GDNF is not required for catecholaminergic neuron survival *in vivo*

To the Editor:

Glial cell line–derived neurotrophic factor (GDNF) has been tested in clinical trials to treat Parkinson's disease with promising, but variable, results. Improvement of therapeutic effectiveness requires solid understanding of the physiological role of GDNF in the maintenance of the adult brain catecholamine system. However, existing data on this issue is contradictory. Using three complementary approaches, we found that, independent of the time of reduction, *Gdnf* is not required for maintenance of catecholaminergic neurons in adult mice.

Neurotrophic factors hold promise as therapeutic tools to treat neurodegenerative diseases. GDNF was identified for its ability to support and maintain the midbrain dopamine and noradrenaline neurons[1,2], which are specifically affected in Parkinson's disease[3]. Clinical trials to treat Parkinson's disease using GDNF or its family member neurturin (NRTN) have yielded promising, yet conflicting, results[3]. Improvement of therapeutic strategies requires substantial understanding of the physiological role of endogenous GDNF in the maintenance of brain catecholamine system in adult life. Knockout mice for *Gdnf*, its binding receptor *Gfrα1* or its main signaling receptor *Ret* die after birth mainly as a result of the lack of kidneys, but with intact catecholamine system, rendering postnatal analysis impossible[4]. However, conditional ablation using a *loxP-Cre* system (referred to as floxed) of GDNF main signaling receptor *Ret* and *Gdnf* have yielded conflicting results. Jain *et al.* reported that *Ret* deletion from dopamine neurons using DAT-Cre has no effect on brain dopamine system until 9 months of age, when the study was concluded[5]. An independent study by Kramer *et al.* using a *de novo* generated *loxP*-flanked

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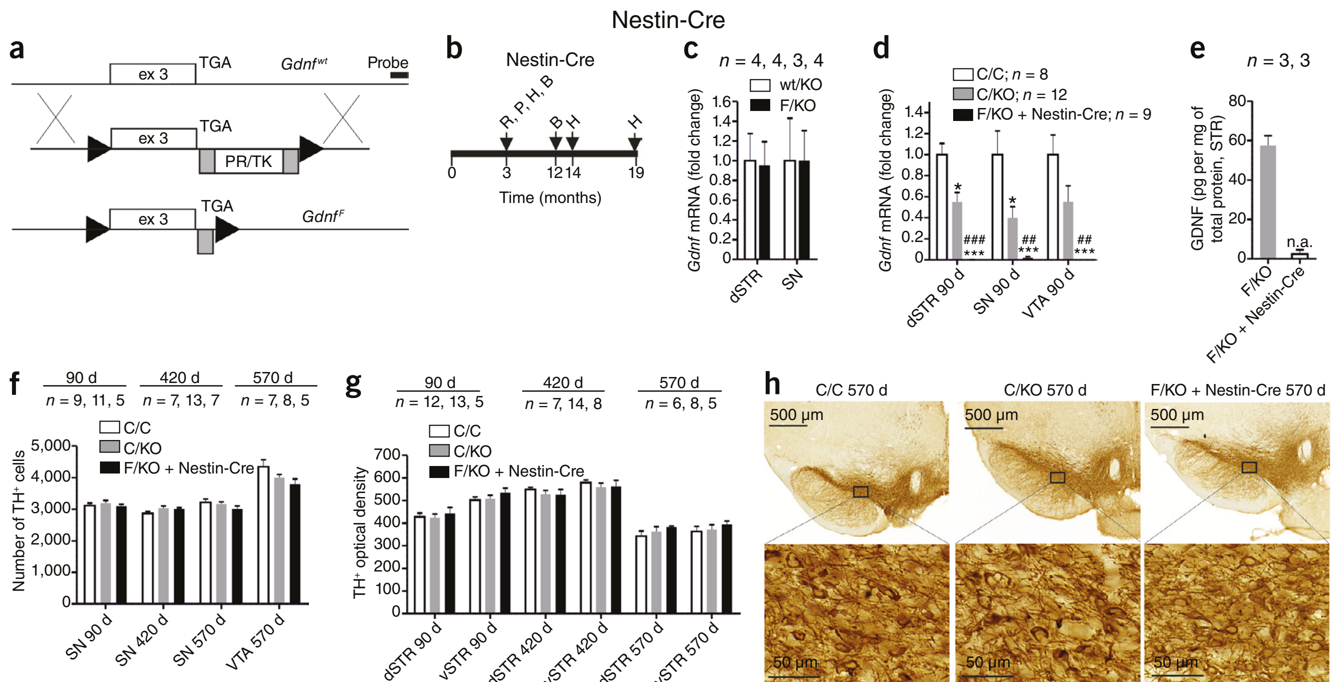

Figure 1 Generation of *Gdnf* floxed mice and catecholamine system analysis after crosses to Nestin-Cre. (**a**) *Gdnf* wild-type ($Gdnf^{wt}$) allele, targeting construct and floxed allele ($Gdnf^{F}$). Triangles indicate *loxP*, gray rectangles indicate FRT sequences. PR/TK is a selectable marker. (**b**) Experimental timeline for analysis of $Gdnf^{F/KO}$ + Nestin-Cre animals showing time points for RNA (R), protein (P), histology (H) and behavioral (B) analysis. (**c**) *Gdnf* mRNA expression from $Gdnf^{F}$ and $Gdnf^{wt}$ allele, determined by real-time quantitative PCR (qPCR). KO, knockout. (**d**) Relative levels of *Gdnf* mRNA after cross to Nestin-Cre line, determined by qPCR ($P < 0.0001$ for dorsal striatum (dSTR) and SN, $P = 0.001$ for VTA, Kruskal-Wallis test). (**e**) GDNF protein levels in the striatum (STR) after cross to Nestin-Cre line. (**f**) Unbiased stereological cell counts of SN and VTA tyrosine hydroxylase (TH)-immunoreactive neurons at indicated time points. (**g**) Unbiased measurement of striatal TH$^+$ optical density reflecting TH$^+$ fiber density. (**h**) Representative TH$^+$ immunostaining of coronal midbrain slices at 19 months of age. C, control containing both wild-type and floxed alleles; n = number of animals analyzed in each experiment, n.a. = not applicable, $^{*}P > 0.05$ or $^{***}P < 0.001$ relative to $Gdnf^{C/C}$, $^{\#\#}P > 0.01$ or $^{\#\#\#}P < 0.001$ relative to $Gdnf^{wt/KO}$. Error bars represent mean ± s.e.m.

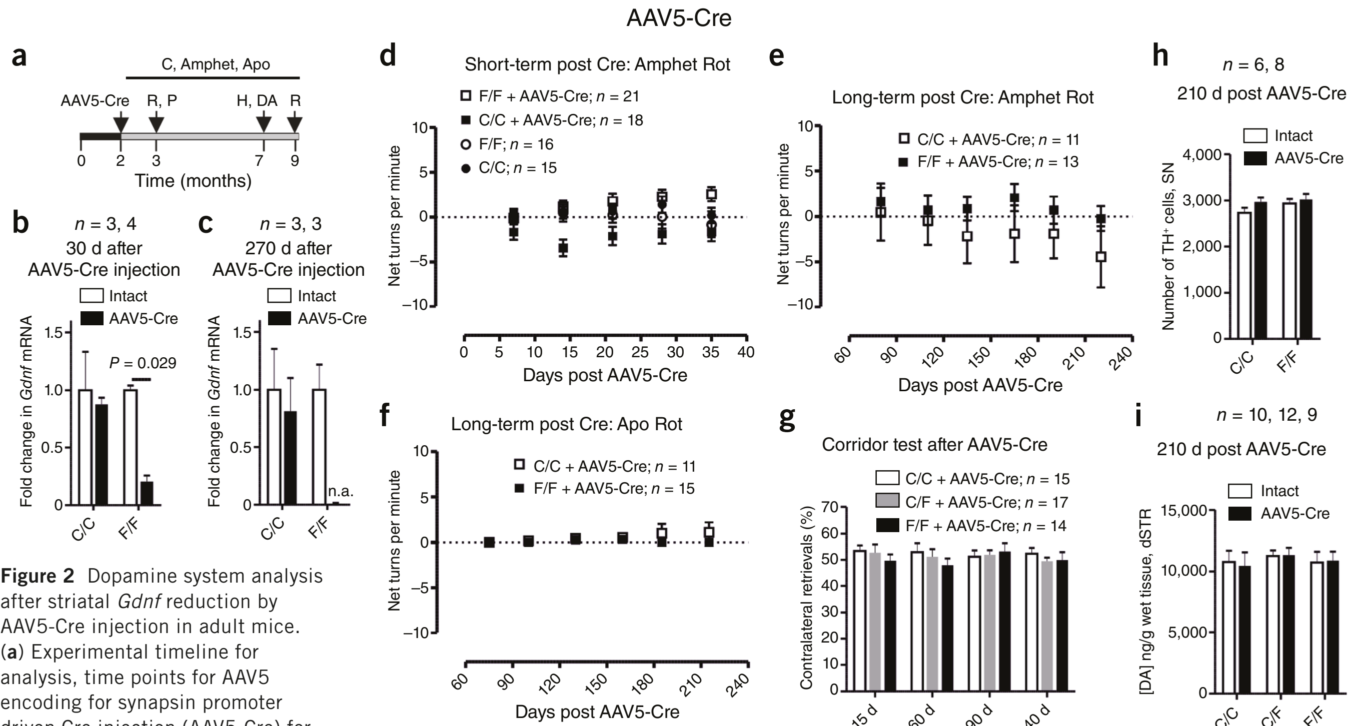

Figure 2 Dopamine system analysis after striatal *Gdnf* reduction by AAV5-Cre injection in adult mice. (**a**) Experimental timeline for analysis, time points for AAV5 encoding for synapsin promoter driven Cre injection (AAV5-Cre) for RNA (R), protein (P), histology (H), striatal dopamine level measurements (DA), and behavior by corridor test (C) and after amphetamine (Amphet) or apomorphine (Apo) injections are indicated. (**b**,**c**) Relative levels of *Gdnf* mRNA determined by qPCR in the intact and AAV5-Cre–injected striata. (**d**–**f**) Measurements of rotations (Rot) after amphetamine (Amphet) and apomorphine (Apo) injection at the indicated time points after AAV5-Cre injection. (**g**) Corridor test at the indicated time points after AAV5-Cre injection. (**h**) Unbiased stereological cell counts of substanti nigra tyrosine hydroxylase (TH)-immunoreactive neurons 7 months (210 d) after *Gdnf* reduction. (**i**) Striatal tissue dopamine (DA) levels 7 months after *Gdnf* reduction. The experiments in **e** and **f** were performed in Helsinki. The experiments in **d** and **g** were performed in Lund. C, control containing only wild-type alleles; *n* = number of animals analyzed in each experiment; n.a. = not applicable. Error bars are mean ± s.e.m.

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Ret allele revealed that the absence of *Ret*, either ubiquitously in CNS or in the dopamine neurons using Nestin-Cre or DAT-Cre, respectively, results in modest, age-dependent dopamine system degeneration in substantia nigra (SN), but not in ventral tegmental area (VTA) or noradrenergic locus coeruleus, starting around 12 months of age[6]. In contrast with the above studies, Pascual *et al.* reported that ubiquitous GDNF reduction in 2-month-old adult mice using a tamoxifen/Esr1-Cre system resulted in marked degeneration of dopamine neurons in both SN and VTA, and a complete degenerative destruction of locus coeruleus 7 months after tamoxifen injection[7]. The existence of alternative GDNF receptors, NCAM and Syndecan-3, and/or onset of developmental compensation in *Ret* floxed mice have been suggested to explain the apparent discrepancy. However, given the obvious clinical relevance of GDNF and the lack of follow-up studies on GDNF conditional deletion since Pascual *et al.*'s report[7] in 2008, the above inconsistency has remained a matter of intensive debate.

Here we report *de novo* generation and analysis of *Gdnf* floxed mice. We used the same strategy as Pascual *et al.*[7] in that we flanked *Gdnf* exon three encoding for GDNF protein with *loxP* sites (**Fig. 1a**). We studied the effect of *Gdnf* deletion on the catecholamine system with a focus on the dopamine system using three different gene deletion methods: Nestin-Cre, AAV5-Cre and Esr1-Cre. Nestin-Cre deletes *Gdnf* from the CNS during embryonic development, whereas intrastriatal injection of adeno-associated virus (AAV) encoding for Cre deletes *Gdnf* from the innervation target of SN dopamine neurons in adult mice. Finally, we used the same tamoxifen-inducible Esr1-Cre mouse line and experimental procedures as Pascual *et al.*[7] to replicate their experiments. *Gdnf* floxed (*Gdnf*$^{F/F}$) mice were generated, genotyped and validated using routine methods (**Fig. 1a** and **Supplementary Methods**, **Supplementary Figs. 1a**,**b** and **3**, and **Supplementary Methods Checklist**). Nestin-Cre mice were analyzed as indicated in **Figure 1b**. *Gdnf* mRNA expression from *Gdnf*^{wt} and *Gdnf*^{F} alleles was comparable (**Fig. 1c**). As expected from earlier work[6,8], cross to Nestin-Cre resulted in CNS-specific *Gdnf* ablation (**Fig. 1d**,**e** and **Supplementary Fig. 1b**,**c**). As in Pascual *et al.*[7], compound heterozygous *Gdnf*$^{F/KO}$ mice were analyzed (**Fig. 1d**). Morphological analysis of the catecholamine system using immunostaining for tyrosine hydroxylase, stereological quantification of tyrosine hydroxylase–expressing cells in the midbrain, and optical density (OD) measurements of dorsal striatum (dSTR) and ventral striatum (vSTR) reflecting density of catecholaminergic innervation revealed no difference between the genotypes at 3, 14 and 19 months of age (**Fig. 1f**–**h**). Locus coeruleus appeared morphologically normal at 19 months of age (**Supplementary Fig. 1d**). Analysis of motor function with open field and rotarod tests revealed no difference in young (2 months) and old (12 months) mice (**Supplementary Table 1**). Given that dopamine system dysfunction is involved in schizophrenia and anxiety, mice were analyzed with pre-pulse inhibition, light-dark and elevated plus-maze tests at both ages. No differences between the genotypes were found (**Supplementary Table 1**).

Next, we tested whether the absence of GDNF is compensated during development by deleting *Gdnf* in adult animals. Mice were unilaterally striatally injected with AAV5-Cre

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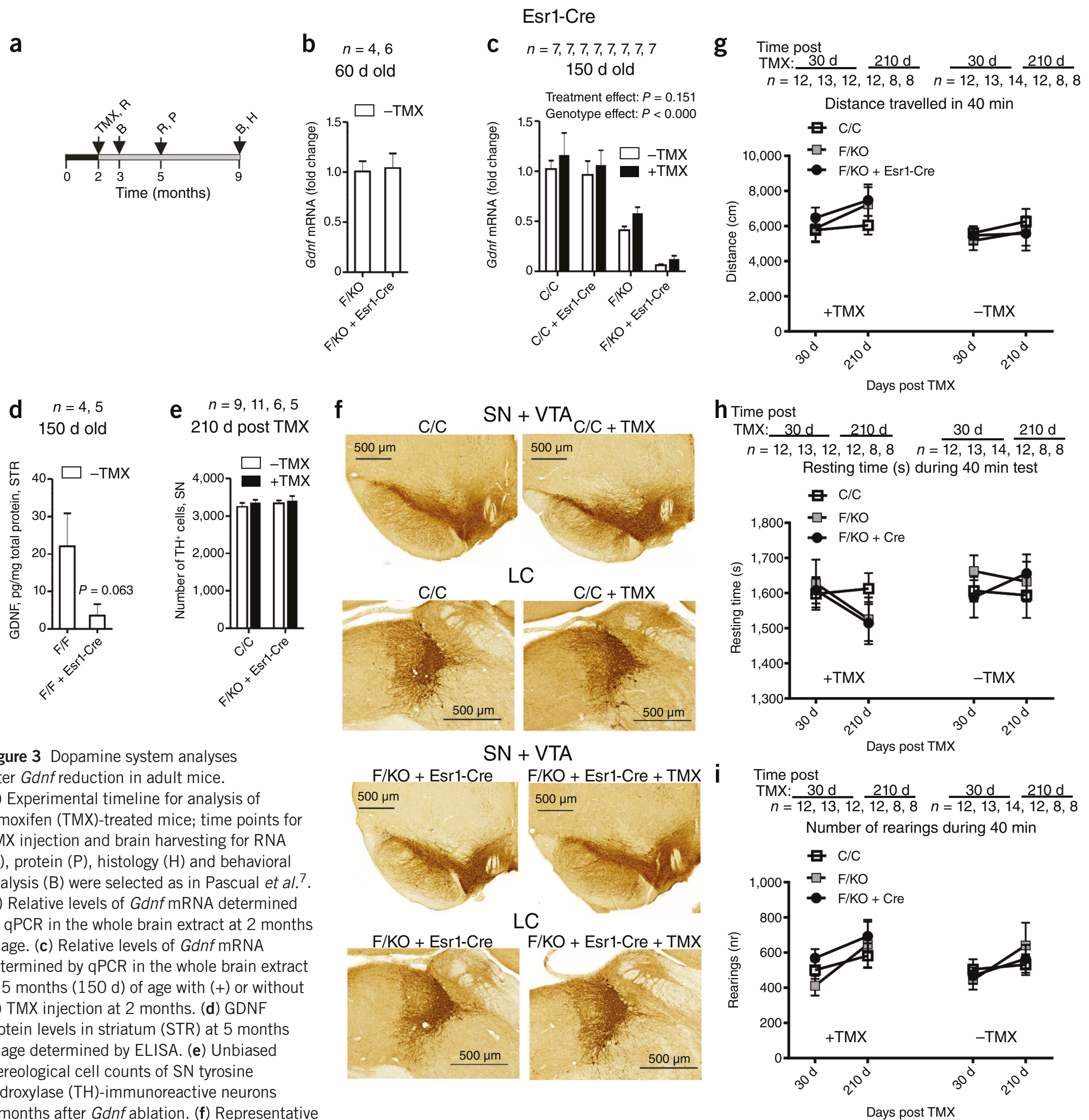

Figure 3 Dopamine system analyses after *Gdnf* reduction in adult mice. (**a**) Experimental timeline for analysis of tamoxifen (TMX)-treated mice; time points for TMX injection and brain harvesting for RNA (R), protein (P), histology (H) and behavioral analysis (B) were selected as in Pascual *et al.*[7]. (**b**) Relative levels of *Gdnf* mRNA determined by qPCR in the whole brain extract at 2 months of age. (**c**) Relative levels of *Gdnf* mRNA determined by qPCR in the whole brain extract at 5 months (150 d) of age with (+) or without (–) TMX injection at 2 months. (**d**) GDNF protein levels in striatum (STR) at 5 months of age determined by ELISA. (**e**) Unbiased stereological cell counts of SN tyrosine hydroxylase (TH)-immunoreactive neurons 7 months after *Gdnf* ablation. (**f**) Representative TH immunostaining of coronal midbrain displaying intact SN, VTA and locus coeruleus (LC) 7 months post *Gdnf* reduction. (**g**–**i**) Open field activity at 1 and 7 months after *Gdnf* reduction was not affected. C, control containing only wild-type alleles; *n* = number of animals analyzed in each experiment. Error bars are mean ± s.e.m.

(**Supplementary Fig. 2a**) and analyzed as indicated in **Figure 2a**. To strengthen the conclusions, we performed behavioral experiments in parallel in two independent laboratories, Lund University and the University of Helsinki. Analysis of striatal *Gdnf* mRNA levels revealed a fivefold reduction at 1 month and an almost complete ablation at 9 months post AAV5-Cre injection (**Fig. 2b**,**c**). We later measured *Gdnf* mRNA and GDNF protein levels using a separate batch of animals and a separate stock of the virus to confirm the acute reduction in striatal GDNF. In this batch, we detected a 40% drop in GDNF protein levels accompanied by a 30% reduction in *Gdnf* mRNA 30 d post injection (**Supplementary Fig. 2b**,**c**). Unilateral decline in dopamine system function is associated with side bias in corridor test[9,10] and by amphetamine (ipsilateral) or apomorphine (contralateral) induced rotational bias[11]. During the 7 month follow-up, unilateral GDNF reduction had no effect on rotational behavior in either laboratory (**Fig. 2d**–**g** and **Supplementary Fig. 2d**–**g**), no effect on catecholaminergic cell survival in SN (**Fig. 2h** and **Supplementary Fig. 2h**) and no effect on striatal tissue dopamine (**Fig. 2i**) or its metabolite levels (**Supplementary Fig. 2i**,**j**).

Finally, we repeated the experiments reported by Pascual *et al.*[7] using the same Esr1-Cre line and experimental conditions (**Fig. 3a**). At 2 months of age, *Gdnf* mRNA levels in

Gdnf$^{F/KO}$ mice and *Gdnf*$^{F/KO}$ mice expressing Esr1-Cre were comparable (**Fig. 3b**). Compared with the controls, mice hemizygous for *Gdnf* allele displayed about 50% reduction in *Gdnf* mRNA levels (**Fig. 3c**). However, consistent with a previous report on 'leakiness' of Esr1-Cre[12], we noted that *Gdnf* exon 3 was spontaneously deleted independently of tamoxifen injection between 2 and 5 months of age, resulting in an 85% drop in *Gdnf* mRNA (**Fig. 3c**). We later measured striatal GDNF protein levels in the presence and absence of Esr1-Cre using a separate batch of *Gdnf*$^{F/F}$ animals and detected an 80% drop in GDNF protein levels at 5 months of age (**Fig. 3d**). Unlike Pascual *et al.*[7], who reported close to 100% destruction of locus coeruleus and around 70% loss of dopamine cells in SN and VTA with concomitant decline in motor activity, we found no morphological changes in any of these structures (**Fig. 3e**,**f**) and no changes in motor activity in *Gdnf*$^{F/KO}$ + Esr1-Cre animals with reduced GDNF independent of tamoxifen application (**Fig. 3g–i**).

Ret-deficient central dopamine neurons do not respond to GDNF[13], suggesting that RET is the main signaling receptor for GDNF in those neurons. Using three complementary approaches, we found that GDNF reduction independent of the time of deletion does not lead to any substantial changes in the number and function of central monoaminergic neurons. Given that GDNF is one of the four ligands activating RET signaling in the CNS[4], our results are consistent with those of *Ret* deletion. We conclude that GDNF reduction is dispensable for maintenance of central catecholaminergic neurons in mice.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. Wiss and K. Rautio for expert technical assistance. J.-O.A. was supported by the Academy of Finland grants 136591, 140983 and 263700, the US National Institutes of Health (NS 0708259) and by the Institute of Biotechnology. M.S. was supported by grants from Sigrid Jusélius Foundation, H. Lundbeck Foundation, M. J. Fox Foundation for Parkinson's Research, Academy of Finland (grant 11186236), US National Institutes of Health (NS 0708259) and the University of Helsinki. T.P.P. was supported by Päivikki and Sakari Sohlberg Foundation. A.B. was supported by grants from the Swedish Research Council. Mouse Behavioural Phenotyping Facility. V.V. is supported by Biocenter Finland. E.C. is supported by the ERC iPlasticity project (322742), Sigrid Jusélius foundation and the Academy of Finland. S.G. was supported by a postdoctoral stipend from the Swedish Brain Foundation (Hjärnfonden). K.V. is supported by a grant from BnM. J.K. was supported by Research Foundation of the University of Helsinki and The Finnish Cultural Foundation.

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AUTHOR CONTRIBUTIONS

J.-O.A. designed and generated the GDNF conditional knockout mice, K.V., C.V., J.-O.A. and M.-A.H. performed qPCR measurements, J.-O.A. and M.-A.H. performed GDNF ELISA, J.K. and C.V. performed immunohistochemistry and stereology, J.K., C.V. and S.G. dissected the brains, J.K. measured dopamine levels and optical density, S.G. carried out AAV-Cre injections, C.V. did tamoxifen injections, J.L., C.V. and S.G. performed all of the behavioral assays, J.-O.A., M.S., T.P.P., A.B., V.V. and E.C. planned the experiments, M.S., J.-O.A., T.P.P. and A.B. provided funding, and J.K. and J.-O.A. prepared the figures and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Pascual and López-Barneo reply:

We have reported that GDNF is required for adult catecholaminergic neuron survival[1]. We generated heterozygous mice with a *loxP*-flanked (floxed) *Gdnf* allele and estrogen receptor–associated Cre recombinase (Esr1-Cre, F/– mice) in which the *Gdnf* gene was ablated in adulthood (2 months of age) by treatment with tamoxifen (TMX). The mice showed a progressive hypokinesia and selective decrease of brain tyrosine hydroxylase (*Th*) mRNA 7 months after GDNF downregulation (to ~40% of the normal striatal protein content). This was accompanied by pronounced catecholaminergic cell death affecting the locus coeruleus, the substantia nigra and the ventral tegmental area. Kopra *et al.*[2] have challenged our results by using a triple approach to inhibit striatal GDNF production. Several issues need to be considered here. First, the Nestin-Cre–driven deletion of the *Gdnf* floxed allele is an interesting model because, as the animals can reach adulthood, it demonstrates dopaminergic nigrostriatal neuron survival in the complete absence of GDNF. However, in this mouse line, *Gdnf* deletion occurs during development and the possibility of embryonic compensation therefore cannot be dismissed. Indeed, although it is well established that GDNF is transiently required during development for defining adult subtypes of Th^+ midbrain neurons[3], animals without GDNF are born with a normal number of dopaminergic neurons, which makes the hypothesis of embryonic compensation highly plausible. Second, Kopra *et al.*[2] also used an Esr1-Cre–driven recombination model to delete the floxed *Gdnf* alleles in adult animals. However, their study is hampered by the fact that, for unknown reasons, *Gdnf* deletion was not induced by TMX treatment. In contrast, they seemed to observe constitutive striatal Cre activation that, nonetheless, did not induce a substantial reduction of striatal GDNF protein. The behavior exhibited by this ESR1-Cre mouse line is unusual, as we have shown with several mouse models carrying floxed alleles that substantial recombination only occurs after TMX treatment[1,4,5]. The efficiency of TMX-activated Esr1-Cre is highly variable, and the distinct effects observed with this Cre line could be a result of variations in the genetic background (F1 hybrid C57bl6/129SvJ in Pascual *et al.*[1] and mixed 129Ola/ICR/C57bl6 in Kopra *et al.*[2]). Third, the final model employed by Kopra *et al.*[2] was the intrastriatal injection of AAV5-Cre in F/F mice (with two floxed *Gdnf* alleles. The reported efficiency of *Gdnf* deletion (measured as *Gdnf* mRNA in the entire striatum 30 d after AAV5 treatment) was highly variable (between 20 and 80% of the control value) and the amount of striatal GDNF protein was maintained above 50% of the normal level. Hence, one could argue that GDNF content was not sufficiently reduced to elicit neurodegeneration in these mice with a mixed genetic background, and that they might be more resistant to GDNF deficiency than other strains. In addition, the possibility that tissue damage produced by stereotaxic AAV injections could have activated glial cells to produce trophic factors that compensate for the lack of GDNF cannot be ruled out[6].