Mouse gender influences brain transduction by intravascularly administered AAV9

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Abstract

Vectors based on adeno-associated virus serotype 9 (AAV9) cross the blood-brain barrier and mediate efficient gene expression in the mammalian brain. We observed that intravenous injection of single-stranded AAV9 encoding the reporter genes Firefly luciferase and green fluorescent protein yielded higher transgene expression in the brain of female mice compared to male mice. This observation was consistent in two strains of mice (nude and C57BL/6) and was correlated with an increased number of AAV genomes in female brain compared to males. Increased numbers of GFP positive astrocytes and neurons were also detected in female brains. These observations stress the importance of carefully matching groups for gender and may also have implications for improving gene expression in animals of the male gender.
**Introduction**

Adeno-associated virus serotype 9 (AAV9) has shown remarkable efficiency in transducing organs *in vivo* including the heart, liver, and brain [1–6]. Recently, AAV9 has gained renewed interest due to its ability in crossing the blood-brain barrier upon intravenous injection [7–12]. In adult mice, AAV9 was shown to preferentially transduce endothelial cells, astrocytes, and neurons after intravenous injection [10]. The implications of these findings are enormous: a noninvasive route of administration and widespread gene delivery to the brain. Complete characterization of the gene transfer properties of AAV9, including kinetics of expression, immunogenicity, and biodistribution in different species will be necessary for clinical translation.

Here we show that intravenous injection of single-stranded AAV9 encoding the reporter genes *Firefly* luciferase (Fluc) and green fluorescent protein (GFP) yielded higher transgene expression in the brain of females compared with male mice. This observation was consistent in two strains of mice (nude and C57BL/6) and was correlated with an increased number of AAV genomes in the female brains. These findings stress the importance of proper matching of mice gender in different experimental groups when using systemic injection of AAV9.

**Methods & Results**

Age-matched (6–8 weeks) male and female athymic NU/NU nude mice and C57BL/6N mice were injected intravenously (through the tail vein) with $1.5 \times 10^{12}$ gene copies (g.c.)/kg of single-stranded AAV9 vector encoding Fluc, driven by the ubiquitously active cytomegalovirus/chicken beta-actin (CBA) hybrid promoter (AAV9-Fluc). The transduction efficiency of AAV9 in the brain and abdomen was monitored 7 and 14 days later using bioluminescence imaging (BLI). We observed a higher bioluminescent signal in the head region of females compared with males in both strains of mice and at both time points (Figure 4.1a,b). In contrast to the bioluminescent signal in the head region, and in support of previous reports [13–15], the Fluc signal from the abdomen (most likely liver) was lower in female mice than in males (Supplementary Figure S4.1).

In another set of mice, we performed a kinetic analysis of luciferase expression in the head and liver in both male and female nude and C57BL/6N mice over a 4-week period. We observed a stable luminescence expression in the head of nude and C57BL/6N mice, whereas the abdomen values showed more variability over time in both strains (Supplementary Figure S4.2). Next we confirmed the BLI results by direct analysis of Fluc enzymatic activity in the brain regions from dead animals, *ex vivo*. Consistent with BLI, the Fluc levels in the cortex, cerebellum, and olfactory bulbs of female mice were significantly higher ($p < 0.05$) compared to the brain of male mice in both strains (Supplementary Figure S4.3).
Figure 4.1: Systemic injection of AAV9 yields higher transduction of the brain of female compared to male mice. (a,b) Male and female nude and C57BL/6 mice were injected via the tail vein with $1.5 \times 10^{12}$ g.c./kg of AAV9-Fluc vector. Mice were injected 7 and 14 days later with D-luciferin and imaged for Fluc-associated light emission. Representative mice from each group ($n = 5$ per group) at day 14 are shown in a. Total flux was calculated following data collection by selecting a region of interest (ROI) around the head (b). (c,d) C57BL/6 mice were injected via the tail vein with $5 \times 10^{13}$ g.c./kg of AAV9-GFP and 2 weeks later killed for histological analysis for GFP expression ($n = 3$ per group). Representative images of GFP immunohistochemistry from male (top) and female (bottom) mouse brain cortex are shown in c (Scale bar: 1 mm). Quantification of GFP-positive astrocytes and neurons revealed higher numbers of both cell types in females (d). Experiments in a and b were repeated twice, and similar results were obtained. (*$p < 0.05$).

We next sought to determine the transduction profile of AAV9 expressing GFP under CBA promoter (AAV9-GFP) in the brains of male and female mice. C57BL/6N mice were injected intravenously with $5 \times 10^{13}$ g.c./kg of AAV9-GFP. A higher dose was utilized in this experiment to allow visualization of GFP, which is a less sensitive reporter than the Fluc enzyme. At 2 weeks after injection, these mice were killed, then perfused with phosphate-buffered saline, and their brains and livers harvested. Immunostaining with an antibody specific for GFP revealed diffuse expression of almost exclusively neurons and astrocytes throughout the brain (Figure 4.1c and Supplementary Figure S4.4). A count of the GFP-positive neurons and astrocytes per square millimeter in the cortex revealed a 1.7–fold ($p = 0.111$) and 2.7–fold ($p = 0.032$) increase, respectively, in the female vs. the male brains (Figure 4.1d). Interestingly, the ratio of GFP-positive astrocytes to neurons was 2.1 and 1.333 for males and females, respectively, which may indicate a
slight difference in AAV tropism in the brain between mice genders. To investigate whether the increase in transduction of the female brain was linked to higher transport of the vector to this organ, quantitative polymerase chain reaction for AAV genome was performed on tissue homogenates from the cortex of mice. In both strains a significantly higher number of AAV genome copies ($p < 0.05$) was detected in the brain tissue of females compared with male mice (Supplementary Figure S4.5).

**Discussion**

This study is the first to provide a comparative analysis of transgene expression in the brain of male and female mice upon systemic injection of a single-stranded AAV9 vector. Previous studies using this vector serotype used either male or female mice exclusively [11, 13] or mixed littermates were used [10], with no direct comparison between different genders. Although the exact mechanism of increased transgene expression in the brain of female mice remains unknown, our findings are important for future AAV9 vector research, specifically when the systemic injection route is chosen. Of immediate application, our results suggest that equal distribution of mouse gender between groups or the use of one gender for a given study is crucial for accurate data interpretation. Additionally, this study warrants further investigation of AAV9–mediated brain transduction of genders of other species (e.g. rats, dogs, nonhuman primates) for possible translation of these findings to human gene therapy.

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References


Supplementary Material

Supplementary Methods

Cell culture. Human 293T cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in high glucose Dulbecco’s Modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) in a humidified atmosphere supplemented with 5% CO₂ at 37 °C.

Vector constructs and production. Briefly, 293T cells were transfected with AAV (AAV9 rep/cap and AAV-ITR containing transgene expression plasmid [single-stranded genome]) and helper plasmid (Fd6) by the calcium phosphate method. Seventy-two hours post-transfection cells were harvested and vector purified using a standard iodixanol density gradient and ultracentrifugation protocol. Iodixanol was removed and vector concentrated in PBS by diafiltration using Amicon Ultra 100 kDa MWCO centrifugal devices (Millipore, Billerica, MA). Vector was stored at -80 °C until use.

Animals. Animal experiments were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital (MGH), and performed in accordance with their guidelines and regulations. Nude athymic nu/nu mice (age 6–8 weeks) were ordered from the MGH in-house COX-7 facility. C57BL/6N mice (age 6–8 weeks) were purchased from Charles River Laboratories (Wilmington, MA). For tail vein injections of AAV vectors, mice were placed into a restrainer, (Braintree Scientific, Inc., Braintree, MA). Next the tail was warmed in 40 °C water for 30 seconds, before wiping the tail with 70% isopropyl alcohol pads. A 100–300 μL volume of vector (in PBS) was slowly injected into a lateral tail vein, before gently clamping the injection site until bleeding stopped.

Bioluminescence imaging. All imaging experiments were performed using the IVIS Spectrum imager outfitted with an XGI-8 gas anesthesia system (Caliper Lifesciences, Hopkinton, MA). Mice were anesthetized and then injected intraperitoneally with 4.5 mg of D-luciferin resuspended in 150 μL of PBS. Five minutes post-substrate injection, mice were imaged for luciferase expression using auto-acquisition. Region of interest were selected and images were analyzed using Living Image software (v4.17, Caliper Life Sciences).

Ex-vivo luciferase assay and quantitative PCR for vector genomes. Four weeks post-vector injection, mice were sacrificed and organs (liver, whole brain or cerebellum, and olfactory bulb) were harvested for analysis of Fluc levels as well as
virus genome copies (g.c.). Tissues were quickly removed from the animals, and immediately frozen on liquid nitrogen and stored at -80 °C. For Fluc assay, 50 mg of each tissue was lysed in a 96-deepwell plate (Nunc/Thermo Fisher Scientific, PA, USA) using a stainless steel bead (Qiagen Inc., CA, USA) and 500 μL of Mammalian Protein Extraction Reagent (M-PER; Pierce Biotechnology, IL, USA). Tissues were homogenized in a TissueLyser II (Qiagen Inc.) at 7.0 Hz 4x three minutes. Next, the plate was centrifuged for 5 minutes at 1200 rpm and 20 μL of tissue homogenate was transferred to 96-well white bottom plate and analyzed using 100 μL of Bright-Glo™ Fluc substrate reagent (Promega, WI, USA) and a plate luminometer (Dynex Technologies, VA, USA). A Bradford assay was performed to normalize each Fluc value to the total amount of protein in the sample. For the quantitative PCR (qPCR) assay to detect AAV genomes in organs, 25 mg of each organ was cut into small pieces using a sterile razor blade. Isolation of mouse genomic and AAV DNA was performed using the DNeasy® Blood and Tissue Handbook (Qiagen, Valencia, CA). Next 100 ng of total DNA was used as a template for a quantitative TaqMan PCR that detects AAV genomes (Poly A region of the transgene cassette).

**Histology and GFP expression.** At two weeks post-injection, mice were given an overdose of anesthesia and transcardially perfused with PBS. The liver and brain were removed. The brain was separated into two hemispheres. One hemisphere was snap frozen in the vapor phase of liquid nitrogen and stored at -80 °C for subsequent qPCR analysis and the other hemisphere was fixed in 4% formaldehyde in PBS and cryoprotected in 30% sucrose for immunohistological analysis of tissue sections. These hemispheres were cut on the sagittal plane in 40 μm sections using a sledging freezing microtome. Double immunohistochemistry was performed in freefloating sections with primary antibodies for GFP (chicken polyclonal, 1:500, Aves Labs, Cat# GFP-1010) and either GFAP (rabbit polyclonal, 1:1000, Sigma-Aldrich, Cat# G9269), or glutamine synthetase (mouse monoclonal, clone GS-6, 1:1000, Millipore, Cat# MAB302). Briefly, the sections were permeabilized in Tris-buffered saline (TBS) with Triton-X 0.5% for 1 h, blocked in 10% normal goat serum for 1 h, and incubated with the primary antibodies overnight at 4 °C. Next day, the sections were thoroughly washed in TBS with Triton-X 0.1%, incubated with the appropriate fluorescently-labeled secondary antibodies for 1 h at room temperature, washed again in TBS with Triton-X 0.1%, and coverslipped with Vectashield mounting media with DAPI (Vector Labs, Cat# H-1200). The number of cortical GFP-positive astrocytes and neurons was counted in every 10th section under the 4x objective of a BXS1 Olympus epifluorescence microscope (Olympus, Tokyo, Japan), equipped with a motorized stage and a DP70 camera that are coupled to a computer through the image analysis software CAST. The region of interest (cortex) was outlined under the 1.25x objective and the number
of GFP-positive astrocytes and neurons were counted under the 4x objective using the appropriate tools of the software. Neurons and astrocytes were easily distinguished from each other using morphological criteria. The volume of interest corresponding to the cortex from every 10th section was estimated with the CAST software. Total numbers of GFP-positive neurons and astrocytes in the whole brain cortical mantle were then estimated based on the fractionator principle.

### Supplementary Figures

#### Supplementary Figure S4.1: Bioluminescent signal in the abdomen region of male and female mice injected systemically with AAV9-Fluc.**

Male and female nude (a) and C57BL/6 (b) mice were injected via the tail vein with $1.5 \times 10^{12}$ g.c./kg of AAV9-Fluc vector. Seven and 14 days later, mice were injected with D-luciferin and imaged for Fluc-associated light emission. At each time point, a representative mouse from each group ($n = 5$ per group) is shown in the panel. Total flux was calculated post data collection by selecting a region of interest (ROI) around the abdomen (ventral view). Experiments were repeated twice and similar results were obtained. (M = male, F = female, D = day. *$p < 0.05$.$n = 5$ per group).
Supplementary Figure S4.2: Bioluminescence kinetic analysis in the head and abdomen region of mice injected with AAV9-Fluc. Nude (a,b) and C57BL6/N (c,d) mice were injected with $5 \times 10^{12}$ g.c./kg of AAV9-Fluc and imaged at the time points depicted in the graph. Bioluminescence signal was quantitated in the head and abdomen region at each time point. (* $p < 0.05$. $n = 5$ per group).

Supplementary Figure S4.3: Fluc activity in brain tissue homogenates in male and female mice injected i.v. with AAV9-Fluc. Male and female nude and C57BL/6N mice were sacrificed four weeks after i.v. injection of AAV9-Fluc ($1.5 \times 10^{12}$ g.c./kg). Tissues were harvested and homogenized. Biochemical assays were performed for Fluc expression and protein content was determined for normalization. Fluc levels in tissue homogenates of brain, liver, cerebellum and olfactory bulb of nude and C57BL/6N mice. All Fluc levels were normalized to total mg of protein in tissue homogenate. Experiments were repeated twice and similar results were obtained. (D = day, RLU = Relative Light Units. *$p < 0.05$; *$ trend. $n = 5$ per group).
Supplementary Figure S4.4: AAV9 transduces primarily neurons and astrocytes in male and female mice upon systemic injection. Image of a section of cortex from female mice injected i.v. with AAV9-GFP represents the typical morphology of the brain cells transduced: the arrow points to a pyramidal neuron, and the arrowheads point to two astrocytes. (Scale bar, 50 μm).

Supplementary Figure S4.5: Quantitation of AAV9 genome copies in the brain of male and female mice after systemic vector injection. DNA was purified from homogenates of brain and liver from C57BL/6N mice injected i.v. with $5 \times 10^{12}$ g.c./kg of AAV9-Fluc (a) and $5 \times 10^{13}$ g.c./kg of AAV9-GFP (b), and analyzed by quantitative PCR to detect AAV genomes (100 ng template). (*p < 0.05. n = 3 per group).
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