

The Noradrenergic System of Aged GDNF Heterozygous Mice

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Glial cell line-derived neurotrophic factor (GDNF) is a trophic factor for noradrenergic (NE) neurons of the pontine nucleus locus coeruleus (LC). Decreased function of the LC-NE neurons has been found during normal aging and in neurodegenerative disorders. We have previously shown that GDNF participates in the differentiation of LC-NE neurons during development. However, the continued role of GDNF for LC-NE neurons during maturation and aging has not been addressed. We examined alterations in aged mice that were heterozygous for the GDNF gene (*Gdnf*^{+/-}). Wild-type (*Gdnf*^{+/+}) and *Gdnf*^{+/-} mice (18 months old) were tested for locomotor activity and brain tissues were collected for measuring norepinephrine levels and uptake, as well as for morphological analysis. Spontaneous locomotion was reduced in *Gdnf*^{+/-} mice in comparison with *Gdnf*^{+/+} mice. The reduced locomotor activity of *Gdnf*^{+/-} mice was accompanied by reductions in NE transporter activity in the cerebellum and brain stem as well as decreased norepinephrine tissue levels in the LC. Tyrosine hydroxylase (TH) immunostaining demonstrated morphological alterations of LC-NE cell bodies and abnormal TH-positive fibers in the hippocampus, cerebellum, and frontal cortex of *Gdnf*^{+/-} mice. These findings suggest that the LC-NE system of *Gdnf*^{+/-} mice is impaired and suggest that GDNF plays an important role in continued maintenance of this neuronal system throughout life.

Key words: Locus coeruleus; Noradrenergic system; Glial cell line-derived neurotrophic factor (GDNF); Aging; Neurotrophic factors

INTRODUCTION

The aging process affects the integrity of the central nervous system (CNS) pathways and these alterations might underlie the onset and progression of various neurological and neurodegenerative disorders. The pontine nucleus locus coeruleus (LC), in the upper part of the floor of the fourth ventricle, the largest group of noradrenergic (NE) neurons, is affected by neurodegenerative diseases associated with aging (8,18). Further, the normal aging process produces significant alterations in the function and morphology of LC-NE neurons. The total number and size of LC neurons, as well as the volume of this nucleus, are inversely correlated with age in humans (44), with neuronal loss greater in rostral than in caudal cells (48). Electrophysiological studies suggest that axonal branching of noradrenergic neurons projecting from the LC to the frontal cortex and dentate gyrus is increased with aging (60). It was proposed that this axonal branching might be a compensatory response (i.e., a reactive collateral sprouting) for the loss of nor-

adrenergic projection neurons to these areas (32), an interpretation consistent with the significant neuroplasticity attributed to the LC-NE system (62). In addition, previous studies have also shown a significant downregulation in both α - and β -adrenoceptor postsynaptic responses to norepinephrine in the hippocampus and cerebellum during aging (13,20). Collectively, these studies suggest that the LC-NE transmitter system undergoes significant alterations both pre- and postsynaptically during the normal aging process.

Neurotrophic factors play an important role in the survival and differentiation of neurons during development, as well as in activity-dependent plasticity and survival following neuronal injury in the adult CNS (1,22, 30,58). Age-related changes in trophic factor levels may be one of the underlying causes of behavioral alterations and neural degeneration observed during senescence (1,5, 41,44). Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor- β superfamily of neurotrophic factors (40,58). GDNF has profound effects on the survival of injured dopaminergic

neurons and has primarily been characterized as a neurotrophic factor for CNS dopaminergic neurons (7,42,56, 63). Interestingly, studies from our laboratory and others have shown that absence of this trophic factor in GDNF null mutant mice (*Gdnf*^{-/-}) does not impair the initial birth or early differentiation of dopaminergic neurons (52, 56,59). However, a significant reduction in the packing density of LC-NE neurons was found at birth in *Gdnf*^{-/-} mice (21). Many other reports also indicate that GDNF has pronounced effects on both developing and mature neuronal populations utilizing norepinephrine as well as other transmitters (2,21,51). These collective studies suggest that LC-NE neurons depend upon GDNF for developmental as well as maintenance of function, but the role of endogenous GDNF in the intact mature nervous system has not been explored.

Because *Gdnf*^{-/-} mice do not survive after birth due to agenesis of the kidneys (56), it is impossible to study postnatal maturation using this null mutation. Therefore, we chose a mouse model with partial deletion of the GDNF gene (*Gdnf*^{+/-}) to study the role of GDNF in the LC-NE system of 18-month-old animals. Due to the reported effects (see above) of GDNF upon both substantia nigra dopamine and LC-NE neurons, we explored effects of the GDNF genotype on locomotor activity, because both of these transmitter systems are known to affect this behavioral parameter. As LC neurons project to frontal cortex, hippocampus, and cerebellum, we evaluated the pattern of monoamine innervation in these areas in the two groups of mice. Thus, the aim of the present study was to explore whether partial deletion of the GDNF gene (*Gdnf*^{+/-}) would alter locomotor activity, LC-NE morphology, norepinephrine levels, noradrenergic transporter (NET) activity, or target innervation pattern in aged mice.

MATERIALS AND METHOD

Animals

GDNF heterozygous (*Gdnf*^{+/-}) mice used in the present study were obtained from the National Institute of Health (Bethesda, MD). A colony was established at the Medical University of South Carolina. For the present study, aged male *Gdnf*^{+/-} mice (18 months old) were studied along with naive aged-matched male C57BL/6 *Gdnf*^{+/+} mice, the background strain for the *Gdnf*^{+/-} line. The animals in the control group were littermates of the *Gdnf*^{+/-} mice.

In constructing the *Gdnf*^{+/-} line, the third exon that encodes the GDNF protein was replaced with a cassette expressing the selectable marker neomycin phosphotransferase to generate a nonfunctional allele of the GDNF gene. Genotyping was completed via standard PCR analysis. Details of these procedures have previously been re-

ported (23,56). All experiments were performed under NIH ethical guidelines, and protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

Motor Activity Assessment

Gdnf^{+/+} and *Gdnf*^{+/-} mice (18 months of age) were used to evaluate the effects of partial GDNF gene deletion on motor activity. Locomotor activity (total distance traveled) and vertical activity were assessed in a Digiscan Animal Activity Monitor system for 1 h [Omnitech Electronics Model RXYZCM (8) TAO, Columbus, OH]. The details of the apparatus have been described previously (25). On the day of testing, the mice were transferred from the vivarium to the laboratory in groups of six and tested immediately in a darkened environment. Data were collected in 15-min intervals for 1 h. Because data were characterized as having increases in group error variance proportional to increases in group means, they were transformed to natural log units to meet the assumption of homogeneity of variance across groups prior to analysis of variance (ANOVA) [see (11)]. Both data sets were subjected to a 2 (Genotype Group) × 4 (15-min Interval) ANOVA with the latter being a repeated measures factor.

Tissue Dissection

Mice were anesthetized and decapitated. Brains were removed and sliced sagittally along the midline. One half of the brain was immersed for 48 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for morphological studies. The other half of the brain was dissected and tissue pieces containing locus coeruleus (LC), cerebellum, and frontal cortex were collected in preweighed microcentrifuge tubes. For the LC, the landmarks were: the lateral fold of the fourth ventricle and 1 mm ventral of the floor of the fourth ventricle; for the cerebellum, only cortical matter excluding the deep cerebellar nuclei was dissected. The tubes were weighed immediately and kept on dry ice before transferring into a -70°C freezer for norepinephrine level measurements by high performance liquid chromatography (HPLC). The brain stem and cerebellum were collected in ice-cold 0.32 M sucrose solution for NE uptake analysis.

HPLC for Norepinephrine Measurements

The tissue levels of norepinephrine in frontal cortex, brain stem containing the LC, and cerebellum were measured using our previously described HPLC method (26). Norepinephrine values were calculated as total nanogram per gram wet weight (ng/g) of tissue. Data were analyzed via ANOVA with genotype as the between-groups factor.

Norepinephrine Transporter (NET): Preparation of Synaptosomes and [³NE] Uptake Assay

Cerebellum and brain stem tissues were dissected and homogenized in ice-cold 0.32 M sucrose at 2000 rpm using a polytron homogenizer and centrifuged at 1000 × *g* for 10 min. The resulting supernatant was centrifuged at 12,500 × *g* for 20 min and the pellet containing the crude synaptosomes was suspended in 0.32 M sucrose. Protein content was determined by the BCA method (Bio-Rad, Hercules, CA) and the tissue was immediately used for norepinephrine uptake assays. For uptake experiments, synaptosomal fractions of 40–80 µg from each group (pooled brain tissue from two animals) were incubated in 0.5 ml Krebs-Ringers-HEPES (KRH) medium (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM HEPES, pH 7.4) containing 1.8 g/L glucose, 100 µM pargyline (Sigma, St. Louis, MO), and 100 µM ascorbic acid (Sigma) with or without nisoxetine (100 nM) for 5 min at 37°C followed by addition of 25 nM 1-[7,8-³H]noradrenaline (Amersham Pharmacia Biotech, Piscataway, NJ; specific activity 28 Ci/mmol) for 3 min. Quantitative studies demonstrate that uptake is linear up to 6 min of incubation (data not shown). We therefore monitored uptake for 3 min. Transport was terminated by rapid filtration (Brandel) over GF/B glass fiber filters (Whatman, Clifton, NJ) presoaked in 0.3% polyethyleneimine (Sigma). Filters were washed in ice-cold PBS and accumulated radioactivity was measured by liquid scintillation counting (Beckman). Assays were performed in triplicate. Non-specific [³H]norepinephrine uptake was defined as the accumulation in the presence of nisoxetine, and these data were subtracted from total counts to yield NET specific uptake. Statistical significance was evaluated using ANOVA.

Immunohistochemistry

Brains were immersion fixed in 4% paraformaldehyde for 48 h at 4°C. They were washed in 0.1 M PB and transferred into 30% sucrose in PB. Serial coronal sections of 45-µm thickness were cut on a cryostat and free-floating sections were collected in 0.1 M PB in 24-well plates. Every 12th section was stained with cresyl violet (Sigma) to visualize brain morphology. Every sixth section from the level of the LC, frontal cortex, hippocampus, and cerebellum was processed for tyrosine hydroxylase (TH, Pel-Freez, Rogers, AR) immunohistochemistry using the ABC method. For TH immunostaining, our standard protocol was followed as described below. Free-floating sections were treated with Tris buffer saline (TBS, 0.01 M, pH 7.4), methanol, and H₂O₂ mixture (7:2:1) for 15 min and, after washing in TBS, sections were treated with 0.1 M sodium *m*-periodate in TBS for

20 min. Sections were washed in TBS with 0.25% Triton X-100 (TBST) and blocked with 10% normal goat serum (NGS, Sigma) in TBST for 30 min to block non-specific binding. Sections were incubated in polyclonal antibody against TH (1:1000) for 48 h at 4°C. After washing the sections in TBST with 3% NGS, sections were treated with biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA, 1:200) for 1 h followed by incubation in avidin-biotin complex (Elite ABC kit, Vector Labs). The reaction was developed with 3,3'-diaminobenzidine (DAB, Sigma) with H₂O₂ (0.5 µl/ml) in imidazole acetate buffer (0.01 M imidazole and 0.05 M sodium acetate) and intensified by adding nickel ammonium sulfate (0.025 g/ml solution). Immunostained sections were mounted on gelatin-coated glass slides, dehydrated, cleared in xylene, and coverslipped using Permount.

RESULTS

Assessment of Spontaneous Locomotor Activity

Both total distance (Fig. 1A) and vertical activity (Fig. 1B) were reduced for *Gdnf*^{+/-} mice in comparison with *Gdnf*^{+/+} controls. ANOVAs on these data demonstrated significant effects of genotype on both total distance, $F(1, 12) = 8.024$, $p < 0.01$, and vertical activity, $F(1, 12) = 8.192$, $p < 0.01$. Although inspection of means for the two genotypes across 15-min intervals suggests that activity declined more rapidly for the *Gdnf*^{+/-} mice than for *Gdnf*^{+/+} mice over the 60-min testing period, an apparent difference was not supported by a significant genotype × time interaction.

Norepinephrine Levels and Noradrenergic Transporter Activity

Norepinephrine levels in brain stem tissue containing the LC were significantly lower ($p < 0.05$) in 18-month-old *Gdnf*^{+/-} mice than in age-matched *Gdnf*^{+/+} mice (mean ± SEM; 132 ± 35, $n = 7$ for the *Gdnf*^{+/-} vs. 280 ± 34, $n = 10$ for the *Gdnf*^{+/+} groups, respectively) (Fig. 2). Note that norepinephrine levels in *Gdnf*^{+/-} mice were reduced to less than half (47%) of the levels seen in age-matched controls. Similar reductions were not observed in frontal cortex (1115 ± 83, $n = 6$ in *Gdnf*^{+/+}, and 1195 ± 13, $n = 3$ in *Gdnf*^{+/-}) or cerebellum (161 ± 13, $n = 10$ in *Gdnf*^{+/+}, and 155 ± 22, $n = 7$ in *Gdnf*^{+/-}) (Fig. 2).

Norepinephrine uptake data from synaptosome preparations are summarized in Figure 3. In comparison to *Gdnf*^{+/+} mice, norepinephrine uptake into crude synaptosomes prepared from aged *Gdnf*^{+/-} mice was significantly decreased in both cerebellum (39%, $p < 0.05$) and in dorsal brain stem tissue containing the LC (38%, $p < 0.05$). The synaptosomal uptake reduction in brain stem

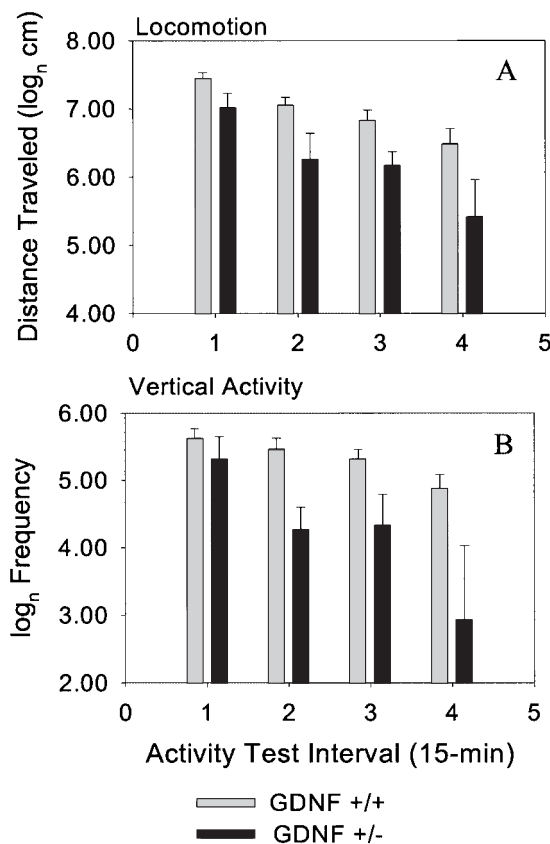


Figure 1. Locomotion (A) and vertical activity (B) for *Gdnf*^{+/+} and *Gdnf*^{+/-} mice recorded at 15-min intervals across a 1-h test session. Both types of motor activities were diminished in the *Gdnf*^{+/-} mice across time ($p < 0.01$).

and cerebellum thus mimicked alterations seen in nor-epinephrine levels in the LC region.

Morphological Alterations in TH-Positive Neurons and Fibers

The general appearance of TH-immunopositive neurons of LC in *Gdnf*^{+/-} mice and control (*Gdnf*^{+/+}) is shown in Figure 4A and B. Because we had already demonstrated quantitative alteration in NE levels and NET activity, we saw no need to also quantify morphological correlations in this study. The LC neurons are exclusively noradrenergic, not dopaminergic; therefore, it is most likely that the TH-immunoreactive neurons and their neurites represent NE neurons and fibers in the LC area, even though the TH antibody does not distinguish between DA and NE neurons and fibers. Gross examination of TH-immunostained sections through the LC nucleus from rostral to caudal regions showed that the packing density of TH-immunopositive LC neurons in *Gdnf*^{+/-} mice (Fig. 4B, D) appeared to be reduced in comparison with *Gdnf*^{+/+} mice (Fig. 4C). The most

conspicuous alteration, however, was in the size of LC NE neurons, which was altered in *Gdnf*^{+/-} animals (Fig. 4D, boxed area), and the morphology of axons and dendrites surrounding the TH-immunoreactive cell bodies was affected. The *Gdnf*^{+/-} mice exhibited a greater variability in LC neuronal size than *Gdnf*^{+/+} mice, with many atrophical neurons interspersed with normal or larger cell bodies (see Fig. 4D). TH-immunoreactive neurites surrounding the LC nucleus in *Gdnf*^{+/+} mice had a distinct morphology, with varicosities and a smooth appearance (Fig. 4A, C) and the pattern of TH-immunoreactive neurites was dense. In contrast, the fibers surrounding LC neurons of *Gdnf*^{+/-} mice were much less dense (Fig. 4B, D) and exhibited a pattern that is usually seen in neurites undergoing active neurodegeneration. Frequent axonal swellings and an interrupted pattern of neurites were seen in all animals that had a partial deletion of the GDNF gene (see example in Fig. 4D).

Areas that receive a dense innervation from the LC-NE neurons were examined next. Fiber density and thickness appeared to be abnormal in the frontal cortex of *Gdnf*^{+/-} mice (Fig. 5). The density of TH-immunoreactive neurites appeared to be greater in *Gdnf*^{+/-} mice compared with *Gdnf*^{+/+} controls, even though the pattern was also suggestive of neurodegenerative changes in this area. Accumulation of immunoreactive material was seen within neurites, indicative of axonal swelling. The frontal cortex is innervated by both DA and NE fibers; therefore, the neuronal plexus seen in Figure 5 could represent both DA and NE innervations.

The dentate gyrus of the hippocampal formation (Fig. 6) was innervated by abnormally thick and nonconvergent TH-positive fibers in *Gdnf*^{+/-} mice (Fig. 6D, arrowheads) compared with *Gdnf*^{+/+} mice. Fiber density and pattern were different between the two groups (Fig. 6A, B). Similarly, there was a noticeable abnormal morphology of TH-positive fibers in the cerebellar molecular layer of *Gdnf*^{+/-} mice (Fig. 7B) compared with *Gdnf*^{+/+} mice (Fig. 7A). The TH-positive fibers in cerebellum of *Gdnf*^{+/-} mice contained axonal swellings (Fig. 7B) with apparent degenerative changes and appeared to exhibit a decrease in density compared with *Gdnf*^{+/+} mice. The cerebellum was the only area examined where the *Gdnf*^{+/-} animals exhibited a decrease in TH-positive fiber density.

DISCUSSION

The present study demonstrated a reduction in locomotor activity as well as alterations in the NE system of aged *Gdnf*^{+/-} mice, suggesting a potential role for endogenous GDNF in the maintenance of noradrenergic neurons with advancing age. Diminished levels of nor-epinephrine in LC and decreased uptake by the nor-epinephrine transporter in cerebellum and brain stem

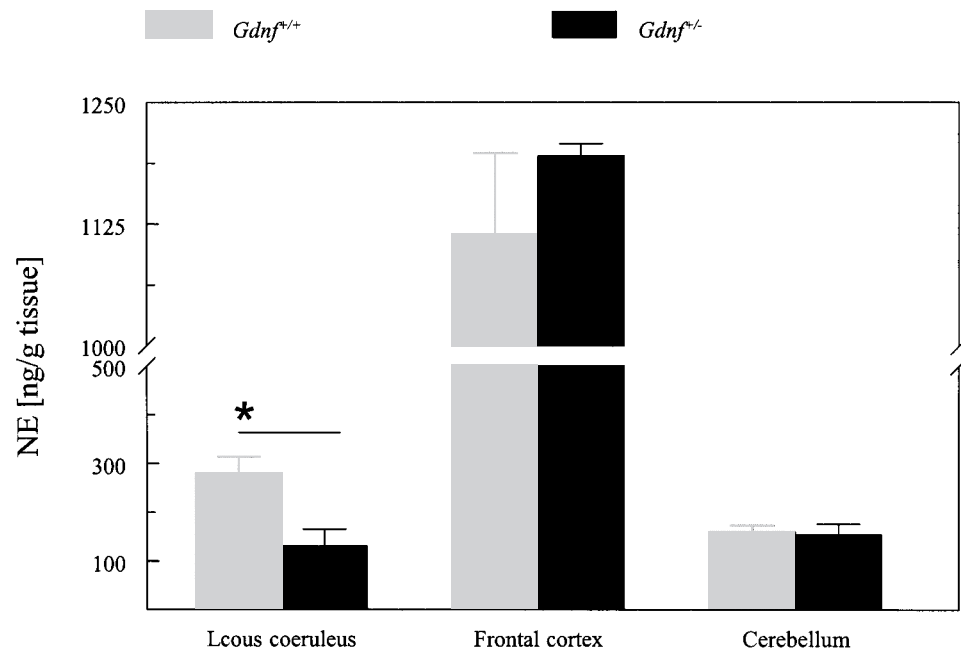


Figure 2. Tissue content of norepinephrine determined by HPLC analysis in brain stem containing locus coeruleus, frontal cortex, and cerebellum. There was a significant decrease ($p < 0.05$) in norepinephrine levels in the locus coeruleus samples of *Gdnf*^{+/-} compared with *Gdnf*^{+/+} mice. However, no alteration in norepinephrine levels was found in the frontal cortex or cerebellum between the groups. The values represent the mean \pm SEM averaged over all animals in each group.

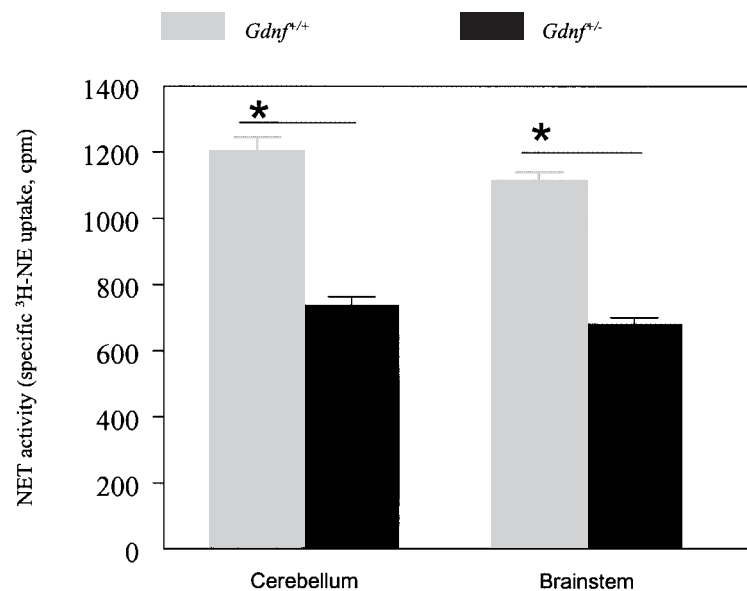


Figure 3. Alterations in norepinephrine transporter (NET) activity in aged *Gdnf*^{+/-} and *Gdnf*^{+/+} mice. Norepinephrine uptake was significantly decreased ($p < 0.05$) in cerebellum and brain stem of *Gdnf*^{+/-} mice compared with *Gdnf*^{+/+} mice. The values represent mean \pm SEM in each group.

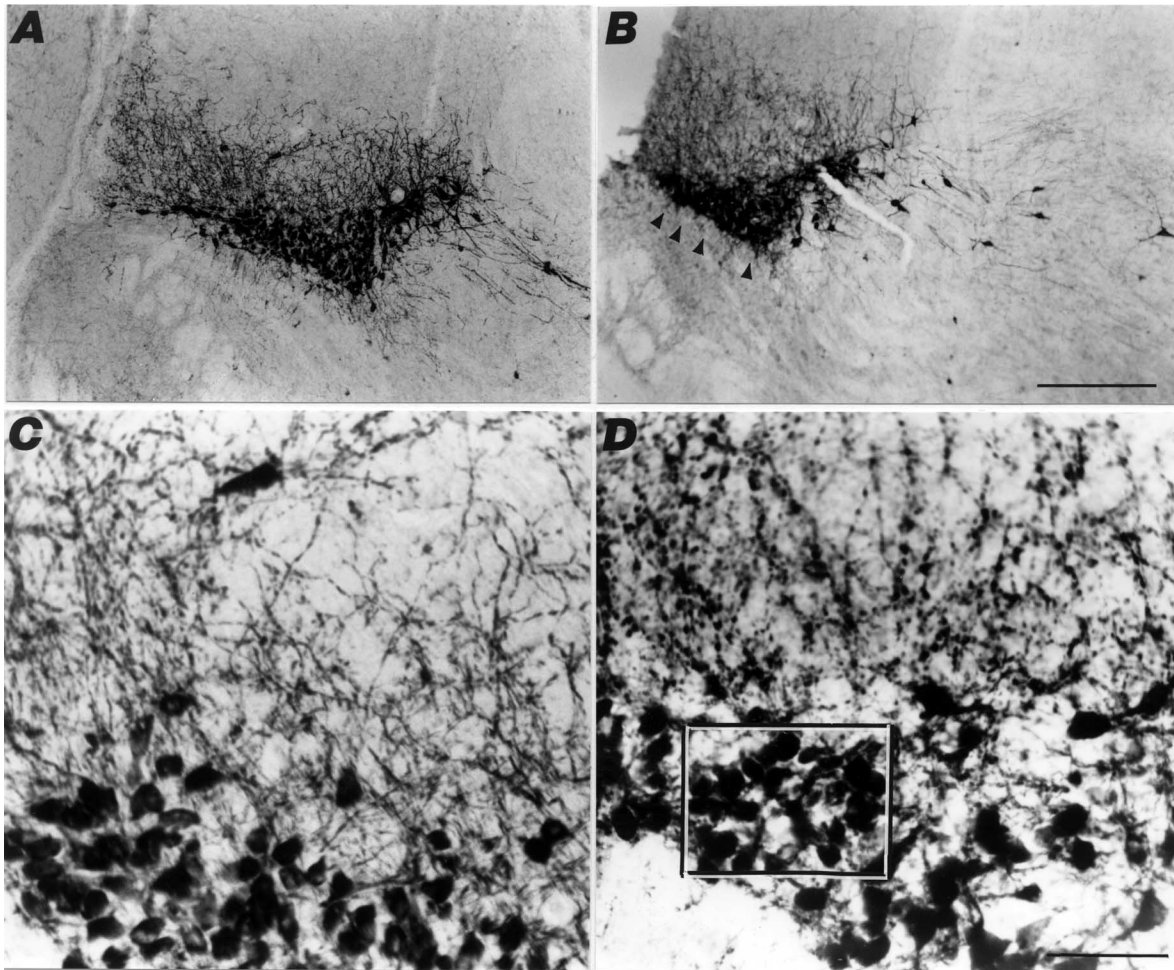


Figure 4. Photomicrograph of brain stem section containing locus coeruleus neurons (LC) from *Gdnf*^{+/+} (A, C) and *Gdnf*^{+/-} mice (B, D) immunostained for TH. Note the reduced density as well as altered pattern of TH-immunostained fibers in the *Gdnf*^{+/-} mice (B, arrowheads). (C, D) Enlarged view from (A) and (B), respectively, exhibiting detailed morphology of LC neurons and fibers. Note the thick accumulations in the TH-stained fibers of *Gdnf*^{+/-} mice (D). It is evident from the high magnification that cell bodies were significantly smaller and more variable in *Gdnf*^{+/-} mice (boxed area) compared with cell bodies in *Gdnf*^{+/+} mice. Scale bar: 200 μ m (A, B), 40 μ m (C, D).

provide a potential biochemical substrate for the impairment seen in the behavioral studies. Importantly, morphological studies demonstrated genotype-specific alterations in TH-immunoreactive innervation of the frontal cortex, hippocampus, and cerebellum, supporting the hypothesis that GDNF plays a role in the continued maintenance of central NE neurons.

Our findings provide direct evidence for an important role of GDNF in the LC-NE transmitter system, because we found that both NET activity and NE levels were altered in aged *Gdnf*^{+/-} animals in different regions of the LC-NE system. Although the LC-NE neurons express mRNA for GDNF only during early development (2,54), the receptor protein for this growth factor (i.e., GFR α -1) is expressed in the developing as well as in

the adult rat (65). However, the target areas innervated by LC-NE neurons express mRNA for GDNF throughout life (2), supporting GDNF's role as a retrogradely transported trophic factor for LC neurons (63). GDNF protein levels undergo an age-related decline in the meso-striatal system of rats (43,70), and it is possible that similar alterations also occur in the LC-hippocampal NE system, even though this has not been investigated. A significant and region-specific upregulation of GDNF and its receptors has been noted in the hippocampus following kindling-evoked seizures in adult rats (39), a physiological phenomenon that directly depends on LC-NE innervation (3,14,38,69). Conversely, it was recently shown that kindling-evoked epileptogenesis is suppressed in mice with a null mutation of the GFR α -2

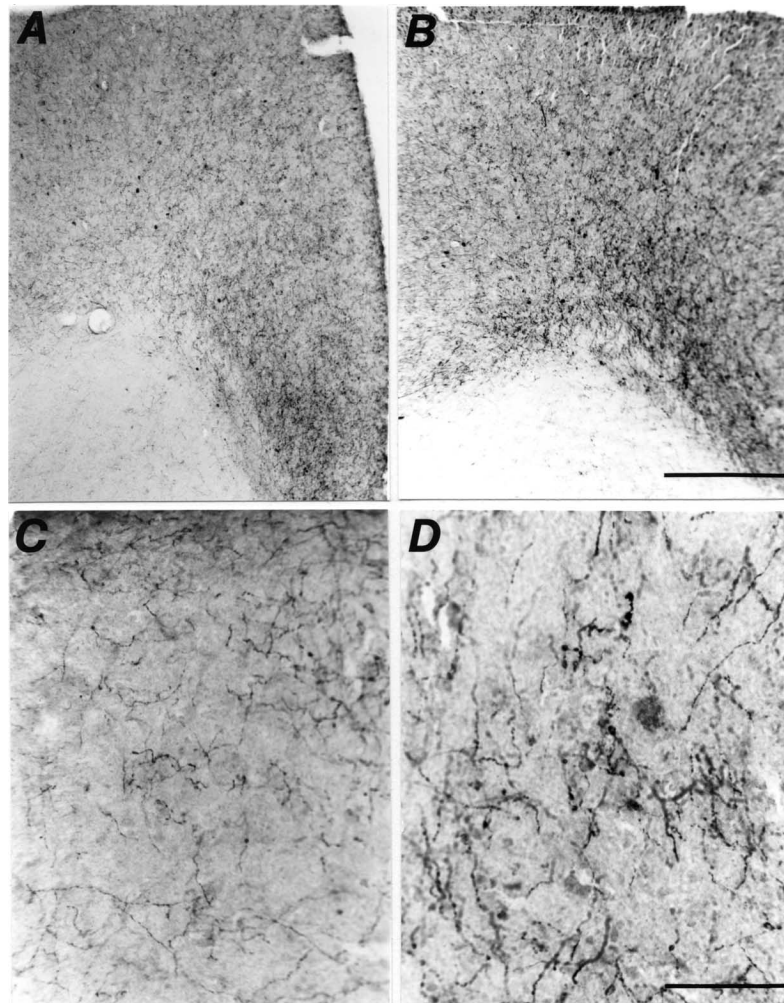


Figure 5. Photomicrograph from frontal cortex of *Gdnf*^{+/+} mice (A, C) and *Gdnf*^{+/-} mice (B, D) demonstrating TH-immunostained fibers. The TH fibers appear to be thicker and denser in the *Gdnf*^{+/-} mice (D) compared with *Gdnf*^{+/+} mice (C). (C) Representative sections of frontal cortex from *Gdnf*^{+/+} and (D) enlarged view of an example of frontal cortex from *Gdnf*^{+/-} mice. Note the thickness and density of TH-stained fibers in the frontal cortex of *Gdnf*^{+/-} mice, with numerous accumulations and interrupted nerve endings (D). Scale bar: 200 μ m (A, B), 50 μ m (C, D).

receptor, further supporting a role for GDNF in the noradrenergic pathway innervating the hippocampus (53). Arenas and collaborators (2) provided direct evidence for an LC-GDNF interaction when they demonstrated GDNF-mediated rescue of adult LC-NE neurons after neurotoxic exposure in the rat. These earlier findings are consistent with our present results, showing a significant decrease in LC-NE levels and activity of the NE transporter in aged *Gdnf*^{+/-} mice. The changes observed might suggest that a partial deletion of the GDNF gene accelerates age-related changes in the GDNF support system, thus jeopardizing transmitter systems that are dependent on this growth factor. The age chosen in the present study, 18 months, usually does not present age-related

alterations in this transmitter system in normal mice. By selecting this age, we would thus be able to tease out early aging signs in the experimental group.

Dysfunction of the LC-NE system has been implicated in behavioral disorders such as depression, opiate dependence, and inability to adapt to environmental changes (29,47,57). Earlier studies have shown that destruction of LC-NE neuronal terminals by the NE neurotoxin DSP-4 leads to deficits in performance of tasks such as exploration in an open field arena and of novel objects (28). Aged (18 months old) *Gdnf*^{+/-} mice in the present study exhibited reductions in horizontal and vertical motor activity. This finding contrasts with the absence of changes in locomotor activity and habituation to

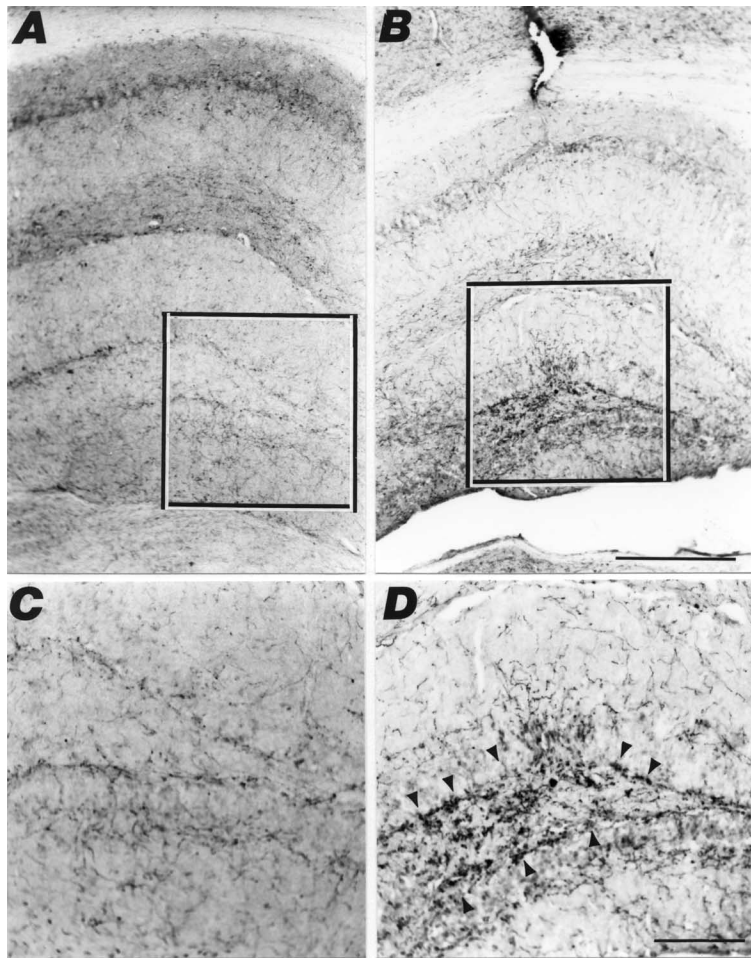


Figure 6. Sections of hippocampus, CA1/dentate area, of *Gdnf*^{+/+} (A, C) and *Gdnf*^{+/-} mice (B, D) immunostained for TH. (C, D) Enlarged view from the boxed area in (A) and (B), respectively. Note the hyperinnervation of TH fibers with axonal swellings and other accumulations of immunoreactive material (D, arrowheads) in the dentate gyrus of *Gdnf*^{+/-} mice. The fibers appear to be abnormal and have lost their varicose and smooth appearance. Scale bar: 200 μ m in (A, B), 100 μ m in (C, D).

a novel environment reported for young adult *Gdnf*^{+/-} mice (17) and further suggests altered function particularly during the later part of the life cycle. The lower activity levels for aged *Gdnf*^{+/-} mice in the present study were due to a more rapid decline over the 60-min test period in comparison with *Gdnf*^{+/+} control mice. This pattern of diminished motor activity suggests that aged *Gdnf*^{+/-} mice are less aroused by a novel environment or that their exploratory behavior dissipates more rapidly over time rather than having a motor dysfunction. Future studies will be focused on a more detailed analysis of behavioral parameters such as exploration and habituation versus motor function to explore the behavioral deficits in this animal model. Selective lesion and pharmacological studies (37,45) have established the importance of the mesolimbic and nigrostriatal dopamine sys-

tems in mediating motor activity; hence, future studies using our animal model will be expanded to include specific alterations in this transmitter system as well.

One of the target areas that is involved in motor coordination and motor learning is the cerebellum (10), and a progressive decline in motor performance and coordination with aging has been noted for both animals and humans (33,49,66). In particular, lesions reducing noradrenergic input to the cerebellum have been shown to cause deficits in motor learning (4,67,68). Compared to other target areas such as the cerebral cortex and the hippocampus, the cerebellum has been shown to exhibit a diminished capacity for reinnervation following loss of LC neurons after a neurotoxic lesion (15). The morphological abnormalities in the cerebellum of *Gdnf*^{+/-} mice observed in our study support these findings, be-

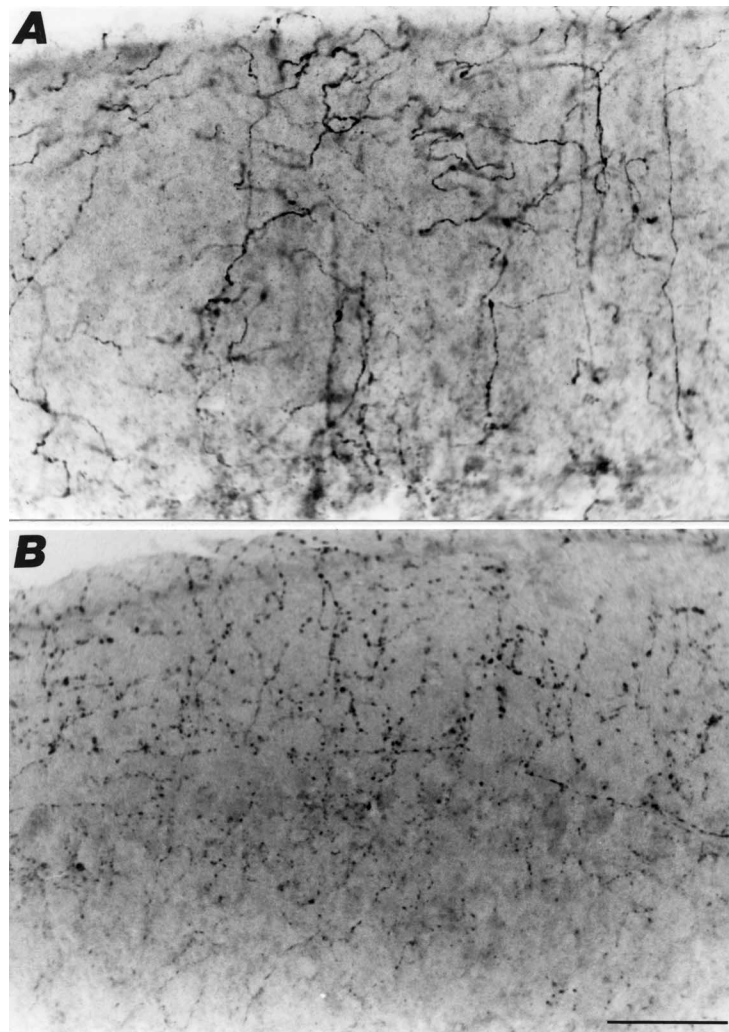


Figure 7. Molecular layer of cerebellum immunostained for TH. (A) The TH-stained fibers in a *Gdnf*^{+/+} mouse. There are obvious alterations in the TH-positive innervation pattern in the heterozygous mouse (*Gdnf*^{+/-}, B), with decreased fiber density, interrupted axonal paths, and axonal swellings as well. Scale bar: 40 μ m.

cause we found reduction in TH fiber innervation in the cerebellum of *Gdnf*^{+/-} mice compared with *Gdnf*^{+/+} mice. The supportive role of GDNF in the maintenance of both LC noradrenergic (2) and Purkinje neurons has been reported (50,51). Thus, Purkinje neurons, which require GDNF for maintenance, might have been directly affected by the diminished levels of this trophic factor in *Gdnf*^{+/-} mice, along with indirect dysfunction resulting from diminished NE input from the LC, adding loss of important innervation modalities to lack of local support within the cerebellum itself.

Catecholamine transporters function as important modulators of neuronal transmission because they are responsible for the removal of catecholamines from the extracellular space to terminate neural transmission (6,

12). Changes in the efficiency of uptake can exert profound effects on intensity, duration, and spread of transmitter action (12). There is a regional difference in uptake of dopamine and norepinephrine in the brain (9). Jonsson and Sachs (34,35) proposed that the quantitative measurement of neurotransmitter uptake could be an early and sensitive indicator of degenerative processes in the CNS. They demonstrated that neurotoxin exposure of LC-NE neurons affects various target regions differently; cortical areas were more affected by the NE neurotoxin (6-hydroxydopamine) than were subcortical areas such as the caudate and the hypothalamus (36). They also found that 6-hydroxydopamine treatments gave rise to significant accumulations of norepinephrine in nerve terminals. They ascribed this process to be part

of the "normal degenerative process." In addition, earlier work has shown that a noradrenergic lesion in the adult rat actually leads to increased norepinephrine levels in target areas (45). Moreover, it has been suggested that the surviving LC neurons increase their synthesis of norepinephrine to compensate for neuronal loss (31), perhaps by decreasing NET mRNA expression and increasing TH mRNA expression (61). Our morphological findings are in line with the work by Jonsson and collaborators (36), because we found significant TH accumulations in axonal swellings in TH-immunoreactive neurites in all target areas, suggestive of an adaptive upregulation of norepinephrine levels in the neurites. This is also in line with the unaltered norepinephrine levels in target regions of *Gdnf*^{+/-} mice in the present study, even though levels were diminished in the LC nucleus itself. The diminished NET activity in cerebellum and brain stem of aged *Gdnf*^{+/-} mice suggests that these brain areas are affected by the ongoing degenerative process and provides an indication of decreased number of NE fibers in these brain areas of *Gdnf*^{+/-} compared with *Gdnf*^{+/+} mice, or decreased transmitter reuptake function within each neurite. A selective damage to the uptake mechanisms or abnormal phenotypic expression of NET in the *Gdnf*^{+/-} mice cannot be ruled out at this point and will be investigated in future experiments.

Morphological evidence showing alterations in TH-positive neurons in LC along with abnormalities in TH fibers in the target areas suggests a perturbed NE system in *Gdnf*^{+/-} mice. TH-positive fibers in target areas, frontal cortex, hippocampus, and cerebellum had lost their normal appearance. The fibers in frontal cortex and hippocampus were thick. The dentate gyrus in *Gdnf*^{+/-} mice was hyperinnervated whereas in cerebellum, fiber density appeared to be reduced. Gustafson and Moore (24) suggested that NE neurons of lateral tegmentum and LC are genetically programmed to produce a predetermined amount of axons and terminals. Hence, denervation of one target area might lead to hyperinnervation in another target area. In addition, Fritschy and Grzanna (15) reported that LC neurons surviving neurotoxic insult have a strong regenerative response with collateral sprouting occurring in some forebrain regions, but not in the cerebellum. Similar findings were already reported by Jonsson and Sachs (36), who were the first to describe collateral sprouting, using 6-hydroxydopamine injections in developing animals. Such an interpretation might also apply to our findings, indicating that loss of LC neurons might lead to collateral sprouting in the dentate gyrus. Thus, it is possible that *Gdnf*^{+/-} mice undergo a compensatory recovery of NE fibers in the dentate gyrus, as has been observed after cerebellar (16) or hippocampal (46,55) denervation. The hyperinnervation of NE fibers in the dentate gyrus projecting from LC could also be the consequence of disinhibition of axonal growth from

the septum (19). This finding supports the idea that the pattern of hyperinnervation is determined by factors intrinsic to the target areas (24,27). In spite of the compensatory changes in certain target areas of *Gdnf*^{+/-} mice, behavioral abnormalities occurred in these mice, perhaps not surprisingly, because the noradrenergic neurites did not have a normal morphological appearance and were most likely not functioning normally. In addition, it is likely that other transmitter systems were affected by the partial GDNF deletion, which is currently under investigation.

The well-documented memory deterioration accompanying the aging process has also been related to a dysfunctional LC-hippocampal noradrenergic pathway. For example, Leslie et al. (41) reported that the extent of LC cell loss was correlated with the degree of memory impairment in aged mice. Adult heterozygous mice with the same genetic background as those in the current study reportedly exhibit cognitive deficits as well (17). Although we did not assess memory in our study, the loss of LC neurons in aged *Gdnf*^{+/-} mice and the degenerative changes in LC-hippocampal neurites reported might at least partially explain the cognitive deficits reported by Gerlai and collaborators (17).

In conclusion, the findings reported here suggest that mice with partial deletion of the GDNF gene have enhanced sensitivity towards alterations in the LC-NE system in the LC with advancement of age, indicating a role for GDNF in the continued maintenance of NE neurons with aging. This disruption of the LC-NE system might have implications in age-related neurodegenerative diseases such as Parkinson's and Alzheimer's disease, because the LC-NE system is affected in those conditions. Therefore, therapeutic intervention involving this growth factor may affect the important LC-NE system throughout the aging brain.

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REFERENCES

1. Alberch, J.; Pérez-Navarro, E.; Arenas, E.; Marsal, J. Involvement of nerve growth factor and its receptor in the regulation of the cholinergic function in aged rats. *J. Neurochem.* 57:1483–1487; 1991.
2. Arenas, E.; Trupp, M.; Akerud, P.; Ibanez, C. F. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* 15:1465–1473; 1995.
3. Bengzon, J.; Kokaia, M.; Brundin, P.; Lindvall, O. Seizure suppression in kindling epilepsy by intrahippocampal locus coeruleus grafts: Evidence for an alpha-2-adrenoreceptor mediated mechanism. *Exp. Brain Res.* 81:433–437; 1990.
4. Bickford, P.; Heron, C.; Young, D. A.; Gerhardt, G. A.;

- de la Garza, R. Impaired acquisition of novel locomotor tasks in aged and norepinephrine-depleted F344 rats. *Neurobiol. Aging* 13:475–481; 1992.
5. Bimonte, H. A.; Nelson, M. E.; Granholm, A.-Ch. Age-related deficits as working memory load increases: Relationship with growth factors. *Neurobiol. Aging* 24:37–48; 2002.
 6. Blakely, R. D.; De Felice, L.J.; Hartzell, H. C. Molecular physiology of norepinephrine and serotonin transporters. *J. Exp. Biol.* 196:263–281; 1994.
 7. Bowenkamp, K. E.; Lapchak, P. A.; Hoffer, B. J.; Bickford, P. C. Glial cell line-derived neurotrophic factor reverses motor impairment in 16–17 month old rats. *Neurosci. Lett.* 21:81–84; 1996.
 8. Chan-Palay, V.; Asan, E. Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. *J. Comp. Neurol.* 287:373–392; 1989.
 9. Coyle, J. T.; Snyder, S. H. Catecholamine uptake by synaptosomes in homogenates of rat brain: Stereospecificity in different areas. *J. Pharmacol. Exp. Ther.* 170:221–231; 1969.
 10. Eccles, J. C.; Ito, M.; Szentogothai, J. The cerebellum as a neuronal machine. New York: Springer; 1967.
 11. Edwards, A. L. Experimental design in psychological research. New York: Holt, Rinehart and Winston; 1966:130.
 12. Eisenhofer, G. The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. *Pharmacol. Ther.* 91:35–62; 2001.
 13. Eriksdotter Jonhagen, M.; Hoffer, B.; Luthman, J. Alterations in alpha-adrenoceptors in aging intraocular hippocampal grafts. *Neurobiol. Aging* 16:633–638; 1995.
 14. Ferraro, G.; Sardo, P.; Sabatino, M.; Caravaglios, G.; La Grutta, V. Anticonvulsant activity of the noradrenergic locus coeruleus system: Role of beta mediation. *Neurosci. Lett.* 169:93–96; 1994.
 15. Fritschy, J. M.; Grzanna, R. 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; 1992.
 16. Gasser, U. E.; Dravid, A. R. Noradrenergic, serotonergic, and cholinergic sprouting in the hippocampus that follows partial or complete transection of the septohippocampal pathway: Contribution of spared inputs. *Exp. Neurol.* 96:352–364; 1987.
 17. Gerlai, R.; McNamara, A.; Choi-Lundberg, D. L.; Armanini, M.; Ross, J.; Powell-Braxton, L.; Phillips, H. S. Impaired water maze learning performance without altered dopaminergic function in mice heterozygous for the GDNF mutation. *Eur. J. Neurosci.* 14:1153–1163; 2001.
 18. Gesi, M.; Soldani, P.; Giorgi, F. S.; Santinami, A.; Bonaccorsi, I.; Fornai, F. The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci. Biobehav. Rev.* 24:655–668; 2000.
 19. Goldowitz, D.; Seiger, A.; Olson, L. Degree of hyperinnervation of area dentata by locus coeruleus in the presence of septum or entorhinal cortex as studied by sequential intraocular triple transplantation. *Exp. Brain Res.* 56:351–360; 1984.
 20. Gould, T. J.; Bickford, P. C. The effects of aging on cerebellar beta-adrenergic receptor activation and motor learning in female F344 rats. *Neurosci. Lett.* 216:53–56; 1996.
 21. Granholm, A. C.; Srivastava, N.; Mott, J. L.; Henry, S.; Henry, M.; Westphal, H.; Pichel, J. G.; Shen, L.; Hoffer, B. J. Morphological alterations in the peripheral and central nervous systems of mice lacking glial cell line-derived neurotrophic factor (GDNF): Immunohistochemical studies. *J. Neurosci.* 17:1168–1178; 1997.
 22. Granholm, A.-C. Trophic influences on neural tissue transplants: Delivery methods and co-graft interaction. In: Dunnett, S. B.; Boulton, A. A.; Baker, G. B., eds. *Neural transplantation methods*. Totowa, NJ: Humana Press Inc.; 2000:385–409.
 23. Granholm, A. C.; Reyland, M.; Albeck, D.; Sanders, L.; Gerhardt, G.; Hoernig, G.; Shen, L.; Westphal, H.; Hoffer, B. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J. Neurosci.* 20:3182–3190; 2000.
 24. Gustafson, E. L.; Moore, R. Y. Noradrenaline neuron plasticity in developing rat brain: Effects of neonatal 6-hydroxydopamine demonstrated by dopamine-beta-hydroxylase immunocytochemistry. *Dev. Brain Res.* 37:143–155; 1987.
 25. Halberda, J. P.; Middaugh, L. D.; Gard, B. E.; Jackson, B. P. DAD1- and DAD2-like agonist effects on motor activity of C57 mice: Differences compared to rats. *Synapse* 26:81–92; 1997.
 26. Hall, M. E.; Hoffer, B. J.; Gerhardt, G. A. Rapid and sensitive determination of catecholamines in small tissue samples by high pressure liquid chromatography coupled with dual-electrode coulometric electrochemical detection. *LC/GC* 7:258–265; 1989.
 27. Haring, J. H.; Miller, G. D.; Davis, J. N. Changes in the noradrenergic innervation of the area dentata after axotomy of coeruleohippocampal projections or unilateral lesion of the locus coeruleus. *Brain Res.* 368:233–238; 1986.
 28. Harro, J.; Orelund, L.; Vasar, E.; Bradwejn, J. Impaired exploratory behaviour after DSP-4 treatment in rats: Implications for the increased anxiety after noradrenergic denervation. *Eur. Neuropsychopharmacol.* 5:447–455; 1995.
 29. Harro, J.; Orelund, L. Depression as a spreading adjustment disorder of monoaminergic neurons: A case for primary implication of the locus coeruleus. *Brain Res. Brain Res. Rev.* 38:79–128; 2001.
 30. Hefti, F. Neurotrophic factor therapy for nervous system degenerative diseases. *J. Neurobiol.* 25:1418–1435; 1994.
 31. Hoogendijk, W. J.; Feenstra, M. G.; Botterblom, M. H.; Gilhuis, J.; Sommer, I. E.; Kamphorst, W.; Eikelenboom, P.; Swaab, D. F. Increased activity of surviving locus coeruleus neurons in Alzheimer's disease. *Ann. Neurol.* 45:82–91; 1999.
 32. Ishida, Y.; Shirokawa, T.; Miyaishi, O.; Komatsu, Y.; Isobe, K. Age-dependent changes in projections from locus coeruleus to hippocampus dentate gyrus and frontal cortex. *Eur. J. Neurosci.* 12:1263–1270; 2000.
 33. Janicke, B.; Wrobel, D. Changes in motor activity with age and the effects of pharmacologic treatment. *Exp. Gerontol.* 19:321–328; 1984.
 34. Jonsson, G.; Sachs, C. Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. *Eur. J. Pharmacol.* 9:141–155; 1970.
 35. Jonsson, G.; Sachs, C. Degenerative and nondegenerative effects of 6-hydroxydopamine on adrenergic nerves. *J. Pharmacol. Exp. Ther.* 180:625–635; 1972.
 36. Jonsson, G.; Sachs, C. Pharmacological modifications of the 6-hydroxy-dopa induced degeneration of central noradrenaline neurons. *Biochem. Pharmacol.* 22:1709–1716; 1973.
 37. Kirik, D.; Rosenblad, C.; Björklund, A. Characterization

- of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastratial 6-hydroxydopamine in the rat. *Exp. Neurol.* 152:259–277; 1998.
38. Kokaia, M.; Cenci, M. A.; Elmer, E.; Nilsson, O. G.; Kokaia, Z.; Bengzon, J.; Björklund, A.; Lindvall, O. Seizure development and noradrenaline release in kindling epilepsy after noradrenergic reinnervation of the subcortically deafferented hippocampus by superior cervical ganglion or fetal locus coeruleus grafts. *Exp. Neurol.* 130:351–361; 1994.
 39. Kokaia, Z.; Airaksinen, M. S.; Nanobashvili, A.; Larsson, E.; Kujamaki, E.; Lindvall, O.; Saarma, M. GDNF family ligands and receptors are differentially regulated after brain insults in the rat. *Eur. J. Neurosci.* 11:1202–1216; 1999.
 40. Kriegstein, K.; Suter-Crazzolaro, C.; Fischer, W. H.; Unsicker, K. TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *EMBO J.* 14:736–742; 1995.
 41. Leslie, F. M.; Loughlin, S. E.; Sternberg, D. B.; McGaugh, J. L.; Young, L. E.; Zornetzer, S. F. Noradrenergic changes and memory loss in aged mice. *Brain Res.* 359: 292–299; 1985.
 42. Lin, L. F.; Doherty, D. H.; Lile, J. D.; Bektesh, S.; Collins, F. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260:1130–1132; 1993.
 43. Ling, Z. D.; Collier, T. J.; Sortwell, C. E.; Lipton, J. W.; Vu, T. Q.; Robie, H. C.; Carvey, P. M. Striatal trophic activity is reduced in the aged rat brain. *Brain Res.* 856: 301–309; 2000.
 44. Lohr, J. B.; Jeste, D. V. Locus ceruleus morphometry in aging and schizophrenia. *Acta Psychiatr. Scand.* 77:689–697; 1988.
 45. Luthman, J.; Fredriksson, A.; Sundstrom, E.; Jonsson, G.; Archer, T. Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: Motor behavior and monoamine alterations at adult stage. *Behav. Brain Res.* 33:267–277; 1989.
 46. Madison, R.; Davis, J. N. Sprouting of noradrenergic fibers in hippocampus after medial septal lesions: Contributions of the central and peripheral nervous systems. *Exp. Neurol.* 80:167–177; 1983.
 47. Maldonado, R. Participation of noradrenergic pathways in the expression of opiate withdrawal: Biochemical and pharmacological evidence. *Neurosci. Biobehav. Rev.* 21: 91–104; 1997.
 48. Manaye, K. F.; Macintire, D. D.; Mann, D. M. A.; German, D. C. Locus coeruleus cell loss in the aging human brain: A non-random process. *J. Comp. Neurol.* 358:79–87; 1995.
 49. Marshall, J. F.; Berrios, N. Movement disorders of aged rats: Reversal by dopamine receptor stimulation. *Science* 206:477–479; 1979.
 50. McAlhany, R. E., Jr.; West, J. R.; Miranda, R. C. Glial-derived neurotrophic factor rescues calbindin-D28k-immunoreactive neurons in alcohol-treated cerebellar explant cultures. *J. Neurobiol.* 33:835–847; 1997.
 51. Mount, H. T.; Dean, D. O.; Alberch, J.; Dreyfus, C. F.; Black, I. B. Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. *Proc. Natl. Acad. Sci. USA* 92:9092–9096; 1995. [Published erratum appears in *Proc. Natl. Acad. Sci. USA* 92:11945; 1995]
 52. Moore, M. W.; Klein, R. D.; Farinas, I.; Sauer, H.; Armanini, M.; Phillips, H.; Reichardt, L. F.; Ryan, A. M.; Carver-Moore, K.; Rosenthal, A. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* 382:76–79; 1996.
 53. Nanobashvili, A.; Airaksinen, M. S.; Kokaia, M.; Rossi, J.; Asztely, F.; Olofsdotter, K.; Mohapel, P.; Saarma, M.; Lindvall, O.; Kokaia, Z. Development and persistence of kindling epilepsy are impaired in mice lacking glial cell line-derived neurotrophic factor family receptor alpha 2. *Proc. Natl. Acad. Sci. USA* 97:12312–12317; 2000.
 54. Nosrat, C. A.; Tomac, A.; Lindqvist, E.; Lindskog, S.; Humpel, C.; Stromberg, I.; Ebendal, T.; Hoffer, B. J.; Olson, L. Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. *Cell Tissue Res.* 286:191–207; 1996.
 55. Peterson, G. M. Sprouting of central noradrenergic fibers in the dentate gyrus following combined lesions of its entorhinal and septal afferents. *Hippocampus* 4:635–648; 1994.
 56. Pichel, J. G.; Shen, L.; Sheng, H. Z.; Granholm, A. C.; Drago, J.; Grinberg, A.; Lee, E. J.; Huang, S. P.; Saarma, M.; Hoffer, B. J.; Sariola, H.; Westphal, H. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 382:73–76; 1996.
 57. Rasmussen, K. Afferent effects on locus coeruleus in opiate withdrawal. *Prog. Brain Res.* 88:207–216; 1991.
 58. Saarma, M. GDNF—a stranger in the TGF-beta superfamily? *Eur. J. Biochem.* 267:6968–6971; 2000.
 59. Sanchez, M. P.; Silos-Santiago, I.; Frisen, J.; He, B.; Lira, S. A.; Barbacid, M. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 382:70–73; 1996.
 60. Shirokawa, T.; Ishida, Y.; Isobe, K. I. Age-dependent changes in axonal branching of single locus coeruleus neurons projecting to two different terminal fields. *J. Neurophysiol.* 84:1120–1122; 2000.
 61. Shores, M. M.; White, S. S.; Veith, R. C.; Szot, P. Tyrosine hydroxylase mRNA is increased in old age and nor-epinephrine uptake transporter mRNA is decreased in middle age in locus coeruleus of Brown-Norway rats. *Brain Res.* 826:143–147; 1999.
 62. Srivastava, N.; Granholm, A. C.; Gerhardt, G. A. Collateral sprouting of central noradrenergic neurons during aging: Histochemical and neurochemical studies in intraocular triple transplants. *Exp. Neurol.* 145:524–535; 1997.
 63. Tomac, A.; Widenfalk, J.; Lin, L. F.; Kohno, T.; Ebendal, T.; Hoffer, B. J.; Olson, L. Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proc. Natl. Acad. Sci. USA* 92:8274–8278; 1995.
 64. Tomac, A.; Lindqvist, E.; Lin, L. F.; Ogren, S. O.; Young, D.; Hoffer, B. J.; Olson, L. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* 373:335–339; 1995.
 65. Trupp, M.; Belluardo, N.; Funakoshi, H.; Ibanez, C. F. Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *J. Neurosci.* 17:3554–3567; 1997.
 66. Wallace, J. E.; Krauter, E. E.; Campbell, B. A. Motor and reflexive behavior in the aging rat. *J. Gerontol.* 35:364–370; 1980.
 67. Watson, M.; McElligott, J. G. 6-OHDA induced effects

- upon the acquisition and performance of specific locomotor tasks in rats. *Pharmacol. Biochem. Behav.* 18:927–934; 1983.
68. Watson, M.; McElligott, J. G. Cerebellar norepinephrine depletion and impaired acquisition of specific locomotor tasks in rats. *Brain Res.* 296:129–138; 1984.
69. Weiss, G. K.; Lewis, J.; Jimenez-Rivera, C.; Vigil, A.; Corcoran, M. E. Antikindling effects of locus coeruleus stimulation: Mediation by ascending noradrenergic projections. *Exp. Neurol.* 108:136–140; 1990.
70. Yurek, D. M.; Fletcher-Turner, A. Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion. *Brain Res.* 891:228–235; 2001.