2.　実用新案登録

なし

3.　その他

なし

研究成果の刊行に関する一覧表

1. Ishida, Y., Okawa, Y., S. Ito, S., Shirokawa, T. and Isobe, K. Age-dependent changes in dopaminergic projections from the substantia nigra pars compacta to the neostriatum. Neuroscience Letters, in press.

2. Nakai, S., Matsunaga, W., Ishida, Y., Isobe, K. and Shirokawa, T. Effects of BDNF infusion on the axon terminals of locus coeruleus neurons of aging rats. Neuroscience Research, 54: 213-219, 2006.

3. Nakai, S., Matsunaga, W., Isobe, K. and Shirokawa, T. Age-dependent interactive changes in serotonergic and noradrenergic cortical axon terminals in F344 rats. Neuroscience Research, 54: 220-229, 2006.

4. Matsunaga, W., Isobe, K. and Shirokawa, T. Involvement of neurotrophic factors in aging of noradrenergic innervations in hippocampus and frontal cortex. Neuroscience Research, 54: 313-318, 2006.

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Age-dependent changes in dopaminergic projections from the substantia nigra pars compacta to the neostriatum

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Abstract

Age-dependent changes in dopaminergic (DA) innervation of the neostriatum (Str) were studied in male F344/N rats. Projections from the substantia nigra pars compacta (SNc) to the neostriatum were quantified using electrophysiological methods at age points from 6 to 24 months. The percentage of DA neurons activated antidromically by electrical stimulation (P-index) of Str increased between 18 and 24 months. Additionally, the percentage of DA neurons showing multiple antidromic latencies from striatal stimulation (M-index), which suggests axonal branching of individual DA neurons, increased significantly between 6 and 12 months and 6 and 24 months. These results suggest that DA neurons exhibit increased axonal branching in the aged brain.
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Keywords: Aging; Substantia nigra pars compacta; Electrophysiology; F344 rat

The substantia nigra pars compacta (SNc) is one of the doperminergic (DA) nuclei in the central nervous system. Nigral dopaminergic neurons innervate many target regions in the brain and supply the main dopaminergic input to the neostriatum (Str) [12]. The axons of DA neurons terminate mainly in the dorsolateral region of the Str [7]. DA neurons play an important role in complex motor control [18] and exhibit decreases in their function during both the normal aging process and in neurodegenerative states such as Parkinson's disease. However, it remains unclear at present how dopaminergic innervation of the neostriatum changes throughout aging. Specifically, it is unclear as to whether the deficits in dopaminergic signaling are due to pre- and/or post-synaptic changes. To investigate the age-dependent changes in the projections from SNc to Str in the rat, we used *in vivo* electrophysiological techniques to antidromically activate their axon terminal fields in the Str to determine whether or not there are changes in the excitability of nigral dopaminergic terminals during aging.

Male F344/N rats (four groups; 6, 12, 18 and 24 months of age, $n=5$ for each age group) were used. Animals were housed with food and water available ad libitum on a 12 h light/dark cycle. All animal procedures complied with the Animal Research Facilities Committee of the National Institute for Longevity Sciences. Animals were anesthetized with urethane (1.2 g/kg, i.p.). Lidocaine was applied locally to all incisions. Rectal temperature was maintained at 36.5 °C by a heating pad. The ECG and EEG were monitored continuously during these experiments. Stimulating electrodes were of the bipolar type, and consisted of two insulated stainless steel wires (diameter 200 μm, tip separation 0.5 mm). Electrical pulses for the stimulus site were 0.5 ms in duration with currents ranging from 0.1 to 5.0 mA, and the cycle of stimulation was 1.5 s. The electrodes were stereotaxically guided into the Str (A: 1.0 from bregma, L: 3.7, D: 4.0). The electrical activity of nigral dopaminergic neurons was recorded extracellularly with glass pipette microelectrodes filled with 2 M NaCl. Electrode resistance ranged from 10 to 20 MΩ. A recording electrode was inserted from a point (A: 2.1 from lambda, L: 2.0). SNc neurons were usually encountered 6.8–8.0 mm below the cortical surface. In each animal, 21–23 SNc neurons were recorded by

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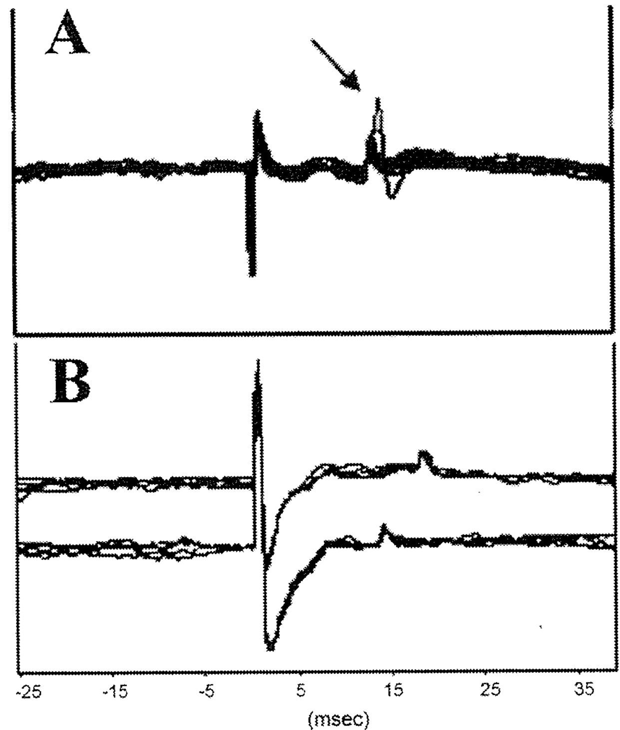
moving a recording electrode within about 200 μm rostrocaudally or mediolaterally to avoid sampling bias. The DA neurons in the SNc were identified according to well-established criteria [4,7,14]. Briefly, nigral dopaminergic neurons exhibited a characteristic wide spike duration (~2 ms), and slow spontaneous firing between 0.5 and 6 Hz. SNc neurons exhibited responses from striatal stimulation considered to be antidromic in nature provided that the following criteria were satisfied: (1) constant latency of the initial segment spike at a low stimulation frequency (1 Hz) (Fig. 1A), (2) ability to follow stimulation at high frequencies (>200 Hz) and (3) collision with spontaneous or orthodromic spikes. The stimulating current was adjusted to a value which was just sufficient to elicit an antidromic response to every stimulus. The threshold current was measured by varying the stimulating current in 0.01 mA steps. The antidromic latency was also measured for individual SNc neurons. A certain proportion of SNc neurons showed two or more discrete antidromic latencies (multiple antidromic latencies) from neostriatal stimulation. In these SNc neurons, if the stimulus current was increased to threshold or beyond, the long latency response with the low threshold often abruptly jumped to short latency responses that occured at higher intensities (Fig. 1B). We measured the threshold current for the long latency response as well as the short ones for all SNc neurons that responded antidromically. Fig. 1C demonstrates histologically (HE staining) that the recording sites and the tracts of the recording electrodes were included in the SNc.

We employed two electrophysiological measurements [1,10,13,17] to quantify the density of SNc axons: (1) the percentage of SNc neurons activated antidromically from Str (P-index: number of SNc neurons with antidromic latencies/number of recorded SNc neurons) and (2) the ratio of SNc neurons that showed two or more discrete antidromic latencies from Str at different intensities of stimulus currents (M-index: number of SNc neurons with multiple antidromic latencies/number of SNc neurons with antidromic latencies). In many systems, these multiple antidromic latencies are regarded as the activation of two or more different axonal branches. Since high frequency stimulation often leads to the blockage of impulse conduction at branch points in invertebrates, we used a frequency of stimulation low enough to avoid impulse conduction failure. In the present study, the stimulus currents (0.1–5.0 mA) always produced 100% SNc antidromic responses, with no instances of impulse conduction failure. The data are expressed as mean ± S.E., and were compared by one-way analysis of variance (one-way ANOVA) with a Bonferroni/Dunn post-hoc analysis.

The total number of SNc neurons recorded from five animals for each age group are as follows: 6 months of age (6 months), $n = 108$; 12 months, $n = 107$; 18 months, $n = 108$; 24 months, $n = 107$. To quantify a change in the density of SNc projections in Str, we first focused on the percentage of SNc neurons activated antidromically from electrical stimulation of the Str (P-index). Fig. 2A shows that the mean P-index was maintained between 6 (57.4 ± 6.0%) and 12 months of age (58.0 ± 3.1%), and then the P-index decreased slightly between 12 and 18 months of age (50.0 ± 6.2%). Unexpectedly, we observed a marked increase in the P-index at 24 months of age (71.9 ± 3.9%). The mean P-index obtained at the age of 24 months was significantly higher than that at 18 months. The utility of the M-index was based on the view that these multiple antidromic latencies resulted from the activation of two or more different axonal branches around a single stimulus locus. Therefore, the M-index provided a phys-



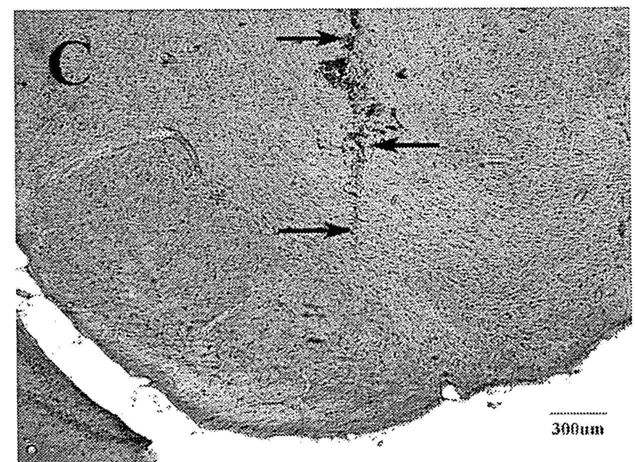


Fig. 1. Antidromic responses of DA neurons to electrical stimulation of Str. (A) At a current of 0.88 mA, an antidromic response with a single antidromic latency was evoked at a latency of 11.4 ms in a 12-month-old rat. Ten consecutive sweeps were superimposed in the noncollision trials. The majority of the antidromic action potentials consist of the initial segment spike only, but one consists of a full initial segment-somatodendritic spike (arrow). (B) The multiple antidromic response to Str stimulation of a single DA neuron in a 12-month-old rat. At a current of 1.25 mA, antidromic response was evoked at a latency of 17.4 ms (upper trace), where at a current of 3.58 mA the latency abruptly jumped to 13.6 ms (lower trace). Five consecutive sweeps were superimposed in the noncollision trials. The stimulus was applied at time 0. (C) The tracts of the recording electrodes (arrows) were included in the substantia nigra pars compacta. Scale bar, 300 μm.

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iological index of axonal branching of individual SNc neurons. Fig. 2B shows the time-course of changes in the M-index in the Str. In Str, we observed a marked increase in the average M-index between 6 (31.4 ± 0.6%) and 12 months of age (69.7 ± 7.8%). The mean M-indices obtained at the age of 12 months were significantly higher than that at 6 months. The M-index slightly leveled off at 18 months of age (48.9 ± 3.4%) and then slightly increased at the age of 24 months (58.4 ± 5.9%). The increase in the average M-index at 12 or 24 months was significantly higher than that at 6 months. These results suggested that nigral dopaminergic neurons exhibit increased axonal branching in the Str during aging.

We classified axon terminals of nigral dopaminergic neurons into three types based on their antidromic responses: (1) single-threshold terminals with single antidromic latency (single-threshold), (2) low threshold terminals with long latency, and (3) the high threshold terminals with short latency. To investigate the age-dependent changes in the physiological properties of axon terminals of individual SNc neurons, we compared the threshold–latency relationship of each terminal type in SNc. Based on our analysis of the threshold–latency relationship of DA neurons (Table 1), we concluded that the dominant terminals varied with advancing age; the single-threshold terminals were dominant until 6 months, then the dominance in threshold shifted from single to low/high after 12 months. The age-dependent changes observed in the excitability of nigral dopaminergic neuron terminals during aging suggests that there may be changes in the excitability of individual terminals during aging.

In the present study, we found age-dependent changes in the excitability of dopaminergic terminals strongly suggestive of changes in the DA innervation of Str over time. Specifically, these results suggest an increase in axonal branching between 6 and 12 months, and an increase in innervation density between 18 and 24 months. The variance seen in Fig. 2A (and also in 2B) differs among individual age groups, but we think these variances are not the result of sampling bias but rather the age-related effect on the animals. In the young animals, there was some variance which decreased at 18 months and finally increased at 24 months. We observed the same trends in our previous work [10] and the work by Suzuki's group [22]. Thus, we speculate the variability among individual age groups may be related to the age-dependent effect on the animals.

Though the P- and M-indices of DA neurons changed with age, other parameters of antidromic responses such as threshold and latency were retained during aging (Table 1). It has also been suggested that there are no significant differences in the electrophysiological parameters such as firing rate, firing rate distribution and firing pattern between 3 months and 24–28 months F344 rats [5]. In addition, the number of TH-positive neurons does not change between 3 and 6 months and 19 and 21 months in F344 rats using unbiased stereology [2]. Thus, we hypothesize that the properties of DA neurons in the SNc may be retained during aging and the age-related changes observed may be due to changes in axonal properties in target regions such as the neostriatum.

The tissue levels of DA and its metabolites in the Str over time still remain to be elucidated. Some reports showed DA and its metabolites levels were decreased in the Str of aged F344 rats [6,8,15]. Others showed no significant differences in DA and its metabolites levels in the Str between young and aged F344 rats [9,16,21]. However, a decrease in DA receptors in the Str of aged F344 rats has been consistently observed [19,22]. Thus, the sprouting of dopaminergic axon terminals in the Str might be in response to the loss of DA receptors with increasing age.

Recently, we reported age-dependent changes in the noradrenergic (NA) innervation of frontal cortex and hippocampus of F344 rats that suggested a decrease in density between 7 and 15 months, and increased axonal branching between 15 and 24 months [10,11]. Quantitative autoradiography reflecting the binding of ligands specific for noradrenergic receptor subtypes in frontal cortex and hippocampus showed no significant differences for any receptor subtype between young and aged-normal rats [3]. Only the binding of ligands for beta-1 receptors in cerebral cortex and hippocampus was likely to decrease between 5 and 24 months. These are partially consistent with our previous data. However, by using inhibitors of NA uptake and NA release in pre-synaptic terminals of LC axons, we found that the release activity mediated by the pre-synaptic autoreceptor

Table 1
The age-dependent changes in mean antidromic latency, and the number of latency jumps of DA neurons

	6 months	12 months	18 months	24 months
Mean latency (ms)				
Single	11.7 ± 1.0	13.6 ± 0.6	13.6 ± 1.0	12.5 ± 0.6
Multiple-short	12.2 ± 0.7	11.9 ± 0.5	12.5 ± 0.4	12.1 ± 0.5
Multiple-long	14.2 ± 0.8	14.4 ± 0.6	15.0 ± 0.6	15.1 ± 0.6
Mean threshold (mA)				
Single	2.24 ± 0.29	2.40 ± 0.32	2.58 ± 0.26	2.77 ± 0.43
Multiple-high	2.16 ± 0.40	2.47 ± 0.22	3.10 ± 0.30	2.75 ± 0.22
Multiple-low	1.13 ± 0.24	1.24 ± 0.17	1.62 ± 0.22	1.63 ± 0.22
Number of latency jumps	20	41	27	44

No significant difference are obtained as follows: Bonferroni/Dunn-test, single latency $F(3.16) = 1.28$, $P = 0.31$; multiple-short latency, $F(3.16) = 0.21$, $P = 0.89$; multiple-long latency, $F(3.16) = 0.47$, $P = 0.71$ single-threshold, $F(3.16) = 0.47$, $P = 0.71$; multiple-high threshold, $F(3.16) = 1.86$, $P = 0.18$; multiple-low threshold, $F(3.16) = 1.39$, $P = 0.28$.

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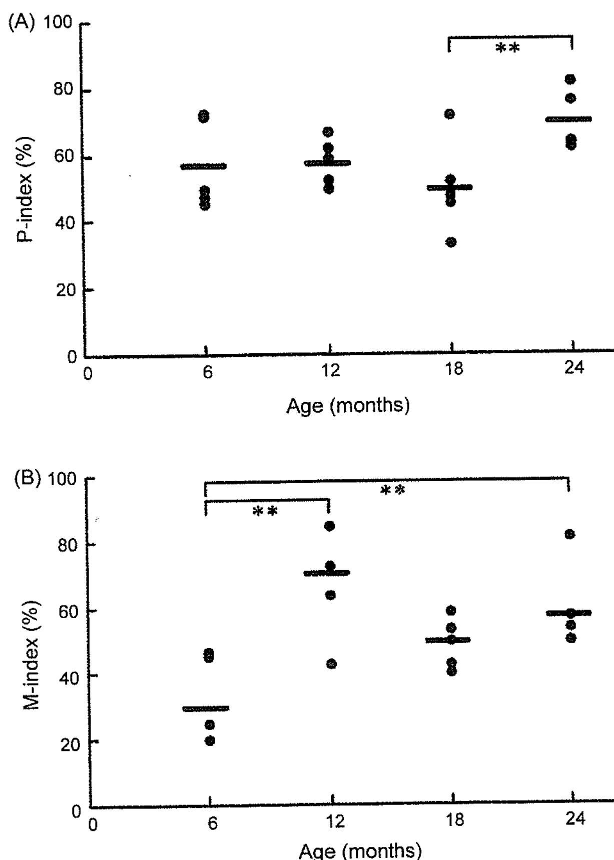

Fig. 2. (A) Age-dependent changes in P-indices (number of DA neurons with antidromic latencies/number of recorded DA neurons) in Str. Each age group consisted of five animals (filled circles). Horizontal bar indicates the mean P-index for each age group. The mean P-indices (mean ± S.D.) in Str did not change between 6 and 12 months. The mean P-indices declined gradually between 12 and 18 months. The P-indices obtained at 24 months were significantly higher than those at 18 months (Bonferroni/Dunn-test: 18 months vs. 24 months, $^{**}P<0.01$). (B) Age-dependent changes in M-indices (number of LC neurons with multiple antidromic latencies/number of DA neurons with antidromic latencies) in Str. Each age group consisted of five animals (filled circles). Horizontal bar indicates the mean M-index for each age group. The mean M-indices in Str increased significantly between 6 and 12 months, and then slightly declined at 18 months (Bonferroni/Dunn-test, 6 months vs. 12 months, $^{**}P<0.01$). The M-indices increased gradually between 18 and 24 months. Finally, the M-index increased significantly between 6 and 24 months (Bonferroni/Dunn-test, 6 months vs. 24 months, $^{**}P<0.01$).

did not change with age, but the uptake activity mediated by the NA transporter declined with age in the axon terminals of LC neurons [20]. In addition, the activity of DA transporters in the Str decreased significantly between young (6 and 12 months) and aged (18 and 24 months) F344 rats [8]. These are also approximately consistent with the timing of the axonal branching of DA neurons. Thus, we speculate that the age-related changes in the innervation of DA or NA neurons correspond most with changes in their respective transporters. Further studies are needed to examine the changes in the expression of the DA transporter in the terminal field of DA neurons during aging.

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Effects of BDNF infusion on the axon terminals of locus coeruleus neurons of aging rats

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Abstract

Using in vivo electrophysiological techniques and continuous local infusion methods, we examined the effects of brain-derived neurotrophic factor (BDNF) and its specific antibody (anti-BDNF) on the noradrenergic axon terminals of the locus coeruleus (LC) neurons in the frontal cortex of aging rats. Recently, we observed that LC neurons with multiple-threshold antidromic responses (multi-threshold LC neurons) increased critically between 15 and 17 months of age. To examine whether the BDNF is involved in this change occurred in the aging brain, we continuously infused BDNF into the frontal cortex for 14 days. Exogenous BDNF produced a marked increase in the multi-threshold LC neurons in the 13-month-old brain, accompanied with a decrease in threshold current. However, no morphological change in the noradrenergic axons was observed in the BDNF-infused cortex. In contrast, infusion of anti-BDNF led to a dose-dependent reduction of the multi-threshold LC neurons in the 19-month-old brain, accompanied with an increase in threshold current. These findings suggest that BDNF may contribute to functional changes in the presynaptic axon terminals of LC neurons in the aging brain.
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Keywords: Aging; Noradrenergic axon; Locus coeruleus; BDNF; Electrophysiology; Frontal cortex; F344 rat

1. Introduction

The age-dependent changes in the neuronal system are important indicators for understanding the functions of aging brain. The locus coeruleus (LC), a major noradrenergic cell group in the brain, is recognized as a dynamic system that has a remarkable capacity for remodeling of innervations. Recently, we found that LC neurons with multiple-threshold antidromic responses (multi-threshold LC neurons) increased critically between 15 and 17 months of age (Ishida et al., 2000; Shirokawa et al., 2000). Although this finding suggests that the LC neurons preserve a capacity for changing their axon terminals even in the aging brain, mechanisms of this plastic change in the aging brain are unknown. Because brain-derived neurotrophic factor (BDNF) influences the axon terminals in the adult brain (Inoue and Sanes, 1997), neurotrophic support of BDNF may be involved in the change of LC axon terminals in the aging brain. Also, BDNF mRNA and its receptor trkB mRNA are expressed in the adult LC (Numan et al., 1998). In addition, it is reported that BDNF is anterogradely (Conner et al., 1997) and retrogradely (Sobreviela et al., 1996) transported by LC neurons.

In the present study, to test whether exogenous BDNF would induce changes in the presynaptic axon terminals of LC neurons in the middle-aged rat, we continuously infused BDNF into the frontal cortex at the age of 13 months, because the multi-threshold LC neurons was not yet increased at the age (Ishida et al., 2000; Shirokawa et al., 2000). In addition, to test whether neutralization of endogenous BDNF would retard the BDNF-induced changes in the presynaptic activity of LC axon terminals, we infused a neutralizing antibody to BDNF (anti-BDNF) into the frontal cortex at the age of 19 months, because the multi-threshold LC neurons was already increased and maintained at the age (Ishida et al., 2000; Shirokawa et al., 2000).

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2. Materials and Methods

2.1. Animals

Male F344/N rats (13 months of age, $n = 22$; 19 months of age, $n = 30$) were used. Animals were obtained from the aging colony at the National Center for Geriatrics and Gerontology (NCGG). They were housed with food and water available ad libitum on a 12-h light/dark cycle. All animal procedures complied with the National Institutes of Health guidelines and were approved by the Laboratory Animal Research Facilities Committee of the NCGG.

2.2. BDNF and anti-BDNF antibody infusion

Under sterile conditions 3–6 h before surgery, an Alzet (Alza, Palo Alto, CA) pump/cannula ensemble was filled with one of the following solutions: (1) BDNF (500 μg/ml in PBS-containing 0.1% BSA), (2) anti-BDNF antibody (50 or 500 μg/ml in PBS-containing 0.1% BSA) or (3) control IgG (500 μg/ml in PBS-containing 0.1% BSA) stored in sterile PBS at 37 °C until surgical implantation. Under anesthesia with ketamine (80 mg/kg, i.m.) and isoflurane (0.5–1.5%) in oxygen, a 28 G stainless steel cannula (Brain Infusion Kit) connected to an osmotic minipump (Alzet 2002) was implanted in the right frontal cortex (anterior, 3.0 mm; lateral, 1.5 mm; depth from cortical surface, 1.5 mm) of the rats (Paxinos and Watson, 1986). The frontal cortex was infused continuously at a rate of 0.5 μl/h for 14 days with (1) BDNF (6 μg/day), (2) a specific antibody to BDNF (AB1779SP, rabbit IgG fraction, Chemicon, CA) at low (0.6 μg/day) or high (6 μg/day) concentration, or with (3) control IgG (6 μg/day, non-immune rabbit IgG, Santa Cruz, CA) in vehicle solution (PBS-containing 0.1% BSA). To prevent infection, animals were administrated an antimicrobial agent (enrofloxacin, 5 mg/kg) every day after the surgery until recording.

2.3. Immunohistochemistry

To demonstrate the extent of BDNF diffusion, we infused the biotin-labeled BDNF into the frontal cortex of rat. Labeling of BDNF was done using Biotin Protein Labeling Kit (Molecular Probe). At the day of termination of infusion for 14 days, rats (13 months of age, $n = 3$) were killed with an overdose of Somnopentyl (100 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected out and then post-fixed with 4% paraformaldehyde overnight at 4 °C. After post-fixation, brains were immersed in 30% sucrose in 0.05 M phosphate-buffered saline (PBS) overnight at 4 °C. Frozen sections were cut with a CM1800 cryotome (Leica, Heerburg) at a thickness of 25 μm. Sections were pretreated with 1% H_2O_2 in PBS for 20 min at room temperature, and 0.1% $NaBH_4$ in PBS for 5 min and then incubated with ABC reagent (dilution 1:200 Vector Labs) for 2 h at room temperature. Visualization was performed with 0.02% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.4).

For immunohistochemistry of noradrenergic axon terminals, sections were incubated with MAB308 monoclonal anti-dopamine beta-hydroxylase (DBH) antibody (Chemicon) diluted 1:4000 in PBS-containing 0.3% Triton X-100 (PBST) and 1% normal horse serum for 2 days at 4 °C. After incubation with the primary antibody, sections were incubated with biotinylated anti-mouse IgG (dilution 1:400 in PBST, Vector Labs, Burlingame, CA) for 2 h at room temperature, and were incubated with ABC reagent (dilution 1:200 in PBST, Vector Labs) for 2 h at room temperature. Visualization was performed with 0.02% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.4).

2.4. Quantitative image analysis

Quantitative image analysis of noradrenergic axons in the frontal cortex was conducted by WinRoof ver. 3.13 software (Mitsutani Shoji, Tokyo, Japan), and DBH-immunoreactivity was used as the morphological marker of noradrenergic axons. The sections containing the frontal cortex were filmed by DP-11 CCD camera (Olympus, Tokyo, Japan), and stored as JPEG files. Biotin-conjugated BDNF infusion (Fig. 1B) showed that infused BDNF diffused to 1–1.5 mm away from cannula lesion, therefore, the area of 1–1.5 mm from the implant lesion was regarded as the BDNF or vehicle infusion site. For the quantitative image analysis for each animal, we randomly selected 3 or 4 sections from 20 to 25 sections, which contained the cannula lesion. In each section, total length of DBH-immunopositive axons was measured on one microscope field (340 × 425 μm square) in the BDNF or vehicle-infused site. The animal means were derived from section means, and the mean total length of DBH-positive axons per area was regarded as the density.

2.5. LC unit recordings

Animals were anesthetized with urethane (1.2 g/kg, i.p.). The anesthetic was supplemented as necessary during the experiments. Lidocaine (4% xylocaine) was applied locally to all incisions. Rectal temperature was maintained at 36.5 °C. After removing the cannula from the infusion site, a stimulating electrode of two insulated stainless steel wires (200 μm in diameter) was implanted within 1.0 mm from the infusion site. The infused site was subjected to electrical stimulation consisting of single square pulses of 0.5 ms duration with currents ranging from 0.1 to 6.0 mA. The electrical activity of LC neurons was recorded extracellularly by means of a glass pipette filled with 2 M NaCl, with impedance ranging from 10 to 18 MΩ. The location of the LC was determined by the appearance of a short train of multiple units with small amplitudes following electrical stimulation of the frontal cortex. The single-unit activity of LC neurons was superimposed upon the multi-unit response. The LC neurons were identified according to the criteria used by several authors (Nakamura, 1977; Aston-Jones et al., 1980; Nakamura et al., 1989; Ishida et al., 2000; Shirokawa et al., 2000). Briefly, the LC neurons revealed wide spike duration (~2 ms), slow and tonic spontaneous firing (0.5–6 Hz), and excitation by tail pinches followed by a long-lasting suppression of firing. In each animal, recordings were made from 46 to 58 neurons from the right LC that satisfied the above criteria. Responses of LC neurons were considered to be antidromic provided that the following criteria were satisfied: (1) fixed latency, (2) ability to follow high-frequency stimulation, and (3) collision with spontaneous, orthodromic action potentials (Nakamura, 1977; Nakamura et al., 1989; Ishida et al., 2000; Shirokawa et al., 2000). The antidromic latencies were determined for all LC neurons that responded antidromically.

2.6. Multi-threshold LC neurons

The stimulating current, i.e., threshold current, was adjusted to a value that was just sufficient to elicit an antidromic response to every stimulus. The threshold currents were measured by varying the stimulating current (0.1–6.0 mA) in 0.01 mA steps. Based on these threshold currents, we defined the "multi-threshold LC neuron" as follows. In young rats, the great majority of LC neurons showed single-threshold antidromic responses. In contrast, LC neurons with two or more discrete thresholds (i.e., multi-threshold) increased critically between 15 and 17 months of age (Ishida et al., 2000; Shirokawa et al., 2000). An example of multi-threshold responses in a LC neuron is shown in Fig. 1A. In these LC neurons, when the stimulus current was increased to threshold or beyond, the long latency response with the low threshold often abruptly jumped to the short latency responses that occurred at the high intensities. We calculated the percentage of multi-threshold LC neurons, as an electrophysiological measurement to quantify the age-dependent changes of LC axon terminals (Nakamura et al., 1989; Ishida et al., 2000; Shirokawa et al., 2000).

2.7. Data analysis

Data in the text and figures are expressed as means ± S.E.M. Two-group comparisons were analyzed by the two-tailed *t*-test for independent samples. $P < 0.05$ was considered statistically significant.

3. Results

The extent of diffusion of BDNF was examined in the frontal cortex that was infused with the biotin-labeled BDNF (Fig. 1B). The biotin-labeled area was very limited; the mean extent of diffusion was 1.15 ± 0.13 mm ($n = 3$) from the infusion center. This diffusion range is consistent with the spread of stimulating

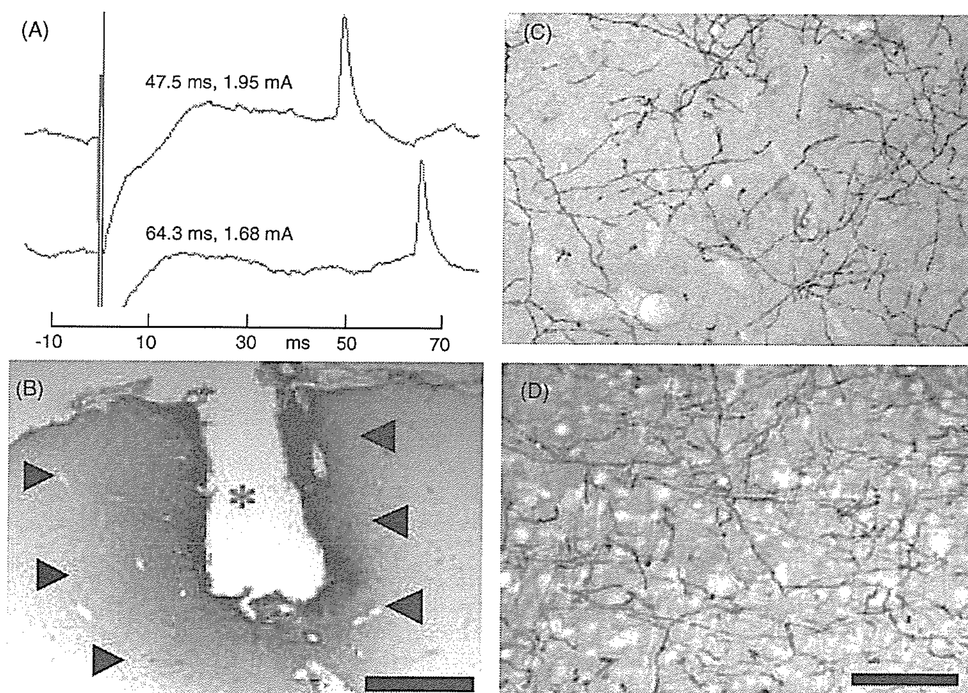

Fig. 1. (A) An extracellular recording of multi-threshold antidromic responses in a single LC neuron in a 13-month-old rat. When the stimulus was adjusted to the minimum current necessary to evoke antidromic responses on every trial (1.68 mA), an antidromic response was evoked at a fixed, discrete latency (64.3 ms, bottom). When the stimulus was increased to 1.95 mA, the antidromic response occurred at a shorter latency (47.5 ms, top). Further increases in stimulus currents (up to 6.0 mA) did not cause any latency change. (B) Diffusion of biotin-labeled BDNF infused for 14 days into the frontal cortex of a 13-month-old rat. The extent of diffusion of biotin-labeled BDNF (arrowheads) is approximately 1 mm from the infusion center (asterisk). Scale bar = 500 μm. (C and D) DBH-immunopositive axons in the frontal cortex of 13-month-old rats which were infused for 14 days with BDNF (C) and vehicle solution (D). No visible difference was observed in the density of DBH-immunopositive axons between the BDNF- and vehicle-infused groups. Scale bar = 50 μm.

current generated by the same type of bipolar electrode (Sakaguchi and Nakamura, 1987). Thus, it is likely that the LC axon terminals in the cortical area infused with BDNF are subjected to electrical stimulation.

In our recent study, local infusion of drugs that act on autoreceptor and transporter located on the presynaptic axons were effective on the threshold currents for antidromic activation of LC axons (Shirokawa et al., 2003). Since the infusion cannula was implanted within a lateral displacement of approximately 100 μm away from the stimulating electrode, this gave us evidence that responsive axon terminals would be present in the vicinity of the stimulating electrode. This is supported by the observations using an electron microscope that noradrenergic axons in the frontal cortex consist of a large number of "varicosities", which are characterized by the synaptic specialization with noradrenaline-containing vesicles (Seguela et al., 1990). Since the density of noradrenergic varicosities is 64.5–79.5 counts per field (100 × 100 μm) between 13 and 25 months of age (Ishida et al., 2001a,b), it is likely that a large number of varicosities in the vicinity of the stimulating electrode are subjected to electrical stimulation.

3.1. BDNF increases multi-threshold LC neurons

We first examined the effects of BDNF (6 μg/day) on the LC axon terminals in the 13-month-old rats. Infusion of BDNF produced a significant increase in the percentage of multi-threshold LC neurons in the 13-month-old rats (Fig. 2A). The percentage of multi-threshold LC neurons obtained in the BDNF-infused group (50.0 ± 3.24%, $n = 6$) was significantly greater than that obtained in the vehicle control group (28.0 ± 4.60%, $n = 6$) ($t = 4.028$, $p < 0.01$).

In contrast with the electrophysiological data, exogenous BDNF has little effect on the morphology of noradrenergic axons (Fig. 1C, D). The results of quantitative image analysis of the DBH-positive axons in the frontal cortex are shown in Fig. 2B. There was no significant differences in the total length of DBH-positive axons between the BDNF-infused group (13,583 ± 858.1 μm, $n = 6$) and vehicle-infused group (12,837 ± 1002 μm, $n = 4$) ($t = 0.522$, $p > 0.1$). However, this lack of effects of BDNF on the cortical noradrenergic axons in the 13-month-old rats was consistent with our recent result (Matsunaga et al., 2004).

3.2. Anti-BDNF decreases multi-threshold LC neurons

Next we examined the effects of anti-BDNF on the LC axon terminals in the 19-month-old rats. As expected, the retarding effect of anti-BDNF on multi-threshold LC neurons was notable (Fig. 3A). At the high dose (6 μg/day), the percentage of multi-threshold LC neurons was significantly reduced (filled column, 30.5 ± 3.71%, $n = 6$), as compared with that in the IgG control group (open column, 59.6 ± 3.40%, $n = 6$) ($t = 5.787$, $p < 0.01$). At the low dose (0.6 μg/day), the percentage of multi-threshold LC neurons was also significantly reduced (hatched bar, 42.4 ± 3.89%, $n = 6$), as compared with the IgG control group

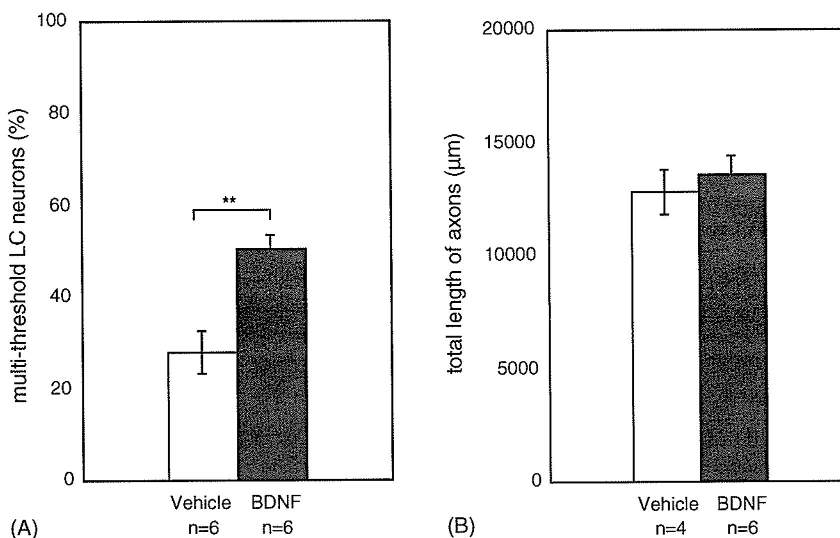

Fig. 2. (A) Effect of BDNF infusion on the percentage of multi-threshold LC neurons in the 13-month-old rats. The percentage of multi-threshold LC neurons obtained in the BDNF-infused group (filled column) was significantly greater than that in the vehicle control group (open column) ($^{**}p < 0.01$). Data are the mean ± S.E.M. of six 13-month-old rats for each group. (B) Effect of BDNF infusion on the total length of DBH-positive axons in the 13-month-old rats. There were no significant differences in the total length of DBH-positive axons between the BDNF-infused group (filled column) and vehicle-infused group (open column) ($p > 0.1$). Data are the mean ± S.E.M. of six BDNF-infused rats and four vehicle-infused rats.

($t = 3.321$, $p < 0.01$). Significant difference was found between the low dose and high dose group ($t = 2.225$, $p < 0.05$).

The results of quantitative image analysis of the DBH-positive axons in the frontal cortex are shown in Fig. 3B. In contrast with the electrophysiological data, the anti-BDNF showed a threshold dose on the morphology of noradrenergic axon terminals. The total length of DBH-positive axons was significantly reduced at the high dose of anti-BDNF (filled column, 5511 ± 1642, $n = 4$), as compared with that in the IgG control group (open column, 11,012 ± 452, $n = 4$) ($t = 3.229$ $p < 0.05$). On the other hand, no significant reduction was observed at the low dose (hatched column, 9920 ± 1201, $n = 4$), as compared with the IgG control ($t = 0.850$, $p > 0.1$).

3.3. BDNF decreases threshold current of LC neurons

We finally examined the effects of BDNF and anti-BDNF on the excitability of axon terminals of LC neurons (Fig. 4).

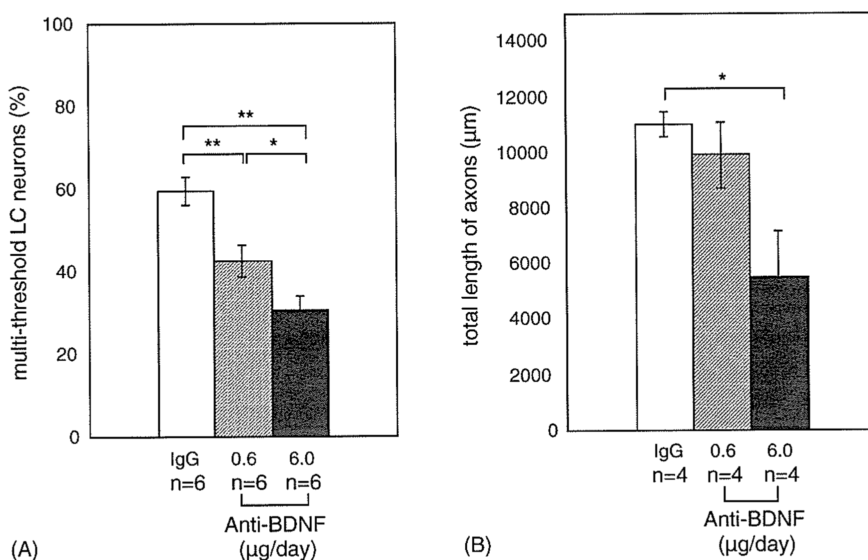

Fig. 3. (A) Effects of anti-BDNF on the percentage of multi-threshold LC neurons in the 19-month-old rats. At the high dose (6 μg/day), the percentage of multi-threshold LC neurons was significantly reduced (filled column), as compared with that in the IgG control group (open column) ($^{**}p < 0.01$). At the low dose (0.6 μg/day), the percentage of multi-threshold LC neurons was also significantly reduced (hatched bar), as compared with the IgG control group ($^{**}p < 0.01$). Significant difference was also found between the low dose and high dose group ($^{*}p < 0.05$). Data are the mean ± S.E.M. of six 19-month-old rats for each group. (B) Effect of anti-BDNF infusion on the total length of DBH-positive axons in the 19-month-old rats. The total length of DBH-positive axons was significantly reduced at the high dose of anti-BDNF (filled column), as compared with that in the IgG control group (open column) ($^{*}p < 0.05$). No significant reduction was observed at the low dose (hatched column), as compared with the IgG control ($p > 0.1$). Data are the mean ± S.E.M. of four 19-month-old rats for each group.

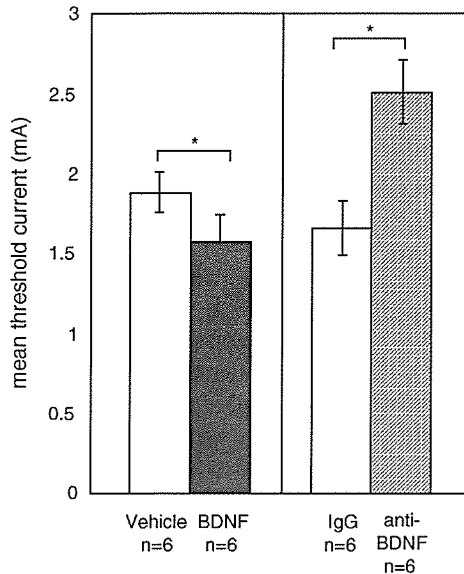

Fig. 4. Opposite effect of BDNF and anti-BDNF on the excitability of LC axon terminals in the aging rats. The mean threshold current in the BDNF-infused group (filled column) was significantly lower than the vehicle control group (open column) ($^*p < 0.05$) in the 13-month-old rats (left panel). On the other hand, the mean threshold current in the anti-BDNF-infused group (hatched column) was significantly higher than the IgG control group (open column) ($^*p < 0.05$) in the 19-month-old rats (right panel). Data are the mean ± S.E.M. of six 13-month-old rats for each group (left panel) and six 19-month-old rats for each group (right panel).

Infusion of BDNF (6 μg/day) and anti-BDNF (0.6 μg/day) produced opposite effects on the mean threshold current to elicit antidromic action potentials at the LC axon terminals. The mean threshold current in the BDNF-infused group (filled column, 1.57 ± 0.16 mA, $n = 6$) was significantly lower than the vehicle control group (open column, 1.88 ± 0.12 mA, $n = 6$) ($t = 2.655$, $p < 0.05$) in the 13-month-old rats (left panel). On the other hand, the mean threshold current in the anti-BDNF-infused group (hatched column, 2.51 ± 0.20 mA, $n = 6$) was significantly higher than the IgG control group (open column, 1.65 ± 0.17 mA, $n = 6$) ($t = 3.148$, $p < 0.05$) in the 19-month-old rats (right panel).

4. Discussion

4.1. Effects of BDNF on LC axon terminals of aging brain

Recently, we showed that the multi-threshold LC neurons increased critically between 15 and 17 months of age. These findings suggest that the LC neurons maintain a strong capacity to remodel their axon terminals even in the aged brain (Ishida et al., 2001b); however, the mechanisms of plasticity in the aging brain are largely unclear.

If BDNF has a trophic action on the LC axon terminals, one can expect that the infusion of exogenous BDNF would induce the plasticity of LC noradrenergic axon terminals in the aging brain. Exogenous BDNF was infused into the frontal cortex at the age of 13 months, because the multi-threshold LC neurons were not yet increased at the age (Ishida et al., 2000; Shirokawa et al., 2000). But, we did not observe any BDNF-induced morphological changes in the noradrenergic axon terminals in the 13-month-old brain. One likely interpretation of this finding is that the LC neurons in the 13-month-old brain have no sufficient sensitivity to BDNF for sprouting their axon terminals. This lack of morphological effect of BDNF on the noradrenergic axons in the middle-aged brain is consistent with our recent study. Exogenous BDNF infusion caused a marked increase in the density of noradrenergic axons in the 25-month-old brain, but no significant morphological change was observed in the 6- and 13-month-old brain (Matsunaga et al., 2004).

4.2. Effects of BDNF on electrophysiological properties of LC axon terminals

The present electrophysiological data clearly showed that exogenous BDNF produced a significant increase in the multi-threshold LC neurons in the aging brain. This increase in multi-threshold LC neurons was accompanied with a decrease in threshold current for antidromic activation. Therefore, a decrease in threshold current should be expected between 13 and 19 months of age, if the increase in multi-threshold LC neurons caused by exogenous BDNF shares the same process with the natural increase of those neurons previously reported (Ishida et al., 2000; Shirokawa et al., 2000). As shown in Fig. 4, the mean threshold current in the 19-month-old rat (1.65 mA) was lower than that of 13-month-old rat (1.88 mA), but this difference was not statistically significant. In contrast, the naturally occurring increase in the multi-threshold LC neurons was obvious (the percentage of multi-threshold LC neurons was 60% at 19 months, while it was 28% at 13 months). These suggest that endogenous BDNF may contribute to two electrophysiological changes; increase of multi-threshold LC neurons and decrease of threshold current. These electrophysiological changes by endogenous BDNF may be due to the increase of sprouting of LC axon terminals (Ishida et al., 2000; Shirokawa et al., 2000). On the other hand, exogenous BDNF may not have promoted the sprouting of LC axon terminals. It is unclear the reason that the morphological change was not induced by the exogenous BDNF. One possible explanation is that the period of BDNF infusion, 2 weeks, may not be sufficient for the morphological change. Thus it may be sufficient for physiological change (increase of excitability), but not for induction of sprouting. This is supported by our present results from infusion with anti-BDNF. At the low dose (0.6 μg/day), anti-BDNF significantly reduced the multi-threshold LC neurons and increased the threshold current, but it did not induce any significant changes in morphology of LC axons. In contrast, a remarkable loss of LC axons occurred at the high dose (6 μg/day), associated with a reduction of multi-threshold LC neurons. Thus we suggest that BDNF acts on the electrophysiological properties of LC axon terminals first, and then the morphology of LC axons.

4.3. Effects of BDNF on excitability of axon terminals of LC neurons

Regarding the electrophysiological roles of BDNF in the adult brain, it is reported that BDNF plays a role in the modulation of synaptic transmission and plasticity (McAllister et al., 1999). Inhibition of BDNF signaling with antibodies specific for BDNF and its receptor attenuates long-term potentiation in the hippocampus of adult rats (Figurov et al., 1996) and in the visual cortex of young rats (Jiang et al., 2001). Our present data strongly suggest that endogenous BDNF is necessary for changes in the excitability of LC axon terminals in the aging brain. In support of this notion, the attenuating effect of anti-BDNF on terminal excitability of LC neurons has been notable (Fig. 4). Terminal excitability is estimated by measuring the threshold current, and it is closely related to the presynaptic activities of axon terminals of LC neurons (Nakamura et al., 1981; Tepper et al., 1985). Noradrenaline release is locally regulated by presynaptic mechanisms such as noradrenergic α_2 autoreceptor and noradrenaline transporter, both located on the axon terminals of LC neurons (Nakamura et al., 1981; Aghajanian and VanderMaelen, 1982; Egan et al., 1983; Tepper et al., 1985; Washburn and Moises, 1989). Thus, the synaptic levels of noradrenaline in the frontal cortex may be determined by the activity of these presynaptic mechanisms for release and uptake.

Recently, we showed that the release activity mediated by the autoreceptor did not change with age, whereas the uptake activity mediated by the transporter declined with age (Shirokawa et al., 2003). It was reported that noradrenaline transporter mRNA in the LC was decreased in the middle age (Shores et al., 1999). The decrease in noradrenaline uptake activity in the aging brain may be an adaptive response to loss of noradrenergic innervations, because the reduction in uptake activity could increase synaptic levels of noradrenaline. Thus, the decrease in uptake activity may be due to the stable noradrenaline levels in the aging brain (Ishida et al., 2001a).

Endogenous BDNF may have been involved in another age-dependent regulation of presynaptic mechanisms of LC neurons. Since expression of c-fos in the LC after activation of L-type calcium channels was reported (Jinnah et al., 2003), analysis of mRNA-expression profiles of L-type calcium channels in the LC during aging would be informative.

4.4. Effects of BDNF on maintenance of noradrenergic innervations

BDNF influences the branching of axonal arbors in the mammal brain (Inoue and Sanes, 1997; Hata et al., 2000) and effectively promotes axonal outgrowth (Bamber et al., 2001). The neutralization of BDNF reduces the arborizations of axons (Streppel et al., 2002) and retards the length of regenerated axons (Zhang et al., 2000) in the adult brain. In our recent study, we have observed that infusion of high dose of anti-BDNF caused a significant reduction in the noradrenergic innervations (Matsunaga et al., 2004). If BDNF is necessary for maintaining noradrenergic innervations in the aging brain, its neutralization with a high dose of anti-BDNF should have a greater effect on the axon terminals of LC neurons. A specific antibody to BDNF was infused into the frontal cortex at the age of 19 months, because the multi-threshold LC neurons were already increased and maintained at the age. Depletion of endogenous BDNF with a high dose of anti-BDNF led to reduction both in the multi-threshold LC neurons and in the noradrenergic innervations. In contrast, treatment with a low dose of anti-BDNF reduced the multi-threshold LC neurons, without affecting the noradrenergic innervations. Thus, we conclude that endogenous BDNF is crucial for the maintenance of multi-threshold LC axon terminals as well as the noradrenergic innervations in the aging brain. These results may reflect the multiple functions of BDNF that can be regulated with advancing age.

Therapeutic application of BDNF was suggested by our recent work. The infusion of BDNF caused a marked increase in the density of cortical noradrenergic axons in the aged (25-month-old) rats, but not in the young (6-month-old) and middle-aged (13-month-old) rats (Matsunaga et al., 2004). This morphological effect of BDNF may be a clue for the therapeutic application to the loss of noradrenergic axons in the aged brain. It was reported that the regenerative sprouting of 5-HT axons was associated with an increase in expression of BDNF in the cortical cells (Liu et al., 2004). This finding suggests that BDNF may play an important role for the successful outcome of antidepressant therapy.

In the present study, BDNF did not stimulate the morphological change in noradrenergic axons in the middle-aged brain, but it acted on the presynaptic excitability of LC axon terminals. If we can take advantage of this trophic action of BDNF on the excitability of LC axon terminals, it may well provide more effective therapeutic support for the neurodegenerative disease with advancing age.

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Age-dependent interactive changes in serotonergic and noradrenergic cortical axon terminals in F344 rats

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Abstract

In the frontal cortex of aging rats, we found an increase in sprouting of the noradrenergic (NA) axons originated from the locus coeruleus (LC). The serotonergic (5-HT) axons originating from the dorsal raphe (DR) share the same cortical area and their age-dependent changes and interactions with NA axons were still unclear. To compare quantitatively the extent of axonal sprouting of DR and LC neurons in the frontal cortex, we extracellularly recorded from both DR and LC neurons in the same animals and antidromically stimulated 32 cortical sites (a pair of stimulating electrodes was moved at 100-μm intervals from 500 to 2000 μm in depth). In addition, to examine the effects of degeneration of 5-HT axons on NA axons, and vice versa, we used specific neurotoxins for 5-HT (PCA) or NA (DSP-4) axons. We also used noradrenaline uptake inhibitor (maprotiline) to verify the effects of NA on degeneration of 5-HT axons. Results suggested that 5-HT axons sprouted between 15 and 17 months of age and noradrenaline accelerated the age-dependent change of 5-HT axons.
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Keywords: Electrophysiology; Dorsal raphe; Locus coeruleus; Monoamine; Interaction; Aging

1. Introduction

In the rat frontal cortex, two ascending monoaminergic axons which originated from the brain stem innervate throughout the cortical layers (Fuxe et al., 1968; Morrison et al., 1978; Vertes, 1991). The serotonergic (5-hydroxytryptamine; 5-HT) axons are from the dorsal raphe (DR) nucleus, and the noradrenergic (NA) axons from the locus coeruleus (LC). These monoaminergic projections are involved in anxiety and depressed behavior, and their impairment is implicated in the mechanism of clinical depression (Mongeau et al., 1997; Beaufour et al., 1999; Cryan et al., 2004). Recently, the extent of 5-HT axonal sprouting has been evaluated in the prefrontal cortex of depressed patients who had committed suicide. Significant loss of 5-HT axons was found in deep layers of the patients' prefrontal cortex (Austin et al., 2002). This suggested that the involvement of cortical 5-HT axon in depression. However, it is still unclear whether the reduction of cortical 5-HT innervations is the only cause of depression.

Antidepressant drugs are known to elicit their effects by increasing synaptic concentrations of 5-HT and/or NA (Frazer, 1997). Although the effects of these drugs are mainly due to an inhibition of uptake of released monoamines, the monoamine uptake inhibitors also induce regenerative sprouting of monoamine axons in the frontal cortex (Nakamura, 1990, 1991; Kitayama et al., 1994). This suggests that the therapeutic effects of antidepressants may be due to the sprouting of monoaminergic axons. Therefore, investigation of the possible interaction between 5-HT and NA axon terminals may well provide insight into the remediation of depression.

Sprouting of monoaminergic axons is a likely anti-aging mechanism to prevent loss of innervations which are concerned with attention, cognition and mood alterations in the terminal fields (Glennon, 1990; Harley, 1991; Van de Kar, 1991; Ishida et al., 2000, 2001a; Shirokawa et al., 2000a,b). Recently, we have reported that the axon terminals of LC neurons sprout in middle age, following the age-dependent loss of innervations in the frontal cortex and hippocampus (Ishida et al., 2000, 2001a;

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Shirokawa et al., 2000a,b). Since concentration of NA was maintained in the frontal cortex in the aged rats (Ishida et al., 2001b), the increased sprouting of LC axons may compensate the loss of their innervation in the aged brain (Shirokawa et al., 2003). If this age-dependent sprouting simultaneously occurs in the axon terminals of DR-5-HT neurons during aging, interactions are plausible among monoaminergic axons.

We studied, therefore, the age-dependent changes in 5-HT and NA axon terminals in rat frontal cortex. In addition, to investigate possible interaction between the two monoaminergic axon terminals, we quantify the extent of axonal sprouting in the cortical layers using in vivo electrophysiological techniques to antidromically activate the axon terminals of individual DR and LC neurons. Moreover, maprotiline, a selective NA uptake inhibitor, *n*-(2-chloroethyl)-*n*-ethyl-2-bromobenzylamine hydrochloride (DSP-4) and DL-*p*-chloroamphetamine hydrochloride (PCA) are used to evaluate interaction between 5-HT and NA axon terminals in middle-aged rat. The results of the present study demonstrate an important role for NA in activation and/or maintaining of 5-HT axon terminals in normal aging rats.

2. Materials and methods

2.1. Animals

Male F344/N rats ranging 3–24 months old were used. They were kept with a 12 h/12 h light/dark cycle, and had free access to food and water. All animal procedures complied with the National Institutes of Health guidelines and were approved by the Laboratory Animal Research Facilities Committee of National Center for Geriatrics and Gerontology.

2.2. Experimental procedures

Animals were anaesthetized with urethane (1.2 g/kg, i.p.) which was supplemented as necessary during these experiments. Lidocaine (4% xylocaine) was applied locally to all incisions. Rats were fixed in a stereotaxic apparatus. Rectal temperature was maintained at 36.5 °C by a heating pad (Bio Research Center Co., Japan). The electrocardiogram was monitored continuously during these experiments. Stimulating electrodes of the bipolar type consisted of the insulated stainless steel wires (diameter 80 μm, tip separation 40 μm). Electrical pulses of 0.5 ms duration with currents ranging from 0.1 to 5.0 mA were given to these stimulus sites, and the cycle of stimulation was 1.5 s. A pair of stimulating electrodes was moved up and down between 500 and 2000 μm below the cortical surface with an interval of 100 μm. Thirty-two cortical sites (16/track) were stimulated by antidromic activation of DR or LC cells. The stimulating current was adjusted to a value just sufficient to elicit an antidromic response to every stimulus. For each DR/LC neuron activated antidromically from the frontal cortex, the threshold currents were measured by varying the stimulating current in 0.01 mA steps (Fig. 1A). The threshold current was defined as the minimum stimulating current sufficient to elicit an antidromic response. The antidromic latency was also measured for individual DR/LC neurons. The electrical activities of DR and LC neurons were extracellularly recorded with a glass pipette microelectrode filled with 2 M NaCl. Electrode resistance ranged from 10 to 20 MΩ. Recording electrodes were introduced in the cerebellum, usually 0.2 mm from the midline and 2.4 mm posterior to lambda for DR neurons, and 1.2 mm from the midline and 3.0 mm posterior to lambda for LC neurons. Each electrode track was angled 16° anteriorly from the vertical for LC unit recording and 30° for DR unit recording, respectively. For each animal, we first recorded 10–12 antidromically driven units from the DR, and then recorded from the LC.

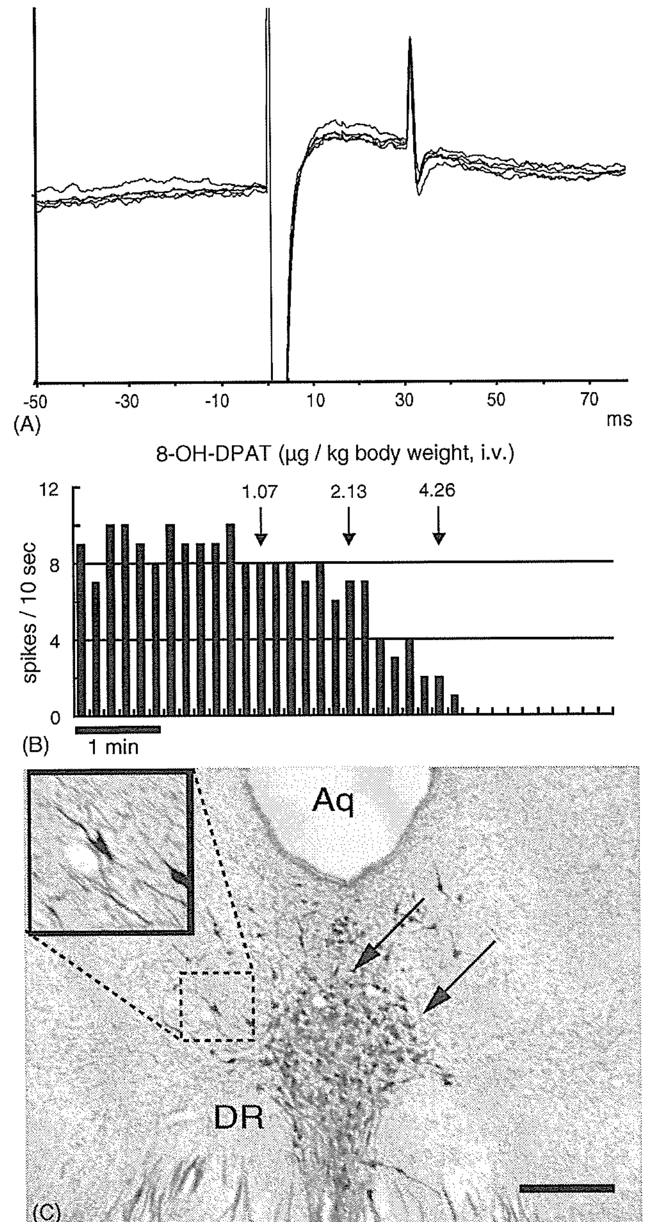

Fig. 1. (A) Antidromic responses of serotonergic neurons to electrical stimulation of FC. At a current of 2.05 mA, antidromic response with single antidromic latency was evoked at a latency of 30.9 ms in a 6-month-old rat. Five consecutive sweeps were superimposed in the non-collision trials. (B) The effect of intravenous administration of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, on the spontaneous firing of a representative serotonergic neuron. After the recording of basic spontaneous activities for 2 min, 8-OH-DPAT was injected every minute (1.07, 2.13, and 4.26 μg/kg i.v.). (C) Serotonin immunoreactivity in the DR. The part of an interrupted circle is shown at higher magnification. Arrows indicate serotonergic neurons. DR: dorsal raphe nucleus. Aq: aqueduct (bar = 100 μm).

2.3. Confirmation of LC noradrenergic neuron

The LC noradrenergic neurons were identified according to the criteria used by several authors (Nakamura, 1977; Aston-Jones and Bloom, 1981; Ishida et al., 2000). Briefly, the LC neurons were identified by their characteristic wide spike duration (≈2 ms), slow and tonic spontaneous firing (0.5–6 Hz) and activation by tail pinches followed by a long-lasting suppression of firing (data not shown).

2.4. Confirmation of DR serotonergic neuron

The DR serotonergic neurons were identified according to the criteria used by several authors (Sawyer et al., 1985; Wang and Aghajanian, 1977). Briefly, the DR neurons were identified by (i) a regular spontaneous firing rate (0.1–3.0 spikes/s), (ii) a characteristic di- or triphasic extracellular waveform, and (iii) the antidromic responses with a long latency to the stimulation of neostriatal or medial forebrain bundle in some case. Finally, some neurons were confirmed as serotonergic by examining if the spontaneous firing was abolished by the injection of a selective 5-HT_{1A} autoreceptor agonist, 8-hydroxy-2 (di-*n*-propylamino)tetralin (8-OH-DPAT, Biomol, PA, 1.07, 2.13, and 4.26 μg/kg, i.v.). After total inhibition of this spontaneous activity, electrical pulses were applied to check the existence of the neuron.

2.5. Drug treatments

DSP-4 (*n*-(2-chloroethyl)-*n*-ethyl-2-bromobenzylamine hydrochloride) and PCA (DL-*p*-chloroamphetamine hydrochloride) (Sigma–Aldrich, MO) were intraperitoneally injected into rats at the age of 15 months in doses of 50 mg/kg or 20 mg/kg. These solutions were dissolved in 1.0 ml isotonic saline, and prepared just prior to use. As a control, isotonic saline was intraperitoneally administered to rats. After 1 month of injections, the electrical activity of DR neurons and LC neurons was recorded (Fig. 3A).

After the 2-week DSP-4 treatment at the age of 15 months, the rats received maprotiline (Sigma–Aldrich, MO) dissolved in tap water via light-protected bottles for 1 week or 2–4 weeks, and solutions were renewed on alternate days. The concentration of maprotiline in the solution was controlled, ranging from 8 to 11 mg/kg/day, which is comparable with previously reported doses that inhibited NA reuptake (Garcha et al., 1985).

2.6. Immunohistochemistry

Dopamine-β-hydroxylase (DBH) immunohistochemistry was performed as follows. Rats were anesthetized with an overdose of urethane (3 g/kg, i.p.) and perfused through the ascending aorta with saline followed by 500 mL of chilled 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (pH 7.4). The brain of each rat was removed and postfixed overnight in 4% paraformaldehyde in 0.1 M PB and immersed in 30% sucrose in 0.1 M PB at 4 °C. The brain was sectioned in the coronal plane at 20-μm thickness between the level from 1.75 to 2.25 mm anterior to bregma, which included the stimulation site in the frontal cortex, and the level from −7.04 to −9.30 mm posterior to bregma, which included the recording site in the dorsal raphe, by a CM1800 cryotome (Leica, Heerburg, Switzerland). Sections were pretreated with 1% H_2O_2 in PBS for 20 min at room temperature (RT), and 0.1% $NaBH_4$ in PBS for 5 min. After pretreatment, sections were incubated with MAB308 monoclonal anti-DBH antibody (Chemicon International, Inc., CA) diluted 1:4000 in PBS containing 0.3% Triton X-100 (PBST) and 1% normal horse serum for 2 days at 4 °C. After incubation with the primary antibody, sections were incubated with biotinylated anti-mouse IgG made in horse (dilution 1:400 in PBST, Vector Labs) for 2 h at RT, and then incubated with ABC elite reagent (dilution 1:200 in PBST, Vector Labs) for 2 h at RT. Visualization was performed with 0.02% 3,3′-diaminobenzidime and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.4). Sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped out of xylene. Staining for 5-HT was conducted successively using a similar procedures for DBH. Briefly, non-specific binding was blocked with the 1% normal goat serum (Chemicon International, Inc., CA) in PBST for 3 days at RT. Endogenous peroxidase activity was blocked as described above, then the sections were incubated with polyclonal antibody against 5-HT (1:100,000) (Diasorin, MN) in PBST containing at 37 °C for 3 days. This was followed by incubations with biotinylated anti-rabbit IgG(H + L) (1:400) (Vector Labs, CA) in PBST at RT for 2 h, and then with ABC reagent (1:200, ABC Elite, Vector Labs, CA) at RT for 2 h. Visualization was performed with 0.02% 3,3′-diaminobenzidime and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.4). Sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped out of xylene.

2.7. Sites/cell and P-index

We employed two electrophysiological measurements to quantify the extent of axonal sprouting of DR/LC neurons: (i) the site activated antidromically for each DR/LC neuron through the cortical layers in the frontal cortex (*sites/cell*: number of sites activated antidromically for individual DR/LC neurons); and (ii) the percentage of DR/LC neurons activated antidromically from the frontal cortex for each animal (*P*-index: number of DR/LC neurons with antidromic response/ number of recorded DR/LC neurons) (Nakamura et al., 1989; Ishida et al., 2000).

2.8. Data analysis

Results are expressed as mean ± S.E.M. Statistical analysis of differences between groups was performed using post hoc Games-Howell. Significance levels were set at $p < 0.05$.

3. Results

3.1. Effects of 8-OH-DPAT on spontaneous activity of DR serotonergic neuron

Seven antidromically activated neurons, presumed to be serotonergic DR neurons on the basis of their waveform and spontaneous activity, were checked for a dose-dependent inhibition of the spontaneous firing by intravenous injection of 8-OH-DPAT. Fig. 1B shows a representative putative serotonergic neuron. Every presumed serotonergic neuron was inhibited by 8-OH-DPAT dose-dependently. After each injection of 8-OH-DPAT, the existence of neurons was checked by applying electrical pulses (data not shown). Fig. 1C shows the recording site of DR serotonergic neurons.

3.2. Effects of aging on cortical axon terminals of DR and LC neurons

Fig. 2A shows the age-dependent changes in cortical projections of DR neurons, which were antidromically activated through the cortical layers at least at one site. To quantify the changes in the extent of axonal sprouting for individual DR neurons, we examined the number of sites activated antidromically (maximum value: 32 sites/cell). No significant change was observed between 3 months (11.3 ± 0.8 sites/cell) and 15 months of age (12.1 ± 0.9 sites/cell), but the number of activated sites significantly increased at 17 months of age (Fig. 2A; 16.1 ± 1.1 sites/cell, $p < 0.05$). This increase returned to the initial level at 24 months of age (12.1 ± 1.0 sites/cell).

Fig. 2B shows the age-dependent changes in cortical projections of LC neurons, which were antidromically activated through the cortical layers. The extent of LC axonal sprouting did not show any significant change between 3 and 11 months of age (13.7 ± 0.8 and 15.5 ± 1.0 sites/cell, respectively, $p > 0.05$). The number of activated sites decreased at 15 months of age (12.2 ± 1.1 sites/cell), and then the sites returned to the initial level at 17 months of age (14.7 ± 1.3 sites/cell). The increased sites/cell gradually decreased thereafter and reached the lowest level at 24 months of age (9.7 ± 0.8 sites/cell).

We employed another electrophysiological measurement, the *P*-index (Nakamura et al., 1989; Ishida et al., 2000), to quantify the amount of cortical projections from each nucleus,

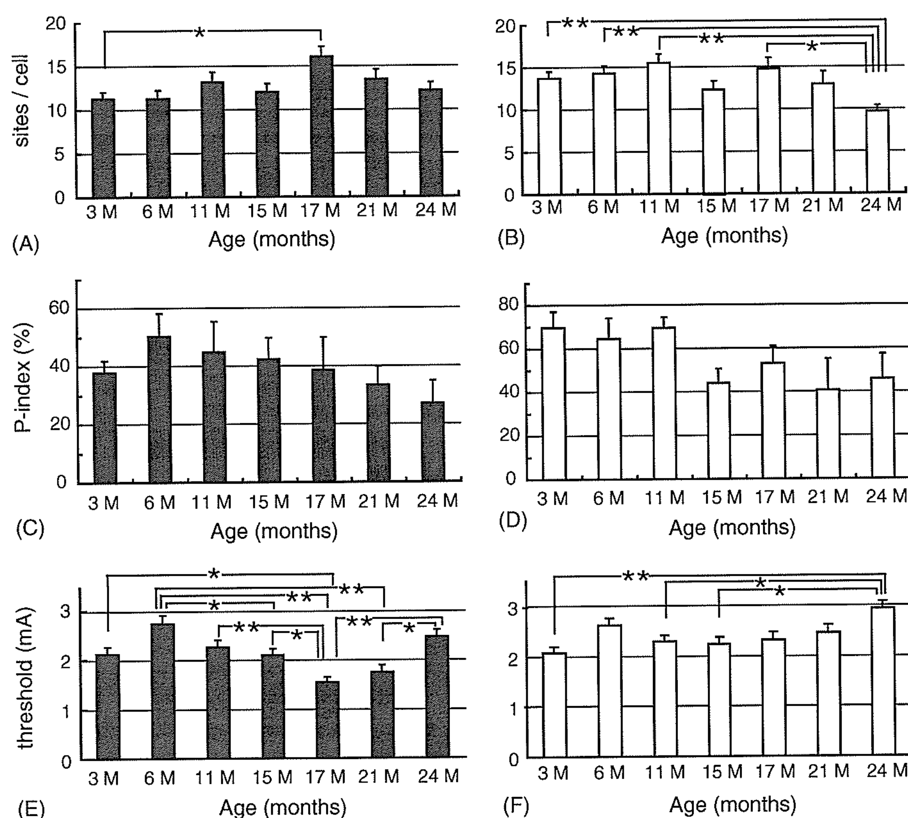

Fig. 2. The effect of aging on the projections from DR (A) and LC (B) to frontal cortex. (A) The extent of 5-HT axonal sprouting represented by the number of stimulation sites evoking antidromic responses in DR neurons was plotted against age in months. The extent of 5-HT axonal sprouting in the frontal cortex critically increased between 15 and 17 months of age, and gradually declined thereafter. The total number of DR neurons recorded for each age group is as follows: 3 months of age, $n = 50$; 6 months, $n = 51$; 11 months, $n = 51$; 15 months, $n = 57$; 17 months, $n = 38$; 21 months, $n = 40$; 24 months, $n = 43$. (B) The extent of NA axonal sprouting in the frontal cortex declined between 11 and 15 months of age. The extent of axonal sprouting increased again between 15 and 17 months of age, and gradually declined thereafter. Vertical axis shows the antidromically activated numbers of sites of each neuron (maximum value: 32 sites/cell) from the electrical stimulation of frontal cortex. The total number of LC neurons recorded for each group is as follows: 3 months, $n = 60$; 6 months, $n = 51$; 11 months, $n = 51$; 15 months, $n = 40$; 17 months, $n = 34$; 21 months, $n = 34$; 24 months, $n = 49$. (C) Age-dependent changes in P-indices (number of DR neurons with antidromic latencies/number of recorded DR neurons) in the frontal cortex. The P-indices in DR neurons increased between 3 and 6 months of age, and gradually declined thereafter. $n = 5$–6 rats per group of age. (D) Age-dependent changes in P-indices (number of LC neurons with antidromic latencies/number of recorded LC neurons) in the frontal cortex. The P-indices in LC neurons decreased between 11 and 15 months of age, and did not change thereafter. $n = 4$–6 rats per group of age. (E) Age-dependent changes in threshold of axon terminals of DR neurons in frontal cortex. The threshold currents of DR neurons in frontal cortex showed the lowest value at 17 months of age (3 months, $n = 80$; 6 months, $n = 90$; 11 months, $n = 84$; 15 months, $n = 98$; 17 months, $n = 80$; 21 months, $n = 68$; 24 months, $n = 79$). (F) Age-dependent changes in threshold of axon terminals of LC neurons in the frontal cortex. The threshold currents of LC neurons in frontal cortex showed the lowest value at 15 months of age, and increased thereafter (3 months, $n = 118$; 6 months, $n = 106$; 11 months, $n = 110$; 15 months, $n = 75$; 17 months, $n = 72$; 21 months, $n = 67$; 24 months, $n = 78$). Data are means ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$; post hoc Games-Howell.

LC and DR, for individual animals. As shown in Fig. 2C, the mean P-indices for DR projections peaked at 6 months of age (50.5 ± 7.7%), and then gradually decreased until 24 months of age (27.0 ± 8.0%). In contrast, the mean P-indices for LC projections indicated different aging pattern as shown in Fig. 2D. The P-indices were maintained until 11 months of age (69.4 ± 4.7%). They rapidly decreased at 15 months of age (44.0 ± 6.5%), and then did not return to the initial level there after. Thus, we obtained two different types of age-dependent changes for DR and LC projections as follows: (i) the axon terminal arborization of individual DR neurons, evaluated by sites/cell, showed a marked increase at middle age (Fig. 2A), while the amount of DR projections for each animal, assessed by the P-index, indicated a gradual decrease from adult to aged (Fig. 2C); and (ii) the axon terminal arborization of individual LC neurons showed a decrease/increase/decrease cycle from middle-age to aged (Fig. 2B), while the amount of LC projections indicated a critical decrease at middle age (Fig. 2D). Focusing on the changes in DR/LC projections in middle age, we observed the following sequence of changes: (i) a decrease in LC projections at 15 months of age, (ii) an increase in LC projections at 17 months of age, and (iii) an increase in DR projections at 17 months of age.

To investigate age-dependent changes in excitability of axon terminals of individual DR neurons, we focused on the mean threshold currents for antidromic activation of DR neurons during aging, as shown in Fig. 2E. The threshold current for antidromic activation of axon depends on the distance between stimulating electrodes and activated axons as well as axon excitability (Nakamura et al., 1981; Ishida et al., 2000). If axonal sprouting increases at the stimulus site, the distance between the stimulating electrodes and activated axons is

relatively smaller, so that the threshold current for the antidromic activation would be decreased. Therefore, we have a notion that the threshold current may reflect not only the excitability of axons but also the extent of sprouting of axons.

The threshold current showed the highest value at 6 months of age (2.76 ± 0.16 mA), and then significantly decreased with age. It reached the lowest value (1.55 ± 0.11 mA) at 17 months of age, and then increased again until 24 months of age (2.47 ± 0.15 mA). Similar age-dependent changes were observed in the threshold current of LC neurons (Fig. 2F). After 6 months of age, the threshold current showed the lowest value (2.23 ± 0.14 mA) at 15 months of age, and then increased until 24 months of age (2.93 ± 0.14 mA). These results clearly indicate the middle-aged decrease in threshold currents for terminal activation.

3.3. Effect of PCA and DSP-4 on cortical axon terminals of DR and LC neurons

The extent of axonal sprouting of individual DR/LC neurons (sites/cell) indicated a critical peak at the same age of 17 months. Thus, we focused on this age to examine the interaction between noradrenergic and serotonergic axon terminals. For the investigation of the interaction between these axons, we used two neurotoxins specific to serotonergic (PCA) and noradrenergic (DSP-4) axons (see Fig. 3A). In the PCA-treated rats, the extent of DR axonal sprouting significantly decreased in the frontal cortex (8.3 ± 0.9 sites/cell, Fig. 3B, right column, $p < 0.05$), whereas there was no change in the extent of LC axonal sprouting (14.8 ± 1.3 sites/cell, Fig. 3C, right column, $p > 0.05$). Unlike the effects of PCA, in the DSP-4-treated rats, a significant decrease occurred in the extent of DR axonal sprouting (7.8 ± 1.1 sites/cell, Fig. 3B, middle column, $p < 0.05$), as well as in the LC axon terminals (6.9 ± 0.7 sites/cell, Fig. 3C, middle column, $p < 0.01$).

Fig. 3D shows typical images of 5-HT-positive serotonergic axons (left) and DBH-positive noradrenergic axons (right). The respective degenerative effects of DSP-4 and PCA on the noradrenergic axons (right, middle) and serotonergic ones (left, bottom) were clear compared with the control (right and left, top). The PCA did not affect the noradrenergic axons (right, bottom), but the effects of DSP-4 were significant on the serotonergic axons (left, middle). Note that the serotonergic axons were obviously decreased in the DSP-4-treated frontal cortex (left, middle). These immunohistological results were consistent with the present electrophysiological data.

3.4. Effects of maprotiline on decreased axon terminals of DR neurons

To test whether noradrenaline is responsible for the restoration of serotonergic axon terminals, we used maprotiline, a noradrenaline uptake inhibitor, to restore the extracellular level of noradrenaline in the DSP-4-treated brain (Fig. 4A). Fig. 4B shows the effects of maprotiline on the axon terminals of DR neurons. After 2 weeks of DSP-4 treatment (7.8 ± 1.1 sites/cell), maprotiline treatment for 1 week caused a marked increase in the number of axon terminals of DR neurons (14.0 ± 1.1 sites/cell, $p < 0.01$). This increase was maintained for 2–4 weeks of maprotiline treatments (13.4 ± 0.9 sites/cell). However, as shown in Fig. 4C, maprotiline treatments either for 1 week (7.9 ± 0.9 sites/cell) or 2–4 weeks (7.4 ± 1.4 sites/cell) did not affect the axon terminals of LC neurons in the DSP-4-treatment brain (6.9 ± 0.7 sites/cell). No significant effect of maprotiline was observed on either DR or LC axon terminals of DSP-4-free control (13.6 ± 1.1 and 13.2 ± 1.3 sites/cell, respectively, $p > 0.05$).

Fig. 4D shows the typical images of the 5-TH-positive serotonergic axons (left) and DBH-positive noradrenergic axons (right). After 2 weeks of DSP-4 treatment (left, middle), maprotiline treatment for 1 week caused a marked increase in the axons of DR neurons (left, bottom). This increase was maintained for 2–4 weeks of maprotiline treatments (data not shown), but maprotiline treatments either for 1 week (right, bottom) or 2–4 weeks did not affect the axons of LC neurons in the DSP-4-treatment brain (right, middle). These immunohistological results were consistent with the present electrophysiological data. No significant effect of maprotiline was observed on either LC or DR axons of DSP-4-free control (left and right, top, respectively).

4. Discussion

4.1. Aging pattern in two monoaminergic axon terminals

Analysis of the number of activated sites of the axon terminals for individual monoaminergic neurons (sites/cell) indicates that the activated axon terminals increase between 15 and 17 months of age in both monoaminergic projections in rat frontal cortex (Fig. 2A and B). This result strongly suggests that both DR and LC neurons give rise to axonal sprouting in middle age. This was particularly significant for the axon terminals of DR neurons, while the LC-NA neurons showed a decrease in their axon terminals between 11 and 15 months of age. This early middle-aged decline in the LC axon terminals is consistent with our previous studies (7–15 months of age, Ishida et al., 2000; 9–13 months of age, Ishida et al., 2001a). These results indicate that the LC-NA innervations in the rat frontal cortex start to decrease around 12 months of age. This decline may cause the following age-dependent plasticity in the two monoaminergic systems, i.e., the sprouting of axon terminals between 15 and 17 months of age. The increased axon terminals gradually declined with advancing age; however, a significant decrease was found only in the LC neurons at 24 months of age (Fig. 2B), but not in the DR neurons (Fig. 2A). This implies that the axon terminals of DR neurons are more robust than those of LC neurons in the aging brain.

Analysis of the *P*-index, which reflects the amount of cortical projections from each nucleus, LC and DR, revealed a different pattern of aging in two monoaminergic projections. The 5-HT projections from DR exhibited its peak at 6 months of age, and then gradually decreased with age (Fig. 2C), while the LC-NA projections clearly decreased at 15 months of age without recovery of the projections after that (Fig. 2D). For the

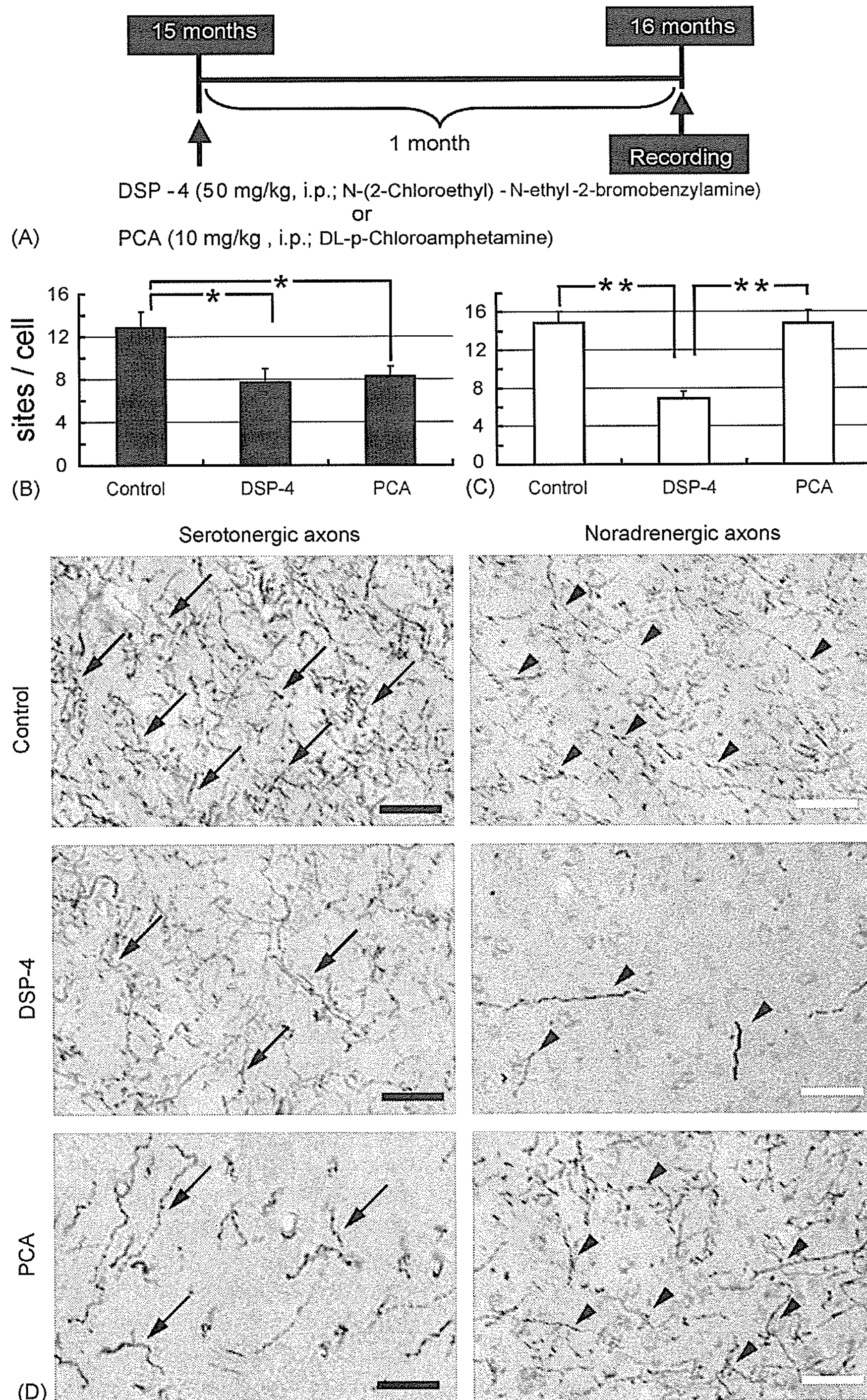

Fig. 3. (A) Specific neurotoxin treatments for 5-HT (PCA) or NA (DSP-4). Fifteen-month-old rats were injected with PCA or DSP-4. At 16 months of age, the rats were used for recording. (B) Effects of specific neurotoxin for 5-HT (PCA) and NA (DSP-4) on the extent of 5-HT axonal sprouting at 16 months of age. The extent of 5-HT axonal sprouting of PCA-treated neurons was significantly lower than those saline treated. Note that the extent of 5-HT axonal sprouting of DSP-4-treated neurons was also significantly lower than that of saline-treated controls (control, $n = 31$; DSP-4, $n = 23$; PCA, $n = 25$). (C) Effects of DSP-4 and PCA on the extent of NA axonal sprouting at 16 months of age. The extent of NA axonal sprouting of DSP-4-treated neurons was significantly lower than that of saline-treated control neurons. On the other hand, there was no significant difference in the extent of NA axonal sprouting between saline-treated neurons and PCA-treated ones (control, $n = 32$; DSP-4, $n = 17$; PCA, $n = 32$). (D) Immunohistochemical visualizations of a coronal section (20 μm thick) of the frontal cortex (treated with saline, DSP-4 and PCA) stained with an antibody against serotonin to identify the serotonergic DR axons and dopamine-β-hydroxylase (DBH) to identify the noradrenergic LC axons. Arrows indicate serotonergic axons. Arrowheads indicate noradrenergic axons. $^{*}p < 0.05$, $^{**}p < 0.01$; post hoc Games-Howell (black bar = 50 μm, white bar = 25 μm).

DR neurons that remain innervated, they maintained their axon terminals even in the aged brain (Fig. 2A), though the cortical projections gradually decreased with age (Fig. 2C). For the LC neurons that remain innervated, they maintained their axon terminals until late middle age (Fig. 2B), though the cortical projections rapidly decreased in early middle age (Fig. 2D).

Another aging pattern was observed on threshold currents for activation of axon terminal (Fig. 2E and F). In the DR