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

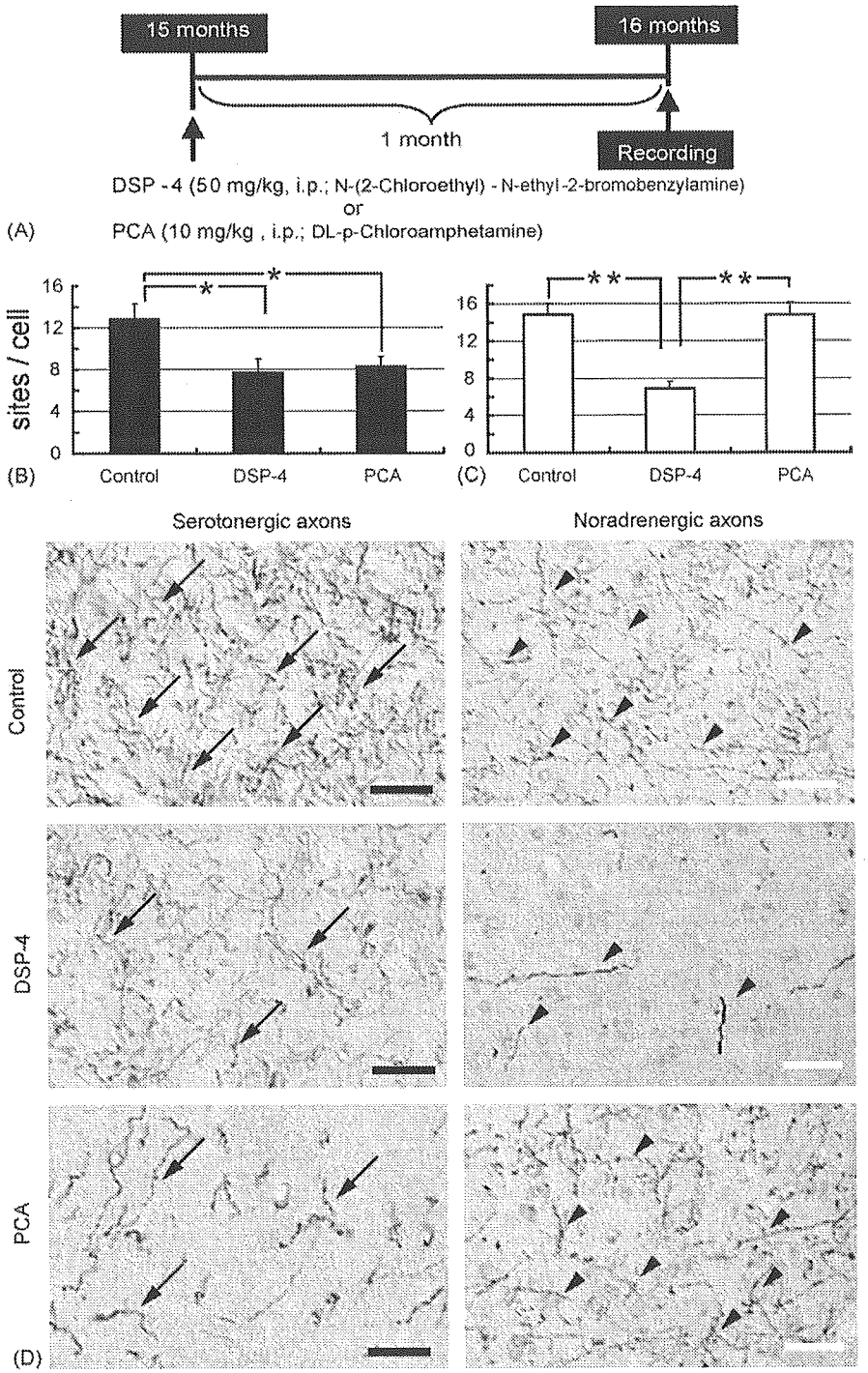

Fig. 3. (A) Specific neurotoxin treatments for 5-HT (PCA) or NA (DSP-4). Fifteen-month-old rats were injected with PCA or DSP-4. At 16 months of age, the rats were used for recording. (B) Effects of specific neurotoxin for 5-HT (PCA) and NA (DSP-4) on the extent of 5-HT axonal sprouting at 16 months 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

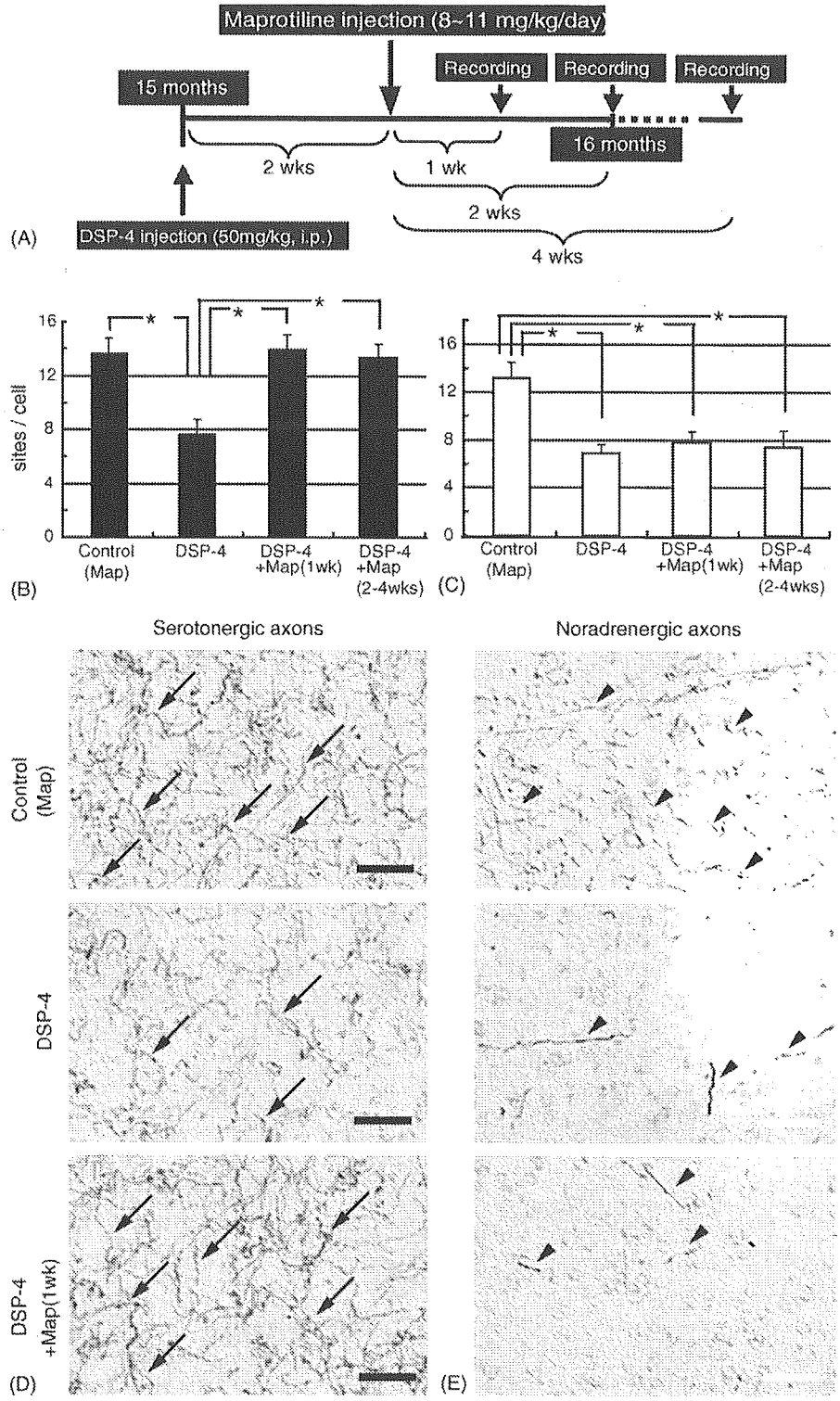

Fig. 4. (A) Chronic antidepressant treatments after DSP-4 treatment. Fifteen-month-old rats were treated with DSP-4. Two weeks later, maprotiline, a noradrenaline uptake inhibitor, was administered for 1, 2, or 4 weeks. Effects of a specific neurotoxin for NA (DSP-4) and maprotiline (Map, a NA uptake inhibitor) on the extent of 5-HT axonal sprouting. (B) Control, $n = 34$; DSP-4, $n = 23$; DSP-4 + Map(1w), $n = 45$; DSP-4 + Map(2w), $n = 68$ and NA axon terminals. (C) Control, $n = 30$; DSP-4, $n = 17$; DSP-4 + Map(1w), $n = 20$; DSP-4 + Map(2w), $n = 13$ at 16 months of age. The extent of 5-HT axonal sprouting of 5-HT neurons treated with maprotiline for 1 week was significantly higher than that of DSP-4-treated neurons. There was no significant effect of maprotiline (1 week) on the extent of NA axonal sprouting of DSP-4-treated neurons. Similarly, there was no significant effect of maprotiline on the extent of NA axonal sprouting of saline-treated control neurons ($p > 0.05$). Immunohistochemical visualizations of a coronal section (20 μm thick) of the frontal cortex (treated with saline, DSP-4 and maprotiline) stained with an antibody against serotonin to identify the serotonergic DR axons (D) and dopamine-β-hydroxylase (DBH) to identify the noradrenergic LC axons (E). 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).

neurons, the threshold current peaked at 6 months of age and gradually decreased thereafter, reaching its lowest value at 17 months of age. Again, it gradually increased to reach its highest level at 24 months of age that is the final age for present examination (Fig. 2E). Obviously, there is a mirror image at the age of 17 months; the lowest level of threshold current (Fig. 2E) in contrast to the highest extent of axonal sprouting (Fig. 2A). This finding supports our notion that the threshold current reflects the excitability of axons as well as the extent of sprouting of axons. Thus, we conclude that DR serotonergic neurons have more sprouted and excitable axon terminals at the middle age. Similar changes are also observed in the LC neurons, in which the threshold current is maintained at low value in middle age, and then gradually increased with advancing age (Fig. 2F). Thus, it is most likely that the increase in excitability and sprouting of axon terminals plays a crucial role in maintaining stable monoamine levels in the middle-aged brain.

4.2. Interactions between the two monoaminergic axon terminals

In the present study, we hypothesized that interaction between the two monoaminergic systems is necessary for the maintenance of their innervations in the frontal cortex. We questioned whether two monoaminergic axon terminals interact during aging, focusing on the extent of axonal sprouting of individual DR and LC neurons projecting to the frontal cortex. In the present study, we considered "*an affecting change in the extent of axonal sprouting of 5-HT (NA) on NA (5-HT)*" as an interaction between the two. A recent study reported that the role of 5-HT axons in the regeneration of NA axons is opposite to that of NA axons in the regeneration of 5-HT axons (Liu et al., 2003). Thus, the NA axons enhanced the sprouting of 5-HT axons, whereas the 5-HT axons suppressed the sprouting of NA axons. If such asymmetric interaction is the case in the normal aging of monoaminergic innervations, the sprouting of NA axons should promote sprouting of the 5-HT axons. In the present study, since the extent of LC and DR axonal sprouting increased at the same time, 17 months of age, we examined whether interaction took place between these two monoaminergic terminals. If the LC axon terminals have some sprouting-enhancing influence on the DR axons, the lesion of the LC axon terminals may cause a failure in the age-dependent sprouting of DR axon terminals. We tested two neurotoxins specific to NA (DSP-4) and 5-HT (PCA) axons. The treatment with PCA did not have any effect on the LC-NA terminals (Fig. 3C), while DSP-4 induced a profound loss of DR-5-HT axon terminals (Fig. 3B). This might have been due to a non-specific effect of DSP-4 on 5-HT axons. However, it is known that DSP-4 is highly specific to LC-NA axons, and the concentration used in this experiment is comparable with previously reported doses that effect LC-NA axons (Fritschy and Grzanna, 1991, 1992). Therefore, we concluded that the lesion of NA terminals caused the impairment of a sprouting of 5-HT axons.

The lesion of NA axon terminals failed to induce the sprouting of 5-HT axon terminals in the aging brain. One interpretation of this finding is that the NA axon terminals have a trophic action on the 5-HT axon terminals, and more likely via releasing of a neurotrophic factor. Since the brain-derived neurotrophic factor (BDNF) strongly promotes 5-HT axonal sprouting or regeneration in the adult brain (Mamounas et al., 2000), it is likely that BDNF might act on the lesioned 5-HT axon terminals. Since BDNF was synthesized in LC neurons (Fawcett et al., 1997), it may be released from the LC axon terminals (Thoenen, 1995; Fawcett et al., 1998). However, this notion is not consistent with our present finding that the 5-HT axon terminals were restored in the brain treated with an NA uptake inhibitor, maprotiline. In the maprotiline-treated brain, NA content is presumably recovered, despite the absence of NA axon terminals. Thus, the recovery of 5-HT axons may not have depended on the NA axons themselves, but instead may have depended on NA content. However, we cannot rule out an alternative interpretation that NA acts on the cortical residential cells to enhance their release of BDNF affecting the 5-HT axons. It is supported by a recent report that the regenerative sprouting of 5-HT axons coincides with an increase in expression of BDNF in cortical cells (Liu et al., 2004).

It was reported that regeneration of NA axons is impeded by the 5-HT axons in rat frontal cortex (Liu et al., 2003). If the suppressive influence of 5-HT axons on NA axons is the case in the aging brain, the lesion of 5-HT axons should markedly induce the sprouting of NA axons. However, the lesion of 5-HT axons by PCA did not have any effects on the sprouting of NA axons. Although the reason for this discrepancy is unclear at present, it may be due to the difference in the ages of rats used in the previous (2 months old) and present (15 months old) studies. Further studies are needed to clarify this age-related matter.

4.3. Therapeutic implications for depression

In postmortem brain of suicide victims with depression, a profound loss of 5-HT innervations was reported in the deep layers of their prefrontal cortex (Austin et al., 2002). Furthermore, in patients on antidepressant treatment, the loss was less (8%) than in those not on treatment (31%). This result suggests that antidepressant treatment may facilitate 5-HT axonal regeneration. In the present study, we found that loss of serotonergic axon terminals induced by DSP-4 was restored by the treatment with an antidepressant, maprotiline. Current antidepressant therapies mainly target the concentrations of 5-HT and/or NA at synaptic sites. However, they have not considered that the pattern of innervations might be changes as a result of the treatments with the antidepressant. If this sprouting-enhancing action of maprotiline on the 5-HT axons is the case, it is possible that the drug is useful for therapeutics for depression induced by the loss of 5-HT axons. This may account for the therapeutic effect of another type of antidepressant, 5-HT/NA reuptake inhibitor (SNRI), which leads to regeneration of 5-HT axons as well as recovery of the 5-HT/NA concentration. Thus, the therapeutic action of SNRI may depend on this interaction between NA and 5-HT axon terminals.

These recovery processes of clinical symptoms associated with axonal sprouting may require more time than the immediate changes in the release mechanism induced by antidepressants (Nakamura, 1990). In the rats received with the forced walking, a depression model, significant retraction or loss of NA axons were found in the frontal cortex (Kitayama et al., 1994). Treatment with a common antidepressant usually leads to regrowth or reinnervation of NA axons in the frontal cortex and to behavioral recovery (Nakamura, 1991). The extent with behavioral recovery is positively correlated with the extent of NA axonal regrowth (Kitayama et al., 1997). Involvement of neurotrophic factors, including BDNF has been suggested in the mechanism of antidepressant action (Altar, 1999). In support of this notion, it was reported that the regenerative sprouting of 5-HT axons coincides with an increase in expression of BDNF in the cortical cells (Liu et al., 2004). This suggests that neurotrophic factors may play an important role for the successful outcome of antidepressant therapy.

Interestingly, maprotiline treatments have no effect on either the intact 5-HT axons or the intact or DSP-4-treated NA axons. This means that 5-HT axons can be restored in the absence of the NA axons in situ. With DSP-4-induced denervation of NA axons, reinnervation indeed occurs, but slowly (Nakai et al., 1994; Liu et al., 2003). In these previous studies, DSP-4-induced denervation of NA axons was nevertheless nearly complete 60 days after the treatment. Therefore, we conclude that the NA itself, instead of the NA axons, may be necessary for the reinnervation of 5-HT axons. It is well known that NA can induce some types of synaptic plasticity such as ocular dominance plasticity in the visual cortex (Kasamatsu, 1991), and long-term potentiation in the hippocampus dentate gyrus (Harley, 1987, 1991). NA also has growth-promoting properties that affect the development and extension of neurons (Laifenfeld et al., 2002). If maprotiline treatment is sufficient to restore normal NA concentration in the DSP-4-treated rats, it may promote the sprouting from the lesioned 5-HT axons as a result of neuronal plasticity.

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References

Altar, C.A., 1999. Neurotrophins and depression. Trends Pharmacol. Sci. 20, 59–61.

Aston-Jones, G., Bloom, F.E., 1981. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J. Neurosci. 1, 876–886.

Austin, M.C., Whitehead, R.E., Edgar, C.L., Janosky, J.E., Lewis, D.A., 2002. Localized decrease in serotonin transporter-immunoreactive axons in the prefrontal cortex of depressed subjects committing suicide. Neuroscience 114, 807–815.

Beaufour, C.C., Ballon, N., Le Bihan, C., Hamon, M., Thiebot, M.H., 1999. Effects of chronic antidepressants in an operant conflict procedure of anxiety in the rat. Pharmacol. Biochem. Behav. 62, 591–599.

Cryan, J.F., O'Leary, O.F., Jin, S.H., Friedland, J.C., Ouyang, M., Hirsch, B.R., Page, M.E., Dalvi, A., Thomas, S.A., Lucki, I., 2004. Norepinephrine-deficient mice lack responses to antidepressant drugs, including selective serotonin reuptake inhibitors. Proc. Natl. Acad. Sci. U.S.A. 101, 8186–8191.

Fawcett, J.P., Aloyz, R., McLean, J.H., Pareek, S., Miller, F.D., McPherson, P.S., Murphy, R.A., 1997. Detection of brain-derived neurotrophic factor in a vesicular fraction of brain synaptosomes. J. Biol. Chem. 272, 8837–8840.

Fawcett, J.P., Bamji, S.X., Causing, C.G., Aloyz, R., Ase, A.R., Reader, T.A., McLean, J.H., Miller, F.D., 1998. Functional evidence that BDNF is an anterograde neuronal trophic factor in the CNS. J. Neurosci. 18, 2808–2821.

Frazer, A., 1997. Antidepressants. J. Clin. Psychiatry 58, 9–25.

Fritschy, J.M., Grzanna, R., 1991. Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? Prog. Brain Res. 88, 257–268.

Fritschy, J.M., Grzanna, R., 1992. Restoration of ascending noradrenergic projections by residual locus coeruleus neurons: compensatory response to neurotoxin-induced cell death in the adult rat brain. J. Comp. Neurol. 321, 421–441.

Fuxe, K., Hamberger, B., Hokfelt, T., 1968. Distribution of noradrenaline nerve terminals in cortical areas of the rat. Brain Res. 8, 125–131.

Garcha, G., Smokcum, R.W., Stephenson, J.D., Weeramanthri, T.B., 1985. Effects of some atypical antidepressants on beta-adrenoceptor binding and adenylate cyclase activity in the rat forebrain. Eur. J. Pharmacol. 108, 1–7.

Glennon, R.A., 1990. Serotonin receptors: clinical implications. Neurosci. Biobehav. Rev. 14, 35–47.

Harley, C.W., 1987. A role for norepinephrine in arousal, emotion and learning? Limbic modulation by norepinephrine and the Kety hypothesis. Prog. Neuropsychopharmacol. Biol. Psychiatry 11, 419–458.

Harley, C.W., 1991. Noradrenergic and locus coeruleus modulation of the perforant path-evoked potential in rat dentate gyrus supports a role for the locus coeruleus in attentional and memorial processes. Prog. Brain Res. 88, 307–321.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2000. Age-dependent changes in projections from locus coeruleus to hippocampus dentate gyrus and frontal cortex. Eur. J. Neurosci. 12, 1263–1270.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2001a. Age-dependent changes in noradrenergic innervations of the frontal cortex in F344 rats. Neurobiol. Aging 22, 283–286.

Ishida, Y., Shirokawa, T., Komatsu, Y., Isobe, K., 2001b. Changes in cortical noradrenergic axon terminals of locus coeruleus neurons in aged F344 rats. Neurosci. Lett. 307, 197–199.

Kasamatsu, T., 1991. Adrenergic regulation of visuocortical plasticity: a role of the locus coeruleus system. Prog. Brain Res. 88, 599–616.

Kitayama, I., Nakamura, S., Yaga, T., Murase, S., Nomura, J., Kayahara, T., Nakano, K., 1994. Degeneration of locus coeruleus axons in stress-induced depression model. Brain Res. Bull. 35, 573–580.

Kitayama, I., Yaga, T., Kayahara, T., Nakano, K., Murase, S., Otani, M., Nomura, J., 1997. Long-term stress degenerates, but impramine regenerates, noradrenergic axons in the rat cerebral cortex. Biol. Psychiatry 42, 687–696.

Laifenfeld, D., Klein, E., Ben-Schchar, D., 2002. Norepinephrine alters the expression of genes involved in neuronal sprouting and differentiation: relevance for major depression and antidepressant mechanisms. J. Neurochem. 83, 1054–1064.

Liu, Y., Ishida, Y., Shinoda, K., Nakamura, S., 2003. Interaction between serotonergic and noradrenergic axons during axonal regeneration. Exp. Neurol. 184, 169–178.

Liu, Y., Ishida, Y., Shinoda, K., Furukawa, S., Nakamura, S., 2004. Opposite morphological responses of partially denervated cortical serotonergic and noradrenergic axons to repeated stress in adult rats. Brain Res. Bull. 64, 67–74.

Mamounas, L.A., Altar, C.A., Blue, M.E., Kaplan, D.R., Tessarollo, L., Lyons, W.E., 2000. BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. J. Neurosci. 20, 771–782.

Mongeau, R., Blier, P., de Montigny, C., 1997. The serotonergic and noradrenergic systems of the hippocampus: their interactions and the effects of antidepressant treatments. Brain Res. Brain Res. Rev. 23, 145–195.

Morrison, J.H., Grzanna, R., Molliver, M.E., Coyle, J.T., 1978. The distribution and orientation of noradrenergic fibers in neocortex of the rat: an immunofluorescence study. J. Comp. Neurol. 181, 17–39.

Nakai, K., Niiyama, K., Kasamatsu, T., Naka, Y., Itakura, T., Komai, N., 1994. Regeneration of norepinephrine-containing fibers in occipital cortex of adult cats. Brain Res. Bull. 35, 409–412.

Nakamura, S., 1977. Some electrophysiological properties of neurons of rat locus coeruleus. J. Physiol. 267, 641–658.

Nakamura, S., 1990. Antidepressants induce regeneration of catecholaminergic axon terminals in the rat cerebral cortex. Neurosci. Lett. 111, 64–68.

Nakamura, S., 1991. Axonal sprouting of noradrenergic locus coeruleus neurons following repeated stress and antidepressant treatment. Prog. Brain Res. 88, 587–598.

Nakamura, S., Tepper, J.M., Young, S.J., Groves, P.M., 1981. Neurophysiological consequences of presynaptic receptor activation: changes in noradrenergic terminal excitability. Brain Res. 226, 155–170.

Nakamura, S., Sakaguchi, T., Aoki, F., 1989. Electrophysiological evidence for terminal sprouting of locus coeruleus neurons following repeated mild stress. Neurosci. Lett. 100, 147–152.

Sawyer, S.F., Tepper, J.M., Young, S.J., Groves, P.M., 1985. Antidromic activation of dorsal raphe neurons from neostriatum: physiological characterization and effects of terminal autoreceptor activation. Brain Res. 332, 15–28.

Shirokawa, T., Ishida, Y., Isobe, K., 2000a. Age-dependent changes in axonal branching of single locus coeruleus neurons projecting to two different terminal fields. J. Neurophysiol. 84, 1120–1122.

Shirokawa, T., Ishida, Y., Isobe, K., 2000b. Changes in electrophysiological properties of axon terminals of locus coeruleus neurons with age in F344 rat. Neurosci. Lett. 289, 69–71.

Shirokawa, T., Ishida, Y., Isobe, K., 2003. Age-related changes in the release and uptake activity of presynaptic axon terminals of rat locus coeruleus neurons. Neurosci. Lett. 344, 212–214.

Thoenen, H., 1995. Neurotrophins and neuronal plasticity. Science 270, 593–598.

Van de Kar, L.D., 1991. Neuroendoçrine pharmacology of serotonergic (5-HT) neurons. Annu. Rev. Pharmacol. Toxicol. 31, 289–320.

Vertes, R.P., 1991. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. J. Comp. Neurol. 313, 643–668.

Wang, R.Y., Aghajanian, G.K., 1977. Antidromically identified serotonergic neurons in the rat midbrain raphe: evidence for collateral inhibition. Brain Res. 132, 186–193.

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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.

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

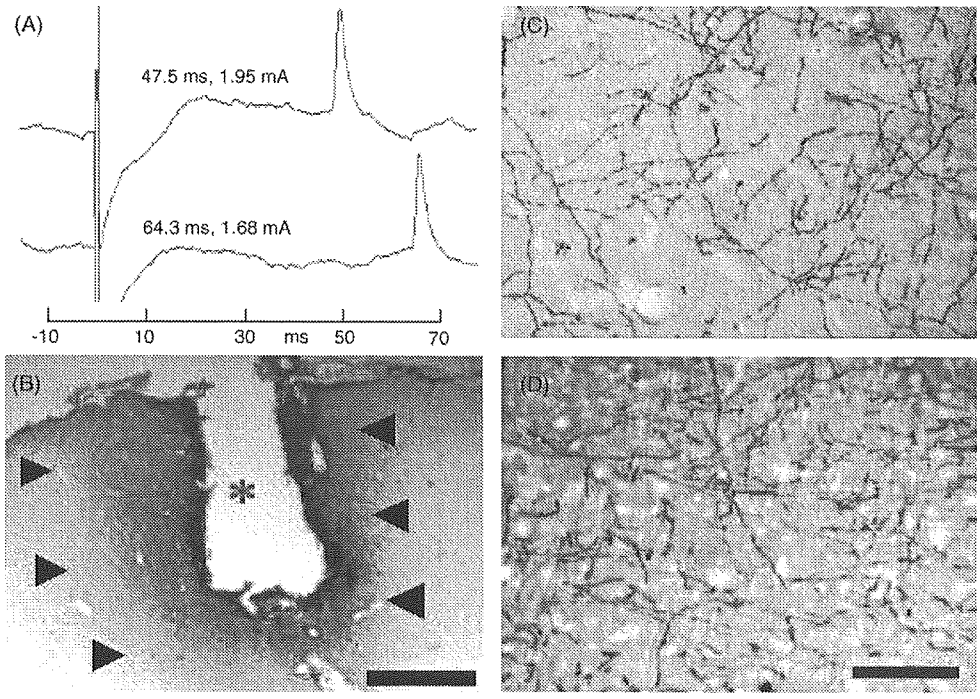

Fig. 1. (A) An extracellular recording of multi-threshold antidromic responses in a single LC neuron in a 13-month-old rat. When the stimulus was adjusted to the minimum current necessary to evoke antidromic responses on every trial (1.68 mA), an antidromic response was evoked at a fixed, discrete latency (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

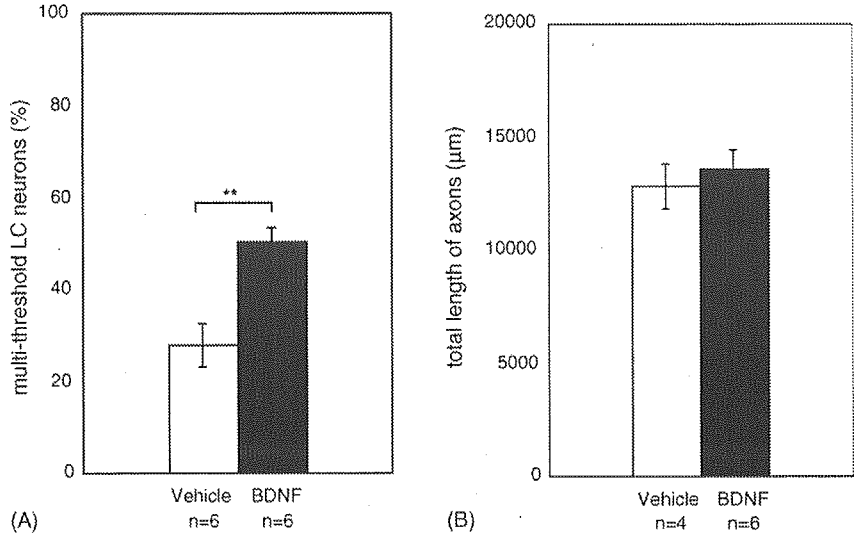

Fig. 2. (A) Effect of BDNF infusion on the percentage of multi-threshold LC neurons in the 13-month-old rats. The percentage of multi-threshold LC neurons obtained in the BDNF-infused group (filled column) was significantly greater than that in the vehicle control group (open column) ($^{**}p < 0.01$). 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).

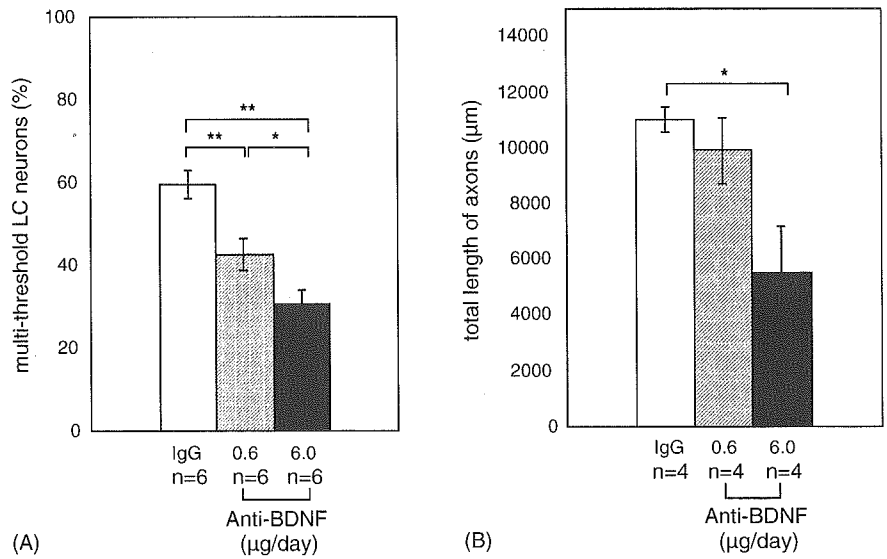

Fig. 3. (A) Effects of anti-BDNF on the percentage of multi-threshold LC neurons in the 19-month-old rats. At the high dose (6 μg/day), the percentage of multi-threshold LC neurons was significantly reduced (filled column), as compared with that in the IgG control group (open column) ($^{**}p < 0.01$). 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.

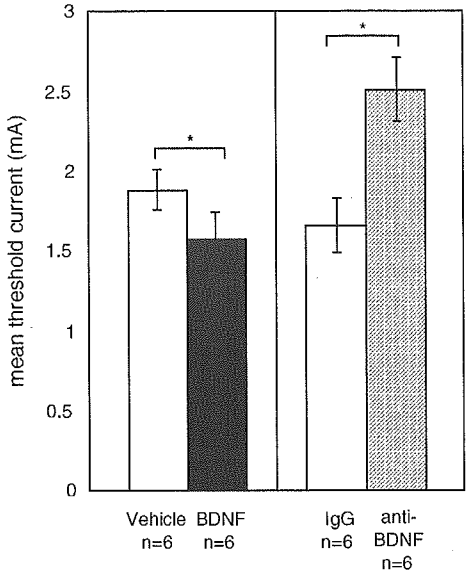

Fig. 4. Opposite effect of BDNF and anti-BDNF on the excitability of LC axon terminals in the aging rats. The mean threshold current in the BDNF-infused group (filled column) was significantly lower than the vehicle control group (open column) ($^{*}p < 0.05$) in the 13-month-old rats (left panel). On 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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References

Aghajanian, G.K., VanderMaelen, C.P., 1982. Alpha 2-adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: intracellular studies in vivo. Science 215, 1394–1396.

Aston-Jones, G., Segal, M., Bloom, F., 1980. Brain aminergic axons exhibit marked variability in conduction velocity. Brain Res. 195, 215–222.

Bamber, N.I., Li, H., Lu, X., Oudega, M., Aebischer, P., Xu, X.M., 2001. Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels. Eur. J. Neurosci. 13, 257–268.

Conner, J.M., Lauterborn, J.C., Yan, Q., Gall, C.M., Varon, S., 1997. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J. Neurosci. 17, 2295–2313.

Egan, T.M., Henderson, G., North, R.A., Williams, J.T., 1983. Noradrenaline-mediated synaptic inhibition in rat locus coeruleus neurons. J. Physiol. 345, 477–488.

Figurov, A., Pozzo-Miller, L.D., Olafsson, P., Wang, T., Lu, B., 1996. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381, 706–709.

Hata, Y., Ohshima, M., Ichisaka, S., Wakita, M., Fukuda, M., Tsumoto, T., 2000. Brain-derived neurotrophic factor expands ocular dominance columns in visual cortex in monocularly deprived and nondeprived kittens but does not in adult cats. J. Neurosci. 20, RC57.

Inoue, A., Sanes, J.R., 1997. Lamina specific connectivity in the brain: regulation by *N*-cadherin, neurotrophins, and glycoconjugates. Science 276, 1428–1431.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2000. Age-dependent changes in projections from locus coeruleus to hippocampus dentate gyrus and frontal cortex. Eur. J. Neurosci. 12, 1263–1270.

Ishida, Y., Shirokawa, T., Komatsu, Y., Isobe, K., 2001a. Changes in cortical noradrenergic axon terminals of locus coeruleus neurons in aged F344 rats. Neurosci. Lett. 307, 197–199.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2001b. Age-dependent changes in noradrenergic innervations of the frontal cortex in F344 rats. Neurobiol. Aging 22, 283–286.

Jiang, B., Akaneya, Y., Ohshima, M., Ichisaka, S., Hata, Y., Tsumoto, T., 2001. Brain-derived neurotrophic factor induces long-lasting potentiation of synaptic transmission in visual cortex in vivo in young rats, but not in the adult. Eur. J. Neurosci. 8, 1219–1228.

Jinnah, H.A., Egami, K., Rao, L., Shin, M., Kasim, S., Hess, E.J., 2003. Expression of c-FOS in the brain after activation of L-type calcium channels. Dev. Neurosci. 25, 403–411.

Liu, Y., Ishida, Y., Shinoda, K., Furukawa, S., Nakamura, S., 2004. Opposite morphological responses of partially denervated cortical serotonergic and noradrenergic axons to repeated stress in adult rats. Brain Res. Bull. 64, 67–74.

McAllister, A.K., Katz, L.C., Lo, D.C., 1999. Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. 22, 295–318.

Matsunaga, Y., Shirokawa, T., Isobe, K., 2004. BDNF is necessary for maintenance of noradrenergic innervations in the aged rat brain. Neurobiol. Aging 25, 341–348.

Nakamura, S., 1977. Some electrophysiological properties of neurons of rat locus coeruleus. J. Physiol. 267, 641–658.

Nakamura, S., Tepper, J.M., Young, S.J., Groves, P.M., 1981. Neurophysiological consequences of presynaptic receptor activation: changes in noradrenergic terminal excitability. Brain Res. 226, 155–170.

Nakamura, S., Sakaguchi, T., Aoki, F., 1989. Electrophysiological evidence for terminal sprouting of locus coeruleus neurons following repeated mild stress. Neurosci. Lett. 100, 147–152.

Numan, S., Lane-Ladd, S.B., Zhang, L., Lundgren, K.H., Russell, D.S., Seroogy, K.B., Nestler, E.J., 1998. Differential regulation of neurotrophin and trk receptor mRNAs in catecholaminergic nuclei during chronic opiate treatment and withdrawal. J. Neurosci. 18, 10700–10708.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, 2nd ed. Academic Press, San Diego.

Sakaguchi, T., Nakamura, S., 1987. The mode of projections of single locus coeruleus neurons to the cerebral cortex in rats. Neuroscience 20, 221–230.

Seguela, P., Watkins, K.C., Geffard, M., Descarries, L., 1990. Noradrenaline axon terminals in adult rat neocortex: an immunocytochemical analysis in serial thin sections. Neuroscience 35, 249–264.

Shirokawa, T., Ishida, Y., Isobe, K., 2000. Age-dependent changes in axonal branching of single locus coeruleus neurons projecting to two different terminal fields. J. Neurophysiol. 84, 1120–1122.

Shirokawa, T., Ishida, Y., Isobe, K., 2003. Age-related changes in the release and uptake activity of presynaptic axon terminals of rat locus coeruleus neurons. Neurosci. Lett. 344, 212–214.

Shores, M.M., White, S.S., Veith, R.C., Szot, P., 1999. Tyrosin hydroxylase MRNA is increased in old age and norepinephrine uptake transporter mRNA is decreased in middle age in locus coeruleus of Brown-Norway rats. Brain Res. 826, 143–147.

Sobreviela, T., Pagcatipunan, M., Kroin, J.S., Mufson, E.J., 1996. Retrograde transport of brain-derived neurotrophic factor (BDNF) following infusion in neo- and limbic cortex in rat: relationship to BDNF mRNA expressing neurons. J. Comp. Neurol. 375, 417–444.

Streppel, M., Azzolin, N., Dohm, S., Guntinas-Lichius, O., Haas, C., Grothe, C., Wevers, A., Neiss, W.F., Angelov, D.N., 2002. Focal application of neutralizing antibodies to soluble neurotrophic factors reduces collateral axonal branching after peripheral nerve lesion. Eur. J. Neurosci. 15, 1327–1342.

Tepper, J.M., Groves, P.M., Young, S.J., 1985. The neuropharmacology of the autoinhibition of monoamine release. Trends Pharmacol. Sci. 6, 251–256.

Washburn, M., Moises, H.C., 1989. Electrophysiological correlates of presynaptic alpha 2-receptor-mediated inhibition of norepinephrine release at locus coeruleus synapses in dentate gyrus. J. Neurosci. 9, 2131–2140.

Zhang, J.Y., Luo, X.G., Xian, C.J., Liu, Z.H., Zhou, X.F., 2000. Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. Eur. J. Neurosci. 12, 4171–4180.

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Involvement of neurotrophic factors in aging of noradrenergic innervations in hippocampus and frontal cortex

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Abstract

In the present study, we investigated the age-dependent changes in the axon terminals of the locus coeruleus (LC) neurons in the frontal cortex and hippocampus, in which a high degree of axonal branching in the middle-aged brain was suggested to occur in our previous electrophysiological study. We used 6-, 13- and 25-month-old male F344/N rats, and performed Western blot analysis of the norepinephrine transporter (NET), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). NET expression level increased in the 13-month-old hippocampus, but was not altered by aging in the frontal cortex. BDNF expression level increased in the hippocampus, but did not change with age in the frontal cortex. On the other hand, GDNF expression level was increased with age in the frontal cortex, but was not in the hippocampus. These results suggest that the LC noradrenergic innervations may be locally regulated by different neurotrophic factors that exert their trophic actions at different target sites.

Keywords: Noradrenergic innervation; Locus coeruleus; BDNF; GDNF; Aging; F344/N rat

1. Introduction

The locus coeruleus (LC) is a densely-packed cluster of noradrenergic neurons in the brain stem (Amaral and Sinnamon, 1977). These neurons innervate widely different target sites, such as the frontal cortex, cerebellum, medulla oblongata and hippocampus (Moore and Bloom, 1979; Morrison et al., 1979; Segal and Bloom, 1976; Swanson and Hartman, 1975). In particular, the LC is known as a major noradrenergic source of the hippocampus (Haring and Davis, 1985; Swanson and Hartman, 1975) and frontal cortex (Morrison et al., 1979). However, it is not clear at present how these multiple innervations of LC neurons are maintained with advancing age.

Our previous electrophysiological study suggested that the LC noradrenergic terminals are maintained during aging in the polymorphic layer of the hippocampus dentate gyrus (PoDG) (Ishida et al., 2000). In the PoDG, the densities of LC axon terminals did not decrease during aging, but the sprouting of LC axon terminals gradually increased between 17 and 25 months of age (Ishida et al., 2000). On the other hand, in the frontal cortex, the LC projections gradually decreased with age (Ishida et al., 2000). The sprouting in the frontal cortex rapidly increased after 15 months of age, and it was maintained at a high level until 25 months of age (Ishida et al., 2000). Thus, these data suggest that the hippocampus PoDG differs from the frontal cortex in the process of aging, despite both sites having the same noradrenergic source originating from the LC. Therefore, we hypothesized that the aging patterns of the LC noradrenergic innervations depend on its terminal sites. In this study, we investigated the age-dependent changes in the norepinephrine transporter (NET) expression in the hippocampus and frontal cortex. NET is located on the noradrenergic presynaptic axon terminals, and it uptakes the released norepinephrine to regulate synaptic activity (Matsuoka et al., 1997). Age-dependent changes were reported for the uptake activity of presynaptic axon terminals of LC neurons (Shirokawa et al., 2003) and for the NET expressions in the LC (Shores et al., 1999). Thus, we examined whether the age-dependent changes in NET expression occur in the terminal areas of LC noradrenergic neurons.

Neurotrophic factors have an important role for neuronal survival or in the formation of axonal branching (Arenas et al.,

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1995; Holm et al., 2002). Previous studies showed that neurotrophic factors are taken from axonal terminals and transported retrogradely (Leitner et al., 1999; Mufson et al., 1994; Sobreviela et al., 1996; Yan et al., 1988). If the axon terminals of LC noradrenergic neurons take up neurotrophic factors at their terminal sites, the different aging patterns of LC innervations between the hippocampus and frontal cortex may be due to the different neurotrophic factors at each terminal site. The brain-derived neurotrophic factor (BDNF) is thought to have a close relationship with the LC noradrenergic system. Previous studies showed that BDNF promotes the survival of noradrenergic neurons (Friedman et al., 1993) and the up-regulation of noradrenaline uptake (Sklair-Tavron and Nestler, 1995). In our recent study, continuous local infusion of BDNF caused an increase in the sprouting of the LC axon terminals in the frontal cortex of aged rats, but the sprouting-enhancing effect was not observed in young or middle-aged rats (Matsunaga et al., 2004). Therefore, we examined whether the glial cell line-derived neurotrophic factor (GDNF) which is known to be distributed in the LC (Choi-Lundberg and Bohn, 1995), changes during aging in the frontal cortex and hippocampus. Some previous studies showed that GDNF enhances noradrenergic innervations (Granholm et al., 2001) and protects LC neurons from 6-hydroxydopamine-induced degeneration (Arenas et al., 1995). Moreover, a relationship between GDNF expression and the aging process of LC neurons was suggested by GDNF heterozygous mice study (Zaman et al., 2003).

In the present study, we considered the NET expression as an index of the density of noradrenergic innervations in the hippocampus and frontal cortex. We first performed Western blot analysis of the hippocampus and frontal cortex to determine the age-dependent changes in NET expression. Next, Western blot analysis was also performed to examine the relationship between the aging of noradrenergic innervations and the expressions of GDNF and BDNF.

2. Materials and methods

2.1. Animals

Six-month-old (young), 13-month-old (middle-aged) and 25-month-old (aged) male F344/N rats were used in this study. They were maintained in a 12 h light:12 h 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 the National Center for Geriatrics and Gerontology.

2.2. Immunohistochemistry

Rats were anesthetized with an overdose of Somnopentyl (100 mg/kg i.p.), and were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C. After postfixation, the brains were immersed in 30% sucrose in 0.01 M phosphate buffered saline (PBS, pH 7.2) overnight at 4 °C. Frozen sections were made using a CM1800 cryotome (Leica, Heerburg, Switzerland) at a thickness of 25 μm. Sections were preincubated with 1% H_2O_2 in PBS for 20 min; then, sections were treated with 0.1% $NaBH_4$ in PBS for 5 min. After preincubation, the sections were incubated with MAB308 monoclonal anti-dopamine beta-hydroxylase (DBH) antibody (Chemicon, Temecula, CA, USA) 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, the sections were incubated with biotinylated horse anti-mouse IgG (dilution 1:400 in PBST, Vector Labs, Burlingame, CA, USA) for 2 h, and were incubated with ABC reagent (dilution 1:200 in PBST, Vector Labs) for 2 h. 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).

2.3. Western blotting

The brain tissues containing the whole hippocampus and frontal cortex were removed from 6-month-old ($n = 4$), 13-month-old ($n = 4$) and 25-month-old ($n = 4$) rats. Tissues were homogenized with a glass homogenizer in a four-fold amount of PBS containing Complete Protease inhibitor tablets (Roche Diagnostics, Tokyo, Japan) on crushed ice. Protein determination was performed using a Proteostain–Protein Quantification Kit-CBB (Dojindo, Kumamoto, Japan), and tissue homogenates were dissolved in electrophoresis sample buffer at a protein concentration of 1 mg/ml, then boiled for 90 s. The protein samples (protein content of 10 μg per lane) were separated on 8% sodium dodecylsulfate (SDS)-polyacrylamido gel for NET and GDNF analysis, and on 18% gel for BDNF analysis. Electroblotting was performed on a polyvinylidene difluoride membrane (pore size = 0.45 μm) in 25 mM Tris and 192 mM glycine containing 20% methanol. The blotted PVDF membrane was pretreated with 5% skim milk and 0.2% bovine serum albumin in Tris-buffered saline containing 0.5% Tween 20 (TBST) overnight at 4 °C. The transferred PVDF membrane was then incubated with polyclonal anti-NET antibody (NET11-A, Alpha Diagnostic, San Antonio, TX, USA; diluted 1:200 in TBST), anti-BDNF antibody (N-20 sc-546, Santa Cruz Biotechnology; diluted 1:2000 in TBST) or anti-GDNF antibody (D-20 sc-328, Santa Cruz Biotechnology; diluted 1:200 in TBST) for 2 h at 37 °C. The PVDF membrane was then further incubated with donkey HRP-labeled anti-rabbit IgG (Amersham Bioscience, Little Chalfont, UK; diluted 1:5000 in TBST) for 2 h at 37 °C. HRP was visualized using the ECL Plus Kit (Amersham Bioscience) according to the manufacturer's protocol. The PVDF membranes of chemiluminescence preparations were scanned with a FAS-1000 Lumino Image Analyzer (Toyobo, Tokyo, Japan). The resulting 2D images were analyzed with a Gel-pro Analyzer Version 4.0 (Media Cybernetics, Silver Spring, USA). Western blotting was performed four to nine times in each experiment, and the relative expression level was given by the mean index of band density × band area ± S.E. The statistical significance of the density was evaluated by one-way ANOVA, and p-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Immunohistochemistry

Fig. 1 shows the DBH-immunopositive fibers in the hippocampus and frontal cortex. In the hippocampus, dense DBH-immunopositive fibers were observed in the PoDG, but only a few DBH-immunopositive fibers were observed in other areas. The density of DBH-immunopositive fibers in the PoDG of 13-month-old rats appeared higher than that of 6- and 25-month-old rats (Fig. 1A, C and E). In the frontal cortex, in contrast, DBH-immunopositive fibers were distributed equally, and no visible differences in the density of DBH-immunopositive fibers were observed among the 6-, 13- and 25-month-old rats (Fig. 1B, D and F).

3.2. Age-dependent changes in NET expression levels

Fig. 2 shows the Western blot analysis of the relative NET expression levels in the hippocampus and frontal cortex. The relative NET expression levels in the whole hippocampus were

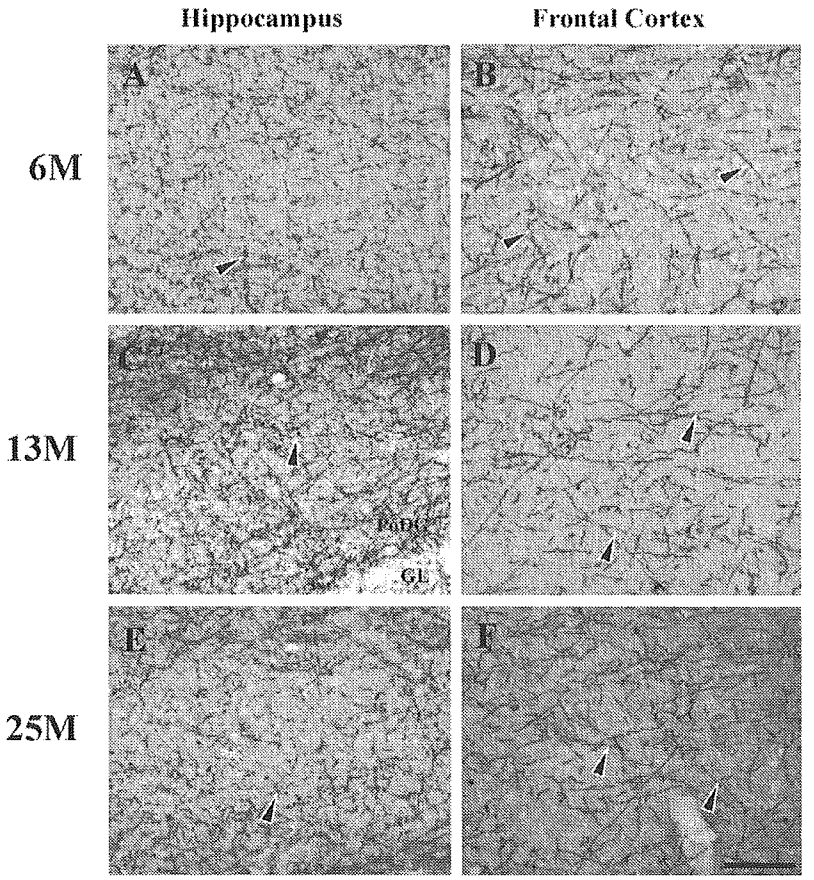

Fig. 1. DBH immunopositive fibers of 6-month-old (A and B), 13-month-old (C and D) and 25-month-old (E and F) rats in the hippocampus (left column) and the frontal cortex (right column). In the hippocampus, dense DBH-positive fibers were observed in the polymorphic dentate gyrus (PoDG, arrowheads), but DBH-positive fibers were very few in the granular layer (GL) or other regions. In 13-month-old PoDG (C), DBH-positive fibers were visibly denser than those in 6-month-old (A) and 25-month-old rats (E). In the frontal cortex, many DBH-positive fibers were observed in each experimental age (arrowheads), and no visible differences were observed in the density of DBH-positive fibers among the experimental ages (B, D and F). Scale bar = 100 μm, magnification = 130×, all images were adjusted for brightness and contrast.

30.27 ± 2.05 in 6-month-old rats, 36.49 ± 1.04 in 13-month-old rats and 33.24 ± 1.01 in 25-month-old rats. The NET expression level in the 13-month-old rats was significantly higher than that in the 6-month-old rats ($n = 4$, $F(2, 9) = 4.61$ $p < 0.05$), but no significant difference was observed between the 13- and 25-month-old rats (Fig. 2A). In the frontal cortex, the NET expression levels were 35.40 ± 1.04 in the 6-month-old rats, 35.61 ± 0.49 in the 13-month-old rats and

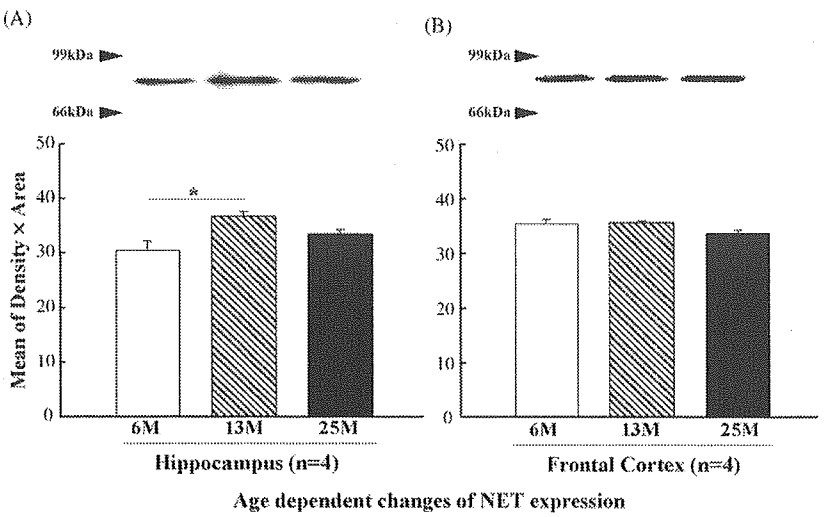

Fig. 2. Western blot analysis of relative NET expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The NET expression levels were shown as the mean density × area of detected protein band ($N = 4$). NET expression levels in the hippocampus increased significantly in the 13-month-old rats (A). However in the frontal cortex, NET expression level was not altered significantly by aging (B). Data are expressed as mean ± S.E. $^{*}p < 0.05$.

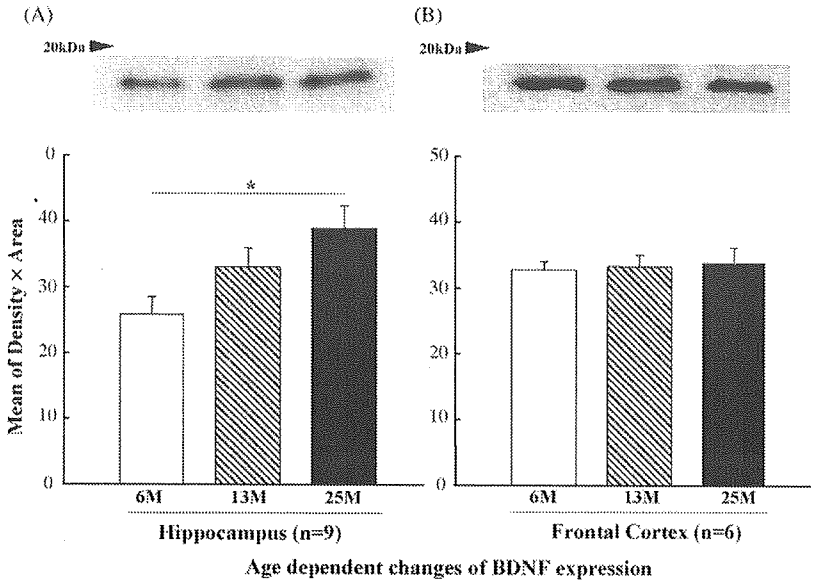

Fig. 3. Western blot analysis of relative BDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The BDNF expression levels were shown as the mean density × area of detected protein band. In the hippocampus, the BDNF expression level of 25-month-old rats was significantly higher than that of 6-month-old rats (A). In the frontal cortex, no significant differences were observed among all the experimental ages (B). Data are expressed as mean ± S.E. $^{*}p < 0.05$.

33.70 ± 0.66 in the 25-month-old rats. There were no significant differences in the NET expression levels between any experimental age groups (Fig. 2B, $n = 4$, $F(2, 9) = 2.50$, $p > 0.10$).

3.3. Age-dependent changes in BDNF and GDNF expression levels

Fig. 3 shows the Western blot analysis of the relative BDNF expression levels in 6-, 13- and 25-month-old rat brains. In the whole hippocampus, the relative BDNF expression levels were 26.47 ± 2.83 in the 6-month-old rats, 33.70 ± 3.22 in the 13-month-old rats and 39.83 ± 3.54 in the 25-month-old rats. The BDNF expression level of the 25-month-old hippocampus was significantly higher than that of the 6-month-old hippocampus (Fig. 3A, $n = 9$, $F(2, 24) = 4.34$, $p < 0.05$). In the frontal cortex, the relative BDNF expression levels were 32.87 ± 1.38 in the 6-month-old rats, 33.33 ± 1.93 in the 13-month-old rats and 33.80 ± 2.63 in the 25-month-old rats. No significant differences were observed between any experimental age groups (Fig. 3B, $n = 6$, $F(2, 15) = 0.05$, $p > 0.10$).

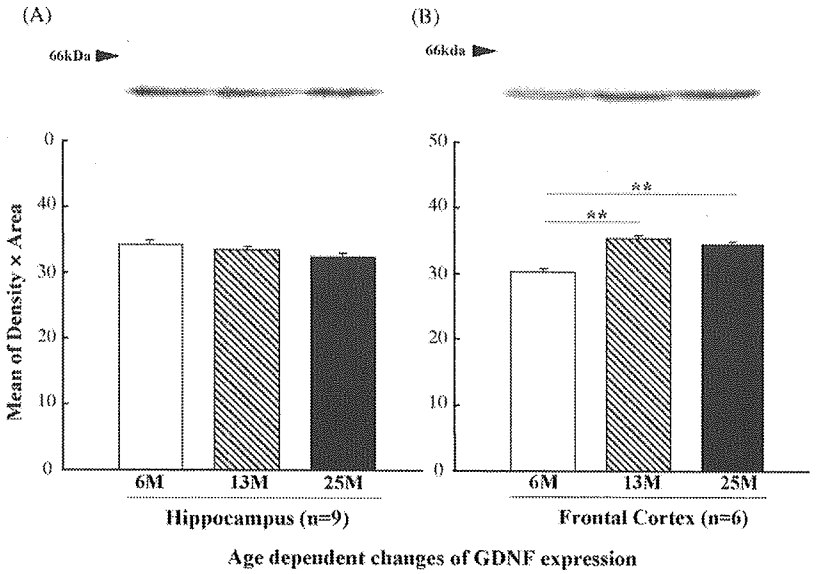

Fig. 4. Western blot analysis of GDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The GDNF expression levels were shown as the mean density × area of detected protein band. The GDNF expression level in the hippocampus did not change with aging (A). On the other hand, in the frontal cortex, the GDNF expression levels of 13- and 25-month-old rats were significantly higher than that of 6-month-old rats (B). Data are expressed as mean ± S.E. $^{**}p < 0.01$.

Fig. 4 shows the Western blot analysis of the relative GDNF expression levels in 6-, 13- and 25-month-old rat brains. The relative GDNF expression levels in the whole hippocampus were 34.24 ± 0.76 in 6-month-old rats, 33.40 ± 0.61 in 13-month-old rats, and 32.36 ± 0.64 in 25-month-old rats, and no significant differences were observed between the experimental age groups (Fig. 4A, $n = 9$, $F(2, 24) = 1.96$, $p > 0.10$). The relative GDNF expression levels in the frontal cortex are shown in Fig. 4B. The expression levels were 30.25 ± 0.62 in 6-month-old rats, 35.31 ± 0.60 in 13-month-old rats and 34.44 ± 0.48 in 25-month-old rats. The GDNF expression levels in the 13- and 25-month-old frontal cortex were significantly higher than that in the 6-month-old frontal cortex (Fig. 4B, $n = 6$, $F(2, 15) = 22.52$, $p < 0.0001$), but no significant difference between that of 13- and 25-month-old rats was observed.

4. Discussion

In the hippocampus, our Western blot analysis indicated that the NET expression level was significantly increased in 13-month-old rats compared with 6-month-old rats. Although the function of this transient increase in the 13-month-old hippocampus is unclear, NET is closely associated with the regulation of noradrenalin reuptake at the axon terminals (Galli et al., 1995). Our previous electrophysiological study suggested that the noradrenergic projection from LC to the hippocampus dentate gyrus is not changed significantly by aging (Ishida et al., 2000). Moreover, the sprouting of LC noradrenergic axons in the dentate gyrus increased rapidly in the middle-aged brain, and sprouting increased continuously until the rats were 24-month-old (Ishida et al., 2000). Thus, we believe that the results of our NET expression analysis correspond with our previous electrophysiological study, and the hippocampal NET expression level is likely to show the maintenance of noradrenergic innervations in the hippocampus of aged brain.

In the frontal cortex, the NET expression level was not altered by aging. This suggests that the aging pattern of cortical LC noradrenergic terminals might be different from that of hippocampal LC noradrenergic terminals. Our electrophysiological study suggested that the noradrenergic projection from LC to the frontal cortex decreases gradually between 7 and 15 months of age (Ishida et al., 2000), and following this decrease, a rapid increase in the sprouting of LC noradrenergic axon terminals occurs in the middle-aged brain (Ishida et al., 2000). This is consistent with our present finding that the density of noradrenergic axons was maintained in the aged brain. This might be an adaptive response to the loss of noradrenergic innervations. The increase in sprouting may be sufficient to maintain a stable noradrenaline level if the synaptic noradrenaline is increased at the sprouted LC axon terminals in the aged brain, and this may account for the stable noradrenaline levels in the frontal cortex during aging (Ishida et al., 2001). Therefore, the present results of NET expression during aging in the frontal cortex are in good agreement with our previous electrophysiological studies, and suggest that noradrenergic activity in the frontal cortex is not impaired during aging.

The target dependency of LC noradrenergic innervations during aging was suggested in our previous study (Shirokawa et al., 2000), and we hypothesized that neurotrophic factors may be associated with this property if they are taken from LC axon terminals retrogradely (Mufson et al., 1994; Yan et al., 1988). The trophic effect of BDNF on LC noradrenergic neurons was previously reported (Friedman et al., 1993). In the present study, we found that the BDNF expression level in the hippocampus was gradually increased by aging, but this increase was not observed in the frontal cortex. It has been reported that the BDNF concentration increases with age in the hippocampus (Katoh-Semba et al., 1998), and our previous study showed that the BDNF expression level in the frontal cortex is not changed significantly by aging (Matsunaga et al., 2004). These results agreed well with the results of our previous study. Another neurotrophic factor, GDNF, was also reported to have trophic effects on survival (Arenas et al., 1995) and axonal sprouting (Holm et al., 2002) of LC noradrenergic neurons in vivo. As GDNF mRNA is expressed at high levels in the LC (Choi-Lundberg and Bohn, 1995), and as GDNF heterozygous mice show morphological abnormalities of LC noradrenergic innervations in the frontal cortex (Zaman et al., 2003), it is likely that GDNF also plays a trophic role in the maintenance of LC noradrenergic innervations in the aging brain (Granholm et al., 2001; Ishida et al., 2000). In fact, in the frontal cortex, GDNF expression level significantly increased between 6 and 13 months of age, but no significant change was observed in the hippocampus.

Therefore, it is reasonable to assume that LC noradrenergic innervations are regulated by different neurotrophic factors: BDNF is involved in the hippocampus and GDNF in the frontal cortex. This notion may be partly supported by our recent finding that the intracortical infusion of BDNF has no trophic action on noradrenergic axons in the middle-aged brain (Matsunaga et al., 2004). In the hippocampus PoDG, our previous electrophysiological study showed that the LC noradrenergic axonal sprouting gradually increases between 7 and 24 months of age (Ishida et al., 2000), and our present Western blotting analysis of the hippocampus revealed that the BDNF expression level gradually increased between 6 and 25 months of age. On the other hand, in the frontal cortex, LC axonal sprouting rapidly increases in middle age (Ishida et al., 2000), and our present analysis of the GDNF expression level also showed a similar aging pattern. Thus, we conclude that LC noradrenergic innervations are maintained by BDNF in the hippocampus and GDNF in the frontal cortex.

In the hippocampus, BDNF mRNA expression was observed in all cell layers (Smith et al., 1995), and GDNF mRNA expression was also widely localized in the cerebral cortex (Pochon et al., 1997). Therefore, it is difficult to specify the type of cells target on the noradrenergic axon terminals. However, GFRalpha-2 receptor mRNA expression was not observed in dentate gyrus but was observed in the cortex (Burazin and Gundlach, 1999), and this difference in distribution of GDNF receptors is consistent with our present results.

In conclusion, the age-dependent changes in LC noradrenergic innervations are different between the hippocampus and

the frontal cortex despite both regions originating from the same noradrenergic source. Moreover, noradrenergic activities in the hippocampus and in the frontal cortex were not impaired in aged brain. Therefore, the difference in noradrenergic innervations with age between the hippocampus and the frontal cortex might be due to the age-related changes in the expression of neurotrophic factors for each terminal area: BDNF for the hippocampus, and GDNF for the frontal cortex.

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References

Amaral, D.G., Sinnamon, H.E., 1977. The locus coeruleus: neurobiology of a central noradrenergic nucleus. Prog. Neurobiol. 9, 147–196.

Arenas, E., Trupp, M., Akerud, P., Ibanez, C.F., 1995. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron 15, 1465–1473.

Burazin, T.C., Gundlach, A.L., 1999. Localization of GDNF/neurturin receptor (c-ret, GFRalpha-1 and alpha-2) mRNAs in postnatal rat brain: differential regional and temporal expression in hippocampus, cortex and cerebellum. Brain Res. Mol. Brain Res. 73, 151–171.

Choi-Lundberg, D.L., Bohn, M.C., 1995. Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in the rat. Brain Res. Dev. Brain Res. 85, 80–88.

Friedman, W.J., Ibanez, C.F., Hallbook, F., Persson, H., Cain, L.D., Dreyfus, C.F., Black, I.B., 1993. Differential actions of neurotrophins in the locus coeruleus and basal forebrain. Exp. Neurol. 119, 72–78.

Galli, A., DeFelice, L.J., Duke, B.J., Moore, K.R., Blakely, R.D., 1995. Sodium-dependent norepinephrine-induced currents in norepinephrine-transporter-transfected HEK-293 cells blocked by cocaine and antidepressants. J. Exp. Biol. 198, 2197–2212.

Granholm, A.C., Helt, C., Srivastava, N., Backman, C., Gerhardt, G.A., 2001. Effects of age and GDNF on noradrenergic innervation of the hippocampal formation: studies from intraocular grafts. Microsc. Res. Tech. 54, 298–308.

Haring, J.H., Davis, J.N., 1985. Differential distribution of locus coeruleus projections to the hippocampal formation: anatomical and biochemical evidence. Brain Res. 325, 366–369.

Holm, P.C., Akerud, P., Wagner, J., Arenas, E., 2002. Neurturin is a neuritogenic but not a survival factor for developing and adult central noradrenergic neurons. J. Neurochem. 81, 1318–1327.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2000. Age-dependent changes in projections from locus coeruleus to hippocampus dentate gyrus and frontal cortex. Eur. J. Neurosci. 12, 1263–1270.

Ishida, Y., Shirokawa, T., Miyaishi, O., Komatsu, Y., Isobe, K., 2001. Age-dependent changes in noradrenergic innervations of the frontal cortex in F344 rats. Neurobiol. Aging 22, 283–286.

Katoh-Semba, R., Semba, R., Takeuchi, I.K., Kato, K., 1998. Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3. Neurosci. Res. 31, 227–234.

Leitner, M.L., Molliver, D.C., Osborne, P.A., Vejsada, R., Golden, J.P., Lampe, P.A., Kato, A.C., Milbrandt, J., Johnson Jr., E.M., 1999. Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin suggests that in vivo signaling for the GDNF family is GFRalpha coreceptor-specific. J. Neurosci. 19, 9322–9331.

Matsuoka, I., Kumagai, M., Kurihara, K., 1997. Differential and coordinated regulation of expression of norepinephrine transporter in catecholaminergic cells in culture. Brain Res. 776, 181–188.

Matsunaga, W., Shirokawa, T., Isobe, K., 2004. BDNF is necessary for maintenance of noradrenergic innervations in the aged rat brain. Neurobiol. Aging 25 (3), 341–348.

Moore, R.Y., Bloom, F.E., 1979. Central cateholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annu. Rev. Neurosci. 2, 113–168.

Morrison, J.H., Molliver, M.E., Grzanna, R., 1979. Noradrenergic innervation of cerebral cortex: widespread effect of local cortical lesions. Science 205, 313–316.

Mufson, E.J., Kroin, J.S., Sobreviela, T., Burke, M.A., Kordower, J.H., Penn, R.D., Miller, J.A., 1994. Intrastriatal infusions of brain-derived neurotrophic factor: retrograde transport and colocalization with dopamine containing *Substantia nigra* neurons in rat. Exp. Neurol. 129, 15–26.

Pochon, N.A., Menoud, A., Tseng, J.L., Zurn, A.D., Aebischer, P., 1997. Neuronal GDNF expression in the adult rat nervous system identified by in situ hybridization. Eur. J. Neurosci. 9, 463–471.

Segal, M., Bloom, F.E., 1976. The action of norepinephrine in the rat hippocampus. IV. The effects of locus coeruleus stimulation on evoked hippocampal unit activity. Brain Res. 107, 513–525.

Shirokawa, T., Ishida, Y., Isobe, K., 2000. Age-dependent changes in axonal branching of single locus coeruleus neurons projecting to two different terminal fields. J. Neurophysiol. 84, 1120–1122.

Shirokawa, T., Ishida, Y., Isobe, K., 2003. Age-related changes in the release and uptake activity of presynaptic axon terminals of rat locus coeruleus neurons. Neurosci. Lett. 344, 212–214.

Shores, M.M., White, S.S., Veith, R.C., Szot, P., 1999. Tyrosine hydroxylase mRNA is increased in old age and norepinephrine uptake transporter mRNA is decreased in middle age in locus coeruleus of Brown-Norway rats. Brain Res. 826, 143–147.

Sklair-Tavron, L., Nestler, E.J., 1995. Opposing effects of morphine and the neurotrophins, NT-3, NT-4, and BDNF, on locus coeruleus neurons in vitro. Brain Res. 702, 117–125.

Smith, M.A., Makino, S., Kvetnansky, R., Post, R.M., 1995. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J. Neurosci. 15, 1768–1777.

Sobreviela, T., Pagcatipunan, M., Kroin, J.S., Mufson, E.J., 1996. Retrograde transport of brain-derived neurotrophic factor (BDNF) following infusion in neo- and limbic cortex in rat: relationship to BDNF mRNA expressing neurons. J. Comp. Neurol. 375, 417–444.

Swanson, L.W., Hartman, B.K., 1975. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J. Comp. Neurol. 163, 467–505.

Yan, Q., Snider, W.D., Pinzone, J.J., Johnson Jr., E.M., 1988. Retrograde transport of nerve growth factor (NGF) in motoneurons of developing rats: assessment of potential neurotrophic effects. Neuron 1, 335–343.

Zaman, V., Li, Z., Middaugh, L., Ramamoorthy, S., Rohrer, B., Nelson, M.E., Tomac, A.C., Hoffer, B.J., Gerhardt, G.A., Granholm, A.C.H., 2003. The noradrenergic system of aged GDNF heterozygous mice. Cell Transplant 12, 291–303.