Aged Tgfβ2/Gdnf double-heterozygous mice show no morphological and functional alterations in the nigrostriatal system

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Abstract Loss of dopaminergic neurons in the substantia nigra pars compacta and the resulting decrease in striatal dopamine levels are the hallmarks of Parkinson’s disease. Tgfβ and Gdnf have been identified as neurotrophic factors for dopaminergic midbrain neurons in vivo and in vitro. Haploinsufficiency for either Tgfβ or Gdnf led to dopaminergic deficits. In this study we therefore analyzed the nigrostriatal system of aged Tgfβ2+/−/Gdnf+/- double-heterozygous mice. Unexpectedly, we found no morphological changes in the nigrostriatal system as compared with age-matched wild-type mice. There were no significant differences in the number of TH-positive midbrain neurons and no changes in the optical density of TH immunoreactivity in striata of Tgfβ2+/−/Gdnf+/- double-heterozygous mice. Moreover, we found no significant differences in the striatal levels of dopamine and its metabolites dihydroxyphenylacetic acid and homovanillic acid. Our results indicate that a combined haploinsufficiency for Tgfβ2 and Gdnf has no impact on the function and the survival of midbrain DA neurons under normal aging conditions.

Keywords Tgfβ2 · Gdnf · Dopamine · Nigro-striatal system · Midbrain

Introduction

The deleterious loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the resulting decrease in dopamine (DA) levels in the striatum, the main target projection area of SNpc DA neurons, are the hallmarks of Parkinson’s disease (PD; Dauer and Przedborski 2003). Although the exact mechanisms for degeneration of DA neurons in the SNpc have not yet been elucidated, many in vivo and in vitro attempts have been made to identify neuroprotective factors. Tgfβ and Gdnf have attracted scientific attention as they are able to increase the numbers of cultured midbrain neurons (Farkas et al. 2003; Krieglstein et al. 1995; Lin et al. 1993). Moreover, Tgfβ and Gdnf are able to protect DA neurons in different models of PD, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Krieglstein and Unsicker 1994; Krieglstein et al. 1995; Tomac et al. 1995; Tomac et al. 1995; Kordower et al. 2000) and 6-hydroxydopamine (6-OHDA) toxicity (Hoffer et al. 1994; Bowenkamp et al. 1995; Kearns and Gash 1995). Recently, it has been shown that Gdnf-mediated neuroprotection in the MPTP-lesioned nigrostriatal system is Tgfβ-dependent (Schober et al. 2007). These
results suggest a Tgfβ/Gdnf synergism, which is further underlined by the fact that Tgfβ increases Gdnf responsiveness in neurons by recruiting the Gdnf receptor GFRζ1 to the cell membrane (Peterziel et al. 2002).

However, the role of Tgfβ and Gdnf for the development of midbrain DA neurons and their maintenance under normal conditions remains elusive. Mice deficient for either Tgfβ2 (Sanford et al. 1997) or Gdnf (Pichel et al. 1996) die perinatally. As a consequence, the nigrostriatal system can only be investigated prenatally. Interestingly, the double-deficiency of both Tgfβ2 and Gdnf does not affect the differentiation and survival of midbrain DA neurons during embryonic development (Roussa et al. 2008; Sánchez et al. 1996). Tgfβ2−/−/Gdnf−/− mice showed no loss of midbrain DA neurons at E14.5 (Roussa et al. 2008) and E18.5 (Rahhal et al. 2009), indicating that Tgfβ2/Gdnf synergism is not important for the induction of the dopaminergic phenotype in vivo. In contrast, recent studies found that Tgfβ2+/− heterozygous mice have 12% fewer dopaminergic neurons at 6 weeks of age and 30% less dopamine in the striatum than controls (Andrews et al. 2006). Gdnf+/− heterozygous mice showed an accelerated decline of dopaminergic neurons during aging along with functional deficits (Boger et al. 2006). These studies thus imply that Tgfβ2 and Gdnf might serve as factors regulating the function and the survival of adult midbrain DA neurons.

In the present study we thus analyzed the nigrostriatal system of aged Tgfβ2+/−/Gdnf+/− double-heterozygous mice. Using tyrosine hydroxylase immunohistochemistry, we demonstrate that double-heterozygous animals had no morphological changes in the nigrostriatal system as compared with age-matched wild-type mice. Moreover, we found no significant differences in the striatal levels of dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Our results indicate that a combined haploinsufficiency for Tgfβ2 and Gdnf, in contrast to the single haploinsufficiencies, has no impact on the function and the survival of midbrain DA neurons under normal aging conditions.

Materials and methods

Animals

The generation of Tgfβ2 and Gdnf single mutant animals was described previously (Sanford et al. 1997, Pichel et al. 1996). To generate Tgfβ2+/−/Gdnf+/− animals, single-heterozygous mice were crossbred. Mice were bred locally in the Central Animal Facility (University of Göttingen) on a C57BL/6 × 129S background. The experimental setup was approved by the local chief veterinarian. Animals were housed in cages (1–4 mice per cage) and had ad libitum access to food and water. Mice were maintained in a 12 h dark/light cycle with light from 7:00 am to 7:00 pm and a constant temperature of 20–22°C. For experiments carried out during this study, adult mice with ages ranging from 20 to 25 month were used and are from now on referred to as aged.

Genomic DNA isolation and genotyping

Genomic DNA was isolated from tail biopsies. Heterozygous Tgfβ2 mice were detected by PCR using the exons 6 specific primers p5 (5′-AATGTGCGAGGATAATGCTG-3′) and p3 (5′-AACCTCATAGATATGGGCATG-3′) as described by Sanford et al. (1997). Gdnf heterozygous animals were detected by PCR using the wild-type allele specific primers GdnF (5′-GACTACGGGAGGATAGAGAAG-3′) and GdnFR (5′-TATCGTCTCTGCTTGTCC-3′) as well as the neomycin cassette specific primers NEOF (5′-CCAGAAACTCCAGGGAAAGTCA-3′) and NEOR (5′-CCATACATCCACCGTTTACGG-3′). PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining (Fig. 1).

RNA isolation and RT-PCR

RNA was isolated from striatal tissue of aged single-heterozygous mice using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. 1 μg of each sample was reverse transcribed using Fermentas Reverse Aid™ according to the manufacturer’s instructions. RT-PCR was performed using the following primers: Gdnf for 5′-GGCCACTTTGAGTTAAATGTC-3′, Gdnfrev 5′-CCGATTCCTCTCCTCA TCGAG-3′, Tgfβ2for 5′-GGTGGATTTCCATCTACAA-3′, Tgfβ2rev 5′-GGAAGTATCCACACCGTTTACCG-3′. Gapdh was used as loading control (Gapdhhfor 5′-TGACCAGGCGCCCAATAC-3′, Aaid™ 338-bp fragment amplified from the wild-type

![Fig. 1](image-url) Genotyping results for Tgfβ2 and Gdnf mutant animals. a A 132-bp fragment of the Tgfβ2 wild-type allele and a 1,300-bp fragment of the NEO-cassette served to determine wt and heterozygous animals. b For the detection of Gdnf−/− animals a 480-bp fragment was amplified from the NEO-cassette in combination with a 338-bp fragment amplified from the wild-type Gdnf allele.
C. Nigral and striatal free-floating sections (40 μm) were stained using the PaP-DAB protocol. Densitometric analysis was performed with FluorChem 8800 software (AlphaInnotech, Biozym). Densitometric analysis was performed with FluorChem 8800 software (AlphaInnotech, Biozym) and values were normalized to Gapdh.

Determination of striatal DA and metabolites

Concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) from striatal tissue samples were analyzed using HPLC with electrochemical detection. Briefly, animals were killed by cervical dislocation, brains were rapidly removed and chilled on ice. Left side striata were dissected, weighed and stored in homogenator vials containing ceramic pearls. 0.1 M perchloric acid was added (40 μl/mg tissue) and tissue samples were stored at −80°C until measurements. Prior to measurements, tissues were homogenized using Homogenator Precellys24 (PEQLAB, Erlangen, Germany) to measure DA and its metabolites. After washing three times with TBS; 0.9% NaCl, 0.01 M phosphate buffer) containing 3% hydrogen peroxide (H2O2) to quench any remaining endogenous activity, samples were homogenized using the Precellys24 homogenizer (PEQLAB, Erlangen, Germany) for 1 minute with two shaking of 30 s each. Afterwards, all vials were centrifuged (5 min, 1,000 g, 4°C) and 20 μl of supernatant were loaded onto a reverse-phase column (prontosil 120-3-C18, Bischoff, Leonberg, Germany). Dopamine was detected electrochemically using an ESA Coulorchem II detector with a 5011A analytical cell (400 mV). Each liter of the mobile phase contained 105 ml methanol (MERK) 6.973 g of sodium acetate; 0.048 g of EDTA; 7.355 g of citric acid monohydrated; 0.105 g octane sulfonate. pH 4.3 was reached using concentrated citric acid. For each run, standards of 0.15 or 0.3 or 1.5 μM of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured at the beginning, in the middle and at the end of the sample sequences. Finally, DA levels were normalized to wild-type levels and are given in percentages.

Immunohistochemistry

Brains from animals sacrificed for striatal DA analysis were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight and stored in phosphate buffered saline (PBS; 0.9% NaCl, 0.01 M phosphate buffer) containing 30% sucrose at 4°C. Nigral and striatal free-floating sections (40 μm) were stained using the PaP-DAB method. After rinsing with tris-buffered saline (TBS; 0.05 M Tris, 0.9% NaCl) three times for 10 min, sections were quenched with 3% hydrogen peroxide (H2O2) for 5 min and rinsed again in TBS three times for 10 min. Sections were incubated for 3 h at room temperature with blocking solution containing 10% normal goat serum (NGS; Gibco), 0.5% bovine serum albumin (BSA; Roth, Karlsruhe, Germany). Thereafter, sections were incubated overnight with rabbit anti-TH polyclonal antibody (Millipore, AB152) diluted 1:3,000 in blocking solution at room temperature with gentle shaking. After rinsing three times with TBS for 10 min, sections were incubated for 1 h with goat anti-rabbit antibody (Nordic Immunology) diluted 1:500 in blocking solution gently shaking at room temperature. Again, sections were rinsed three times with TBS for 10 min and incubated for 1 h at room temperature with PaP-rabbit (Nordic Immunology) diluted 1:800 in blocking solution. Immunoreactivity was visualized using diaminobenzidine (Sigma) as described by Adams (1981). Sections were mounted on gelatine-coated slides, air-dried overnight, dehydrated through graded ethanols, cleared in xylene and finally coverslipped with Entellan mounting medium (Merck). Images were taken with Biozero BZ-8000 microscope (KEYENCE, Germany).

Striatal TH fiber density measurements

Striatal TH-positive fiber staining was assessed by optical density (OD) analysis. Images of stained striatal sections were captured using a NIKON SMZ 1500 (Nikon, Düsseldorf, Germany). ODs were measured using AlphaEase Software (AlphaInnotech, Biozym). For every animal, the ODs from three different striatal and cortical sections were determined and the final reading was calculated as an average of the ratio of striatal and cortical TH-staining from the same section. OD values are expressed as percentages of control.

Stereological analysis of TH-positive cells

The number of TH-positive cells of the substantia nigra pars compacta (SNpc) was assessed using the StereoInvestigator (Microbrightfield Europe, Magdeburg, Germany) with optical fractionator according to the optical dissector rules (Gundersen et al. 1988). The medial terminal nucleus (MTN) of the accessory optical tract was used as the medial border of the SNpc, as described by Sauer et al. (1995). For each animal, five sections with an evaluation interval of 3 were selected and all TH-positive neurons lateral to the MTN were counted. The nucleus of each TH-immunoreactive neuron was chosen as the counting unit. The optical fractionator was optimized to reach a coefficient of error ≤0.1. Cell counts were performed using a 63× oil objective (Zeiss, Göttingen) and AxioImager (Zeiss, Göttingen).

Western blot

Total proteins were isolated from midbrain and striatum samples from 3-month-old Tgfl2+/+Tgfl2−/− and
4.5-month-old Gdnf<sup>+/+</sup>/Gdnf<sup>-/-</sup> animals using M-PER Mammalian Protein Extraction Reagent (Pierce) supplemented with Complete Protease Inhibitor cocktail (Roche). 10 μg protein was loaded per lane of 9% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred onto PVDF membranes (Immobilon, Milipore, Schwalbach, Germany). After transfer, the membranes were washed with TBST and blocked with 10% dry milk (Roth, Karlsruhe, Germany) in TBST for 2 h at room temperature. Membranes were incubated with primary antibodies (anti-Tgfβ, SantaCruz, 1:200; anti-Gdnf, R&D Systems, 1:1,000) at 4°C overnight with gently shaking. After incubation with horseradish peroxidase-conjugated secondary antibodies (GaR-HRP/GaM-HRP, Cell Signaling Technology, 1:10,000), labeled proteins were detected using the SuperSignal<sup>TM</sup> detection Kit (Pierce, USA). To confirm equal protein loadings, membranes were reprobed with a Gapdh antibody (1:10,000, Abcam). All blots were captured with Amersham Hyperfilm<sup>TM</sup> ECL (GE Healthcare). Densitometric analysis was performed using FluorChem 8800 software (AlphaInnotech, Biozym, Olendorf, Germany).

Statistics

Statistical analysis was performed using the software GraphPad Prim 4 (GraphPad Software Inc.). For determination of significant differences the unpaired t test or one-way ANOVA and Bonferroni’s post hoc test for multiple comparisons was used. P values < 0.05 were considered to be significant.

Results

Reduced mRNA levels of Tgfβ2 and Gdnf in heterozygous animals

To learn more about the role of Tgfβ2 and Gdnf for the survival of dopaminergic neurons, we used their combined deficiency in Tgfβ2<sup>+/−</sup>/Gdnf<sup>−/−</sup> double-heterozygous mice. For the interpretation of our findings it is important to know the extent of Tgfβ2/Gdnf deficiency in these animals. We therefore determined the expression levels of Tgfβ2 and Gdnf by semiquantitative RT-PCR. To determine the mRNA levels of Tgfβ2 and Gdnf in the dopaminergic system of heterozygous animals, aged mice were killed and total RNA was isolated from striatum. As shown in Fig. 2a the levels of Tgfβ2 mRNA in heterozygous mice were significantly reduced compared to age-matched wild-type littermates. Figure 2b shows the corresponding results for Gdnf RNA levels. Again, the levels of Gdnf RNA were significantly reduced in heterozygous animals.

Reduced protein levels of Tgfβ2 and Gdnf in heterozygous mice

After we have shown that the mRNA levels of Tgfβ2 and Gdnf were reduced in heterozygous mice we further analyzed if the corresponding protein levels were also reduced. As shown in Fig. 3 protein levels of Tgfβ2 were reduced in midbrain (Fig. 3a, c) and striatum (Fig. 3d, f) samples from Tgfβ2 heterozygous mice. Interestingly, we observed increased protein levels of Gdnf in striatal samples from Tgfβ2 heterozygous mice (Fig. 3e, f), whereas the levels of Gdnf in midbrain samples were similar to wild-type littermates (Fig. 3b, c).

In accordance with mRNA levels we observed significantly reduced Gdnf protein levels in midbrain and striatum samples from Gdnf heterozygous mice (Fig. 4a, c, d, f). Moreover, we detected significantly increased Tgfβ2 protein levels in striatum samples from Gdnf heterozygous mice. Tgfβ2 protein levels in midbrain samples from these mice were slightly increased without reaching significance.

Morphology, distribution and number of TH-ir neurons in SNpc of aged Tgfβ2<sup>+/−</sup>/Gdnf<sup>−/−</sup> mice

For the analysis of the morphology of TH-immunoreactive neurons in the SNpc, brains of aged wild type, Tgfβ2 and Gdnf single-heterozygous as well as Tgfβ2/Gdnf double-heterozygous animals were cut in 40-μm slices and stained with anti-TH antibody. Figures 5a–d show that all genotypes analyzed displayed a normal distribution of dopaminergic neurons in the SNpc. Detailed images (Fig. 5e–h) show a normal cellular morphology of TH-ir neurons indicating that haploinsufficiency for Tgfβ2, Gdnf or Tgfβ2 and Gdnf does not alter dopaminergic neuron distribution and morphology in the SNpc of aged mice. Stereological...
Fig. 3 Protein levels of Tgfb2 and Gdnf in Tgfb2−/− mice. Proteins were isolated from midbrain and striatum samples of 3-month-old Tgfb2−/− mice and wild-type littermates. After electrophoresis and protein transfer, membranes were probed with anti-Tgfb2 and anti-Gdnf antibodies. Gapdh was used to determine equal protein loading. Protein levels of Tgfb2 were reduced in midbrain (a, c) and striatum samples (d, f) in Tgfb2−/− mice. Gdnf levels in the midbrains of Tgfb2−/− mice were similar to wild-type littermates (b, e). Interestingly, the protein levels of Gdnf in striatum samples were increased in Tgfb2−/− mice (e, f). Densitometric analysis of protein band intensities was performed using FluorChem 8800 software.

Fig. 4 Protein levels of Gdnf and Tgfb2 in Gdnf−/− mice. Proteins were isolated from midbrain and striatum samples of 4.5-month-old Gdnf−/− mice and wild-type littermates. After electrophoresis and protein transfer, membranes were probed with anti-Tgfb2 and anti-Gdnf antibodies. Gapdh was used to determine equal protein loading. Protein levels of Gdnf were significantly reduced in midbrain (a, e) and striatum samples (d, f) in Gdnf−/− mice. Tgfb2 levels in the midbrains of Gdnf−/− mice were slightly increased compared to wild-type littermates (b, c). Surprisingly, the protein levels of Tgfb2 in striatum samples of Gdnf−/− mice were significantly increased compared to wild-type littermates (e, f). Densitometric analysis of protein band intensities was performed using FluorChem 8800 software. **, *P values derived from student’s t test are <0.05 and <0.01.

counting showed that aged Gdnf+ mice had slightly reduced numbers of TH-ir neurons in the SNpc. However, this reduction in neuron number was not significant (Fig. 5i).

Striatal TH fiber density

DA neurons of the SNpc project to the dorsal striatum via the nigrostriatal pathway. In order to analyze the density of
TH-positive nerve terminals in striata of aged mice, we dissected the right forebrain (the left was used to isolate striata for DA analysis). Figures 6a–d show the TH immunohistochemistry results for the different genotypes analyzed. There were no substantial differences in the staining intensities of striatal slices of different genotypes. Further, the analysis of the optical densities confirmed these impressions (Fig. 6e).

Normal striatal dopamine levels in aged $\text{Tg}\beta 2^{+/−}; \text{Gdnf}^{+/−}$ mice

As a functional readout for the nigrostriatal system we analyzed the levels of dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum by HPLC. As shown in Fig. 7a, there were no significant differences in the DA levels of aged double-heterozygous and single-heterozygous animals compared with age-matched wild-type mice. Further, the levels of DOPAC and HVA (Fig. 7b, c), as well as the DA turnover (data not shown) were not altered in aged mice in all genotypes analyzed. These results indicate that haploinsufficiency for $\text{Tg}\beta 2$, Gdnf or $\text{Tg}\beta 2$ and Gdnf has no effect on striatal DA synthesis and metabolism in aged animals.

Discussion

In the present study we analyzed the effect of double haploinsufficiency for $\text{Tg}\beta 2$ and Gdnf on the maintenance
of the nigrostriatal system in aged mice. Our results demonstrate that aged Tgfβ2+/−/Gdnf+/− mice had the same numbers of TH-positive midbrain DA neurons as age-matched wild-type animals. Further, there were no differences in the density of TH-positive fibers in striata of Tgfβ2+/−/Gdnf+/− mice. As a functional readout for the nigrostriatal system we analyzed the levels of dopamine and its metabolites DOPAC and HVA in aged Tgfβ2+/−/Gdnf+/− mice. Again, we found no significant differences between double-haploinsufficient and wild-type mice. These data thus indicate that the combined haploinsufficiency for Tgfβ2 and Gdnf has no effect on the morphology and function of midbrain DA neurons under normal aging conditions.

The observed lack of an age-dependent impairment of morphology and function of midbrain DA neurons in Tgfβ2 and Gdnf double haploinsufficiency was a highly unexpected. Tgfβ2 heterozygous animals were reported to have a 12% reduction of DAergic neurons already at 6 weeks of age, and striatal dopamine declined to 70% within 6 months (Andrews et al. 2006). This finding was not reproduced in our study, as the aged Tgfβ2+/−/Gdnf+/− mice did not show a smaller number of TH-ir neurons in the SNc or reduced striatal markers. Gdnf heterozygous animals were documented to have an increasing age-dependent reduction of the number of DA neurons, altered fiber and cell body morphology, as well as reduced levels of TH (Boger et al. 2006). In our study, the morphology of DA neurons was normal. The mean number of TH-positive neurons in the SNc was slightly smaller than controls, but this difference did not reach statistical significance (Fig. 5i). Consistently, mice deficient for RET receptor, which forms the Gdnf receptor together with GDNF family receptor α, have normal numbers of dopaminergic neurons up to 12 months of age (Jain et al. 2006), and the same sensitivity to MPTP as WT mice (Kowsky et al. 2007). The RET receptor (and thus, likely Gdnf) were only required for sprouting of axon collaterals in the striatum after MPTP treatment (Kowsky et al. 2007), suggesting that Gdnf might be more important for regeneration of dopaminergic neurons than for survival per se. However, RET-deficient mice did show some loss of dopaminergic markers after 12 months of age (Kramer et al. 2007).

Taken together, our findings suggest that a reduction in Tgfβ2 and Gdnf mRNA and protein are not sufficient to affect the survival of dopaminergic neurons in the SNc in aged (20–25 months old) mice. These findings are in line with the normal dopaminergic system in Tgfβ2−/−/Gdnf−/− embryos (Roussa et al. 2008). The discrepant findings by others in “middle-aged” (12 months old) mice (Andrews et al. 2006; Boger et al. 2006) might result from a stronger depletion of Tgfβ2 and Gdnf in these studies. Alternatively, the normal, aging-related decline in dopamine neuron numbers might arrive at a “floor” level at 20–25 months of age, so that the difference between wild-type and heterozygous animals is less pronounced than in “younger” animals.

Based on the previous findings by others, we had expected that haploinsufficiency for both Tgfβ2 and Gdnf would result in the same kind, or even in a more pronounced age-dependent impairment of morphology and function of DA neurons. The lacking phenotype of the combined haploinsufficiency for both Tgfβ2 and Gdnf could be interpreted as a requirement for balanced levels of both factors. Availability of both is required to promote
and maintain the survival of midbrain DA neurons. A partial depletion of one of the factors may affect the balance of both proteins and might therefore result in an age-dependent impairment of the system. Partial depletion of both factors may then result in the establishment of a new balance of the amounts of Tgfβ2 and Gdnf, however, on a lower level. Interestingly, we found that Gdnf protein levels in striatal tissue from Tgfβ2 heterozygous and Tgfβ2 protein levels in striata from Gdnf heterozygous mice were higher than in wild-type littermates. This phenomenon could be interpreted as a compensatory mechanism after partial depletion of one factor. Moreover, this hypothesis would also support the fact that Gdnf and Tgfβ2 cooperate to promote and maintain the survival and status of differentiation of midbrain DA neurons (Krieglstein et al. 1998; Schober et al. 2007). A combined or balanced requirement for Tgfβ2 and Gdnf would have strong implications for therapeutic strategies for PD patients based on Gdnf administration.

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