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Differential transgene expression profiles from rAAV2/1 vectors using the tetON and CMV promoters in the rat brain.

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Running title:

Specificity of CMV and tetON promoters in the CNS.

ABSTRACT

The biodistribution of transgene expression in the CNS after localized stereotaxic vector delivery is an important issue for safety of gene therapy for neurological diseases. The cellular specificity of transgene expression from rAAV2/1 vectors using the tetON expression cassette in comparison with the CMV promoter was investigated in the rat nigrostriatal pathway. After intrastriatal injection, although GFP was mainly expressed into neurons with both vectors, the percentage of GFP^+ astrocytes was 5-fold higher with the CMV vector. The relative proportions of DARPP-32^+ projection neurons and parvalbumin$^+$ interneurons were respectively 13:1 and 2:1 for the CMV and tetON vectors. $DARP32^+$ neurons projecting to the globus pallidus were strongly GFP^+ with both vectors, whereas those projecting to the substantia nigra pars reticulata (SNpr) were efficiently labeled by the CMV but poorly by the tetON vector. Numerous GFP^+ cells were evidenced in the subventricular zone with both vectors. However, in the olfactory bulb (OB), GFP^+ neurons were observed with the CMV but not the tetON vector. We conclude that the absence of significant amounts of transgene product in distant regions (SN and OB) constitutes a safety advantage of the AAV2/1-tetON vector for striatal gene therapy.

Midbrain injections resulted in selective GFP expression in tyrosine hydroxylase$^+$ neurons by the tetON vector whereas with the CMV vector, GFP^+ cells covered a widespread area of the midbrain. The biodistribution of GFP protein corresponded to that of the transcripts and not of the viral genomes. We conclude that the rAAV2/1-tetON vector constitutes an interesting tool for specific transgene expression in midbrain dopaminergic neurons.

INTRODUCTION

An AAV serotype 2 vector constitutively expressing Neurturin, a glial cell line-derived neurotrophic factor (GDNF) analog has shown promising results in a phase I clinical trial (Marks *et al*., 2008). However, adverse effects which have been described after administration of GDNF recombinant protein delivery (Nutt *et al*., 2003; Hovland *et al*., 2007) or uncontrolled GDNF gene transfer (Kirik *et al*., 2000a; Georgievska *et al*., 2002; Eslamboli *et al*., 2005) should be taken into account for future developments. These clinical trials and animal studies have shown that key factors for the success of this therapeutical approach are: a widespread distribution of GDNF in the putamen (Kirik *et al*., 2000b; Peterson and Nutt , 2008) ; the absence of significant GDNF tissue concentrations in the substantia nigra which has been shown to induce aberrant sprouting (Georgievska *et al*., 2002) as well as an optimal dose of GDNF providing neuroprotection in the absence of metabolic effects (Eslamboli *et al*., 2005). Therefore, an ideal viral vector for GDNF gene delivery in Parkinson's disease should: -diffuse to a significant portion of the striatum; -not transduce projecting neurons, in particular the gaba-ergic neurons projecting to the substantia nigra pars reticulata; -not be retrogradely transported, in particular to the substantia nigra pars compacta; -allow to modulate and eventually switch-off transgene expression.

Different AAV serotypes show different cellular tropisms in the brain (Davidson *et al*., 2000; Passini *et al*., 2003; Burger *et al*., 2004; Paterna *et al*., 2004; McCown, 2005; Cearley *et al*., 2006; Taymans *et al.,* 2007; Klein *et al*., 2008). Cross-packaging of vectors using ITRs from AAV2 with capsids from other serotypes (Rabinowitz *et al*., 2002) allowed to evaluate the contribution of the capsid to the cellular transduction specificity in the brain (Wang *et al*., 2003; Burger *et al*., 2004; Paterna *et al*., 2004; Cearley *et al*., 2006; Klein *et al*., 2006; Klein *et al*., 2008). It has been suggested that receptors differentially expressed on the cell surface account for the observed differences. Indeed, AAV2 utilizes heparan sulfate proteoglycans as

receptor (Summerford and Samulski, 1998) and FGF-R (Qing *et al*., 1999) and αVβ5-integrin (Summerford *et al*., 1999) as co-receptors, AAV5 utilizes PDGF receptor (Di Pasquale *et al*., 2003) and sialic acid (Walters *et al*., 2001) for internalization whereas AAV1 and AAV6 require alpha2,3 and alpha2,6 N-linked sialic acids for efficient binding (Wu *et al*., 2006). As a consequence of these different mechanisms of cell entry, the biodistribution of trangene expression in the brain depends on the serotype. In particular, AAV2-mediated transduction of the striatum is restricted to the vicinity of the delivery site, whereas a widespread distribution of the transgene product can be obtained with a single injection of AAV1, AAV5, AAV7 or AAV8 (Taymans *et al*., 2007).

However, the promoter used to drive transgene expression also influences the cellular specificity as well as the time course of transgene expression. Klein and coll. (Klein *et al*., 1998) have first demonstrated that the decrease of transgene expression initially observed with AAV2 vectors using the CMV promoter can be overcome by using the cellular neuron-specific enolase (NSE) promoter. Furthermore, discrepancies observed between the cellular specificity of transgene expression from rAAV vectors of a defined serotype, suggest that capsid entry is not the only factor involved. For example, in the mouse striatum, transgene was expressed in both neurons and astrocytes when using rAAV2/1-CMV (Wang *et al*., 2003) whereas transgene expression was restricted to neurons when using rAAV2/1 with the GUSB promoter (Paterna *et al*., 2004). A direct demonstration that promoter usage affects the cellular specificity of transgene expression came from a study of Haberman and coll. (Haberman *et al*., 2002) showing that rAAV2/2 vectors using respectively the CMV promoter and the tetOFF system were driving transgene expression in distinct subpopulations of neurons resulting in opposite physiological effects.

The tet promoter consists in a minimal CMV promoter in which the enhancer sites have been replaced by tetracycline operator sites (Gossen and Bujard, 1992). These operator

sites bind the tTA/rtTA transactivator (Gossen and Bujard, 1992; Gossen *et al*., 1995), a synthetic transcription factor in which the transactivating domain derives from the Herpes VP16 transactivator and the DNA-binding domain from the bacterial tetracycline repressor. Both humoral and cytotoxic immune responses directed against the tTA/rtTA transactivator and severely impairing transduction efficiency have been reported after intramuscular (Favre *et al*., 2002) but not intracerebral administration (Fitzsimons *et al*., 2001; Xiong *et al*., 2008).

Direct gene transfer into the brain also has potential for studying gene function. However, because of the heterogenous cellular composition of the brain, cell type-specific gene expression would constitute a valuable tool for studying functional genomics as well as for better controlled gene therapy applications.

MATERIAL AND METHODS

Plasmids

The pAC1-M2-WPRE plasmid comprising AAV ITRs bracketing the bidirectional tetracycline-responsive cassette expressing both rtTAM2 and EGFP and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Donello *et al*., 1998) introduced immediately downstream to the rtTAM2 sequence has been described previously (Chtarto *et al*.,2007).

Recombinant viruses

High titers of recombinant rAAV2/1-rtTAM2- EGFP, rAAV2/1-rtTAM2-WPRE-EGFP and recombinant rAAV2/1-CMV-EGFP viral stocks were produced at the Gene Vector Production Network (Laboratoire de Thérapie Génique, Nantes, France) as described (Salvetti *et al*.,1999). Titers expressed as viral genomes per ml were: rAAV2/1-rtTAM2-WPRE-EGFP, 1.7 X 10^{11}; rAAV2/1-rtTAM2-EGFP, 2 X 10^{11}, rAAV2/1-CMV-EGFP, 2.0 X 10^{11}, rAAV1-rtTAM2-GDNF, 7.0 X 10^{11}.

Stereotaxic injections

Adult female Wistar rats of 250g (Harlan, Indianapolis, Indiana) were used for unilateral intracerebral injections as previously described (Tenenbaum *et al*., 2000). The animals were anesthetized using a mixture of ketamine (Ketalar, Pfizer, New York City, NY; 100 mg kg^{-1} ip) and xylazin (Rompun, Bayer, Leverkusen, Germany; 10mg kg^{-1} ip). Injections were made according to coordinates defined by Paxinos and Watson (Paxinos and Watson, 1997) using a Kopf stereotaxic apparatus (David Kopf, Tujunga, California). The injection coordinates were respectively for the striatum, 0.5 mm anterior, 2.8 mm lateral to bregma and 5 mm below the dural surface and 5.3 mm posterior, 2.2 mm lateral to bregma and 6.6 mm below the dural

surface for the midbrain. Viral particles diluted in 2 µl D-PBS (BioWittaker, Walkersville, MD) were infused using a motor-driven Hamilton syringe (Bonaduz, Switserland; 0.2 µl/ min.) with a 30G needle. After injection, the needle was left in place for 5 minutes in order to allow diffusion of the viral suspension in the parenchyma. The needle was then slowly lifted 1 mm up and left in place 5 minutes, then slowly removed. Since after the surgery, the animals were given water containing 3% sucrose and doxycycline (600 µg/ml or 1g/l for qPCR analysis).

Animals were killed at the indicated time after surgery by an overdose of anesthetic (200 mg kg^{-1} ketamine and 20 mg kg^{-1} xylazine). For immunohistological analysis, animals were perfused through the ascending aorta first with 150-200 ml of saline (NaCl 0.9%), then with 300 ml 4% paraformaldehyde in 0.1M phosphate buffer (PF4). After overnight post-fixation in PF4 at 4°C, brains were transferred to phosphate buffer saline (PBS) and stored at 4°C.

GDNF ELISA assay

For determination of GDNF brain tissue levels, animals were decapitated and the brains were removed, gradually frozen in isopentane/dry ice (-10°C for 10 sec. then -20°C for 20 sec.) and stored at –80°C. Two hundred micron coronal slices were cut using a cryostat and striata and midbrains were dissected out, weighed and processed for ELISA assay. Tissue was sonicated (Branson Sonifier 250, output 2, 30% duty cycle, 10 sec(Branson Ultrasonics, Danbury, CT) in M-Per buffer (Pierce, Thermo Fisher Scientific Inc., Rockford, IL) supplemented with protease inhibitors cocktail (Boehringer Ingelheim GmbH, Ingelheim, Germany). Samples were centrifuged at 10,000 x g and supernatants were harvested and their protein concentration analysed using the BCA Assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL). GDNF tissue levels were measured according to the manufacturer's protocol (BioSource, Nivelles, Belgium) and expressed in pg per mg of tissue. Recombinant human

GDNF (R&D System, Minneapolis, MN) was used to establish the standard curve. A cross reaction of 100% was demonstrated with recombinant rat GDNF (Oncogene Research Products, Calbiochem, Merck, Frankfurter, Germany).

Immunofluorescence

Coronal sections (50 µm) obtained using a vibratome (Leica Microsystems, Wetzlar, Germany) were sequentially incubated in: i) THST (50mM Tris, 0.5 M NaCl, 0.5% Triton X100 (Merck, Frankfurter, Germany) pH7.6) containing 10% horse serum for 2 hours.; ii) rabbit anti-GFP IgG (1:3000, Molecular Probes, Invitrogen, Carlsbad, CA) diluted in THST containing 5% horse serum for 16 hours at 4°C; iii) donkey anti rabbit IgG conjugated with biotin (Amersham, GE Healthcare, Munich, Germany) diluted 1:600 in THST containing 5% horse serum, 2 hour at room temperature; iv) streptavidin conjugated to cyanine 2 (1:300; Jackson ImmunoResearch, West Grove, PA) in THST containing 5% horse serum, 2 hours at room temperature. Three washings in TBS (Tris 10mM, NaCl 0.9%, pH7.6) of 10 min. were performed between each step.

For double immunofluorescence, these incubations were combined with mouse monoclonal antibodies (anti-NeuN (1:200, Chemicon, Millipore, Billerica, MA); anti-glutamic acid decarboxylase (1:200, Chemicon, Millipore, Billerica, MA); anti-parvalbumin (1:2000, Sigma-Aldrich, St Louis, MO) or anti-glial fibrillary acid protein (GFAP, 1:200, Chemicon, Millipore, Billerica, MA)) (step ii); and donkey anti-mouse IgG coupled to cyanine 3 (1:200; Jackson ImmunoResearch, West Grove PA) in THST containing 5% horse serum) (step iv).

For DARPP-32/GFP immunofluorescence, a mouse monoclonal anti-gfp antibody (a gift from J.D.Franssen, Euroscreen, Gosselies, Belgium) at a 1:50 dilution was combined with rabbit anti DARPP-32 IgG (1:200, Chemicon, Millipore, Billerica, MA) in THST containing 5% horse serum 16 hours at 4°C (step ii). The primary antibodies were detected using a donkey

anti-rabbit IgG conjugated with biotin (1:200, Amersham, GE Healthcare, Munich, Germany) and a goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) antibody (1:100, from tyramide signal amplification (TSA) kit, Molecular Probes, Invitrogen, Carlsbad, CA) diluted in Blocking Reagent provided by the TSA kit (Molecular Probes, Invitrogen, Carlsbad, CA), 1 hour at room temperature (step iii). Step iv was performed as described above. Step v: the monoclonal anti-gfp then revealed using the TSA kit according to manufacturer's protocol.

Sections were mounted using FluorSave mounting fluid for fluorescence (Calbiochem, Merck, Frankfurter, Germany) and photographed using a Zeiss Axiophot 2 microscope equipped with FITC and TRITC filters (Car Zeiss, Göttingen, Germany) as well as an AxioCam digital camera (Carl Zeiss, Gottingen, Germany). Images were acquired as jpeg files using the KS300 software (Car Zeiss, Gottingen, Germany).

Confocal microscopy

Co-labelling analysis were performed on pictures taken on at least three different sections within the transduction zone using an automatic image analysis system (Lasersharp version 3.2 (Biorad, Hercules, CA) coupled to Axiovert 100 microscope, (Carl Zeiss, Gottingen, Germany)). Pictures were then processed and analysed with the Image J software (NIH, USA)

Stereology

The number of GFP-positive cells and the volume of the labelled brain area were evaluated by stereological procedures based on the Cavalieri principle (Sterio, 1984). For each animal, serial sections with an interval of 500 µm were analyzed by means of the optical fractionator

of the Stereoinvestigator software (MBF Bioscience, Williston, VT) connected with a CCD video camera to the microscope (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

For GFP stainings, cryostat sections (50 μm) were sequentially incubated in: i) 3% H_2O_2 in TBS (Tris 10mM, 0.9% NaCl, pH7.6) for 30 min.; ii) THST (50mM Tris, 0.5 M NaCl, 0.5% Triton X100 pH7.6) containing 10% horse serum for 1 hour.; iii) rabbit anti-GFP IgG (Clonetech, Palo Alto, CA) diluted 1:3000 in THST containing 5% horse serum overnight at 4°C; iv) donkey anti rabbit IgG conjugated with biotin (Amersham, GE Healthcare, Munich, Germany) 1:600 in THST containing 5% horse serum, 2 hours at room temperature. The peroxidase staining was revealed using the ABC Elite vectastain kit and diaminobenzidine (Vector, NTL Laboratories, Brussels, Belgium), according to the manufacturer's protocol. Sections were mounted on gelatin-coated slides, dehydrated and mounted using DPX mounting fluid (Sigma-Aldrich, St Louis, MO). Sections were photographed using a Zeiss Axiophot 2 microscope (Carl Zeiss, Gottingen, Germany).

For CD5 and CD11b stainings, an identical protocol was used for steps i) and ii), then sections were incubated with mouse anti-CD5 IgG (1:500, Serotec, MorphoSys, Dusseldorf, Germany) or mouse anti-CD11b IgG (1:500, Serotec, MorphoSys, Dusseldorf, Germany) (step iii), then goat anti-mouse IgG conjugated with HRP (Molecular Probes, Invitrogen, Carlsbad, CA, from TSA kit) diluted in Blocking Reagent provided with the TSA kit (Molecular Probes, Invitrogen, Carlsbad, CA) was added for 2 hours at room temperature (step iv); and v) diaminobenzidine (Vector, NTL Laboratories, Brussels, Belgium), according to the manufacturer's protocol. Sections were photographed using a Zeiss Axiophot 2(Carl Zeiss, Gottingen, Germany) microscope and optical density of defined regions at short distance from the needle tract (10 μm) measured using the Image J Software (NIH, USA).

The ratio of the mean optical densities of 3 sections on the injected side and symmetrical sections on the intact side was taken as a measure of microglial cells activation (CD11b) or T cells infiltration (CD5) induced by vector injection and transgene expression.

Tissue sample collection

Animals injected with 4.0 X 10^8 viral particles of rAAV2/1-rtTAM2-EGFP or rAAV2/1-CMV-EGFP were anesthetized using an overdose of anesthetic (200 mg kg^{-1} ketamine and 20 mg kg^{-1} xylazine i.p.) and then decapitated. The brains were dissected out and frozen to -20°C in a bath of methyl-butane using dry ice, before transfer to -80°C for conservation. The brains were equilibrated 1 hour at -20°C before being sliced into 1 mm thick sections using a brain matrix (Alto, Roboz, Gaithersburg, MD). Tissue punches corresponding to the substantia nigra or to the dorsal midbrain were collected from 3 slices around the injection site. Samples were then kept at -80°C before RNA and Hirt DNA extraction.

mRNA extraction

Messenger RNA extractions were performed using the MagNA Pure LC Instrument (Roche Applied Bioscience, Basel, Switserland) using the MagNA Pure LC mRNA Isolation kit II (Roche Applied , Basel, Switserland) according to the manufacturer's recommendations.

Hirt low molecular weight DNA extraction

Tissue samples (approx. 10 mg) were homogenized in 300 µl lysis buffer (Tris 10 mM pH 8.0, EDTA 10 mM, SDS 1%) containing 1 µl of DNase-free RNase (10 mg/ml; Roche Applied Bioscience, Basel, Switserland) and Proteinase K (Roche Applied Bioscience, Basel, Switserland) were added to final concentrations of 0.5 and 1 mg/ml, respectively. The reaction was continued for 120 min at 37°C, after which NaCl was added to a final

concentration of 1.1 M. Following overnight precipitation at 4°C, samples were centrifuged at 14,000 rpm in a tabletop microcentrifuge for 1 hour and low molecular weight DNA (hDNA) (Hirt, 1967) was purified from the supernatant by standard phenol-chloroform extractions (twice), chloroform extractions (twice) and ethanol precipitation. The final DNA pellet was resuspended in 50 µl of TE buffer. Fuether RNase digestion was performed for 30 min at room temperature by adding RNase (DNase free, Roche Applied Bioscience, Basel, Switserland) at a final concentration of 0.2 ng/ µl).

Reverse transcription

mRNA samples were converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Bioscience, Basel, Switserland) according to the manufacturer's recommendations using 3 µl of the RNA sample and anchored-oligo $(dT)_{18}$ primers.

Real-time quantitative PCR

Gfp sequences were quantified using real time SYBRgreen quantitative PCR analysis (Roche Applied Bioscience, Basel, Switserland) on a LightCycler 1.5 apparatus (Roche Applied Bioscience, Basel, Switserland). Tyrosine 3-monooxygenase/tryptophan 5 monooxygenase activation protein, zeta polypeptide (YWHAZ) and actin γ2 house keeping genes were used to normalize *gfp* expression levels. Primers used were: for *gfp*: forward: 5'-GCAGAAGAACGGCATCAAGGT-3'; reverse: 5'-ACGAACTCCAGCAGGACCATG-3', for actin γ2: forward: 5'-TACCCTATTGAGCACGGCAT-3'; reverse: 5'-CGCAGCTCGTTGTAGAAGGT-3' and for YWHAZ: forward: 5'-CAAGCATACCAAGAAGCATTTGA-3'; reverse: 5'-GGGCCAGACCCAGTCTGA-3'. PCR conditions were: 95°C for 10 min, 45 cycles of 95°C 10 sec, 60°C 30 sec, 72°C 20 sec using 5 µl of the sample. Hirt DNA samples were analyzed for *gfp* copy number using a

standard curve based on serial dilutions of plasmids containing a rAAV genome harbouring the *gfp* sequence. cDNA samples were analyzed expression levels of *gfp,* actin γ2 and YWHAZ. Data analysis and normalizations were performed using the qBase software (Ghent University, Belgium).

Statistical analyses

For all data, exept qPCR results, the means ± standard deviations are shown. For qPCR data, the mean ± standard error of the mean are given.

Data were analyzed using the Graph Pad Software Prism 3.0 (Graph Pad Software, La Jolla, CA). Unpaired Student t tests or one-way ANOVA were performed as indicated.

Animal housing and ethics

Animals were maintained, 4 in each cage, in a 12:12 h light–dark cycle with free access to rat chow and water. All experimental procedures were conducted in accordance with the Belgium Biosafety Advisory Committee and with the ethical committee of the Faculty of Medicine (CEBEA, ULB).

RESULTS

1. Intrastriatal injection

1.1.Striatum

We have previously shown that the rAAV2/1-tetON vector drives doxycycline (dox)-dependent GDNF expression in the rat striatum (Chtarto *et al*., 2007). We have extended these data and shown that the striatal amount of GDNF could be modulated in function of the dox dose (Fig. 1).

Since GDNF is secreted, it does not allow an immuno-histological identification of transduced cells. Therefore, in the present study, we have used vectors expressing GFP, which have an intracellular localisation.

Four 10^8 viral particles of crosspackaged rAAV2/1-rtTA-M2-WPRE-EGFP and rAAV2/1-CMV-EGFP were infused into the right striatum of adult rats.

GFP-positive cells were observed in a widespread region of the forebrain (Fig.. 2 a,b) covering a volume of 32.06 ± 10.84 mm^3 and 28.48 ± 3.49 mm^3 for the CMV and tetON vectors, respectively. GFP-positive cells were mainly located in the striatum but also in the globus pallidus, the sub-ventricular zone and the external capsule. The total number of GFP-positive cells per animal was significantly higher ($p< 0.001$; Student t test) for the CMV vector (103,131 ± 8,469; n=4) than for the tetON vector (46,298 ± 5,545; n=4).

The majority of GFP-positive cells colabeled with the NeuN neuronal marker (85.1±4.7% and 89.2 ±6.1% for the CMV and the tetON vector, respectively; Fig.3). However, the relative proportion of DARPP-32 gaba-ergic neurons and parvalbumin-positive interneurons varied between the 2 vectors. Significantly more DARPP-32-positive cells were expressing GFP with the CMV vector (91.6±2.3% of the GFP-positive cells versus 66.7±5.1 % for the tetON

vector; p< 0.001, Student t test; Fig. 3b) whereas significantly more parvalbumin-reactive neurons were expressing GFP with the tetON vector (29.0±10.9% versus 7.1±1.3% for the CMV vector; p< 0.05, Student t test; Fig.3b).

The CMV vector lead to GFP expression in relatively more astrocytes (5.9 ± 1.9 % of GFP-positive cells) than the tetON vector (1.2 ± 2.2%) (p < 0.05; Fig.3b).

1.2.Anterograde transport to the globus pallidus and substantia nigra pars reticulata

GFP-positive fibers were observed in the substantia nigra pars reticulata (SNr) after intrastriatal injection of the CMV (Fig. 4a) but not of the tetON vector (data not shown). These GFP-positive fibers were co-labeled using an antibody directed toward glutamic acid decarboxylase (GAD) (Fig. 4a) characterizing the efferent projections of striatal gaba-ergic inhibitory neurons. These data suggest that striato-nigral gaba-ergic projection neurons were efficiently expressing GFP when using the CMV but not the tetON vector. It should be noted that using a sensitive peroxidase biotin/streptavidin amplified immunohistochemistry for GFP, stained fibers could be evidenced in the SNr of rAAV2/1-tetON-injected animals (Fig. 4b). The staining was however weaker than for the CMV vector (Fig. 4b). To compare the intensity of the stainings, the ratio of the mean optical densities of 3 sections on the injected versus intact sides was taken as a measure of fiber density and was found to be significantly lower for the tetON vector (Fig. 4c). The extent of the staining was also more restricted for the tetON vector. Indeed, it covered a smaller part of the SNr (Fig.4b). The antero-posterior distribution of the labeled region was similar for both vectors (data not shown).

Accordingly, intrastriatal injection of AAV2/1 vectors expressing the GDNF cDNA, resulted in low amounts of GDNF (not significantly higher than the endogenous level) in the substantia nigra (Fig.1).

In contrast, GFP-positive projections of DARPP-32-positive gaba-ergic neurons in the globus pallidus were evidenced with both the CMV and the tetON vector (Fig. 4b) and the fiber densities were not significantly different for the 2 vectors (Fig. 4c).

1.3.Retrograde transport to substantia nigra pars compacta

After intrastriatal injection of rAAV2/1-CMV-EGFP, GFP-positive cells (111.7±30.7 per animal; n=3) co-labeling with the dopaminergic neuron marker tyrosine hydroxylase were evidenced in the SNpc (Fig. 5), suggesting that rAAV2/1 viral particles were retrogradely transported from the injection site in the striatum to the SNpc. In contrast, no GFP-positive cell was detected by immunofluorescent labeling in the tetON vector group (data not shown). To determine if a weak GFP expression from the tetON vector could be evidenced using a more sensitive detection technique, midbrain sections were labeled by immunohistochemistry for GFP using a sensitive biotin-streptavidin amplification step (see Material and Methods). Few weakly labeled GFP-positive cells were evidenced in the SNpc of AAV-tetON injected animals (data not shown).

1.4. Subventricular zone

It has been previously reported that, injection of rAAV2/1 vectors using the CMV promoter in the striatum of mice (Wang *et al*., 2003) resulted in efficient transduction of the subventricular zone. In this study, GFP-positive cells were found in the olfactory bulb, the region in which neural stem cells of the subventricular zone terminally differentiate (Alvarez-Buylla and Lim, 2004).

In most animals, intrastriatal injections of rAAV2/1-rtTA-M2-WPRE-EGFP and rAAV2/1-CMV-EGFP resulted in efficient GFP expression in the subventricular zone (Fig. 6a,b). To determine whether neural progenitors cells present in the subventricular zone (Alvarez-Buylla

and Lim, 2004) could possibly be transduced, we examined the olfactory bulbs for GFP expression. We detected numerous GFP-positive cells (5406.7±5049.9 per animal; n=3), 5 weeks after intrastriatal injection of rAAV2/1-CMV-EGFP) (Fig. 6c) but not rAAV2/1-rtTA-M2-WPRE-EGFP (2.0±2.7 per animal; n=3) (data not shown).

1.5. Immune response

Markers for infiltrating lymphocytes (CD5) and microglial cells (CD11b) were absent or restricted to the needle tract for both rAAV vectors, whereas a strong labeling for both markers was observed after injection of an adenoviral vector expressing LacZ . The ratio of the mean optical densities of 3 sections on the injected side and intact sides was taken as a measure of microglial cells activation (CD11b) or T cells infiltration (CD5) induced by vector injection and transgene expression (data not shown). The optical density of sections labeled for the astrocytic marker GFAP (known to be upregulated during inflammation) was also similar for sham-injected animals and for both AAV vectors (data not shown).

2. Midbrain Injections

2.1 Substantia nigra pars compacta

Four 10^8 viral particles of rAAV2/1-rtTA-M2-WPRE-EGFP and rAAV2/1-CMV-EGFP were infused into the right midbrain of adult rats dorsal to the substantia nigra. To determine if the WPRE sequence could possibly affect the biodistribution of transgene expression, 4 X 10^8 viral particles of rAAV2/1-rtTA-M2- EGFP were injected at the same coordinates. Whereas injections of rAAV2/1-CMV-EGFP resulted in a wide GFP-positive area including various structures i.e. SNpc, SNr, VTA and more dorsal structures (Figs 7a,b), the tetON vector exclusively expressed GFP in the SNpc and VTA, regardless of the presence of the WPRE sequence (rAAV2/1-rtTA-M2-WPRE-EGFP : see Figs.7c,d,e ; rAAV2/1-rtTA-M2-

EGFP : data not shown). For the 3 vectors, the GFP-positive area covered the entirety of the SNpc and VTA.

To analyze the specificity and the efficiency of rAAV2/1 vectors for dopaminergic neurons of the SNpc, colabeling for GFP and tyrosine hydroxylase (TH) was performed. Double-labeled cells were identified by confocal microscopy. For each animal a total number of about 150 cells was counted in 3 sections at 250-µm intervals, taken around the injection site.

Specificity: Among the GFP-positive cells observed in the SNpc, a large proportion were TH-positive for the three vectors. Indeed, 54.05 ± 11.46% , 72.78 ± 17.39% and 76.75 ± 5.70% of the GFP-positive cells were also TH-positive for rAAV2/1-CMV-EGFP, rAAV2/1-rtTA-M2-WPRE-EGFP and rAAV2/1-rtTA-M2- EGFP, respectively. The percentage of TH-positive cells among GFP-positive cells was not different between the 3 vectors (one-way ANOVA).

Efficiency: A large proportion the tyrosine hydroxylase-positive cells were expressing GFP. Indeed, respectively 42.72 ± 20.57 % , 65.51 ± 18.05 % and 73.35± 7.80% of the tyrosine hydorxylase-positive neurons of the SNpc were also GFP-positive when using the rAAV2/1-CMV-EGFP, rAAV2/1-rtTA-M2-WPRE-EGFP and rAAV2/1-rtTA-M2- EGFP vectors. The percentage of GFP-positive cells among TH-positive cells was not different between the 3 vectors (one-way ANOVA).

In order to address the mechanism of the observed differential biodistribution of GFP protein with the CMV and tetON vectors, the *gfp* mRNA and DNA were quantified in the substantia nigra and in the dorsal midbrain. Hirt low molecular weight DNA (hDNA) (Hirt, 1967) and messenger RNAs (mRNA) were extracted from tissue punches and mRNAs were converted to cDNA. Real-time quantitative PCR showed that the levels of *gfp* transcripts normalized versus the levels of two different house keeping genes (YWHAZ and actinγ2) and relative to the levels of hDNA were significantly higher (5-fold; p=0.0074) in the SN versus the midbrain (Fig.8). A ~2.5-fold higher relative level of *gfp* transcripts in the SN versus the

midbrain was also observed for the CMV vector (Fig.8). However, the difference was not statistically significant (p=0.086).

2.2 Striatum

GFP-postive fibers were evidenced in the striatum (Fig.9) suggesting that GFP was anterogradely transported from dopaminergic cell bodies of the SNpc to dopaminergic fibers in the striatum.

DISCUSSION

Key factors for the success of neuroprotective gene therapy for Parkinson's disease are: a widespread distribution of the neurotrophic factor in the putamen, the absence of significant amounts of transgene product in connected regions which can induce adverse effects as well as an optimal dose of the neurotrophic factor providing neuroprotection in the absence of other, undesired biological effects.

We have previously shown that a single injection of the autoregulatory rAAV2/1-tetON vector drives doxycycline-dependent GDNF expression in a widespread area of the rat striatum (Chtarto *et al*., 2007). We have extended these data and shown that the striatal amount of GDNF could be modulated in function of the doxycycline dose. The GDNF concentrations (~33 and 75 pg/mg tissue at doxycycline doses of 300 and 600 mg/l of drinking water, respectively) were similar to that obtained by Eslamboli and coll. (Eslamboli *et al*., 2005), i.e. 40 pg/mg tissue, providing neuroprotection and behavioural improvements in the absence of effects on the dopamine metabolism.

In the present study, we compared the biodistribution and cellular tropism of rAAV2/1-tetON-EGFP and rAAV2/1-CMV-EGFP in the striatum and in the midbrain.

After striatal injection, although the vast majority of the GFP-positive cells were neurons with both vectors, the percentage of GFP-positive astrocytes was significantly higher when using the CMV vector. Furthermore, a different subset of neurons was expressing GFP when using the tetON and the CMV vectors. Indeed, while rAAV2/1-CMV-EGFP expressed the transgene in parvalbumin-positive interneurons and DARPP-32 projection neurons in proportions roughly reflecting the presence of these two subpopulations of neurons (the vast majority, i.e. 90–95%, of striatal neurons being DARPP-32-positive medium spiny projection neurons) (Graveland and DiFiglia, 1985), rAAV2/1-tetON-EGFP targeted transgene

expression in proportionally more parvalbumin-immunoreactive neurons (approx. 1 parvalbumin-positive for 2 DARPP-32-positive neurons). In addition, among DARPP-32 immunoreactive neurons, those projecting to the globus pallidus were expressing GFP similarly with both vectors, whereas those projecting to the substantia nigra pars reticulata were expressing GFP with the CMV but much less efficiently by the tetON vector as evidenced by the GFP-positive projections observed in these structures. Retrograde transport of viral particles from the striatum to the SNpc has been already reported with rAAV2/1 vectors using the CBA (Burger *et al*., 2004) and the CMV promoters (Wang *et al*., 2003). The present study confirms retrograde transport when using the CMV promoter. However, it was inefficient (approx. 100 cells per animal) as compared to the number of transduced cells around the delivery site in the striatum (approx. 100,000 per animal). In contrast, only few weakly labeled GFP-positive cells were detected in the SNpc of animals injected with rAAV2/1-tetON in the striatum. This could be due to a limited transgene inducibility in dopaminergic neurons. However, after direct injection in the midbrain, very efficient GFP expression in tyrosine hydroxylase-positive neurons was obtained with both vectors. Altogether these data could suggest that high multiplicities of rAAV-tetON are required to obtain an intracellular concentration of transactivator sufficient to induce the tetON system and that the threshold number of viral genome copies could not be reached after retrograde transport. Additional experiments, using for example labeled capsids, will be necessary to address this question. In accordance with the very inefficient retrograde transport of AAV1 viral particles as well as anterograde transport of the transgene product, injection of AAV2/1 vectors expressing the human GDNF cDNA, resulted in low amounts of GDNF (not significantly higher than the endogenous level) in the substantia nigra.

Another striking difference between the CMV and the tetON vectors consisted in the differential transgene expression in the neural stem cells of the subventricular zone (SVZ).

Indeed, although both vectors very efficiently expressed GFP in the SVZ, numerous GFP-positive cells were evidenced in the olfactory bulb (in which neural stem cells of the SVZ migrate and differentiate (Alvarez-Buylla and Lim, 2004)) 5 weeks after injection of rAAV2/1-CMV-EGFP but not rAAV2/1-tetON-EGFP. The absence of GFP-positive cells in the olfactory bulb suggests that either the cells migrating to the olfactory bulb did not express a detectable level of GFP due to low transcription efficiency or gene expression from the tet-responsive promoter was switched-off during migration and/or differentiation of neuroblasts. The full characterization of initially-infected cells in the SVZ will require analysis shortly after infection, before migration takes place. Regardless, the lack of GFP expression in the migrating neuroblasts together with the efficient GFP expression in the neighbouring cells in the SVZ, suggests that the rAAV2/1-tetON-EGFP could be used to express diffusible factors acting in a paracrine way on neural stem cells while avoiding transgene expression in olfactory bulb neurons.

Altogether, the data obtained after striatal injection support the potential use of rAAV2/1-tetON for Parkinson's disease GDNF gene therapy. Indeed, the inefficient retrograde transport of rAAV2/1 to the SNpc and anterograde transport of the transgene product to the SNr, ensure a minimal amount of GDNF in the SN after striatal viral delivery. Since the presence of high amounts of GDNF in the SN has been associated with aberrant sprouting (Georgievska *et al*., 2002), the here described vector constitutes a safer alternative for striatal GDNF gene delivery. In addition, the absence of transgene expression in the olfactory bulb despite transduction of the subventricular zone when using the tetON but not the CMV promoter-bearing rAAV2/1 vector, also renders the former safer than the latter.

Another important finding of this study is that, after injection in the midbrain, rAAV2/1 vectors using the autoregulatory tetON transcription cassette provides preferential transgenic expression in tyrosine hydroxylase-immunoreactive neurons of the substantia nigra

pars compacta and ventral tegmental area. This property will be useful to evaluate the function of genes potentially involved in the etiology of Parkinson's disease as well as in the physiology of dopaminergic neurons. Quantification of *gfp* transcripts relative to the amounts of viral DNA evidenced a 5-fold higher expression level in the substantia nigra versus the dorsal midbrain. Recombinant AAV2/2 vectors using constitutive promoters also efficiently drive transgene expression into dopaminergic neurons when injected in the midbrain (Kirik *et al*., 2002; Burger *et al*., 2004). Kirik and coll. (Kirik *et al*., 2002) using the CBA promoter reported that the expression profile was not restrictive to dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area but also affected the pars reticulata of the substantia nigra and the mesensephalic reticular formation.

We can not exclude that the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) could contribute to the observed transgene expression profile. Indeed, the WPRE sequence has been reported to contain enhancer activity (Kingsman *et al*., 2005). Furthermore, it has been shown to enhance the transgene expression efficiency from rAAV2/2 vectors using different cellular (Paterna *et al*., 2000) and viral (Passini *et al*., 2003) promoters. Whether the WPRE sequence modifies the cellular specificity of these vectors has not been studied. In the present study, the WPRE sequence, placed downstream to the rtTA-M2 transactivator coding sequence did not significantly modify neither the efficiency nor the profile of trangene expression from the autoregulatory AAV-tetON vector in the SNpc. These data are in accordance with a previous study using the same vectors expressing the GDNF cDNA (Chtarto *et al*., 2007), in which we have shown that the WPRE sequence did not modify the GDNF tissue levels in the induced (+ doxycycline) state but resulted in a higher basal level of GDNF expression (-doxycycline). It could be that, the potentially increased rtTAM2 expression in the rAAV2/1-rtTA-M2-WPRE relative to the rAAV2/1-rtTA-M2 vector, results in an increased expression of the transgene under the control of the

tetracycline-responsive promoter in the non-induced but not in the induced state. In favour of this hypothesis, the rtTA-M2 transactivator harbour a high affinity for doxycycline and a higher stability in eukaryotic cells than the original rtTA, suggesting that it might saturate the tetracycline operator sites in the induced state not allowing further improvements by increasing its intracellular concentration.

Overall, the restricted transgene expression of rAAV2/1-tetON as compared to rAAV2/1-CMV could be due to i) lack of activation of the tetON system in cells which do not accumulate enough transactivator in the absence of tetracycline; ii) absence of cellular factors binding to the VP16 activation domain of the rtTA; iii) inefficient penetration or rapid metabolisation of doxycycline in cells not expressing the transgene. However, some cell types were expressing GFP as efficiently (TH-positive SNpc neurons) or more efficiently (parvalbumin-immunoreactive striatal neurons) by the tetON than by the CMV vector.

Differential transgene expression profiles of rAAV2/2-CMV and rAAV2/2-tTA (tetOFF) has also been reported (Haberman *et al*., 2002). The authors showed that the same transgene had opposite functional effects when expressed respectively under a CMV or tetOFF transcriptional control and demonstrated using a *gfp* and a *lacZ* reporter gene respectively, that the vectors were expressed mainly in different subpopulations of neurons.

Immune reaction is a major issue in gene therapy. The rtTA-M2 transactivator is a composite protein harbouring a viral as well as bacterial subdomains. Several studies report immune responses directed against the rtTA after injection of tetracycline-regulatable viral vectors in the muscle (Favre *et al*., 2002). However, in the brain of healthy animals, no cellular immune reaction to the tTA/rtTA transactivator has been described using rAAV2/2 and adenoviral vectors (Fitzsimons *et al*., 2001; Xiong *et al*., 2008). These conclusions were confirmed in the present study using rAAV2/1 pseudotyped vectors. In addition Xiong and

coll. showed that systemic immunity against the rtTA-M2 transactivator did not lead to inflammation or reduction of transgene expression in the striatum (Xiong *et al*., 2008).

In conclusion, targeted transgene expression in specific brain cell populations might be achieved by a combination of local injection and adequate choice of serotype and promoter. In this respect, the here described rAAV2/1-tetON vector is an interesting tool i) to study the function of genes in dopaminergic neurons of the SNpc; ii) to conditionally deliver diffusible factors in the subventricular zone for the paracrine modification of neural stem cells. In addition, regulatable transgene expression in the SNpc is of great interest for the study of the function of Parkinson's disease-related genes such as α-synuclein, Parkin, etc… in order to characterize and distinguish permanent and reversible effects of the overexpression of the wild-type and mutated forms of these genes.

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LEGENDS TO FIGURES

Figure 1. Dox-dependent concentration of GDNF in the striatum and the substantia nigra after intrastriatal injection of rAAV2/1-rtTAM2-GDNF.

Eight weeks after injection of rAAV2/1-rtTAM2-GDNF, brain extracts were analysed by ELISA to detect GDNF concentration in the striatum (White boxes) and in the substantia nigra (Black boxes). Striatal levels of GDNF at both Dox concentration in drinking water (300mg/L and 600 mg/L) were highly significantly different from PBS control injection and significantly and highly significantly different from the no Dox control condition for the animals receiving 300mg of Dox per litre and 600 mg of Dox per litre respectively. GDNF concentrations measured in the substantia nigra were not significantly different from the endogenous level of PBS injected animals (one way ANOVA).

Figure 2. Comparison of the transduction efficiency of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum.

Five weeks after injection of rAAV2/1 CMV (a) or tetON (b) vector into the striatum, 50 μm vibratome brain sections were labeled with anti-gfp antibodies. GFP-immunopositive cells were counted on every tenth section over a distance of 3mm (AP= +1.0 to AP=-2.0 from bregma) [40] using an unbiased stereology method (40). Scale bar = 3mm.

Figure 3. Cellular tropism of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum.

Panel a. Five weeks after injection of rAAV2/1 CMV (n= 4) or tetON vector (n=4) into the striatum, brain sections were double-labeled with gfp (green fluorescence) and NeuN, GFAP, parvalbumin or DARPP-32 (red fluorescence). Confocal pictures showing GFP and cell-

specific markers double-labeled cells (yellow). Arrowheads indicate double-labeled cells. Scale bar = 50 μm.

Panel b. The proportions of colabeled cells were significantly different for the 2 vectors for GFAP (5.9 ± 1.9 % of GFP-positive cells for the CMV vector versus 1.2 ± 2.2% for the tetON vector; $p < 0.05$), parvalbumin (29.0±10.9% for the tetON versus 7.1±1.3% for the CMV vector; $p< 0.05$) and DARPP-32 (91.6±2.3% for the CMV versus 66.7±5.1 % for the tetON vector; $p< 0.001$); but not for the NeuN marker. (Student t test). White boxes, CMV vector; black boxes, tetON vector. Data are expressed as mean + SD.

Figure 4. Differential anterograde transport of GFP in the SNr and globus pallidus after injection of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum.

Five weeks after injection of rAAV2/1 CMV or tetON (a) vector into the right striatum, midbrain sections were co-labeled with anti-GAD (red fluorescence) and anti-GFP (green fluorescence) antibodies. Double-labeled fibers (yellow) were evidenced in the right SNr. Alternatively, sections were labeled by anti-GFP antibodies using a sensitive biotin-streptavidin/peroxidase/DAB staining method (b). Scale bar = 1mm (a) and 0.5 mm (b).

Panel c. Five weeks after injection of rAAV2/1 CMV or tetON vector in the striatum, sections at the level of the globus pallidus or substantia nigra were labeled with anti-GFP antibody. The optical density of the fibers (panels d,e for substantia nigra and f,g for globus pallidus) was measured on greyscale pictures with the software Image J on at least 5 different sites per section into the transduction area and in the contralateral structure. Measures were made on 1 or 2 sections per animal (n=4) for the globus pallidus and on 2 or 3 sections per animal (n=4) for the substantia nigra pars reticulata. The fiber labeling for CMV and tetON vectors was significantly different in the substantia nigra ($p=0.0119$) but not in the globus pallidus ($p>0.05$). Data are expressed in mean ± SD.

Figure 5. Retrograde transport in the substantia nigra pars compacta.

Five weeks after injection of rAAV2/1 CMV-EGFP into the right striatum, midbrain sections were co-labeled with anti-TH (red fluorescence) and anti-GFP (green fluorescence) antibodies. Double labeled cell bodies (yellow) were evidenced in the right SNpc. Arrowhead indicates a double-labeled cell. Scale bar = 50 μm.

Figure 6. Differential transgene expression from rAAV2/1-CMV and rAAV2/1-tetON vectors in the olfactory bulb.

Five weeks after injection of rAAV2/1 CMV or tetON vector into the striatum, sections at the level of the striatum (a, b) or olfactory bulb (c) were labeled with anti-GFP antibodies. GFP-positive cells were evidenced in the subventricular zone with both the CMV (a) and the tetON (b) vectors. Sections at the level of the olfactory bulb showed GFP-positive cells in animals injected with the CMV vector (c) but not the tetON vector (data not shown). Scale bar = 125 μm.

Figure 7. Specificity of rAAV2/1-tetON vector for dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area.

Five weeks after injection of rAAV2/1 CMV (a, b) or tetON (c, d, e) vector into the midbrain, sections were labeled with anti-GFP (green fluorescence) and anti-TH (red fluorescence) antibodies. The proportion of TH-positive cells that were also GFP-positive (yellow fluorescence) was not significantly different for the 2 vectors (Student t test). Arrowheads indicate double-labeled cells. Panels a, c, d, fluorescence microscopy; b, e, confocal microscopy. Scale bar = 500 μm (a, c), 125 μm (d) and 50 μm (b, e).

Figure 8. Normalized gfp expression levels measured by qPCR in the dorsal midbrain and substantia nigra after injection of rAAVA2/1-CMV or rAAV2/1-tetON vectors in the midbrain.

Five weeks after injection of rAAV2/1-CMV-EGFP or rAAV2/1-rtTAM2-EGFP, mRNA and low molecular weight DNA were extracted from substantia nigra or dorsal midbrain tissue. mRNAs were reverse transcribed to cDNA. *Gfp* sequences were quantified by qPCR. mRNA levels were normalised for house keeping genes and then relative to hDNA levels. The level of *gfp* transcripts expressed by the tetON vector was 5-fold higher in the SN than in the dorsal midbrain (p=0.0074), whereas the level of *gfp* transcripts expressed by the CMV vector was not significantly different in the SN and in the dorsal midbrain (p=0.08624). Data are expressed in *gfp* relative expression units, defined as the *gfp* expression level of the less expressed sample in the experiment after normalisation for house keeping genes and amounts of hDNA (*gfp* copies/mg tissue). White boxes, midbrain; black boxes, substantia nigra. Data are expressed in mean + S. E. M. (n=7).

Figure 9. Anterograde transport of GFP in the striatum after injection of rAAV2/1-CMV and rAAV2/1-tetON vectors in the midbrain.

Five weeks after injection of rAAV2/1 CMV or tetON vectors into the right midbrain, striatum sections were labeled with anti-GFP (green fluorescence) antibodies. GFP-positive fibers were evidenced in the right striatum. Scale bar = 50µm.

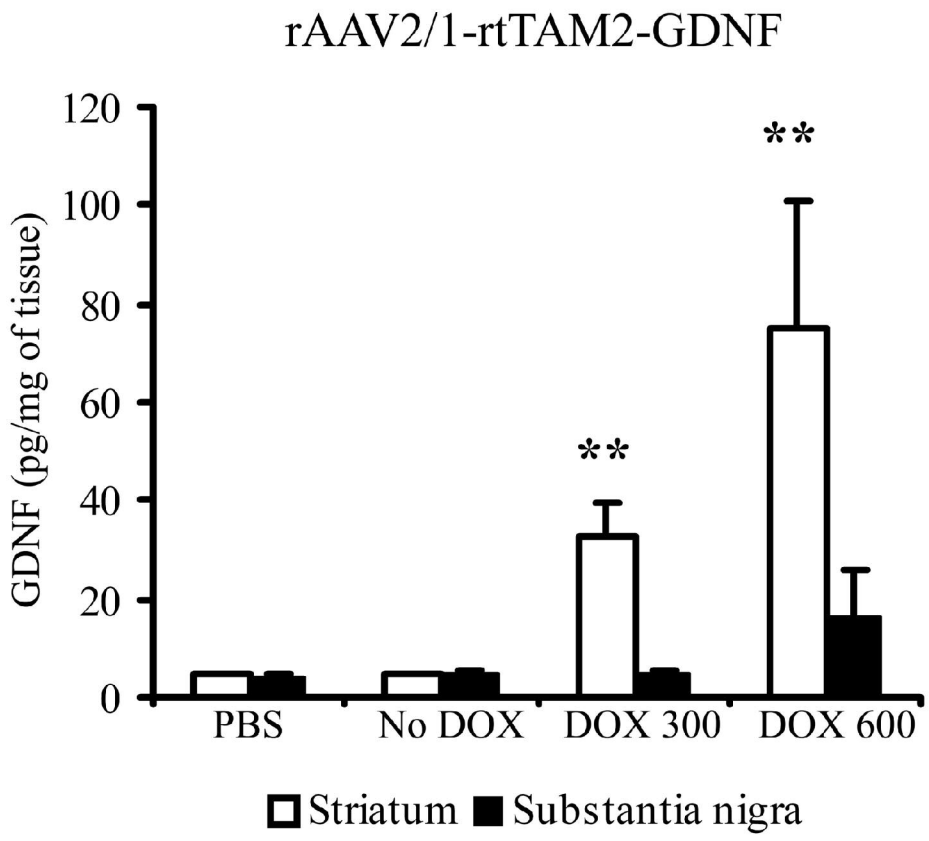

Figure 1. Dox-dependent concentration of GDNF in the striatum and the substantia nigra after intrastriatal injection of rAAV2/1-rtTAM2-GDNF. Eight weeks after injection of rAAV2/1-rtTAM2-GDNF, brain extracts were analysed by ELISA to detect GDNF concentration in the striatum (White boxes) and in the substantia nigra (Black boxes). Striatal levels of GDNF at both Dox concentration in drinking water (300mg/L and 600 mg/L) were highly significantly different from PBS control injection and significantly and highly significantly different from the no Dox control condition for the animals receiving 300mg of Dox per litre and 600 mg of Dox per litre respectively. GDNF concentrations measured in the substantia nigra were not significantly different from the endogenous level of PBS injected animals (one way ANOVA).

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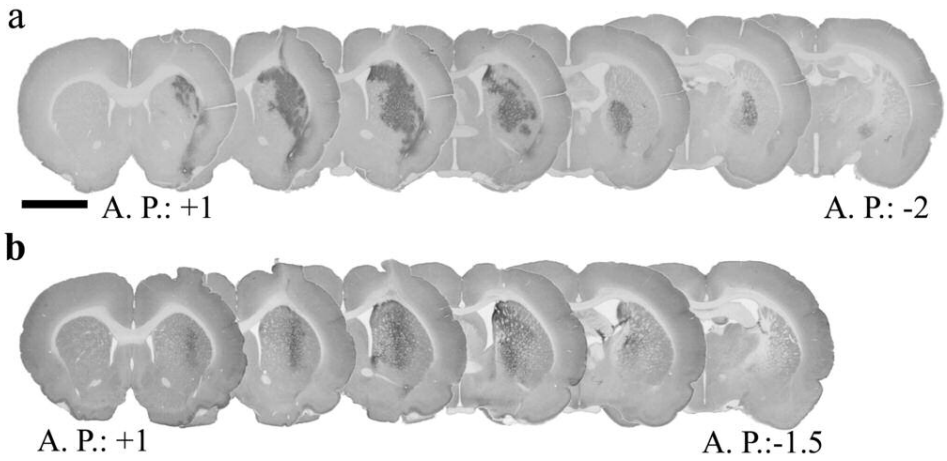

Figure 2. Comparison of the transduction efficiency of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum. Five weeks after injection of rAAV2/1 CMV (a) or tetON (b) vector into the striatum, 50 μm vibratome brain sections were labeled with anti-gfp antibodies. GFP-immunopositive cells were counted on every tenth section over a distance of 3mm (AP= +1.0 to AP=-2.0 from bregma) [40] using an unbiased stereology method (40). Scale bar = 3mm.

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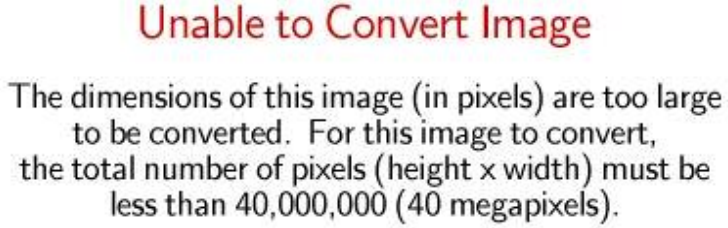
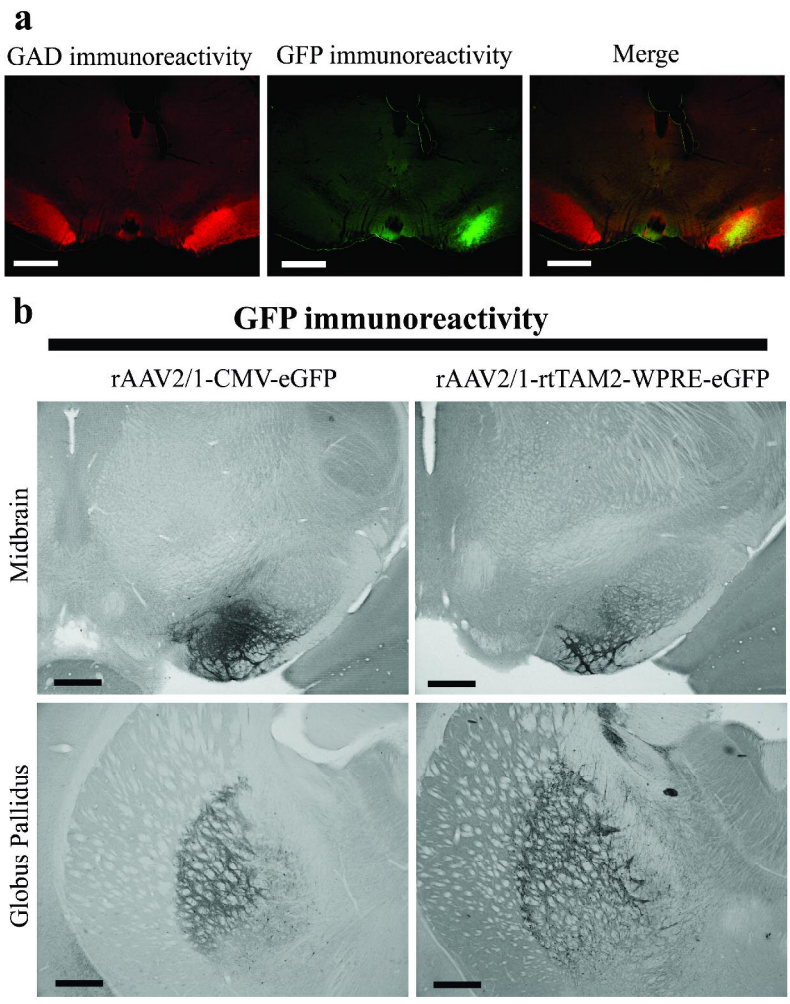


Figure 3. Cellular tropism of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum. Panel a. Five weeks after injection of rAAV2/1 CMV (n= 4) or tetON vector (n=4) into the striatum, brain sections were double-labeled with gfp (green fluorescence) and NeuN, GFAP, parvalbumin or DARPP-32 (red fluorescence). Confocal pictures showing GFP and cell-specific markers double-labeled cells (yellow). Arrowheads indicate double-labeled cells. Scale bar = 50 μm. Panel b. The proportions of colabeled cells were significantly different for the 2 vectors for GFAP (5.9 ± 1.9 % of GFP-positive cells for the CMV vector versus 1.2 ± 2.2% for the tetON vector; $p < 0.05$), parvalbumin (29.0±10.9% for the tetON versus 7.1±1.3% for the CMV vector; $p< 0.05$) and DARPP-32 (91.6±2.3% for the CMV versus 66.7±5.1 % for the tetON vector; $p< 0.001$); but not for the NeuN marker. (Student t test). White boxes, CMV vector; black boxes, tetON vector. Data are expressed as mean + SD.



c

(O. D. ipsilateral / O. D. contralateral) X 100	Globus pallidus	Substantia Nigra
AAV1-pCMV-EGFP	391.39% ±82.88	380.8% ±28.83
AAV1-rtTaM2-EGFP-WPRE	309.54% ±44.86	234.51% ±65.80

Figure 4. Differential anterograde transport of GFP in the SNr and globus pallidus after injection of rAAV2/1-CMV and rAAV2/1-tetON vectors in the striatum. Five weeks after injection of rAAV2/1 CMV or tetON (a) vector into the right striatum, midbrain sections were co-labeled with anti-GAD (red fluorescence) and anti-GFP (green fluorescence) antibodies. Double-labeled fibers (yellow) were evidenced in the right SNr. Alternatively, sections were labeled by anti-GFP antibodies using a sensitive biotin-streptavidin/peroxidase/DAB staining method (b). Scale bar = 1mm (a) and 0.5 mm (b). Panel c. Five weeks after injection of rAAV2/1 CMV or tetON vector in the striatum, sections at the level of the globus pallidus or substantia nigra were labeled with anti-GFP antibody. The optical density of the fibers (panels d,e for substantia nigra and f,g for globus pallidus) was measured on greyscale pictures with the software Image J on at least 5 different sites per section into the transduction area and in the contralateral

structure. Measures were made on 1 or 2 sections per animal (n=4) for the globus pallidus and on 2 or 3 sections per animal (n=4) for the substantia nigra pars reticulata. The fiber labeling for CMV and tetON vectors was significantly different in the substantia nigra (p=0.0119) but not in the globus pallidus (p>0.05). Data are expressed in mean ± SD.

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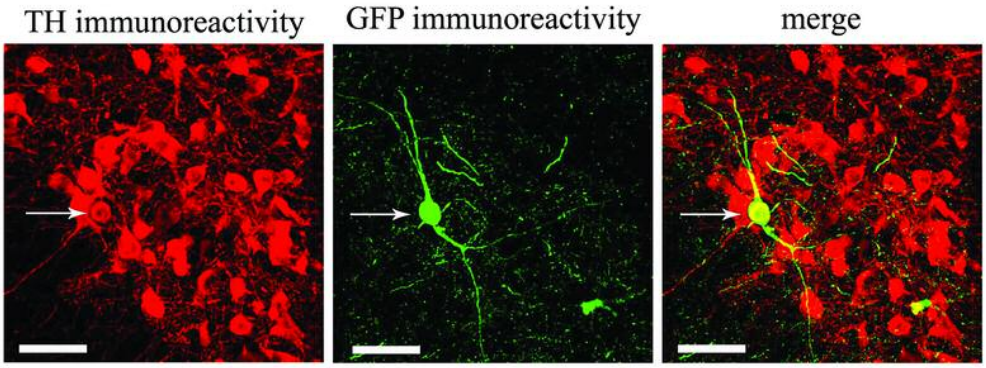

Figure 5. Retrograde transport in the substantia nigra pars compacta. Five weeks after injection of rAAV2/1 CMV-EGFP into the right striatum, midbrain sections were co-labeled with anti-TH (red fluorescence) and anti-GFP (green fluorescence) antibodies. Double labeled cell bodies (yellow) were evidenced in the right SNpc. Arrowhead indicates a double-labeled cell. Scale bar = 50 μm.

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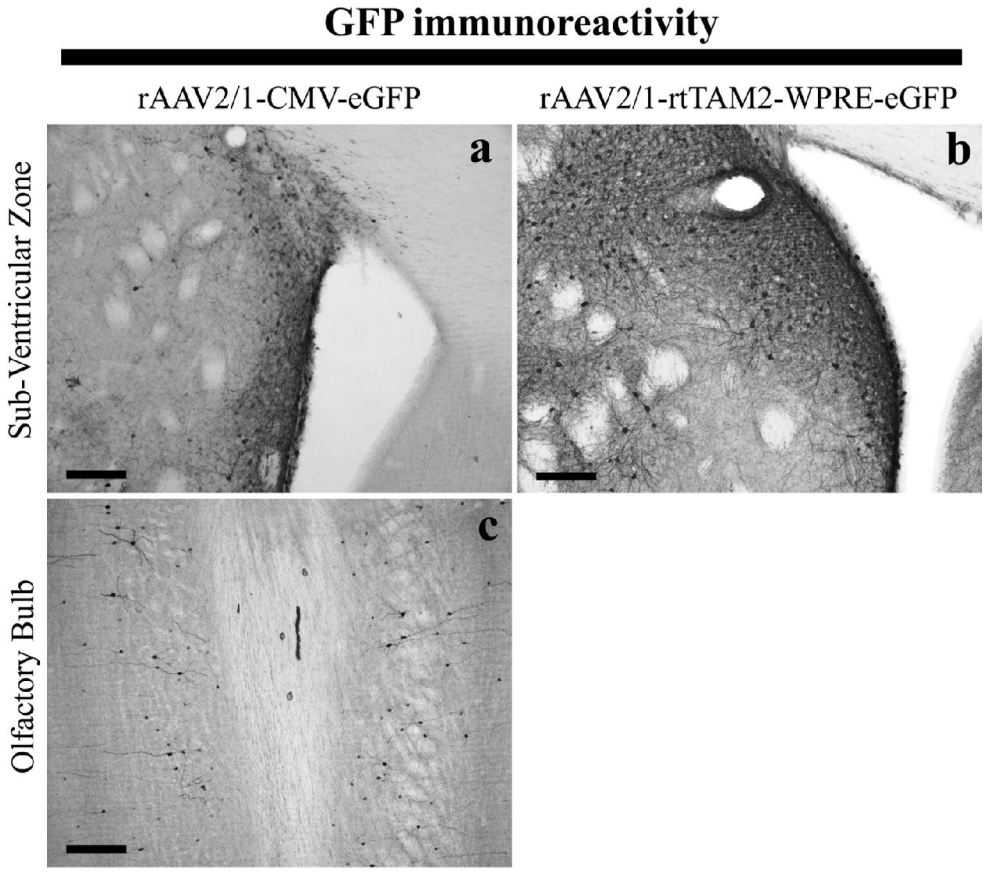

Figure 6. Differential transgene expression from rAAV2/1-CMV and rAAV2/1-tetON vectors in the olfactory bulb. Five weeks after injection of rAAV2/1 CMV or tetON vector into the striatum, sections at the level of the striatum (a, b) or olfactory bulb (c) were labeled with anti-GFP antibodies. GFP-positive cells were evidenced in the subventricular zone with both the CMV (a) and the tetON (b) vectors. Sections at the level of the olfactory bulb showed GFP-positive cells in animals injected with the CMV vector (c) but not the tetON vector (data not shown). Scale bar = 125 Figure 6. Differential transgene expression from rAAV2/1-CMV and rAAV2/1-tetON vectors in the olfactory bulb. Five weeks after injection of rAAV2/1 CMV or tetON vector into the striatum, sections at the level of the striatum (a, b) or olfactory bulb (c) were labeled with anti-GFP antibodies. GFP-positive cells were evidenced in the subventricular zone with both the CMV (a) and the tetON (b) vectors. Sections at the level of the olfactory bulb showed GFP-positive cells in animals injected with the CMV vector (c) but not the tetON vector (data not shown). Scale bar = 125 μm.

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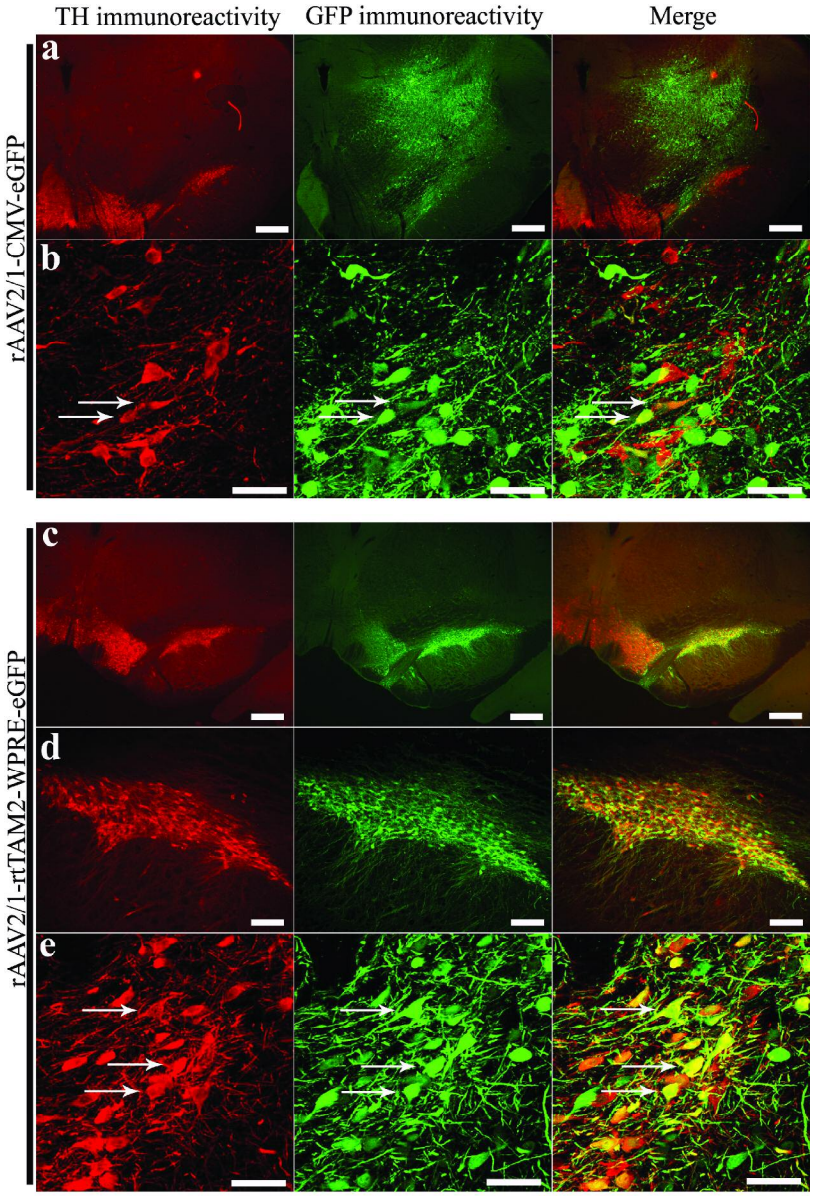

Figure 7. Specificity of rAAV2/1-tetON vector for dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area. Five weeks after injection of rAAV2/1 CMV (a, b) or tetON (c, d, e) vector into the midbrain, sections were labeled with anti-GFP (green fluorescence) and anti-TH (red fluorescence) antibodies. The proportion of TH-positive cells that were also GFP-positive (yellow fluorescence) was not significantly different for the 2 vectors (Student t test). Arrowheads indicate double-labeled cells. Panels a, c, d, fluorescence microscopy; b, e, confocal microscopy. Scale bar = 500 μm (a, c), 125 μm (d) and 50 μm (b, e).

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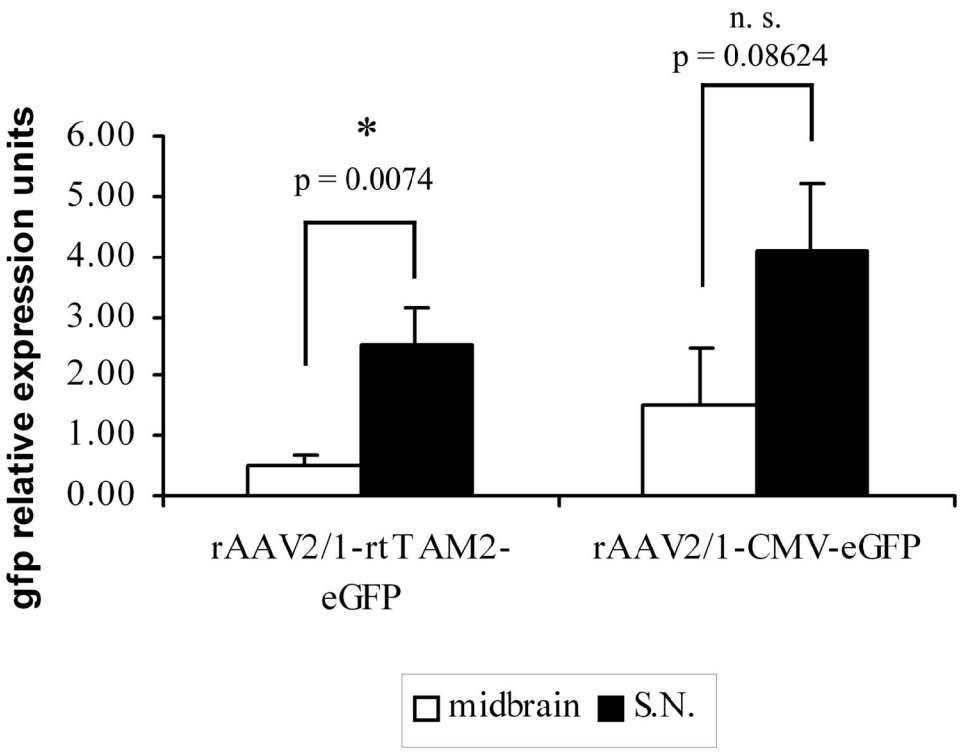

Figure 8. Normalized gfp expression levels measured by qPCR in the dorsal midbrain and substantia nigra after injection of rAAVA2/1-CMV or rAAV2/1-tetON vectors in the midbrain. Five weeks after injection of rAAV2/1-CMV-EGFP or rAAV2/1-rtTAM2-EGFP, mRNA and low molecular weight DNA were extracted from substantia nigra or dorsal midbrain tissue. mRNAs were reverse transcribed to cDNA. Gfp sequences were quantified by qPCR. mRNA levels were normalised for house keeping genes and then relative to hDNA levels. The level of gfp transcripts expressed by the tetON vector was 5-fold higher in the SN than in the dorsal midbrain (p=0.0074), whereas the level of gfp transcripts expressed by the CMV vector was not significantly different in the SN and in the dorsal midbrain (p=0.08624). Data are expressed in gfp relative expression units, defined as the gfp expression level of the less expressed sample in the experiment after normalisation for house keeping genes and amounts of hDNA (gfp copies/mg tissue). White boxes, midbrain; black boxes, substantia nigra. Data are expressed in mean + S. E. M. (n=7).

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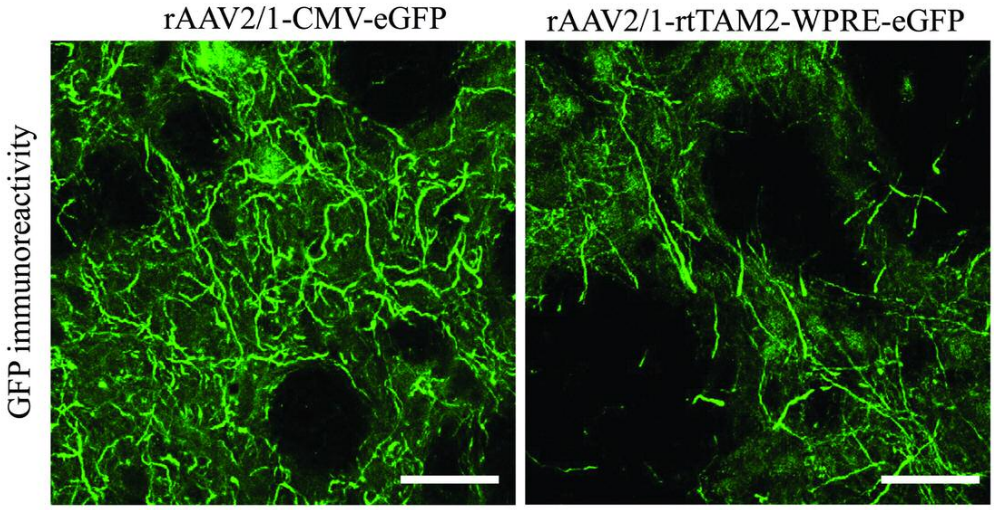

Figure 9. Anterograde transport of GFP in the striatum after injection of rAAV2/1-CMV and rAAV2/1-tetON vectors in the midbrain. Five weeks after injection of rAAV2/1 CMV or tetON vectors into the right midbrain, striatum sections were labeled with anti-GFP (green fluorescence) antibodies. GFP-positive fibers were evidenced in the right striatum. Scale bar = 50 µm.

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