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Therapeutic efficacy of regulable GDNF expression for Huntington´s and Parkinson´s disease by a high-induction, background-free "GeneSwitch" vector

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Abstract

Gene therapy is currently an irreversible approach, without possibilities to fine-tune or halt the expression of a therapeutic gene product. Especially when expressing neurotrophic factors to treat neurodegenerative disorders, options to regulate transgene expression levels might be beneficial. We thus developed an advanced single-genome inducible AAV vector for expression of GDNF, under control of the approved small molecule drug mifepristone. In the rat brain, GDNF expression can be induced over a wide range up to three hundred-fold over endogenous background, and completely returns to baseline within 3-4 weeks. When applied with appropriate serotype and titre, the vector is absolutely free of any non-induced background expression. In the BACHD model of Huntington´s disease we demonstrate that the vector can be kept in a continuous ON-state for extended periods of time. In a model of Parkinson´s disease we demonstrate that repeated short-term expression of GDNF restores motor capabilities in 6-OHDA-lesioned rats. We also report on sex-dependent pharmacodynamics of mifepristone in the rodent brain. Taken together, we show that wide-range and high-level induction, background-free, fully reversible and therapeutically active GDNF expression can be achieved under tight pharmacological control by this novel AAV - "Gene Switch" vector.

Keywords:

GeneSwitch; Regulated expression; GDNF; Parkinson´s disease; Huntington´s disease; AAV; Mifepristone

Introduction

Gene therapy in its current layout is an irreversible treatment: a curative gene is introduced into patient´s tissues by means of a viral or non-viral vector and produces its gene product (a protein or regulatory nucleic acid) from the time of vector application onward. In case of unwanted side effects or sufficient therapeutic success, transgene expression cannot be stopped nor can the level of expression be modified according to individual patient´s needs.

Thus, gene therapy systems allowing for external control over the expressed transgene by pharmacological means would be highly beneficial under certain circumstances. Several such gene transfer systems are available, and especially tetracycline-controlled gene expression has been developed into very advanced systems [1], but none has advanced into clinical applicability yet [2]. This unfortunate fact at least partially depends on the finding that the bacteria-derived Tet-operator provoked substantial immune responses when exploited in non-human primate muscle, although it worked almost perfectly in immune restricted compartments like the retina [3-5]. Another highly promising inducible gene therapy concept, based solely on human components and thereby by default preventing immune issues, did not advance to application in patients due to unavailability of clinically approved analogues to its small molecule drug, rapamycin, a strong immunosuppressant [6]. Furthermore, rapamycin crosses the blood-brain-barrier inefficiently, requiring clinically non-acceptable dosages for regulation of transgene expression in the brain [7].

In order to drive forward development of alternative regulated gene therapy vectors, we recently adopted the "GeneSwitch" system to AAV vectors, where regulated GDNF expression is under control of the clinically approved synthetic steroid mifepristone (Mfp) [8, 9]. In this system, Mfp application activates the GeneSwitch (GS) protein by dimerization, resulting in transgene expression from the responsive minimal UAS-TATA promoter [10]. GS is a fusion of a short fragment of the yeast Gal4-DNA binding domain with a truncated human progesterone receptor and the human p65 transactivator. As it does not contain bacterial or viral components, it is anticipated to be less immunogenic than the Tet repressor and thus might become clinically exploitable.

Our earlier adaptions of the GS system to AAV vectors had to rely on a dual vector layout in order to achieve relatively low background expression of GDNF. Furthermore, the rate of induction of GDNF expression was only moderate, and the promoters used to drive GS expression were active in both neurons and glia. Here we show that an advanced single

vector design allowed for high-level GDNF induction in the absence of any non-induced background expression. We also demonstrate a very good dose response for this system in the rat brain, allowing us to achieve a wide variety of GDNF levels by different Mfp dosing regimens. Importantly, we found gender-specific pharmacodynamics of Mfp in the rat brain. We further demonstrate that the vector can be kept in the ON-state for prolonged periods of time and shows biological effects in a mouse model of Huntington´s disease. Finally, we show that in an established rat model of Parkinson´s disease GDNF expression from this AAV-GS-GDNF vector restored motor capabilities.

Results

Design of a single-vector layout for Mfp-induced GDNF expression

It was the first aim of our present study to design an improved vector layout allowing for incorporation of all necessary elements into a single AAV vector genome, with high rate of induction and without any detectable background expression in absence of Mfp. Figure 1A shows some of the iteratively tested vector constructs, differing mainly in the orientation of the two expression cassettes to each other. In all these constructs GS expression is driven by the strictly neuron-specific human synapsin 1 gene (hSyn) promoter [11]. Vectors were evaluated for GDNF expression in absence and presence of the inducer Mfp, by injecting the respective recombinant AAV-5 viruses unilaterally into the striatum, application of Mfp or solvent at 3 weeks after vector injection, and collection of striatal tissue for GDNF-ELISA at 7 days after the first Mfp application (Figure 1B). Levels of GDNF achieved by the various vectors are shown in Figure 1C.

These experiments demonstrated, that in case of a head-to-head orientation of GS to GDNF expression cassettes, i.e. when hSyn and inducible promoter are directly side-by-side, background expression of GDNF in absence of Mfp was not significantly lower than GDNF expression after induction by Mfp: the GS-GDNF-HtH construct expressed 650 ± 192 pg GDNF/mg tissue in absence of Mfp, and 1094 ± 879 pg GDNF/mg tissue after induction by Mfp. Isolation of both promoters from each other by incorporation of one or three synthetic transcription blocker sequences reduced background expression somewhat, allowing for a roughly 10-fold induction of GDNF expression: the GS-GDNF-HtH-TB construct expressed 174 ± 25 pg GDNF/mg tissue in absence of Mfp, and 868 ± 552 pg GDNF/mg tissue after induction by Mfp. However, non-induced GDNF levels were still about 30-fold higher as compared to 5-6 pg GDNF/mg tissue in non-treated control brains, or in brains injected with a GS-EGFP vector in absence or presence of Mfp (construct GS-EGFP-HtT in figure 1A).

This situation changed completely by flipping the GS expression cassette around, so that GS and GDNF were in a head-to-tail configuration (construct GS-GDNF-HtT in figure 1A). Now, non-induced GDNF levels were found to be at 10 ± 4 pg/mg tissue, while Mfp-induced GDNF levels reached 1942 ± 793 pg/mg tissue, i.e. an almost 200-fold induction above non-induced background and almost 400-fold above endogenous background levels. Induced levels of GDNF achieved by the novel one-vector construct were at least in the same order of magnitude as from a constitutive vector designed for maximum expression. They were also about one order of magnitude higher than those recently

achieved by the best suited two-vector approach [8, 9]. All these experiments were carried out with a vector titre of $3x10^9$ vg / striatum.

Fine-tuning: influences of AAV titre and serotype

Given the significant background expression seen with certain constructs, we next sought to establish Mfp-induced GDNF expression, which is absolutely free from any leaky, non-induced GDNF production, meaning that without Mfp application only the endogenous 5-6 pg GDNF per mg of striatal tissue are detectable. To achieve this, we tested titres of $1x10^9$ and $3x10^9$ vg of AAV-5-GS-GDNF and AAV-1/2 GS-GDNF vectors, both in the head-to-tail (HtT) configuration, which was used in all following experiments. The mosaic AAV-1/2 serotype was chosen as an alternative to AAV-5, as this serotype allows for similar tissue penetrance as AAV-5, but uses different primary receptors and is purified by heparin affinity chromatography, while AAV-5 was purified by AVB sepharose chromatography. These experiments should also allow for a correlation between vector titre and GDNF levels.

A side-by-side comparison of $3x10^9$ and $1x10^9$ vg/striatum of AAV-5-GS-GDNF revealed that: i) at 7 days after Mfp induction the 3-fold higher vector titre resulted in 1.5-fold increased levels of GDNF (1942 ± 793 pg/mg tissue versus 1298 ± 450 pg/mg tissue, $p < 0.05$, power (1-ß err prob) = 0.55); ii) at 28 days after Mfp induction GDNF levels have declined close to endogenous background with both $3x10^9$ and $1x10^9$ vg/striatum, but were still higher than endogenous GDNF levels ($p < 0.05$; power (1-ß err prob) = 0.6); iii) at 7 days non-induced GDNF levels were significantly higher than endogenous background with $3x10^9$ vg/striatum (10.4 ± 4 pg/mg tissue versus 5.5 ± 1 pg/mg tissue; $p < 0.01$; power (1-ß err prob) = 0.83), but after application of $1x10^9$ vg/striatum GDNF levels were indistinguishable from background (5.3 ± 1.6 pg/mg tissue versus 5.5 ± 1 pg/mg tissue). Thus, with minor modifications of AAV-5 vector titre, we achieved a 260-fold induction of GDNF levels over endogenous background, while without Mfp application absolutely no GDNF above endogenous background was detectable (Fig 2A, B).

Since in these animal groups GDNF levels did not completely return to endogenous background levels at 28 days after Mfp application, we performed another animal group with striatal injection of $1x10^9$ vg of AAV-5-GS-GDNF, and followed the decay of GDNF in more detail and over longer time (Fig 2C). These results demonstrate that GDNF levels drop rapidly after withdrawal of Mfp application, and completely decay to endogenous

background between 28 and 42 days after Mfp application. Thus, the novel one-vector GS-GDNF system proves to be fully reversible in terms of induction of GDNF expression.

The rate of induction and the levels of non-induced GDNF levels could only partially be repeated with the same vector genome packed into an AAV-1/2 capsid, indicating that the choice of AAV serotype has some influence on the performance of the GeneSwitch system (Fig 2D). An AAV-1/2 vector constitutively expressing GDNF resulted in GDNF levels of 9917 ± 3102 pg/mg tissue, which is significantly more GDNF as compared to 5013 ± 1102 pg/mg tissue achieved with an AAV-5 at the same titre of 3x10e9 vg/striatum ($p < 0.001$; power (1-ß err prob) = 0.95). Application of $3x10^9$ vg of AAV-1/2 GS-GDNF resulted in 1012 ± 553 pg GDNF/mg tissue, which is significantly less GDNF as compared to 1942 ± 793 pg/mg tissue achieved with the equivalent AAV-5 vector ($p < 0.05$; power (1-ß err prob) = 0.65). Strikingly, reducing the titre of AAV-1/2-GS-GDNF to $1x10^9$ vg/striatum achieved only $1/10^{th}$ of GDNF as compared to the equivalent AAV-5 (135 ± 75 pg/mg tissue versus 1298 ± 450 pg GDNF/mg tissue; $p < 0.001$; power (1-ß err prob) = 1).

Furthermore, in absence of Mfp, background GDNF expression was moderately but significantly higher with any titre of AAV-1/2-GS-GDNF as compared to the corresponding AAV-5 vector (Fig 2D).

Thus, while on principle the one-vector GeneSwitch system reacted similarly in both serotypes, usage of AAV-5 allowed for higher levels of induction and elimination of non-induced background expression.

Dose-response analysis: dependence of Mfp-induced GDNF levels on dosage and application route

So far, all experiments in this and our previous studies [8, 9] have been carried out by inducing GDNF expression through application of Mfp intraperitoneally (i.p.), dissolved in DMSO. Mfp is insoluble in aqueous solutions; therefore, we preferred dissolving Mfp in an organic solvent over the suspension in an aqueous carrier. Furthermore, in order to guarantee for sufficient bioavailability of the drug, 3 i.p. injections of 20 mg/kg each were performed on three consecutive days, based on the fact that in rodents half-life and bioavailability of Mfp is significantly reduced as compared to humans ($T_{1/2}$ rat = 4h, human = 30h; bioavailability rat = 39%, human = 69% [12].

We now aimed to determine if GDNF production from the new one-vector GS-GDNF system can be modulated by different dosages of Mfp, allowing to achieve full external control not only over ON-OFF states but also for gradual changes in neurotrophin levels

(Figure 3). These studies revealed a favourable dose-response curve for Mfp applied 3x by the i.p. route, with dosages as low as 1 mg/kg already causing therapeutically meaningful levels of GDNF of about 155 ± 52 pg/mg striatal tissue. Application of only a single dose of Mfp resulted in proportionally less activation of the system: while 3x 20 mg/kg Mfp achieved about 1600 ± 500 pg GDNF per mg of striatal tissue, 1x 20 mg/kg Mfp achieved 540 ± 247 pg GDNF per mg tissue.

These results were on principle confirmed by oral application of Mfp, which was suspended in either an aqueous carrier including micelle forming organic compounds (ASV) or in sesame oil, both of which delivered an identical outcome. Oral administration required higher dosages of Mfp to achieve similar levels of GDNF induction, indicating a lower bioavailability of the drug through this route. Again, a single oral administration of Mfp resulted in less pronounced GDNF induction as compared to 3x Mfp application: 3x 50 mg/kg resulted in 1010 ± 260 pg GDNF per mg of striatal tissue, while 1x 50 mg/kg achieved 525 ± 198 pg GDNF per mg of striatal tissue.

Overall, using different routes and dosages of Mfp application allowed for a wide range of GDNF levels to be induced in rat brains, ranging from 30-fold up to 300-fold over endogenous levels.

Mfp pharmacodynamics in female versus male rat brain: robust differences in GDNF induction

All experiments in this and our previous studies have been performed in female rats, as due to their reduced body weight gain they are easier to maintain and to handle as compared to male rats. Unexpectedly, we found a limitation of Mfp pharmacodynamics in male rats, with significant impact on therapeutic applications: as shown in Fig. 4 A, Mfp applied into male rats resulted in roughly 8-fold less induction of striatal GDNF expression as compared to the same dose in female rats (20 mg/kg i.p., or 50 mg/kg oral, applied on three consecutive days).

It has been demonstrated that certain AAV vectors have different transduction properties in male versus female animals, e.g. in liver or brain of rodents, in some cases even independent from promoters and serotypes, in other cases depending on application routes [13, 14] . We thus injected the constitutively expressing AAV-5-syn-GDNF vector into male and female rat brains and determined GDNF levels obtained in both genders at 3 weeks after injection. This experiment showed that GDNF levels obtained in male and female striata vary by no more than 5% (males: 8446 ± 2728 pg/mg tissue; females: 8176

± 3277 pg/mg tissue; n=7 per group; not shown), indicating that transduction efficacy of AAV-5 vectors is identical in male and female rat striatum. Thus, gender-specific transduction is not responsible for the differences in GDNF induction after Mfp application.

We thus determined plasma levels of Mfp in both genders after intraperitoneal application of 20 mg/kg Mfp each on three consecutive days. As shown in Fig. 4 C, the first injection resulted in identical plasma levels of Mfp in males and females at 1 hour after Mfp injection. However, while the second application of Mfp resulted in substantially increased levels of plasma Mfp in female rats as compared to the first injection, this was not true for male rats, and likewise the third injection of Mfp did not raise plasma Mfp levels above those achieved with the first injection in male rats. As a consequence, plasma Mfp levels in male rats were robustly lower as compared to females over the course of the application, providing the basis for the substantially lower rate of GDNF induction in male brains. Given that in female rats a lowering of the applied Mfp dosage from 3x 20 mg/kg to 3x 10 mg/kg results in a 4-fold reduced induction of GDNF in the brain (Fig 3 B), it seems reasonable to argue that the decreased pharmacodynamic action of Mfp in male versus female rodent brains is directly correlated to its reduced absorption, increased clearance or sequestration in male animals as compared to females.

Sustained long-term term expression of GDNF in the Huntington´s disease BACHD mouse model

Given the long time span of development and progression of neurodegenerative disorders in man, it is necessary to demonstrate that this regulable system can be kept in the ON-state for extended periods of time. This issue was addressed in a transgenic animal model of Huntington´s disease, the BACHD mouse [15]. These mice develop a subtle striatal pathology resembling human HD, but also gain significantly more weight than non-transgenic littermates due to expression of the full-length human *HTT* gene. Reduction of weight gain (or even loss of weight) is a well-studied side-effect of continuous GDNF overexpression [16]. The second aim of this trial was to determine if GDNF acts as a potential therapeutic factor in this model of HD.

At 2 months of age, BACHD mice were injected bilaterally into striatum with $3x10^9$ vg of AAV-5-GS-GDNF, AAV-5-GS-EGFP or AAV-5-syn-GDNF (the control vector constitutively expressing GDNF). An additional group was injected with a higher titre of the constitutive GDNF expressing vector ($9x10^9$ vg), in order to achieve high level GDNF expression. This

group was included into the study as neuroprotective effects of GDNF have not been investigated in this model before, and it was unclear which GDNF levels might be necessary to achieve protective effects. Starting from two weeks after vector injection, animals were given a single i.p. application of Mfp in sesame oil once a week over the time course of the experiment (dosage: 20 mg/kg). Animals were subjected to weighing every week. From about 4 weeks after initiation of GDNF expression by Mfp onward, animals showed less weight gain as compared to animals injected with AAV-5-GS-GDNF but treated only with vehicle, or injected with AAV-5-GS-EGFP and treated with Mfp. This reduction in weight gain was similar in animals expressing GDNF from the constitutive vectors (Fig 5 F) or from the GeneSwitch vector (Fig 5 G).

Mice were subjected to assessment of motor control capabilities on the accelerating rotarod at 9, 14, 27, 40 and 46 weeks of age. These experiments demonstrated a positive training effect from week 9 to week 14 for all groups of BACHD mice, except for those animals that were injected with the high dose of constitutively expressing AAV-5-syn-GDNF (Fig. 5 H). This finding suggests that at least early in disease progression high doses of GDNF might rather exert negative effects on motor control in this animal model. At later time points, animals that received AAV-5-GS-GDNF and weekly induction by Mfp showed a tendency to perform better as compared to AAV-5-GS-GDNF animals without induction or AAV-5-GS-GDNF controls (Fig 5 I). However, despite the relatively high numbers of animals per group (n = 15 - 19) statistical analysis by ANCOVA over all groups and time points and taking into account the reduced weight gain of animals treated with constitutive or induced GDNF, did not reveal statistical significant improvements of motor control through expression of GDNF.

After sacrifice at 10.5 months of age, the quantitative analysis of striatal sections stained with dopamine- and cAMP-regulated phosphoprotein 32 kDa (DARPP-32), a GABAergic marker altered in HD patients [17], also failed to reveal a neuroprotective effect of GDNF (not shown). However, immunohistochemical analysis for GDNF expression confirmed the continuous ON-state of the GeneSwitch system, by revealing a staining level of similar intensity as compared to GDNF expression from the constitutively active vectors (Fig 5 B - E).

These data clearly demonstrate continuous expression of physiologically relevant levels of GDNF from AAV-5-GS-GDNF under continuous activation by Mfp. Thus, Mfp-induced GDNF expression can be kept “ON” for prolonged periods of time.

Functional test of AAV-5-GS-GDNF in the partial 6-OHDA model of Parkinson´s disease

The vector was further tested for therapeutic efficiency in the partial 6-OHDA lesion model of Parkinson´s disease. AAV-5 vectors expressing constitutive GDNF or GS-GDNF were injected into the lesioned striata at 3 weeks after 6-OHDA application at a titre of $1x10^9$ vg. Animals were subjected to apomorphine-induced rotation behaviour before 6-OHDA application (timepoint t=0), and at 2, 7, 10, 12, 15, 18, 21, 24, 27 and 31 weeks after lesion. One group of AAV-5-GS-GDNF injected animals was not treated with Mfp, a second group was given Mfp once at 2 weeks after AAV injection (i.e. at 5 weeks after 6-OHDA application), and a third group received Mfp application at 2, 7,12 and 17 weeks after AAV injection (i.e. at 5, 10, 15 and 20 weeks after 6-OHDA application) (Fig 6 A).

As expected, all animals lack rotation behaviour before 6-OHDA lesion, and showed about 200 contralateral turns per hour at 2 weeks after lesion. Animals with constitutive GDNF expression returned to about 70 rotations/h at time point 7 weeks after 6-OHDA lesion. The animals of this group showed a tendency in increase in rotations up to about 100 rotations/h over the time course of the experiment, although this effect was not statistically significant.

Animals injected with AAV-5-GS-GDNF that were not induced with Mfp remained at 200 rotations/h over the time course of the experiment, demonstrating absence of any endogenous recovery or leaky GDNF expression at biologically relevant levels. Animals induced only once by Mfp showed a decline from 200 rotations/h at time point 7 weeks after 6-OHDA to about 110 rotations/h at time point 10 weeks after 6-OHDA. These animals also showed a moderate increase in rotation behaviour from about 12 weeks after 6-OHDA lesion onward (i.e. at 7 weeks after induction of GDNF expression), which became significantly different from rotations at 7 weeks after 6-OHDA at the endpoint of the experiment at 31 weeks (i.e. at 26 weeks after the single induction of GDNF expression).

Animals that received 4 inductions of GDNF expression at intervals of roughly 4 weeks showed the same initial decline of numbers of rotations as compared to animals with only one induction. In contrast to the group with only one induction the numbers of rotations in the four times induced group showed stable rotation behaviour over the time course of the experiment. Comparing rotation behaviour between 1x and 4x induced animals clearly demonstrated significantly fewer rotations for the 4x induced animals at time points later than 12 weeks after 6-OHDA lesion. Notably, rotation behaviour of animals

injected with AAV-GS-GDNF, which were repeatedly induced by Mfp, was not significantly different from that of animals expressing GDNF constitutively.

Animals of all groups were monitored for weight gain, and, when analysed by 2-way ANOVA, failed to demonstrate significant differences between groups. However, it appears noteworthy that animals under constitutive GDNF expression presented with about 10% less body weight at all time points after vector injection, suggesting that a subtle negative effect of GDNF on body weight might be present in this group (Suppl. Fig. 1). Analysis of rotation behaviour was stopped at 31 weeks after 6-OHDA lesion, as due to some loss of experimental animals numbers in some groups had dropped to only 5 rats. All remaining animals were thus used for two purposes: firstly, we aimed to prove that the AAV-5-GS-GDNF vector can be reactivated even after long-term inactivity, and secondly, we quantified levels of dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in striata of all animals. To demonstrate reactivation of the system, we applied Mfp at week 45 after 6-OHDA lesion, in the group of animals that had already received 4 inductions of GDNF expression at weeks 5, 10, 15 and 20 after 6-OHDA lesion. Thus, the system was reactivated after about 6 months of inactivity, in rats that have reached an age of about 12 months. As shown in Fig 6 C, this reactivation resulted in GDNF expression at a level of about 10.000 pg/mg tissue in the ipsilateral striatum, and significant leakage to the contralateral striatum. The level of GDNF expression obtained in these brains is roughly 8-fold higher as compared to levels obtained in rats with an age of about 2-3 months, although the same dosage of 20 mg/kg Mfp, applied on three consecutive days, was used. This result suggests that pharmacodynamics of Mfp do not only depend on the gender of the experimental animals, but also on their age. Furthermore, our results show that in animals that were not re-induced or did not receive any Mfp throughout the course of the study, GDNF levels did not differ from endogenous control levels, demonstrating that the system stays absolutely background-free for extended periods of time when not activated by Mfp.

In order to relate the behavioural assessment of motor capabilities to molecular data, we also determined levels of DA, DOPAC and HVA in the striata of all animals (Fig 6 D). We found that after constitutive GDNF expression levels of DA were not significantly different from contralateral controls. In the group that had received only one induction of GDNF expression DA levels were equal to those in animals that had not received any induction of GDNF expression. DA levels of animals that were repeatedly induced to express GDNF

were significantly higher than those that were not induced, but also significantly lower than those obtained by consecutive GDNF expression. Thus, the assessment of available dopamine reflects the results obtained by apomorphine-induced rotation analysis. It needs to be noted, however, that rotation behaviour was recorded only until week 31 after 6-OHDA lesion, but DA and metabolites were determined at week 46 after 6-OHDA lesion.

Discussion:

Is there a need for a regulated GDNF gene therapy vector?

Finding the optimal dosage is a key requisite for successful application of any drug, and genetic medicines are no exception. So far, this can only be achieved by varying the number of viral particles used for injection into patients. Thus, ability to regulate the dosage of the therapeutic gene product through external pharmacological controls might facilitate dose-escalation studies considerably, as this dosage modification can be tailored according to responses of individual patients. Given the enormous variability in clinical phenotypes seen with PD [18], an individual dosage regime for neurotrophic factor expression may be highly beneficial. Ageing-related neurodegenerative diseases like PD and Alzheimer´s disease (AD) are multifactorial in etiology and multifaceted in progression of pathology. Mono-causal treatment opportunities are unlikely to emerge soon, or may even not become available at all. Thus, neurotrophic factors as "symptomatic" treatment options, aiming to enhance neuronal survival capabilities and, if applied early enough, enabling restorative attempts of the lesioned CNS, are still valid candidates for gene therapy approaches targeting these devastating disorders [19]. In case of PD, gaining control over expression levels of neurotrophic factors might help to make such therapies acceptable for patients very early after diagnosis, at times when effective medication is available in form of L-DOPA. These patients benefit from conventional L-DOPA substitution therapy for a few years, but it appears that during this time their dopaminergic output into caudate/putamen ceases completely, leaving little to no template for any restoration of the dopaminergic projection [20]. Thus, availability of a pharmacologically controllable gene therapy system might encourage patients to accept a neurotrophic factor gene therapy at times when it has still a good chance to lead to protective and probably restorative effects, because it offers a distinct level of control over an otherwise irreversible and non-controllable therapeutic approach.

Functional verification of Mfp-regulated GDNF expression in BACHD mice

The new AAV-5-GS-GDNF vector was functionally tested in two different paradigms, transgenic BACHD mice and 6-OHDA lesioned rats. BACHD mice are a slowly progressive model of Huntington's disease (HD), that show low to moderate HD-related pathology, accompanied by robust increase in weight gain as compared to non-transgenic littermates [21]. A major side-effect of continuous nigro-striatal GDNF overexpression is weight loss or at least diminished weight gain due to stimulation of corticotrophin releasing hormone

(CRH) release from a subgroup of hypothalamic neurons [16, 22]. This issue was exploited to demonstrate that the GS-GDNF system can be kept in activated ON-state for prolonged times: continuous weight recording served as a live monitoring system for the presence of this side-effect of GDNF. The reduction of weight gain in BACHD mice that was achieved with Mfp-activated GS-GDNF was indistinguishable from constitutive GDNF expression. Thus, a weekly application of Mfp allowed for continuous biological effects of GDNF over a period of over 7 months. Although we had not determined GDNF levels during different time points of this study, our results strongly suggest that no evident saturation effects due to repeated Mfp application might impact on long-term GDNF expression from this vector.

Therapeutic effects of GDNF in mouse models of HD have been shown to crucially depend on the respective model under investigation: GDNF demonstrated neuroprotective effects in toxin-based models [23, 24], in N171-82Q mice [25, 26], but not in R6/2 mice [27]. Given this discrepancy, it would thus be informative to know if GDNF could achieve any protective or restorative effects in BACHD mice, which are clinically relevant due to their expression of full length mutant HTT protein. While the robust impact of GDNF on body weight of BACHD mice presented a serious confounding effect on the subtle improvements seen in rotarod analysis, no therapeutic effect of GDNF beyond that on body weight could be found in this model of HD. Given the fact that high level constitutive GDNF expression initially had rather negative effects on motor performance might be interpreted as a disadvantage of GDNF, as well as the lacking effect on numbers of striatal GABAergic neurons.

Functional verification of Mfp-regulated GDNF expression in 6-OHDA lesioned rats

The second animal model that was used for functional verification of the novel AAV-5-GS-GDNF construct was the partial striatal 6-OHDA lesion model of PD [28]. Here we could show robust improvements of the apomorphine-induced rotation phenotype already by a single induction of GDNF expression, which was performed at 5 weeks after the 6-OHDA lesion. However, these animals showed increased numbers of rotations at several weeks after GDNF induction, indicating that the one-time application of 6-OHDA has long-lasting toxic impact, which is not fully counteracted by just one short GDNF induction. In agreement with this hypothesis, stable improvement of rotation behaviour and striatal dopamine content was obtained by 4 cycles of GDNF induction, resulting in a lasting therapeutic effect. These data confirm our earlier study with a previous version of AAV-

5-GS-GDNF, where intermitted induction of GDNF expression rescued striatal dopamine levels and prevented degeneration of nigral dopaminergic neurons to the same extent as continuous GDNF expression [8]. The moderately lower gain of weight of animals with constitutive GDNF expression as compared to those with induced or no GDNF expression was not significant in appropriate statistical tests (2-way ANOVA), but if considered translatable to human patients might already be of serious concern.

Comparison of background expression and induction efficacy with other regulated systems for GDNF expression

It is not trivial to quantitatively compare the efficacy of induction of GDNF expression that is achieved by different vector systems, due to differences in vector type and serotype, quality of vector production (i.e. transducing units per vector genomes), applied vector titre, dosage and timing of pharmacological control, duration of the experiment, etc). However, a few studies using regulable GDNF expression in rodent striatum using an ON-switch for induction (e.g. a Tet-ON rather than a Tet-OFF system, as in the latter the drug is used to turn off transgene expression) are available and can be put into context with our new vector: a dual LV vector using the Tet-ON system achieved about 1000 pg GDNF / mg tissue, but demonstrated high background of about 250 pg GDNF / mg tissue in non-induced state [29]. Early Tet-ON AAV vectors showed no GDNF expression in non-induced states, but achieved only very moderate rates of induction of about 60 pg/mg tissue [30, 31]. A recently developed AAV-Tet-ON-GDNF vector demonstrated up to 1000 pg GDNF / mg tissue after induction, while background expression was relatively low (about 15-fold over background) at 75 pg/mg tissue [32]. In all these studies, the inducer doxycycline was applied for many days or even weeks over the drinking water, it is thus unknown if short-term doxycycline applications would result in similar effects. In an alternative system depending on stabilization of GDNF fusion proteins coupled to destabilizing domains of *E.coli* dihydrofolate reductase (DHFR), expressed from a LV vector, striatal GDNF levels of about 125 pg/mg tissue were achieved in the trimethoprim-activated ON-state, while background was low at about 13 pg/mg tissue [33]. In yet another approach, a rapamycin-regulated AAV vector expressed GDNF up to 450 pg/mg tissue, without any non-induced background, although by exploiting clinically not acceptable dosages of rapamycin [7].

While all of these systems for regulated GDNF expression clearly have potential for further optimizations, the Mfp-regulated vector presented here represents, at least for the time being, a superior combination of zero background expression, favourable dose-

response curve to the inducer, and high-level induction at clinically acceptable dosages of an approved small molecule drug.

Mifepristone: dose responses and sex-specific pharmacodynamics

Mifepristone is a clinically approved drug with excellent safety profile [34], that is in use for emergency contraception / early pregnancy termination (in combination with prostaglandins) for more than two decades, and has also been used successfully and safely in several off-label situations, i.e. psychiatric threats or Cushing syndrome [35]. Our study has shown that a wide range of pharmacodynamics in terms of induction of GDNF expression could be achieved in rodent´s brains. As such, the system has the proven capability to be able to adapt levels of neurotrophic factors to individual situations: for example, early after diagnosis of PD and vector application, high levels of GDNF might be most beneficial in order to preserve as much of the dopaminergic projection as possible, and to potentially induce a certain level of re-sprouting of dopaminergic termini in the basal ganglia. Later, after having achieved sufficient restoration or in case of any side-effects, the dosage may be reduced to a lower level maintenance supply of GDNF or completely turned off.

The lowest dosage of Mfp that induced GDNF levels with proven neuro-restorative capabilities in our rat model of PD (3 x 1 mg/kg, i.p, resulting in about 150 pg/mg GDNF, which restored motor performance in this model in earlier studies [8]) corresponds to a human dose of 3 x 0.15 mg/kg, or a total dose of 31.5 mg in a person weighing 70 kg, according to the FDA approved normalization to body surface area [36]. The Mfp dose used for high level expression in rats (3 x 20 mg/kg) corresponds to a total daily dose of 210 mg in humans. In clinical pilot trials targeting breast cancer [37], meningioma [38], endometriosis [39] or uterine leiomyomata [40] Mfp has been safely administered in daily doses of 50-200 mg per patient over several months , indicating that the dose used in our study is very well within acceptable clinical ranges.

It was an unexpected finding that in male rat brain Mfp induces a roughly 8-fold lower response in GDNF expression as compared to females. No literature is available that quantitatively compares pharmacodynamics of Mfp in males versus females, presumably because Mfp has been developed exclusively for use in women.

Mfp is primarily degraded by cytochrome P450 3A4 [41], the activity of which can be readily modified by drugs like ketoconazole or simply grapefruit juice [34], thus offering

opportunities to prolong the presence of pharmacologically active levels without increasing Mfp dosage directly. In any case, induced striatal GDNF levels were well within therapeutic range in male rat brains after oral application of Mfp, indicating that gender differences might be important to consider in further development of GS-GDNF gene therapeutic applications, but do not preclude males from application of Mfp-regulated neurotrophic factor gene therapy. In addition, our finding that in aged rats Mfp apparently has a higher pharmacodynamic potency as compared to young animals, probably due to reduced activity of cytochrome P450 systems in the liver of aged individuals, may allow to make use of lower dosage application in the aged patients that would be considered a target for GDNF gene therapy.

Conclusions

We present an advanced regulated GDNF construct with zero background in non-induced state, high level of induction, and well-suited dose-response curve. We show that this system can be kept in activated state for extended periods of time, can be readily re-activated after long-term off-status, and has biological and therapeutic effects in two animal models of disease. Neither regulated nor constitutive GDNF provided therapeutic effects in the BACHD model of Huntington´s disease, but low-frequency induced GDNF restored motor pathology in the 6-OHDA model of Parkinson´s disease. Thus, the concept of Mfp-regulated neurotrophin gene therapy has considerably evolved towards applicability in clinically well accepted AAV vectors.

Materials and Methods

Vector construction

The basic properties of the Mfp-regulated "GeneSwitch (GS)" system [42] and its adaption for AAV vectors-mediated transgene expression in the brain have been described [9]. For the current study, we assembled the components of the GS system in a single vector genome in different configurations (Fig 1A). Vector genome instability was prevented by reducing the high GC nucleotide content of the genuine GS cDNA by chemical synthesis of an appropriate cDNA.

All constructs evaluated in this study make use of the small and strictly neuron-specific human synapsin 1 gene promoter to drive expression of the GeneSwitch (GS) fusion protein [43]. In our earlier vectors, other small promoters, i.e. a thymidine kinase (TK) promoter or a GfaABC1D promoter were exploited, in order to achieve either neuron- or astrocyte-specific expression of GS [8]. However, both promoters turned out to be not entirely cell-type specific when used in AAV vectors in rodent brain. While attempts to render the small GfaABC1D promoter astrocyte-specific were principally successful by incorporation of mir124 target sequences [44], the expression level of the resulting constructs was too low to be used for further developments of the "GeneSwitch" system in AAVs, and was thus abandoned. Therefore, GS-GDNF vectors now express GDNF exclusively in neurons.

Recombinant adeno-associated viral vectors of serotype 5 (AAV-5) were prepared by transient transfection of vector genome plasmids with the DP5 helper plasmid in HEK293 cells, viral particles were purified from cell lysates by iodixanol gradient centrifugation and AVB affinity chromatography. After extensive dialysis against PBS particles were concentrated on Amicon 100 kDa spin concentrators and frozen in single use aliquots at -80°C. Genome titres were determined by qPCR and >98% purity was confirmed by SDS-PAGE.

For functional evaluation in a rat model of Parkinson´s disease the vector was produced by a baculovirus packaging system in insect cells [45]. This production scale allows to generate sufficient material for clinical application and was used here simply to verify that this vector can be produced at large scale.

Ethical approval of animal experimentations

All experimental animal procedures in rats were conducted according to approved experimental animal licenses (16/2074 and 11/0408) issued by the responsible animal welfare authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES) and controlled by the local animal welfare committee and veterinarians of University Medical Center Göttingen. BACHD mice were kindly provided by Dr. Wiliam Yang, University of California, Los Angeles, USA [15]. All experimental procedures in BACHD mice were performed in strict accordance with Swiss regulations concerning the care and use of laboratory animals (veterinary authorization 2888).

Huntington's disease model: BACHD mice

2 months old female BACHD mice [15] were injected bilaterally with 3x10e9 vg / striatum (AP: +1, ML: +1.8, DV: -3.5 mm) with AAV-5-GS-GDNF, AAV-5-GS-EGFP or AAV-5-syn-GDNF (constitutively expressing vector). One group of animals received a higher dose of AAV-5-syn-GDNF (9x10e9 vg / striatum). From two weeks after vector injection onwards animals received weekly i.p. applications of Mfp (20 mg/kg). Animals´ weight was recorded weekly. FVB mice (the genetic background of BACHD mice) were used as controls for weight gain. BACHD and wild-type littermates (FVB) were trained on accelerating rotarod for 2 days at 9 weeks of age. We then performed a 3-day accelerated rotarod test on BACHD and WT littermates and recorded the average latency to fall on each day. Animals were tested at 14, 27, 40 and 46 weeks of age.

Parkinson´s disease model: partial striatal 6-OHDA lesion

All surgical operations were performed under ketamine 10% (95 µl per 100 g) / xylazin 2% (25 µl per 100 g) anaesthesia as described[8] . Animals received two injections of 5µg 6-OHDA in 0.02% ascorbic acid into the left striatum at the coordinates: AP: +0.05, ML: +0.21, DV (from brain surface): -0.5 cm and AP: -0.05, ML: +0.38, DV (from brain surface): -0.5 cm. At 3 weeks after 6-OHDA injection AAV-5-GS-GDNF or AAV-5-syn-GDNF were injected at a total titre of 1x10e9 vg / striatum at the same coordinates.

Apomorphine-induced rotational behaviour

Animals were injected intraperitoneally with 0,4 mg/kg apomorphine and rotation asymmetry was monitored for 60 minutes. Analysis was performed blinded to the

experimental groups. Rotational asymmetry score is expressed as full contralateral body turns/hour.

Quantification of GDNF, dopamine, DOPAC and HVA

Striatal samples for GDNF ELISA were rapidly collected on dry ice. For all experiments shown in figures 1, 2, 3 and 4, the whole striatum was used as one sample. For experiments shown in figure 6 C and D, each striatum was divided into two equal parts, one being used for GDNF ELISA, the other for HPLC to determine dopamine and metabolites. Samples were stored at -80^{0}C until analysis. The GDNF ELISA was performed following the manufacturer´s instructions (Promega, cat. Nr.: G7621). Samples were lysed using a Precellys homogenizer with beads in buffer consisting 137mM NaCl, 20mM Tris (pH 8.0), 1% Nonidet P40, 10% glycerol and protease inhibitor cocktail (Complete mini, Roche). GDNF levels are expressed in pg/mg tissue throughout the text. For comparison with data from other studies we used a conversion of 1 pg/mg tissue = 6 pg/mg protein. Dopamine and its metabolites DOPAC and HVA were quantified by HPLC with electrochemical detection exactly as described [8].

Mifepristone application and quantification

For intraperitoneal (i.p.) injections in rats the synthetic steroid (Sigma # M8046) was diluted in DMSO; control animals received only DMSO. For i.p. injections in mice Mfp was suspended in sesame oil. For oral administration Mfp was suspended in aquous suspension vehicle (ASV) consisting of 0.9% NaCl, (w/v), 0.5% carboxymethyl-cellulose MW 250.000 (w/v), 0.4% polysorbate 80 (v/v), 0.9%benzyl alcohol (v/v).

For quantification of mifepristone from plasma samples a UPLC-MS/MS method with a stable isotope labelled D3-mifepristone as internal standard (IS) was used. Preparation of Calibrators, quality controls and samples were performed by adding acetonitrile containing IS D3-mifepristone. After vigorous vortexing and centrifugation, supernatant was placed into the autosampler for injection.

All experiments were conducted using a triple quadrupole mass spectrometric instrument (Xevo TQS; Fa Waters) equipped with an electrospray interface (ESI) operated in positive ion mode. The analytes were fragmented using argon 5.0 as collision gas and the optimal MRM transition was monitored. The data were collected and processed using MassLynx 4.1 software. All calculations were based on peak area ratios of the given mifepristone and the stable isotope labelled D3-mifepristone. For quantitation, the

following MRM transitions were used: m/z 430/134 (quantifier) for mifepristone, m/z 433/137 for internal standard (IS). The MRM transition 430/372 was used as qualifier. The chromatographic separation was performed within 3 minutes on a BEH Shield RP18 column (100 mm × 2.1 mm i.d., 1.7 µm particle). 1% acetic acid as mobile phase A and acetonitrile as mobile phase B were used at a flow rate of 0.4 mL/min. The column temperature was set to 45 °C and the injection volume was 7 µL. A sharp linear gradient from 30% B 0.3 min to 75% within 1.0 minute was performed.

Statistical analysis

Experimental data were analysed for statistically significant differences between groups by 1-way or 2-way ANOVA with Tukey´s or Bonferroni´s post hoc test´s, or in pairwise comparisons by unpaired, one- or two-tailed T-test (as appropriate), given a normal distribution of values with similar variances. Statistical powers of all such comparisons were analysed by G*Power3.1 [46] with the following settings: test family = t-tests; statistical tests = difference of means between 2 independent groups; type of power analysis = post hoc; effect size *d* computed from means and standard deviations of groups to compare; α error probability = 0.05; with given sample sizes the power of the statistical assessment was computed as (1 – ß error probability). A reasonable statistical power of the respective statistical analysis can be assumed at 1 – ß error probability > 0.8.

Figures and legends

Figure 1

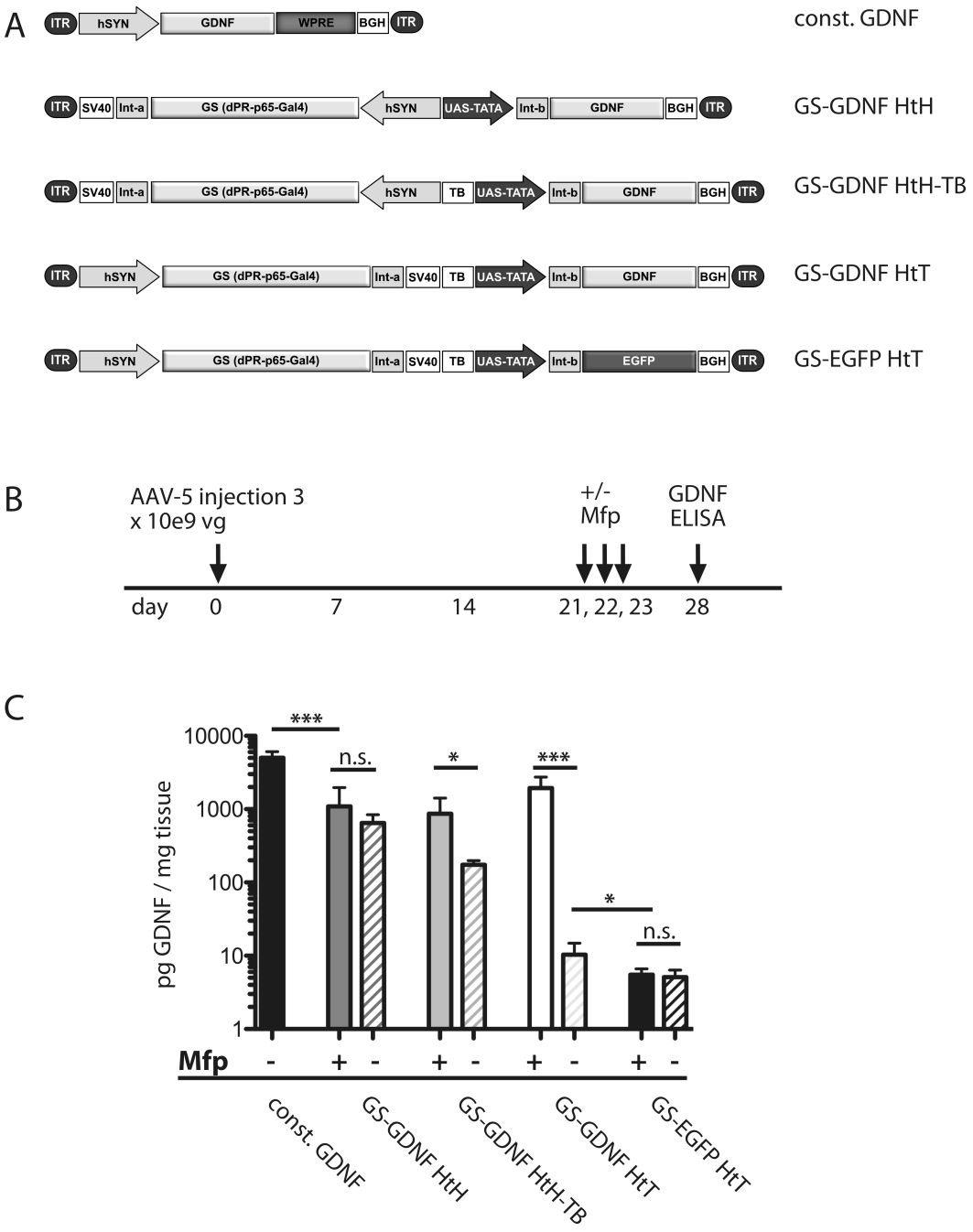


Figure 1: Development of a high induction, low background mifepristone-controlled AAV vector

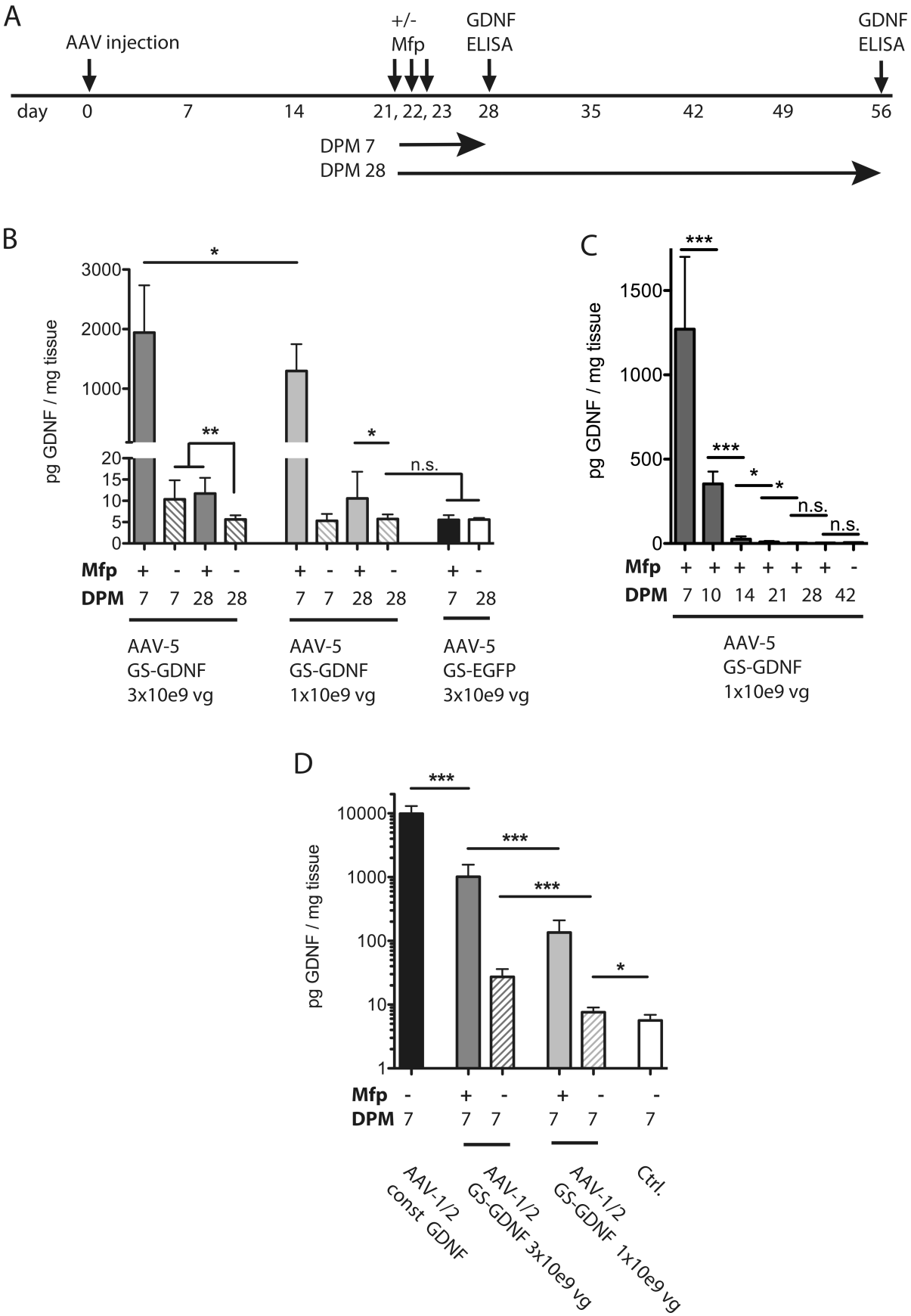
(A) Vector genomes as described in this study.

(B) Time scale of experiments shown in panel (C).

(C) GDNF levels in striatum as measured by ELISA, in pg/mg tissue ± SD. Groups with 3x i.p. application of Mfp are labeled (Mfp +, solid bars), groups with 3x i.p. DMSO application are labelled (Mfp -, hatched bars).

ITR = inverted terminal repeat from AAV-2; GDNF = glial cell line derived neurotrophic factor; WPRE = woodchuck hepatitis virus posttranscriptional regulatory element; BGH = bovine growth hormone polyadenylation site; SV40 = simian virus 40 polyadenylation site; Int-a and Int-b = synthetic introns; GS = fusion protein consisting of Gal4 DNA binding domain, truncated human progesterone receptor and human p65 transactivation domain; hSyn = 420 bp fragment of human synapsin 1 gene promoter; TB = synthetic transcription blocker [47]; UAS-TATA = minimal TATA promoter with 6 upstream Gal4 binding sites; EGFP enhanced green fluorescent protein; HtH = expression cassettes for GS and GDNF/EGFP in head-to-head configuration; HtT = expression cassettes for GS and GDNF/EGFP in head-to-tail configuration; Mfp = mifepristone; * = $p < 0.05$, *** = $p < 0.001$ in pairwise comparisons of groups with normal distribution and similar variance by non-paired, two-tailed T-tests; n.s. = no significant difference. Statistical power P= (1-ß err prob) > 0.9 for each tested condition. N = 7 animals for each group.

Figure 2


A
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AAV-5
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AAV-1/2
GS-GDNF 1x10e9 vg
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Figure 2: Background-free Mfp-controlled GDNF expression depending on viral titre and serotype

(A) Time scale of experiments shown in panel (B).

(B) Striatal GDNF levels in pg/mg tissue ± SD as achieved by AAV-5-GS-GDNF or AAV-5-GS-EGFP at titres of $3x10^9$ vg/striatum or $1x10^9$ vg/striatum, determined at 7 days or 28 days after Mfp application of 20 mg/kg on three consecutive days. Solid bars = application of Mfp, hatched/white bars = application of DMSO. ** $p< 0.01$, * $p < 0.05$; for statistical powers see text.

(C) Decay kinetics of striatal GDNF after application of 20 mg/kg Mfp on three consecutive days. GDNF levels in pg/mg tissue ± SD after injection of $1x10^9$ vg/striatum of AAV-5-GS-GDNF. *** $p < 0.001$, power (1-ß err prob) = 0.99; * $p < 0.05$, power (1-ß err prob) = 0.77.

(D) Striatal GDNF levels in pg/mg tissue ± SD as achieved by different titres of AAV-1/2-GS-GDNF, measured at 7 days after Mfp application (20 mg/kg on three consecutive days). Solid bars = application of Mfp, hatched/white bars = application of DMSO. *** $p< 0.01$, power (1-ß err prob) > 0.93; * $p < 0.05$; power (1-ß err prob) = 0.77.

All vectors used in head-to-tail (HtT) configuration. Mfp + = i.p. application of 20 mg/kg Mfp on 3 consecutive days; Mfp - = i.p. application of DMSO on 3 consecutive days; DPM = days post Mfp application; tissue for GDNF ELISA was isolated at the indicated times after the first Mfp application.

Statistics by pairwise comparisons of groups with normal distribution and similar variance by non-paired, two-tailed T-tests. n.s. = no significant difference. N = 7 animals for each group.

Figure 3

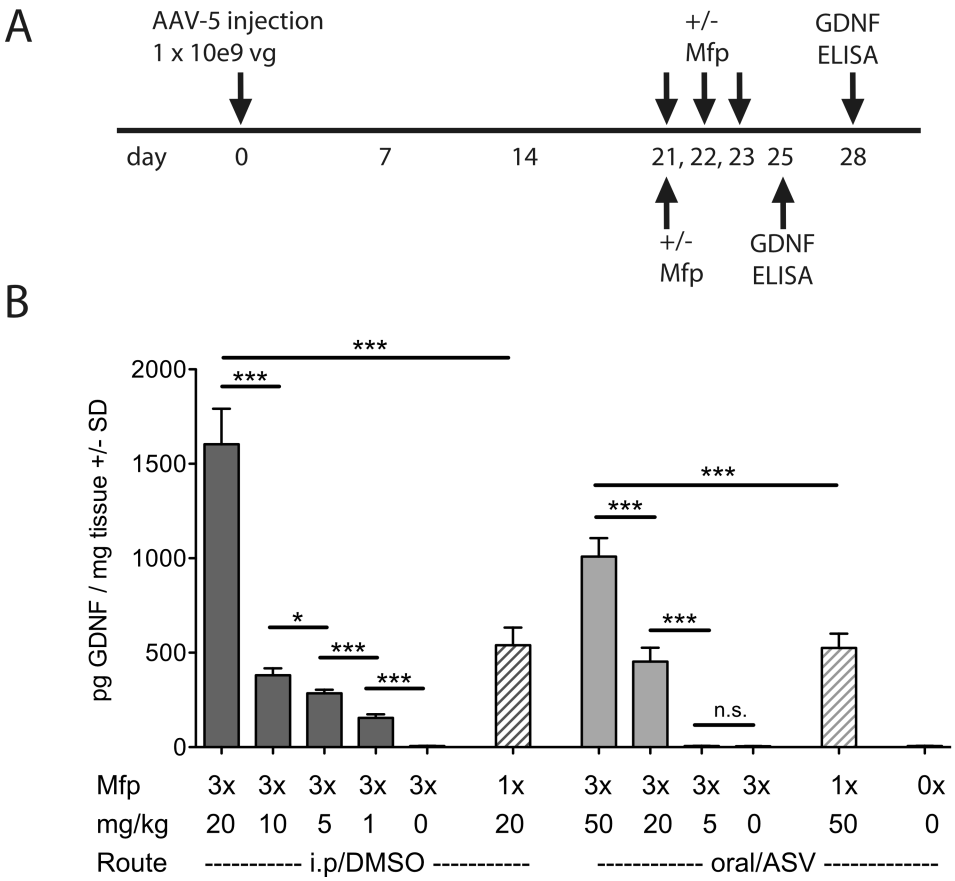

Figure 3: Dose responses to i.p. or oral Mfp application

(A) Time scale of experiments for application of Mfp on three consecutive days or on one day only.

(B) Striatal GDNF levels in pg/mg tissue ± SD achieved by either i.p. application of different doses of Mfp, dissolved in DMSO, or by oral application of different doses of Mfp suspended in an aqueous suspending vehicle (ASV). Mfp was either given on three consecutive days (3x), on one day (1x) or not at all (0x). The dose applied per day is given in mg/kg. * = $p < 0.05$, *** = $p < 0.001$ in pairwise comparisons of groups with normal distribution and similar variance by non-paired, two-tailed T-tests; n.s. = no significant difference. N = 7 animals for each group. Power (1-ß err prob) = 1 for all conditions shown, except for the difference between 3x10 mg/kg i.p and 3x5 mg/kg i.p., where power (1-ß err prob) = 0.72.

Figure 4

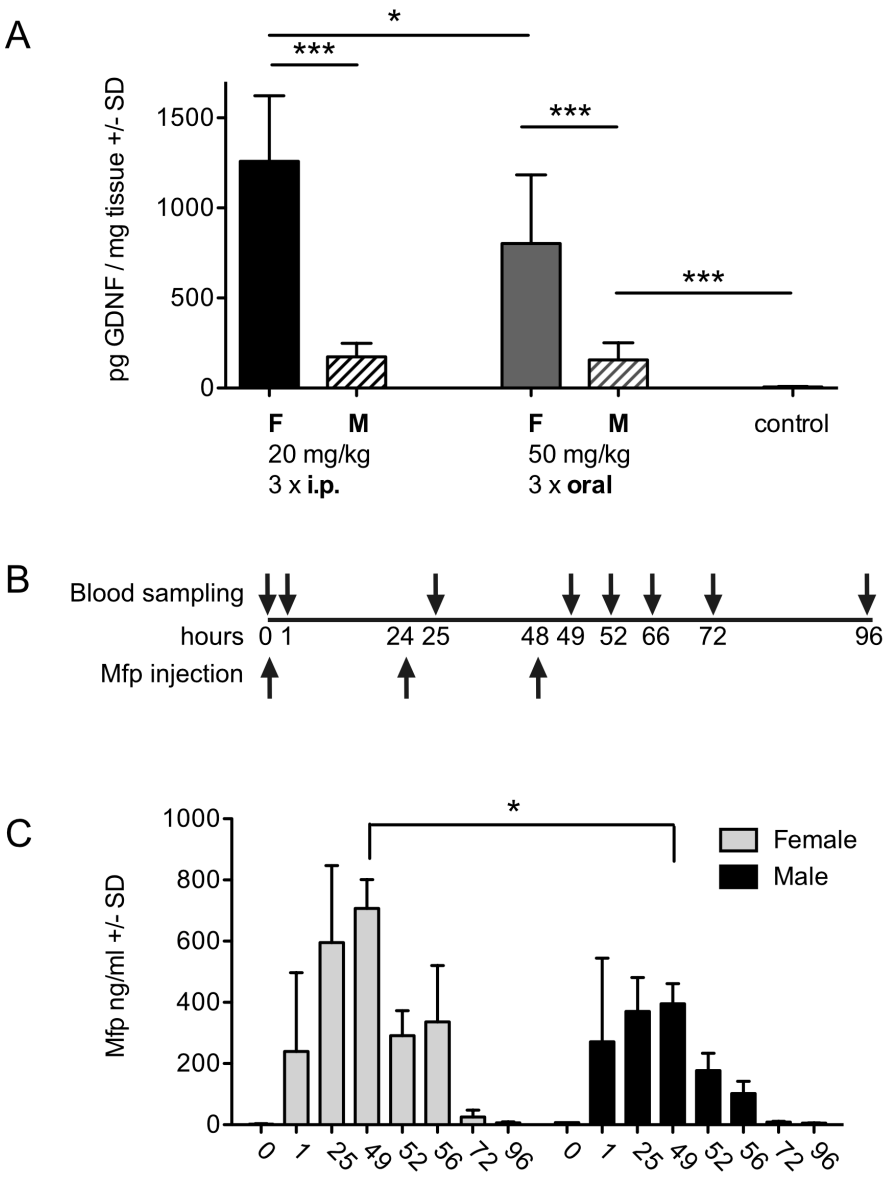

Figure 4: Sex-dependent induction of GDNF expression in rodent brain.

(A) Striatal GDNF levels in pg/mg tissue ± SD after i.p. or oral application of Mfp in female (F) or male (M) rats. * = $p < 0.05$ (power (1-ß err prob) = 0.7; *** = $p < 0.001$ (power (1-ß err prob) = 0.99) in pairwise comparisons of groups with normal distribution and similar variance by non-paired, two-tailed T-tests. N = 7 animals for each group

(B) Time scale of experiments shown in panel (C).

(C) Plasma Mfp levels in ng/ml ± SD after application of Mfp (20 mg/kg, i.p). as outlined in panel (B). Mfp was injected at time points 0, 24 and 48 hours, plasma samples were prepared at time points 1, 25, 49, 52, 66, 72 and 96 hours. Plasma Mfp levels are shown

for females as grey bars and for males as black bars. Satistical analysis by 2-Way ANOVA with Bonferroni´s post hoc test, * = $p<0.05$ (1-ß err prob) = 0.99 N = 5 animals per group.

Figure 5

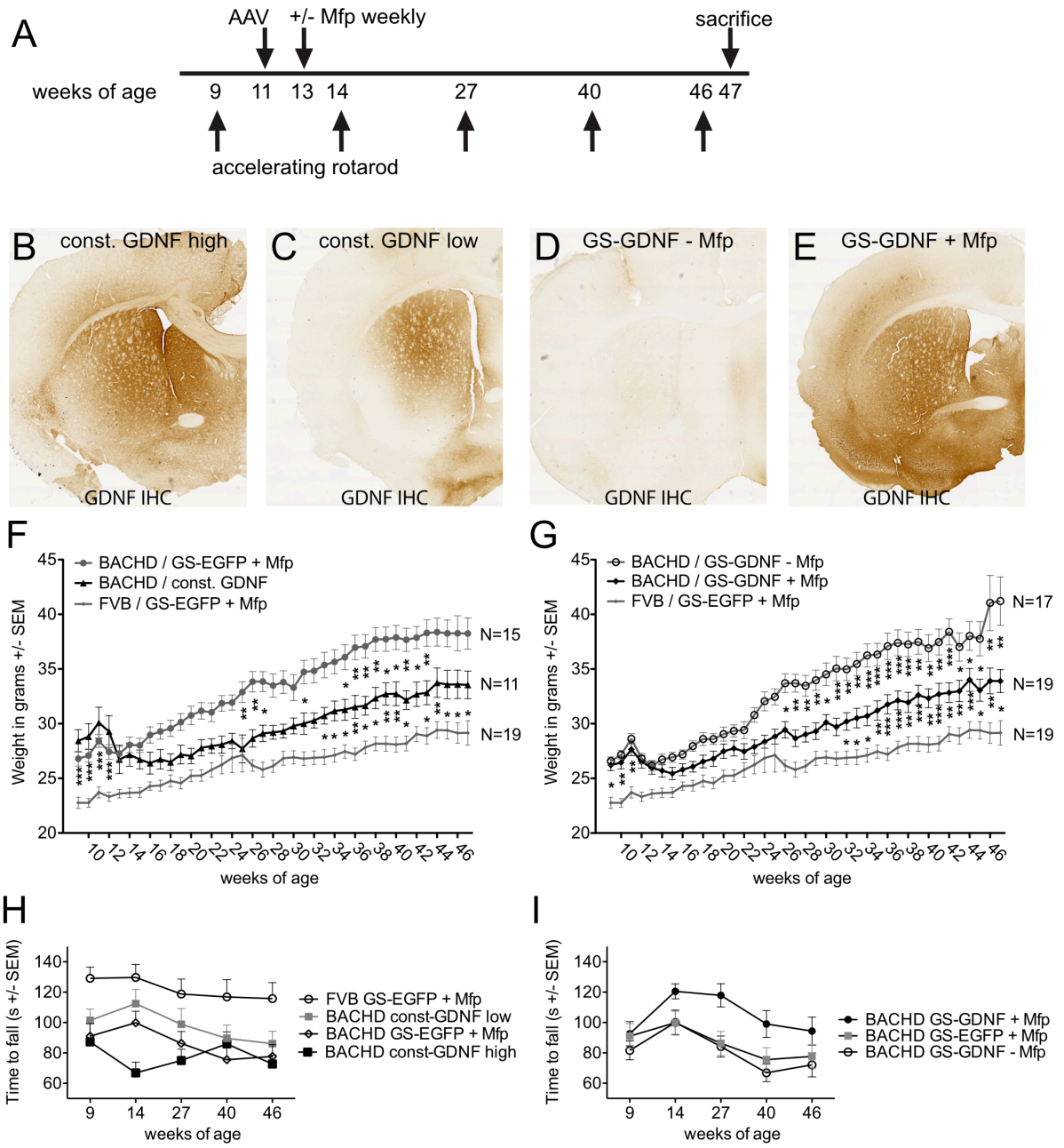

Figure 5: Long-term activation of GDNF expression in the BACHD model of Huntington´s disease

(A) Time scale of experiments shown in (B - I)

(B - E) Histological assessment of GDNF expression by immunohistochemistry at the endpoint of the study at 47 weeks of age. Representative brain sections at the anterior-posterior level of the striatum stained for GDNF are shown for BACHD mice injected with (B) $9x10^9$ vg AAV-5-syn-GDNF (continuous expression, high titre), (C) $3x10^9$ vg AAV-5-

syn-GDNF (continuous expression, low titre), (D) $3x10^9$ vg AAV-5-GS-GDNF and treated with vehicle and (E) $3x10^9$ vg AAV-5-GS-GDNF and treated with Mfp weekly.
(F - G) Weight of animals was recorded weekly from 9 to 47 weeks of age. (F) Comparison of BACHD mice injected with AAV-5-GS-EGFP and treated with Mfp (upper row of data) with BACHD mice injected with AAV-5-syn-GDNF and treated with vehicle (middle row of data) and with FVB mice injected with AAV-5-GS-EGFP and treated with Mfp (lower row of data). (G) Comparison of BACHD mice injected with AAV-5-GS-GDNF and treated with vehicle (upper row of data) with BACHD mice injected with AAV-5-GS-GDNF and treated with Mfp (middle row of data) and with FVB mice injected with AAV-5-GS-EGFP and treated with Mfp (lower row of data). Statistical analysis was performed by 2-way ANOVA with Bonferroni post-tests, significance levels of differences between groups at individual time points are shown between the respective curves. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; Numbers of animals in each group are outlined besides the curves. Power (1-ß err prob) > 0.9 for each statistically significant difference.
(H, I) Motoric capabilities were assessed at 9, 14, 27, 40 and 46 weeks of age on the accelerating rotarod and are shown as the time to fall for FVB control mice injected with AAV-5-GS-GDNF (+ Mfp), and BACHD mice injected with AAV-5-syn-GDNF at high or low titre, or AAV-5-GS-EGFP (+Mfp) in panel (H), and for BACHD mice injected with AAV-5-GS-GDNF (+ or - Mfp) and AAV-5-GS-EGFP (+ Mfp) in panel (I). These groups were analysed by ANCOVA (analysis of co-variance) in order to detect statistically significant differences of GDNF expression on rotarod performance independent from GDNF´s influence on body weight gain, but did not reveal any such effects on a significant level.

Figure 6 revised

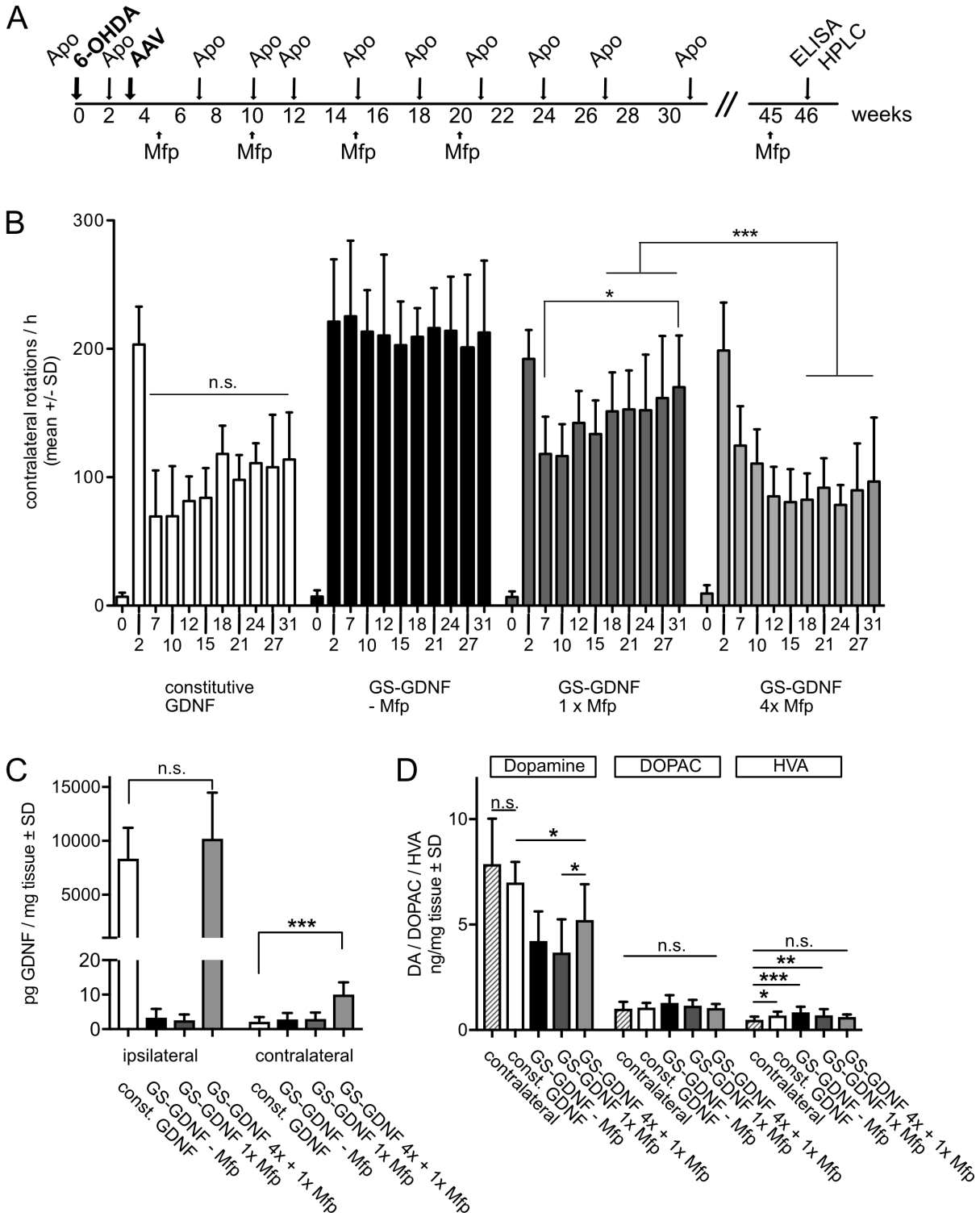

Figure 6: Restoration of motor impairments in the 6-OHDA model of Parkinson´s disease.

A) Time scale of experiments shown in panel (B, C, D). Apo = apomorphine-induced rotations; 6-OHDA = striatal 6-OHDA injection; AAV = striatal AAV injection; Mfp = i.p.

application of mifepristone; ELISA = quantification of striatal GDNF levels; HPLC = quantification of striatal dopamine, DPOPAC and HVA levels. Animals received Mfp either at 5 weeks, or at 5, 10, 15 and 20 weeks and once again at 45 weeks after the 6-OHDA lesion.

B) Apomorphine-induced rotation behaviour in rats injected with constitutively GDNF expressing vector (white bars), in rats injected with GS-GDNF but receiving only vehicle (black bars), in rats injected with GS-GDNF and one induction of GDNF expression by Mfp (dark gray bars), and in rats injected with GS-GDNF and three inductions by Mfp (light gray bars), as determined at 0, 2, 7, 10, 12, 15, 18, 21, 24, 27 and 31 weeks after 6-OHDA lesion. The number of contralateral rotations per hour is shown as mean +/- SD.

Statistical analysis by 1-way ANOVA with Tukey´s post hoc test. * $p < 0.05$; *** $p < 0.001$; n.s. = not significant. Power (1-ß err prob) > 0.98 for all shown comparisons. N = 5 - 7 animals per group at the latest time point of the experiment.

C) Re-application of Mfp at week 45 after 6-OHDA in animals of the group that had received 4 inductions of GDNF expression at weeks 5, 10, 15 and 20. Striata were collected one week after Mfp application, the amounts of GDNF detected in ipsilateral and contralateral striatum of all groups is shown as pg GDNf / mg tissue ± SD. Statistical analysis by 1-way ANOVA with Tukey´s post hoc test. *** $p < 0.001$; n.s. = not significant. Power (1-ß err prob) > 0.98 for all shown comparisons. N = 5 -7 animals per group.

D) Determination of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in brains of animals at week 46 after 6-OHDA. Amounts of neurotransmitter and metabolites are shown in ng/mg tissue ± SD. Statistical analysis by 1-way ANOVA with Tukey´s post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = not significant. N = 5 - 7 animals per group.

White bars = AAV-syn-GDNF, constitutive GDNF expression (const. GDNF); black bars = AAV-GS-GDNF, without any application of mifepristone (- Mfp); dark gray bars = AAV-GS-GDNF, with one application of Mfp at week 5 after 6-OHDA (1x Mfp); light gray bars = AAV-GS-GDNF, with 4 applications of Mfp at weeks 5, 10, 15 and 20 (4x Mfp) plus one application of Mfp at week 45 (4x + 1x Mfp); hached bars in (D) = DA, DOPAC, HVA amounts in contralateral striata. Quantification of GDNF and DA/DOPAC/HVA was performed from tissue samples of the same animals, by using 50% of each striatum for ELISA or HPLC, respectively.

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Conflicts of interest

The authors state that they have no conflicts of interest to declare. J.L. and P.K. are employees of uniQure, a company interested in the commercialisation of gene therapy medicines.

Author contributions

Conceptualization, P.K., N.D., S.K.; Methodology, J.T., F. S.; Formal analysis, A.M., N.D., S.K.; Investigation, S.C., J.T., V.Z., G.V., C.P., M.R., A.R. J.L., F.S., S.K.; Writing - Original Draft, P.K, N.D., S.K.; Supervision, M.B., P.K., N.D., S.K.; Funding Acquisition, P.K., N.D., S.K.

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