ARTICLE

A regulatable AAV vector mediating GDNF biological effects at clinically-approved sub-antimicrobial doxycycline doses

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Preclinical and clinical data stress the importance of pharmacologically-controlling glial cell line-derived neurotrophic factor (GDNF) intracerebral administration to treat PD. The main challenge is finding a combination of a genetic switch and a drug which, when administered at a clinically-approved dose, reaches the brain in sufficient amounts to induce a therapeutic effect. We describe a highly-sensitive doxycycline-inducible adeno-associated virus (AAV) vector. This vector allowed for the first time a longitudinal analysis of inducible transgene expression in the brain using bioluminescence imaging. To evaluate the dose range of GDNF biological activity, the inducible AAV vector (8.0 × 10^9 viral genomes) was injected in the rat striatum at four delivery sites and increasing doxycycline doses administered orally. ERK/Akt signaling activation as well as tyrosine hydroxylase downregulation, a consequence of long-term GDNF treatment, were induced at plasmatic doxycycline concentrations of 140 and 320 ng/ml respectively, which are known not to increase antibiotic-resistant microorganisms in patients. In these conditions, GDNF covered the majority of the striatum. No behavioral abnormalities or weight loss were observed. Motor asymmetry resulting from unilateral GDNF treatment only appeared with a 2.5-fold higher vector and a 13-fold higher inducer doses. Our data suggest that using the herein-described inducible AAV vector, biological effects of GDNF can be obtained in response to sub-antimicrobial doxycycline doses.

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INTRODUCTION

Neuroprotective and neurorestorative effects of glial cell line-derived neurotrophic factor (GDNF) gene delivery have been demonstrated in phenotypical models of Parkinson’s disease (PD).1–14 Partially encouraging clinical data have been obtained in clinical trials using the closely related Neurturin (NTN) factor.15–19 Despite these studies, the precise mechanism of action of these neurotrophic factors in adult diseased brain still needs to be fully elucidated. Indeed, numerous studies have suggested that, in addition to enhanced dopaminergic neuron survival in phenotypical models of PD, poorly understood neurochemical effects on dopaminergic neurotransmission and dopamine biosynthesis are likely to participate to the clinical outcome of GDNF-based treatments. The latter vary with the time, dose, and mode of administration, further complicating the interpretation of the data. A recent study suggests that GDNF and Ret are dispensable for the maintenance of dopaminergic neurons.15 However, in a lesional context, a clear GDNF-mediated protection of the dopaminergic connectivity has been demonstrated.1,4 Regardless of the mechanism of GDNF-mediated neuroprotective activity, side effects of long-term, high-dose or off-target delivery, such as aberrant sprouting, downregulation of tyrosine hydroxylase and loss of weight have been described.12,15–17 Altogether, it will be very interesting to have available clinically-relevant regulatable viral vectors in order (i) to provide a tool to dissect the diverse mechanisms involved in the observed enhancement of the dopaminergic system and (ii) to optimize the outcome of clinical trials by modulating GDNF dose and period of administration.

The most widely used and best characterized regulatable system is the tetracycline-dependent (tet) system. Two versions exist: (i) the tetOFF system based on a fusion between the wild-type tet repressor (tetR) and the activation domain of the HSV VP16 transcription factor16 and (ii) the tetON system in which the tetR sequence has

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been replaced by a mutant which reverses the phenotype from a tet-repressible to a tet-inducible system. Since long-term exposure to antibiotics is undesirable, repeated punctual treatments using an inducible rather than a repressible system seems to be the most clinically-relevant option. The initial tetON system proved to be less sensitive to antibiotic inducers than the tetOFF system. However, new rtTA mutants selected through viral evolution in the presence of a low dose of doxycycline (Dox), have been isolated.

In the brain, an additional concern is the low bioavailability of inducers which poorly pass the blood–brain barrier. Therefore, a sensitive transactivator is a prerequisite for applications in this organ.

Efficient ON/OFF kinetics with a single-cassette AAV-tetOFF system in the rat brain has been demonstrated. Complete extinction of GDNF transgene expression required a plasmatic Dox concentration of ~300 ng/ml (100 mg Dox/kg diet).

We have conducted a similar effort to develop a single-cassette AAV-tetON system using the reverse tetracycline transactivator, rtTA-2M2 mutant mounted in a single autoregulatory AAV vector. In this study, Dox-dependent neurochemical effects as well as behavioral improvements have been obtained at a Dox dose of 600 mg/l in drinking water (corresponding to a plasmatic concentration of 5,400 ng/ml; unpublished data). Although a tight regulation of GDNF levels in the brain, allowing to assess the reversibility of the observed biological effects has been demonstrated using this vector, its clinical applicability was hampered by the high amount of antibiotic required (higher than the plasmatic Dox doses measured in patients treated for infectious diseases).

Other genetic switches have been successfully applied to modulate GDNF delivery. Interestingly, the mifepristone (Mfp)-regulated Gene Switch which also uses an approved drug, was introduced in AAV vectors. Neuroprotective effects were demonstrated at a Mfp dose which, when adjusted using a FDA-approved rat to human conversion factor, was 2.5-fold lower than the dose administered for 7 days to patients for psychiatric indications in a randomized clinical trial in which no adverse effect was reported. This system, however, was tightly regulated only when used in a 2-vectors configuration.

The rapamycin-regulated system, another dual-component system also showed a tight regulation of GDNF transgene expression but the level of expression was low as compared to a constitutive vector. GDNF could also be regulated at the post-transcriptional level, using a fusion with a destabilizing domain.

The herein described highly Dox-sensitive AAV inducible vector combines the advantages of the high Dox-sensitivity of the rtTA-V16 mutant and the tightness of the autoregulatory tet-inducible AAV vector. Therapeutically-relevant biological effects of GDNF were demonstrated in the rat brain, in response to orally administrated Dox at clinically-approved sub-antimicrobial doses. Indeed, the active plasmatic Dox concentration was below the levels measured in patients treated for inflammatory diseases of the skin and of the teeth surrounding tissue. Finally, these levels were proven not to increase the occurrence of Dox-resistant microorganisms and not to affect the intestinal flora.

RESULTS

In vitro Dox dose-dependent transgene expression driven by a highly sensitive autoregulatory inducible AAV using the V16 rtTA mutant

Starting from a previously described autoregulatory tet-inducible AAV vector plasmid (pAC1-M2), we have replaced the rtTA-2M2 mutant by the V16 mutant (3 mutations conferring improved Dox sensitivity) (Figure 1a). Hereafter, the new vector is referred to as pAC1-V16.

In order to compare the Dox sensitivity and overall induction amplitude of pAC1- M2 and pAC1-V16, two different transgenes were used luciferase (LUC) and human GDNF (hGDNF) cDNA.

The constructs were encapsidated into serotype 1 AAV capsids to generate rAAV2/1-tetON-V16 recombinant virus (hereafter referred as rAAV2/1-V16) and used at a multiplicity of infection of 1,000 viral genomes (vg) per cell to infect HEK-293T in the presence of adenovirus type 5 (50 infectious units per ml). Cells were treated with increasing Dox doses, ranging from 1 to 1,000 ng/ml. The amount of secreted hGDNF was measured in the medium of infected cells by enzyme-linked immunosorbent assay (ELISA) (Figure 1b). The expression of the LUC reporter gene was measured in extracts from infected cells by luminometry (Figure 1c). The dose range of transgene expression was slightly different for hGDNF and LUC but both reflected the higher sensitivity of the V16 construct. Expression driven by rAAV2/1-M2 was detectable from 100 ng/ml (GDNF) or 300 ng/ml (LUC) but resulted in significant levels of transgene expression only at the dose of 1,000 ng/ml whereas with the rAAV2/1-V16 vectors, expression was detectable from 1 ng/ml but significant expression levels were readily observed from 10 and 30 ng/ml for the hGDNF and LUC transgenes, respectively. Differences in protein stability and/or in the sensitivity of the measurement methods could account for the different detection thresholds observed with the GDNF and LUC transgene products.

Furthermore, the amplitude of the induction was significantly higher with rAAV2/1-V16 (approximately 15 and 60 pg GDNF/ml for the M2 and V16 vectors, respectively; Figure 1b).

Longitudinal analysis of transgene expression using in vivo bioluminescence imaging in the brain

In order to determine if the higher sensitivity of the V16 rtTA mutant to Dox can be confirmed in vivo in the brain, rAAV2/1-M2-LUC and rAAV2/1-V16-LUC (8.3 × 107 vg each) were injected in the striatum of adult mice which were subsequently fed with diet supplemented with Dox amounts ranging from 0.025 to 6 g/kg diet during 5 weeks (seven mice were injected for each group). First, brain protein extracts were analyzed using luminometry (n = 7). Figure 2a shows that, with the M2 mutant, no bioluminescent signal was detectable at any of the doses tested. In contrast, with the V16 mutant, the signal was significantly increased at the two highest doses: 1 and 6 g/kg diet. In order to determine if the expression of the transgene was stable, we compared the intensity of the bioluminescent signal in mice continuously fed with Dox-supplemented (6 g/kg) or unsupplemented diet for 6 weeks (n = 5 for the no Dox group and n = 4 for Dox group) and 30 weeks (n = 7). Figure 2b shows that the relative light units per mg of proteins were similar at these two time points.

Thanks to the high sensitivity and efficiency of the rAAV2/1-V16 vector, LUC reporter gene expression could be detected using in vivo bioluminescence imaging. Mice were stereotaxically-injected with rAAV2/1-V16-LUC and treated with a high Dox dose: 6 g/kg diet. Figure 2c shows the bioluminescent signal present in Dox-treated (n = 4) but not in untreated (n = 7) mice, 4 weeks post-vector injection. Quantitative analysis of the signal revealed a significant difference between Dox-treated and untreated mice. Thanks to this technology, the kinetics of transgene expression upon Dox withdrawal and readdition (ON/OFF/ON) could be performed without sacrificing animals at each time point (Figure 2d). We evaluated the delay after Dox withdrawal/adddition necessary for full extinction/induction of the vector. After feeding the mice (n = 4) for 1 month with Dox-supplemented food (Figure 2c,d), unsupplemented diet...
was given for 7 weeks. Bioluminescence imaging was performed every second day starting 1 day post-Dox withdrawal. The signal was reduced from 3 days post-Dox removal but full extinction of the system required approximately 9 days. A second Dox treatment was implemented after 7 weeks of unsupplemented food. A bioluminescent signal was detected from 1 day after Dox administration but full induction of the system required approximately 5 days.

Measurements of Dox concentrations in rat’s plasma
In order to determine the threshold of clinically-acceptable doses in our experiments, rats (n = 8 per group) were given food containing Dox at doses ranging from 0.025 to 1 g/kg. After 5 weeks of treatment, plasmatic Dox concentrations, measured using a previously described bioassay, were varying in function of the Dox dose in the rat diet (Figure 3). At the doses of 0.075 g and 0.2 Dox/kg diet, the plasmatic Dox concentrations were 139 ± 50 ng/ml, respectively. These levels are respectively below those found in patients treated for Rosacea (200–500 ng/ml), previously demonstrated not to increase the incidence of Dox-resistant micro-organisms. Furthermore, these plasmatic Dox concentrations are one order of magnitude lower than those found in patients treated for bacterial infections (2.6–5.9 mg/ml).24

Dose-dependent hGDNF transgene expression in the rat striatum induced by Dox treatment
Recombinant AAV2/1-V16-hGDNF was injected in the right striatum at a total dose of 8.0 × 10^9 or 2.0 × 10^10 vg. Rats were given food containing Dox at doses ranging from 0.025 to 1 g/kg. Six weeks after vector injection, the rats (n = 6) were sacrificed and GDNF striatal tissue concentration was measured using an ELISA (Figure 4a). In the untreated rats, the GDNF level was not significantly increased as compared to the control rAAV2/1-V16-EGFP (enhanced green fluorescent protein)-injected rats. In the Dox-treated rats, a dose-dependent GDNF concentration was observed for both vector doses. Significant increases of GDNF concentration were observed from a Dox dose of 0.2 and 0.025 g/kg, respectively for the low (8.0 × 10^9 vg) and the high (2.0 × 10^10 vg) vector dose. Since it has been previously demonstrated that 200 pg/mg tissue of GDNF were sufficient to induce neuroprotective/neurorestorative effects of rAAV-mediated GDNF delivery, we decided to focus on the low vector dose, which provided a GDNF concentration of 219.0 ± 46.6 pg/mg tissue at a Dox dose of 0.2 g/kg, for further characterizations.

Spatial distribution of GDNF in the striatum of Dox-treated rats
In order to optimize GDNF-mediated neuroprotection/neurorestoration of dopaminergic neurons, a distribution of the neurotrophic...
GDNF could be evidenced throughout the striatum over at least 5 weeks of treatment (n = 7 for each Dox dose and for the two vectors). Five weeks postinjection, bioluminescence was evaluated in striatal protein extracts. ***P < 0.001 versus untreated mice. (b) Mice were stereotaxically-injected with rAAV2/1-M2-LUC (circles) or rAAV2/1-V16-LUC (squares) and treated with increasing Dox doses starting immediately after injection until the end of the experiment (n = 7 for each Dox dose and for the two vectors). Five weeks postinjection, bioluminescence was evaluated in striatal protein extracts. *P < 0.05; ***P < 0.01 versus untreated mice. (c) Mice stereotaxically injected with rAAV2/1-V16-LUC were continuously treated with Dox at a dose of 6 g/kg diet starting immediately after injection until the end of the experiment. Six weeks (n = 5 for no Dox and n = 4 for Dox) or 30 weeks (n = 7 for no Dox and Dox) postinjection, bioluminescence was evaluated in striatal extracts. *P < 0.01 versus untreated mice. (d) Mice stereotaxically injected with rAAV2/1-V16-LUC were continuously treated with Dox at a dose of 6 g/kg diet starting immediately after injection (n = 7 and n = 4 for no Dox and Dox groups, respectively). Bioluminescence imaging was performed at 5 weeks postinjection. *P < 0.05 using a Student t-test. (d) After 5 weeks of treatment with Dox diet (6 g/kg), mice (n = 4) were fed with unsupplemented food (Dox withdrawal) and bioluminescence imaging was performed every 2 days, starting 1 day post-Dox withdrawal. After an additional period of 5 weeks, the mice received Dox diet (6 g/kg) and bioluminescence imaging was performed at the additional indicated days until day 34. RLU, relative light units; cpm/cm², counts per minute per cm².

**Figure 2** Ex vivo and in vivo evaluation of luciferase expression in the striatum of recombinant adeno-associated virus (rAAV)2/1-tetON-injected mice. rAAV2/1-LUC vector (8.3 × 10⁷ vg) was stereotaxically injected in the striatum of CD1 mice (n = 7). (a) Mice were stereotaxically-injected with rAAV2/1-M2-LUC (circles) or rAAV2/1-V16-LUC (squares) and treated with increasing Dox doses starting immediately after injection until the end of the experiment (n = 7 for each Dox dose and for the two vectors). Five weeks postinjection, bioluminescence was evaluated in striatal protein extracts. ***P < 0.001 versus untreated mice. (b) Mice were stereotaxically-injected with rAAV2/1-V16-LUC and treated with Dox at a dose of 6 g/kg diet starting immediately after injection until the end of the experiment. Six weeks (n = 5 for no Dox and n = 4 for Dox) or 30 weeks (n = 7 for no Dox and Dox) postinjection, bioluminescence was evaluated in striatal extracts. *P < 0.05; ***P < 0.01 versus untreated mice. (c) Mice stereotaxically injected with rAAV2/1-V16-LUC were continuously treated with Dox at a dose of 6 g/kg diet starting immediately after injection (n = 7 and n = 4 for no Dox and Dox groups, respectively). Bioluminescence imaging was performed at 5 weeks postinjection. *P < 0.05 using a Student t-test. (d) After 5 weeks of treatment with Dox diet (6 g/kg), mice (n = 4) were fed with unsupplemented food (Dox withdrawal) and bioluminescence imaging was performed every 2 days, starting 1 day post-Dox withdrawal. After an additional period of 5 weeks, the mice received Dox diet (6 g/kg) and bioluminescence imaging was performed at the additional indicated days until day 34. RLU, relative light units; cpm/cm², counts per minute per cm².

**Figure 3** Plasmatic Dox concentrations in function of the Dox dose in the rats diet. Rats (n = 8) were treated with increasing Dox doses for 5 weeks. Plasma was harvested as described in Material and Methods and Dox concentrations evaluated using a previously described cell-based assay.²³ Although the two needle tracts, visible on some of the rats were confirmed to be located at the expected coordinates in the striatum, a strong GDNF staining was observed in a region located outside the striatum beyond the borders between the striatum and the cortex, more specifically at the ventro-lateral side (see Figure 4b). Such a strong tropism of rAAV2/1 vectors for neurons located at distance from the injected site, presumably in the external capsule, has already been previously reported.²⁴

Dox dose range of GDNF-mediated induction of ERK pathway-dependent S6 ribosomal protein phosphorylation on Ser-235 and Ser-236

We then sought to determine at which Dox dose a significant induction of GDNF-mediated biological effects could be observed. Therefore, we evaluated the amounts of Ser-235 and Ser-236 phosphorylation as described in Material and Methods.²³

Moreover, the spatial distribution of GDNF protein after injection of rAAV2/1-V16-hGDNF at the low vector dose was examined (four rats per group were analyzed). At Dox doses of 0.075, 0.2, and 1 g/kg, 70, 75, and 90% of the striatum was respectively labeled. Figure 4b illustrates the antero-posterior distribution of the staining in a representative treated with 0.2 g Dox/kg diet. It shows that GDNF could be evidenced throughout the striatum over at least a 2.4 mm range (Figure 4b). In contrast, at the dose of 0.025 g/kg as well as in untreated rats, the GDNF staining harbored a low but detectable intensity covering 30% of the striatum. The quantification of the stainings reveals that at the two highest Dox doses (0.2 and 1 g/kg diet), the GDNF immunoreactivity was significantly increased as compared with the untreated group and the EGFP control group (Figure 4c). In contrast, for the two lower doses (0.075 and 0.025 g Dox/kg diet), it was not significantly different from the untreated and control groups. These data are in accordance with the results of the ELISA (Figure 4a).
phosphorylated S6 ribosomal protein (p-S6) in dopaminergic neurons as an indicator of GDNF-induced ERK/Akt signaling cascade.\(^\text{41}\)

Recombinant AAV2/1-V16-hGDNF, was injected in the right striatum at a dose of 8.0\(\times\)10\(^6\) vg as described above (\(n=6\) rats per Dox dose). Peroxidase staining of midbrain sections using antibodies against p-S6 allows to evidence unilateral activation of the ERK signaling pathway in the substantia nigra of the injected hemisphere.\(^\text{42}\)

A Dox dose-dependent increase of p-S6 staining was observed. This increase reached significance at Dox doses of 0.2\,0.075, 0.2, or 1 g Dox/kg or with unsupplemented food. The figure illustrates an example of GDNF immunoreactivity in striatum of a rat treated with 0.2 g Dox/kg of diet. Scale bar: 2 mm. (c) The GDNF labeling was quantified by multiplying the mean of grayscale by the stained area on four slices at the level of injection. \(n=4\) rats/Dox dose. ***\(P<0.001\) versus the no Dox group).

**Figure 4** Striatal glial cell line-derived neurotrophic factor (GDNF) expression after doxycycline induction. (a) Quantification of GDNF tissue levels was performed 6 weeks after injection of 8.0\(\times\)10\(^6\) or 2.0\(\times\)10\(^7\) vg of recombinant adeno-associated virus (rAAV2/1-V16-hGDNF) in the right striatum. Rats were continuously fed with Dox supplemented diet containing 0.025, 0.075, 0.2, or 1 g Dox/kg or with unsupplemented food. Results are expressed as mean of GDNF concentration in striatal protein extracts; \(n=6\) rats/Dox dose for low dose of vector and \(n=8\) for high dose of vector (\(\text{***}P<0.001; \text{**}P<0.001\); ***\(P<0.001\) versus the enhanced green fluorescent protein group). (b) Spatial distribution of the GDNF transgene product. GDNF immunostaining was performed 9 weeks following injection of 8.0\(\times\)10\(^6\) vg of rAAV2/1-V16-hGDNF in the right striatum. Rats were continuously fed with Dox-supplemented diet containing 0.025, 0.075, 0.2, or 1 g of Dox/ kg or with unsupplemented food. The figure illustrates an example of GDNF immunoreactivity in striatum of a rat treated with 0.2 g Dox/kg of diet. Scale bar: 2 mm. (c) The GDNF labeling was quantified by multiplying the mean of grayscale by the stained area on four slices at the level of injection. \(n=6\) rats per Dox dose. **\(P<0.01\) versus the enhanced green fluorescent protein group).

Dox dose range of GDNF-induced downregulation of tyrosine hydroxylase

We next evaluated the effect of GDNF transgene expression induced by Dox on TH protein level in the striatum using immunohistochemistry (Figure 6). A densitometric analysis of TH labeling in the striatum revealed a significant downregulation at doses of 0.2 and 1 g Dox/kg diet as compared to the untreated ("no Dox") and to the EGFP control groups (\(n=6\) rats per Dox dose). Thus, the minimal Dox dose provoking TH downregulation was approximately threefold higher than the minimal dose inducing S6 phosphorylation. The optical densities in the "no Dox": 0.025 and 0.075g Dox /kg diet experimental groups were not significantly different from the EGFP group.

Absence of glial cells activation

When delivered into the muscle, the rtTA transactivator elicits a strong immune response leading to the elimination of transduced cells.\(^\text{43}\) The long-term stability of transgene expression mediated by rAAV2/1-V16 (see Figure 2) suggests the absence of a strong cellular immune response. To determine if a mild inflammatory response could nevertheless take place, we examined the expression level of astrocytic (glial fibrillary acidic protein (GFAP)) and microglial (Iba1) markers which are upregulated when these cells are activated. Sections from rats injected with rAAV2/1-V16-hGDNF (8.0\(\times\)10\(^7\) vg) and fed with Dox diet or unsupplemented food were labeled with anti-GFAP and anti-Iba1 antibodies (two rats per group). The stainings were of similar intensity and the glial cells morphology were similar in the injected and intact sides, for all Dox doses tested (see Figure 7d, panels e versus f (Iba1) and Figure 7j, panels k versus l (GFAP) for a representative example of a rat treated with 1g Dox/kg diet), indicating that the vector did not induce any detectable activation of glial cells 9 weeks after vector injection. In addition, the staining were also similar in rats fed with unsupplemented food (Figure 7a–c and g–i as compared to rats fed with a high Dox dose (1 g/kg diet; Figure 7d–f and j–l) expressing a high level of GDNF (see Figure 4) and thus presumably of rtTA-V16.

Dox dose range of GDNF-mediated motor abnormalities

Contralateral amphetamine-induced rotations were reported after unilateral injection of AAV vectors constitutively expressing GDNF in healthy rats.\(^\text{1}\) We first analyzed the motor behavior after intrastriatal injection of rAAV2/1-V16-hGDNF at a vector dose of 8.0\(\times\)10\(^6\) vg, which induced ERK signaling at a Dox dose of 0.075 g/kg diet (see above). No significant difference in the number of amphetamine-induced rotations was observed between the EGFP group and all the Dox-treated groups (from 0.025 to 1 g Dox/kg diet; \(n=8\) rats per group) (Figure 8a).
In order to determine the safety limits of rAAV2/1-V16-GDNF, a 2.5-fold higher vector dose was injected in four deposits at the same antero-posterior level (in order to obtain a high local GDNF tissue concentration) \( n = 8 \) rats per group. Amphetamine-induced rotations significantly different from the EGFP group appeared between 20 and 50 minutes post-amphetamine injection at a dose of 1 g Dox/kg diet. This perturbation of the motor behavior thus only appeared at a 2.5-fold higher vector dose combined with a 13-fold higher Dox dose than the minimal dose inducing ERK/Akt signaling (Figure 8b).

**DISCUSSION**

We describe a single autoregulatory AAV vector using a mutant of the reverse tetracycline transactivator selected for its high sensitivity to Dox, which mediates therapeutically relevant biological effects of GDNF in response to sub-antimicrobial Dox doses. These transgenic vectors were then evaluated for their therapeutic activity in several rodent models.

Absence of weight loss

Important weight loss was described when delivering AAV vectors constitutively expressing GDNF, in the rat and monkey substantia nigra. Since we previously demonstrated a low level of anterograde transport of the transgene product in the substantia nigra pars reticulata using the AAV2/1-M2 vector, we compared the weight curves of rAAV2/1-V16-GDNF-treated rats to rAAV2/1-V16-EGFP at both vector doses \( (8.0 \times 10^9 \text{ vg} \text{ and } 2.0 \times 10^{10} \text{ vg}; n = 8 \) rats per group). In accordance with previous studies, we did not observe any significant reduction of the weight when the vectors were injected in the striatum and furthermore the weight gain was not different between the GDNF-treated and control rats (Figure 8c,d).

**Figure 5** Nigral pS6 immunoreactivity after doxycycline induction. Serine 235/236 phosphorylated S6 ribosomal protein (p-S6) immunostaining was performed 9 weeks following intra-striatal injection of \( 8.0 \times 10^9 \text{ vg} \) of recombinant adenovirus (rAAV2/1-V16-hGDNF). Rats were fed with Dox-supplemented diet containing 0.025, 0.075, 0.2, or 1 g of Dox/kg or with unsupplemented food during 9 weeks. (a,b) Example of p-S6 immunoreactivity in substantia nigra pars compacta (SNc) of a rat treated by 0.2 g Dox/kg of diet (a) and an untreated rat (b). Scale bar: 2 mm (c) The p-S6 immunoreactivity is reported as the mean of the optical densities multiplied by the stained area measured on two to three slices at the level of SNc. \( n = 6 \) rats/Dox dose. **\( P < 0.01 \), ***\( P < 0.001 \) versus no Dox group and "\( P < 0.05 \), ""\( P < 0.001 \) versus enhanced green fluorescent protein group. GDNF, glial cell line-derived neurotrophic factor.

**Figure 6** Tyrosine hydroxylase (TH) immunoreactivity after doxycycline induction. TH immunostaining was performed 9 weeks following intra-striatal injection of \( 8.0 \times 10^9 \text{ vg} \) of AAV2/1-V16-hGDNF. (a–d) Representative pictures of TH immunoreactivity of rats fed with unsupplemented food (a) or Dox-supplemented diet containing 0.025 (b), 0.075 (c), 0.2 (d), or 1 g of Dox/kg during 9 weeks. Scale bar: 2 mm (e) Values represent percentage of decrease of TH immunoreactivity relative to the striatum contralateral to the viral injection. \( n = 6 \) rats/Dox dose. ""\( P < 0.001 \) versus no Dox group and **\( P < 0.01 \), ***\( P < 0.001 \) versus enhanced green fluorescent protein group. GDNF, glial cell line-derived neurotrophic factor.
data were obtained using a clinically-relevant protocol. Indeed, the rAAV vector was stereotaxically-infused at four delivery sites along two needle tracts at a dose of $8.0 \times 10^9$ vg/striatum ($3.0 \times 10^9$ vg/mm$^3$), i.e., fourfold-higher than that used in the rAAV2-NTN clinical trial which was demonstrated to be safe$^{5,6,44,45}$ and similar to the highest dose approved for the ongoing rAAV2-GDNF clinical trial (https://clinicaltrials.gov/ct2/show/NCT01621581).

Dox at a dose of 200 mg per day, combined with other antibiotics, is recommended for the treatment of patients with brucellosis, a bacterial infection.$^{66}$ However, long-term treatment raises concerns both in terms of adverse effects for the treated patient (phototoxicity, hepatotoxicity, bone toxicity, etc.) and epidemiologically, in terms of increased incidence of Dox-resistant microorganisms.$^{67}$ In contrast, Dox, administrated at lower sub-antimicrobial doses elicit no or very mild adverse effects and has been approved for the treatment of benign inflammatory diseases. In particular, it has been demonstrated, in phase 3 clinical trials involving several hundreds of patients with Rosacea, a skin disease, that a dose of 40 mg Dox has anti-inflammatory effects, while not increasing the number of Dox-resistant microorganisms in fecal and vaginal samples.$^{32,36,39,48}$ Similarly, in the treatment of periodontitis with 20 mg Dox daily administration, no changes in antibiotic susceptibility have been reported.$^{33}$

Since the metabolism of antibiotics is different in rodents and human, the orally administrated Dox dose might not provide a reliable basis for comparison. It is thus preferable, to use plasmatic concentrations for inter-species comparisons. In patients treated for bacterial infections (200 mg per day), the peak plasmatic concentration ranges from 2,600 to 5,900 ng/ml$^{24}$ whereas in patients treated for Rosacea (40 mg per day), it was found to vary between 200–500 ng/ml$^{36}$ and in patients with periodontitis treated with 20 mg Dox daily for long periods, it reached 400 ng/ml.$^{33}$

In our previous studies using the tetracycline-inducible rAAV2/1-M2 vector, a Dox dose of 600 mg/l in drinking water was necessary to obtain vector-mediated biological effects of hGDNF, including TH downregulation in healthy rats$^{31}$ and partial behavioral recovery in the partial 6-hydroxydopamine PD rat model.$^{11}$ In these conditions, the plasmatic Dox concentration reached 5,378 ng/ml (L. Tenenbaum and A.T. Das, data not shown), thus similar to the highest level in patients treated for bacterial infections and 13-fold higher than the levels found in clinical trials for Rosacea.

In contrast, using the herein described rAAV2/1-V16-hGDNF, induction of ribosomal S6 protein phosphorylation on Ser-235/236 (p-S6) (a downstream product of mTOR and ERK signaling pathways mediated by GDNF$^{41,49}$) were induced at a plasmatic Dox
concentration of 140 ng/ml, thus lower than the levels measured in the plasma of patients treated for Rosacea. The neuroprotective/neurorestaurative effects of rAAV2/1-V16-hGDNF remain to be demonstrated in a PD model. However, in a previous study using GDNF gene transfer in the intrastriatal 6-hydroxydopamine PD model,30 a reduction of the motor symptoms, evaluated using three different behavioral tests was associated to a 1.5-fold increase of the p-S6 immunolabeling in the substantia nigra. In the present study, a similar densitometric quantification revealed a four- to fivefold increased p-S6 labeling in rats treated with 0.075 g Dox/kg diet. Our data strongly suggest that rAAV2/1-V16-hGDNF can mediate neuroprotection at this dose, corresponding to a plasmatic dose of 140 ng Dox/ml.

Several other systems have been proposed to regulate GDNF administration in the brain.

A single rAAV2/5-TetOFF vector administered at a very high titer (7.3 × 10^{11} vg/ml) was shown to deliver high amounts of GDNF in the striatum in the absence of Dox, provoking TH downregulation as well as a reduction of weight gain.21 A Dox dose of 100 mg/kg diet was sufficient to fully extinguish GDNF expression.

The mifepristone-responsive gene switch system has been demonstrated to provide neurorestoration after a 6-hydroxydopamine partial lesion in response to an inducer dose of 20 mg/kg. This dose, when corrected for the rat-human conversion factor using the body surface area, was lower than the mifepristone dose administered for 7 days to bi-polar patients in a randomized clinical trial in which no adverse effect was reported. However, with this system, two separate vectors with a precisely adjusted ratio were necessary to obtain a tight regulation. Interestingly, these authors have demonstrated that pulses of GDNF administration, not provoking TH downregulation, were as efficient as a continuous treatment in terms of neurorestoration.40 Therefore, repeated short-term expression of the therapeutic transgene might constitute the treatment of choice for slowly degenerative diseases.

Another dual transcriptionally-regulated vector system is the rapamycin-inducible cassette.28 This regulatable system was however far less efficient than a constitutive promoter and did not allow to cover a sufficient proportion of the striatum, despite very high vector doses.

An alternative interesting system consists in regulating GDNF at the protein level by using a fusion with the destabilizing domain (DD) of Escherichia coli dihydrofolate reductase.46,47 In this system, the antibiotic trimethoprim stabilizes the DD-domain allowing to secrete functional GDNF. Trimethoprim is used to treat urinary infections at a dose of 200 mg/day (approximately 30 mg/kg/day). The concentration necessary to induce the stabilization of the GDNF-DD in rats was within the range of antimicrobial activity (0.5 mg/ml in drinking water, i.e., approximately 50 mg/kg/day).

For all these regulatable systems, an additional challenge is to obtain therapeutical effects in the ON state, using an acceptable vector dose within the safety and tolerability limits, while keeping a negligible transgene product activity in the OFF state.

We have tested two doses of rAAV2/1-V16 vector: 8.0 × 10^9 vg (moderate dose) and 2.0 × 10^{10} vg (high dose) per rat striatum (approximately 25 mm^3) corresponding to 3.2 × 10^8 and 8.0 × 10^8 vg/mm^3, respectively. When extrapolated to the volume of the human...
putamen (3,500 mm³), these vector doses correspond respectively to 4-fold and 10-fold more than the amount of vg that was administered in the Phase II CERE-120 clinical trial (2.7×10¹¹ rAAV2-NTN vg/hemisphere, i.e., 7.7×10⁴ vg/mm³). It should be noted that at the moderate vector dose, a small amount of GDNF was evidenced in the untreated (no Dox) group (see Figure 3). This leakiness of the system, however, was not sufficient to significantly induce ERK signaling, nor to downregulate TH.

Despite the distribution of the vector delivery in the central part of the striatum, an intense GDNF staining was observed outside the striatum, particularly in a ventrolateral area located beyond the striato-cortical border. This accumulation of GDNF at distance from the injection area could result either from an unequal diffusion of viral particles or from heterogeneous diffusion of GDNF. Since a similar tropism of rAAV2/1 vectors for neurons located at distance from the injected site, in a similar area, has already been previously observed using an intracerebral transgene product (EGFP) and a single needle tract (see reference 32, Figure 5), it is likely that the diffusion and/or tropism of rAAV2/1 viral particles rather than that of GDNF, explain this uneven transduction pattern. In order to reduce the risk of off-target GDNF effects, other AAV serotypes could possibly be used.

Induction of ERK signaling promoting neuronal survival appeared at a Dox concentration of 140 ng/ml plasma (Figure 7), thus lower than that found in patients treated with the FDA-approved dose for anti-inflammatory treatments. TH downregulation, previously described as a feed-back effect of long-term GDNF treatment in rats, appeared at a dose of 320 ng/ml plasma. It should be noted however that this undesired effect of GDNF has never been described in non-human primates, questioning its relevance. Behavioral abnormalities only started to appear with a 2.5-fold higher vector dose and at a dose of 960 ng Dox/ml plasma which, due to the risk of anti-biotic resistance, will not possibly be administered to patients for safety reasons. Finally, no significant reduction of weight gain was observed even at the highest vector and Dox doses.

Another limiting factor of available regulatable systems is the immune response to proteic components. Intramuscular injections of a previous version of the rtTA transactivator have been repeatedly shown to induce a strong immune response resulting in the rejection of transduced cells in macaques2,3,10 but not in mice.11 Replacement of the minimal VP16 activation domain of the rtTA by a humanized moiety while retaining the tetR bacterial DNA-binding domain did not reduce immune rejection.3 In contrast, by fusing to the rtTA codomain while retaining the tetR bacterial DNA-binding domain did of the minimal VP16 activation domain of the rtTA by a humanized [41x281]in vitro assay for human antigen presentation.53 On the other hand, the rtTA immunogenicity has been demonstrated in the brain. Accordingly, our data showing that long-term overexpression of rtTA-V16 does not lead to the abolition of transgene expression suggesting that no cellular immune response eliminating the transduced cells has occurred. Furthermore, no activation of glial cells has been observed, suggesting that no local inflammatory response to the rtTA-V16 protein occurred. The mifepristone gene switch consists in a humanized transactivation domain but contains a prokaryotic DNA binding domain (Gal 4).27 Successful repeated induction of transgene expression at the same level, suggests that there was no cellular immune response in the rat brain. Similarly, the DHFR destabilizing domain used to regulate GDNF protein by trimethoprim is originating from E. coli and thus also potentially immunogenic.

In conclusion, the safe therapeutic window of our novel doxycycline-inducible AAV vector, in terms of number of vector genomes and Dox dose range is wide: at least between 140 ng/ml plasma (efficiency threshold) and 1 mg/ml plasma (antimicrobiial threshold) for Dox and between 3×10⁴ vg/mm³ (efficiency threshold) and 8×10⁴ vg/mm³ (vector dose provoking motor symptoms at high Dox dose). Hence, our data suggests that a pharmacologically-regulated protocol for GDNF-mediated neuroprotective treatment of Parkinson’s disease could be established by combining the administration of rAAV2/1-V16-hGDNF in a single stereotoxic neurosurgery with oral administration of Dox at a dose which is safe for both the patients and the environment. Whether neuroprotective/neurorestorative effects of rAAV2/1-V16-hGDNF can be obtained at similar Dox doses remains to be demonstrated. It also remains to be determined in relevant animal or cellular models whether immune response against the rtTA-V16 transactivator will raise a safety concern.

**MATERIALS AND METHODS**

**Plasmids**

The pAC1-M2 plasmid comprising AAV ITRs bracketing the bidirectional tetracycline-responsive cassette expressing both rtTAM2 and GFP has been previously described21 (Figure 1a). To obtain pAC1-V16, the rtTA transactivator present in pAC1 (ref. 55) has been replaced by rtTAV16, a rtTA mutant derived from rtTA-2-S2 (ref. 23) and harboring three additional mutations (V9I, F67S, and R171X), selected in the presence of a low Dox dose26 (Figure 1a). The human GDNF (hGDNF) and the luciferase cDNA, respectively, have been introduced in this autoregulatory tetracycline inducible vector at the 5’ end of the bi-directional tetracycline promoter (pTetbidi).

**Cell line**

The HEK-293T Human Embryonic Kidney cell line was obtained from Q-One Biotech (Glasgow, UK). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Sigma-Aldrich, Diegem, Belgium).

**Viral production**

To produce recombinant AAV2/1 viral stocks, HEK-293T cells (5.0×10⁶ cells per 10 cm plates) were cotransfected, in a 1:1 molar ratio, with the vector plasmid (3 μg/plate) together with the helper/packaging plasmid pd1rs (10 μg/plate) expressing the AAV viral genes (rep gene from AAV serotype 2 and cap gene from AAV serotype 1) and the adenoviral genes required for AAV replication and encapsidation (Plasmid Factory, Heidelberg, Germany).

Fifty hours post-transfection, the medium was discarded and the cells were harvested by low-speed centrifugation and resuspended in Tris pH 8.0, NaCl 0.1 M. After three cycles of freezing/thawing, the lysate was clarified by 30 minutes centrifugation at 10,000 g, treated with benzonase (50 units/ml, Sigma) at 37 °C for 30 minutes, and centrifugated at 10,000 g for 30 minutes to eliminate the residual debris.

The virus was further purified by iodixanol gradient, QXL sepharose chromatography, buffer exchange, and microconcentration according to a previously-described method.26

**Viral genomes (vg)** were titrated using quantitative polymerase chain reaction as previously described.22 Titer were 6.8×10¹⁰ vg/ml for AAV2/1-V16-LUC, 2.3×10¹³ vg/ml for AAV2/1-V16-EGFP, 5.0×10¹³ vg/ml for AAV2/1-V16-hGDNF, 8.9×10¹⁰ vg/ml for AAV2/1-M2-LUC and 1.4×10¹⁰ vg/ml for AAV2/1-M2-GDNF.

**Infection of HEK 293T cells**

Fifty thousand cells seeded in 48-well plates were incubated in the presence of rAAV2/1 at a multiplicity of infection of 10³ vg/cell in serum-free medium containing human adenovirus 5 (HS) reference material (ATCC VR-1516; litter 5.8×10¹⁰ particle forming units (pfu)/ml) (LG Standards, Teddington, Middlesex, UK) at the multiplicity of infection of 50 particle forming units (pfu)/cell. After 2 hours, the cells were rinsed with serum-supplemented medium and further cultured for 72 hours without passaging in culture medium containing or not Dox at the indicated concentration.
GDNF ELISA
For determination of GDNF concentrations in rAAV-infected HEK-293T cells, the culture medium was harvested 72 hours postinfection.
For determination of GDNF brain tissue levels, animals were decapitated and the brains were rapidly isolated, gradually frozen in isopentane/dry ice (~10 °C for 10 seconds then ~20 °C for 20 seconds) and stored at ~80 °C. Two-hundred-micrometer coronal slices were cut using a cryostat and striata were dissected out, weighed and processed for ELISA. For the preparation of the tissue extract, small pieces of striatum were triturated in T-per buffer (Pierce Fisher Scientific, Lucens, Switzerland) supplemented with protease inhibitors cocktail (Roche Diagnostics, Rotkreuz, Switzerland). Samples were centrifuged at 10,000xg and supernatants were harvested and their protein concentration analyzed using a nanodrop machine.
GDNF concentrations were measured using a commercial ELISA (Human GDNF CytoSets, catalog #CHC2422, BioSource, Nivelles, Belgium) and expressed in picograms per milligram tissue. Recombinant human GDNF (provided by the manufacturer) was used to establish the standard curve.

In vitro luminometry
Seventy-two hours postinfection a volume of 50 µl of diluted passive-lysis buffer (Promega, Duebendorf, Switzerland, Cat. # E194A) was added to the HEK-293T cell monolayer. Plates were incubated for 30 minutes under shaking at room temperature. The detached and lysed cells were harvested and centrifuged at 12,000g for 10 minutes. Twenty microliters of supernatant were loaded on a OpitplateTM-96 well, White Opague 96-well Microplate (Perkin Elmer, Cat.# 6005290) for luciferase firefly activity measurements in the presence of luciferase assay substrate (Promega, Duebendorf, Switzerland, Cat. # E1501) using a Mithras LB 940 Multilabel reader (Berthold Technologies, Zug, Switzerland).

Mice
Swiss CD1 mice purchased from Janvier Labs (Le Genest Ste Isle, France) were housed and treated according to the Belgian law. The protocols were in accordance with national rules on animal experiments and were approved by the Ethics Committee of the Faculty of Medicine of the "Universite Libre de Bruxelles". Animals were anesthetized with a mixture of ketamine (Imalgène 1000, Merial, Diegem, Belgium; 100 mg/kg) and xylazin (Rompun, Bayer, Diegem, Belgium; 10 mg/kg) and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA). The injection coordinates in the striatum were 0.8 mm anterior to the bregma, 1.5 mm lateral to the bregma (right side) and 3.5 mm (first site) and 2.5 mm (second site) below the dural surface. At each injection site, 1 µl of viral suspension diluted in Dulbecco’s phosphate-buffered saline (Lonza, Verviers, Belgium) to the titer of 8.3 × 1010 vg/ml. The injection rate was 0.2 µl/minute. The needle was left in place for 5 minutes before a slow withdrawal over an additional minute.
When indicated, mice were fed with doxycycline-supplemented food (Dox diet, Safe, Augy, France) at the indicated dose after the injection. The food was replaced every two days in order to keep a stable Dox dose. Animals were then decapitated and the brains were rapidly dissected and frozen in methylbutane with dry-ice and then kept at ~80 °C until use.

In vivo bioluminescence imaging
Mice were anesthetized with isoflurane (3%) vaporized in compressed air (3 l/minute). Under anesthesia, the skin covering their skull was shaved using a hair shaver (Braun EP15) and hair-removal cream (Veet) the day before imaging, and shaving was repeated when necessary during the study period.
Animals were injected intraperitoneally with 3 mg of luciferin (Promega, Leiden, The Netherlands) resuspended in 150 microliters of sterile saline (0.9% NaCl) and placed in the Photon Imager (Biospace Lab, France), where they were kept warm and anesthetized (~1-1.5% isoflurane in compressed air (0.4 l/minute) delivered through a nose cone).
Animal experiments were in accordance with all institutional and national guidelines for the care and use of laboratory animals (LA 1500020, protocol MU/09/01).
Bioluminescent signal was collected using the Photon Acquisition software and quantified in cpm (counts per minute) 15 minutes after luciferin injection. Percent transduction was calculated as the ratio of the signal obtained with the M3 Vision software (both softwares were bundled with the Photon Imager).

Ex vivo luminometry (striatal extracts)
Striata were dissected from 100 µm thick cryostat brain sections. Striata pieces were kept in frozen microfuge tubes (~80 °C) until use. Tissue pieces were covered with 10 µl of ice cold Promega’s “Passive lysis buffer” per mg of striatum. Tissue was then mechanically disrupted by pumping the mix up and down twenty times on ice. The tubes were then centrifuged at max speed for 2 minutes at 4 °C and the supernatant was collected into a new microfuge tube.
Protein content of the extracts was quantified using the BCA kit (Thermo Scientific Life Technologies, Zug, Switzerland, Cat.# 23259) according to the manufacturer’s recommendations. Fifty microliters of protein extract of each sample were loaded on a Optiplate-96 well, White Opague 96-well Microplate (Perkin Elmer, Cat.# 6005290) for luciferase firefly activity measurements in the presence of luciferase assay substrate (Promega, Duebendorf, Switzerland, Cat. #. E1501) using a Mithras LB 940 Multilabel reader (Berthold Technologies, Zug, Switzerland).

Rats
For data in Figures 3, 4, 5, and 6a,c, the experiments were conducted on 38 female Wistar rats weighting approximately 200g (7 weeks old) (Janvier-Europe laboratories, France). All animal procedures were approved by the State Veterinary Office of Switzerland (authorization no. VD2759). Animals were housed in a 12-12 hour light–dark cycle room at 22 °C with free access to tap water and were fed ad libitum. They were anesthetized with a mixture of ketamine (75 mg/kg, Ketalar, Graeub AG, Basel, Switzerland) and xylazin (10 mg/kg, Rompun, Bayer, Zürich, Switzerland) and placed in a Kopf stereotaxic frame (Kopf Instruments). All rats underwent an injection of a viral suspension of rAAV1/2-V16-hGDNF (n = 32) or rAAV2/1-V16-EFGP (n = 6), diluted in Dulbecco’s phosphate-buffered saline at a titer of 2.0 × 1012 vg/ml at four delivery sites into the right striatum at the following coordinates: (i) anterior-posterior (AP) = + 0.0 mm, mediolateral (ML) = −3.6 mm, dorso-ventral (DV) = −4.0 and −5.0 mm and (ii) (AP) = + 1.1 mm, (ML) = −2.8 mm, dorso-ventral (DV) = −4.5 and −5.5 mm, relative to the bregma and the dural surface. One µl of viral suspension was injected at each injection site at the rate of 0.2 µl/min. After injection, the needle was left in place for 5 minutes in order to allow diffusion of the viral suspension in the parenchyma. When indicated, rats were fed with Dox-supplemented food (Dox diet) at the indicated dose. The food was replaced every two days in order to keep a stable Dox dose. At the end of the experiment, euthanasia was performed using pentobarbital injection at the dose of 300 mg/kg. When indicated, blood was harvested in heparinized tubes by intracardiac puncture under anesthesia. Animals were perfused transcardially with 100 ml of PBS pH 7.4 followed by 200 ml of ice-cold 4% paraformaldehyde (PF4) in PBS pH 7.4. After overnight postfixation in PF4 at 4 °C, brains were transferred successively to sucrose 20 and 30% at 4 °C and stored at ~80 °C.
For data in Figure 6b,d, 45 female Wistar rats weighing approx. 250g were purchased from Janvier Labs (France) and were housed and treated according to the Belgian law. The protocols were in accordance with national rules on animal experiments and were approved by the Ethics Committee of the Faculty of Medicine of the "Universite Libre de Bruxelles". Animals were anesthetized with a mixture of ketamine (imalgène 1000, Merial, Diegem, Belgium; 100 mg/kg) and xylazin (Rompun, Bayer, Diegem, Belgium; 10 mg/kg) and placed in a Kopf stereotaxic frame (Kopf Instruments). The injection in the striatum followed two injections tracks perpendicular to the skull surface: trajectory 1: 0.2 mm anterior, 2.2 mm lateral to the bregma; trajectory 2: 0.2 mm anterior, 3.8 mm lateral to the bregma. Vector deposits were made at two different positions along those injection tracks at 4.5 and 6.0 mm below the dural surface. The needle was carefully lowered to the first level and left in place 5 minutes before starting the injection. One microliter of vector was injected at each injection site at the rate of 0.2 µl/min. At the end of the injection, the needle was left in place for 5 minutes before being slowly moved to the next injection coordinate. When indicated, rats were fed with Dox-supplemented food (Dox diet, Safe, Augy, France) at the indicated dose after the injection. The food was replaced every second day in order to keep a stable Dox dose.
When indicated, at the end of the experiment, blood was harvested in heparinized tubes by intracardiac puncture under anesthesia and animals were decapitated. Blood was centrifuged 10 minutes at 2000 rpm and plasma was kept at ~80 °C until use.

Bioassay of doxycycline concentrations in plasma
The Dox level in plasma samples was determined using a previously described cell-based assay.21 In brief, HeLaDOX cells were plated at a density of 105 cells per well in a 96-well plate in a total volume of 180 µl. After 24 hours, 20 µl of diluted plasma sample was added to the cells. As a standard, cells were treated with 20 µl culture medium containing 0 to 100 ng/ml Dox.
After 48 hours, cells were washed with phosphate-buffered saline and lysed in passive lysis buffer (Promega, Leiden, The Netherlands). Firefly luciferase activity was determined using the Luciferase Assay System (Promega, Leiden, The Netherlands). The Dox concentration in the experimental samples was determined by extrapolation from the standard curve established in parallel.

Immunohistochemistry and quantification
For all GDNF immunostaining, 50 μm cryostat coronal sections were collected at 10 weeks post-vector injection. One of six brain sections was sequentially incubated for 20 minutes in 3% H2O, and 10% methanol in Tris buffer saline (10 mmol/l Tris, 0.9% NaCl; pH 7.6), incubated 1 hour in Tris buffer high salt Triton (50 mmol/l Tris, 0.5 M NaCl, 0.5% Triton X-100; pH 6) containing 5% bovine serum albumine and incubated overnight at 4°C with the primary antibody diluted in Tris buffer high salt Triton containing 1% bovine serum albumine. After washing, slices were incubated with a biotinylated secondary antibody during 1 hour at room temperature. The peroxidase staining was revealed with a VECTASTAIN Elite ABC kit and diaminobenzidine (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Sections were disposed on slides, dehydrated, and mounted with Eukitt mounting fluid (Sigma-Aldrich, VWR International, Nyon, Switzerland).

For GDNF immunostaining, a strongly sensitive method was set up by performing an additional step to a classical immunohistochemical labeling.1 This step allowed retrieving antigen by heating brain slices for 10 minutes at 70°C in sodium citrate buffer (10 mmol/l Na citrate, 0.5% tween 20, pH 6). The primary incubation with a goat anti-GDNF antibody (1:500; R & D systems, Cat. #BAF212, Minneapolis, MN) was followed by an incubation with a biotinylated anti-goat antibody (1:500, Cat. #105605, Jackson Laboratories, PA). For TH immunostaining, the primary antibody was a mouse monoclonal anti-TH (dilution 1:500; Cat. #MAB 318, Chemicon, Temecula, CA) and the secondary antibody was a biotinylated anti-mouse (1:400; Cat. #BD553441, BD Pharmingen, BD Biosciences, Allschwil, Switzerland). For p-S6 staining, the primary antibody was a rabbit anti-phospho-ribosomal protein S6 (Ser 235 and 236) antibody (1:500; Cat. #2211, Cell signaling Technology, Beverly, MA) and the secondary antibody was a biotinylated goat anti-rabbit antibody (1:1000, Cat. #BA1000, Vector Laboratories). For GFAP staining, a mouse anti-GFAP antibody (1:400, Cat. MAB360, Chemicon), followed by a biotinylated secondary antibody (1:500; Cat. #BAF212, Minneapolis, MN) was followed by an incubation with a biotinylated anti-mouse antibody (1:2000, Cat.019-19741, Wako Chemicals), followed by a biotinylated anti-rabbit antibody (1:1000, Cat. #BA1000, Vector Laboratories) were used.

Images acquired using an Olympus BX40 microscope, at a 2-fold or 20-fold magnification were saved as TIFF files using the Morpho Strider software (NIH).

Amphetamine-induced rotation test
Rats were harnessed and connected to the automated rotomoter bowls (Sandown Scientific, Hampton, UK). 2.5 mg/kg of D-amphetamine sulphate (Federa, Brussels, Belgium) was injected intraperitoneally. Right and left full body turns were recorded over 60 minutes. Mean net rotations are expressed as full body turns per 10 minutes ipsilateral minus contralateral to the lesion.

Statistical analysis
All the statistical analysis were performed using the GraphPad Software. Results were expressed as mean ± standard error of means. In vitro and in vivo comparison between M2 and V16 at increasing doses of doxycycline was analysed using two-way analysis of variance followed by a Dunnett’s post-hoc analysis. TH, p-S6 and GDNF immunostaining measurements were analysed using one-way analysis of variance followed by a Dunnett’s post-hoc analysis. Weight of rats and net turns performed on rotameter were analysed using a repeated measure one-way analysis of variance, followed by a Dunnett’s post-hoc test.

Differences were considered as significant when P < 0.05.

CONFLICT OF INTEREST
The rTA-V16 variant has been filed in patent WO2007058527 (inventors: B.Berkhout and A.T.Das).

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