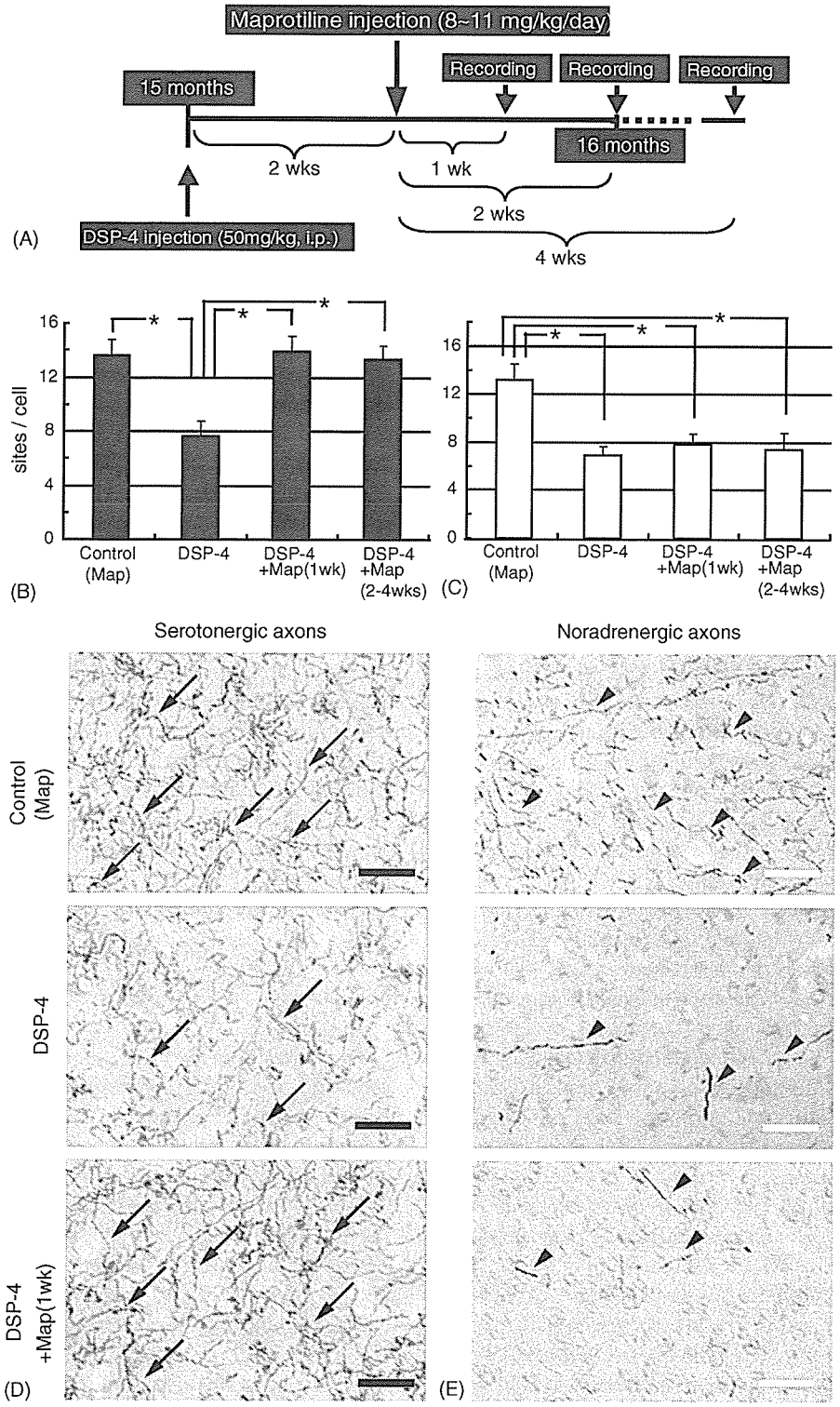

Fig. 4. (A) Chronic antidepressant treatments after DSP-4 treatment. Fifteen-month-old rats were treated with DSP-4. Two weeks later, maprotiline, a noradrenaline uptake inhibitor, was administered for 1, 2, or 4 weeks. Effects of a specific neurotoxin for NA (DSP-4) and maprotiline (Map, a NA uptake 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. Neuroendocrine 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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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

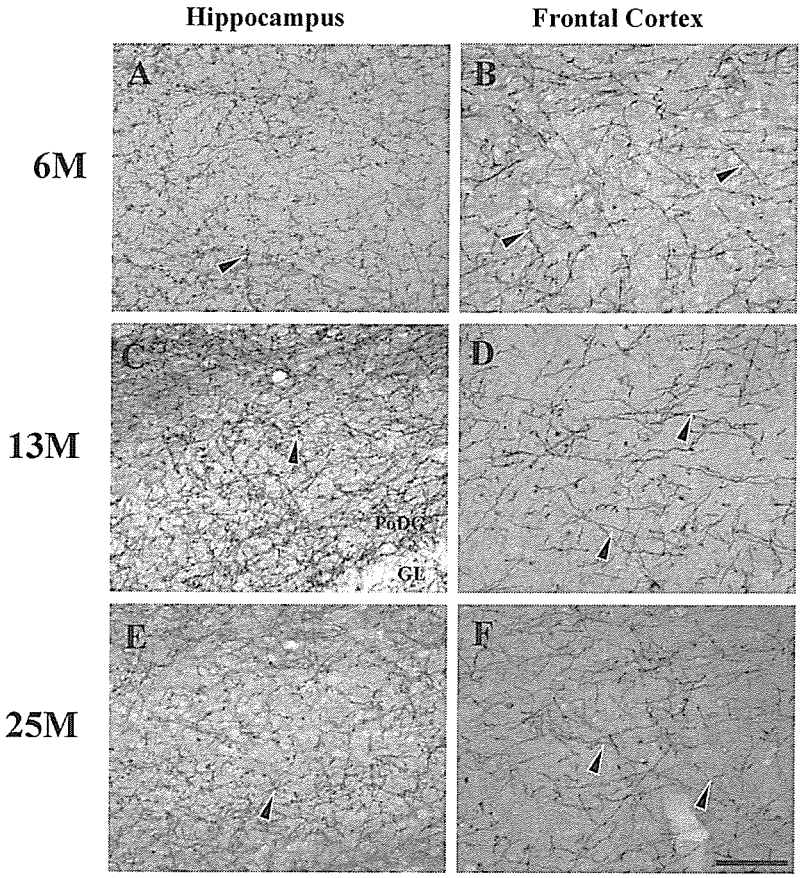

Fig. 1. DBH immunopositive fibers of 6-month-old (A and B), 13-month-old (C and D) and 25-month-old (E and F) rats in the hippocampus (left column) and the frontal cortex (right column). In the hippocampus, dense DBH-positive fibers were observed in the polymorphic dentate gyrus (PoDG, arrowheads), but 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

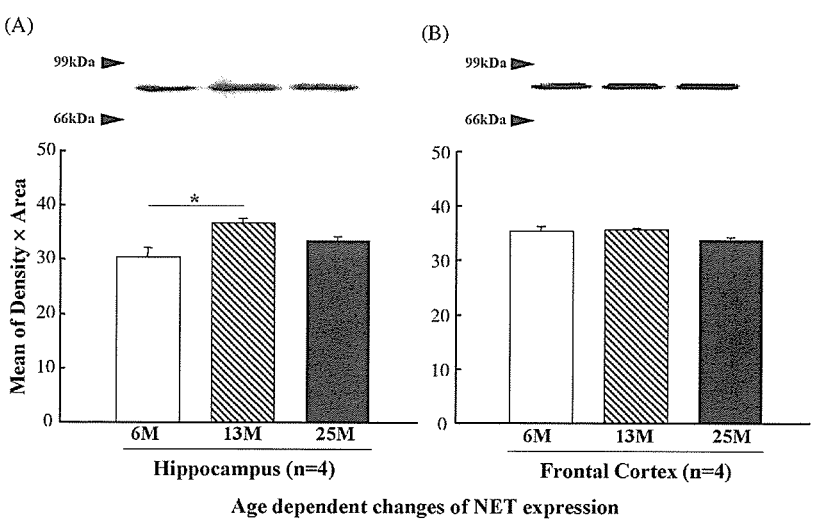

Fig. 2. Western blot analysis of relative NET expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The NET expression levels were shown as the mean density × area of detected protein band ($N = 4$). NET expression levels in the hippocampus increased significantly 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. $^{*}p < 0.05$.

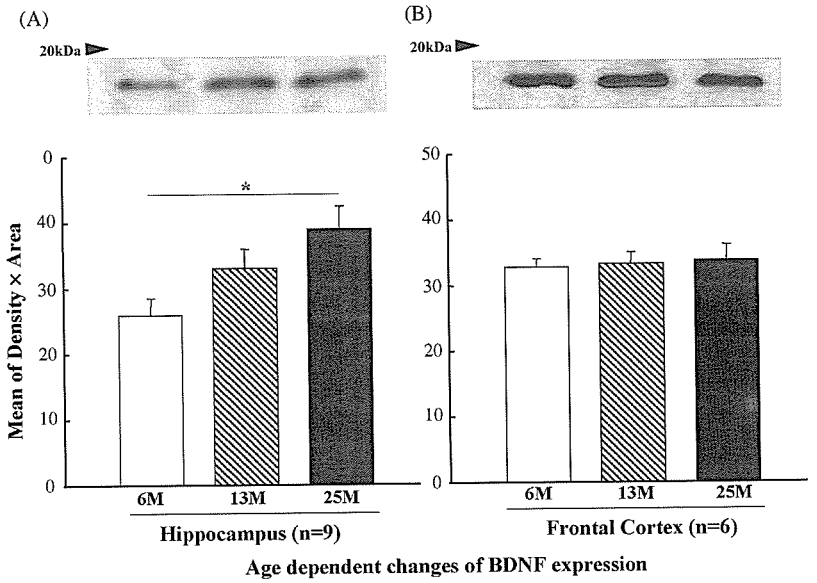

Fig. 3. Western blot analysis of relative BDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The BDNF expression levels were shown as the mean density × area of detected protein band. In the hippocampus, the BDNF expression level of 25-month-old 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, $n = 6$, $F(2, 15) = 0.05$, $p > 0.10$).

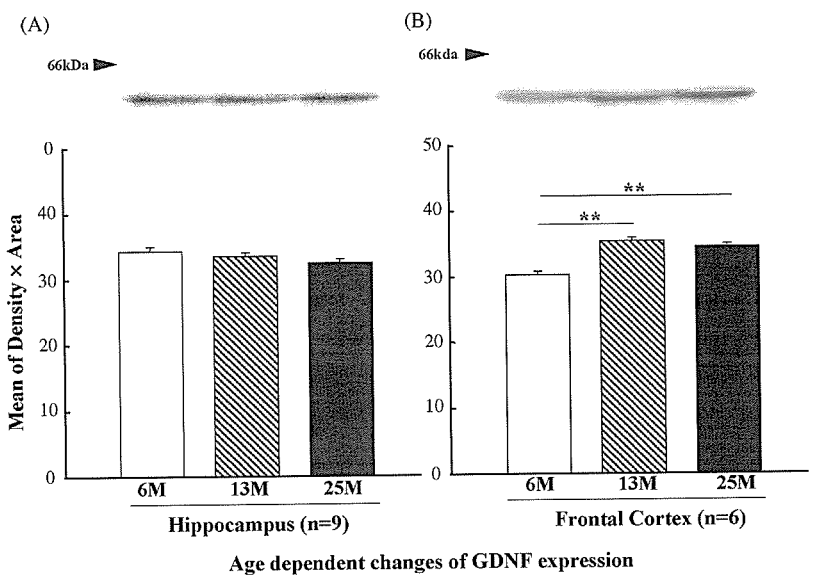

Fig. 4. Western blot analysis of GDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The GDNF expression levels were shown as the mean density × area of detected protein band. The GDNF expression level in the hippocampus did not change with aging (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.