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METHODS: ORIGINAL ARTICLE

Adeno-associated Virus-mediated, Mifepristone-regulated Transgene Expression in the Brain

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Gene therapy, in its current configuration, is irreversible and does not allow control over transgene expression in case of side effects. Only few regulated vector systems are available, and none of these has reached clinical applicability yet. The mifepristone (Mfp)-regulated Gene Switch (GS) system is characterized by promising features such as being composed of mainly human components and an approved small-molecule drug as an inducer. However, it has not yet been evaluated in adeno-associated virus (AAV) vectors, neither has it been tested for applicability in viral vectors in the central nervous system (CNS). Here, we demonstrate that the GS system can be used successfully in AAV vectors in the brain, and that short-term induced glial cell line-derived neurotrophic factor (GDNF) expression prevented neurodegeneration in a rodent model of Parkinson's disease (PD). We also demonstrate repeated responsiveness to the inducer Mfp and absence of immunological tissue reactions in the rat brain. Human equivalent dosages of Mfp used in this study were lower than those used safely for treatment of psychiatric threats, indicating that the inducer could be safely applied in patients. Our results suggest that the GS system in AAV vectors is well suited for further development towards clinical applicability.

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Introduction

The adeno-associated virus (AAV)-based gene transfer vectors are promising tools for gene therapy in several tissues and allow for potentially unlimited duration of transgene expression in nondividing cells.[1] However, this feature represents a two-edged sword as in the current setting of gene therapy as a nonreversible technology, transgene expression can not be turned off in case of adverse side or off-target effects. Thus, the ability to turn on and off expression of a therapeutic transgene or to modulate its expression levels according to individual patient's needs would substantially reinforce forthcoming gene therapy vectors.

A few regulated transgene expression systems have been developed for research purposes in the central nervous system (CNS),[2,3] but so far none of these has entered clinical applications. By far the most established regulated system is based on the tet-operon.[4,5] Recent versions of this system have replaced the viral VP16 domain by mammalian zinc fingers[6] and have been shown to function well and without immunological consequences in the immune-privileged site of nonhuman primate retina.[7] However, they were still immunogenic in the periphery even after substantial engineering of the bacteria-derived rtTA transactivator moiety.[8,9] This demonstrates the cellular and humoral immune responses to rtTA in nonhuman primates, which warrants the development of alternatives.

A major advantage of the Gene Switch (GS) system is that it is composed mainly of human components. The system is activated by binding of the synthetic steroid mifepristone (Mfp) to a truncated human progesterone receptor, which is fused to a human p65 transactivation domain and a yeast Gal4 DNA-binding domain. Earlier studies have compared the basal properties of regulated systems expressed from adenoviral vectors and suggested that Mfp-regulated transgene expression is characterized by particular low noninduced background expression.[10] As neurotrophic factors are biologically active at very low dosage, we argued that this system would be particularly suitable for neurotrophic factor-based gene therapy.

However, different gene therapy vectors persist in transduced mammalian cells in different configurations: classical lentiviral vectors randomly integrate into the host cell genome, while adenoviral vectors, both first generation and gutless, mainly persist as episomal linear monomers.[11] AAV vector genomes also persist predominantly episomally, but their genomes form concatemers,[12] in which transcriptional control elements from one genome can be brought to proximity of elements from another genome and, thus, might interfere with each other. As AAV vectors are very promising and well-characterized vehicles for CNS gene therapy, we, therefore, aimed at characterizing the Mfp system in the framework of AAV vectors for the first time. This study is also the first attempt to demonstrate applicability of the GS system in the brain by means of viral vectors.

We demonstrate that Mfp-regulated AAV vector system can be used in the rat CNS with low, although not with zero background expression. The system can be induced consecutively several times and provides sufficient glial cell line-derived neurotrophic factor (GDNF) expression to protect the dopaminergic projection from 6-hydroxydopamine (6-OHDA) toxicity. Levels of GDNF in the noninduced state were 1.4-fold

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increased as compared with controls, but did not exert any neuroprotection in this rat model of Parkinson's disease (PD). Importantly, the short-term induction of neuroprotective GDNF expression did not result in downregulation of tyrosine hydroxylase (TH) expression in nigral dopaminergic neurons as observed with the constitutively GDNF-expressing control vector, demonstrating both efficient and safe application of the Mfp-regulated AAV vector system. Safety was also confirmed by absence of immunological tissue reactions in rat brains after multiple inductions. Dosages of Mfp necessary for induction of transgene expression in the brain were considerably below the human equivalent doses used for treatment of psychiatric threats.

Our results provide the first evidence that AAV-mediated, Mfp-regulated neurotrophic factor expression has substantial potential to be developed further towards clinical applicability for treatment of CNS disorders.

Results

Assessment of vector genome configurations *in vitro*

The Mfp-regulated "GS" system is composed of the synthetic steroid Mfp, the GS protein, and a minimal regulatable promoter (**Figure 1i**). GS is a fusion of the truncated human progesterone receptor, the human p65 transactivator domain, and the yeast-derived GAL4 DNA-binding domain. Upon Mfp binding, GS dimerizes and activates the minimal UAS-TATA promoter that is used to control transgene expression. GS can be expressed under control of ubiquitous or cell-type–specific promoters, and in the course of this study, it was used with the pTK promoter (enhanced green fluorescent protein (EGFP) expressed in neurons: 99.4%; in astrocytes: 0.6%) or the $GfaABC_1D$[13] promoter (EGFP expressed in neurons: 71.3%; in astrocytes: 28.7%). Several different genome configurations in AAV-6 vectors were tested in primary cortical neurons for their ability to achieve reasonable induction in combination with lowest possible background expression in the noninduced state. This goal was finally achieved under the conditions shown in **Figure 1a–h**. Besides splitting the system in two vectors and using them in a 1:3 ratio, two further issues were important to achieve background-free expression: isolation of the inducible UAS-TATA promoter from the promoter activity of left inverted terminal repeat (ITR) by three synthetic transcription blocker elements and omitting enhancer sequences such as the WPRE from the transgene expression cassette. The latter clearly diminished the level of induced transgene expression (**Figure 1a**,**c**,**e**,**g**), but this decrease of expression levels appeared to be necessary to reduce background expression in the absence of Mfp to almost undetectable levels.

Assessment of vector genome configurations in the rodent brain

AAV-6 vectors were also used initially for transduction of the rat brain, namely the striatum. As shown in **Figure 2a**,**b**, the two-vector system demonstrated not only robust EGFP expression in the induced state, but also clearly detectable background expression in the absence of Mfp. This background expression did not result from aberrant binding of the GS protein to the UAS-TATA promoter as it also occurred in the absence of the GS-expressing vector (**Figure 2c**). Thus, despite the presence of three transcription blockers isolating it from the promoter activity of the left ITR,[14] the UAS-TATA promoter revealed significant inherent activity.

We then argued that probably an even better isolation of the regulated promoter from the ITR would be necessary. Thus, we also tested a one-vector, head-to-head configuration of GS and the regulated expression cassette after high vector dose (9×10^9 vg, **Figure 2d**,**e**) and low vector dose (3×10^9 vg, **Figure 2f**,**g**). This vector demonstrated robust EGFP expression in the induced state, although with considerable background in the noninduced state when applied at high dosage. Background was low in the noninduced state after reducing the dosage to 3×10^9 vg, but EGFP-expressing neurons were still clearly detectable under this condition. This residual background expression was also detectable when the pTK promoter was exchanged for the $GfaABC_1D$

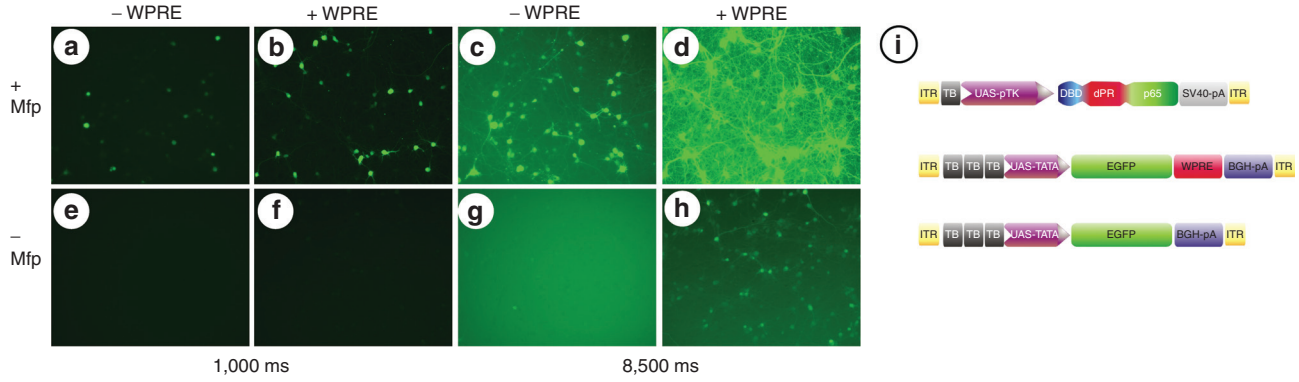

Figure 1 ***In vitro*** **assessment of vector configurations for low background expression.** Primary cortical neurons were cotransduced at day 3 *in vitro* (DIV3) with (**a,c,e,g**) AAV-6-pTK-GS and AAV-6-UAS-TATA-EGFP or (**b,d,f,h**) AAV-6-UAS-TATA-EGFP-WPRE and expression was (**a–d**) induced with Mfp or (**e–h**) was not induced on DIV6. EGFP expression was recorded on DIV10 with (**a,b,e,f**) 1,000-ms exposure time or (**c,d,g,h**) 8,500-ms exposure time. (**i**) Vector genome elements. $N = 6$ independent transductions per condition. AAV, adeno-associated virus; BGH-pA, bovine growth hormone-polyadenylation site; DBD, Gal4 DNA-binding domain; dPR, truncated human progesterone receptor, ligand binding domain; ITR, AAV2-inverted terminal repeat; Mfp, mifepristone; p65, human NF-κB activation domain subunit; TB, transcription blocker/synthetic polyadenylation site; UAS-pTK, minimal thymidine kinase promoter with 4 gal4 upstream activation sites; UAS-TATA, minimal regulatable promoter (6 gal4 upstream activation sites/adenovirus E1A-TATA box).

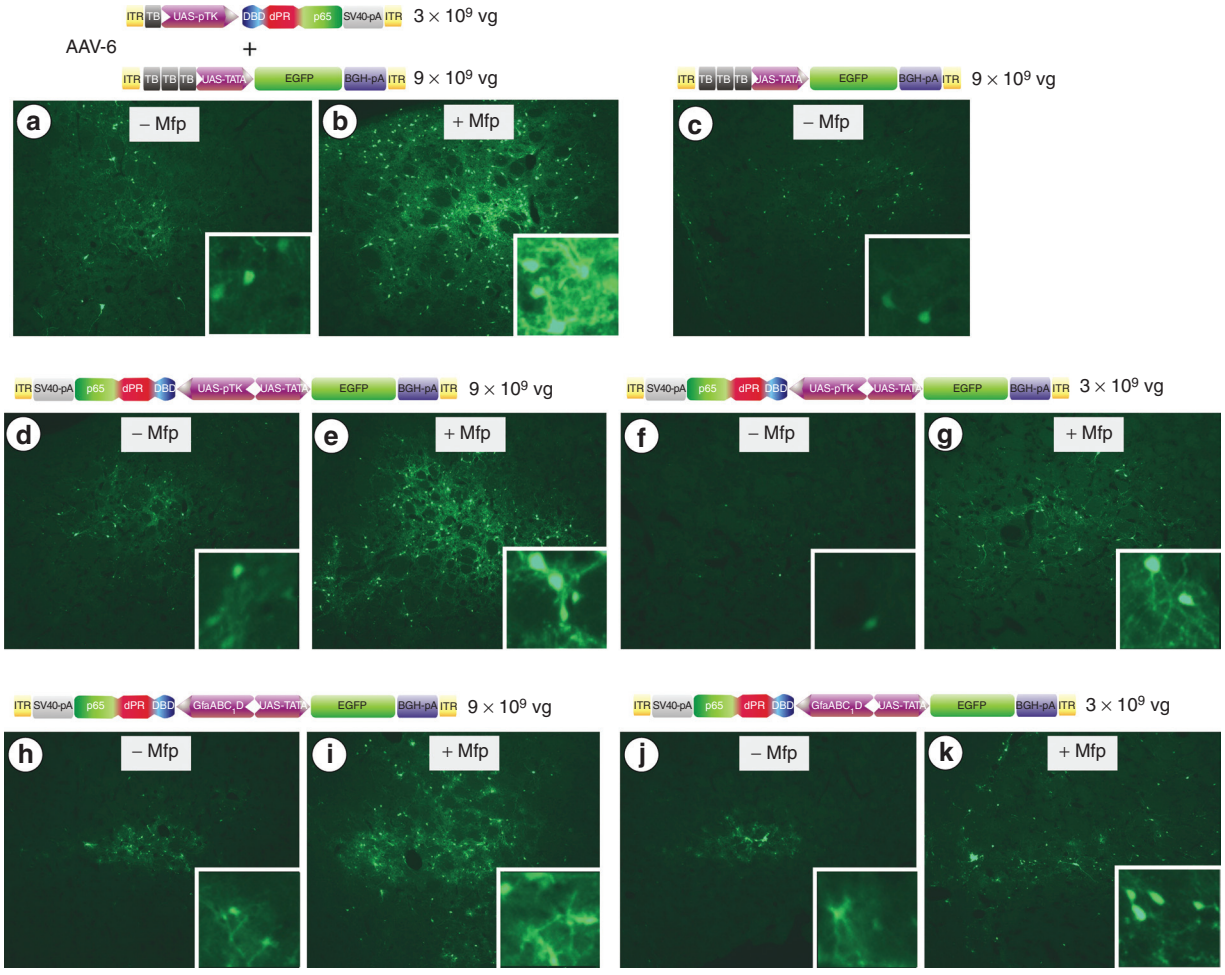

Figure 2 AAV-6–based Mfp-regulated EGFP expression in the rat striatum. Vector genome layouts and the total injected titer are depicted on top of the respective photomicrographs, which show EGFP expression in striatal tissue sections at 3 weeks after vector injection and 4 days after application of Mfp (20 mg/kg, applied on three consecutive days) or dimethyl sulfoxide. (**a**,**b**) EGFP expression of the two-vector system (without and with Mfp induction, respectively). (**c**) EGFP expression after injection of the UAS-TATA-EGFP vector alone. (**d**–**g**)Application of the one-vector, head-to-head configuration using the pTK promoter to drive GS expression, for (**d,e**) high vector dosage and (**f,g**) low vector dosage, (**d,f**) without Mfp induction, and (**e,g**) after Mfp induction. (**h**–**k**) Application of the one-vector, head-to-head configuration using the GfaABC$_1$D promoter, for (**h,i**) high vector dosage, (**j,k**) low vector dosage, (**h,j**) without Mfp induction , and (**i,k**) after Mfp induction. $N = 5$ rats per condition. All pictures recorded with identical exposure time. Insets in each figure show randomly selected cells at higher magnification. AAV, adeno-associated virus; BGH-pA, bovine growth hormone-polyadenylation site; DBD, DNA-binding domain; dPR, truncated human progesterone receptor; ITR, inverted terminal repeat; Mfp, mifepristone; TB, transcription blocker.

promoter, demonstrating background expression in astrocytes as well (**Figure 2h**–**k**). Reducing the viral titer further did not improve the ratio of background-to-induced EGFP expression (data not shown). Thus, in contrast to cultured rat brain cells *in vitro*, the goal of a background-free expression in the rat brain was not met with the exploited AAV-6 vectors, prompting us to test a different serotype of AAV.

Evaluation of an alternative serotype: AAV-5

AAV-6 vectors deliver their transgenes very efficiently to their target cells in the CNS, while AAV-5 particles do not bind to high-affinity receptors in the brain and, thus, diffuse over larger areas and deliver fewer genome copies per cell.[15] Thus, this serotype was tested in further experiments in the two-vector configuration. Very low background expression and repeated responsiveness of this AAV-5–based Mfp-regulated vector system is shown in **Figure 3**. As demonstrated in **Figure 3a**–**d**, the drug dosages used to induce EGFP expression from this vector system in the rat brain were in the range of 5–20 mg/kg body weight. In the course of this study, 20 mg/kg was routinely applied on three consecutive days for each induction. Higher dosages of 50 mg/kg resulted in higher levels of EGFP expression, but animals displayed a hunch-backed gait and horrent fur directly after application, and thus this dosage was not used further (data not shown).

Repeated responsiveness of the system was demonstrated immunohistochemically in brain sections from animals that received up to three inductions in monthly intervals (**Figure 3f**–**l**). These experiments demonstrated that the system repeatedly returned to background expression within 4 weeks after short-term induction by Mfp. It also became obvious that the background level of noninduced expression was indeed very low, but again it was possible to detect weak EGFP fluorescence in the absence of induction

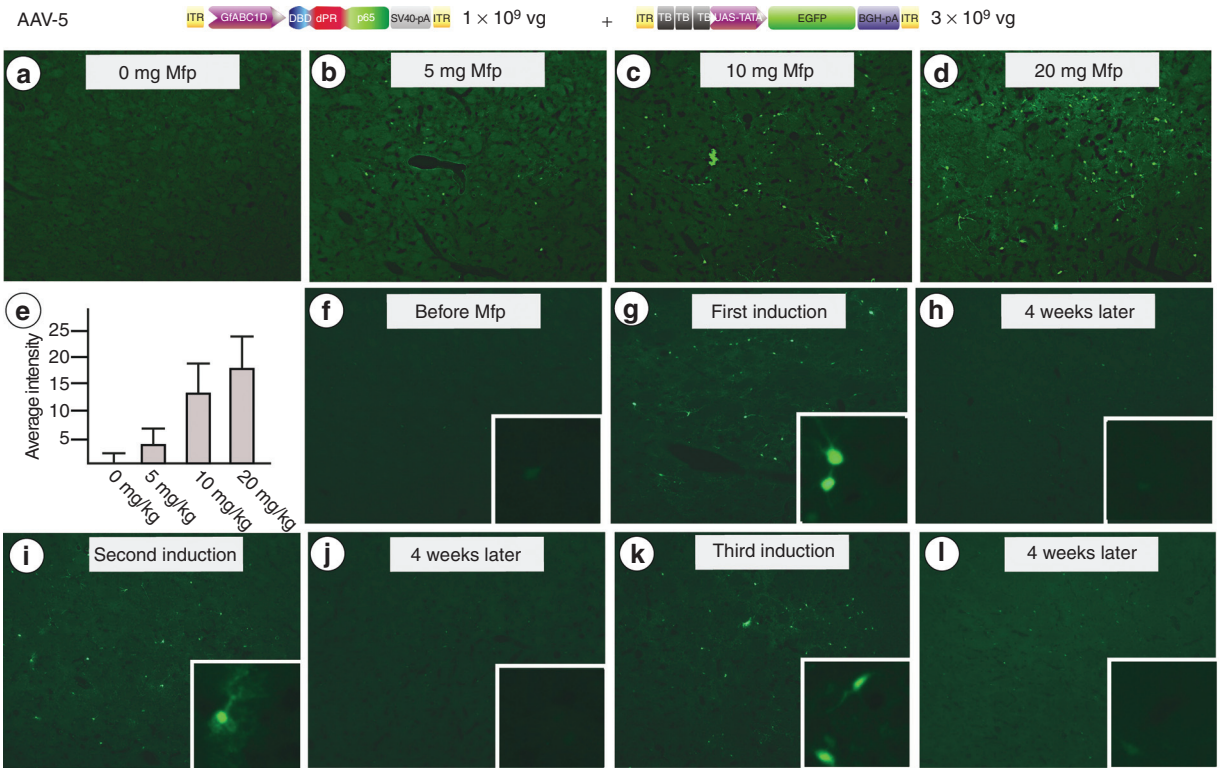

Figure 3 A two-vector AAV-5–based Mfp system resulted in very low background expression, and can be induced repeatedly. Vector genome layouts and the total injected titer are depicted on top of the respective photomicrographs. (**a**–**e**) A dosage response to Mfp, for (**a**) 0 mg, (**b**) 5 mg/kg, (**c**) 10 mg/kg, and (**d**) 20 mg/kg. (**e**) The relative EGFP fluorescence levels obtained for the different Mfp dosages from brain sections. (**f**–**l**) Repeated responsiveness, at (**f**) 3 weeks after vector application and before Mfp injection, (**g**) at 3 days after the first induction, (**h**) at 4 weeks after the first induction, (**i**) at 3 days after the second induction, (**j**) at 4 weeks after the second induction, (**k**) at 3 days after the third induction, and (**l**) at 4 weeks after the third induction. Insets in **f**–**l** show randomly picked cells in the respective brains at higher magnification. *N* = 4–5 rats per condition. AAV, adeno-associated virus; BGH-pA, bovine growth hormone-polyadenylation site; DBD, DNA-binding domain; dPR, truncated human progesterone receptor; ITR, inverted terminal repeat; Mfp, mifepristone; TB, transcription blocker.

(Figure 3f,h,j,l), suggesting that even at low transduction efficacy and transgene expression levels, the UAS-TATA promoter in its current layout is not absolutely silent in the context of AAV-transduced brain cells.

The rapid activation of the two-vector system was also proven by quantification of EGFP transcripts. For these experiments, two different promoters were exploited to drive GS expression: the neuron-specific synapsin 1 gene promoter and the GfABC1D promoter. As shown in Figure 4a, EGFP mRNA was readily detectable already at 1 day after induction by Mfp, and then decayed within 2–3 weeks to background levels, with only 10–20% residual mRNA copies at 1 week after induction.

As a two-vector system may be considered less effective than a one-vector system, we quantified stereologically the amount of EGFP-expressing striatal cells after injection of (i) the constitutively expressing AAV-5-GFAP-EGFP vector (3×10^9 vg) or (ii) AAV-5-GfABC1D-GS (1×10^9 vg) plus AAV-5-UAS-TATA-EGFP (3×10^9 vg). Mfp was applied at 3 weeks after vector injection, and brain slices were sampled 3 days after induction. This experiment demonstrated that the regulated vector system achieved 29.5±4% of the amount of EGFP-fluorescent cells of the constitutively expressing vector (Figure 4b). It should be noted that the regulated vector system was used in a way that only 1×10^9 vg of the GS-expressing vector were injected, as compared with 3×10^9 vg of the constitutively expressing vector, and thus only one-third transgene-expressing cells could be expected.

The repeated responsiveness of the Mfp-regulated AAV vector system was confirmed by live imaging of EGFP expression in the cortex of mice, where up to six consecutive cycles of induction were recorded in the same animal after transduction with high dosages of AAV-6 vectors (**Supplementary Figure S1** and **Supplementary Materials and Methods**).

Absence of adverse tissue reactions

The GS protein is composed of the most part of human protein domains and, thus, should not present an immunological target in gene therapeutic approaches. However, it still contains the small yeast-derived Gal4 DNA-binding domain. We investigated a putative immunological response in rat brain sections by immunohistochemistry for IBA1, an antigen marking microglia and for GFAP, an antigen marking activated astrocytes. Brains were analyzed at 3 weeks after a single activation (Mfp for three consecutive days) and at 15 weeks after a total of three activations (3 × Mfp for three consecutive days at 4-week intervals). As shown in Figure 5a–d,j, astroglia activation is detectable in vicinity to the needle injection tract, but this increased staining intensity has faded after 15 weeks. We could not detect

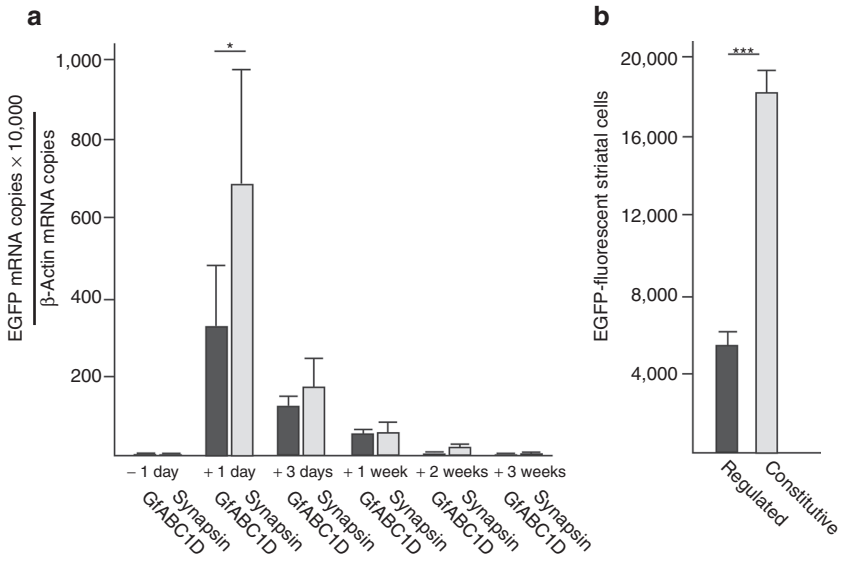

Figure 4 Transcriptional control and cotransduction efficacy of the mifepristone (Mfp)-regulated two-vector system. (**a**) AAV-5-GfABC1D-GS ("GfABC1D"; 1×10^9 vg) or AAV-5-hSYN-GS ("Synapsin"; 1×10^9 vg) and AAV-5 UAS-TATA-EGFP (3×10^9 vg) were coinjected into the rat striatum and EGFP expression was induced by Mfp 3 weeks later. At the indicated time points (–1 day = 1 day before induction; +1 day = 1 day after induction; +3 day = 3 days after induction; +1 week = 1 week after induction; +2 weeks = 2 weeks after induction; and +3 weeks = 3 weeks after induction), total striata were prepared and analyzed for EGFP mRNA copies by quantitative PCR. Transcript numbers are shown in relation to β-actin mRNA molecules multiplied by 10,000 ± SD. *N* = 4–5 striata per time point and promoter. (**b**) The numbers of EGFP-expressing striatal cells are shown after injection of the regulated two-vector system (AAV-5-GfABC1D-GS, 1×10^9 vg + AAV-5-UAS-TATA-EGFP, 3×10^9 vg; = "regulated") versus injection of 3×10^9 vg of AAV-5-GFAP-EGFP (= "constitutive"). Cell numbers were stereologically quantified from *n* = 5 brains per condition. AAV, adeno-associated virus.

any difference in the activation state of microglia between vector-injected and control brains, in that, all microglial cells demonstrated even tissue distribution and a ramified morphology. Stereological assessment of IBA1 immunoreactive cells demonstrated a very minor increase in numbers (4–7% more microglia in an area including the needle injection tract) but no persisting increase of microglial cells was detectable (**Figure 5i**). Notably, short-term astrogliosis and increase of microglial cell numbers were identical in mock-injected animals and in animals injected with GS vectors and receiving Mfp, indicating that these minor tissue reactions are due to the injection procedure itself. These findings suggest that at least at the moderate expression level used in this study, GS expression and repeated Mfp application did not provoke immune responses in the rat brain.

Proof of concept: low background, induced GDNF expression is neuroprotective in a rodent model of PD

For a functional analysis, we substituted the reporter EGFP with the neurotrophin GDNF and tested the vectors in serotype 5 capsids for neuroprotective potency in the partial striatal 6-OHDA model of PD. GDNF expression could be assessed quantitatively by enzyme-linked immunosorbent assay (ELISA) and, thus, allowed a better addressing of background expression versus induced expression levels and comparison to a constitutively expressing vector than EGFP.

AAV vectors were unilaterally injected into the striatum at a ratio of 1 ($GfaABC_1D$–GS; 1×10^9 vg) and 3 (UAS-TATA–GDNF; 3×10^9 vg). A corresponding EGFP-expressing vector (3×10^9 vg) served as negative control, whereas a vector constitutively expressing GDNF from a full-length GFAP promoter[16] (3×10^9 vg) served as control for maximal achievable effects of GDNF. Two and a half weeks after vector injection, transgene expression of the regulated vectors was induced by application of Mfp on three consecutive days. A group of rats was killed 4 days after the last Mfp application and striatal tissue was analyzed for GDNF levels (prelesion timepoint). A second group of animals was subjected to the partial striatal lesion at this timepoint, and postlesion GDNF levels, apomorphine-induced rotations, and striatal dopamine (DA) levels were assessed at 5–7 weeks after vector application (*i.e.,* 2–4 weeks after 6-OHDA lesion).

At prelesion assessment, the constitutively active vector achieved GDNF levels of 209 ± 32 pg/mg tissue, while the Mfp-induced regulated vector achieved GDNF levels of 36 ± 7.6 pg/mg tissue. In the absence of Mfp induction, the regulated GDNF vector expressed GDNF levels of 7.3 pg/mg tissue in the injected hemisphere, which was significantly more GDNF than in the contralateral hemisphere (5.1 pg/mg) (**Figure 6b**, left panel). No influence of GDNF expression or Mfp application was detectable on striatal DA levels (**Figure 6c**, left panel). The same was true for levels of the metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid (data not shown).

At 4 weeks after 6-OHDA lesion, Mfp-induced GDNF levels (18.3 ± 1.2 pg/mg) were still twice as high comparing to the contralateral side (10 ± 0.6 pg/mg). Under these postlesion conditions, noninduced GDNF levels (11.7 ± 1.1 pg/mg) did not differ from the contralateral side (11.6 ± 1.2 pg/mg). The constitutive vector expressed 119.4 ± 57.8 pg/mg GDNF in the injected side at this time point (**Figure 6b**, right panel).

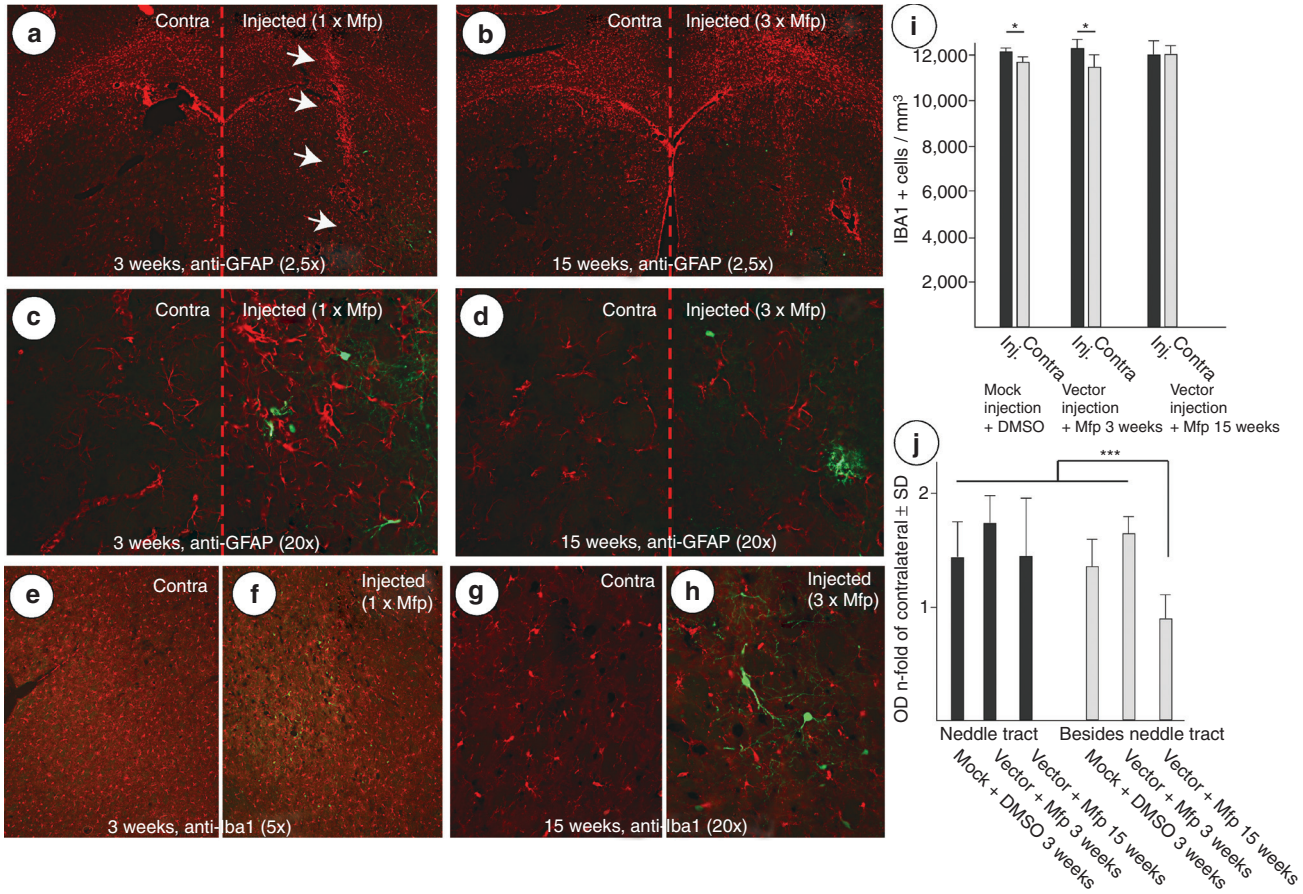

Figure 5 Assessment of astrocyte and microglia reactions. AAV-5-pTK-GS and AAV-5 UAS-TATA-EGFP were coinjected into the rat striatum in a ratio of 1:3 and tissue sections were prepared at (**a,c,e,f**) 3 weeks after Mfp application or at (**b,d,g,h**) 15 weeks after vector injection and Mfp application every 4 weeks. Arrows in **a** demarcate the needle injection tract. Immunohistochemistry for the astrocytic marker GFAP is shown in low magnification for 3 weeks after vector injection (**a**, the stippled line demarcates the midline), for (**b**) 15 weeks after vector injection , and in higher magnification for (**c**) 3 weeks and (**d**) 15 weeks after vector application. Immunohistochemistry for the microglia marker Iba1, for the (**e**) contralateral hemisphere and in for the (**f**) injected hemisphere at 3 weeks after vector injection, and (**g**,**h**) at higher magnification at 15 weeks after vector injection. Pictures are overlays of the vector expressed EGFP (green) with the Cy3 (red) detected antigens. (**i**) Stereological quantification of IBA1 immunoreactive cells after mock injection and DMSO application or after vector injection and Mfp application for the injected (inj.) and the contralateral (contra) hemisphere. (**j**) Analysis of the optical density of GFAP immunoreactivity, for the needle tract and in a distance of 250–500 µm to the needle tract after mock injection and DMSO application or after vector injection and Mfp application in the injected hemisphere as *n*-fold intensity to the respective contralateral hemisphere. *N* = 4 or 5 brains per condition. AAV, adeno-associated virus; DMSO, dimethyl sulfoxide; Mfp, mifepristone; OD, optical density.

Constitutive GDNF expression resulted in almost complete protection of striatal DA levels from 6-OHDA toxicity at 4 weeks after lesion. The induced GDNF expression was equally effective as constitutive GDNF expression, despite the significantly lower level of GDNF as compared with constitutive expression. In 6-OHDA–treated striata, which did not receive any vector injection or which were injected with an EGFP control vector, DA levels dropped to 50% of contralateral DA levels (**Figure 6c**, right panel). Importantly, the minor elevated GDNF expression from the noninduced regulated vector demonstrated no protective effect on DA levels.

Rotation behavior due to overstimulation of hypersensitive DA receptors by apomorphine in the 6-OHDA–lesioned brains was detectable only in animals which were injected with EGFP-expressing vectors or in animals which were injected with the regulated GDNF-expressing vector but not induced by Mfp. In contrast, both constitutive and short-term–induced GDNF expression was sufficient to completely prevent onset of motor impairments, *i.e.,* apomorphine-induced rotation behavior (**Figure 6d**, right panel).

Weight loss as a prominent side effect of GDNF expression did not occur in any of the experimental groups (data not shown). Striatal application of 2×5 µg 6-OHDA results in a protracted degeneration of nigral dopaminergic neurons with about 50% of cell loss at 14 weeks after lesion[16] but no significant cell loss at 4 weeks after lesion, as analyzed in this study (control nigra: 13,631 ± 642; 6-OHDA: 12,900 ± 514; constitutive GDNF + 6-OHDA: 14,173 ± 636; regulated GDNF + 6-OHDA: 13,997 ± 560 TH immunoreactive neurons per nigra). However, the extent of DA loss and drug-induced rotations was the same as reported for harsher lesions with substantial dopaminergic neuron loss at this time point.[17] Absence of dopaminergic cell loss facilitated the analysis of an important impact of GDNF on DA synthesis: at 4 weeks after the lesion, cell bodies of many dopaminergic nigral neurons in the constitutively GDNF-expressing brains showed

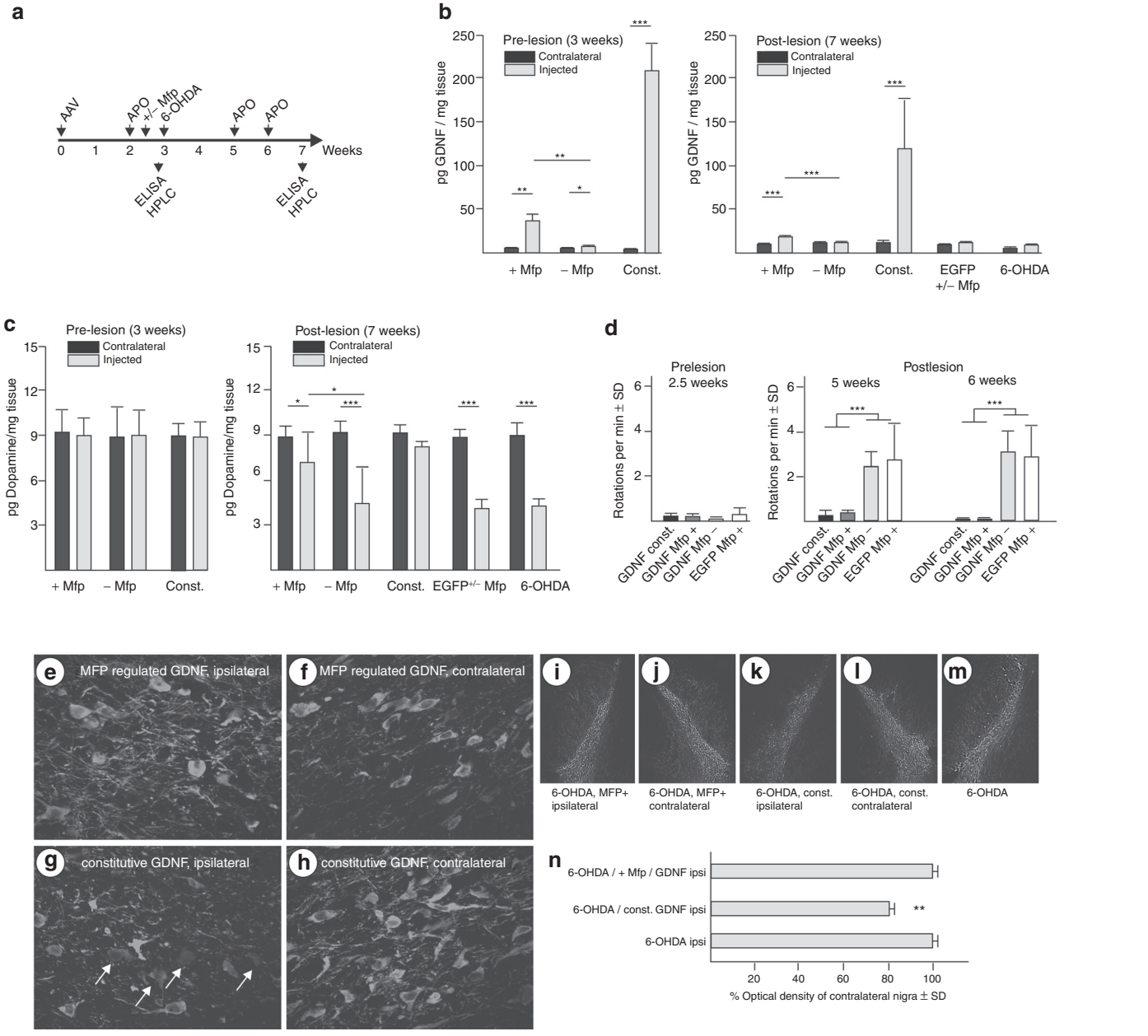

Figure 6 Adeno-associated virus (AAV)-5–mediated Mfp-induced expression of GDNF is neuroprotective in a rodent model of Parkinson's disease without affecting TH levels. (**a**) The time schedule of the experiment in weeks. AAV = vector injection; APO = apomorphine-induced rotations; ± Mfp = application of Mfp or DMSO; 6-OHDA = induction of a partial striatal 6-hydroxydopamine lesion; ELISA/HPLC = tissue sampling for GDNF and dopamine quantification. (**b**) Levels of GDNF were assessed after Mfp induction but before the 6-OHDA lesion (left panel) and at 4 weeks after 6-OHDA lesion (right panel). (**c**) At the same, prelesion and postlesion times levels of striatal dopamine were quantified. +Mfp = AAV-GfaABC$_1$D-GS + AAV-UAS-TATA-GDNF, induced by 20 mg/kg Mfp; – Mfp = AAV- GfaABC$_1$D -GS + AAV-UAS-TATA-GDNF, DMSO application; const. = AAV-GFAP-GDNF; EGFP ± = AAV-GfaABC$_1$D + AAV-UAS-TATA-EGFP, with or without Mfp induction; 6-OHDA = 6-OHDA lesion w/o vector application; black bars = contralateral hemisphere, gray bars = lesioned/injected hemisphere. (**d**) Apomorphine-induced rotation behavior was assessed at the indicated times before (left panel) and after 6-OHDA lesion (right panel). (**e**–**m**) Immunohistochemical detection of tyrosine hydroxylase (TH) expression in dopaminergic nigral neurons at 4 weeks after 6-OHDA lesion. TH staining after constitutive GDNF expression is shown in low magnification in the (**k**) injected and (**l**) contralateral hemisphere, and in high magnification in the (**g**) injected hemisphere and (**h**) contralateral hemisphere. Arrows in **g** point to TH immunoreactive neurons with weak staining intensity. TH staining after Mfp-induced GDNF expression is shown in low magnification in the (**i**) injected and (**j**) contralateral hemisphere, and in high magnification in the (**e**) injected and (**f**) contralateral hemisphere. (**n**) Optical density analysis for intensity of TH immunostaining. $^*P < 0.05$; $^{**}P < 0.05$; $^{***}P < 0.01$. $N = 6$–8 animals per group. HPLC, high-performance liquid chromatography.

only weak staining intensity for TH, the rate limiting enzyme in DA synthesis (**Figure 6g**,**k**). Optical density analysis of TH immunoreactivity demonstrated that over the area of the whole nigra, a moderate but significant reduction of 21 ± 2% in immunoreactivity (as compared with the contralateral nigra) was detectable after constitutive GDNF expression. This well

documented side effect of excessive GDNF supply[16,18] was absent in the short-term–induced GDNF-expressing group (**Figure 6e,i**).

Discussion

Several features would be desirable for an optimal regulated gene therapy tool: (i) basal expression in noninduced state should be negligible or should at least not have physiological relevance, (ii) regulation should be achieved in a way that application of a small-molecule drug should activate rather than suppress transgene expression, (iii) the system should be repeatedly responsive, allowing for several cycles of induction, (iv) as many components of the regulation machinery should be of human origin to avoid putative immunological consequences, and (v) the inducing drug should have a proven safety profile in humans.

The first goal was met in a way that in the rat model of PD, the regulated GDNF-expressing vector had no neuroprotective effects in the absence of Mfp induction. As clearly seen with both the reporter EGFP and with the better-to-quantify GDNF, the currently used regulated UAS-TATA promoter demonstrated low but significant leakiness, even if isolated from the promoter activity of the AAV-2 ITRs. This feature is shared with almost all described tet-ON vectors.[19–22] GDNF levels in the prelesion, noninduced state were elevated by 1.4-fold over the contralateral side but these levels were not sufficient to provide any measurable effect on DA content or motor behavior in the 6-OHDA lesion paradigm. In contrast, the sevenfold increase in GDNF levels after induction by Mfp was sufficient to completely prevent loss of DA and onset of rotation behavior in this functional assay. This level of induced GDNF is similar to the reported 7- to 12-fold increases in GDNF over endogenous levels achieved with tet-ON lentiviral or AAV vectors.[19,23,24] Importantly, this GDNF level did not result in a reduced staining intensity for TH in dopaminergic neuron's cell bodies, as it was observed with the 50-fold increase in GDNF levels as obtained with the constitutively expressing control vector and in previous studies.[16,18] Furthermore, in the postlesion condition after 6-OHDA application, noninduced GDNF expression was identical to contralateral controls due to the small increase in endogenous GDNF levels, presumably as a result of the lesion. In comparison to tet-regulated AAV-GDNF vectors, the leakiness of the Mfp-regulated systems appeared to be similar or slightly better than the tet-ON GDNF vector[23] but not as low as a tet-OFF GDNF vector.[25] Both tet-ON and tet-OFF vectors were used under continuous delivery of the inducing drug (minocycline or doxycycline) for several weeks in these studies. To our knowledge, the Mfp-induced GDNF expression as demonstrated in the present study represents the first attempt to provide bioactive neurotrophin levels with a short-term application of a drug.

Kinetics of activation of EGFP or GDNF expression appear to be relatively fast, in that at 3 days after induction, already substantial protein expression is achieved. Decay of transgene expression was found to be complete after 2 weeks by live imaging of EGFP fluorescence in the mouse cortex (AAV-6 vectors, **Supplementary Figure S1**), while ELISA-based determination of GDNF in the rat striatum showed that at 4 weeks after induction, tissue levels were still twice as high as in controls. This discrepancy may be explained by different metabolic activities between mice and rats and by different stabilities of the respective proteins. None the less, a short-term induction of the AAV-5 two-vector–regulated Mfp system was sufficient to provide neuroprotective GDNF levels for a period of several weeks, suggesting that an intermitted application of the inducer Mfp might be sufficient to maintain such neuroprotective levels in patients. Proof of principle that such a discontinuous induction modus is applicable was provided by up to three consecutive induction cycles in rats and up to six induction cycles in mice, suggesting that this inducible vector system remains responsive over extended periods of time and induction cycles. Decay of Mfp in humans is fast (600 mg decayed within 7 days, ref. 26), and we, thus, assume that in rats, this decay is even faster due to their higher metabolic rates. Thus, the prolonged presence of GDNF after short-term induction seems to depend on slow degradation of the protein or on its sequestration in the extracellular matrix rather than on prolonged presence of Mfp.

While regulated gene therapy vectors are considered to deliver a substantial safety improvement for gene therapies targeting major neurodegenerative disorders like PD,[27] others discourage the use of such systems.[28] A major concern is the composition of the regulation machinery, which has to be introduced into the target tissue together with the transgene of interest. These regulatory proteins in most cases contain virus or nonmammalian-derived components to a significant extent. The tet-operon–derived regulated systems have already been shown to provoke severe peripheral immune responses, which could not be prevented by engineering the rtTA, an *Escherichia coli*-derived protein.[9] While the GS protein in this respect is clearly favorable over any version of the tet system, absence of astrogliosis and microglia activation in our rodent study do not finally prove nonimmunogenicity of this protein in humans, but need further investigations in the brain of nonhuman primates. However, the available combination of a clinically approved small-molecule drug and a regulatory protein composed of the most part of human components warrants further efforts to evaluate if Mfp-regulated neurotrophin expression in the brain can serve as a valid alternative to current vector designs.

The Mfp dosage applied in most experiments of this study to induce gene expression in the rat brain was 20 mg/kg, this dose was injected intraperitoneally on three consecutive days. Converting this dose to humans by applying the Food and Drug Administration-approved normalization to body surface area[29,30] results in a corresponding human equivalent dose of 3 mg/kg. This dose is still considerably below those safely tested in psychiatric patients (600 mg/day for 7 days; corresponding to 8.6 mg/kg/day for a person with a body weight of 70 kg), which resulted in subtle changes in plasma cortisol levels, an effect which might explain the proposed positive action of Mfp in psychiatric threats.[31,32] These dosages have also been tested in chronic applications over several months, demonstrating only mild side effects such

as fatigue and rash, but no disturbance of immune parameters.[26] Thus, the Mfp-regulated AAV vectors investigated in this study may be applicable in humans by using an already approved dosage of the inducing drug. Given that the system was reactive in the rat brain also at dosages as low as 5 mg/kg (corresponding to a human equivalent dosage of 0.7 mg/kg), forthcoming improvements in Mfp delivery modus or vector layout may further decrease the necessary dosage for human gene therapy trials. Mfp is orally available for patients, and forthcoming studies in rats and nonhuman primates will now have to evaluate the minimum oral dosage for induction of sufficient GDNF expression from the Mfp-regulated vector system.

Over all, it is evident that our current Mfp-regulated AAV vectors provide a solid basis for development of neurotrophin-based gene therapeutic approaches in the brain and can be regarded as valuable alternative to tet-operon–based vector systems. The vector system is characterized by low background and moderate-induced expression levels and has shown safety and efficacy in the rat 6-OHDA model of PD. In addition, sustained neuroprotective effects were achieved by only short-term induction of the system. Further studies are now warranted to investigate oral application schemes for Mfp, optimization of the UAS-TATA promoter, application in preclinical paradigms after onset of the lesion and evaluation of immunogenicity of GS expression in nonhuman primates. These studies will open new venues for regulated, therapeutic transgene expression in the CNS and putatively in many further tissues.

Materials and methods

Viral vector production. The GS system was purchased from Invitrogen (now Life Technologies, Darmstadt, Germany) and the two cassettes were cloned in either two separate or in one AAV vector genome. The GS expression cassette contained four GAL4 UAS (upstream activating sequences): the pTK promoter (herpex simplex virus thymidine kinase minimal promoter), an IVS8 synthetic intron, the GS protein, and the SV40 polyadenylation sequence. The GS protein was composed of a fusion of the yeast GAL4-binding domain, a truncated human progesterone receptor that binds Mfp, and the human p65 transactivator domain. The responsive expression cassette contained a minimal promoter composed of six GAL4 UAS and the adenovirus E1b TATA sequence, followed by either EGFP or human GDNF cDNA, the WPRE (woodchuck hepatitis virus post-translational regulatory element) in some vectors, and a bovine growth hormone polyadenylation sequence.

The GAL4 UAS and the pTK promoter were isolated from the AAV-2 left ITR using transcription blocker (synthetic mRNA termination/polyadenylation) sites. In some constructs, the pTK promoter was substituted with the GfaABC1D promoter, which directs GS expression partially to astrocytes.

Recombinant AAV vectors of serotypes 5 or 6 were produced as previously described[16] by transient cotransfection of HEK 293 cells using pDP6 or pDP5 helper plasmids; viral particles were purified by iodixanol gradient centrifugation, fast protein liquid chromatography, and dialysis against phosphate-buffered saline. The titer of the preparation was measured by quantitative PCR and the purity (>99%) was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining.

Vectors in cell culture. Primary cortical neurons cultures were prepared as previously described.[33] Neurons were infected with viruses on day 3 *in vitro* by diluting the proper amount of viruses in 10 µl of phosphate-buffered saline which was then directly added to the wells; 3×10^8 vg/well of AAV-6-pTK-GS and 9×10^8 vg/well of the responsive vectors were used. Expression was induced by Mfp on day 6 *in vitro* with a final concentration of 10 nmol/l. Images were acquired on day 10 *in vitro* using an Zeiss inverted microscope and the Axiovision 4.7 software (Carl Zeiss, Göttingen, Germany).

Virus injection into the rat brain. All animal procedures were performed according to approved experimental animal licenses issued by the responsible animal welfare authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES) and controlled by local veterinarians. Female Wistar rats weighting between 220 and 280 g were housed in 12-hour light/dark cycle and were provided with metamizol (1.5 mg/ml) in the drinking water 3 days before and for 1 week after surgery.

Animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) and fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). For the EGFP experiments, one injection was performed in the middle of the striatum at the following coordinates (in mm relative to bregma): mediolateral (ML), +0.25; anterioposterior (AP), +0.12; dorsoventral (DV), –0.55. Viruses were diluted in 2 µl phosphate-buffered saline and injected using a Nanoliter 2000 injector (World Precision Instruments, Sarasota, FL) at 500 nl/minute; the needle was left in place for 4 minutes both prior and after the injection.

Vector injections before the partial 6-OHDA lesion were performed at coordinates ML, +0.21; AP, +0.05; DV, –0.5 and ML, +0.38; AP, +0.05; DV, –0.5 mm.

Partial 6-OHDA lesion. 6-OHDA (5 µg in 0.5% ascorbic acid) was injected into the same two sites where the viruses have been injected before.

Mfp application. Mfp induction was performed at 3 weeks after vector injection. The steroid was diluted in dimethyl sulfoxide; control animals received only dimethyl sulfoxide; 20 µg/kg body weight (or other dosage as stated) were injected intraperitoneally on three consecutive days.

Behavioral analysis. At 2, 5, and 6 weeks after virus injection, animals were subjected to drug-induced rotation behavior. Animals were injected intraperitoneally with 0.4 mg/kg apomorphine and rotation asymmetry was monitored for 60 minutes. Analysis was performed in a manner blind in regards to the experimental group. Rotational asymmetry score is expressed as full contralateral body turns per minute.

Quantification of GDNF and DA. Striatal samples for ELISA and high-performance liquid chromatography were rapidly collected on ice in a way that each striatum was dissected

in two equal parts: one for ELISA and second for high-performance liquid chromatography. Samples were stored at –80 °C until analysis. The GDNF ELISA was performed following the manufacturer's instructions (Promega, Madison, WI; cat. Nr.: G7621). Samples were lysed using a Precellys homogenizer with beads in buffer consisting 137 mmol/l NaCl, 20 mmol/l Tris (pH 8.0), 1% Nonidet P40, 10% glycerol, and protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland). High-performance liquid chromatography samples were lysed with 50 µl of 0.1 mol/l perchloric acid/mg of tissue using a Precellys homogenizer. After centrifugation with 13,000*g* for 10 minutes at 4 °C, the supernatant was injected onto a C8 reverse-phase Acclaim 120 column (ESA, Bedford, MA). DA, 3,4-dihydroxyphenylacetic acid, and homovanillic acid were quantified by electrochemical detection. The mobile phase (pH 4.3) consisted of 85 mmol/l sodium acetate, 0.2578 mmol/l EDTA, 70 mmol/l citric acid, 0.8962 mmol/l octane sulfonic acid, and 10.5% methanol. Flow rate was 0.4 ml/minute.

Quantification of mRNA expression. AAV-5-pTK-GS or AAV-5-hSYN-GS was coinjected with AAV-5-UAS-TATA-EGFP into the rat striatum and gene expression was induced as described above. Total RNA was extracted from the complete striatum using Trizol (Qiazol lysis reagent; Qiagen, Venlo, Netherlands) at 1 day before induction and at 1 day, 3 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after induction. cDNA was synthesized with RevertAid reverse transcriptase (Thermo Fischer Scientific, Waltham, MA) using random primers and followed by quantitative PCR (Step One Plus real time PCR System; Applied Biosystems, Foster City, CA). Primers for amplification of EGFP mRNA were: forward = 5′-gccacaagttcagcgtgtcc-3′, reverse = 5′-ctaccccgaccacatg aagc-3′, probe = 5′-cggcaagctgaccctgaa-Tamra-3′. β-Actin mRNA served as internal standard.

Immunostaining and stereology. Immunostaining for TH was performed essentially as described.[16] Stereology was performed as described in detail elsewhere.[34]

Statistical analysis. Data are expressed as means ± SD. Multiple comparisons were made by one-way analysis of variance test. The unpaired *t*-test was used for comparison between two groups. Differences were considered significant at $P < 0.05$ and less.

Supplementary material

Figure S1. Repeated induction of the AAV-6 two-vector system in mouse cortex.
Materials and Methods.

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