Absolute Requirement of GDNF for Adult Catecholaminergic Neuron Survival

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SUMMARY

GDNF is a potent neurotrophic factor that protects catecholaminergic neurons from toxic damage and induces fiber outgrowth. However, the actual role of endogenous GDNF in the normal adult brain is unknown, despite GDNF-based therapies are considered promising for neurodegenerative disorders. We have generated a conditional GDNF-null mouse to suppress GDNF expression in adulthood, hence avoiding the developmental compensatory modifications masking its true physiologic action. After *GDNF* ablation animals showed a progressive hypokinesia and a selective decrease of brain *tyrosine hydroxylase* (*TH*) mRNA, accompanied of pronounced catecholaminergic cell death, affecting most notoriously the locus coeruleus (LC), which practically disappears, the substantia nigra (SN) and the ventral tegmental area (VTA). These data unequivocally demonstrate that GDNF is indispensable for adult catecholaminergic neuron survival, and also show that in physiologic conditions down-regulation of a single trophic factor can produce massive neuronal death.

Key words: endogenous GDNF, conditional gene deletion, neurodegeneration, dopaminergic cell death, noradrenergic neuronal death, Parkinson's disease, mouse model

INTRODUCTION

In recent years, neurotrophic factors have emerged as promising therapeutic tools for neurodegenerative diseases due to their effects on the promotion of survival, differentiation and phenotype of neurons. Among these agents, the dopaminotrophic glial cell line-derived neurotrophic factor (GDNF)[1], has raised special attention because of its possible usefulness in the treatment of Parkinson's disease (PD)[2]. In rodent and primate models, GDNF has been shown to protect dopaminergic nigrostriatal neurons from neurotoxins and to induce fiber outgrowth when administered directly into the brain[3–8]. GDNF also preserves other neurons from neurotoxic damage; particularly noradrenergic cells in the locus coeruleus (LC), which are affected in early stages of PD as well as in Alzheimer's disease and other brain disorders[9]. The therapeutic effects of intrastriatal GDNF infusion on PD patients have been evaluated in two open-label clinical trials with encouraging clinical and neurochemical results[10,11]. However, a randomized, placebo-controlled trial performed on PD patients was halted due to safety concern[12]. GDNF-based therapies are still considered to be promising for PD and other neurodegenerative diseases[13]; although improvement of their effectiveness requires an understanding of the actual physiologic role of GDNF in the maintenance of catecholaminergic neurons in adult life, a fact that remains essentially unknown.

GDNF mRNA is found in several regions of the adult rodent brain[9,14] and, within the peripheral nervous system, GDNF is highly concentrated in the adult carotid body (CB)[15,16], a dopaminergic organ used for autotransplantation studies in PD[17–19]. Research on genetically modified animal models in which the GDNF signalling pathway is disrupted have thus far failed to provide definitive

information on the physiological neuroprotective action of GDNF in adult life. Homozygous *GDNF* knockout animals die during the early postnatal period due to agenesis of the kidneys and myenteric plexus. At birth, *GDNF*$^{-/-}$ animals show normal numbers of catecholaminergic neurons in the substantia nigra (SN) and LC, thus suggesting that compensatory neuroprotective mechanisms may be activated during development[20-22]. Heterozygous (*GDNF*$^{+/-}$) animals are fertile and develop normally, although they manifest an accelerated decline in spontaneous motor activity and coordination with age[23]. Nevertheless, this embryonic GDNF deficit seems to have little impact in the adult as, at twenty months of age, the animals show only a 15% decrease of tyrosine hydroxylase (TH) positive SN neurons and no differences in striatal TH+ fiber density with respect to controls[23]. *GDNF*$^{+/-}$ mice, however, appear to exhibit a higher susceptibility to neurotoxin-induced long-term degeneration of monoaminergic neurons than wild type littermates[24]. Region-specific genetic deletion (driven by the dopamine transporter promoter) of Ret, a component of the GDNF receptor complex, in dopaminergic neurons has provided conflicting results regarding the role of this pathway in maintenance of adult neurons. A comparative morphometric and biochemical analysis of dopaminergic nigrostriatal neurons in adult Ret-null mice versus controls showed no differences[25]. Another group has reported that embryonic deletion of *Ret* in catecholaminergic neurons resulted in a significant decrease of TH+ SN neurons and striatal nerve terminals at two years of age, although, surprisingly, neurons in the ventral tegmental area (VTA) and LC remained unaffected[26].

Here, we report the successful generation of a conditional GDNF-null mouse in which GDNF expression was markedly reduced in adulthood, hence

avoiding the establishment of developmental compensatory modifications masking the true physiologic action of GDNF in the adult nervous system. These animals show selective and extensive catecholaminergic neuronal death, most notably in the LC, SN and VTA. Gabaergic and cholinergic pathways appear to be unaffected. The neurochemical and histological alterations in GDNF-deprived animals induce the progressive appearance of behavioral motor disturbances. These data unequivocally indicate that endogenous GDNF is absolutely required for trophic maintenance of catecholaminergic neurons in normal adult rodents.

RESULTS

Neurochemical alterations in conditional GDNF KO mice

Generation of the floxed *GDNF* allele ($GDNF^{F}$) is shown schematically in Fig. 1a. We designed a targeting construct containing the mouse *GDNF* exon 3 flanked by *loxP* sites and *neomycin* resistance markers. Embryonic stem (ES) cells were electroporated with this construct and homologous recombination with the wild type locus resulted in the targeted (floxed) allele $GDNF^{F}$. Positive clones of ES cells were selected by neomycin resistance and analyzed by Southern blotting for proper integration of the floxed allele (**Fig. 1a** and **b**). Routine genotyping of mice carrying the wild type and floxed alleles was done by PCR (**Fig. 1a** and **c**). $GDNF^{F/+}$ animals were interbred with $GDNF^{+/-}$ mice carrying the *Cre-Esr1* transgene[27]. In the resulting $GDNF^{F/-}$, *Cre* (*cGDNF*) offspring the ubiquitously expressed Cre recombinase was activated at two months of age by administration of tamoxifen (TMX) to switch off GDNF expression. The appearance of the GDNF-null allele in the germ line of TM-treated animals was experimentally tested (see Methods). Animals were sacrificed at one, three or seven months after TMX treatment for GDNF protein measurements (P), DNA and RNA analyses, and histological studies (H) (**Fig. 1d**). Confirmation for the excision of the floxed allele in adulthood was done by PCR of striatal DNA using specific probes (**Fig. 1a** and **e**). The proportion of wild type *GDNF* alleles that remained in striatal neurons after TMX treatment was estimated by qPCR to be 18% of that in control animals (relative values, 0.18 ± 0.06, $n = 5$ TMX+ mice *versus* 1 ± 0.22, $n = 3$ TMX– mice; $p < 0.05$).

GDNF mRNA expression in the various mouse strains studied was evaluated by quantitative RT-PCR performed on whole brain extracts (**Fig. 1f**).

Embryonic *GDNF* heterozygosity resulted in a mild, non-significant decrease of total brain *GDNF* mRNA that was similar in $GDNF^{+/-}$ and $GDNF^{F/-}$ strains thus indicating that the floxed and wild type *GDNF* alleles were transcribed with similar efficiency. In these two embryonic heterozygous animals, however, *GDNF* mRNA was 75% the amount seen in the wild type mice thus indicating compensatory activation by the remaining half normal alleles. Administration of TMX had no effect on wild type animals, but in *cGDNF* mice it induced a marked (~60% with respect to +/+ animals; $p < 0.01$) reduction of *GDNF* mRNA (**Fig. 1f**). Besides these mRNA measurements we also estimated the concentration of GDNF protein in striatal homogenates, a region of the adult rodent brain with high GDNF expression level[9,16], using a highly sensitive ELISA assay. In fair agreement with the mRNA data, TMX induced an ~60% decrease of striatal GDNF content with respect to controls (**Fig. 1g**). These results suggest that although TMX treatment of *cGDNF* mice left only ~18% of alleles unaffected, the animals were able to produce up to ~40% of the normal amount of GDNF mRNA and protein. As explained above, this probably reflects compensatory transcriptional up-regulation of the functional *GDNF* allele in the embryonic $GDNF^{F/-}$ animals. GDNF depletion produced a selective decrease of brain *TH* mRNA content, leaving *choline acetyltransferase* (*CHAT*) and *glutamic acid decarboxylase* (*GAD1*) mRNA levels unaltered (**Fig. 1h**). The data demonstrated a substantial decrease of brain GDNF in the conditional *GDNF* knockout mouse and suggested the existence in these mice of catecholaminergic cell damage without affectation of cholinergic or gabaergic neurons.

Selective neuronal death in GDNF-depleted adult brain

To evaluate the impact of TMX treatment and subsequent GDNF depletion on neuronal viability we performed stereological cell counts in brain regions, such as the SN, VTA and LC, containing catecholaminergic neurons whose vulnerability to neurotoxins is prevented by GDNF[3,8,9,24]. The most representative data obtained from these experiments are illustrated in **Figs. 2** and **3** and a quantitative summary is given in **Table 1**. Embryonic *GDNF* heterozygous animals (*GDNF*$^{F/-}$ strain) showed no effect regarding the number of TH+ neurons in the SN, VTA and LC compared with controls (*GDNF*$^{+/F}$ strain, **Table 1**). Deletion of the floxed *GDNF* allele with TMX gave rise, however, to a progressive reduction in the number of TH+ cells in SN and VTA. On average, the decrease in TH+ neurons was small and statistically non-significant at one month after TH treatment but was very pronounced (~60-70% of TH+ cell reduction) at seven months after *GDNF* gene deletion (**Fig. 2a**–**d** and **Table 1**). In parallel with the reduction in TH+ cell number we observed a clear and significant decrease in the total number of neurons both in the SN and VTA (**Fig. 2e**–**h** and **Table 1**). These observations indicated that the decrease of TH+ cells in *GDNF* knockout animals does not simply reflect down-regulation of *TH* gene expression. The disappearance of mesencephalic TH+ neurons was accompanied by diminishment of TH+ striatal nerve terminal density, particularly in the ventral region (**Table 1**). Progressive neuronal death in GDNF-deprived animals was particularly dramatic in the LC since evidence of TH+ neurons in this nucleus almost disappeared in *cGDNF* at seven months after TMX treatment and the total number of Nissl+ neurons in the nucleus decreased markedly (**Fig. 3a**–**f** and **Table 1**). The death of mesencephalic

catecholaminergic neurons in GDNF-deprived animals was paralleled by a decrease of TH+ cell numbers in structures of the peripheral nervous system, such as the carotid body (CB; **Fig. 4a**,**b** and **Table 1**) or the superior cervical ganglion (SCG; **Table 1**).

Neuronal loss observed in *cGDNF* mice was not general but restricted to the aforementioned brain areas. For example, the number of TH+ cells in the arcuate nucleus (AN), a component of the hypothalamic dopaminergic system, was unchanged (**Fig. 4c**–**e** and **Table 1**) and no apparent gross histological alterations were observed in the hippocampus or in neocortical regions (**Supplementary Fig. 1**). TMX treatment did not elicit macroscopic anatomical modifications in gut or kidney in animals studied up to 8 months after TMX treatment. Similarly, we did not observe changes in weight of TMX-treated *cGDNF* mice, which was maintained unaltered during the first 3–4 months despite the ongoing neurodegenerative process (respectively, 22.3 ± 1 g, $n = 3$ *versus* 22.6 ± 0.4, $n = 4$ wild type and GDNF-depleted animals studied 134 days after TMX treatment). Although not studied in detail yet, animal weight did, however, appear to decrease in older (> 200 days after TMX treatment) *GDNF* knockout animals.

Given the striking dependence on GDNF of mesencephalic dopaminergic and noradrenergic neuronal survival, we searched for the location of putative targets constitutively expressing high levels of GDNF and therefore critical for the trophic maintenance of the cells. To this end we used the heterozygous $GDNF^{LacZ}$ mice, a tool that allowed us to identify cells with high levels of GDNF expression by the characteristic blue X-gal staining[16,22]. After systematic analysis of the brain we consistently found X-gal+ deposits in cells distributed

throughout the striatum, in the anteroventral (particularly the ventrolateral region) and anteromedial nuclei of the thalamus as well as in the septum (mainly in the mediolateral region) (**Supplementary Fig. 2a**–**e**). These structures are innervated by projections from catecholaminergic neurons of the SN, VTA and LC[28,29]. Surprisingly, a high level of GDNF expression was also observed in the subcommissural organ (SCO; **Supplementary Fig. 2f** and **g**), an ependymal secretory gland located in the roof of the third ventricle that participates in cerebrospinal fluid homeostasis[30].

Behavioral motor abnormalities in GDNF-depleted mice

To assess the motor performance of the conditional *GDNF* knockout mice, animals were subjected to open field tests[31-34]. We analyzed the horizontal and vertical locomotor activity, with estimation of the distance traveled and time spent walking, as well as the number and duration of rearings. These are well-established behavioral tests used to characterize animals with deficit in the dopaminergic nigrostriatal pathway[31–34].

GDNF-depleted animals showed a clear hypokinetic syndrome, progressively aggravated with time after TMX treatment. In fair agreement with the histological data (see **Figs. 2**–**4** and **Table 1**), open field impairments were mild during the first weeks after TMX treatment but became quite obvious after three to four months of GDNF deprivation (**Fig. 5**). Although the number of cases studied to date is still small, the data available strongly suggest that the motor deficits continue to progress as TMX treated *cGDNF* animals get older.

DISCUSSION

The results demonstrate that endogenous GDNF exerts an indispensable neurotrophic effect on catecholaminergic neurons of the adult mouse nervous system, particularly with respect to the ventral mesencephalic dopaminergic neurons in the SN and VTA as well as the noradrenergic cells in the LC. The dependence of neuronal survival on GDNF is observed despite, on average, the trophic factor is down-regulated to only 40% of its content in normal adult brain. It had previously been shown that intracerebral administration of exogenous GDNF can protect catecholaminergic neurons from toxic damage, and that striatal over-expression of GDNF induces sprouting of dopaminergic fibers[3,4,6–9]. It had also been reported that postnatal survival and phenotypic expression of ventral mesencephalic dopaminergic neurons in tissue explants is favored by GDNF[35]. However, the actual physiologic role of GDNF in preserving adult brain catecholaminergic cells *in vivo* has, until now, been unknown. Our results contrast with the practically absence of neuronal damage in GDNF heterozygous or in animals without canonical GDNF (Ret) receptors[20,23,25,26]. Hence, it seem highly likely that embryonic gene disruption of the GDNF signaling pathway induces developmental compensatory mechanisms involving other neurotrophic factors and/or GDNF receptors. The existence of Ret-independent GDNF receptors in adult mammalian brain is well documented[14,36] and, additionally, it has been shown that both *in vitro* and *in vivo* effects of GDNF on midbrain dopaminergic neurons can be antagonized with anti-NCAM function blocking antibodies[37]. Thus, our findings support the idea that GDNF receptors other than Ret could play a significant physiologic role in adult brain.

The strict dependence of adult catecholaminergic neuronal survival on endogenous GDNF prompted us to search for brain areas with high GDNF expression levels. Using a genetic marker[16,22], we have demonstrated that besides in the striatum, GDNF is highly expressed in the septum as well as in the anteroventral/anteromedial thalamus, preferential targets of projections from catecholaminergic neurons of the SN, VTA and LC[28,29]. These same brain areas had been shown before to express high *GDNF* mRNA levels using Northern analysis and *in situ* hybridization techniques[9,14]. Interestingly, we have also seen a previously unnoticed robust GDNF expression in the SCO, an enigmatic glial-derived secretory gland in the third ventricle that participates in cerebrospinal fluid homeostasis[30,38]. The actual physiologic role of GDNF in SCO cells is an open, and quite appealing, question that must be addressed in future experimental work.

The striking reliance of adult mammalian mesencephalic catecholaminergic neurons on the continuous supply of GDNF further supports its potential use as a therapeutic agent in Parkinson's disease (PD), and possibly other neuronal disorders[2]. In this regard, GDNF delivery to multiple targets (striatum, thalamus and septum) rather than solely to the striatum, mimicking the physiologic trophic activation of catecholaminergic cells, may improve the efficacy of current clinical protocols. On the other hand, the pattern of cell death induced by GDNF down-regulation in adult brain recapitulates many of the neuropathological hallmarks of PD. Besides progressive dopaminergic cell death in SN and VTA, this phenotype is particularly evident in the LC, a region known to contribute to the pathophysiology of PD that degenerates early in the progression of the disease[9,39]. Interestingly,

dopaminergic neurons in the hypothalamic arcuate nucleus, unaffected in PD[40], are also preserved in GDNF-null adult animals. In parallel with the histological modifications, GDNF-depleted animals progressively develop a hypokinetic phenotype, similar to that reported in mice with toxic damage of the nigrostriatal pathway and LC[31–33,41], which is fully compatible with the behavioral changes seen in PD. Hence, the conditional *GDNF* knockout mice model should facilitate the understanding of neuroprotective pathways induced by GDNF, the study of PD pathogenesis, as well as the development of novel therapeutic tools to fight neurodegeneration.

METHODS

Generation of animal models. Animals were housed under temperature-controlled conditions (22 °C) in a 12 h light/dark cycle. All experiments were performed in accordance with institutional guidelines approved by the ethics committee of the "Hospital Universitario Virgen del Rocio". To generate the conditional *GDNF* animals, we engineered a targeting construct that could be homologously recombined into the third coding exon of the *GDNF* locus. The targeting construct contained *GDNF* exon3 (E3) flanked by *loxP* sites ($GDNF^{F}$) and the *neomycin* resistance marker. For homologous recombination, we used 129Sv-derived R1 ES cells. Southern blotting using a DNA 3' probe was used to identify the recombined allele. $GDNF^{F}$ mice were further propagated and used for experiments on a mixed genetic background (129/SvJ:C57BL/6). Routine genotyping to detect the wild-type or floxed allele was performed using PCR. Floxed PCR was performed with the primers loxP15 (5'-TCACGTGTCTATGTGCTAAA-3') and loxP13 (5'-AATGATCATTTCGGGCAGTC-3'). To obtain the experimental model used in this study, $GDNF^{LacZ}$ animals ($GDNF^{+/-}$)[22] were mated with *Cre-Esr1*/+[27] mice to obtain a F1 $GDNF^{+/-}$*; Cre*/+ progeny. F1 animals were mated with $GDNF^{F/+}$ mice to generate control ($GDNF^{+/F}$*;* +/+) and experimental littermates ($GDNF^{F/-}$*; Cre/* or *cGDNF*). Two-month-old animals were intraperitoneally injected with

TMX (Sigma; 0.2 mg/g/day) for four consecutive days. TMX was prepared and used as previously described[42]. c*GDNF* mice without TMX treatment were used as control when indicated. To test for the successful conditional deletion of *GDNF in vivo*, excised PCR was used with the primers loxP15 and loxP23 (5'-GAACTCCAGGTAAATAATCC-3'). To verify the effect of TMX and the proper excision at the $GDNF^{F}$ locus, $GDNF^{+/F}$*; Cre-Esr1/+* mice treated with TMX were mated with wild-type animals. Heterozygous *GDNF*-null mice in the progeny were crossed and, as expected, the resulting offspring carrying the two excised alleles died during the first postnatal day due to renal agenesis.

DNA extraction from brain paraffin slices. Striatal paraffin-embedded slices 20 µm thick were deparaffined twice with xylene and hydrated by washing with solutions containing decreasing concentrations of ethanol. Four slices were digested for 2 hours at 55ºC in 200 µl of directPCR reagent (Viagen) containing proteinase K (15 µg/ml). Proteinase K was inactivated by heating samples at 85ºC for 2 hours. 1 µl from the resulting DNA solution was used for PCR analysis.

Quantitative RT-PCR. To determine *GDNF*, *TH*, *Chat*, and *Gad1* mRNA levels, brain RNA was extracted using TRIzol reagent (Invitrogen) in a homogenizer (Omni 2000). RNA samples (5 µg) were treated with DNase-RNase free (GE Healthcare) and copied to cDNA using SuperScriptII reverse transcriptase (Invitrogen) in a final volume of 20 µl. Real time PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied-Biosystems) using SYBR Green PCR Master mix (Applied-Biosystems) and the thermocycler conditions recommended by the manufacturer. PCR reactions were performed in duplicates in a total volume of 25 µl containing 1 µl of the RT reaction. In each sample, *Actb* and *Ppia* RNA levels were estimated to normalize for RNA input amounts. Relative quantifications using both housekeeping genes produced similar results. To normalize mRNA levels in knockout mice to those in control samples, an average *Ct* of the control samples was calculated and all the samples in the experiment were processed relative to this average *Ct*. To estimate DNA recombination at the *GDNF* locus qPCR was performed on DNA

samples extracted from striatal paraffin-embedded slices. The excision of the floxed allele was calculated estimating *Neo* gene dosage after TMX treatment. DNA amounts were normalized to *beta glucuronidase* (*Gusb*) levels. Primers used for qPCR appear online (**Supplementary Methods**).

GDNF ELISA. Striatal GDNF protein content was estimated using a commercial ELISA kit (GDNF Emax Immunoassay System; Promega, WI). Brain was removed and immediately frozen in liquid nitrogen. Left and right striata were dissected out, placed in 200 µl of 20 mM Tris-HCl, pH = 7.6, 150 mM NaCl, 0.05% (v/v) NP-40 and proteinase inhibitor cocktail (SIGMA), and sonicated with a Branson-150 sonifier for 10 s (output 2–3). Samples were centrifuged at 4ºC for 10 min at maximal speed in a microcentrifuge and supernatant were removed and frozen. Protein concentration was performed with Bio-Rad protein quantification kit and 100 µg of protein were used in the assay. ELISA was performed following the manufacturer's protocol. Neonatal (P1) protein extracts of forebrains from wild-type and *GDNF*$^{-/-}$ animals were used as positive and negative controls. Absorbance from *GDNF*$^{-/-}$ extracts was subtracted to each individual measurement. GDNF content was estimated in left and right striata and an average per animal was used to perform comparative analysis.

Tissue preparation and histochemistry. Animals were anesthetized and fixed with 4% paraformaldehyde. The brain and the carotid bifurcation were extracted and paraffin embedded. Coronal mouse brain sections (thickness 20 µm) were used for tyrosine hydroxylase (TH), NeuN-TH immunohistochemistry, or Nissl staining (0.1% cresyl violet) in quantitative and morphometric studies. The Envision^{+} kit (DAKO) was used for immunohistochemistry according to the recommended manufacturer's protocol. Antibodies and the dilution factors used were as follows: anti-TH polyclonal antibody (Pel-Freez), 1:1000; anti-NeuN monoclonal antibody (Chemicon), 1:200. For double labeling with anti-TH and anti-NeuN antibodies we incubated the slices with antibodies against mouse antibodies and the signal was developed with DAB (DAKO). To increase contrast, a DAB enhancer (DAKO) was used. Antibodies against rabbit antibodies were added in a second step and the reaction developed with AEC

substrate (DAKO). ß-galactosidase staining of 50 µm-thick brain slices was performed as described previously[16,22].

Stereological cell counts. Counts of TH-, NeuN-immunoreactive, and Nissl-positive neurons were performed on 20 µm-thick coronal microtome sections spaced 40 µm apart throughout the extent of the AN, SN, VTA and LC. We followed procedures similar to those previously used in our laboratory[43]. Striatal TH+ fiber density was quantified in a 20 µm-thick slice at Bregma 0.86[44]. The striatum was divided into dorsal and ventral areas and 10-20 random 3,565.2 μm^2 dissectors per hemisphere were counted using the C.A.S.T. Grid System. CB TH+ cell counting was performed across the entire CB parenchyma. SCG TH+ neurons were counted on 30 µm-thick coronal sections spaced 90 µm apart throughout the extent of the structure. Random dissectors of 5,347.7 μm^2 were used.

Open field. We used N7 C57BL/6 animals in open field analysis. Behavioral assessment for motor dysfunction was performed in a box of 22.5 x 22.5 cm^2 floor and with 42-cm-high walls. Four boxes were monitored at the same time using an automatic tracking system (SMART, Panlab, Spain). Animals were recorded for 60 min and several horizontal motor parameters were calculated using the SMART software (v2.5.14). Vertical movements were estimated by visual inspection of the recorded videos using the specific tool provided by the software (SMART).

Statistical analysis. Data were presented as mean ± s.e.m., and were analyzed with either Student's t test or one-way ANOVA followed by Tukey test. $p < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTIONS

A. P. and M. H.-F. designed and conducted most of the experiments. A.P., C.O.P., R.G. and J.I.P. generated the GDNF conditional knockout animals. J.L.-B. supervised the project. A.P. and J.L.-B. wrote the manuscript.

REFERENCES

1. 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).
2. Kirik, D., Georgievska, B. & Bjorklund, A. Localized striatal delivery of GDNF as a treatment for Parkinson disease. *Nat. Neurosci.* **7**, 105–110 (2004).
3. Akerud, P., Canals, J. M., Snyder, E. Y. & Arenas, E. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. *J. Neurosci.* **21**, 8108–8118 (2001).
4. Choi-Lundberg, D. L. et al. Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* **275**, 838–841 (1997).
5. Gash, D. M. et al. Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* **380**, 252–255 (1996).
6. Kordower, J. H. et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* **290**, 767–773 (2000).
7. Rosenblad, C., Martinez-Serrano, A. & Bjorklund, A. Intrastriatal glial cell line-derived neurotrophic factor promotes sprouting of spared nigrostriatal dopaminergic afferents and induces recovery of function in a rat model of Parkinson's disease. *Neuroscience* **82**, 129–137 (1998).
8. Tomac, A. et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* **373**, 335–339 (1995).
9. 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).
10. Gill, S. S. et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat. Med.* **9**, 589–595 (2003).
11. Slevin, J. T. et al. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. *J. Neurosurg.* **102**, 216–222 (2005).
12. Lang, A. E. et al. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann. Neurol.* **59**, 459–466 (2006).

13. Check, E. Second chance. *Nat. Med.* **13**, 770–771 (2007).
14. 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).
15. Toledo-Aral, J. J., Mendez-Ferrer, S., Pardal, R., Echevarria, M. & Lopez-Barneo, J. Trophic restoration of the nigrostriatal dopaminergic pathway in long-term carotid body-grafted parkinsonian rats. *J. Neurosci.* **23**, 141–148. (2003).
16. Villadiego, J. et al. Selective glial cell line-derived neurotrophic factor production in adult dopaminergic carotid body cells in situ and after intrastriatal transplantation. *J. Neurosci.* **25**, 4091–4098 (2005).
17. Arjona, V. et al. Autotransplantation of human carotid body cell aggregates for treatment of Parkinson's disease. *Neurosurgery* **53**, 321-328; discussion 328–330 (2003).
18. Espejo, E. F., Montoro, R. J., Armengol, J. A. & Lopez-Barneo, J. Cellular and functional recovery of Parkinsonian rats after intrastriatal transplantation of carotid body cell aggregates. *Neuron* **20**, 197–206. (1998).
19. Minguez-Castellanos, A. et al. Carotid body autotransplantation in Parkinson disease: A clinical and PET study. *J. Neurol. Neurosurg. Psychiatry* **78**, 825–831 (2007).
20. Moore, M. W. et al. Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76–79 (1996).
21. Pichel, J. G. et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73–76 (1996).
22. Sanchez, M. P. et al. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70–73 (1996).
23. Boger, H. A. et al. A partial GDNF depletion leads to earlier age-related deterioration of motor function and tyrosine hydroxylase expression in the substantia nigra. *Exp. Neurol.* **202**, 336–347 (2006).
24. Boger, H. A. et al. Long-term consequences of methamphetamine exposure in young adults are exacerbated in glial cell line-derived neurotrophic factor heterozygous mice. *J. Neurosci.* **27**, 8816–8825 (2007).
25. Jain, S. et al. RET is dispensable for maintenance of midbrain dopaminergic neurons in adult mice. *J. Neurosci.* **26**, 11230–11238 (2006).
26. Kramer, E. R. et al. Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. *PLoS Biol.* **5**, e39 (2007).
27. Hayashi, S. & McMahon, A. P. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* **244**, 305–318 (2002).
28. Bjorklund, A. & Hokfelt, T. *Handbook of Chemical Neuroanatomy: Classical Neurotransmitters in fhe CNS, Part I*, 123–156. Elsevier, Amsterdam (1984).

29. Lindvall, O. & Stenevi, U. Dopamine and noradrenaline neurons projecting to the septal area in the rat. *Cell Tissue Res.* **190**, 383–407 (1978).
30. Galarza, M. Evidence of the subcommissural organ in humans and its association with hydrocephalus. *Neurosurg. Rev.* **25**, 205–215 (2002).
31. Fleming, S. M. et al. Early and progressive sensorimotor anomalies in mice overexpressing wild-type human alpha-synuclein. *J. Neurosci.* **24**, 9434–9440 (2004).
32. Meredith, G. E. & Kang, U. J. Behavioral models of Parkinson's disease in rodents: a new look at an old problem. *Mov. Disord.* **21**, 1595–1606 (2006).
33. Sedelis, M., Schwarting, R. K. & Huston, J. P. Behavioral phenotyping of the MPTP mouse model of Parkinson's disease. *Behav. Brain Res.* **125**, 109–125 (2001).
34. Zhou, Q. Y. & Palmiter, R. D. Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. *Cell* **83**, 1197–1209 (1995).
35. Granholm, A. C. et al. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J. Neurosci.* **20**, 3182–3190 (2000).
36. Paratcha, G., Ledda, F. & Ibanez, C. F. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* **113**, 867–879 (2003).
37. Chao, C. C., Ma, Y. L., Chu, K. Y. & Lee, E. H. Integrin alphav and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. *Neurobiol. Aging* **24**, 105–116 (2003).
38. Tome, M. et al. The subcommissural organ expresses D2, D3, D4, and D5 dopamine receptors. *Cell Tissue Res.* **317**, 65–77 (2004).
39. Zarow, C., Lyness, S. A., Mortimer, J. A. & Chui, H. C. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch. Neurol.* **60**, 337–341 (2003).
40. Matzuk, M. M. & Saper, C. B. Preservation of hypothalamic dopaminergic neurons in Parkinson's disease. *Ann. Neurol.* **18**, 552–555 (1985).
41. Srinivasan, J. & Schmidt, W. J. Potentiation of parkinsonian symptoms by depletion of locus coeruleus noradrenaline in 6-hydroxydopamine-induced partial degeneration of substantia nigra in rats. *Eur. J. Neurosci.* **17**, 2586–2592 (2003).
42. Guo, C., Yang, W. & Lobe, C. G. A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. *Genesis* **32**, 8–18 (2002).
43. Mejias, R. et al. Neuroprotection by transgenic expression of glucose-6-phosphate dehydrogenase in dopaminergic nigrostriatal neurons of mice. *J, Neurosci,* **26**, 4500–4508 (2006).
44. Franklin, B. J., Paxinos, G. T. The Mouse Brain in Stereotaxic Coordinates. New York: Academic Press (1996).

FIGURE LEGENDS

Figure 1 Molecular characterization of the conditional *GDNF* knockout mice. (**a**) *GDNF* wild-type locus, targeting construct, floxed and excised alleles are shown. *B*, *Bam*HI. Probe and genotyping PCRs are indicated. Gray rectangles represent *Neo* marker and triangles *loxP* sequences. (**b**) ES cell clone showing correct targeting at the *GDNF* locus by Southern blotting. *Bam*HI-digested DNA was hybridized with the probe indicated in (**a**). An additional band is present in $GDNF^{+/F}$ ES cells (+/F). (**c**) Floxed PCR used for genotyping of $GNDF^{+/+}$ (+/+), $GDNF^{+/F}$ (+/F), and $GDNF^{F/F}$ (F/F) mice are shown. The ES $GDNF^{+/F}$ cell line is showed as a control (+/F). (**d**) Experimental protocol used for analysis of TMX-treated animals. Time points of TMX injection and brain harvesting for DNA, protein (P), RNA, and histological analyses (H) are indicated (m, months). (**e**) PCR used to demonstrate the presence of the excised GDNF allele in the striatum after TMX treatment. $GDNF^{F/–}$*; Cre-Esr1* animals without (–TMX) and with (+TMX) TMX treatment are shown. (**f**) Relative levels of *GDNF* mRNA determined by qRT-PCR. (+/+: $GDNF^{F/+}$*;* +. +/–: $GDNF^{+/–}$*; Cre-Esr1*. F/–: $GDNF^{F/–}$*; Cre-Esr1*). (**g**) Levels of striatal *GDNF* protein determined by ELISA. (+/+: $GDNF^{F/+}$*;* +. F/–: $GDNF^{F/–}$*; Cre-Esr1*). (**h**) Relative levels of *tyrosine hydroxylase* (*TH)*, *choline acetyltransferase* (*CHAT)*, and *glutamic acid decarboxylase* (*GAD1)* mRNA in whole brain extracts determined by qRT-PCR. $GDNF^{F/–}$*, Cre-Esr1* mice without (black bars) and with (gray bars) TMX treatment. Means ± s.e.m.; *, $p < 0.05$; **, $p < 0.01$ (Student *t*-test). $n = 3–6$ samples per group.

Figure 2 Mesencephalic dopaminergic neuronal death after *GDNF* ablation in adult mouse. (**a,b**) Mesencephalic brain slices stained with an antibody against TH. SN, substantia nigra; VTA, ventral tegmental area. $GDNF^{F/–}$*, Cre-Esr1* mice without (**a**) and with (**b**) TMX treatment. (**c,d**) Unbiased stereological cell counts of SN (**c**) and VTA (**d**) TH immunoreactive neurons at 30 days (30 d) and 210 days (210 d) after TMX treatment. $GDNF^{+/+}$ treated with TMX (black bars), $GDNF^{F/–}$*, Cre-Esr1* mice without (dark gray bars) and with (light gray bars) TMX treatment. (**e,f**) Double immunostaining of mesencephalic brain slices with antibodies against NeuN (nuclear) and TH (cytoplasmic). The stippled lines

surround the SN and VTA. Inset in (**e**) represents a magnification of the indicated zone. *GDNF$^{F/-}$, Cre-Esr1* mice without (**e**) and with (**f**) TMX treatment. (**g,h**) Unbiased stereological cell counts of SN (**c**) and VTA (**d**) NeuN immunoreactive neurons after 210 days of TMX treatment. *GDNF$^{F/-}$, Cre-Esr1* mice without and *GDNF$^{+/+}$* with TMX treatment (black bars), and *GDNF$^{F/-}$, Cre-Esr1* mice with TMX treatment (light gray bars). Means ± s.e.m.; *, $p < 0.05$; **, $p < 0.01$ (Student *t*-test). $n = 6–8$ samples per group. Scale bars, 100 µm.

Figure 3. Locus coeruleus noradrenergic neuronal death after *GDNF* ablation in adult mouse. (**a,b**) Hindbrain slices stained with an antibody against TH. At the bottom panels, the respective right locus coeruleus (LC) are shown at higher magnification. (**c,d**) Nissl stainning of brain slices containing LC. *GDNF$^{F/-}$, Cre-Esr1* mice without (**a,c**) and with (**b,d**) TMX treatment. The stippled lines highlight the LC. (**e**) Unbiased stereological cell counts of LC TH immunoreactive neurons at 30 days (30 d) and 210 days (210 d) after TMX treatment. *GDNF$^{+/+}$* treated with TMX (black bars), *GDNF$^{F/-}$, Cre-Esr1* mice without (dark gray bars) and with (light gray bars) TMX treatment. (**f**) Unbiased stereological cell counts of LC Nissl positive neurons after 210 days of TMX treatment. *GDNF$^{F/-}$, Cre-Esr1* mice without and *GDNF$^{+/+}$* with TMX treatment (black bars), and *GDNF$^{F/-}$, Cre-Esr1* mice with TMX treatment (gray bars). Means ± s.e.m.; *, $p < 0.05$; **, $p < 0.01$ (Student *t*-test). $n = 5–8$ samples per group. Scale bars are 100 µm in all panels except panels **e**,**f** (top) where they represent 500 µm.

Figure 4 Peripheral and hypothalamic dopaminergic cell survival in conditional GDNF knockout mice. (**a,b**) Carotid body (CB) of *GDNF$^{F/-}$, Cre-Esr1* mice without (**a**) and with (**b**) TMX treatment as revealed by TH immunoreactivity. (**c-e**) TH+ cells of the arcuate nucleus (AN). (**c**) Brain section immunostained for TH indicating the location of the arcuate nucleus (AN). The encircled zone represents the area shown in **d** and **e**. (**d,e**) TH+ cells of AN from *GDNF$^{F/-}$, Cre-Esr1* mice without (**d**) and with (**e**) TMX treatment. Scale bars are100 µm in all panels except panel **c** where it represents 500 µm.

Figure 5. Behavioral analysis of adult GDNF-depleted mice. Spontaneous activity was measured in wild-type (black bars: *GDNF*$^{+/+}$*;* + mice treated with TMX; *n* = 13) and GDNF depleted animals (gray bars: *GDNF*$^{F/-}$*; Cre-Esr1* mice treated with TMX; *n* = 7) 3 days before TMX injection and 37, 60, 100, and 126 days after TMX injection. Animals were recorded during 60 min in an open field chamber. (**a**) Four traces from wild-type and GDNF-depleted animals are shown as representative of the time points 100 and 126 days after TMX injection. Activity trace during minutes 16 to 30 is presented. (**b**) Traveled distance (cm) was calculated by following the centre of gravity of the subject. (**c**) Resting time (s) was the time spent in resting state (with reference to the default velocity threshold of 2.57 cm/s). (**d,e**) Vertical movements quantified in three periods of five minutes from each animal and time point. Averaged number of rearings (N, **d**) and the accumulated time spent with both forepaws without contacting the floor (**e**) are shown. Each individual point represents 3–8 animals. Means ± s.e.m.; *, $p < 0.05$; **, $p < 0.01$ (Tukey test).

Table 1. Catecholaminergic cell death in adult conditional GDNF-null mice				
		GDNF$^{F/+}$ (TMX+)	*GDNF*$^{F/–}$, *Cre* (TMX–)	*GDNF*$^{F/–}$, *Cre* (TMX+)
SN	TH+ (30 d)	**4,252 ± 336$^{\$}$ (*n* = 8)**		**3,440 ± 209 (*n* = 6)**
	TH+ (210 d)	**4,787 ± 480 (*n* = 8)**	**4,931 ± 865 (*n* = 6)**	**2,029 ± 469**/* (*n* = 6)**
	NeuN+ (210 d)	**11,289 ± 510$^{\$}$ (*n* = 6)**		**8,356±720** (*n* = 6)**
VTA	TH+ (30 d)	**2,763 ± 193$^{\$}$ (*n* = 8)**		**2,481 ± 150 (*n* = 6)**
	TH+ (210 d)	**2,561 ± 427 (*n* = 6)**	**2,919 ± 238 (*n* = 6)**	**872 ± 381*/** (*n* = 6)**
	NeuN+ (210 d)	**5,855 ± 421$^{\$}$ (*n* = 6)**		**2,156 ± 586** (*n* = 6)**
LC	TH+ (30 d)	**892 ± 138$^{\$}$ (*n* = 7)**		**785 ± 137 (*n* = 6)**
	TH+ (210 d)	**873 ± 131 (*n* = 5)**	**850 ± 301 (*n* = 5)**	**5 ± 3**/** (*n* = 6)**
	Nissl+ (210 d)	**2,489 ± 363$^{\$}$ (*n* = 7)**		**1,537 ± 169* (*n* = 6)**
AN	TH+ (210 d)	**264 ± 99$^{\$}$ (*n* = 8)**		**268 ± 109 (*n* = 6)**
dST	TH+ (210 d)	**21.4 ± 1.6$^{\$}$ (*n* = 12)**		**15.4 ± 1.4* (*n* = 6)**
vST	TH+ (210 d)	**26.9 ± 2.2$^{\$}$ (*n* = 9)**		**17.1 ± 1.7** (*n* = 6)**
CB	TH+ (210 d)	**1,102.1 ± 67.2$^{\$}$ (*n* = 3)**		**418.1 ± 122.5** (*n* = 3)**
SCG	TH+ (210 d)	**10,036 ± 1,105$^{\$}$ (*n* = 3)**		**4,736 ± 571** (*n* = 5)**

Data are given as cell numbers except in the case of striatum (ST, fiber density in fibers/μm^2 x 1000). dST: dorsal striatum; vST: ventral striatum. Means ± s.e.m.

$^{\$}$ *GDNF*$^{F/+}$ treated with TMX and *GDNF*$^{F/–}$, *Cre-Esr1* control samples were counted together. Asterisks indicate significant differences between *GDNF*$^{F/–}$, *Cre-Esr1* (TMX+) mice and *GDNF*$^{F/+}$, + (TMX+) (*/*); *GDNF*$^{F/–}$, *Cre-Esr1* (TMX–) (/*), or pooled groups (*).

***: $p < 0.05$; **: $p < 0.01$. Cells counts are referred to single, unilateral, structures. Between parentheses are either the time (days, d) after TMX injection or the number of animals studied.**

SI Guide

Journal: Nature Neuroscience

Article Title:	Absolute Requirement of GDNF for Adult Catecholaminergic Neuron Survival
Corresponding Author:	José López-Barneo

Supplementary Item & Number **(add rows as necessary)**	Title or Caption
Supplementary Figure 1	Cortex and hippocampus in GDNF-depleted mice
Supplementary Figure 2	GDNF expression in catecholaminergic brain targets
Supplementary Methods	Primers used for quantitative RT-PCR

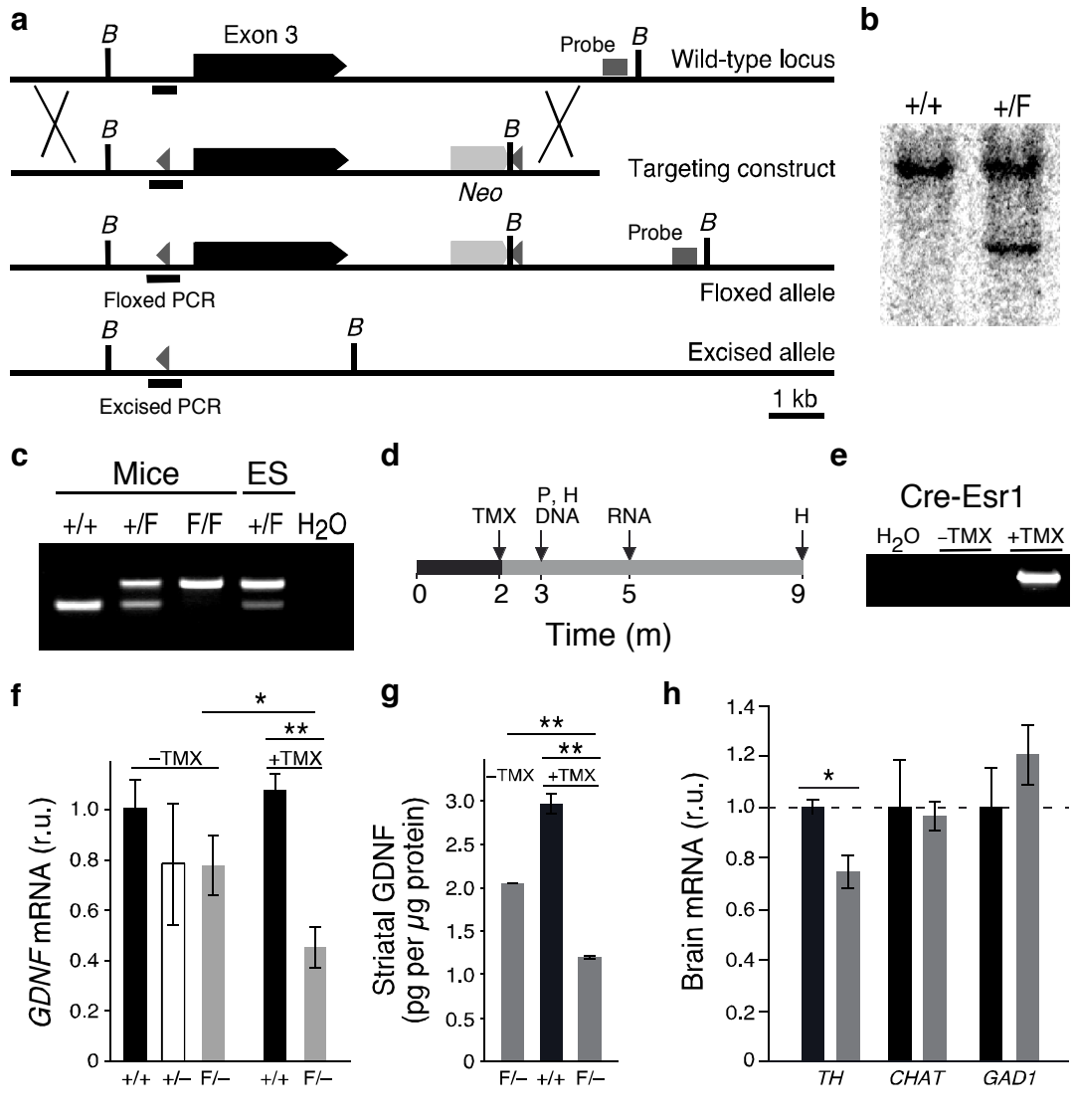

Figure 1
Pascual *et al.*

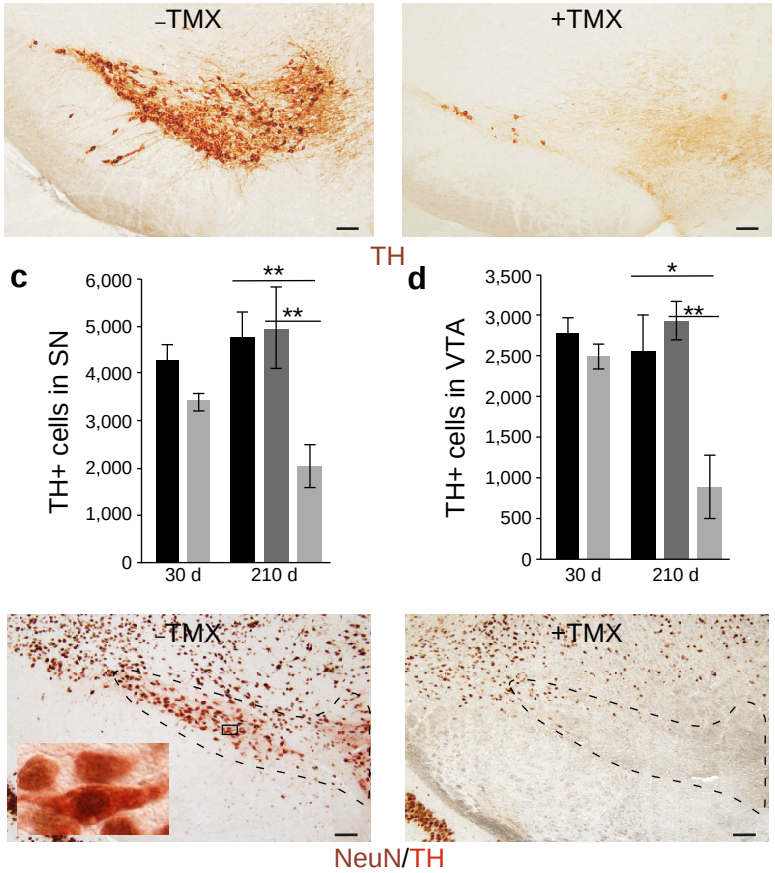

Figure 2
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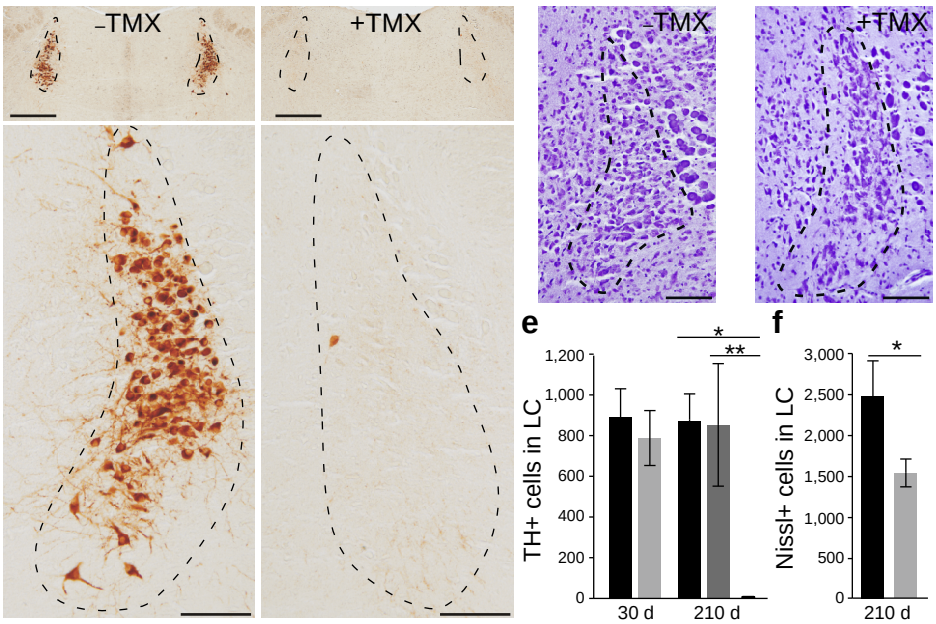

Figure 3
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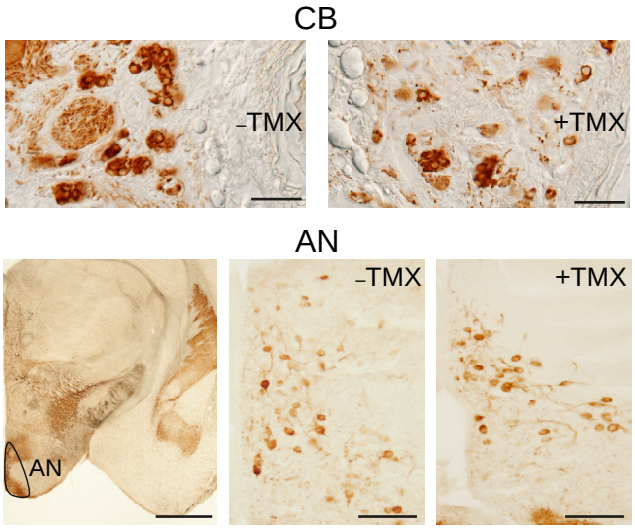

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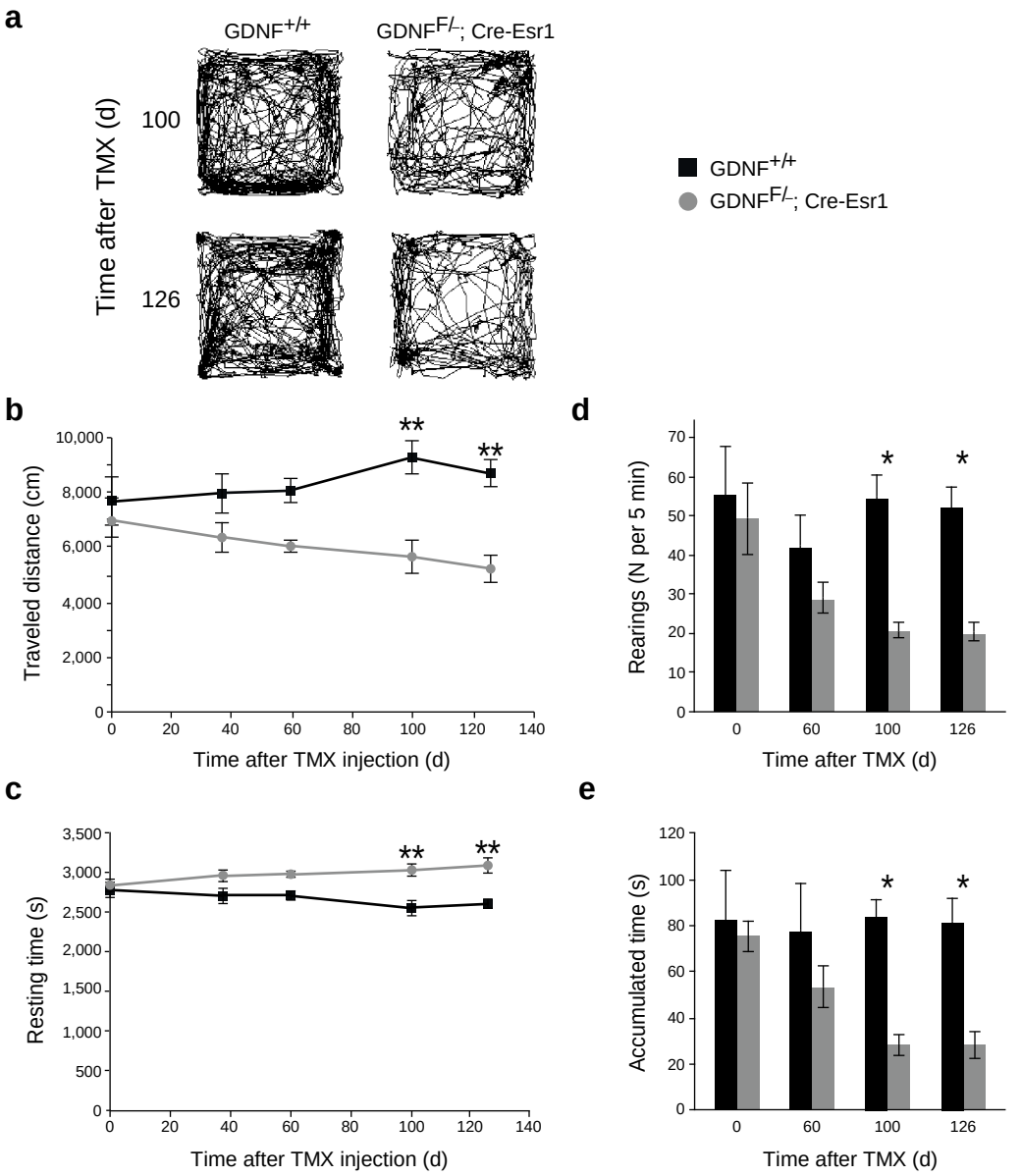

Figure 5
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SUPPLEMENTARY INFORMATION

Absolute Requirement of GDNF for Adult Catecholaminergic Neuron Survival

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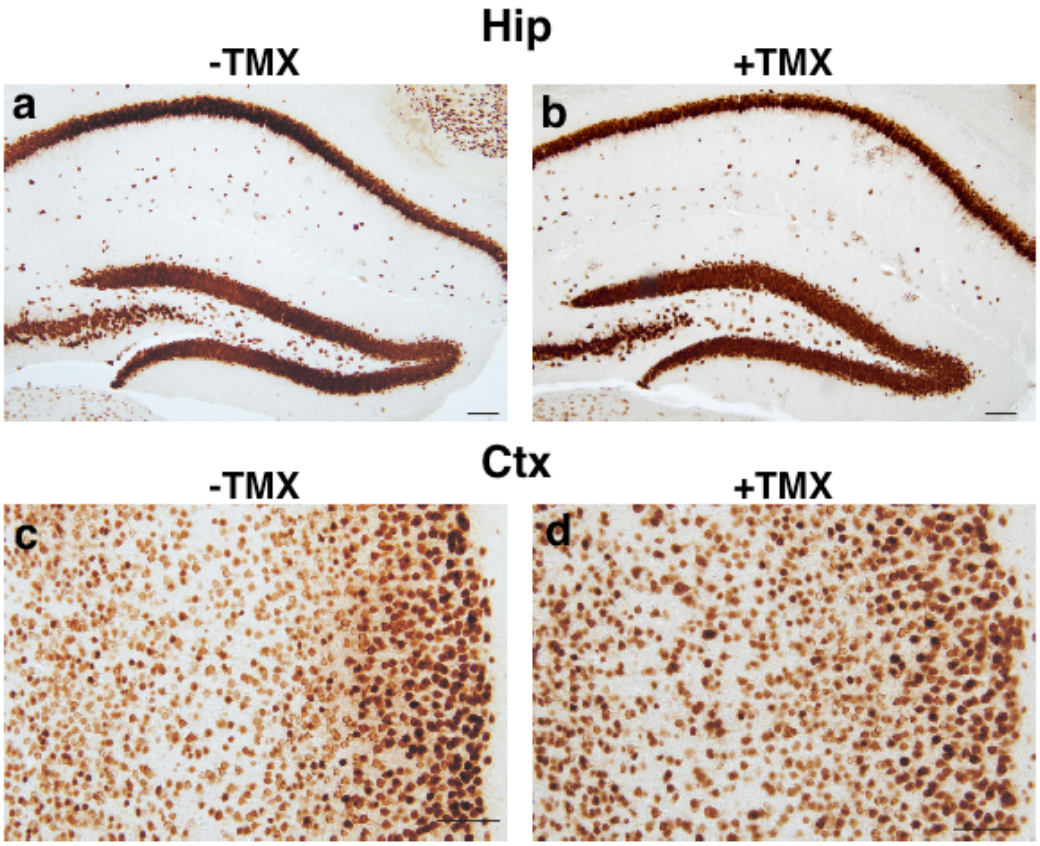
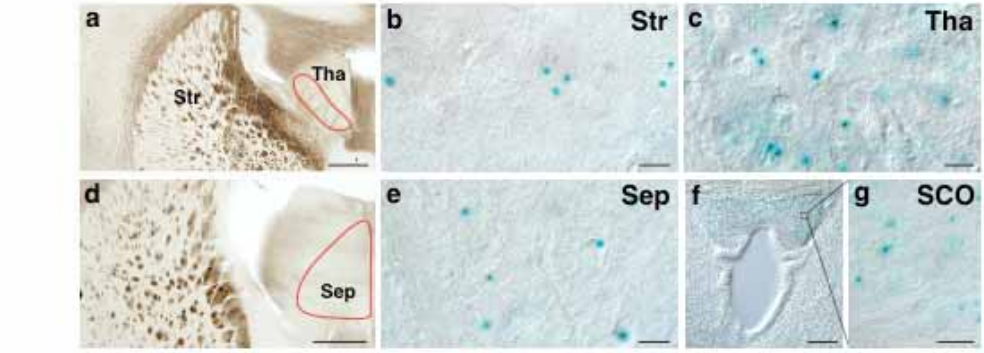


Supplementary Figure 1. Cortex and hippocampus in GDNF-depleted mice. (**a-d**) Brain slices from *GDNF*$^{F/-}$, *Cre-Esr1* mice without (**a,c**) and with (**b,d**) TMX treatment stained with an antibody against NeuN. (**a,b**) hippocampus (Hip); (**i,j**) cortex (Ctx). Scale bar indicates 100 μm.



Supplementary Figure 2. GDNF expression in catecholaminergic brain targets. GDNF promoter activity as estimated by ß-galactosidase enzymatic detection in brains from heterozygous *GDNF*^{LacZ} mice (Sanchez et al., 1996; Villadiego et al., 2005). (**a-c**) Coronal brain section demonstrating high GDNF expression in the striatum (**b**, Str) and thalamus (**c**, Tha). The region encircled by the red line (**a**) represents the area in thalamus with a high density of X-gal+ cells. (**d-e**) Coronal brain section demonstrating high GDNF expression in the septum (**e**, Sep). The region encircled by the red line (**d**) represents the septal area with a high density of X-gal+ cells (**f-g**) GDNF expression in the subcommissural organ (SCO). Scale bar indicates 500 µm in **a** and **d**; 10 µm in **b, c, e** and **g**; and 50 µm in **f**.

Supplementary Methods

Primers used for quantitative RT-PCR

Primers were designed using Primer Express software (Applied-Biosystems). The following primers were used:

ß-actin (*Actb*), 5'-GGCCCAGAGCAAGAGAGGTA-3' and 5'-CATGTCGTCCCAGTTGGTAACA-3′;

cyclophilin A (*Ppia*), 5′-ATGGCAAATGCTGGACCAAA-3′ and 5′-TGCCATCCAGCCATTCAGT-3′*;*

tyrosine hydroxylase (*TH*), 5′-GGCTTCTCTGACCAGGCGTAT-3′ and 5′-GCTCACCCTGCTTGTATTGGA-3′;

choline acetyltransferase (*Chat*), 5′-GGAGCGAATCGTTGGTATGAC-3′ and 5′-ATCTCGGCCCACCACAAA-3′;

glutamic acid decarboxylase (*Gad1*), 5′-ACTCCTCAACTATGTCCGCAAGA-3′ and 5′-TCCAAATTAAAGCCTTCCATGC-3';

GDNF, 5′-GGATGGGATTCGGGCCACT-3′ and 5′-AGCCACGACATCCCATAACTTC-3′.

ß-glucuronidase (*Gusb*), 5'-CATTCAGTTCTGGATCAGAAACGTA-3' and 5'-CATGAAGTCGGCGAAATTCC-3′;

Neomycin (*Neo*), 5′- GGATGGAAGCCGGTCTTGT-3′and 5′-CCTGATGCTCTTCGTCCAGATC-3′.