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The nigrostriatal dopamine system of aging $GFR\alpha$ -1 heterozygous mice: neurochemistry, morphology and behavior

Vandana Zaman¹, Heather A. Boger¹, Ann-Charlotte Granholm^{1,2}, Baerbel Rohrer^{1,3}, Alfred Moore^{1,2}, Mona Buhusi¹, Greg A. Gerhardt⁴, Barry J. Hoffer⁵, and Lawrence D. Middaugh^{1,6}

¹Department of Neurosciences, Medical University of South Carolina, Basic Science Bldg Suite 411, 173 Ashley Ave, Charleston, SC 29425, USA

²Center on Aging, Medical University of South Carolina, Charleston, SC, USA

³Department of Ophthalmology, Medical University of South Carolina, Charleston, SC, USA

⁴Department of Anatomy & Neurobiology, Chandler Medical Center, University of Kentucky, Lexington, KY, USA

⁵Intramural Research Program, NIDA, Baltimore, MD, USA

⁶Psychiatry and Behavioral Science, Medical University of South Carolina, Charleston, SC, USA

Abstract

Given the established importance of glial cell line-derived neurotrophic factor (GDNF) in maintaining dopaminergic neurotransmitter systems, the nigrostriatal system and associated behaviors of mice with genetic reduction of its high-affinity receptor, GDNF receptor ($GFR\alpha$)-1 ($GFR\alpha$ -1^{+/-}), were compared with wild-type controls. Motor activity and the stimulatory effects of a dopamine (DA) D1 receptor agonist (SKF 82958) were assessed longitudinally at 8 and 18 months of age. Monoamine concentrations and dopaminergic nerve terminals in the striatum and the number of dopaminergic neurons in the substantia nigra (SN) were assessed. The results support the importance of $GFR\alpha$ -1 in maintaining normal function of the nigrostriatal dopaminergic system, with deficits being observed for $GFR\alpha$ -1^{+/-} mice at both ages. Motor activity was lower and the stimulatory effects of the DA agonist were enhanced for the older $GFR\alpha$ -1^{+/-} mice. DA in the striatum was reduced in the $GFR\alpha$ -1^{+/-} mice at both ages, and tyrosine hydroxylase-positive cell numbers in the SN were reduced most substantially in the older $GFR\alpha$ -1^{+/-} mice. The combined behavioral, pharmacological probe, neurochemical and morphological measures provide evidence of abnormalities in $GFR\alpha$ -1^{+/-} mice that are indicative of an exacerbated aging-related decline in dopaminergic system function. The noted deficiencies, in turn, suggest that $GFR\alpha$ -1 is necessary for GDNF to maintain normal function of the nigrostriatal dopaminergic system. Although the precise mechanism(s) for the aging-related changes in the dopaminergic system remain to be established, the present study clearly establishes that genetic reductions in $GFR\alpha$ -1 can contribute to the degenerative changes observed in this system during the aging process.

Keywords

monoamines; neuropharmacology; neurotrophic factors; nigrostriatal system; growth factor receptors; SKF 82958

Introduction

Neurotrophic factors promote survival of dopaminergic neurons and influence their metabolic and electrophysiological properties (Pothos *et al.*, 1998; Wang *et al.*, 2001). Glial cell-line derived neurotrophic factor (GDNF) promotes the survival of dopaminergic neurons in the substantia nigra (SN) during normal development and after neurotoxic lesions (Akerud *et al.*, 2001; Borlongan *et al.*, 2001). GDNF is a target-derived growth factor (Tomac *et al.*, 1995) synthesized in the striatum (Trupp *et al.*, 1997), and exerts its trophic effects on nigral neurons via a two-component receptor complex, GDNF receptor α -1 (GFR α -1) and c-Ret (Trupp *et al.*, 1996). GFR α -1 functions as the ligand-binding, and c-Ret as the signal transduction, component of the receptor complex (Trupp *et al.*, 1997). Consistent with its mRNA localization (Nosrat *et al.*, 1997; Trupp *et al.*, 1997; Glazner *et al.*, 1998), GFR α -1 protein is expressed in brain areas with dopaminergic cell bodies, the SN and ventral tegmental area (VTA), and in their target areas, the striatum and nucleus accumbens, whereas c-Ret mRNA is expressed only in cell body areas (Glazner *et al.*, 1998). Immunohistochemical mapping of rat brain indicates abundant expression of GFR α -1-like immunoreactivity on dopaminergic and other neurons in the pars compacta and pars reticulata of the SN and striatum (Perez-Navarro *et al.*, 1999; Matsuo *et al.*, 2000), suggesting that GDNF exerts neurotrophic effects on dopaminergic and non-dopaminergic cells.

Dopaminergic system dysfunction characterizes Parkinson's disease (PD) and normal aging. The noted reduction of GDNF in the SN of PD patients (Chauhan *et al.*, 2001; Mogi *et al.*, 2001) suggests that either this trophic factor or its receptor complex might contribute to the dopaminergic system dysfunction associated with the disease, and perhaps normal aging. Genetic deficiency of GDNF in mice (GDNF^{+/-} mice) has been reported to be associated with an accelerated aging-related decline in motor activity and nigrostriatal dopaminergic function (Boger *et al.*, 2006), elevated extracellular dopamine (DA) levels (Airavaara *et al.*, 2004), and impaired performance on a spatial version of the Morris water maze (Gerlai *et al.*, 2001). In addition, studies on rats and non-human primates indicate that GDNF delivery reduces the dopaminergic neuron damage associated with acute lesions and normal aging (Bowenkamp *et al.*, 1995; Choi-Lundberg & Bohn, 1995; Granholm *et al.*, 1997; Mandel *et al.*, 1997; Date *et al.*, 1998; Gash *et al.*, 1998; Kordower *et al.*, 2000; Ericson *et al.*, 2005). GDNF administration to the intact striatum of young adult rats via recombinant lentiviral vector reduced tyrosine hydroxylase (TH) mRNA levels in SN and VTA neurons and the optical density of the TH-positive fiber innervation in the striatum, but did not alter striatal DA neurotransmission (Rosenblad *et al.*, 2003). A protective role for GDNF and its receptor complex is also suggested by increased expression of endogenous GDNF, GFR α -1 and c-Ret mRNA in several brain areas after systemic exposure to kainic acid (Reeben *et al.*, 1998; Chen *et al.*, 2001) and during ischemia (Arvidsson *et al.*, 2001; Sarabi *et al.*, 2001). Finally, the neuroprotective effect of GDNF during cerebral ischemia is reduced in GFR α -1^{+/-} mice (Tomac *et al.*, 2000).

The impact of reduced GFR α -1 signaling on dopaminergic system function was assessed in the present study using GFR α -1^{+/-} mice. The overall hypothesis was that the decline in dopaminergic systems accompanying the aging process would be exacerbated in GFR α -1^{+/-} mice. Behavior was examined by spontaneous locomotor activity at 8 months (young adult) and 18 months (aged) of GFR α -1^{+/-} and wild-type mice and their responses to a challenge with a DA D1 receptor agonist, SKF 82958 (SKF). Neurochemical assessment of DA and other monoamine neurotransmitters in striatal tissue, and immunohistochemical analysis of TH and dopamine transporter (DAT), were performed at both ages in additional groups of mice.

Materials and methods

Animals

Young adult *GFRα-1^{+/-}* and wild-type mice (8 months old) obtained from Dr Barry Hoffer at the National Institutes on Drug Abuse were used for all experiments. Different groups of mice were used for the behavioral and the neurochemical and morphological assessments described below. The *GFRα-1^{+/-}* mice were created by replacing the first coding exon of the murine *GFRα-1* gene with a phosphoglycerate kinase (pgk) neo-resistance expression cassette in reverse orientation to *GFRα-1* mRNA transcription, as described earlier (Tomas *et al.*, 2000; Sarabi *et al.*, 2003). Animals were housed in the MUSC Animal Care Facility under a 12:12 h light/dark cycle, with access to food and water *ad libitum*. All experiments were performed in accordance with the Society for Neuroscience Statement for the Use of Animals in Research, and were approved by the Institutional Animal Care and Use Committee, Medical University of South Carolina.

Assessment of locomotor activity

Locomotor activity (total distance traveled) was assessed with a Digiscan Animal Activity Monitor system (Omnitech Electronics Model RXYZCM (8); TAO, Columbus, OH, USA), details of which have been described previously (Halberda *et al.*, 1997). Animals of each age (wild type; *GFRα-1^{+/-}*) were assigned to one of three drug groups in a 2 (genotype) × 3 (drug dose) × 2 (age) factorial design, and were tested initially at 8 months of age, and then after aging to 18 months. After a 15-min habituation period, the mice were assigned to one of three groups for each genotype, injected intraperitoneally with saline or with SKF at 0.05, or 0.10 mg/kg, and returned to their respective activity monitors for an additional 30-min test to assess the effect of the DA D1 agonist. The resulting six groups included: (i) wild-type (*n* = 8) or (ii) *GFRα-1^{+/-}* mice (*n* = 8) injected with saline; (iii) wild-type (*n* = 8) or (iv) *GFRα-1^{+/-}* mice (*n* = 7) injected with the 0.05 mg/kg dose of SKF; and (v) wild-type (*n* = 8) or (vi) *GFRα-1^{+/-}* mice (*n* = 6) injected with the 0.10 mg/kg dose of SKF. Data were analysed with a 2 (genotype) × 3 (dose) × 2 (age) ANOVA, with age as a repeated measure factor.

Tissue collection and monoamine assessment (high-performance liquid chromatography)

Aged (18 months) *GFRα-1^{+/-}* (*n* = 4) and wild-type (*n* = 10) mice and young adult (8 months) *GFRα-1^{+/-}* (*n* = 7) and wild-type (*n* = 7) mice were decapitated under halothane inhalation anesthesia, and brain tissues were collected. Brains were removed immediately, and the striatum (right hemisphere) was dissected according to previously published anatomical landmarks (Zaman *et al.*, 2003). Samples were weighed in pre-weighed microcentrifuge tubes, and kept on dry ice before transfer into a -70 °C freezer until analysis. Levels of DA, serotonin and norepinephrine (NE) were determined using a previously described standard protocol (Hall *et al.*, 1989). Data for the monoamines were analysed with ANOVAs across the four groups for each monoamine. Newman-Keuls *post hoc* analysis was used for comparison of individual means.

Measurement of GDNF and GFRα-1 levels in brain tissues

Fresh frozen cortical tissues were collected from 8-month-old and 18-month-old *GFRα-1^{+/-}* and wild-type mice for analysis of GDNF and *GFRα-1* tissue levels. Tissue samples were homogenized in 5–10 volumes of lysis buffer (RIPA buffer, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4); after protein quantitation using a BCA protein assay (Pierce, Rockford, IL, USA), equal protein amounts (20 µg) were loaded and separated onto 4–12% NuPAGE gradient gels (Invitrogen, Carlsbad, CA, USA). After transfer onto nitrocellulose membranes, proteins were visualized using antibodies against GDNF (R&D Systems, Minneapolis, MN, USA; 1 : 500 dilution) or *GFRα-1* (Santa Cruz Biotechnology,

Inc., Santa Cruz, CA, USA; 1 : 500 dilution), horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and an ECL detection kit (Pierce, Rockford, IL, USA). Quantification of major products was done using a FluorChem 9900 system (Alpha Innotech, San Leandro, CA, USA). Data were analysed with a 2 (genotype) $\times$ 2 (age) ANOVA.

Morphological analysis of the dorsolateral and ventromedial striatum and of the SN and VTA

After dissection of the striatum from the right hemisphere for the monoamine measurements described above, the remaining brain tissues from mice of the four groups – aged (18 months) *GFR α -1^{+/-}* ($n = 4$) and wild-type ($n = 6$) mice and young adult (8 months) *GFR α -1^{+/-}* ($n = 5$) and wild-type ($n = 6$) mice – were prepared for histological analysis. Tissue was immediately immersed in 4% paraformaldehyde (in 0.1 M phosphate buffer at pH 7.4) for 48 h, and then transferred into 30% sucrose at 4 °C. Forty-five-micrometer coronal cryosections taken through the left striatum, and bilaterally from the SN and VTA, were collected into 24-well plates containing phosphate buffer. Every sixth section from the striatum and midbrain was processed for immunohistochemical localization of TH (Pel-Freez, Rogers, AR, USA), using our routine protocol (Zaman *et al.*, 2003). In brief, the free-floating sections were treated for 15 min with H₂O₂ (30%), methanol (100%), and 0.01 M Tris-buffered saline (TBS) (pH 7.6; 1 : 2 : 7, respectively) to quench endogenous peroxidase activity. After a wash with TBS, sections were then treated for 20 min with sodium *m*-periodate (0.1 M) in TBS. Sections were washed in TBS and incubated for 30 min in 10% normal goat serum (Sigma, St Louis, MO, USA) in TBS + 0.25% Triton X-100 to block non-specific binding sites, and this was followed by 48 h of incubation with the TH antibody (1 : 1000; rabbit anti-TH; Pel-Freez) in 3% normal goat serum/TBS + 0.25% Triton X-100 at 4 °C. Sections were then incubated with a goat anti-rabbit biotinylated secondary antibody (1 : 200; Vector Laboratories, Burlingame, CA, USA) for 1 h, and then in avidin–biotin complex for 1 h (ABC kit; Vector Laboratories). The reaction was developed by a common chromagen, 3',3'-diaminobenzidine (Sigma, St Louis, MO, USA), and 0.05% of 3% H₂O₂. For the SN and VTA, the reaction was enhanced with nickel ammonium sulfate (2.5%; Sigma). Adjacent sections were stained with cresyl violet for evaluation of neuronal morphology.

Immunofluorescent labeling was used for co-localization of DAT protein on three to four representative sections through the SN as another marker for dopaminergic neurons and fibers to complement the TH marker. Free-floating sections were washed in phosphate-buffered saline (PBS, pH 7.4) and incubated in a cocktail of 10% normal donkey serum, 0.5% fish skin gelatin and 0.3% Triton X-100 for 1 h at room temperature to block non-specific binding and permeabilize the membrane; sections were incubated in primary antibodies (for TH, 1 : 1000; sheep anti-TH; Abcam, Cambridge, MA, USA; and for DAT, 1 : 500; rabbit anti-DAT; Millipore, Billerica, MA, USA) in blocking cocktail for 48 h at 4 °C. Sections were washed in PBS with Tween-20 (0.05%) and incubated in secondary antibodies – donkey anti-rabbit fluorescein isothiocyanate (1 : 200) and donkey anti-sheep tetramethyl rhodamine isothiocyanate (1 : 100) (Jackson ImmunoResearch) – for 2 h at room temperature. Section were washed in PBS, mounted on glass slides, and protected with ProLong Gold (Invitrogen). Images were acquired with a Zeiss confocal microscope (LSM 510 META), using appropriate bandpass filters for fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate.

Morphometric analysis

Quantitation of dopaminergic fiber innervation density was determined from every sixth section through the dorsolateral and ventro-medial (shell and core) striatum, according to a previously described routine protocol (Boger *et al.*, 2006). In brief, four to six images of TH-stained sections of the two brain areas per animal were digitized in gray scale using a 20 $\times$ lens. All samples were digitized in the same time frame, to ensure a constant light intensity across

all groups. The density of TH-immunostained fibers was determined using NIH Image Software (Scion Image, Fredrick, Maryland, USA). The software measures gray scale values within the range of 0–256, with 0 representing white, and 256 black. Measurements were performed blinded, and the values from the four to six images were averaged to obtain one value per animal for each brain area. Subtracting background-staining values from these calculated means provided staining intensities for every sixth section through the two brain areas for each animal. Data were analysed with 2 (genotype) $\times$ 2 (age) ANOVAs for each brain area and Newman–Keuls *post hoc* tests.

Quantitative estimates of total numbers of TH-positive neurons in the SN and VTA were determined by unbiased stereological cell counts from serial sections passing through each nuclear group. The method is described in detail by Gundersen *et al.* (1988) and West (1993). The optical fractionator system used in the present study was Stereo Investigator stereological software (MicroBrightfield, Colchester, VT, USA) coupled to a Prior H128 computer-controlled *x–y–z* motorized stage. Outline contours were drawn at low magnification (10 $\times$), and the outlined region was measured with a systematic random design of dissector-counting frames. The counting frame area was 2500 μm^2 , and the sampling grid area was 100 $\times$ 100 μm (Zaman *et al.*, 2004). The counting brick was approximately 20 μm thick after excluding the upper and lower guard zones of 2.5 μm each (Granholt *et al.*, 2002). TH-positive cells were counted using a 40 $\times$ objective lens with a 1.4 numerical aperture. The first section from the SN in the rostral position was selected randomly; subsequently, every sixth section was counted, and the total number of neurons was calculated. Outline contours for SN cell counts included areas predominantly containing TH-labeled dopaminergic neurons (i.e. SN pars compacta and lateralis), and excluded the SN pars reticulata area, which contains mostly TH fiber network. Similarly, cell counts were determined for the VTA area as defined by the *Mouse Brain Stereotaxic Atlas* (Franklin *et al.*, 1997). Data from each group were analysed with 2 (genotype) $\times$ 2 (age) ANOVAs.

Results

Aged $GFR\alpha-1^{+/-}$ mice are hypoactive but hypersensitive to a DA D1 receptor agonist

Locomotor activities of young and aged wild-type and $GFR\alpha-1^{+/-}$ mice following injections of saline or SKF are summarized in Fig. 1. The 2 (genotype) $\times$ 3 (drug dose) $\times$ 2 (age) ANOVA established that age influenced the effect of both genotype (age $\times$ genotype: $F_{1,39} = 4.408$, $P = 0.05$) and SKF (age $\times$ SKF: $F_{2,39} = 4.344$, $P = 0.05$) on motor activity. Subsequent follow-up ANOVA on data generated when the mice were young adults indicated that SKF clearly elevated motor activity (SKF: $F_{2,39} = 18.549$, $P = 0.01$). Although the mean motor activity levels in Fig. 1 appear to be lower for young $GFR\alpha-1^{+/-}$ than for wild-type mice, particularly for those injected with saline, this was not supported statistically ($F_{1,39} = 3.655$, $P = 0.06$). ANOVA of data generated after the animals had aged indicated that the effect of SKF on motor activity was influenced by genotype (SKF $\times$ genotype: $F_{2,39} = 3.157$, $P = 0.05$), with the aged $GFR\alpha-1^{+/-}$ mice exhibiting a greater response to the drug. Subsequent *post hoc* drug group comparisons at the older age indicated that motor activity was lower for $GFR\alpha-1^{+/-}$ than for wild-type mice injected with saline ($t_{14} = 2.57$, $P = 0.01$), but was higher for $GFR\alpha-1^{+/-}$ than for wild-type mice injected with a high dose (0.10 mg/kg) of SKF ($t_{12} = 2.08$, $P = 0.03$).

$GFR\alpha-1$ protein level is lower in the cortex of aged $GFR\alpha-1^{+/-}$ mice

The amounts of GDNF and $GFR\alpha-1$ in samples of cortical tissue from $GFR\alpha-1^{+/-}$ and wild-type mice at the two ages are summarized in Table 1. The amounts of GDNF did not differ according to either age or genotype; however, the $GFR\alpha-1$ levels for $GFR\alpha-1^{+/-}$ mice depended on an interaction of genotype and age ($F_{1,17} = 4.131$, $P = 0.05$). When the $GFR\alpha-1$ levels in the cortex of 8-month-old wild-type mice were used as a baseline, levels were 19% lower in

the 18-month-old *GFRα-1^{+/-}* mice, and there were no significant differences from those of aged wild-type mice or younger *GFRα-1^{+/-}* mice.

DA level is reduced in the striatum of aged *GFRα-1^{+/-}* mice

Monoamine levels in striatal tissue (ng/g tissue) of young adult and aged *GFRα-1^{+/-}* and wild-type mice are shown in Fig. 2. ANOVAS established group differences for DA ($F_{3,20} = 3.887$, $P = 0.02$) and NE ($F_{3,20} = 3.404$, $P = 0.04$), but not serotonin ($F_{3,20} = 0.44$, $P > 0.05$). *Post hoc* comparison of means indicated that the older *GFRα-1^{+/-}* mice had lower tissue levels of DA than younger mice of either genotype, and higher levels of NE than all other groups ($P = 0.05$, Newman–Keuls test).

Morphological alterations in the nigrostriatal and mesolimbic system of aged *GFRα-1^{+/-}* mice

Immunohistochemical assessments of the nigrostriatal dopaminergic system for *GFRα-1^{+/-}* and wild-type mice at the two ages are summarized in Fig 3–Fig6. Quantification of TH-positive fiber density in the dorsolateral striatum, and the core and shell areas of the ventromedial striatum, is summarized in Fig. 3. The 2 (genotype) × 2 (age) ANOVAS of data from each region indicated that TH-positive fiber density in the core of the ventromedial striatum depended on a genotype × age interaction ($F_{1,18} = 5.129$, $P = 0.03$). *Post hoc* Newman–Keuls tests indicated that TH-positive fiber density was significantly elevated for older *GFRα-1^{+/-}* mice in comparison to all other groups ($P = 0.05$). TH-positive fiber density in the shell region of the ventromedial striatum or the dorsolateral striatum was unaffected by genotype, age, or their interaction.

Figure 4 summarizes photomicrographs of TH immunostaining in sections from the SN (top row) and VTA (bottom row) of young wild-type and *GFRα-1^{+/-}* mice (first two columns), and aged wild-type and *GFRα-1^{+/-}* mice (last two columns). Evaluation of the photomicrographs of the two genotypes suggests that there was no TH-immunoreactive cell loss for young *GFRα-1^{+/-}* mice, a conclusion supported by comparison of the adjacent sections stained with cresyl violet noted in the middle row of Fig. 4. Evaluation of the photomicrographs taken from the SN of aged mice, however, clearly indicates fewer TH-positive neurons for aged *GFRα-1^{+/-}* mice than for wild-type mice of either age. This interpretation is also supported by comparing the adjacent cresyl violet-stained sections in the middle row. This TH-positive neuronal loss for aged *GFRα-1^{+/-}* mice was more evident in the dorsal than in the ventral tier of the SN pars compacta neurons, and was not observed for the VTA, which is shown in the bottom row of Fig. 4 for the four groups.

Figure 5 summarizes estimates of the number of TH-positive neurons in the SN (top graph) and VTA (bottom graph). A genotype × age ANOVA on data from the VTA indicated no significant effects of genotype, age, or their interaction. Analysis of data from the SN, however, indicated that the TH-positive cell count for aged *GFRα-1^{+/-}* mice was reduced ($F_{1,16} = 4.574$, $P = 0.05$), and that it was substantially lower for the aging *GFRα-1^{+/-}* mice than for young or aged wild-type mice (25% and 30%, respectively), or for young *GFRα-1^{+/-}* mice (18%).

The co-localization of DAT and TH is noted in Fig. 6, and confirms that the reduction in TH immunostaining accompanies loss of DAT. DAT located in dopaminergic terminals and cell bodies is independent of TH synthesis or accumulation. It is a reliable marker for functionally viable dopaminergic neurons. The magnified images in the figure are from the SN pars compacta. DAT activities for the four genotype × age groups as labeled in the left margin are summarized in the green panels on the left of Fig. 6. TH immunostaining for the different genotype × age groups are presented in the middle panels (red). Examination of the TH images for the young adult wild-type group (top panel) indicates a dense network of TH-positive fibers in addition to the neurons in the SN pars compacta. In comparison, images from aged

GFRα-1^{+/-} mice (bottom panel) have not only reduced TH-positive fiber density, but also smaller neurons than aged wild-type or young adult mice of either genotype. The confocal images are merged in the panels on the right side of Fig. 6 to demonstrate the co-localization of neurons positive for both DAT (green) and TH (red) for the four groups of mice. We could not find cells in any group that were positive for DAT and not for TH. This co-localization of DAT and TH further confirmed that the loss of TH-positive neurons, measured by unbiased stereological cell counts, was indeed functional neuronal loss.

Discussion

Five findings of this study indicate the importance of the high-affinity GDNF receptor, GFRα-1, in maintaining normal function of the nigrostriatal dopaminergic system. First, motor activity declined with aging and was lower for the older *GFRα-1^{+/-}* mice than for their wild-type age-matched controls, which is consistent with hypofunction of either the nigrostriatal and/or mesolimbic dopaminergic systems. Second, the enhanced locomotor stimulation produced by the DA D1 receptor agonist SKF 82958 in the older *GFRα-1^{+/-}* mice is consistent with the upregulation of this receptor system that occurs with prolonged reductions in synaptic DA levels (Narang & Wamsley, 1995). Third, the reduction of striatal DA levels commonly noted during the aging process (Collier *et al.*, 2007) was even greater in *GFRα-1^{+/-}* mice. Fourth, the number of TH-positive neurons in the SN was lower for *GFRα-1^{+/-}* mice than for wild-type mice, most notably for the older *GFRα-1^{+/-}* mice. Finally, TH immunostaining in the core of the ventromedial striatum was elevated in the older *GFRα-1^{+/-}* mice. The combined behavioral, pharmacological, neurochemical and morphological measures provide evidence of abnormalities in *GFRα-1^{+/-}* mice indicative of an exacerbated aging-related decline in dopaminergic system function. The noted deficiencies, in turn, suggest that GFRα-1, the primary signaling mechanism for GDNF, is necessary for GDNF to maintain normal function of the nigrostriatal dopaminergic system. Although the precise mechanism(s) for the aging-related changes in the dopaminergic system are not clear, the present study clearly establishes that genetic reductions in GFRα-1 can contribute to the degenerative changes observed in this system during the aging process.

GFRα-1 deficiency, aging and motor activity reduction

Motor activity declined with age for both genotypes, and was lower for older *GFRα-1^{+/-}* mice than for age-matched wild-type controls. The decline in motor activity with age is consistent with a number of reported studies on rats (Roth, 1986; Emerich *et al.*, 1993; Dorce & Palermo-Neto, 1994; Hebert & Gerhardt, 1998; Yurek *et al.*, 1998; Ingram, 2000; Leussis & Bolivar, 2006) and several mouse strains (Boger *et al.*, 2006; Colebrooke *et al.*, 2006; Golan *et al.*, 2006; Kopp *et al.*, 2006; Serradj & Jamon, 2007; Zambrana *et al.*, 2007) using the characteristic 20–24 months of age criterion for senescence. Although the older mice in the present study were tested at 18 months of age, motor activity at this age was clearly lower than that obtained when they were young adults, and the reduction was greater for the *GFRα-1^{+/-}* mice. The observed lower motor activity for *GFRα-1^{+/-}* mice is similar to that reported for *GDNF^{+/-}* mice (Zaman *et al.*, 2003; Boger *et al.*, 2006), and provides additional evidence that this neurotrophic factor is involved in the maintenance of neural systems mediating motor behavior. The lower motor activity for *GFRα-1^{+/-}* mice is not likely to be due to compromised motor function, as their motor activity was equal to or greater than that of wild-type controls after DA D1 agonist injections. Perhaps a more likely explanation for the lower motor activity for *GFRα-1^{+/-}* mice in our study is reduced arousal. Arousal, indexed as heightened motor activity, is commonly observed when mice are introduced to a novel environment (Misslin *et al.*, 1981), declines with aging (Willig *et al.*, 1987; Emerich *et al.*, 1993; Dorce & Palermo-Neto, 1994; Shukitt-Hale *et al.*, 2001), and is associated with declining dopaminergic system function (Fink & Smith, 1980; Dulawa *et al.*, 1999). These results and a report that GDNF infusion

improves motor function deficits in aged non-human primates, probably due to improved DA levels and release in the striatum and SN (Grondin *et al.*, 2003), indicate the importance of this trophic factor in aging-related decline in motor activity. Alternatively, the ascending NE system with cell bodies in the locus coeruleus mediates exploratory activity (Harro *et al.*, 1995), and dysfunction of this system might also mediate the aging-related changes observed in *GFRα-1^{+/-}* mice. Interestingly, locus coeruleus NE neurons degenerate with age in *GDNF^{+/-}* mice (Zaman *et al.*, 2003), again indicating the importance of this trophic factor for systems associated with aging-related changes in motor activity.

GFRα-1 deficiency, aging and DA D1 agonist hypersensitivity

The enhanced response of the older *GFRα-1^{+/-}* mice to the DA D1 agonist suggests upregulation of DA D1 receptor activity, perhaps related to prolonged reductions in DA exposure. Synaptic DA influences the number and/or function of DA D1 and D2 receptors (Gnegy & Costa, 1980), with partial dopaminergic system lesions producing compensatory increases in DA turnover (Greenwood *et al.*, 1991), sprouting of striatal fibers (Ho & Blum, 1998), and hypersensitivity (Reader & Dewar, 1999), as well as upregulation of D1 and D2 receptor expression in the striatum (Raisman *et al.*, 1985; Narang & Wamsley, 1995; Betarbet & Greenamyre, 2004). Similar compensatory increases are reported for aged animals with SN neuronal loss, whose surviving neurons increased DA synthesis and turnover (Greenwood *et al.*, 1991; Stokes *et al.*, 1999). In addition, previous work from our group indicates that *GDNF^{+/-}* mice have increased levels of D2 receptor mRNA expression in the striatum at 12 months (Boger *et al.*, 2004). Finally, PD patients are reported to have higher striatal levels of D1 and D2 receptors (Raisman *et al.*, 1985; Seeman *et al.*, 1987), and hypersensitivity of DA receptors is hypothesized to account for the dyskinesias of striatal origin during L-DOPA therapy of PD patients (Bernheimer *et al.*, 1973). Collectively, these studies indicate that prolonged deficiency of DA in the striatum leads to D1 receptor hypersensitivity. The reduced activity of both young adult and aged *GFRα-1^{+/-}* mice in our experiment is consistent with reduced DA levels in the mesolimbic or nigrostriatal system. Although altered drug metabolism cannot be ruled out, the hypersensitivity to the DA D1 agonist in the aged mice is consistent with compensatory increases in DA D1 receptor activity, perhaps related to prolonged reductions in their exposure to DA. This interpretation is consistent with the reduced tissue level of DA and the reduction in TH-positive cells in the SN of the older *GFRα-1^{+/-}* mice in our experiment, and with reports that the extent and duration of DA reduction influences the response to DA D1 agonists (Herrera-Marschitz *et al.*, 1985; Starr *et al.*, 1987; Narang & Wamsley, 1995; Bishop & Walker, 2003). Thus, the enhanced stimulatory effect of the DA D1 agonist on aged *GFRα-1^{+/-}* mice relative to age-matched wild-type or younger mice of either genotype is probably due to upregulated activity of DA D1 receptors resulting from the long-term reduction in available DA.

GFRα-1 deficiency, aging and striatal DA system abnormalities

The reduced DA concentration in the striatum and the enhanced TH-positive fiber density in the core of the ventromedial portion of this structure, combined with the lower cell number in the pars compacta of the SN of *GFRα-1*-deficient aged mice, implicate the importance of *GFRα-1* and *GDNF* in maintaining the normal structure and function of dopaminergic systems with aging. The aging-related reduction in striatal DA levels and the reduced TH-positive cell number in the SN of the *GFRα-1*-deficient aged mice are consistent with previous reports indicating a decline in the nigrostriatal dopaminergic system with aging (Greenwood *et al.*, 1991; Dorce & Palermo-Neto, 1994; Stark & Pakkenberg, 2004). The striatal DA level was lower in *GFRα-1^{+/-}* mice of both ages; however, the TH-positive cell number in the SN was reduced for only the older *GFRα-1^{+/-}* mice (30%). Our evaluation of TH and DAT co-localization in histological sections from all groups indicated differences according to age and genotype. Given that the sodium- and chloride-dependent DA uptake protein is expressed by

SN dopaminergic neurons, it has been proposed that DAT co-localization establishes the dopaminergic nature of TH-positive neurons, and indicates that the TH-positive neurons contain an essential part of the functionally viable machinery (Betarbet *et al.*, 1997). The SN dopaminergic neurons express many transcription factors (e.g. *Pitx3*, *Engrailed-1/2*, *Nurr1* and *Lmx1a/b*) that are essential for the generation, survival and maintenance of these neurons during development and throughout life (Alavian *et al.*, 2008). Because we did not use these markers to label the SN dopaminergic neurons in the present study, we cannot rule out the possibility that the neurons not staining for TH and DAT observed in the older *GFRα-1*^{+/-} mice still survived but lost the ability to express TH. The loss of DAT expression in the neurons suggests that they were not functionally viable. Despite the 30% TH-positive cell loss in the SN of older *GFRα-1*^{+/-} mice, TH-positive fiber density in projection areas for these dopaminergic neurons was increased by 14% in the ventromedial striatum core region, suggesting the possibility of a compensatory sprouting of dopaminergic terminals in the striatum. Dopaminergic fiber sprouting is an established important compensatory response following partial dopaminergic denervation, and can derive from dopaminergic neurons of either the SN or the VTA (Song & Haber, 2000; Hansen *et al.*, 1995; Ho & Blum, 1998). The significantly increased TH-positive fiber density in the core region of the ventromedial striatum for the older *GFRα-1*^{+/-} mice could reflect compensatory increased branching of projections from the surviving SN dopaminergic neurons in this group. Another possible explanation for the increased TH-positive fiber density in the older *GFRα-1*^{+/-} mice is increased sprouting of NE neurites into the striatum as a result of reduced numbers of dopaminergic terminals, an interpretation consistent with the elevated NE levels observed in this genotype × age group. A similar effect has been reported for transmitter loss in other brain regions, such as the hippocampus (Crutcher *et al.*, 1981; Gage *et al.*, 1983). Future studies utilizing tract-tracing will be necessary to establish the cellular origin of the sprouted fibers observed in the present study. Although further investigation will be necessary to establish the detailed mechanisms, the reductions in striatal DA levels and TH-positive neurons in the SN provide a reasonable mechanism for upregulation of DA receptors and resultant hypersensitivity to the DA D1 agonist for the older *GFRα-1*^{+/-} mice as compared to wild-type mice, and is consistent with the reported relationship between DA level and motor activity after acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure (Bezard *et al.*, 2001; Kurosaki *et al.*, 2004). The lower striatal DA levels and morphological alterations in aged *GFRα-1*^{+/-} mice than in wild-type mice and young *GFRα-1*^{+/-} mice also provide a potential mechanism for the hypoactivity of the older *GFRα-1*^{+/-} mice as well as their enhanced response to the DA D1 agonist.

GFRα-1, GDNF and the aging process

GDNF and its receptor components (c-Ret and GFRα-1) have both been reported to be elevated in spinal motor neurons of aged rats (Bergman *et al.*, 1999), and GDNF has been reported to be elevated in the striatum of aged primates (Collier *et al.*, 2005). The present study further defines the relationship of this growth factor and its primary receptor to the aging process. At the ages assessed (8 and 18 months), GDNF levels in the cortex did not differ according to either genotype or age. In contrast to the lack of changes in cortical GDNF levels, GFRα-1 protein levels in the cortex were lower for aging *GFRα-1*^{+/-} mice than for either their younger counterparts or for age-matched wild-type mice. At the younger adult age (8 months), GFRα-1 protein levels were similar to those of wild-type mice. This result is consistent with an earlier report (Sarabi *et al.*, 2003) that the basal level of GFRα-1 mRNA was similar for *GFRα-1*^{+/-} and wild-type mice. In that study, the increase in GFRα-1 mRNA produced by ischemic challenge was attenuated for the *GFRα-1*^{+/-} mice, as it was for the older GFRα-1-deficient mice in our experiment. The results suggest that the aging process can impact on GFRα-1 protein levels, and that further study of the interaction of GFRα-1 function and the aging process is warranted.

GFR α -1/GDNF deficiency, aging and striatal dopaminergic system abnormalities

In addition to aging-related changes in GFR α -1, noted above, the protective effects of intranigral injections of GDNF against 6-hydroxydopamine neurotoxicity has been reported to be reduced for aged as compared to young adult rats (Fox *et al.*, 2001). Although the report can be interpreted as showing possible aging-related changes in the growth factor signaling pathway at the receptor level, aging-related reductions in the GDNF increase in response to the striatal 6-hydroxydopamine lesion might also be a possibility (Yurek & Fletcher-Turner, 2001). The protective effect of GDNF has also been shown in age-related macular degeneration, which is caused by oxidative damage (Dong *et al.*, 2007). These reports suggest the possibility that the dopaminergic system abnormalities noted for the older GFR α -1^{+/-} mice might be mediated by an aging-related inability to compensate for oxidative stress and inflammatory irritants. Alternative possibilities include the contribution of prolonged insufficient activity of high-affinity GFR α -1 in GFR α -1^{+/-} mice, or perhaps developmental alterations imposed by the deficiency that become evident only after normal DA function subsides with the aging process. Finally, as is common to all transgenic experiments, the possible impact of unforeseen gene changes other than those for GFR α -1 itself might contribute to the dopaminergic system changes. A conditional GFR α -1 knockout study, or time-dependent experiments with GDNF antisense, may be able to resolve which alternative is true, but these models have not yet been developed for the GDNF family of growth factors. Although the mechanism remains to be established, the current results indicate that GFR α -1 is important for maintaining the numbers of DA neurons, and also suggest that GFR α -1 expression is not the primary regulator of GDNF protein production in the brain.

Conclusions

The combined behavioral, pharmacological, neurochemical and morphological changes in dopaminergic systems of GFR α -1^{+/-} mice in this study indicate an exacerbated aging-related decline in dopaminergic system function. The reductions in motor activity and enhanced response to the DA D1 agonist are consistent with the observed reductions in striatal DA concentrations and TH-positive SN neuron number. These deficiencies, in turn, suggest that GFR α -1 is necessary for GDNF to maintain normal function of the nigrostriatal dopaminergic system, as it is probably the primary signaling mechanism for this growth factor. Although the precise mechanism(s) for age-related alterations in the dopaminergic transmitter system in the brain are not clear, the present study clearly establishes that genetic reductions in GFR α -1 can contribute to the degenerative changes observed in this system during the aging process.

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Abbreviations

DA, dopamine
 DAT, dopamine transporter
 GDNF, glial cell-line derived neurotrophic factor
 GFR, glial cell-line derived neurotrophic factor receptor
 NE, norepinephrine
 PBS, phosphate-buffered saline
 PD, Parkinson's disease
 SKF, SKF 82958

SN, substantia nigra
 TBS, Tris-buffered saline
 TH, tyrosine hydroxylase
 VTA, ventral tegmental area

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FIG. 1.

Locomotor activity of young adult (8 ± 1 months) and aged (18 ± 2 months) *GFR α -1^{+/-}* and wild-type mice over a 30-min interval following injections of saline or the D1 receptor agonist SKF 82958 (SKF). Following saline control injections, *GFR α -1^{+/-}* mice were less active than wild-type controls at both ages, and activity declined with age. SKF elevated motor activity at both ages, and to a greater extent in the older than in the young adult animals. Importantly, despite the lower basal activity levels for the older *GFR α -1^{+/-}* mice following saline injections, their response to the D1 agonist was greater than that of comparable aged wild-type mice at the highest SKF dose. Follow-up ANOVA results are indicated (* $P < 0.05$). (See Results for further information on statistical analysis.)

FIG. 2.

Brain monoamine levels (ng/g tissue) in striatal tissue of young adult and aged *GFRα1*^{+/-} and wild-type mice. Dopamine levels (A) were reduced in older *GFRα1*^{+/-} mice as compared to all other groups (* $P = 0.02$). Norepinephrine (NE) levels (B) varied according to genotype, with NE levels being significantly higher ($P < 0.05$) in the striatum of *GFRα1*^{+/-} mice than in that of wild-type mice for both age groups. Serotonin concentrations (C) did not differ across genotype or the ages assessed.

FIG. 3.

Immunohistochemical localization of tyrosine hydroxylase (TH) in the dorsolateral and ventromedial striatum of young and aged wild-type (WT) and *GFRα-1^{+/-}* mice. Insets show the magnified view of the dorsal striatum from the respective images, and the boxed area shows the ventromedial striatum (scale bar 125 μ m, inset scale bar 10 μ m). TH-positive fiber density in the dorsolateral striatum and the shell of the ventromedial striatum did not vary according to genotype or the ages examined. However, the core of the ventromedial striatum of the aged *GFRα-1^{+/-}* mice exhibited a significant increase (* $P < 0.05$) in TH-positive fiber density as compared to all other groups.

FIG. 4.

The top row shows tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (SN) pars compacta of the SN of young and old wild-type (wt) and *GFRα-1*^{+/-} mice. Adjacent sections were stained with cresyl violet, and are presented in the middle row. The magnified view of cresyl violet staining is from the boxed area in TH-stained sections from respective groups in the SN. Cresyl violet-stained sections revealed fewer neurons in the SN pars compacta of aged *GFRα-1*^{+/-} mice. The bottom row shows TH-positive neurons in the ventral tegmental area of young and aged wild-type and *GFRα-1*^{+/-} mice. There was no significant cell loss in this area of aged wild-type or *GFRα-1*^{+/-} mice. Scale bar: 25 μm.

FIG. 5.

Stereological cell counts in the substantia nigra pars compacta stained with tyrosine hydroxylase (TH) show fewer TH-positive neurons in aged *GFR α -I^{+/-}* mice (30% cell loss; **P* < 0.038). Although the number of TH-positive neurons in the ventral tegmental area was reduced (24%) in aged *GFR α -I^{+/-}* mice as compared to aged wild-type mice, the difference was not significant.

FIG. 6.

Photomicrograph showing representative fields obtained using confocal microscopy at 63× oil magnification. Images of dopamine transporter (DAT), tyrosine hydroxylase (TH) and co-localization (merged images) from examples of respective age and genotypes (labeled in the left-hand margin) are presented in the right-hand panel. All DAT-positive cells express TH. There was 100% co-localization of DAT-positive and TH-positive neurons. Scale bar: 10 μm.

TABLE 1GFR α -1 and GDNF levels in cortical tissue from *GFR α -1*^{+/-} and wild-type mice

Genotype	GDNF level [*]		GFR α -1 level [*]	
	8 months	18 months	8 months	18 months
Wild type	1.00 $\pm$ 0.029	1.02 $\pm$ 0.38	0.84 $\pm$ 0.015	0.81 $\pm$ 0.75
<i>GFRα-1</i> ^{+/-}	1.01 $\pm$ 0.020	0.99 $\pm$ 0.026	0.82 $\pm$ 0.015	0.68 $\pm$ 0.016

* Integrated density normalized to the control.