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Comparison of AAV Serotypes for Gene Delivery to Dopaminergic Neurons in the Substantia Nigra

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1. Introduction

Targeted viral vector-mediated gene transfer to specific population of neurons in the central nervous system (CNS) is a relatively novel, but fast developing approach to study gene function in a number of neurodegenerative diseases (reviewed in Korecka, Verhaagen, & Hol, 2007; Manfredsson & Mandel, 2010). Moreover, several early phase clinical trials based on viral vector-mediated therapeutic gene transfer have been completed or are underway for neurological disorders (Kaplitt et al., 2007; reviewed in Korecka et al., 2007; Marks, Jr. et al., 2010; Muramatsu et al., 2010; Tuszynski et al., 2005). Gene therapy is especially attractive for diseases where neuronal degeneration is largely restricted to a single neuronal population in a specific anatomical area. Parkinson disease (PD) is a neurodegenerative disease mainly characterized by a progressive degeneration of dopaminergic (DAergic) neurons in the Substantia Nigra (SN) (Dauer & Przedborski, 2003). It would be desirable to direct transgene expression to the dopaminergic neurons in animal models for neurodegenerative diseases, allowing for a range of investigations into the function of that gene in normal, adult DAergic neurons or following neurotoxic insult.

Lentiviral vectors (LV) and adeno-associated viral vectors (AAV) are increasingly regarded as the two most useful gene therapy vectors for the CNS. Both vectors have been successfully used to express a foreign gene in a variety of brain regions and neuronal cell types (Lim, Airavaara, & Harvey, 2010; Manfredsson & Mandel, 2010; Papale, Cerovic, & Brambilla, 2009; Schneider, Zufferey, & Aebischer, 2008). LV vectors have been shown to direct long-lasting expression of a number of transgenes in the brain (Lundberg et al., 2008) including in neurons in the rat SN (Deglon et al., 2000). AAV vectors are considered to be the most appealing vectors for transgene expression in the CNS, due to their efficient neuronal transduction, their capacity to direct long-term transgene expression and their safety profile (Kaplitt et al., 2007; Mandel et al., 2006; McCown, 2005). The early AAV vectors were based on AAV serotype 2 (Kaplitt et al., 1994; Peel & Klein, 2000), but

subsequent vectors have been generated with novel serotypes that differ in their tissue and cellular tropism (Wu, Asokan, & Samulski, 2006).

So far, three studies engaged in exploring the possibility of AAV transgene expression in the mouse SN. In two studies only AAV vectors based on serotype 2 were used inducing either alpha-synuclein (St Martin et al., 2007) or dual leucine zipper kinase (Chen et al., 2008) expression in the DAergic neurons of the mouse SN. In the third study AAV serotypes 1, 2, 5, 7 and 8 were injected into the mouse SN and compared for their tropism for DAergic neurons (Taymans et al., 2007). Although, AAV1 and 5 displayed the most promising transduction rates, this data was qualitatively assessed. In contrast, the rat dopaminergic system has been studied much more extensively with five studies investigating the performance of various AAV serotypes tropism. These studies compared the levels of GFP expression in the rat SN after injection of AAV vector serotype 1, 2 and 5 (Burger et al., 2004; Paterna, Feldon, & Bueler, 2004), AAV8 (Klein et al., 2006; McFarland, Lee, Hyman, & McLean, 2009), and AAV9 and 10 (Klein, Dayton, Tatom, Diaczynsky, & Salvatore, 2008). A general conclusion for all these studies establishes AAV2 as the lowest transducing vector of dopaminergic neurons in the rat SN. Finally and most recently, one AAV serotype study has been performed in primate CNS, where AAV1 to 6 viral vectors were injected into the SN (Markakis et al., 2010). In the primate AAV5 displays the most promising transduction of neurons in this area.

In the following study, we have compared multiple AAV serotypes for transduction of mouse and rat mesencephalic DAergic neurons. AAV vectors were developed to contain either a cytomegalovirus (CMV) promoter or the human synapsin 1 (SYN) promoter. We demonstrate that the synapsin promoter leads to higher nigral transduction compared to the CMV promoter in mice. Additionally we also show that in our setting, AAV serotype 5 and 7 give the highest transduction rate of DAergic neurons in the mouse SN, where as rat SN can be equally well transduced with all serotypes tested. We compare our study with the published data and underline the differences in the methodology and outcome measures.

2. Methods

2.1 AAV constructs and production

Lentiviral vectors were produced as described before (Hendriks, Eggers, Verhaagen, & Boer, 2007). Two plasmids, designated pTRCGw and pTRUF20B-SEW, were used for the production of AAV. The pTRCGW plasmid contained inverted terminal repeats of AAV2 flanking a cytomegalovirus (CMV) promoter driving expression of GFP, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and a polyadenylation signal (Ruitenberg, Eggers, Boer, & Verhaagen, 2002). The second plasmid, designated pTRUF20B-SEW, was a generous gift from Prof. Deniz Kirik (Lund University, Sweden). This plasmid also contained two inverted terminal repeats of AAV2 flanking a human synapsin 1 (SYN) promoter driving expression of GFP, a WPRE, and a polyadenylation signal. For the production of different serotypes helper plasmids were used provided by J.A. Kleinschmidt (AAV1 to 6) (Grimm, Kay, & Kleinschmidt, 2003) and J.M. Wilson (AAV7 and 8) (Gao et al., 2002). For each serotype eight 15 cm petridishes containing 1×10^7 HEK293T cells were transfected with the use of polyethylenimine (PEI, MW25000; Polysciences Inc., Warrington, PA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (GIBCO-Invitrogen Corp, New York, NY, USA). pTRCGW or pTRUF20B-SEW AAV plasmids were cotransfected with packaging plasmids in different

ratios as follows: AAV1 to 6 in a ratio of AAV plasmid over capsid plasmid 1:3 with a total amount of 50μg of DNA per plate, and AAV7 and 8 in a ratio of 1:2:2 of AAV plasmid over helper plasmid pAdΔF6 and capsid plasmid with total amount of 62.5μg of DNA per plate. Two days after the transfection cells were harvested in D-phosphate buffered saline (PBS) (Gibco) containing 10μg/ml DNAseI (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 1 hour at 37°C. Cells were lysed by three freeze-thaw cycles, spun down 30 min at 4000rpm and crude lysate was collected. Virus was purified by the iodixanol gradient ultra-centrifugation method (Hermens et al., 1999; Zolotukhin et al., 1999), diluted in D-PBS/5% sucrose and concentrated using Amicon 100kDA MWCO Ultra-15 device (Millipore, Billerica, MA, USA). All AAV vectors were stored at -80°C until use. Titers were determined by repeated quantitative PCR for viral genomic copies extracted from DNAse-treated viral particles using WPRE directed primers (forward: CAGGTGTATTGCCACAAGACAAA and reverse: TGCACAGGTGAAGACCAAGCAA). Table 1 provides an overview of all viral stocks and their titers used in this study.

Serotype	Titer (GCs/ml)	Injection coordinates in mice	Injection coordinates in rats
LV-CMV	9.0×10^9	AP -2.8, L -1.3, VD -4.2	
AAV1-CMV	6.6×10^{12}	AP -2.8, L -1.3, VD -4.1	
AAV2-CMV	1.0×10^{12}	AP -2.8, L -1.3, VD -4.1	
AAV5-CMV	7.1×10^{11}	AP -2.8, L -1.3, VD -4.1	
AAV6-CMV	1.5×10^{12}	AP -2.8, L -1.3, VD -4.1	
AAV7-CMV	3.2×10^{12}	AP -2.8, L -1.3, VD -4.2	
AAV8-CMV	9.9×10^{11}	AP -2.8, L -1.3, VD -4.1	
AAV5-SYN***	1.2×10^{13}	AP -2.8, L -1.3, VD -4.3	AP -5.2, L -2.0, VD -7.2
AAV6-SYN	3.7×10^{12}	AP -2.8, L -1.3, VD -4.3	AP -5.2, L -2.0, VD -7.2
AAV7-SYN	3.0×10^{12}	AP -2.8, L -1.3, VD -4.3	AP -5.2, L -2.0, VD -7.2
AAV8-SYN	1.4×10^{12}	AP -2.8, L -1.3, VD -4.3	AP -5.2, L -2.0, VD -7.2

Table 1. Viral vectors, titers and injection coordinates. All AAV batches have a similar range of titer with an exception of AAV5-SYN, which has a significantly higher titer than the other AAV-SYN viruses (after multiple testing, P<0.001, one way ANOVA). The injection coordinates are indicated for mice and rats respectively. Abbreviations: CMV-cytomegalovirus, SYN-synapsin, GCs/ml- genomic copies per milliliter, AP- Anterior Posterior, L- lateral, and VD- Ventral Dorsal distances from bregma.

2.2 Experimental animals and surgical procedures

A total of 43 male C57BL/6 mice weighing 20-25g and 16 female Sprague-Dawley rats weighing 200-250g were used (Harlan, Zeist, The Netherlands). Animals were housed with food and water *ad libitum*, with 12 hour light and dark cycles. All the experimental procedures and postoperative care was carried out in accordance with the local animal experimental ethical committee.

The viral injections were carried out with the use of glass capillaries (0.78/1.0mm internal/external diameter; Harvard Apparatus, Holliston, MA, USA) with an 80μm tip. These glass capillaries were connected to Portex polyethylene tubing in turn connected to a Hamilton syringe fixed in a micro-infusion pump (PHD2000, Harvard Apparatus). The system was filled with water and a target volume of 1μl and an infusion rate of 0.2μl/min

was set for mice injections and target volume of 2μl and an infusion rate of 0.4 μl/min for rats. The glass needles were mounted on a stereotactic device (David Kopf Instruments, Tujunga, CA, USA). A total of 1.1μl of virus was loaded for mice and 2.3 μl for rats for each injection separately.

Mice were intraperitoneally (IP) injected with FFM mix made of Hypnorm (0.1 mg/kg Fentanyl citrate/ 3.3 mg/kg Fluanisone HCl, Janssen Pharmaceuticals) and Dormicum (8.3 mg/kg Midazolam, Roche) and placed into a stereotactic device where they were fixed and the skull was exposed. The skull was leveled based on the heights of bregma, lambda and two additional and very crucial lateral measurements of 2.0 mm from bregma. The injection coordinates were calculated from bregma with anterior posterior (AP) being -2.8 mm and lateral (L) -1.3 mm. Ventral dorsal (VD) coordinate was measured from the dura of either -4.1, -4.2 or -4.3 mm depending on the viral injection (see table 1). Subsequently, the needle was lowered into the brain 0.1 mm below the VD coordinate and retracted back up to the correct level. After the infusion, the needle was left in place for 5min before retraction.

Rats were anesthetized by intramuscular injection of 0.08ml/100g of Hypnorm and mounted in the stereotact. The skull level was controlled by measurement of bregma and lambda. 2μl of each AAV virus was injected at the following coordinates with VD being measured from dura: AP -5.2, L -2.0, and VD 7.2 (Ulusoy, Sahin, Bjorklund, Aebischer, & Kirik, 2009).

All of the animals recovered on a heating pad at 37°C and were allowed to survive for 4 weeks post surgery after which they were sacrificed by an IP overdose with Pentobarbital (50mg/μl) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA, Sigma-Aldrich Co., St. Louis, MO, USA) in PBS pH 7.4. The brains were further post-fixed overnight and 4 series of 30μm thick coronal sections were cut on a vibratome. The sections were stored free-floating at 4°C in 1% PFA in PBS pH 7.6.

2.3 Immunohistochemistry and histological quantification

All immunohistochemical (IHC) stainings were performed on free-floating sections. Prior to the staining, one series of sections was pre-blocked in 1x tris buffered saline (TBS) (Sigma) with 2.5% fetal calf serum (FCS) and 0.2% Triton-X (Sigma) for 1 hour at room temperature. Sections were then incubated with anti-tyrosine hydroxilase (TH) rabbit polyclonal antibody (Institute Jacques Boy SA, Reims, France) at 1:1000 dilution in blocking buffer and anti-GFP (Millipore) chicken monoclonal antibody at 1:1000 for 1 hour at room temperature followed by overnight incubation at 4°C. Secondary goat anti-rabbit antibody Alexa 594 (1:400, Invitrogen, Carlsbad, CA, USA) was used for the detection of the TH antibody and donkey anti-chicken Alexa 488 (1:400, Invitrogen) for the detection of the GFP antibody. These antibodies were incubated for 1 hour at room temperature. Sections were then mounted on chrome-aluin and gelatin coated glass slides.

Images were acquired with an Axioplan microscope (Zeiss, Sliedrecht, The Netherlands). Images for quantification of the transduced neurons of the SN were taken at 10x magnification for mice and 5x magnification for rats with fixed exposure times for both TH and GFP signal. The sections of the striatum area were photographed at 2.5x magnification also with fixed exposure times for both TH and GFP fluorescent signal.

ImagePro Plus Fluorviewer software (Media cybernetics, Bethesda, MD, USA) was used for the SN transduction quantification. All TH-positive neurons were manually counted in a single-blinded setup. Furthermore, TH and GFP colocalization was assessed using cellular morphology and fluorescent intensity parameters. For each section the percentage of GFP-

positive and TH-positive cells was determined, and all values from all of the sections were averaged to give the total percentage of colocalized cells in the whole structure. In the AAV-SYN comparison study one animal in AAV8 group, which the injection had missed the SN structure, was excluded from further statistics unless specified. Caudate quantification was performed with the use of ImagePro Plus Measure Threshold macro. The striatal areas in both hemispheres were outlined based on the TH expression in the striatal fibers. The average GFP background signal measured in cortex areas in both hemispheres was subtracted from each ipsilateral striatum signal. Next, the recalculated intensity from the non-injected striatum was subtracted from the injected side. Finally, this average intensity value in the injected striatum was multiplied by the size of the area resulting in total GFP intensity of the measured area.

3. All AAV serotype vectors with the CMV promoter direct poor transgene expression in DAergic neurons of the mouse SN

Vectors based on AAV serotypes 1, 2, 5, 7, 8 and LV that contained the CMV-GFP expression cassette were injected into the mouse SN. Four weeks after the injection, the number of TH-positive neurons expressing GFP was quantified. All AAV serotypes and the LV vector showed very low numbers of transduced TH-positive neurons (Figure 1B). AAV7 directs the highest transduction rate with 8% of the TH-positive SN cells expressing GFP. Most of the serotypes, with the exception of AAV2, showed high rates of cellular transduction in and around the SN, but the transduced neurons were TH-negative (Figure 1A). AAV1 and LV also transduced glial cells particularly in the area directly surrounding the injection site (data not shown). Glial transduction has not been observed with any other AAV serotypes.

Three groups have reported on AAV-mediated gene transfer to the mouse SN (Chen et al., 2008; St Martin et al., 2007; Taymans et al., 2007). Experimental details, including the serotype and promoter used in these studies are summarized in Table 2.

Study	Viral vector used	Analysis parameters	Used promoter
Taymans et al., 2007	AAV1, 2, 5, 7 and 8	GFP expression in the SN & the striatum	CMV
St Martin et al., 2007	AAV2	GFP and TH colocalization in the SN	CBA
Chen et al., 2008	AAV2	GFP and TH colocalization in the SN	CBA

Table 2. Summary of literature reports using AAV vectors for gene transfer in mouse SN.

A comparative analysis of AAV1, 2, 5, 7 and 8 showed efficient transduction of cells in the SN area (Taymans et al., 2007). AAV 1 and 5 showed robust GFP expression in the fibers of the striatum. In contrast AAV7 and 8 directed only low GFP levels in this projection area of the SN DAergic neurons. Additionally, a few GFP positive cells were observed in the striatum in all of the serotypes. Consistent with our observations, AAV2 showed poor transgene expression in the SN. The efficiency of AAV-CMV driven GFP expression was assessed qualitatively and no specification of the DAergic lineage of the transduced cells was performed. We have also found high numbers of GFP expressing cells with AAV-CMV vectors in and around the SN, however, quantification of the TH-positive GFP-labeled cells revealed very low numbers of GFP expressing DAergic neurons in the SN (Figure 1).
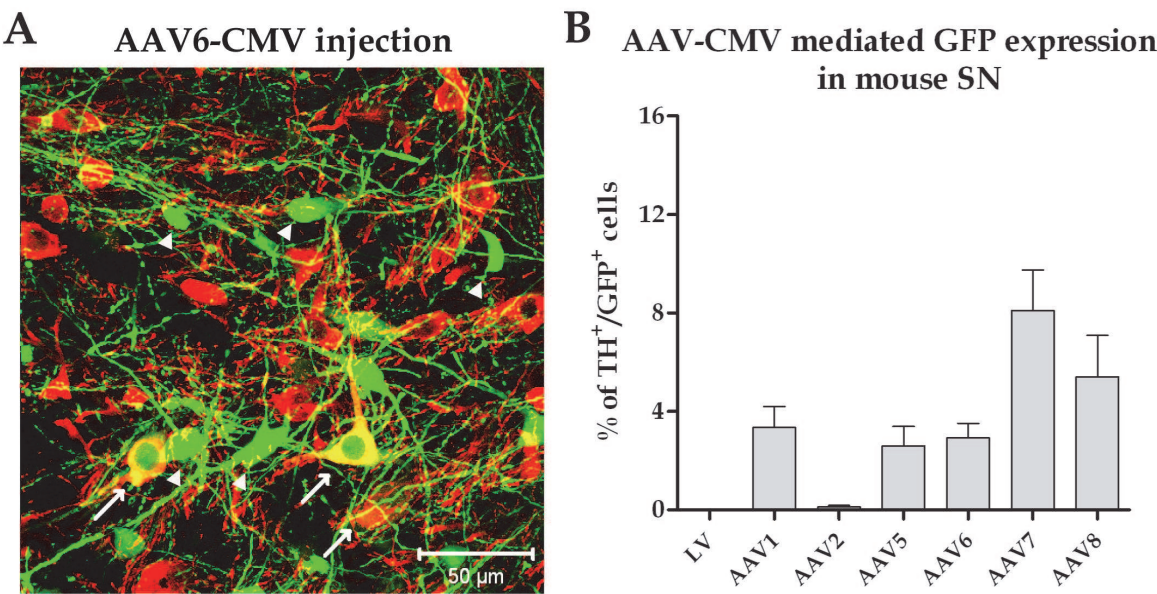
Fig. 1. AAV-CMV-mediated GFP expression in the mouse Substantia Nigra. **A.** Confocal Z-stack image of an immunohistochemical staining of the mouse SN showing GFP transgene expression in a small number of dopaminergic neurons identified by TH staining (arrows indicate the double labeled cells) but also in non-dopaminergic neurons (arrowheads) after AAV6-CMV injection. TH neurons are shown in red, GFP is shown in green. The scale bar indicates 50μm. **B.** Quantification of transduced DAergic neurons in the mouse SN (n=3) using LV-CMV-GFP and AAV-CMV-GFP viral vectors. The bars represent the percentage of TH positive neurons in the SN expressing GFP. AAV7 shows the highest transduction of 8% of DAergic neurons in the SN. Error bars indicate the SEM.

The other two studies, performing AAV mediated gene transfer in mouse SN used only serotype 2. In the first study, injection of AAV2 into the SN showed between 10 and 80% of TH-positive neurons that express GFP in individual sections (St Martin et al., 2007). In the second study AAV2 was injected into the posterior SN, which resulted in transduction of 71.1±6.0% of TH-positive neurons (Chen et al., 2008). The number of GFP transduced cells in both of these studies is much higher compared to our study, where AAV2-CMV led to the lowest transduction rate of SN neurons. Although both studies used slightly different injection coordinates than we did, it is unlikely that this caused significant differences in AAV2 transduction efficiencies. The most likely explanation for this difference is the use of the chicken β-actin (CBA) promoter, a promoter known to drive stronger and more persistent expression in several population of neurons (Fitzsimons, Bland, & During, 2002). Unfortunately there were no other serotypes used in these studies.

4. AAV vectors that harbor the synapsin promoter direct high-level transgene expression in dopaminergic neurons of the mouse SN

Based on our results and the available literature, the CMV promoter appears to direct less efficient transgene expression in different cell types, including striatal neurons (Jakobsson, Ericson, Jansson, Bjork, & Lundberg, 2003), cochlea cells (Liu et al., 2007), human embryonic stem cells (Orban et al., 2009) and finally rat SN neurons (Paterna, Moccetti, Mura, Feldon, & Bueler, 2000; Wang et al., 2005). Therefore we conclude that the CMV promoter leads to a
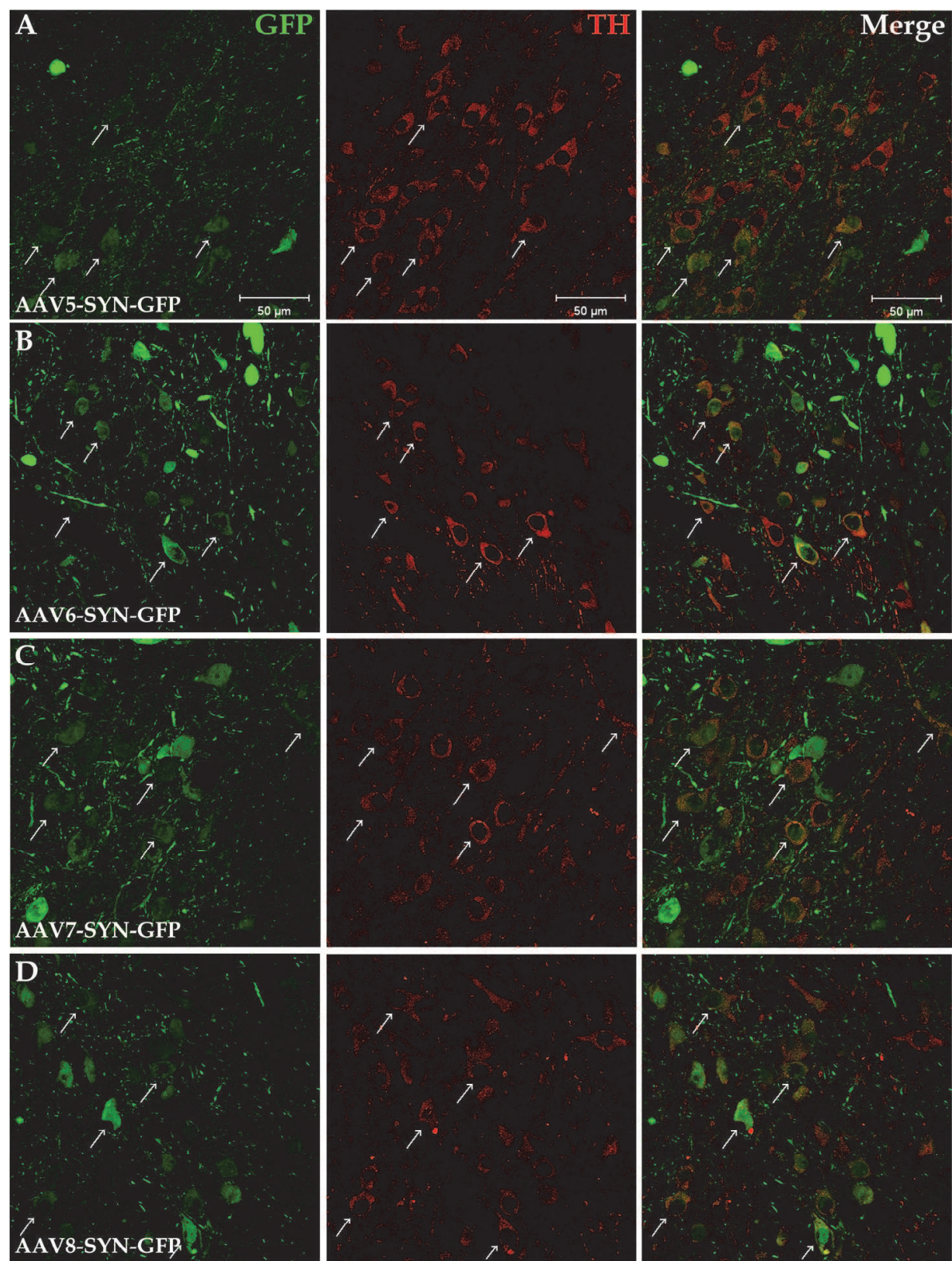
Fig. 2. GFP expression in AAV-SYN injected mouse SN. GFP stained (green) and TH stained (red) neurons are visualized in confocal images. Arrows point to examples of GFP positive and TH positive cells in all viral serotypes. **A.** AAV5-SYN-GFP; **B.** AAV6-SYN-GFP; **C.** AAV7-SYN-GFP; and **D.** AAV8-SYN-GFP. The scale of 50µm is represented by a bar in panel A.

limited transduction in the mouse SN and may not be suitable to drive viral vector-mediated transgene expression in the DAergic neurons of the mouse SN. The human synapsin 1 promoter, on the other hand, has been shown to be an excellent neuron specific promoter in co-cultured primary hippocampal neurons isolated from embryonic brain (Kugler, Lingor, Scholl, Zolotukhin, & Bahr, 2003), in primary dorsal root ganglion cultures (Sims et al., 2008) and *in vivo* following injection of an adenoviral vector in rat brain, including the SN (Hermening, Kugler, Bahr, & Isenmann, 2006; Kugler, Kilic, & Bahr, 2003; Kugler et al., 2003). We therefore produced 4 AAV vectors (AAV5, 6, 7 and 8) that harbored a GFP reporter gene under the human synapsin 1 promoter. The performance of these AAV vectors was tested in mice and rats after injection in the SN.

4.1 AAV-synapsin-GFP drives transgene expression in tyrosine hydroxylase positive neurons

Vectors based on AAV serotypes 5, 6, 7 and 8 that contained a SYN-GFP expression cassette were injected in the SN in the same fashion as the AAV-CMV-GFP viral vectors. All serotypes directed GFP expression in neurons throughout the midbrain, including the SN. AAV7 injected mice showed the most widespread viral transduction with GFP-positive neurons in multiple midbrain areas (data not shown).

Subsequently, we specifically investigated the transduction efficiency of DAergic neurons of the SN. Immunohistochemical staining showed that all serotypes transduce TH positive neurons in the mouse SN (Figure 2). Interestingly, the level of GFP expression in the individual TH positive cells appears to be lower than in other TH-negative neurons in the SN area. This was also observed following transduction with AAV-CMV vectors. In addition, large numbers of GFP positive fibers were observed in the SN. Based on cellular morphology, no other cell types expressed GFP. This indicates that the AAV-SYN-GFP construct drives neuron-specific expression.

4.2 AAV5 and AAV7 mediate the highest transduction of TH positive neurons in the mouse SN

Quantification of the number of TH-positive and GFP-positive neurons in the SN demonstrated a much higher proportion of double labeled neurons with all AAV-SYN vectors compared to the AAV-CMV vectors. AAV5 and AAV7 lead to significantly higher percentage of the GFP labeled DAergic neurons compared to AAV6 and AAV8. These two serotypes directed GFP expression in 76-80% of TH-positive neurons (Figure 3A). The homogeneous distribution of the TH and GFP-positive neurons from the posterior to the anterior side of the SN corroborates the superiority of AAV5 and AAV7 compared to AAV6 and AAV8 at each anatomical level (figure 3B, for more details see supplementary figure 1).

In conclusion, AAV5-SYN and AAV7-SYN are the most effective vectors for transduction of DAergic neurons in mouse SN. Interestingly, even though AAV5 had a significantly higher titer in comparison to AAV7, it transduces a comparable number of DAergic neurons throughout the SN. In contrast, AAV8 injected animals, apart from a relatively low total percentage of TH and GFP-positive neurons, display a decrease in the number of these neurons in the anterior portion of the SN. AAV6 shows quite poor transduction efficiency throughout the SN. This is in accordance with the relative low overall percentage of TH-positive neurons that express GFP (Figure 3A).
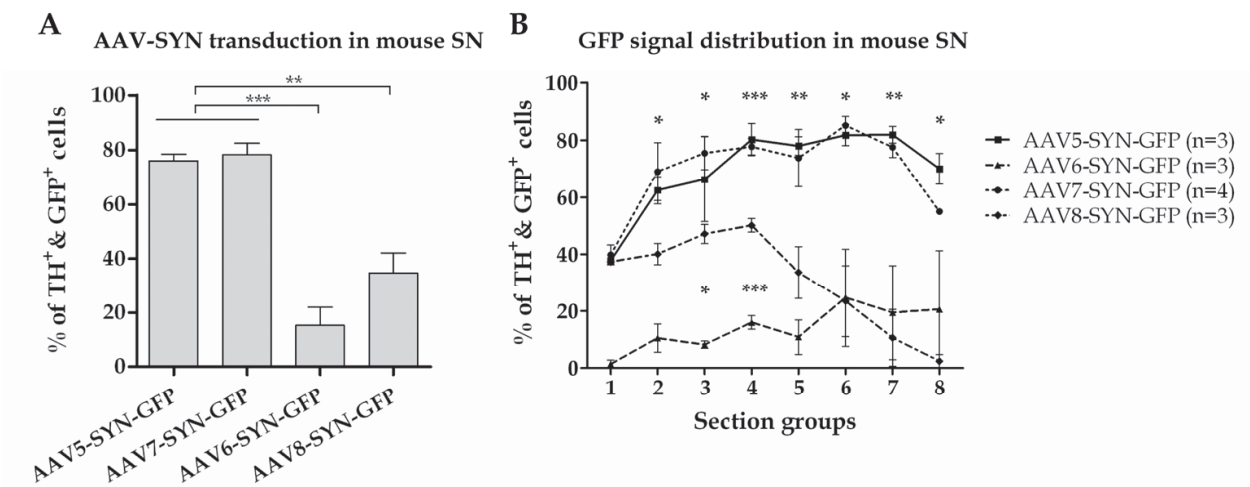
Fig. 3. AAV-SYN-GFP transduction of mouse SN. **A.** Quantification of TH positive (TH+) and GFP positive (GFP+) neurons in mouse SN transduced with different AAV serotypes. In the AAV5 and AAV7 groups, almost 80% of TH+ neurons express GFP, which is significantly higher than the AAV6 group where < 20% of the DAergic neurons are GFP positive (P<0.001), and the AAV8 group where 35% of the DAergic neurons express GFP (P<0.01). **B.** Quantification of GFP expressing TH+ cells per serotype in SN serial sections arranged according to the Allan Brain Atlas TH in situ hybridization (Lein et al., 2007) with 1 being most posterior and 8 being the most anterior part of the SN (supplementary figure 1). Throughout the groups there was no statistical significant difference between AAV5 and AAV7, however in section groups 2, 4, 5, 6 and 7 AAV7 showed significantly higher average of GFP+/TH+ cells than the other two serotypes (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), where as in section 3 only comparing to AAV6 ($P < 0.01$). AAV5 showed a significantly higher GFP expression in comparison to AAV6 and AAV8 serotypes in section groups 4, 5, 6, 7 and 8 and in section 2 and 3 to AAV6 only. AAV8 shows significantly higher GFP expression to AAV6 in group 3 and 4 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

4.3 AAV7 shows the most GFP positive fibers ascending through the nigrostriatal tract

The DAergic neurons of the substantia nigra anatomically project to the striatum creating the nigral-striatal pathway. Accordingly, when the DAergic neurons in the substantia nigra are transduced their fibers in the striatum are expected to be GFP positive. To investigate this relationship, we have quantified the intensity of GFP fluorescent signal in the mouse striatum and compared this to the effectiveness of the AAV serotypes to target the DAergic neurons in the SN (Figure 4). While AAV5-SYN-GFP and AAV7-SYN-GFP display a high fluorescence intensity of GFP, AAV6-SYN-GFP and AAV8-SYN-GFP show a low level of GFP expression in the striatum. Furthermore, AAV7-SYN directs significantly higher levels of GFP expression in the striatum compared to AAV5-SYN (Figure 4 E). Moreover, we have found a significant correlation between the transduction efficiency of the SN and the labeling intensity of the fibers in the striatum (Figure 4F). This corroborates the superiority of the AAV7 and AAV5 serotypes in transducing DAergic neurons in the mouse SN.
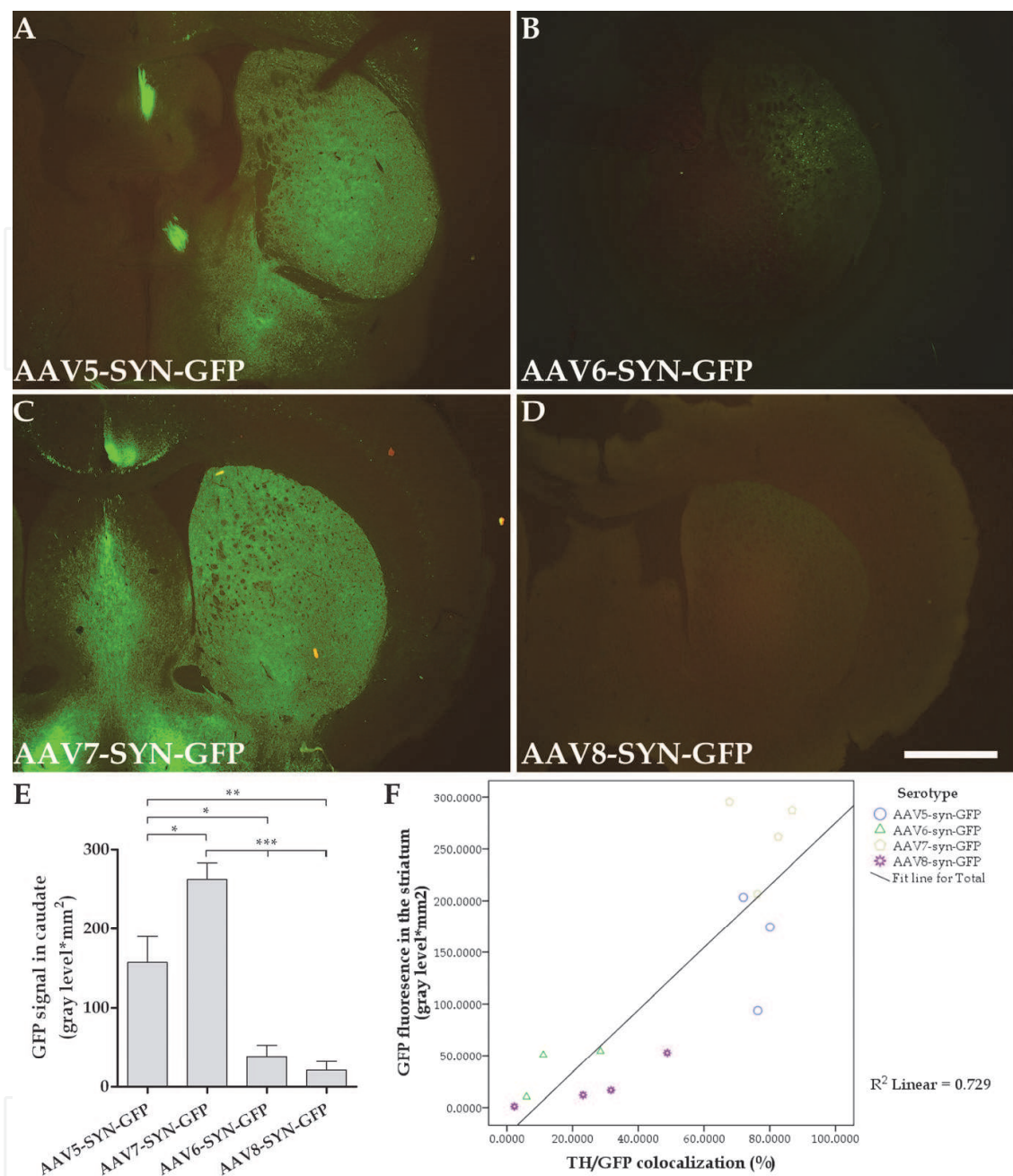
Fig. 4. GFP expression in the mouse striatum after SN AAV transduction. **A-D.** Mouse striatal sections stained with anti-TH (red) and-GFP (green) antibody transduced with different AAV serotype. The scale bar represents 1mm. **E.** Quantification of GFP fluorescent intensity in all AAV-SYN serotypes. Each striatal section was measured for GFP intensity and corrected for the measured area (gray level*mm^2). Statistical analysis indicates AAV7-SYN-GFP result in the highest GFP expression in the striatum, with AAV5 inducing the second best expression ($*P<0.05$, $**P< 0.01$ and $***P<0.001$). **F.** The percentage of TH+ neurons expressing GFP in the SN and the level of GFP fluorescence in the striatum are significantly correlated (Pearson correlation, $R^2=0.729$, $P<0.001$). Each individual animal belonging to a specific AAV serotype injection group is depicted by different marker described in the figure legend. Animal with missed injection in AAV8-SYN group (as described in Methods section) was also included in this analysis.

Taken together, these observations demonstrate that AAV7-SYN is the best vector for the transduction of TH-positive neurons of mouse SN among the tested serotypes regarding the specificity and the transduction rate. Although both AAV5 and AAV7 effectively transduced large numbers of TH-positive neurons in the SN, AAV7 showed significantly higher GFP expression in the striatum. Injection of AAV7-SYN also resulted in a substantial transduction of other neurons in the midbrain. This indicates that AAV7 spreads further than other serotypes and/or has a more ubiquitous neuronal tropism in the mouse brain. It would be worthwhile to investigate whether it is possible to more specifically target only DAergic neurons in the SN by e.g. lowering the volume of the viral vector solution that is injected into the SN. Finally, we have not seen any signs of toxicity following the injection of high titer AAV vectors in the mouse brain.

5. All AAV-vectors harboring the synapsin promoter direct similar level of transgene expression in the rat SN and the striatum

5.1 All AAV-synapsin serotypes show similar GFP expression throughout the rat SN

Female Sprague-Dawley rats were injected with AAV-SYN-GFP viral vectors 5, 6, 7 and 8 into the SN. The viral stocks used were the same as in mouse SN injections. To estimate the transduction efficiency, the TH-positive and GFP-positive neurons of the SN were quantified in the same fashion as described in the mouse study. All AAV serotypes showed similar numbers of GFP-positive neurons in the rat SN (Figure 5A) with no significant
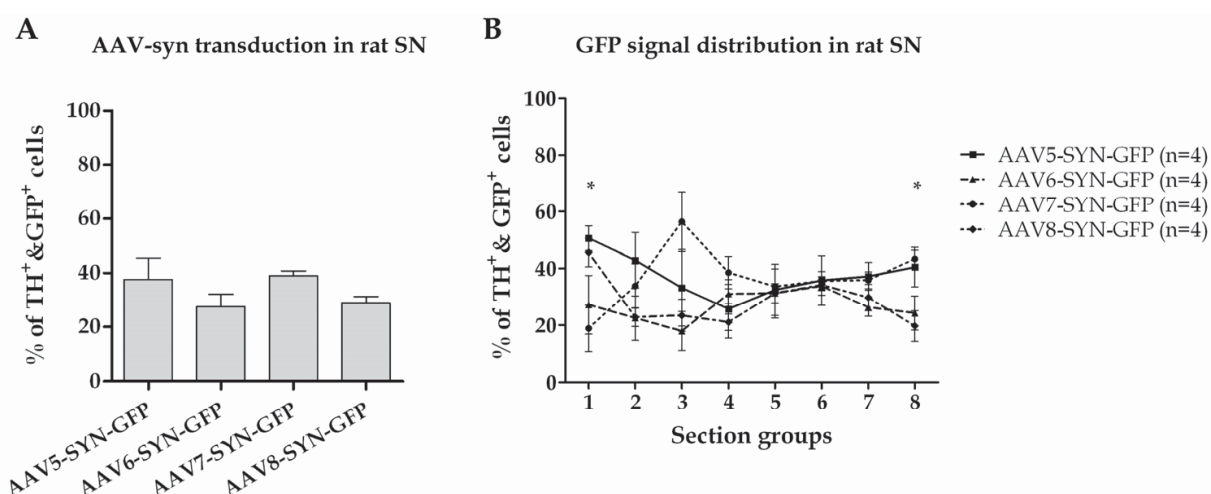

Fig. 5. AAV-SYN-GFP transduction of rat SN. **A.** Quantification of TH positive (TH+) and GFP positive (GFP+) neurons in rat SN transduced by different AAV serotypes. All serotypes showed similar GFP expression in about 30-40% of DAergic neurons. **B.** Quantification of GFP expressing TH-positive cells per serotype in the SN serial sections arranged in posterior-distal direction accordingly to the 'The rat brain atlas' by Paxinos and Watson (2007) (arrangement was based on the stereotact anatomical slides and adjacent Acetylcholinesterase (AChE) stainings with 1 being most posterior area and 8 being the most anterior area of the SN (supplementary figure 2)). All of the serotypes show similar GFP distribution within the SN structure with two exceptions: section 1 shows AAV5 expressing significantly higher amount of GFP + in TH+ cells comparing to AAV7 (* $P < 0.05$) and section 8 where AAV7 has greater amount of GFP+ DAergic cells than AAV8 (* $P < 0.05$).
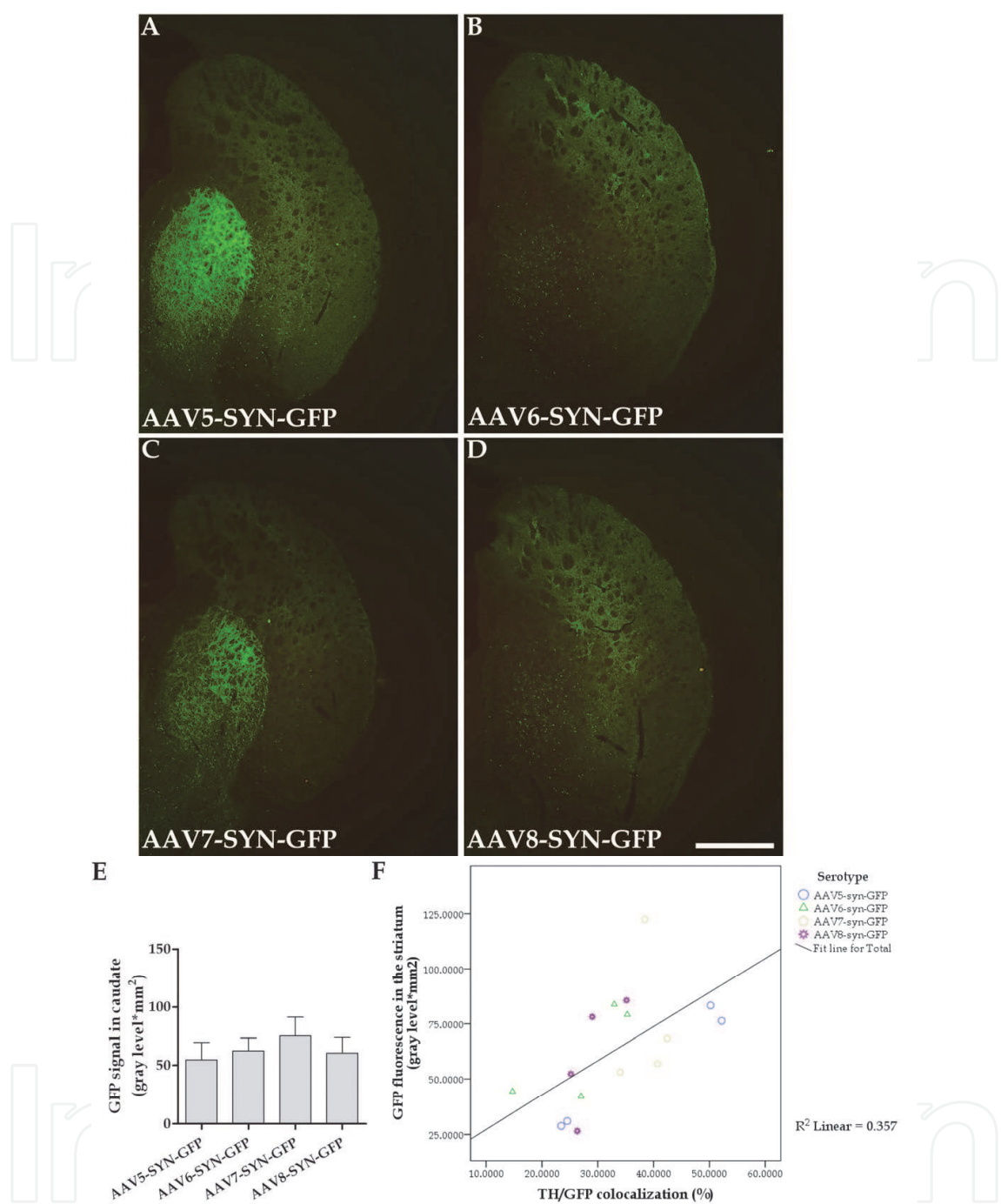
Fig. 6. GFP expression in the rat striatum after SN AAV injection. **A-D.** Rat striatal sections stained with anti-TH (red) and-GFP (green) antibodies. The scale bar represents 1mm. **E.** GFP fluorescent intensity quantification in the striatum for all AAV-SYN serotypes. Whole striatum was cut, stained and each section was measured for GFP intensity and corrected for the measured area (gray level*mm^2). Statistical analysis shows no differences in GFP expression levels between the different AAV serotypes. **F.** The percentage of TH+ neurons expressing GFP in the SN and the level of the GFP fluorescence in the striatum are significantly correlated (Pearson correlation R^2=0.357, P<0.05). Each individual animal belonging to a specific AAV serotype injection group is depicted by different marker described in the figure legend.

Comparison of AAV Serotypes for
Gene Delivery to Dopaminergic Neurons in the Substantia Nigra
217

differences between the serotypes. For AAV5 and AAV7 the proportion of transduced TH-positive neurons is lower in the rat SN compared to the mouse SN. AAV6 directed transgene expression in a higher proportion of DAergic neurons in the rat SN (27%) than in mouse SN (16%), whereas AAV8 shows slightly higher number of GFP-positive neurons in the mouse SN (36%). These results suggest differential AAV tropism for DAergic neurons in rats comparing to mice.

Analysis of the distribution of the GFP-positive neurons within the SN also shows no major differences between the AAV serotypes (Figure 5B) except for two anatomical levels where AAV5 shows significantly higher amount of TH-positive and GFP-positive cells when compared to AAV7 in section 1, and AAV7 shows more GFP-positive DAergic neurons when compared to AAV8 in section 8. Although significant, these differences are not prominent enough to allow us to speculate on either of the serotype superiority in transducing rat DAergic neurons.

5.3 All AAV-SYN serotypes show GFP expression in the fibers of the rat striatum

As in the mouse study, we have quantified the intensity of GFP signal in the rat striatum to compare the effectiveness of AAV serotype transduction of the rat SN anatomical projection (Figure 6). All AAV serotypes showed similar, relatively high levels of GFP expression in the striatum (Figure 6E). A correlation analysis revealed a significant correlation between the numbers of TH and GFP-positive neurons and the levels of GFP expression in striatum (Figure 4F). This further supports that all AAV serotypes are equally effective in targeting rat substantia nigra DAergic neurons. In addition, we have observed a transduction of the globus pallidus fibers by AAV5 and AAV7 serotypes. This may suggest more spread of these viral vectors in the rat brain.

5.4 AAV7 viral vector injection decreases the amount of TH-positive cells in the rat SN

We observed a > 50% decrease of number of TH-positive neurons in rat SN after AAV7 injection when compared to the non injected contra-lateral side of the structure (Figure 7). We also studied the expression of vesicular monoamine transporter-2 (VMAT2), another DAergic phenotype marker, and found its protein levels also strongly decreased in the injected SN (data not shown). Interestingly, the number of TH-positive and GFP-positive neurons in the SN is not less than in the other serotypes (Figure 5) as well as the intensity of GFP-positive fibers in the striatum (Figure 6).

6. Discussion and concluding remarks

Targeted gene delivery to mesencephalic DAergic neurons can be a very valuable approach to study the molecular mechanisms that underlie the development and progression of PD. Gene delivery to DAergic neurons has also a potential to evolve into a new therapeutic strategy for PD. In this study we have compared the capacity of multiple AAV serotypes to deliver a reporter gene to DAergic neurons in the adult mouse and rat SN. We have quantified the transduction efficiency of AAV vectors harboring two different promoters: the CMV and human synapsin 1 (SYN) promoter. We have demonstrated that following stereotactic injection of vectors containing the SYN promoter, a large number of DAergic neurons express GFP in the mouse as well as the rat SN. AAV7 is the most effective serotype for transduction of mouse SN DAergic neurons. AAV5 also displayed high transduction

efficiency for TH-positive neurons, but the GFP expression levels in the striatum were consistently lower when compared to AAV7. In the rat, all AAV-SYN vectors efficiently transduced DAergic neurons in the SN. AAV vectors containing the CMV promoter directed expression only in a small proportion of TH-positive SN neurons in mice, thereby demonstrating superiority of the synapsin promoter in this specific neuronal subtype. Collectively, these observations are useful for future experiments that aim to study the function of specific genes in the mesencephalic DAergic system.
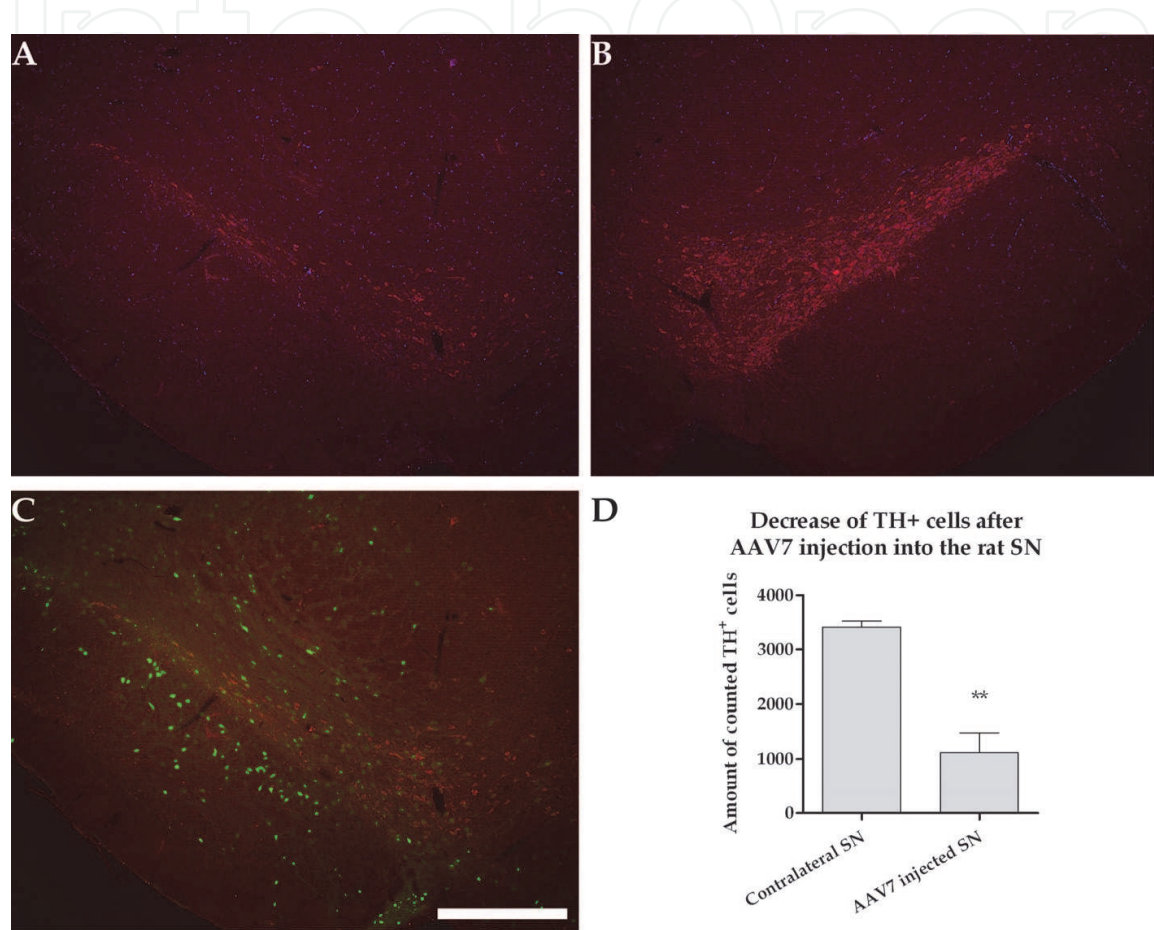
Fig. 7. AAV7 decrease of TH immunohistochemical signal in rat SN. All of the images were obtained from the same brain section. **A.** and **C.** Rat SN injected with AAV7-SYN-GFP **B.** Contra-lateral non injected side of the rat SN. Section was stained for TH (red), a nuclear marker Hoechst (blue) and GFP (green). The scale bar in panel C represents 0.5mm. **D.** Quantification of the TH positive neurons in the rat SN after AAV7 transduction. TH positive neurons were counted in both the AAV7 injected side of the SN and the contra-lateral side in each section. One way ANOVA indicated a significant decrease of TH+ neurons in the AAV7 injected SN (** $P < 0.01$).

So far, one study has compared five AAV serotypes (AAV 1, 2, 5, 7 and 8) for gene delivery to the mouse SN (Taymans et al., 2007). All vectors, except for AAV2, showed positive transduction of the SN area, with AAV1 and AAV5 implicating to direct highest levels of GFP in the striatal fibers. This study did not show quantifications of the numbers of GFP and TH-positive neurons and GFP expression in striatal fibers, and can therefore not be directly compared to our study.

For the rat SN, the four tested AAV serotypes directed equally efficient transduction in DAergic neurons. In comparison, AAV5 and AAV8 appear to display the most consistent transduction efficiencies in the literature (Klein et al., 2006; McFarland et al., 2009; Paterna et al., 2004). Additionally, AAV9 and AAV10 have also been tested and indicated to have higher tau expression in the SN area and higher TH neuronal loss evoked by tau expression comparing to AAV2 and AAV8 (Klein et al., 2008). These observations in combination with our results indicate that multiple AAV serotypes share relatively high tropism for DAergic neurons of the rat SN.

In the mouse SN, AAV vectors with the SYN promoter were superior to the AAV vectors containing the CMV promoter. In contrast, in the rat, a range of promoters have been used very successfully to direct the transgene expression in the DAergic neurons of the SN via an AAV vector including the CMV (McFarland et al., 2009), CBA (Burger et al., 2004; Klein et al., 2006; Paterna et al., 2004; Ulusoy et al., 2009), CMV/CBA hybrid (Klein et al., 2008) and PDGV (Paterna et al., 2000)) promoters. This suggests that for experiments in the rat the choice of the promoter is not as critical as it appears to be for the mouse.

Following AAV7-SYN-GFP injection, a dramatic decline in TH and VMAT2 expression occurred in the DAergic neurons in the rat SN. Klein et al. also observed a decrease in the number of TH-positive cells following high titer AAV8-GFP application, but not after AAV8-empty vector. Moreover, following the application of lower AAV8-GFP viral titer, no decrease of TH signal was noted. Therefore the authors suggest that the high concentrations of GFP can be neurotoxic to the DAergic neurons of the rat SN (Klein et al., 2006). Ulusoy et al. also reported a neurotoxic effect of high titer AAV5-GFP viral vectors. In this study not only TH was diminished, but also the expression of VMAT2. In subsequent experiments with low titer AAV5-GFP injections this effect was not seen anymore (Ulusoy et al., 2009). We speculate that in our case, the high tropism of AAV7 for DAergic neurons induces more GFP expression, and as a consequence causes DAergic neurotoxicity. We do not see this effect on TH expression in mice SN after the AAV7 injection.

As presented here, the specifications of the delivery vehicle can be crucial for successful and accurate cellular transduction. We have demonstrated that targeting SN in the mouse is difficult and could only be successfully achieved in our set up with AAV serotypes 7 and 5 harboring the SYN promoter. In contrast targeting rat SN can be efficiently achieved by multiple AAV viral vectors. It is therefore necessary to determine the vector potential for each animal species before pursuing genetic manipulation in the DAergic system. This can also be valid for human clinical PD gene therapy studies. So far AAV2 has been the only vector injected into the human brain as a delivery vehicle for PD gene therapy (Kaplitt et al., 2007; Marks, Jr. et al., 2010; Muramatsu et al., 2010). As discussed before, AAV2 seams to be the least efficient vector in the transduction of DAergic neurons in the rodent brain. Understandably human serotype studies are not possible, but primate studies may shed more light on the transduction efficiency of different delivery vehicles and can improve the efficiency of the gene therapies dramatically. One such study has recently indicated AAV5 to be the most efficient in transducing neurons in the area of SN but also glial cells, whereas glial transduction by AAV5 is not observed in the rodent brain (Markakis et al., 2010). This clearly illustrates the differences between viral vector transduction preferences between animal species.

Another major concern at the moment in the field of gene therapy is the specification of the target area. Two of the above mentioned clinical studies have targeted the putamen of PD

patients (Marks, Jr. et al., 2010; Muramatsu et al., 2010) and one the subthalamic nucleus (Kaplitt et al., 2007). Depending on the function of the target gene, the location of the target area is crucial for successful therapeutic application. It is therefore rational to apply gene therapy for dopamine synthesis enzymes such as AADC to the putamen to increase the dopamine production at that area to alleviate the motor-related clinical symptoms (Muramatsu et al., 2010). On the other hand, it may not be as beneficial to induce an expression of a neurotrophic factor in the area not significantly affected by neuronal death (Marks, Jr. et al., 2010). It was therefore extensively discussed and suggested for the future to target the SN DAergic neurons when applying pro-survival and regenerative therapeutic agents (Benabid, 2010; Marks, Jr. et al., 2010). Concluding, it is therefore necessary to apply the right vectors in the specific animal species and target the appropriate area of interest, depending on the function of the expression gene, for the most effective targeted gene delivery.

7. Acknowledgements

8. Supplementary figures
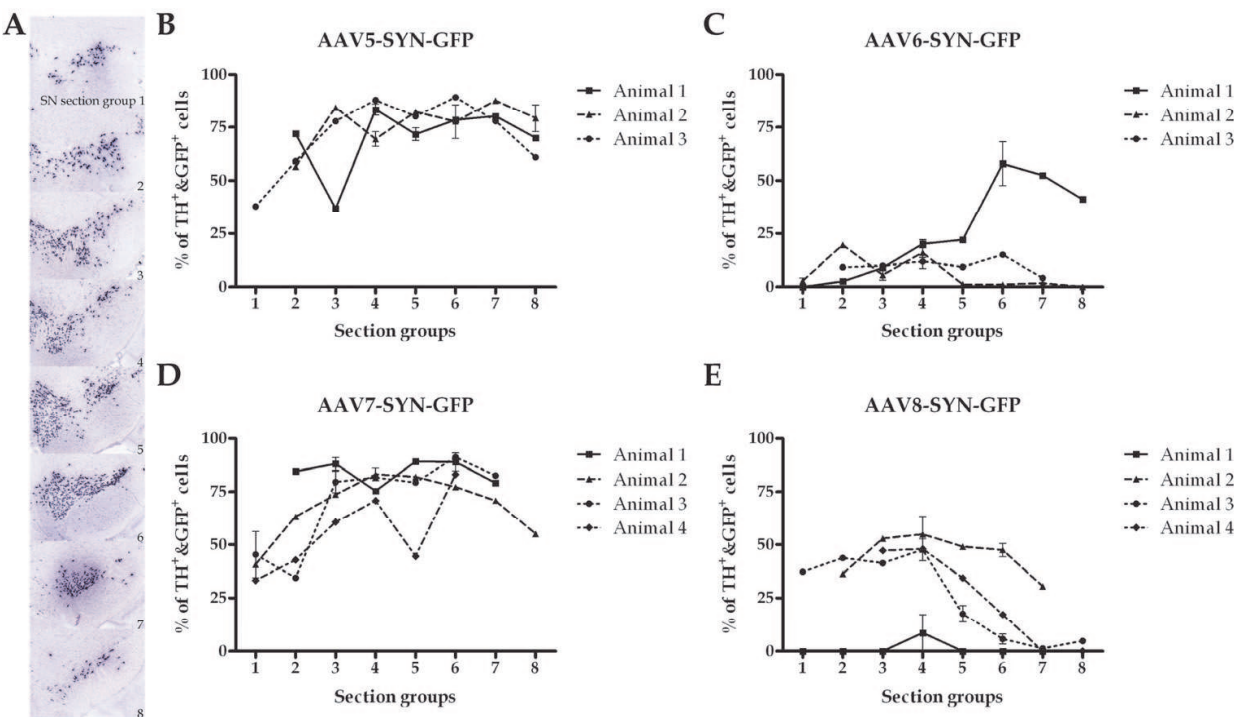
Supplementary Fig. 1. Quantitative overview of AAV-SYN-GFP transduction throughout the mouse SN in 4 different viral serotypes. **A.** The sorting of SN anatomical areas through section groups 1-8 is based on a TH in situ hybridization presented by the Allen Brain Atlas (Lein et al., 2007). Section group 1 is the most posterior group and section group 8 the most anterior. **B-E.** Quantification of SN AAV transduction throughout the structure in 4 different viral serotypes in individual animals. Values represent mean of all quantified sections belonging to the sorted section group and their SEM.
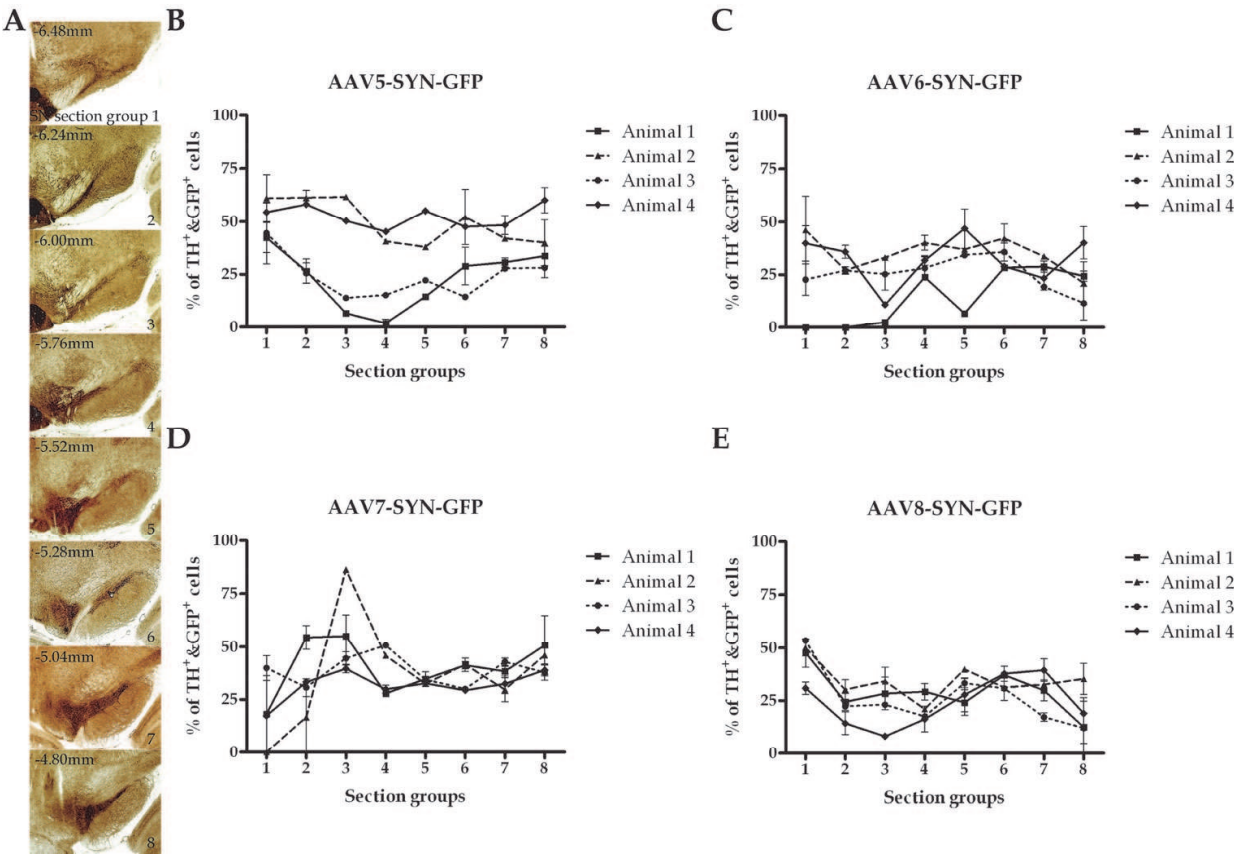
Supplementary Fig. 2. Quantitative overview of AAV-SYN-GFP transduction throughout the rat SN in 4 different viral serotypes. **A.** The sorting of SN anatomical areas through section groups 1-8 is based on the anatomical AChE stainings presented by 'The rat brain atlas' by Paxinos and Watson (2007). Section group 1 is the most posterior group and section group 8 the most anterior with indicated distances from the bregma. **B-E.** Quantification of SN AAV transduction throughout the structure in 4 different viral serotypes in individual animals. Values represent mean of all quantified sections belonging to the sorted section group and their SEM.

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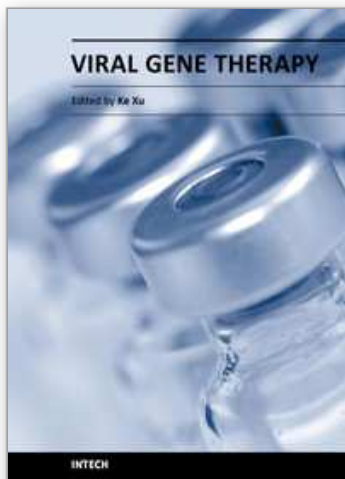
Viral Gene Therapy

Edited by Dr. Ke Xu

The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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