UNIVERSITY OF CALIFORNIA SAN DIEGO

Impact of Age on AAV Gene Transfer to Mouse Brain

A Thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in

Biology

by

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2020

The Thesis of Nabta Bahaeldeen Laz is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

EPIGRAPH

Black as obsidian
Black so ingenious
Black Excellence
Black creativity
Black ingenuity
Blackness
Darker and deeper
Than the mysteries of any ocean abyss
Blacker than black holes that can literally suck up existence
Do you see how amazing your Blackness is?
From our skin to our hair to our eyes
Black is how we were born
And no matter how many times
They try to deny our Black lives (which side note: Do in fact matter)
We must Always embrace
The blessing of being Black and Divine
Fight for every bit of Blackness
Just like the ones before us did while they were alive
Because just like them
Black is how we live and black is how we gone die.
But Unapologetically Black
Is at least how I intend to die.
And that's the Negro spiritual gospel soul truth
Ain't no little white lie.
Shout it Claim it
Because For us
It's the only way of life.

And as a Black person
In America
We never know
Which one of us is next to go
So if I get shot down tomorrow
Let it be known
I said I want it written in stone
That I was an
Unapologetically Black
Race Jones.

All Black Lives Matter.

-Excerpt from “Unapologetically Black”
Tenomewah Murray, Founder, The Blue Poets Society (UCSD Thurgood Marshall College)

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ACKNOWLEDGEMENTS

I would like to thank Professor Mark Tuszynski for giving me the opportunity to work in a lab that inspires, challenges and enriches me and other students. I have learned so much over the past three years, and I look forward to using all the knowledge I learned in this lab in my career.

I would also like to thank Dr. Michael J. Castle for crafting such a stimulating project idea, for performing the surgery throughout the experiments, and taking the time to teach me all the skills I needed to complete this project. I would not have been able to undertake this project without his incredible guidance and mentorship.

Next, I would like to thank my co-chair Professor Brenda Bloodgood, and my thesis committee member Professor Gentry Patrick for their support.

ABSTRACT OF THE THESIS

Impact of Age on AAV Gene Transfer to Mouse Brain

by

Nabta Bahaeldeen Laz

Master of Science in Biology

University of California San Diego, 2020

Professor Mark Tuszynski, Chair
Professor Brenda L. Bloodgood, Co-Chair

Gene therapy is being explored for its potential to treat neurodegenerative diseases such as Alzheimer's Disease and Huntington's Disease through the delivery of therapeutic transgenes with Adeno-Associated Virus (AAV) vectors. The leading candidates for these treatments are AAV9 and AAV-PhP.eB because of their safety, efficacy in rodent and non-human primate studies, and their ability to cross the blood-brain barrier. These vectors have been tested in juvenile mice, but neurodegenerative diseases typically affect older individuals and these vectors have never been tested in aged mice. Therefore, we delivered AAV9-eGFP and AAV-PhP.eB-eGFP intrathecally or intravenously to mice and compared the virus' transduction patterns in the brain across aging, using groups of aged 20-month-old mice versus juvenile 3-month-old mice. We found a reduction in gene expression with aging in mice treated with intravenous AAV9. However, there was no detectable change in gene expression across aging in mice receiving intravenous AAV-PhP.eB or intrathecal AAV9. There is a significant reduction associated with

increased weight when mice are treated with intravenous AAV9 but not with intrathecal AAV9, indicating that the reduced efficacy of intravenous AAV9 in aged mice may be due to the larger size of these animals. These results suggest that the more promising delivery method for AAV9 gene therapy in older individuals is via the intrathecal route.

Introduction

Gene therapy is emerging as a promising way to treat many diseases, as it allows for a single treatment to have a long-lasting impact on a person's health as opposed to multiple treatments from traditional protein-based therapies (Dunbar et al., 2018). It involves the introduction of genes to cells *in vivo* or *ex vivo* via various delivery machineries, leading to the transcription and translation of these genes for therapeutic effect. Gene therapy has been making progress in the biomedical world as the FDA has already approved gene therapy treatments for inherited retinal disease, spinal muscular atrophy, and childhood leukemia (U. S. Food and Drug Administration, 2019). However, there are still hurdles that need to be overcome in gene therapy treatment. These include limiting off-target effects and improving the safety, efficacy and production of gene therapy treatments (Dunbar et al., 2018).

Adeno-Associated Viruses in Gene Therapy

Adeno-associated viruses (AAV) are key delivery candidates for gene therapy treatments. They are the preferred method of *in vivo* gene delivery for non-dividing cells and they are proving to be safe. This virus, derived from non-pathogenic and non-enveloped parvoviruses, can be packaged with the genetic cassette intended to be expressed by the host cells (Hermonat et al. 1984; Samulski et al. 1987). The AAV's capsid interacts with cell surface receptors that allow for a series of downstream reactions that culminate in the expression of the delivered transgene (Schultz et al., 2008). There are multiple AAV serotypes, which differ based on their capsids. These capsids can also be modified to alter their tropism towards certain tissues or cell types (Castle et al., 2016). Along with the ability to target certain tissues by altering the serotype or capsid modifications of a vector, they can be targeted by various delivery methods. These

include by direct organ injection or by systemic delivery intravenously or intra-arterially. After targeting in this way, they can be used for gene replacement to treat loss-of-function mutations, to silence genes in diseases that are gain-of toxicity such as Huntington's, or to express genes which are therapeutic for many acquired diseases such as cardiac, neurological, or infectious diseases by regulating pathways or supplying important molecules (Balazs et al., 2012; Dunbar et al., 2018; Wang et al., 2019). In addition to the CNS, most AAV capsids are able to target the liver and have the ability to treat diseases such as hemophilia (Wang et al., 2019). AAV8 and AAV9 are able to target the muscles so they can be used to treat disorders such as duchenne muscular dystrophy (DMD) (Kawecka et al., 2015). Thus, AAVs can be used to treat a variety of diseases due to their versatility. Recently, they have made their way into phase I and phase II clinical trials in humans with promising results in treating congenital blindness, hemophilia, AADC deficiency and Tay-Sachs disease (Wang et al., 2019).

Additionally, a key benefit to using AAVs is that they do not cause any human diseases (Wang et al., 2019). Their safety in humans has also been proven by two FDA approvals. Nevertheless, the search for improved viral capsids is ongoing as humans can have naturally occurring antibodies against AAV capsid sequences found in humans (Blacklow et al., 1968). But, capsids derived from other organisms may be less effective in transducing human cells (Wang et al., 2019). Furthermore, the parvovirus they are derived from is naturally replication-defective ensuring that the virus will not spread unintendedly (Dunbar et al., 2018). However, this also limits its ability to have a long-term effect on dividing cells through a single administration (Dunbar et al., 2018). Another challenge is that they can only deliver a limited amount of DNA, 5.0 kB, calling for methods to minimize the amount of DNA packaged while still ensuring maximal efficacy (Dunbar et al., 2018; Wang et al., 2019). On the other hand,

improved manufacturing techniques increased yield and purity allowing for proof-of-principle tests in larger animals ensuring their efficacy (Dunbar et al., 2018).

AAV Vectors to Target Neurological Diseases

AAV vectors can be used to treat diseases of the nervous system (Kaplitt et al., 1994). Alzheimer's disease (AD) is a neurodegenerative disorder with growing prevalence but limited treatment options. It is the most common form of dementia, but no treatment has been made available to halt its progression. There are many gene therapy candidates to treat AD using AAV vectors. The apolipoprotein (APOE) gene influences the amount of the amyloid-beta peptide that accumulates with aging, with the E4 allele being a risk factor for the development of late-onset AD (Liu et al., 2013; Sanders et al., 1993). By using AAV gene therapy to express the protective *APOE2* allele in the CNS of those with the E4 variant, the neuroprotective effects of the E2 allele can still protect against the disease, rapidly decreasing amyloid plaques (Dodart et al., 2005; Hudry et al., 2013; Rosenberg et al., 2018; Zhao et al., 2016). This type of therapy has made its way to phase 1 clinical trials (ClinicalTrials.gov #NCT03634007, 2018). AD pathology is also characterized by tau aggregates. By having neurons and glia express anti-tau antibodies delivered by AAV vectors in mice, the spreading of tau in the brain could be blocked, ultimately offering potential therapeutic effect (Boutajangout et al., 2011; Ising et al., 2017; Yanamandra et al., 2013). Similarly, anti-amyloid-beta antibodies delivered by AAV vectors are a potential candidate for gene therapy treatments for AD (Elmer et al., 2019). The use of AAV vectors for this gene therapy offers the potential to greatly reduce immune responses with a limited amount of administrations (Shimada et al., 2013). AAV vectors can also be used to deliver the transgene for Neprilysin, a protease that can cleave beta-amyloid peptides, to reduce total amyloid in mice brain (Carty et al., 2013; Iwata et al., 2001). Furthermore, Huntington's

disease is another neurodegenerative disease that can be treated with gene therapies delivered by AAV vectors. The disease is caused by an extensive amount of polyglutamine repeats in the HTT gene (Ross & Tabrizi, 2011). By AAV delivery of microRNA targeting the HTT gene in mice, the neuronal dysfunction caused by the gene can be reduced (Spronck et al., 2019). Similarly, AAV encoding for microRNA has also been studied to treat amyotrophic lateral sclerosis (ALS) by silencing the mutant SOD1 gene that causes neuronal degeneration in many cases of ALS (Dirren et al., 2015). Thus, AAV vectors play an important role in gene therapy delivery candidates for neurodegenerative diseases, so the search for the optimal capsid, serotype and infusion method is still ongoing.

AAV vectors can be used to target the CNS through direct brain injection, intrathecally via the CSF, or intravenously. Direct brain injection of AAV2 can be used to treat specific brain regions such as the putamen in Parkinson's disease (Dashkoff et al., 2016; Manfredsson et al., 2009). AAV2 cannot cross the blood brain barrier, so it is not used to systemically treat diseases of the CNS. Since the blood brain barrier protects the brain from unwanted molecules, it blocks many AAV serotypes, such as AAV2, from entering the brain (Manfredsson et al., 2009). Therefore, AAV2 must be injected directly into the brain for treatment. However, treating the CNS in a widespread manner would require thousands of direct brain injections of the virus, but this can be quite invasive, so it may not be useful for widespread diseases in the CNS such as AD or Huntington's disease (Chan et al., 2017). Intravenous deliveries combat this issue as they are noninvasive and deliver more widespread gene transfer because the vector is distributed by the blood (Duque et al., 2009; Foust et al., 2009; Inagaki et al., 2006). AAV9 has the ability to cross the blood-brain barrier in rodents and nonhuman primates, offering a good delivery method for a variety of treatments to be delivered to the brain and CNS intravenously (Foust & Kasper, 2009,

Manfredsson., 2009). AAV9 vectors are able to transduce cortical, cerebellar, and spinal neurons as well as glia when delivered systemically or via intraparenchymal injection in mice (Cearly et al., 2006; Foust et al., 2009; Gray et al., 2013; Howard et al., 2008). However, intravenous AAV9 injections also have disadvantages. This method lacks the specificity of intraparenchymal injections as there is no way to target the vector to specific brain regions when delivered systemically. Similarly, it also transduces other tissues such as the spleen, heart, liver and kidneys, potentially leading to off-target effects (Mori et al., 2006). Furthermore, intravenous AAV9 results in relatively lower neuronal transduction, so very high doses of AAV9 are needed to treat the brain, which may be unrealistic given manufacturing capabilities (Foust & Kasper, 2009; Gray et al., 2011). Since the vector is delivered intravenously, it can come into contact with neutralizing antibodies in the blood that can block the efficacy of the gene therapy (Gray et al., 2011). On the other hand, intraparenchymal injections and intrathecal infusions of the vector can evade antibodies in the bloodstream (Gray et al., 2013).

Another form of delivery is via intrathecal injection through the cisterna magna into the CSF. This delivery method is less invasive than direct brain injections and transduces the brain with a higher bias than other organs, offering more localized CNS distribution in non-human primates (Gray et al., 2013; Lukashchuk, 2016). Lower vector doses of AAV9 are required to transduce the brain intrathecally compared to the intravenous approach. However, intravenous and intrathecal delivery of AAV9 display higher biases towards glia than intraparenchymal injections of AAV9 which almost exclusively transduce neurons (Foust et al., 2009; Gray et al., 2013). In addition to increasing the specificity of AAV vectors to the brain using these methods, choosing the optimal serotype and capsid construction can also aid in the effectiveness of gene therapy treatments.

In the search to find an AAV vector that has maximal transduction of cells in the CNS from the bloodstream, a method called Cre recombinase-based AAV targeted evolution (CREATE) has been developed to select the most effective AAV capsid for a specific application (Deverman et al., 2016). This involves testing AAV viruses in-vivo with a variety of random peptide insertions in the capsid and then selecting the virus that transduces the most target cells (Chan et al., 2017). Through this method, an AAV serotype called AAV-PhP.eB was discovered for its ability to transduce the majority of neurons in the brain of adult mice while using a lower dose than other serotypes when delivered intravenously (Dayton et al., 2018). AAV-PhP.eB is able to transduce a significantly higher number of cells in the CNS than AAV9, using a much lower viral load, when delivered intravenously to mice. This is due to AAV-PhP.eB's ability to effectively cross the BBB by interaction with the LY6A receptor (Deverman et al., 2016; Hordeaux et al., 2019). This allows us to use lower doses of AAV-PhP.eB than AAV9 which can reduce off target side effects and also lower production costs. Although AAV-PhP.eB has been shown to transduce a considerably greater number of neurons in mice, only AAV9 has been tested to be safe and effective in humans whereas AAV-PhP.eB has not been. Furthermore, since AAV-PhP.eB was produced through directed evolution in mice, it may not be effective in humans and a similar virus may need to be made tailored using directed evolution to the primate brain.

AAV Use in Aged vs Juvenile Mice Brains

AAV-9 and AAV-PhP.eB are leading candidates for the delivery of gene therapy treatments of neurodegenerative diseases, however, it is unclear whether they perform similarly in young versus aged individuals (Bostick, 2006; Koedam et al., 2010). There are various differences between aged and young individuals across different species. For example, aged

humans demonstrate a loss in grey matter and the shrinkage of neurons (Mooradian, 1988). Furthermore, changes in the blood brain barrier due to aging may affect the transduction of AAV9 and AAV-PhP.eB following systemic delivery as they must cross the BBB to transduce neurons. The BBB normally acts as a tight filter between the blood and the CNS, preventing unwanted molecules from entering the brain formed through tight junctions between its endothelial cells (Oakley & Tharakan, 2014). However, increases in BBB permeability have been observed in aged mice, humans and rats (Blau et al., 2012; Oakley & Tharakan, 2014; Pelegrí et al., 2007). Furthermore, there is also a decrease in cerebral blood flow associated with aging (Oakley & Tharakan, 2014).The BBB impacts the ability of AAV vectors to transduce the brain, and changes in BBB integrity or cerebral blood flow with age may alter AAV transduction from the bloodstream.

Direct brain injection of AAV is effective in aged monkeys, however, this is an invasive method with only local transgene expression so the search for a less invasive method that will produce widespread results in aged species is ongoing (Johnston et al., 2009; Nagahara et al., 2018; Su et al., 2009). Some neurodegenerative diseases like Alzheimer's disease may ultimately affect the entire cerebral cortex, which has a vast surface area of approximately 5 square feet, therefore, widespread gene delivery is needed to treat these diseases (Toro et al., 2008). In search of this method, we aimed to test intrathecal AAV9, intravenous AAV9 and intravenous AAV-PhP.eB in juvenile (3-month-old) mice and aged (20-month-old) mice. We compared the number of transduced neurons and glia in the brains of both age groups. We hypothesized that the efficacy of intravenous AAV9 and AAV-PhP.eB would be altered in aged mice due to changes in the BBB with age, while the efficacy of intrathecal AAV9 would remain unchanged in aged mice due to this route's more direct targeting of the brain.

Results

We tested the effect of aging on AAV gene therapy over three experiments. Gene expression was compared across aging in groups that received intravenous AAV9, intrathecal AAV9 or intravenous AAV-PhP.eB. The young mice were 3 months old and the old mice were 20 months old at treatment. Brains were then sectioned sagittally at 40 µm thickness and immunolabeled for eGFP.

Intravenous AAV9 injections through the retro-orbital sinus were given to twenty mice, eight young mice (four male and four female) and twelve old mice (six male and six female) at a concentration of $1x10_{12}$ vg/ml. Brains were stained against eGFP and the total number of labeled cells was counted in one lateral and one intermediate brain section from each animal (Figure 1). At both levels there were significantly more transduced cells in young than in old mice (Two-Way ANOVA, both $p<0.0001$). At both levels there were also significantly more transduced cells in female than in male mice (Two-Way ANOVA, $p = 0.0119$ for lateral, $p=0.0015$ for intermediate). In lateral sections, Tukey's multiple comparison post-hoc tests found significantly more transduced cells in young female mice than in old male or old female mice ($p \leq 0.001$), as well as significantly more transduced cells in young male mice than in old male mice ($p \leq 0.05$). In intermediate sections, there were significantly more transduced cells in young female mice than in young male, old male, or old female mice ($p\leq 0.001$).

Eight mice were treated with intravenous AAV-PhP.eB ($1.5x10_{11}$ vg/ml) injected through the retro-orbital sinus. Each age group consisted of two females and two males. The tissue was immunostained against eGFP. It was not possible to distinguish individual cells for counting due to the density of labeled cells (Figure 2 A-B), but there were no obvious visual differences in staining across aging (Figure 2).

Ten mice underwent surgery to receive intrathecal AAV9-CAG-eGFP ($1.5x10_{11}$ vg/ml) infusion into cisterna magna. Five of these mice were young and five were old, with three females and two males in each group. The tissue was cut sagittally and eGFP-expressing cells in lateral and intermediate sections were counted from each animal. There was no significant difference in gene expression between young and old animals in lateral (*t*-test, p=0.9319) or intermediate sections (*t*-test, p=0.3816) (Figure 3).

Additionally, we tested the relationship between weight and gene expression in the intravenous AAV9 and intrathecal AAV9 groups (Figure 4). Linear regressions were performed using weight as the dependent variable and the number of transduced cells as the independent variable. After intravenous AAV9 infusion, the number of transduced cells decreased significantly at increasing weights in both intermediate sections ($p < 0.0001$, $R_2=0.6267$) and lateral sections ($p = 0.0001$, $R_2=0.5753$). There was no significant relationship between weight and the number of transduced cells in the groups receiving intrathecal AAV9 in either intermediate sections ($p = 0.7509$, $R_2=0.01332$) or in lateral sections ($p = 0.8988$, $R_2=0.002150$).

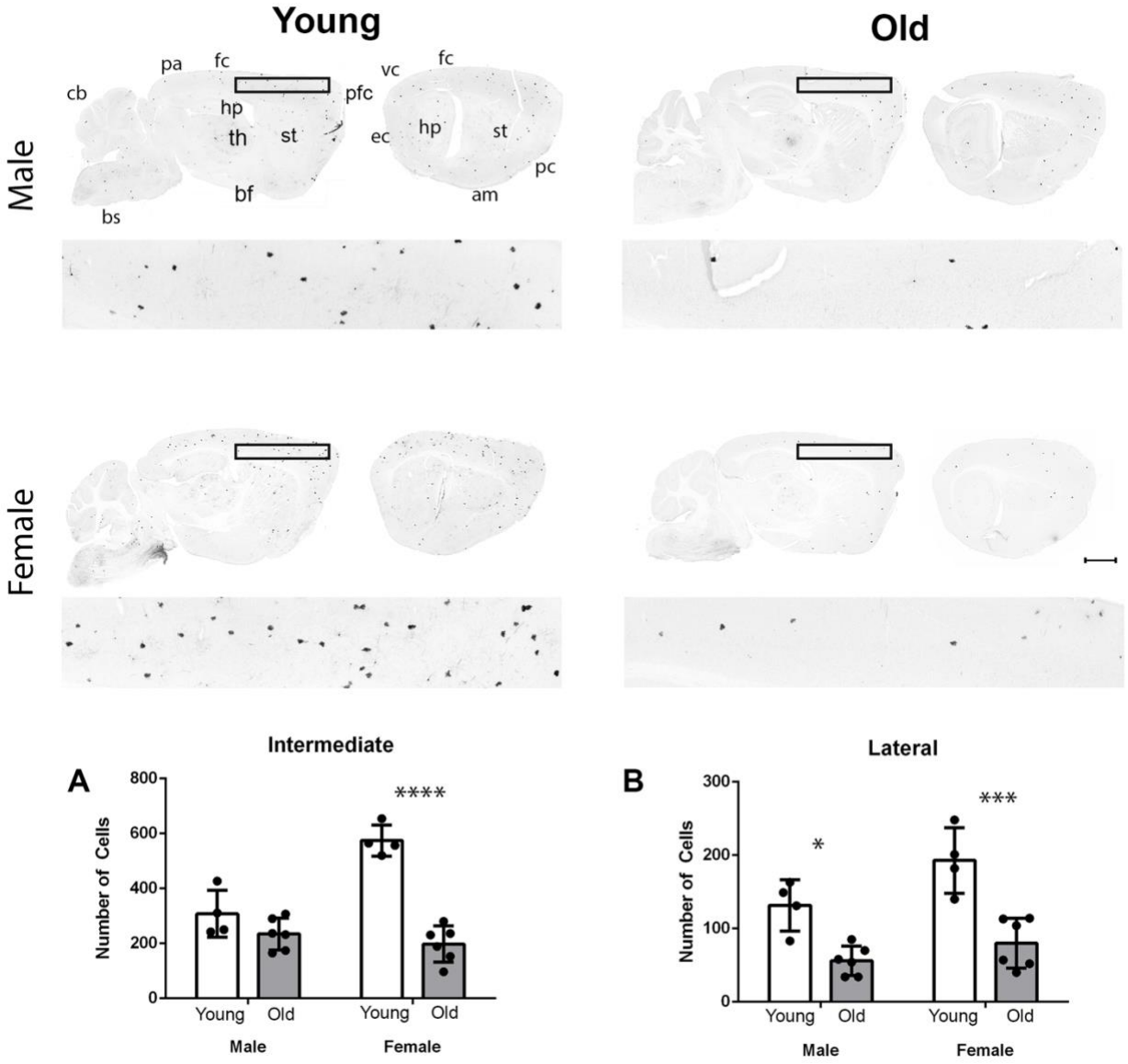

Figure 1. Transgene Expression after intravenous AAV9 is reduced with age.
Immunostaining against eGFP was performed in 40-µm-thick sagittal brain sections from mice that received intravenous infusions of AAV9-CAG-eGFP. Two sections from the same representative mouse are shown for each group. The intermediate sections are roughly 1.5mm from the midline. The lateral sections are roughly 3.25mm from the midline. High magnification images of the cortex from each animal are shown. (**A-B**) Error bars represent the standard deviation from the mean. Stars represent the significance of Tukey's post-hoc tests comparing old and young mice of the same gender after Two-Way ANOVA. ****: $p \leq 0.0001$. ***: $p \leq 0.001$. *: $p \leq 0.05$. Scale bar: 1mm. Abbreviations: anygdala (am), brainstem (bs), basal forebrain (bf), cerebellum (cb), entorhinal cortex (ec), frontal cortex (fc), hippocampus (hp), parietal cortex (pa), prefrontal cortex (pfc), striatum (st), thalamus (th), visual cortex (vc).

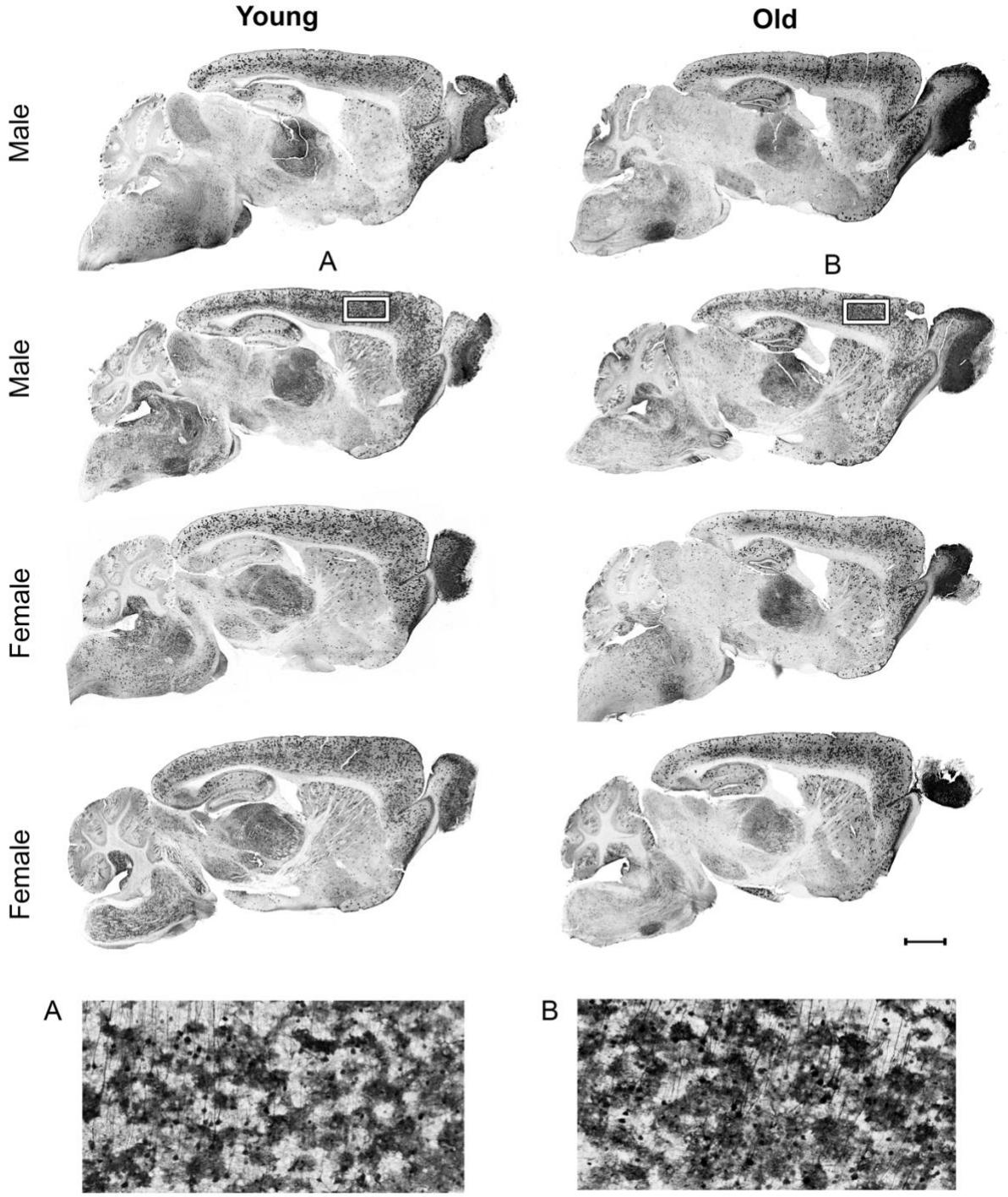

Figure 2. Similar transduction by intravenous AAV-PhP.eB was detected in young and old mice. Immunostained sections against GFP revealed visually similar gene expression across aging. A representative intermediate section from every mouse in the experiment is shown. (**A-B**) High magnification images of frontal cortex show dense labeling of both neurons and astrocytes. Scale bar: 1mm.

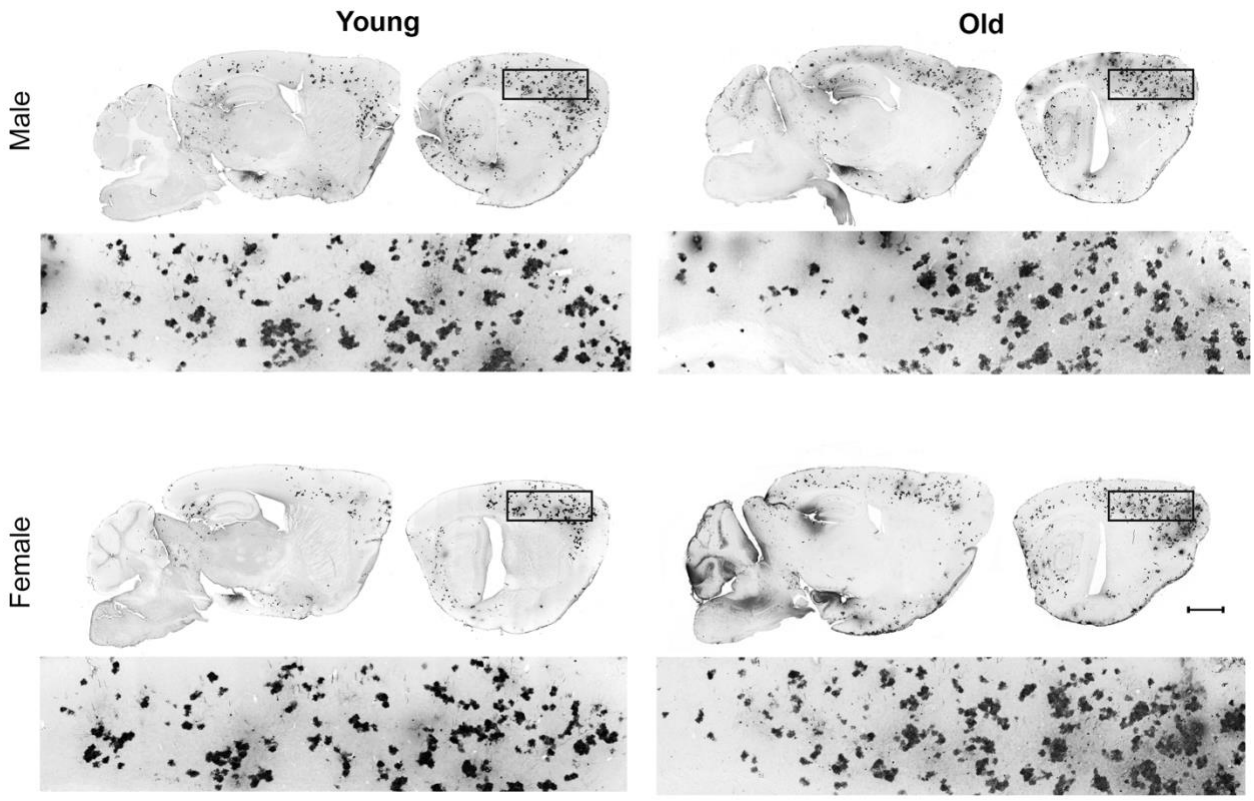

Figure 3. Similar transduction by intrathecal AAV9 was detected in young and old mice. Immunostained sections against GFP revealed similar gene expression across aging in the lateral and intermediate sections. Two sections from the same representative mouse are shown for each group with subsets from the frontal cortex magnified below.

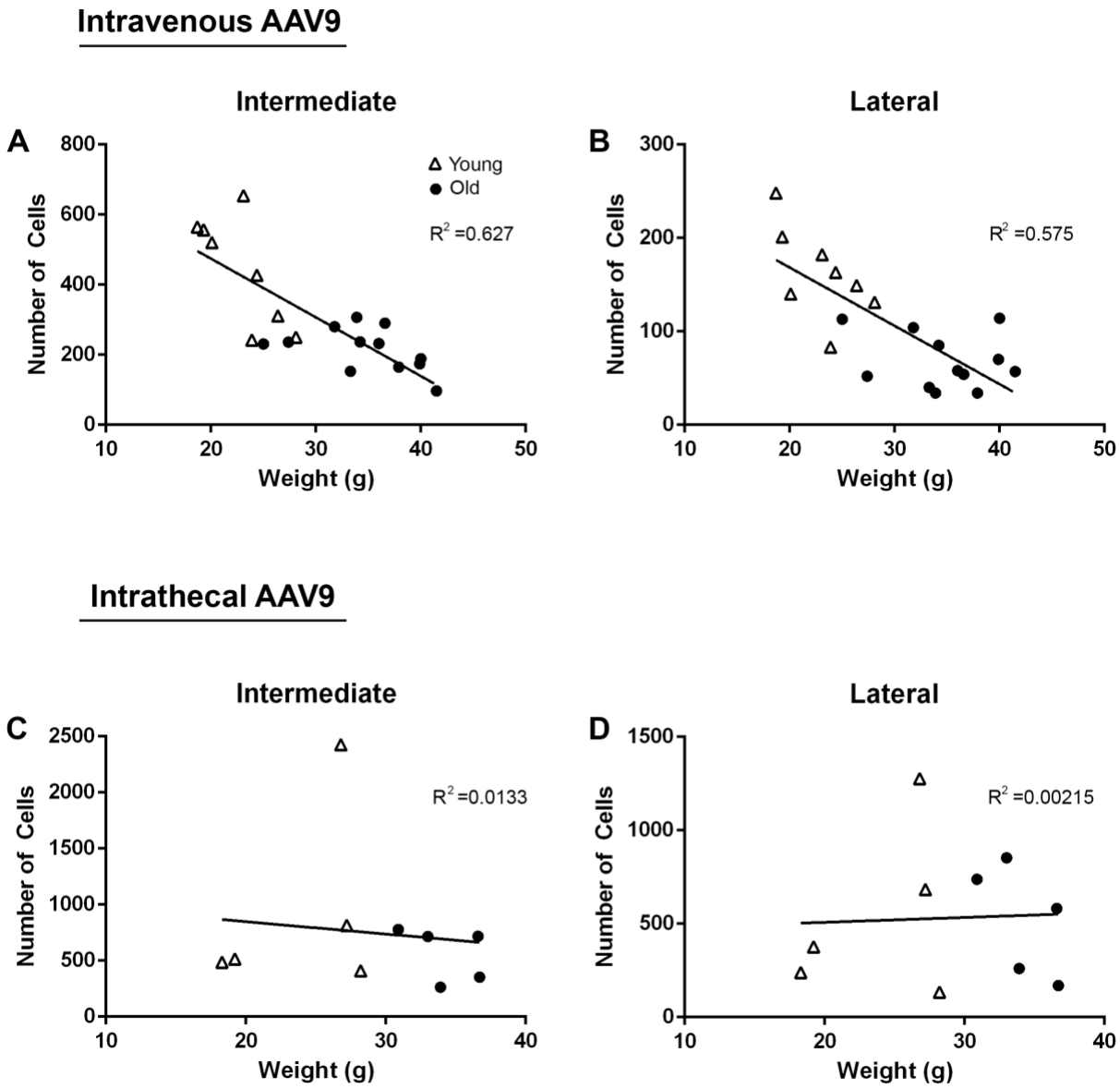

Figure 4. The efficacy of intravenous but not intrathecal AAV9 infusion declines with body weight. There was a negative correlation between age and weight in the group receiving intravenous AAV in the intermediate sections ($p < 0.0001$, $R^2=0.6267$) and lateral sections ($p = 0.0001$, $R^2=0.5753$). There was very weak to no correlation between weight and total cell count in the groups receiving intrathecal AAV9 in the intermediate sections ($p = 0.7509$, $R^2=0.01332$) and in the lateral sections ($p = 0.8988$, $R^2=0.002150$).

Discussion

No difference was detected in aged mice after intrathecal AAV9 or intravenous AAV-PhP.eB, but brain transduction was reduced after intravenous AAV9. The reduced intravenous AAV9 transduction in aged mice may be due to a change in the BBB associated with aging (Figure 1). The exact mechanism that AAV9 uses to cross the BBB is unknown. However, AAV vectors are too large to passively diffuse from the blood or CSF into the brain, suggesting that AAV9 is using an active mechanism to enter the brain (Iliff et al., 2012; Merkel et al., 2017). Furthermore, mannitol is ineffective at enhancing intravenous AAV9 transduction, suggesting AAV uses an active transport mechanism to cross the BBB (Gray et al., 2011). As the BBB ages, there may be less expression of the unknown receptor that is bound by AAV9 for transcytosis, leading to decreased transduction levels. The reduced transduction of intravenous AAV9 across aging may also be due to the greater size of the aged mice. Since the liver is the most strongly transduced tissue by intravenous AAV9, it may act like a sink, and take up more vector in larger mice, leaving less vector for transduction of the brain (Figure 4). Other peripheral tissues are also transduced by AAV9 and may contribute to this effect in larger mice. This latter explanation would also account for the greater brain transduction seen in female than in male mice after intravenous AAV9, as young female mice are smaller than young male mice (Maguire et al., 2013). It would also be interesting to repeat this experiment with vector dosed by weight to account for the increased weight of aged mice. Regardless, intravenous AAV9 infusions may not be the best gene therapy delivery method for larger subjects, as the dose of intravenous AAV9 used in this study was approximately $3x10^{13}$ vg/kg, requiring more than $1x10^{15}$ vg to treat even the smallest adult humans. Further increasing this dose may not be feasible: increased dosage can also pose problems as it is more difficult to manufacture, more expensive, can cause greater

immune responses and increased off-target effects. Additionally, the efficacy of this delivery method is significantly hindered by anti-AAV neutralizing antibodies in the bloodstream.

We concluded that there was no detectable difference in transduction by intravenous AAV-PhP.eB across aging (Figure 2). After intravenous infusion, AAV-PhP.eB uses the LY6A receptor for transcytosis across the BBB into the brain tissue (Deverman et al., 2016; Hordeaux et al., 2019). The similar transduction seen across aging may indicate that the expression of this receptor is not weakened by aging. Furthermore, AAV-PhP.eB is heavily biased towards the brain, meaning that peripheral tissue transduction is not as strong as AAV9. The larger liver in the aged mice is less likely to act as a sink in this case, allowing more virus vector to transduce the brain. This suggests that increasing the specificity of AAV9 to the brain may also decrease the change in transduction seen across aging.

The intrathecal infusion of AAV9 displayed no detectable change in transduction across aging (Figure 3). This suggests that the intrathecal infusion of AAV9 offers a better delivery method of gene therapy treatments to aged individuals than intravenous AAV9. This can be a result of the more direct delivery route intrathecal AAV9 uses to transduce the brain. After the vector is infused into the cisterna magna, it circulates in the CSF for a while before entering the blood. Thus, the vector has a higher chance of transducing the brain before transducing the liver and other peripheral organs. This method of infusion may prevent the liver from acting as a sink in larger aged mice. Since the efficacy of intrathecal AAV9 is not impacted by age, it may serve to be a more promising candidate for gene therapy treatments in aged individuals than intravenous AAV9. Although more invasive, it can still effectively treat the entire brain through a single injection while reducing off-target effects and eliminating the need to increase the vector

dosage for older individuals. However, novel capsids like AAV-PhP.eB also provide a promising way to treat neurological disorders in aged individuals.

In order to better support our conclusions about whether age and weight affect gene transfer, it will be important to repeat the intrathecal experiments with a more refined surgery technique. The infusion of the AAV9 vector into the cisterna magna produced variable results based on the success of the surgery. Using a more concentrated prep of AAV9 will allow for higher success of the surgery as the volume of infusion will be reduced, and ultimately less variability in the results. We can also reduce variability by increasing the number of mice in the experiment. Furthermore, we can more accurately compare the gene expression across aged and young mice by staining the endothelial cells using fluorescent immunostaining, like CD31. Counting the endothelial cells was not possible using light-level immunostaining. Additionally, to confirm that the liver does indeed act as a sink and that weight increases the amount of vector taken up by peripheral tissues, qPCR can be performed. This will quantify the amount of viral DNA in peripheral tissues such as the heart, lung, liver, kidney, spleen, skeletal muscle, and gonad. If the larger and older mice have the same number of vector genomes per cell in the liver as the smaller and younger mice, this would indicate a greater total amount of vector being taken up by the liver due to the liver's larger size in older men.

It will be important to test our conclusions in other animals commonly treated with AAV such as rats and monkeys as different animals experience different changes in brain morphology due to aging (Mooradian, 1988). Mouse models are used to model many of these diseases, notable models exist for Alzheimer's disease as well as ALS and Parkinson's disease (Foust et al., 2013; Hao et al., 2017; Onos et al., 2016). Although these diseases primarily affect older individuals, these diseases can also affect the young. There has been a case of ALS occurring as

young as 11 years old and Alzheimer's disease can onset at 40 years old (Conte et al., 2012; Koedam et al., 2010). There are many advantages to using rodents, including their low cost and short life spans as well as the plethora of research and genetic testing that has already been done on them, so they are commonly used as disease models (Hazzard, 1991). It is rare to use aged mice in AAV studies due to their increased cost and the risk that they may die. Furthermore, rodent disease models display early onset of the diseases so they receive AAV treatment at a young age. These results suggest that intrathecal AAV9 and intravenous AAVPhP.eB gene therapies are appropriate to be tested in young mice, as aging did not cause a difference in transduction or tropism. However, caution should be used when testing intravenous AAV9 in young mice, as efficacy may be reduced in older subjects. Since different species exhibit variable changes in brain morphology across aging, it will be important to test whether these methods work similarly across aging in rats and non-human primates.

Materials and Methods

Intrathecal Infusion of AAV9

Surgery was performed to infuse the AAV9 vectors intrathecally to the mice. At the time of treatment mice were either juvenile (3 months) or aged (20 months old). Groups of 5 juvenile and 5 aged mice were treated, three of which were male and two of which were female. They were anaesthetized using inhaled 1% isoflurane/1.5% oxygen and injected KXA cocktail [ketamine (50 mg/kg), xylazine (2.6 mg/kg), and acepromazine (0.5 mg/kg)]. After their fur was shaved, the head was mounted at a 45-degree angle in a stereotactic frame and a scalpel incision was made from the occipital crest to the C1 vertebra. A surgical retractor held the incision open as a 30 gauge, 45-degree bevel needle was inserted straight into the dura overlying the cisterna magna, creating a hole in the dura for the vector to be injected. Fifteen microliters of AAV9-CAG-eGFP ($1.5x10_{11}$ vg/ml) were then injected using a 30-gauge blunt syringe into the cisterna magna. Human thrombin and fibrinogen were combined over the needle hole to prevent reflux during infusion. The needle was withdrawn after ten minutes to prevent backflow of the vector during withdrawal. The muscle was then sutured, and wound clips were used to seal the skin. To improve transduction in the cortex, the mice were inverted in an incubator at a 25-degree angle with their heads made parallel to the ground using a cotton pad (Castle et al., 2018). They were monitored at this position for two hours with anesthesia boosts of KXA cocktail given periodically.

Intravenous delivery of AAV9 and AAV-PhP.eB

The first experiment using intravenous delivery consisted of eight mice, four of which were juvenile mice (3-month-old) and four of which were aged mice (20-month-old). Each age group

consisted of two females and two males. These mice were all treated with 150uL 150 µL of intravenous AAV-PhP.eB ($1.5x10_{11}$ vg/ml) diluted in PBS. The second experiment consisted of twenty mice, eight young mice (four male and four female) and twelve old mice (six male and six female). These mice received 150 µL of intravenous AAV9 ($1x10_{12}$ vg/ml) diluted in PBS. This Group sizes were larger in this experiment was much larger due to reports the anticipation that intravenous AAV9 shows higher transgene expression greater transduction in female mice mouse brain than male (Maguire et al., 2013). The mice were sedated using 1% isoflurane/1.5% oxygen and the virus was injected into the retro-orbital sinus (Gessler et al., 2019). The needle is inserted at the base of the eye roughly parallel to the rostral/caudal axis of the head. The vector is injected where the ophthalmic veins come together in the sinus.

Tissue collection and Immunohistochemistry

After a period of four weeks, the mice were sacrificed and perfused with saline, then dissected. The brains were severed from the spinal cord and completely removed completely from the skull. The brains and vertebral columns were drop-fixed for 24h in 4% paraformaldehyde in 0.1M phosphate buffer (4% PFA) at 4°C, and cryoprotected for at least 72h in 30% sucrose at 4°C. The brains were sectioned sagittally at 40 µm thickness and stained using light-level immunohistochemistry against the eGFP protein.

Free-floating sagittal brain tissue were washed in TBS and incubated in methanol before being blocked in 5% donkey serum and 0.25% Triton X-100 in TBS for 1 hour. The tissue was incubated in rabbit anti-GFP (Invitrogen #A11122) primary antibody diluted at 1:10,000 in blocking solution overnight at 4°C. The next day, sections were washed in TBS, incubated in Jackson Biotinylated Donkey anti-Rabbit antibody at 1:200 in TBS for an hour. The sections

were incubated in ABC solution and then developed in DAB for exactly 7 minutes. The sections were mounted on gelatin-coated slides and coverslipped in DPX mounting medium.

Microscopy, Analysis, and Statistics

Brightfield microscopy was performed using an AxioScan.Z1 Automated Slide Scanner (Zeiss) and 10X Plan Apo objective. Sections of the intravenous and intrathecal AAV9 experiments were then analyzed using ImageJ, using the cell counter plugin. The cells were counted using one lateral section, approximately 3.25mm lateral of midline, and one intermediate section, approximately 1.5mm lateral, from each animal. All images were blinded prior to counting. The number of cells in the entire section was counted and they were classified as neurons or glia based on their morphology. For intrathecal AAV9, unpaired two-tailed student's *t*-tests compared the total number of cells per lateral section or per intermediate section in aged mice versus young mice. For intravenous AAV9, two-way ANOVAs with Tukey's post-hoc tests compared the total number of cells per lateral section or per intermediate section among young males, young females, old males, and old females. Linear regressions were also performed for lateral and intermediate counts from both experiments, using body weight at the time of surgery as the dependent variable and the total number of GFP-expressing cells as the independent variable.

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