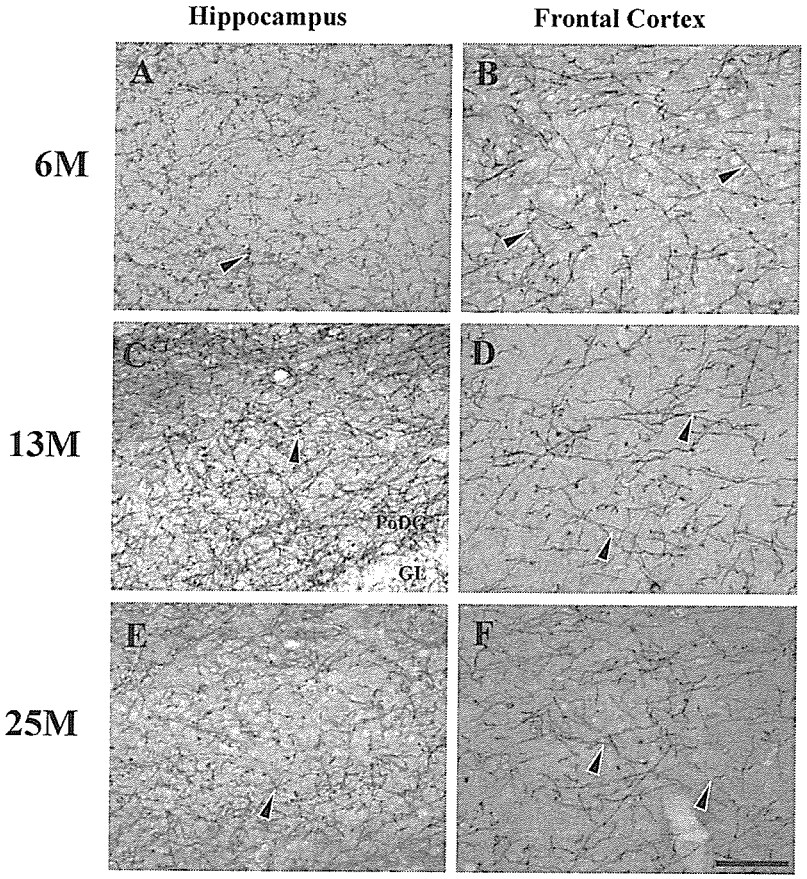

Fig. 1. DBH immunopositive fibers of 6-month-old (A and B), 13-month-old (C and D) and 25-month-old (E and F) rats in the hippocampus (left column) and the frontal cortex (right column). In the hippocampus, dense DBH-positive fibers were observed in the polymorphic dentate gyrus (PoDG, arrowheads), but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

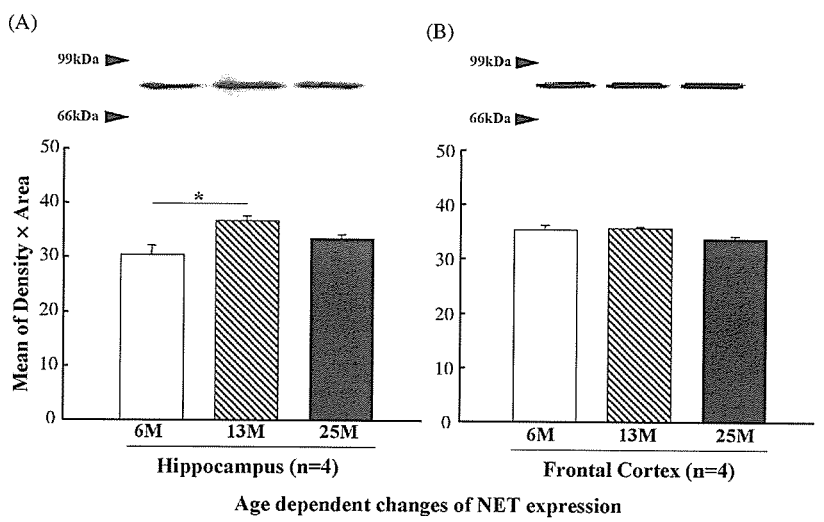

Fig. 2. Western blot analysis of relative NET expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The NET expression levels were shown as the mean density × area of detected protein band ($N = 4$). NET expression levels in the hippocampus increased significantly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

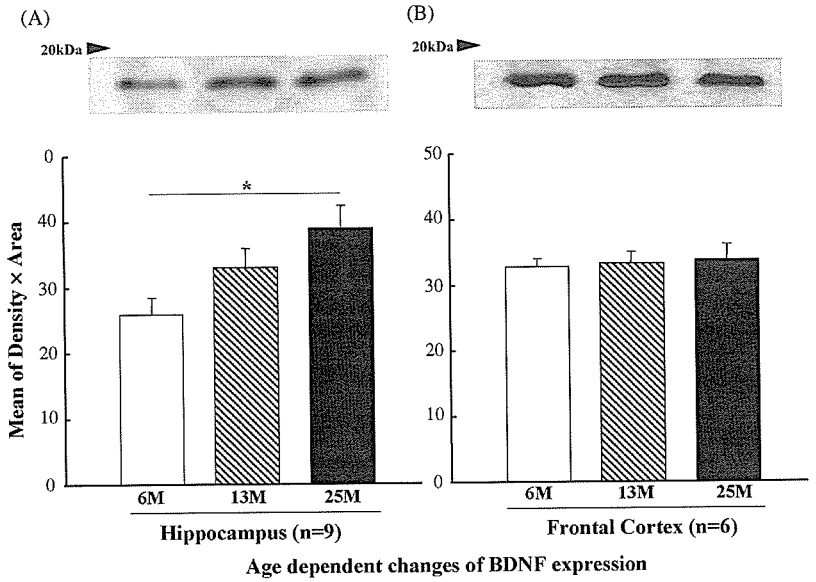

Fig. 3. Western blot analysis of relative BDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The BDNF expression levels were shown as the mean density × area of detected protein band. In the hippocampus, the BDNF expression level of 25-month-old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

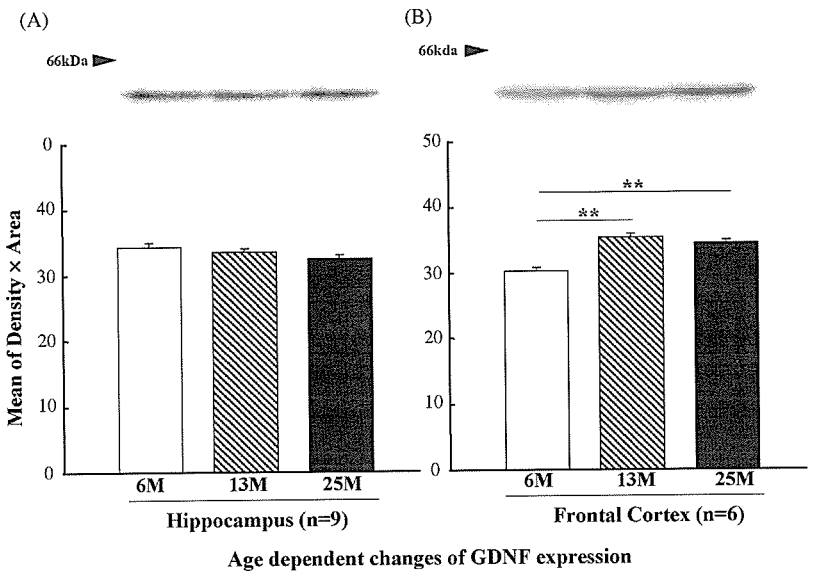

Fig. 4. Western blot analysis of GDNF expression levels in the hippocampus (A) and the frontal cortex (B) of 6-, 13- and 25-month-old rats. The GDNF expression levels were shown as the mean density × area of detected protein band. The GDNF expression level in the hippocampus did not change with aging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