakly stained (Fig. 1I, Fig. 2I).

Double immunohistochemical staining of the hippocampus of PD patients showed many CR3/43-positive microglia in the CA2/3 region and dentate gyrus, whereas only a few GFAP-positive astrocytes were seen in both regions (Fig. 3A, B). Some of CR3/43-positive microglia were associated with α-synuclein-positive Lewy neurites in the CA2/3 region (Fig. 3C) and Lewy body-containing neurons in the dentate gyrus (Fig. 3D). Some CR3/43-positive microglia were associated with weakly BDNF-positive neurons in the CA2/3 region (Fig. 3E) and the dentate gyrus (Fig. 3F).

Distribution of activated microglia

Compared to NC patients, brains from PD and DLB patients had a significantly higher number of CR3/43-positive microglia in all areas of the limbic system, especially the CA2/3 region and the dentate gyrus of the hippocampus. However, there was no statistical differ-

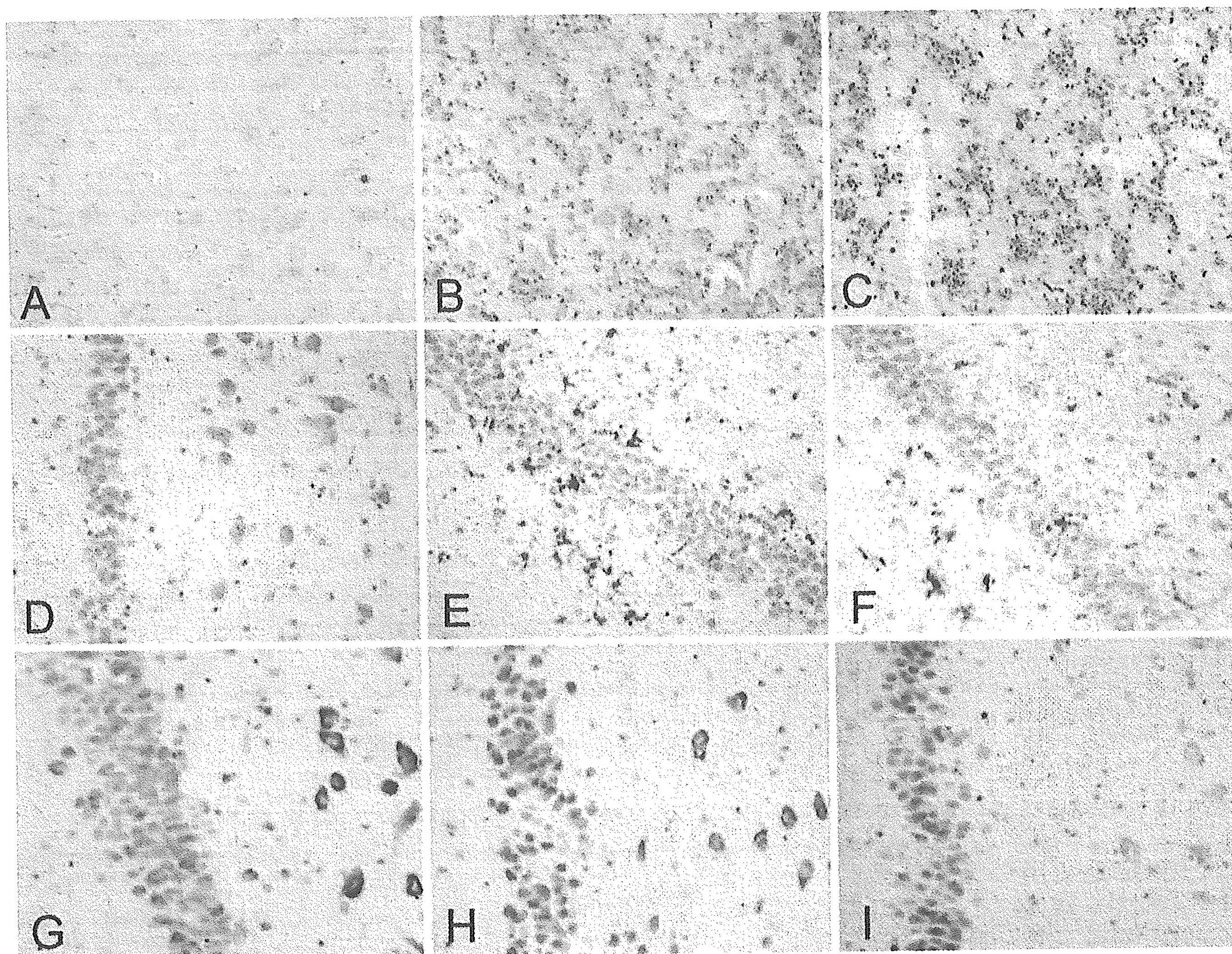


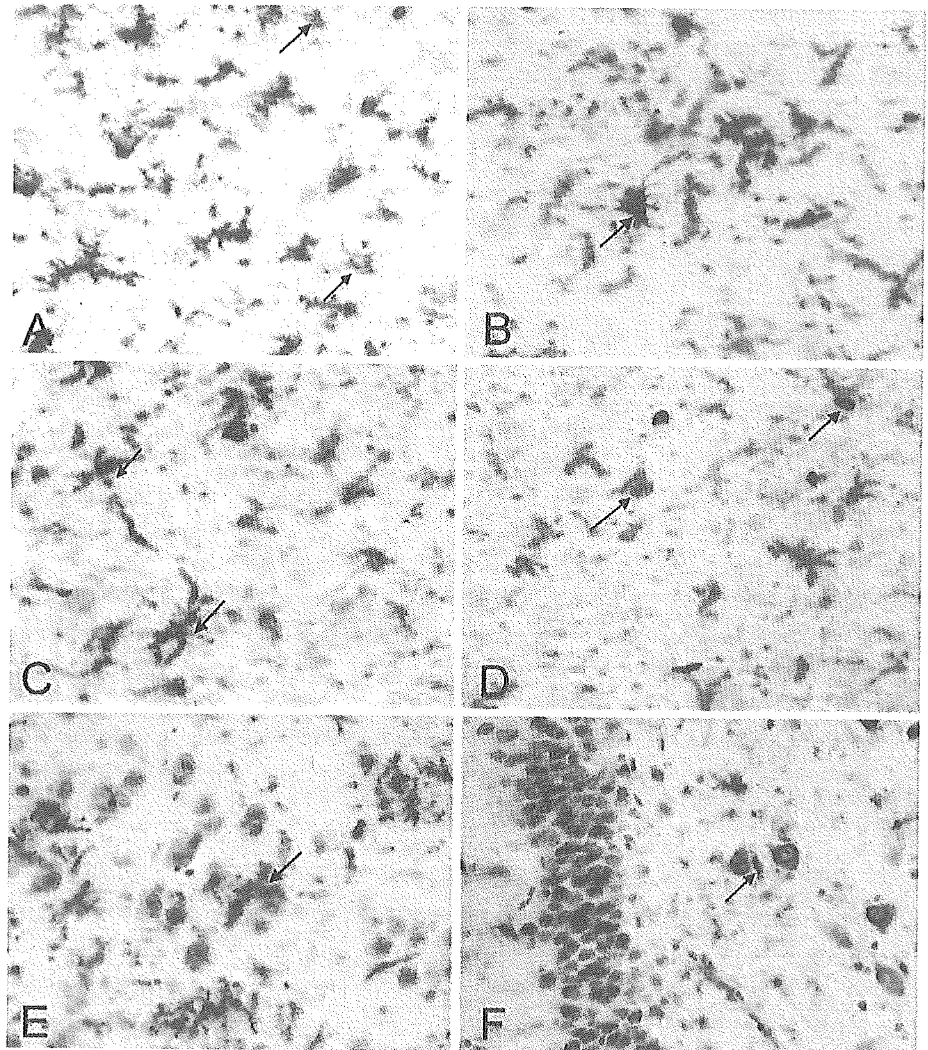
Fig. 2 Immunohistochemical studies of the dentate gyrus in NC (**A**, **D**, **G**), PD (**B**, **E**, **H**), and DLB (**C**, **F**, **I**). The first immunohistochemical study was carried out with anti-α-synuclein (**A–C**). **A** A few α-synuclein-positive presynaptic terminals are seen in NC. **B** There are many α-synuclein-positive mossy fibers in PD. **C** In DLB, more numerous mossy fibers are seen. The second immunohistochemical study was carried out with anti-HLA-DP, DQ, DR (CR3/43) (**D–F**). **D** CR3/43-positive microglia are not seen in NC. **E**, **F** In PD and DLB, many CR3/43-positive microglia were seen. The third immunohistochemical study was carried out with anti-BDNF (**G–I**). **G** Almost all neuronal cytoplasm is stained in NC. **H** Almost all pyramidal neuronal cytoplasm is stained weakly, and nuclei are stained strongly in PD. **I** In DLB, almost all pyramidal neurons cytoplasm and nuclei are stained weakly. ×380

ence in CR3/43-positive microglia counts between PD and DLB (Fig. 4).

RT-PCR method

The average ± SE ratios of the mRNA expression of neurotrophic factors and cytokines to that of GAPDH in the hippocampus, putamen and cingulate cortex of NC, PD and DLB are shown in Fig. 5. As for neurotrophic factors in the hippocampus, the mRNA expression of BDNF, GDNF and NT-3 in PD tended to decrease compared with NC. In DLB, the mRNA expression of these three neurotrophic factors showed a stronger decreasing trend than that in PD, and the mRNA expression of BDNF was significantly decreased compared with NC. As for cytokines in the hippocampus, the mRNA expression of IL-1β in PD and DLB significantly decreased compared with NC, whereas that of IL-6 in PD and DLB significantly increased compared with NC. TNF-α mRNA expression in PD and DLB tended to increase compared with NC. In the putamen, the mRNA expression of BDNF in DLB significantly decreased, and the mRNA expression of NT-3 in DLB tended to decrease compared with NC. In the putamen, the mRNA expression of IL-6 in PD and DLB significantly increased compared with NC. In the cingulate cortex, the mRNA expression of NGFβ in DLB significantly increased as did the mRNA expression of IL-1β and IL-6 in PD and DLB, and that of TNF-α in DLB when compared with NC.

Fig. 3 Double immunohistochemical staining of the hippocampus of PD used anti-HLA-DP, DQ, DR (CR3/43) versus anti-GFAP (**A**, **B**), anti-α-synuclein (**C**, **D**), and anti-BDNF (**E**, **F**). Many CR3/43-positive microglia (*purple*) are seen in the CA2/3 region (**A**) and dentate gyrus (**B**), and a few GFAP-positive astrocytes (*brown*, *arrows*) are seen in both regions. Some CR3/43-positive microglia (*purple*, *arrows*) are associated with α-synuclein-positive Lewy neurites (*brown*) in the CA2/3 region (**C**) and Lewy body (*brown*) -containing neurons in the dentate gyrus (**D**). Some CR3/43-positive microglia (*purple*, *arrows*) are associated with weakly BDNF-positive neurons (*brown*) in the CA2/3 region (**E**) and dentate gyrus (**F**). **A**, **C**, **E**, **F** ×380; **B**, **D** ×450



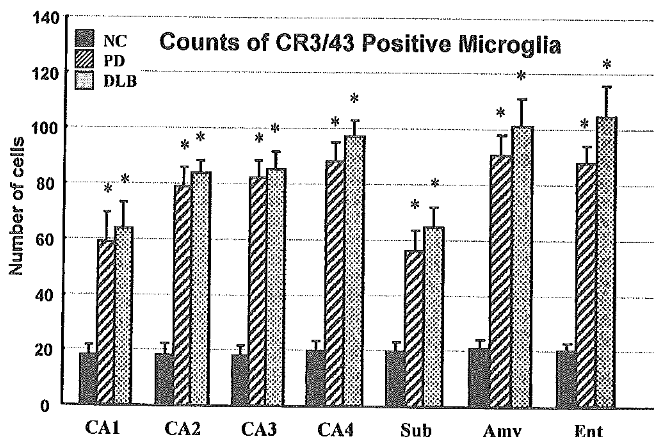


Fig. 4 Bar graphs show a comparison of the average numbers of HLA-DP, DQ, DR (CR3/43)-positive microglia in the limbic system in NC (*black bar*), PD (*striped bar*) and DLB (*dotted bar*) (number/HPF). Patients with PD and DLB have significantly higher numbers of CR3/43-positive microglia in all areas compared with NC (*$P < 0.01$), but there is no statistical difference in CR3/43-positive microglia counts between PD and DLB brains

Discussion

Comparing patients of similar age, those with PD have a higher increased risk of dementia [2, 3, 18, 43]. Mild atrophy of the hippocampus was found in both demented and non-demented cases of PD, consistent with recent postmortems and MRI findings [11, 13, 39]. Subcortical pathologies, including atrophy of the cholinergic nucleus basalis of Meynert, striatonigral degeneration, and involvement of the dopaminergic ventral tegmental area and other monoaminergic nuclei, may also contribute to dementia in PD. In the hippocampus, the decrease in choline acetyltransferase (ChAT) activity in PD with or without dementia has varied from 40% to 62% as compared with controls, and the cognitive decline in PD patients may be associated with reduced ChAT activity in the hippocampus [42].

BDNF is a neurotrophic factor that promotes cholinergic and dopaminergic neuronal differentiation [1, 28, 36, 63]. The protein and mRNA of BDNF are

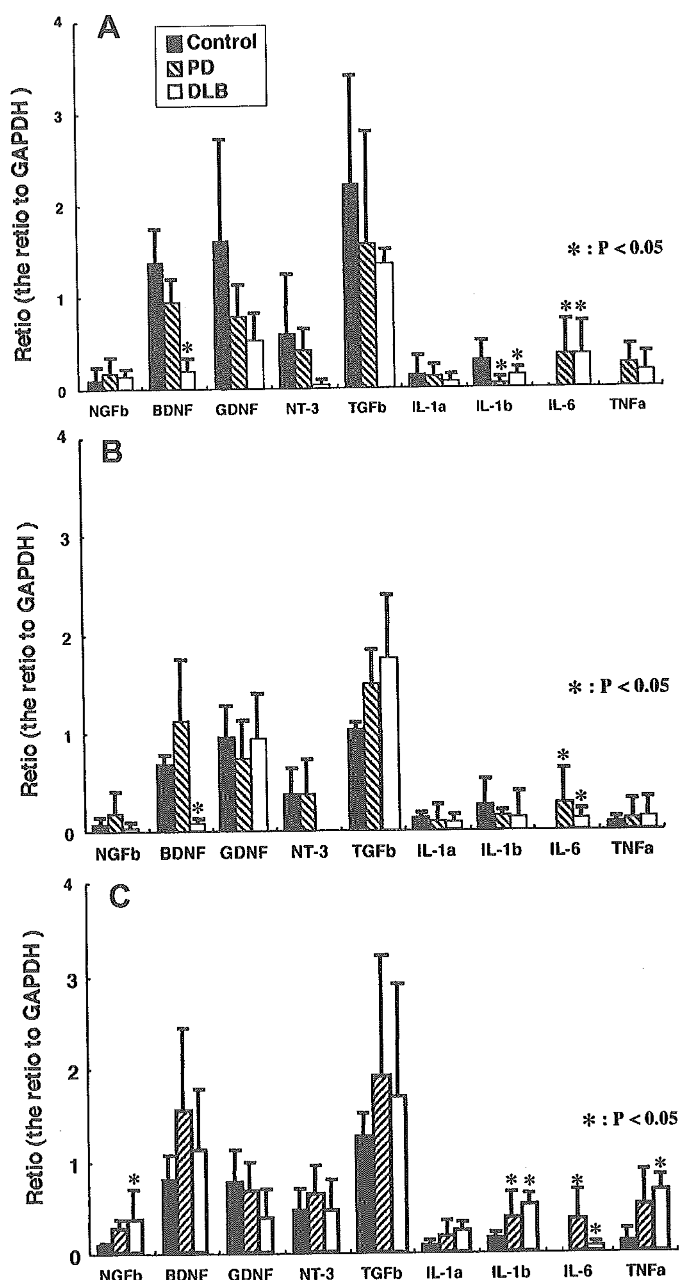


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Fig. 5 Bar graphs show the average ± SE ratio of the mRNA expression of neurotrophic factors and cytokines to that of GAPDH in the hippocampus (**A**), putamen (**B**) and cingulate cortex (**C**) in NC (*black bars*), PD (*striped bars*) and DLB (*dotted bars*). **A** Hippocampus: The mRNA expression of BDNF, GDNF and NT-3 in PD tend to decrease compared with NC. In DLB, mRNA expression of these three neurotrophic factors shows a stronger decreasing trend than that in PD, and mRNA expression of BDNF is significantly decreased compared with NC ($*P<0.05$). mRNA expression of IL-1β in PD and DLB is significantly decreased compared with that in NC ($*P<0.05$), and mRNA expression of IL-6 in PD and DLB is significantly increased compared with that in NC ($*P<0.05$). The TNF-α mRNA expression in PD and DLB tends to increase compared with that in NC. There is no statistical difference between PD and DLB in their mRNA expression of neurotrophic factors and cytokines. **B** Putamen: mRNA expression of BDNF in DLB is significantly decreased compared with that in NC ($*P<0.05$), and mRNA expression of NT-3 in DLB tends to decrease compared with that in NC. mRNA expression of IL-6 in PD and DLB is significantly increased compared with that in NC ($*P<0.05$). There is no statistical difference between PD and DLB in their mRNA expression of neurotrophic factors and cytokines. **C** Cingulate Cortex: mRNA expression of NGFβ in DLB significantly increased compared with that in NC ($*P<0.05$). mRNA expression of IL-1β and IL-6 in PD and DLB, and that of TNF-α in DLB is significantly increased compared with that in NC ($*P<0.05$). There is no statistical difference between PD and DLB in the mRNA expression of their neurotrophic factors and cytokines

located throughout the major target of the basal forebrain cholinergic system, i.e., the hippocampus, amygdala, and neocortex [12, 25, 33, 58, 66]. The level of BDNF protein in PD substantia nigra was reduced [51, 56, 64], and that of BDNF mRNA was decreased in the hippocampus of individuals with Alzheimer's disease [59, 64]. In the present report, we have shown that immunoreactivity and mRNA expression of BDNF were reduced in the hippocampus of PD and DLB patients. These findings may contribute to the reduced ChAT activity in the hippocampus with PD and DLB, as well as to the cognitive decline resulting from these diseases. We have already reported an increased number of activated microglia in the putamen and cingulate cortex [30]. In the putamen, the mRNA expression of BDNF in DLB was significantly decreased, but in the cingulate cortex, there was no difference compared with controls.

As for the neuropathological findings in the hippocampus of Lewy body diseases, the existence of numerous ubiquitin- or α-synuclein-positive Lewy neurites in the CA2/3 region [14, 15, 34], α-, β-synuclein-positive mossy fibers in the dentate gyrus [16], and significant neuronal loss in the presubiculum were reported [21]. In addition, atrophy of the hippocampus correlated with increasing severity in the CA2/3 Lewy neurites grade as well as with the density of Lewy bodies in the frontal lobe [20, 27]. In this report, many MHC class II-positive microglia were seen diffusely, especially in the CA2/3 region and dentate gyrus, in the hippocampus of Lewy body disease, and were associated with α-synuclein-positive Lewy neurites or Lewy body-containing neurons or neurons of reduced BDNF immunoreactivity. Neumann et al. [55] suggested that BDNF secreted by electrically active neurons inhibits the expression of MHC class II molecules on microglia. Thus, we speculate that reduced BDNF secretion of the hippocampal neurons of Lewy body disease may contribute to the up-regulation of MHC class II molecule expression on adjacent microglia.

Many studies indicate the vulnerability of neurons to the potential toxicity of microglia, which produces neurotoxic substances including superoxide anion, nitric oxide (NO), glutamate, and pro-inflammatory cytokines [8, 9, 22, 23, 26, 35, 37, 40, 47]. The presence of microglia expressing TNF-α, IL-1β, and other cytokines in the SN of PD brains has been previously reported, and Nagatsu and co-workers [50, 53] have

reported the presence using ELISA of an increased concentration of TNF-α, IL-1β and IL-6 in the striatum of PD. We have already shown [30] the presence of activated microglia and the absence of reactive astrocytosis in the putamen and hippocampus of PD, and, by immunohistochemical methods, that the activated microglia expressed TNF-α and IL-6; in the present study we have demonstrated increased expression of the mRNA of these cytokines in the putamen and hippocampus of PD and DLB. Although these data may suggest an involvement of the pro-inflammatory cytokines secreted by microglia in the degeneration of dopaminergic neurons in Lewy body disease, it is also recognized that TNF-α and IL-6 have neurotrophic mechanisms [4, 10, 19, 60, 62]. To date, glial cells are acknowledged to possess neurotrophic properties that are essential to the survival of dopaminergic and cholinergic neurons [52]. Among them, BDNF and GDNF, which can be released by activated microglia [5], seem to be the most potent factors supporting the dopaminergic neurons of the SN.

BDNF has protective effects on 6-hydroxydopamine-lesioned rat striatum and enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons [61, 65, 67]. Hippocampal neurons have endogenously abundant BDNF protein, and cultured hippocampal neurons show a response to BDNF [31]. Furthermore, the mRNA of trkB, which is the receptor of BDNF, is preserved in neurons of the putamen and hippocampus of PD [6]. These results suggest that supplements of BDNF to the hippocampus and cholinergic system may be an effective treatment for the cognitive impairment of Lewy body disease.

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Amyloid-β peptides induce cell proliferation and macrophage colony-stimulating factor expression via the PI3-kinase/Akt pathway in cultured Ra2 microglial cells

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Abstract **Alzheimer's disease is characterized by numerous amyloid-β peptide (Aβ) plaques surrounded by microglia. Here we report that Aβ induces the proliferation of the mouse microglial cell line Ra2 by increasing the expression of macrophage colony-stimulating factor (M-CSF). We examined signal cascades for Aβ-induced M-CSF mRNA expression. The induction of M-CSF was blocked by a phosphatidylinositol 3 kinase (PI3-kinase) inhibitor (LY294002), a Src family tyrosine kinase inhibitor (PP1) and an Akt inhibitor. Electrophoretic mobility shift assays showed that Aβ enhanced NF-κB binding activity to the NF-κB site of the mouse M-CSF promoter, which was blocked by LY294002. These results indicate that Aβ induces M-CSF mRNA expression via the PI3-kinase/Akt/NF-κB pathway.**
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Keywords: Microglia; Alzheimer's disease; Amyloid-β; Akt; NF-κB; Macrophage colony-stimulating factor

1. Introduction

Alzheimer's disease (AD) is characterized by the presence of senile plaques in the brain composed primarily of amyloid-β peptide (Aβ). Microglia have been reported to surround the Aβ plaques, which provokes a microglia-mediated inflammatory response that contributes to neuronal cell loss [1]. On the other hand, microglia play an important role in the clearance of Aβ by phagocytosis, primarily through scavenger receptor class A (SR-A, CD204), scavenger receptor-BI (SR-BI) and CD36 [2–4]. Recently, it has been reported that microglia isolated from CD36-deficient mice had marked reductions in Aβ-induced cytokine/chemokine secretion [5]. CD36 binds to Aβ in vitro [6], and is physically associated with members of the Src family tyrosine kinase [7,8], which transduce signals from this receptor [9]. Another receptors such as receptor for advanced glycosylation end-products (RAGE), integrins and heparan sulfate proteoglycans, also have been reported to bind with Aβ [10].

There are many reports that microglia are activated by Aβ, but it has been unclear whether Aβ is associated with the proliferation of microglia. Here we report that Aβ induces proliferation of the microglial cell line Ra2 by increasing macrophage colony-stimulating factor (M-CSF) expression. We also elucidated signal transduction pathways from Aβ-treatment to M-CSF mRNA expression in microglia.

2. Materials and methods

2.1. Materials

Synthetic human Aβ25–35, Aβ1–42 and Aβ1–16 were obtained from Peptide Institute Inc. Aβ35–25 was from AnaSpec Inc. Aβ25–35, Aβ1–16 and Aβ35–25 were dissolved in H_2O and Aβ 1–42 was dissolved in 0.1% NH_3 according to the manufacturer's instructions. Anti-phospho-Akt (Serine 473), anti-Akt, anti-phospho-IκBα (Serine 32), and anti-IκBα antibodies were from Cell Signaling. PP1 was from Biomol. Wortmannin, LY294002 and Akt inhibitor [1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] were from Calbiochem. Piceatannol was from Sigma–Aldrich. Mouse recombinant granulocyte–macrophage colony-stimulating factor (mrGM-CSF) was from Pharmingen. Mouse recombinant M-CSF (mrM-CSF) was from Techne. Aβ25-35 and Aβ1–42 were used at 50 and 10 μM, respectively, in all studies unless otherwise stated.

2.2. Cell culture

Microglial cell line Ra2 was cultured in MGI medium [Eagle's MEM supplemented with 0.2% glucose, 5 μg/ml bovine Insulin (Sigma–Aldrich), and 10% fetal bovine serum (FBS, Invitrogen)] and 0.8 ng/ml mrGM-CSF (Pharmingen) [11]. Before Aβ-treatment, Ra2 cells were cultured in MGI medium without mrGM-CSF for 16 h. Primary microglia and primary astrocytes were prepared using newborn C57BL/6 mice as described previously [12], and cultured in MGI medium containing 0.8 ng/ml mrGM-CSF. The neuroblastoma cell line Neuro2a was cultured in DMEM supplemented with 10% FBS. Primary neurons were obtained from the cortex of 14-day-old C57BL/6 mouse embryos as described previously [13] with some modifications. Neural cells cultured in DMEM supplemented with TIS (5 μg/ml

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Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GM-CSF, granulocyte–macrophage colony stimulating factor; M-CSF, macrophage colony-stimulating factor; PBS, phosphate-buffered saline; PI3-kinase, phosphatidylinositol 3 kinase; RAGE, receptor for advanced glycation end-products

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transferrin, 5 μg/ml insulin, and 5 ng/ml selenite, Sigma), 10% FBS and 5 μM cytosine arabinoside (Ara-C, Sigma). Before Aβ-treatment, primary neurons were cultured in MGI medium for 16 h.

2.3. Cell proliferation (WST-1) assay

Cell proliferation was determined by analyzing the conversion of WST-1 (light red) to its formazan derivate (dark red) using a WST-1 Cell Counting Kit (Dotite). For neutralization of M-CSF, anti-mouse M-CSF antibody (Techne) was added to the culture medium. At the end of the experiments, the media were replaced, and cells were incubated with 10 μl of the WST-1 reagent for 1 h at 37 °C in 5% CO_2. The absorbance at 450 nm was measured by using a microplate reader (Bio-Rad).

2.4. Immunoblotting

Cells were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 5% bromophenol blue). Then 50 μg of total protein was resolved by SDS–PAGE and transferred to PVDF membranes (Millipore). Immunoblotting was performed with the appropriate antibody using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia).

2.5. RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen). For RT-PCR and real-time quantitative PCR, the primers for mouse M-CSF and β-actin genes were as follows (5′ to 3′): M-CSF sense, CCATCGAGACCCTCAGACAT; M-CSF antisense1 for RT-PCR, CCTAAGGGAAAGGGTCCTGA; M-CSF antisense2 for real-time PCR, GATGAGGACAGACAGGTGGA; β-actin sense, AGTGTGACGTTGACATCCGT; and β-actin antisense, GCAGCTCAGTAACAGTCCGC. Conventional RT-PCR was performed using 0.5 μl cDNA, and 30 cycles of amplification for M-CSF or 23 cycles for β-actin at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Quantitative real-time PCR was performed on the Smart Cycler system (Takara) using the following program: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 8 s at 72 °C. The reactions were carried out using 0.5 μl cDNA with Smart Kit for Sybr Green I (Eurogentec). To check the specificity of reactions, a single band of the correct size was visualized by running out on 2% agarose gels. Values were expressed as relative expression of M-CSF mRNA normalized to the β-actin mRNA.

2.6. Nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts of Ra2 cells were prepared as previously described [14]. Three micrograms of nuclear extract was incubated with 5 fmol of ^{32}P end-labeled double-stranded oligonucleotides derived from M-CSF promoter in binding buffer [10 mM Tris, pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.05 μg/μl poly(dI–dC)] for 20 min at room temperature. For competition assays, 1 pmol of unlabeled probe was incubated in the reaction mix before the addition of the ^{32}P-labeled probe. The oligonucleotides used in these experiments were as follows: NF-κB probe, 5′-GCCTTGAGGGAAAGTCCCTAGGGGC-3′; AP1 probe, 5′-GTAGTATGTGTCAGTGCC-3′. For supershift assays, nuclear extracts were preincubated with anti-NF-κB p50 or p65 antibodies (Santa Cruz) for 1 h at 4 °C. The DNA–protein complex was separated on 5% native polyacrylamide gels. The dried gels were visualized using an Image Reader (Fujifilm).

2.7. Statistical analysis

Results are expressed as means ± S.D. Statistical analysis was done by a two-tailed Student's *t* test. A *P* value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Aβ promotes microglial cell proliferation

To investigate the possible role of Aβ in the activation of microglia, we examined if Aβ could sustain the cell proliferation of microglial cell line Ra2. Ra2 cells proliferate in MGI medium containing GM-CSF and stop proliferating without GM-CSF [11]. Under MGI medium without GM-CSF, the effects of M-CSF or Aβ on the proliferation of Ra2 cells were analyzed by the WST-1 assay. The addition of M-CSF induced cell proliferation dose-dependently (Fig. 1A). Aβ25–35 increased Ra2 cell proliferation dose-dependently (Fig. 1B). Aβ25–35 does not occur naturally but has shown to mimic the effects of Aβ1–42 [15–17]. Aβ1–42, which occurs in a brain affected by AD, was more effective in cell proliferation than Aβ25–35 (Fig. 1C). It has been reported that Aβ stimulates the proliferation of microglia to enclose Aβ plaque [18,19]. We examined if M-CSF provoked the cell proliferation with Aβ-treatment. The effect of Aβ on the proliferation was blocked by anti-M-CSF antibody ($P < 0.05$) (Fig. 1D). The treatment with M-CSF was performed as a control. The effect of M-CSF was blocked by anti-M-CSF antibody (Fig. 1D). We found that Aβ induces microglial cell proliferation by M-CSF production.

3.2. Aβ induces M-CSF mRNA expression in microglia

To examine whether Aβ could induce M-CSF mRNA expression in microglia, Ra2 cells were stimulated with Aβ25–35 for 16 h at various concentrations. Aβ25–35 induced M-CSF mRNA expression dose-dependently (Fig. 2A). As a result of real-time quantitative RT-PCR (Fig. 2A, right), M-CSF mRNA induction by 50 μM Aβ25–35 was about sevenfold of non-treated control. Aβ25–35 induced time-dependent increases in M-CSF mRNA expression (Fig. 2B). Aβ1–42 also induced M-CSF expression (Fig. 2A and B). GM-CSF mRNA, on the other hand, was not induced by Aβ25–35 or Aβ1–42 (data not shown). Aβ1–16 did not induce M-CSF mRNA expression (Fig. 2C), nor

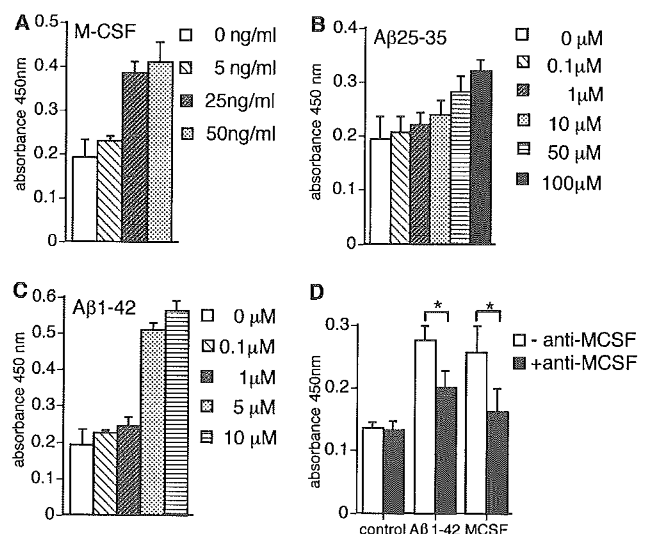

Fig. 1. Aβ promotes Ra2 cell proliferation. Cellular proliferation was measured by WST-1 assay. (A, B and C) Ra2 cells were incubated with the medium containing M-CSF, Aβ25–35 or Aβ1–42 at indicated concentrations for 48 h. (D) Ra2 cells were preincubated with 1 μg/ml anti-M-CSF antibody for 1 h before treatment with 5 μM Aβ1–42 or 25 ng/ml M-CSF for 24 h. Mean ± S.D. values from a single experiment were performed in triplicate. Similar results were obtained in each of two separate experiments (*$P < 0.05$).