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THE EFFECTS OF SEROTYPES AND ROUTE OF ADMINISTRATION ON TRANSDUCTION EFFICIENCY FOR AAV-MEDIATED GENE DELIVERY TO THE CNS

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Abstract

Introduction: *The recent advances in gene-therapy have enabled clinicians to treat diseases that were once deemed untreatable without any effective treatment modalities. Modulation at the genetic level was once thought to be impossible. However, advances such as using recombinant adeno-associated viruses (rAAV), which are viruses that have been re-engineered to be used as a vehicle for transport and subsequent transduction have opened new horizons in replacing defective and under expressed proteins. Various rAAV have been engineered, each with differing susceptibility to different tissues.*

Methods*: In this experiment, we tested all of the aav's available on the market (AAV1, 2, 5,6,7 & 8) with both injection directly into the spinal cord as well in the major muscles (triceps, quadriceps and gastrocnemius) of Naïve C57BL/6J mice. The aav were constructed to express Green Fluorescent Protein (GFP). We analyzed brain and spinal cord from animals harvested at 6 weeks, 6 months and 1 year time point. Using qualitative and quantitate analysis with Immunofluorescence and qPCR, we determined the most efficient AAV type for intraspinal and Intramuscular injection.*

Result: *When measuring Immunofluorescence and qPCR, aav 8 showed the highest transduction of GFP in Intraspinal injection. It also showed high levels of GFP in the spinal cord following intramuscular injection, though qPCR levels were not significant.*

Discussion: *AAV 8 showed a high level of CNS transduction with both immunofluorescent as well as qPCR, showing a preferential transduction capacity in the CNS which may be applicable in treating primary CNS disorders.*

Keywords: *gene-therapy, recombinant adeno-associated viruses, adenovirus, isofluorane*

Introduction

Therapeutic approaches for the treatment of CNS diseases have in part been limited by the blood brain barrier's (BBB) ability to prevent infiltration of most drugs. However, recent studies in the field of gene therapy have given hope to possible interventions and treatments of diseases that were once deemed hopeless. Great strides have been made in this field with the use of rAAV (recombinant adeno-associated viral vectors) which were first constructed nearly 23 years ago [*Tratschin JD, 1985; Mueller C, Flotte TR,2008*] and have since been used in numerous applications. The aav vectors have several advantages over other viral vectors which have made them quite attractive for therapeutic purposes. The wild type virus is nonpathogenic and all of the viral genes can be removed from the recombinant virus. Furthermore, the reversal of aav to a replication-competent virus is dependent on the presence of a lytic helper virus [*Muzyczka N et. al,*

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1984] and studies have shown that aav vectors are safe and non-toxic [*Lo WD et. al, 1999*]. In addition, advances in purification methods have allowed the production of pure rAAV vector stocks [*Kaludov N et. al, 2002; Smith RH et. Al. 2003*]. These attributes, coupled with the unique ability of the aav virus to integrate into a specific region of chromosome 19 [*Kotin RM et. al, 1990*] have encouraged scientists to pursue gene therapy in animal models as well as clinical trials. They have been used to transfer genes into various animal tissues [*Kaplitt MG et. al, 1994, McCown TJ et. al, 1996*], showing maximal transduction in a matter of a few weeks and usually lasting the lifetime of the animal [*McCown TJ et. al, 1996, Snyder RO et. al, 1997*]. The various advantages of using aav vectors allow for a variety of applications and possible therapeutic interventions such as over-expressing certain genes, silencing unwanted and mutated genes, as well as replacing metabolic enzymes [*Elbashir SM et. al., 2001a; Elbashir SM et. al, 2001b*]. Clinical trials using aav vectors have been conducted on patients with cystic fibrosis as well as hemophilia B. Both studies showed positive effects early on in the study, but were limited due to immunological barriers after repeated doses as a result of humoral response to the aav2 capsids [*Halbert CL et. al, 2001; Aitken ML et. al., 2001*].

Currently there are several aav serotypes available, each with different transduction efficiency and tissue specific capabilities. The most common aav is the serotype 2. It has been successfully used to deliver genes to various parts of the central nervous system [*Kaplitt MG et. al, 1994*]. It has been shown to deliver genes mainly to neurons [*McCown TJ et al, 1996*]. However, use of aav2 has been limited by the small volume of transduction [*Burger C et. al, 2004*]. Another serotype has been the aav 1. It has shown to have better and wider transduction efficiency in the mouse brain compared to aav 2 [*Wang Z et al., 2003*]. In addition, the serotype 2/1 behaves very similar to aav 2/5 in that they both transduce the mouse brain; specifically, the hippocampus, striatum, basal ganglia and the spinal cord, with very high transduction of GFP positive cells [*Burger C et. al, 2004*]. New aav serotypes have been manufactured, such as the aav 2/6, 2/7 and 2/8. The aav 2/6 serotype has been shown to be highly efficient in transducing epithelial cells of mouse lungs [*Halbert CL et al., 2001*] whereas the aav 7 has shown to transduce skeletal muscle very effectively with similar levels to aav 2, which is known to be the most efficient at transducing skeletal muscle [*Gao GP et. al, 2002*]. The most recent vector on the market has been the aav 8 serotype. It appears to be very efficient in transducing liver cells and has been shown to be stable for over two years [*Wang L et. al, 2005*]. A recent study has also shown that the aav 8 serotype is more efficient for gene delivery to the neonatal mouse brain than the aav-1 or aav-2 serotypes [*Broekman ML et. al, 2006*].

The potential therapeutic benefits of using raav in treating many neurological conditions are very appealing. However, before adeno associated vectors can be used in alleviating many disorders, optimization of viral vectors and its conditions must be done. With the various aav serotypes discussed, we proposed to determine which serotype was the most efficient and most stable in transducing neurons of the spinal cord. We are also interested in quantifying the distribution pattern of the lowest and highest transduced serotype in the mouse spinal cord. Lastly, we are interested to see whether any of the serotypes discussed would be efficient in retrograde transport from various peripheral muscles to the spinal cord.

Material and Methods

Animals: Naive C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). In each group, mice were gender and litter matched with equal numbers of males and females. The age of the mice ranged from 5-9 weeks. All of the animal studies were done in accordance with NIH mandated guidelines and regulations.

Intraspinal Injections: All intraspinal injections were done under anesthesia (2% Isofluorane for induction and 1.2 % for maintanance). Adequate anesthesia was ensured by testing toe-pinch and tail-pinch reflexes, monitoring respiration rate and

depth, and muscle relaxation. After induction of anesthesia, the animal was placed in the prone position and the dorsal midline shaved and prepped with betadine aseptic solution. To prevent corneal abrasion a small amount of Vaseline was placed across the eyes of the mice using a cotton-tipped applicator. A 2-3 *cm* rostro caudal midline skin incision was then made with straight scissors. Using the operating microscope, blunt and sharp dissection was used to dissect paraspinal muscles of the spinous process and lamina, thereby exposing the intralaminar space. The intralaminar space was then gently distracted with forceps and ligamentous tissue was removed to expose the spinal cord to direct vision. Once exposed, a graduated glass micropipette with a 70-100*uM* diameter tip was slowly and gently inserted using a vertical stereotaxic apparatus through the bony window in the lamina into the midline of the spinal cord to inject the virus. The proper depth of needle delivery was accomplished by measuring the needle insertion via the 1mm graduations on the glass micropipette. In each injection 4*m*l of adenovirus were injected over a period of 4 minutes (1ml/min). The procedure involved four injection sites. The first one was done in the cervical area at approximately in the C5 area. The next injection site was the lower thoracic region. The last two injection sites were done in the lumbar region. Each mouse received a total of 6.25 e^9 particles of AAV-CBA-GFP from the following serotypes: aav 2/1, aav 2/2, aav 2/5, aav 2/6, aav 2/7 and aav 2/8. After delivery of the adenovirus, the needle was removed and moved to the next spinal cord location. After all injections were completed, the paraspinal muscle is closed with a 5-0 chromic gut absorbable suture and the skin overlying the wound was then closed with a 5-0 monofilament nylon suture. The animal was then placed on a heating pad at 37°*C* and allowed to recover from anesthesia under constant observation, and frequent gentle stimulation until awake and fully functional. An animal is considered to have recovered from anesthesia when its breathing rate and depth have returned to normal, has toe-pinch and tail-pinch reflexes, and is mobile and active. Food and water are placed on the bottom of the cage for those animals that are considered weak in the first few days post-surgery. Each procedure takes approximately 1 hour from skin to skin (Figure 1).

Intramuscular Injections: In order to determine the most efficient route of administering the aav vectors, direct spinal cord injections (as outlined above) were compared to intramuscular in-

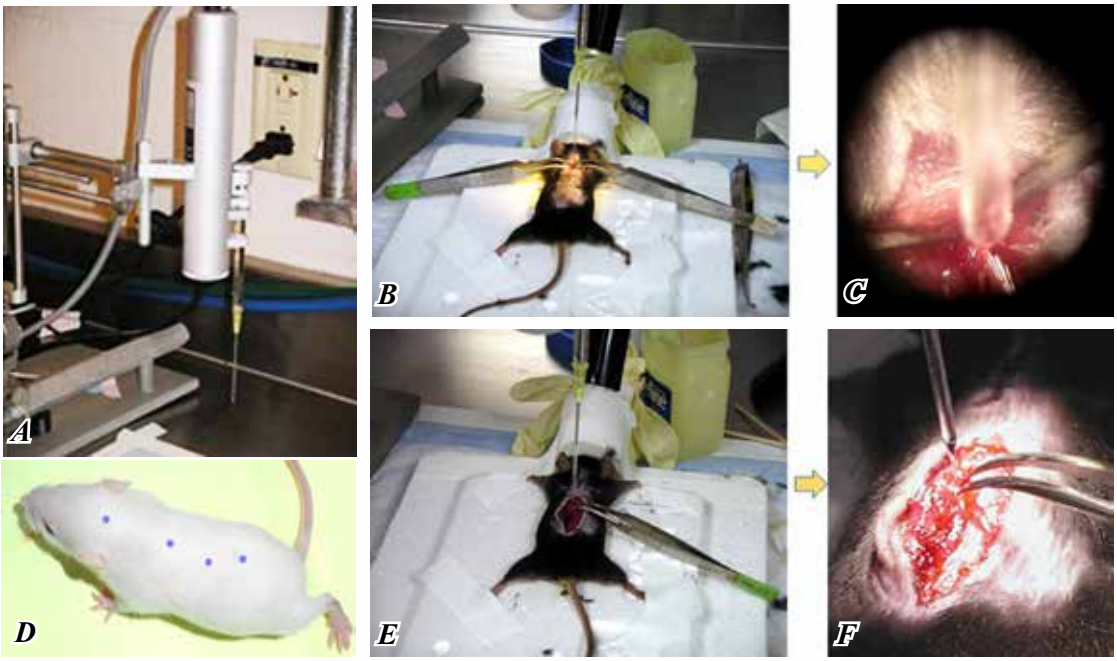


FIGURE 1. *Intraspinal injections. (**A**) Stereotactic Injection Device, (**B**) Stereotactic Cervical Spine Injection, (**C**) Microscope View of Cervical Injection, (**D**) X-marks spot of injection (1 in cervical and 3 in thoraco-lumbar) (**E**) Stereotactic Thoracic Spine Injection , (**F**) Microscope view of Thoraco-Lumbar Injections*

jections with retrograde delivery of the aav's to the spinal cord. For the IM injections, the anesthetic and pre-operative preparatory procedures were identical to the spinal cord injection. Once the animal was anesthetized and in the prone position, the forelimbs and hind limbs were shaved and prepped with betadine ™ antiseptic solution and a 3 *cm* skin incision was made with straight scissors. Blunt dissection was carefully done to locate the Triceps, Quadriceps and Gastrocnemius muscle. With the use of a Hamilton syringe each muscle was given 2 injections, each with a volume 10µl PBS containing $2.08x10^8$ viral particles, totaling 120µl of PBS containing a total of $2.5x10^{10}$ viral particles per mouse. After the injection is complete, the wound is closed with a 5-0 monofilament nylon suture.

Analysis of aav serotype distribution in the spinal cord: After completion of all surgical procedures, the animals were housed in the animal facility with four mice per cage and maintained by the core facility veterinarians and technicians. Animals were sacrificed at 6 week, 6 month and 1 year period after injection with the various serotypes containing GFP (Green Fluorescent Protein) as a marker of viral transduction. After each time period, two sets of animals were sacrificed and tissue was analyzed for qualitative and quantitative distribution of the aav serotype.

Analysis of GFP distribution in mouse spinal cord by immunohistochemistry: For the qualitative analysis, animals were euthanized by CO_2 asphyxiation and were then perfusion-fixed with 4% Para formaldehyde/phosphate buffer saline (PBS). Brain and spinal cords were harvested from the animals and post-fixed in 4% PFA overnight and followed by 24 hour incubation in 30% sucrose solution as a cryo-protectant. Fifteen micron serial sections were taken from the spinal cord. Sections were blocked in PBS containing 0.3% Triton X-100 and 20% normal horse serum for one hour. Immunostaining was done by incubating primary antibodies overnight at 4°*C*. These included mouse anti-NeuN (1:1000; Chemicon), rabbit anti-(GFAP) glial fibrillary acidic protein (1:1000; Calbiochem), anti-green fluorescent protein (GFP)-conjugated Alexa Fluora dye (1:500; Invitrogen), and rat anti-mouse cd11b (1:50; Serotec). After overnight incubation of primary antibodies, slides were washed three times in PBS for 5 minutes each. Sections were then incubated with indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (1:300; Jackson Immuno Research Labs) for 3 hours at room temperature. After secondary incubation sections were washed and counterstained with DAPI-containing mounting medium (Vector Labs). The processed samples were then analyzed using a Nikon E800 microscope with a Nikon-Spot digital camera (Microvideo Instruments, Avon). The above procedures were done on spinal cords from both intraspinal injections as well as intramuscular injections with aav serotypes.

Quantitative analysis by qPCR: For quantitative analysis of the various aav serotypes by qPCR, animals were sacrificed by CO_2 asphyxiation, and brains were harvested. Spinal cords were syringe-extruded from the spinal column using a 28gauge syringe with sterile PBS. To determine the quantity of virus transduced in the muscle tissue from intramuscular injections, Gastrocnemius, Quadriceps and Triceps muscles were harvested bilaterally and analyzed with qPCR. All samples were stored immediately at 80°C. Instruments were depurinated and all working stations were carefully wiped down with RNAase wipes to avoid RNAase contamination (Genzyme, Cambridge, MA).

Quantitative analysis of aav distribution by Immunohistochemistry: In order to determine the distribution of the virus in the spinal cord, transverse serial sections at 20 µm were taken throughout the whole spinal cord. Every 5^{th} section was taken to avoid counting a cell twice. Since thoracic and lumbar segments contained multiple sites of injections, distribution analysis was done on the cervical segment in order to avoid overlap of GFP positive cells that might result from multiple injection sites. The first presence of GFP positive cells to the last GFP positive region indicated the horizontal distribution of each aav vector. For each serotype, two spinal cords were analyzed.

In addition to distribution analysis, cell specificity of the aav serotypes was determined by co-

staining alternating slides of spinal cords sections from aav 2/8 and aav 2/1 with antibodies to NeuN, Cd11b and GFAP. For analysis of viral transduction in neuronal cells, GFP positive cells, NeuN positive cells, as well as cells that were positive for both were counted. To determine the percentage of NeuN positive cells that were transduced with the aav virus, the number of co-localized cells were divided by the sum of the total number of NeuN positive cells and the number of co-localized cells. GFP positive regions in the lumbar segments were used for counting cells. Similar methods were also employed for analysis of microglial cells with Cd11b staining and astrocytic cells with GFAP staining.

Results:

qPCR results: Fresh spinal cords and brain were harvested from 6-wee and 6-month injection of aav-GFP directly into the spinal cord as well as peripheral muscles. All data was expressed in total level of Bovine Growth Hormone DNA (BGH DNA) copies. In the 6-week time period, the greatest expression of GFP was found in spinal cords injected with aav2/8 and aav2/7 serotypes, while the lowest expression of GFP was seen in spinal cords injected with aav2/1. Levels of GFP expression were found to be insignificant in brains harvested from animals at 6-week time point as they were similar to control non-injected brains. Furthermore, injection of the various serotypes into peripheral muscles failed to show similar levels of GFP expression in the spinal cord when compared to levels of GFP expressed from direct spinal cord injections. However, the aav 2/8 serotype did show the greatest amount of GFP expression in the spinal cord as a result of retrograde transport compared to the other serotypes. Tissue analyzed at the 6-month time point generated consistent data with our previous qPCR results. The aav2/8 serotype continued to be the serotype with the greatest expression of GFP while the aav2/1 remained the serotype with the lowest expression of GFP. GFP expression in the spinal cord as a result of retrograde transport from intramuscular injection increased for all serotypes, when compared to spinal cords harvested and analyzed at 6-week time point. However, levels of GFP expression were similar to control levels and were still significantly lower than levels of GFP expressed from direct spinal cord injection (Figure 2).

Quantitative analysis of AAV distribution by immunohistochemistry: Serial sections were taken from spinal cords that were injected with the most efficient transduced virus (aav2/8) and the least efficient transduced virus (aav2/1). The two spinal cords that were analyzed for the aav 2/8 serotype

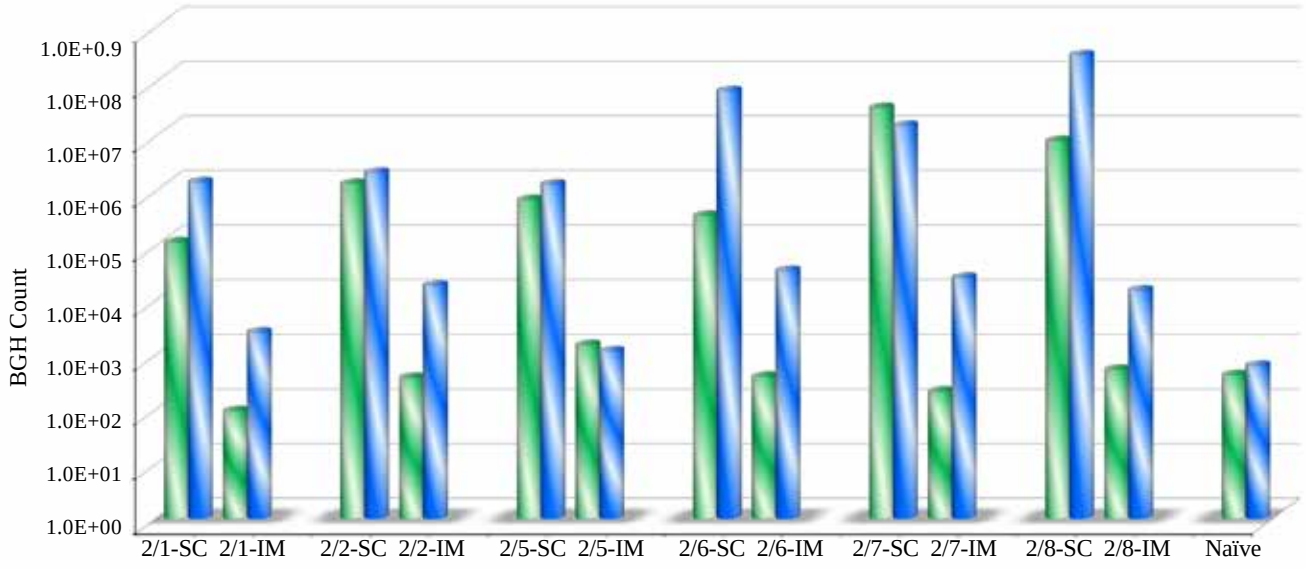
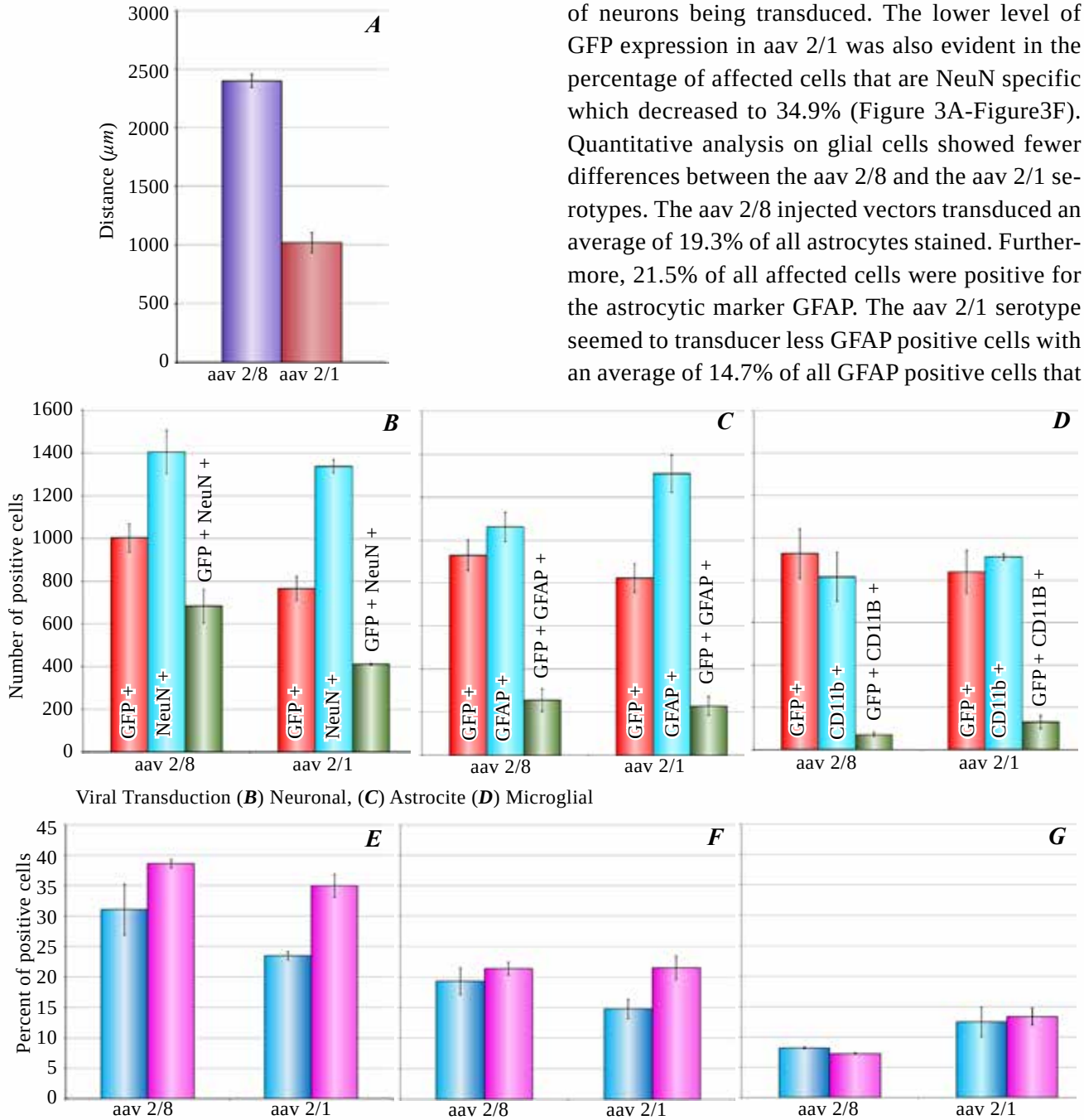


Figure 2. *Spinal cord Spinal Cord BGH levels Taqman PCR results. Green Columns after 6 weeks and blue columns after 6 months.*

showed a longitudinal distribution of 2440µm and 2360μm based on the distance between the first GFP positive cell and the last GFP positive cell in the cervical segment of the spinal cord. The average distance the virus traveled across the spinal cord for the aav 2/8 serotype was 2400µm across the spinal cord with a standard deviation of 56.6µm (Figure 1A).

Quantitative analysis of aav transduction in neuronal and glial cells by Immunohistochemistry: After cell counts were completed from aav 2/8 injected spinal cords, it was shown that 31% of all neurons counted on the visual field had confirmed presence of viral transduction. Furthermore, it was also shown that 38.6% of all GFP positive cells were specific for neuronal cells (Figure 1C). In contrast, the aav 2/1 serotype showed less transduction in neuronal cells with an average of 23.8% of neurons being transduced. The lower level of GFP expression in aav 2/1 was also evident in the percentage of affected cells that are NeuN specific which decreased to 34.9% (Figure 3A-Figure3F). Quantitative analysis on glial cells showed fewer differences between the aav 2/8 and the aav 2/1 serotypes. The aav 2/8 injected vectors transduced an average of 19.3% of all astrocytes stained. Furthermore, 21.5% of all affected cells were positive for the astrocytic marker GFAP. The aav 2/1 serotype seemed to transducer less GFAP positive cells with an average of 14.7% of all GFAP positive cells that



Viral Transduction (***B***) Neuronal, (***C***) Astrocite (***D***) Microglial

Percent of neurons (***E***), of astrocytes (***F***) and of microglia (***G***) transduced VS (Blue columns). Percent of transduced cells that are NeuN (***E***), GFAP (***F***), and CD11b (***G***) specific (Pink columns)

Figure 3. Longitudinal Distribution of aav serotypes

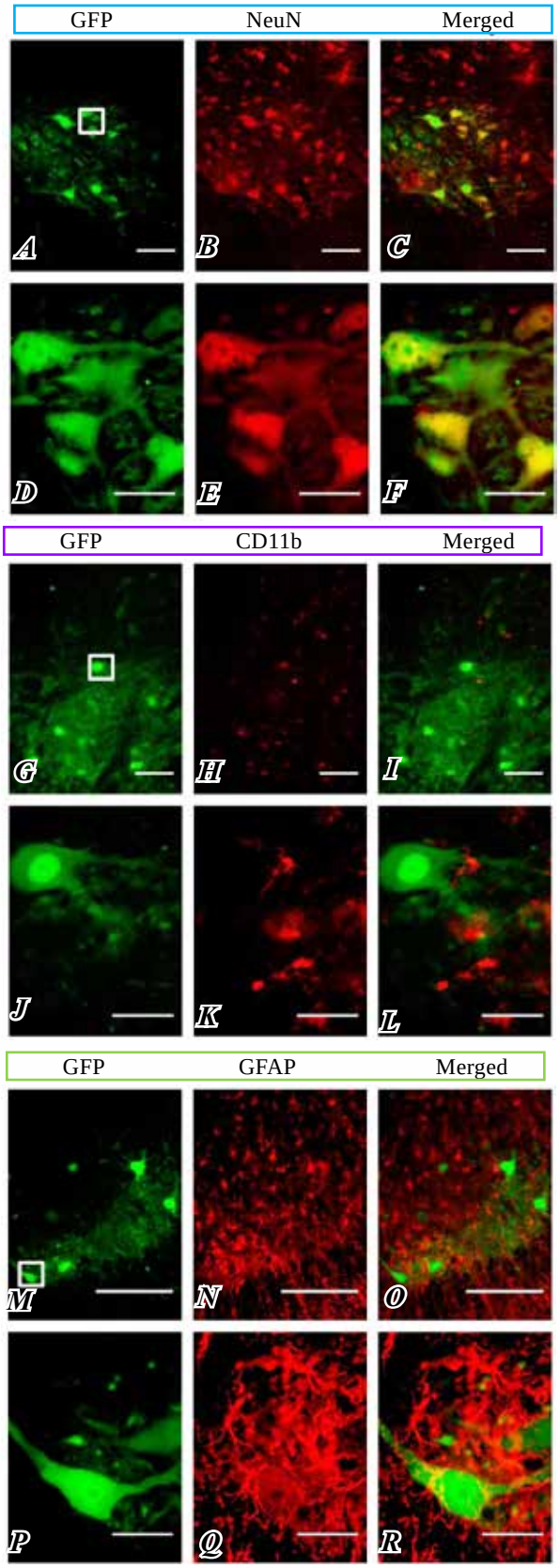


Figure 4. *Double immunofluorescence staining of lumbar segment of spinal cord injected with aav2/1 viral vectors expressing GFP via direct intraspinalcord injection. Bars : A-C=50μm. D-F=20μm,G-I=50μm,J-L=20,M-O=50μm,P-R=10μm.*

were counted (Figure 3M-3R). However, when counting the percentage of all affected cells that are GFAP positive, an average of 22% was determined, which was very close to the data obtained for the serotype aav 2/8 (Figure 3D-3E). Immunofluorescence staining with the activated microglial marker CD11b revealed very little viral transduction in both serotypes (Figure 4G-4L & Figure 4M-5U). In the aav 2/8 injected spinal cord, 8% of all microglial cells counted were transduced and the 7.2% of all affected cells seemed to be positive for the CD11b marker. The aav 2/1 serotype vectors had a slightly higher ability to transduce microglia cells with 12.5 % of all CD11b positive cells were transduced. Furthermore, 13.3 % of all GFP positive transduced cells were specific for the CD11b marker (Figure 3G-3H).

Discussion

After meticulous qualitative and quantitative analysis of the aav serotypes 2/1, 2/2, 2/5, 2/6, 2/7 and 2/8, our data suggests that GFP expression, which reflects levels of viral vector transduction, the aav 2/1 vector is the least transduced in mouse spinal cords and the aav 2/8 serotype shows the highest GFP expression in the mouse spinal cords. Our qPCR data shows consistent higher levels of GFP expression with the aav 2/8 vector from the 6-week and 6-month time period, compared to the aav 2/1 vector. Our data parallels previous studies in showing aav8 as very efficient in neuronal transduction [*Broekman ML, 2006*]. Although on average only 31 % of neurons were positive for GFP expression and 38% of all transduced cells were positive for the neuronal marker NeuN, a majority of the cells transduced appear to be motor neurons in the ventral horns of spinal cords. Smaller neurons did not show robust GFP expression (Figure 5A-5U). Despite the lower presence of astrocytes in the spinal cords transduced with the aav 2/8 vector, a higher percentage of cells were positively co-localized with GFP and GFAP. This finding that GFAP positive cells are also transduced in the CNS is consistent with previous work done with aav vectors (Figure 5G-5L) [*Broekman ML 2006*]. When measuring the levels of activated microglia,

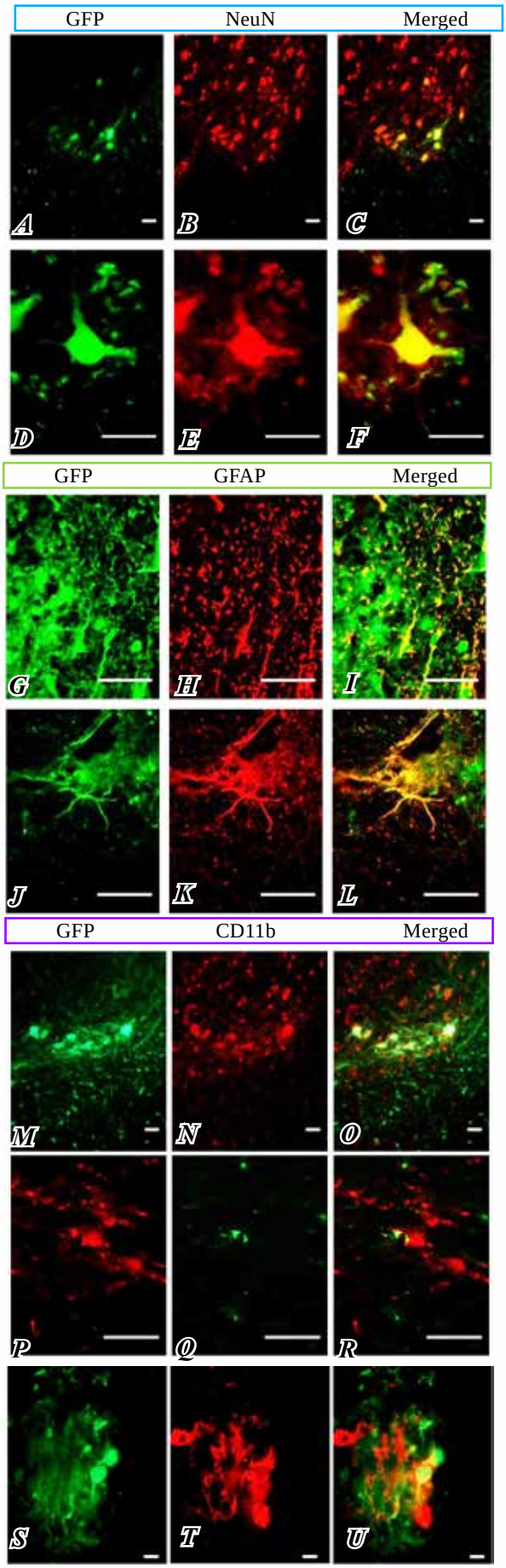


Figure 5. *Double immunofluorescence staining of lumbar segment of spinal cord injected with aav 2/8 viral vectors expressing GFP via direct intraspinal cord injection. Bars=20μm.*

we found that spinal cords injected with aav 8 showed a lower number of activated microglia cells. However, the aav1 serotype showed a slightly higher proportion of CD11b positive cells co-localized with GFP (Figure 1F). The high transduction capability of the aav 8 vector can be attributed to the presence of ubiquitous receptor which allows for rapid release of the vector genome into cells, as well as wide range of transduction. This can be explained by the rapid un-coating of the vector genomes in the nucleus, which facilitates the renaturing of the plus and minus single-stranded genomes into stable and transcribed double stranded vector genomes [*Thomas CE, 2004]*. Previous studies have shown that certain aav serotypes, specifically, the aav8, have a rapid rate of vector genome un-coating and capsid degradation. This property is believed to be responsible for its high viral transduction as well as invoking a lower immune response. [*Peden CS, 2004*]. This may explain why our 6 month data showed lower levels of GFAP positive astrocytes and Cd11b positive microglia cells with the aav8 injected mice. Furthermore, as the CD 11b positive cells stain microglia, which are activated inflammatory cells, it is presumed that by the 6 month time, there is a decrease in the immune system as the immediate perioperative period which is responsible for a robust expression of microglia cells. While the un-coating rate of the aav 2/1 has not been elucidated yet, it is well established that viral gene degradation and hence levels of GFP expression may be attributed to slower rates of viral vector genome un-coating.

Conclusion

Using Adeno-associated viral particles as a vehicle for gene transduction is a very exciting tool and has been used more recently in various fields. Genetic disease that were once deemed untreatable with a poor prognosis may have treatable options in the near future with the advent of gene therapy.

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