Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Nawoo Kim April 15, 2015

Comparing AAV Vector Serotypes for Gene Delivery to the Dorsal Root Ganglion for Pain Relief

by

Nawoo Kim

Dr. Nicholas Boulis
Adviser

Neuroscience and Behavioral Biology

Dr. Nicholas Boulis

Adviser

Dr. Robert Wyttenbach

Committee Member

Dr. Samuel Sober

Committee Member

2015

Comparing AAV Vector Serotypes for Gene Delivery to the Dorsal Root Ganglion for Pain Relief

By

Nawoo Kim

Dr. Nicholas Boulis

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

2015

Abstract

Comparing AAV Vector Serotypes for Gene Delivery to the Dorsal Root Ganglion for Pain Relief

By Nawoo Kim

Chronic peripheral neuropathy is a debilitating condition characterized by distal tingling, numbness, and pain. The current treatments only try to control the pain or treat the underlying cause, but there is a surprisingly low success rate. The dorsal root ganglion (DRG) has been a potential target for administering therapeutics, as spontaneous firing from this cluster of sensory neurons and pain fibers is a characteristic of peripheral neuropathy. Viral vectors, such as adeno-associated virus (AAV), can be used to deliver therapeutic genes to dampen the pain sensations. This study focuses on comparing the transduction efficacy of different AAV serotypes, 1, 2, 5, 6, and 8 expressing green fluorescent protein (GFP) administered to embryonic rat DRGs in culture. The cells were also stained with β-tubulin for co-localization with sensory neurons, TRPM8 (transient receptor potential cation channel, subfamily M, member 8) for co-localization with C and Aδ pain fibers, and IB4 (isolectin-b4) for co-localization with C fibers exclusively. Although significance could not be established for one serotype, all vectors were successful in transduction and co-localization with each type of stain. From an observational standpoint, AAV8 and AAV5 expressed the highest percentage of GFP-positive cells co-localizing with β-tubulin, TRPM8, and IB4. These findings are consistent with previous studies comparing different AAV serotypes *in vivo*. The determination of the most effective AAV serotype will prompt further studies involving gene therapy to the DRG using different delivery methods and therapeutic genes to provide pain relief.

Comparing AAV Vector Serotypes for Gene Delivery to the Dorsal Root Ganglion for Pain Relief

By

Nawoo Kim

Dr. Nicholas Boulis

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

2015

Acknowledgements

I would like to thank Dr. Nicholas Boulis for allowing me to work in his lab, and for giving this unique opportunity for many undergraduates during their years at Emory University. These past three years in the lab have opened my mind to the endless possibilities of research, especially in the exciting field of gene therapy for clinical purposes in neurological diseases.

I would also like to thank Dr. Deidre O'Connor for her guidance and patience every step of the way. Your encouragements and constructive criticisms have grown me as a researcher and writer. I am so grateful to have had you as my mentor!

Special thanks to Zachary McEachin and Dr. Sukreet Raju for spending their days doing the DRG extractions and surgeries. This could not have been possible without your help.

Thank you to the Gross Lab for allowing me to use their inverted microscope. Also special thanks to Dr. Claire-Anne Gutekunst and Jack Tung for being available for questions and helping me with technical difficulties.

And to my mom, for her consistent prayers, encouragements, and love. Thank you for being my number one fan, always telling me to take my vitamins, and being confident in me when I lose hope. I love you so much.

Finally, I give praise and thanks to God who sustains me everyday. This is all for His glory.

Table of Contents

Table of Figures

Introduction

The American Chronic Pain Association estimates that more than 15 million people in the U.S. and Europe experience peripheral neuropathy. This condition is characterized by distal numbness, tingling, weakness, and pain, often starting at the hands or feet. There are many causes, including infections, disease, medications, and toxic exposures (Azhary et al., 2010). When a patient is diagnosed with peripheral neuropathy, the treatment is to subdue the symptoms and control the underlying cause. However, the current treatments offered are disappointing for this crippling condition. Some patients find pain relief with the current treatments such as medicine or surgery, but most usually suffer for a lifetime trying different treatments for pain relief. Invasive techniques such as nerve blocks, electrical stimulation, or surgery are used, but they may permanently damage necessary sensations, only provide short-term relief, or cause a different pain to occur. Another system of pain relief is necessary, and gene therapy may provide a more effective solution.

To develop a successful treatment, it is important to understand the process of peripheral neuropathy. The fibers responsible for pain transmission are the C and Aδ fibers, which have peripheral nerve endings that respond to noxious stimuli. The C fibers are unmyelinated whereas the Aδ fibers are lightly myelinated. Their purpose is to be protective when exposed to stimuli that cannot be ignored (Costigan et al., 2009). Peripheral neuropathy, however, takes form when there is either increased sensitivity of the receptors or when there is spontaneous pain due to depolarizations from the cell body or axon. These conditions occur from the cell's plasticity to survive major changes such as inflammation or the severing of peripheral axons (Woolf and Ma, 2007).

When the periphery experiences an injury or inflammation, various chemicals are produced at the site of injury to increase the responsiveness of the nociceptors, so that non-noxious stimuli now become painful at the specific site (Woolf and Ma, 2007). Continuous production of these signals, however, causes a downstream cascade producing factors that are retrogradely transported to the cell bodies in the DRG. These signals initiate a sensitization of the nerve endings. Severe inflammation or axotomy also retrogradely informs the soma, which increases the transcription of neuropeptides and growth factors to upregulate the nociceptor density or create new pain fibers (Woolf and Ma, 2007). An interesting example of this phenotypic change is the increase of tetrahydrobiopterin (BH4) synthesis in the DRG after axonal injury (Tegeder et al., 2006). BH4 allows the influx of Ca^{2+}, in turn contributing to the heightened excitability and sensitivity of the DRG and its pain fibers.

Peripheral neuropathy can also occur without the presence of stimuli. A nociceptor should only initiate firing from its peripheral terminal; any depolarization originating from the axon or cell body represents ectopic firing. Injured DRG display patterned ectopic firing due to increased ion channel densities and trafficking (Liu et al., 2002). Another source may be from the intact neighboring C fibers, which depolarize spontaneously without direct injury to its own axon (Djouhri et al., 2006). An explanation for this phenomenon could be that the fibers are exposed to certain signal molecules originating from the injured axon. Many channels can contribute to this ectopic firing, such as non-selective cation channels, Ca^{2+}, Na^{+}, or K^{+} channels, all of which are shown to be involved in rats (Gold et al., 2003; Hilaire et al., 2005; Lee et al., 2005). These receptor regulations lower the threshold of formerly high-threshold mechano- and thermoreceptors, and pain can be felt even with the slightest stimuli such as nearby pulsating blood vessels or normal temperatures.

The current treatment for this condition merely manages the pain or treats the underlying disease causing the neuropathy (such as cancer, diabetes, infection, etc.). There is no permanent cure, and most treatments are relatively short-term. The preferred mode of treatment that doctors prescribe is pharmacological, divided into first and second line medications. The first line medications include certain antidepressants, calcium channel ligands (such as gabapentin), and topical lidocaine. If these medications do not provide satisfactory pain relief, doctors are recommended to use the second-line treatments that include opioid analgesics, certain antiepileptic and other antidepressants, topical capsaicin, mexiletine, and N-methyl-D-aspartate receptor antagonists (Dworkin et al., 2007). However, it is important to individualize the treatment combinations for each patient as each person responds differently to various medications. There is a risk of side effects such as dizziness, nausea, and sleepiness that also contribute to the patient's noncompliance. Despite the wide usage of pharmaceuticals, there is a strikingly low success rate for its effectiveness in pain relief. A large randomized study with various medications found only one out of every two or three patients achieving at least 50% pain relief (Finnerup et al., 2005). A follow-up study using the most developed pharmacological treatments found the same results, indicating that there was no improvement in the efficacy of medications for neuropathic pain (Finnerup et al., 2010). Medications continue as the preferred method of treatment due to its relatively safe and versatile nature, even though the majority of patients do not achieve considerable pain relief.

Other treatment options that are utilized are more invasive, such as nerve blocks and electrical stimulation. A nerve block is a local anesthetic injection usually in the spinal cord to interrupt pain signals to the brain. However, the effect is short-term, only lasting weeks to months. Additionally, the evidence of its success is limited and weak, and a recent study

concluded they would not recommend this method in most cases (Mailis and Taenzer, 2012). Electrical stimulation is when a thin, electrical lead is placed beneath the skin in the region of the pain to provide electric currents to, similarly, block pain signals. The device can be controlled like a remote control to adjust the strength, speed, and duration of the currents. Although the effect may last longer than a nerve block, the complications with this method arise from the lead itself: dislocation, infection, breakage, or the battery dying. A comprehensive review of electrical stimulation for neuropathy found that 40% of patients experienced one or more of these issues with the device (Wolter, 2014). These complications may be enough to dissuade patients from using this treatment long-term.

The most invasive method is complete surgical removal of the nerve or part of the system that is causing the neuropathic pain. This is the least preferred option, only recommended when the patient has exhausted the former and other less invasive, treatments and found them ineffective. Radiofrequency nerve ablation is a way to completely lesion the problematic nerves, destroying them with heat and provides a longer pain relief than a nerve block. However, a study found no difference between the control and treatment groups in partial radiofrequency lesioning of the DRG for chronic lumbosacral radicular pain (Geurts et al., 2003). Other surgical methods removing the pain nerves have serious side effects long-term, including sensory or motor deficits, or cause pain elsewhere. For example, cordotomy, which is removing certain pain tracts in the spinal cord, results in complete and instant abolishment of pain lasting 8-12 months. Unfortunately, this can eventually lead to unmasking the pain on the contralateral side, causing dysesthesia. Lesion of the dorsal root entry zone (DREZ) leads to sensory disturbances such as weakness in the ipsilateral leg (Meyerson, 2001). Total DRG eliminations have been performed, as well, that had initial positive outcomes. However in a follow-up study 5 years later, some of

the patients reported a loss of sensory and motor functions, and all had the pain return at the same or higher intensity as before the surgery (North et al., 1991). The conclusion was that a complete DRGectomy is not an effective method of treating neuropathy.

The disappointingly low success rate of the current treatments for chronic pain calls for a novel method of administering pain relief. A fairly recent target emerging in this area is the DRG, as the pathological changes occur within the soma of the sensory neurons as mentioned above. Drugs delivered systematically in the CNS can have very adverse effects, and many neuropathies have local causes, so a treatment within the PNS with a local administration to the DRG may be more beneficial (Sapunar et al., 2012). Gene therapy using herpes simplex virus (HSV) vectors injected subcutaneously to the rat has been performed, expressing a proenkephalin gene, which produce endogenous opioid peptide hormones (Goss et al., 2001; Lee et al., 2006). However, the analgesic effects diminished after ~4 weeks, but the use of a therapeutic gene targeting the DRG for pain relief was seen as a possibility. A more straightforward approach to the DRG, such as direct injection, and another viral vector may prove to be more successful. Direct injection to the DRG has already been performed in rat models with success, although it requires bone removal due to the intervertebral foramen enclosing the DRG (Fischer et al., 2011). Targeting the DRG is desirable because spontaneous depolarizations and sensitization can arise from these neurons. The treatment of the hyperexcitable DRG with gene therapy that is not available currently is important to further investigate, as the local treatment to the PNS is less likely to produce undesirable side effects (Chung and Chung, 2002).

One of the most frequently used viral vector for gene therapy is the adeno-associated virus (AAV). AAV come from the family Parvoviridae and has the ability to infect both dividing

and non-dividing cells. It is a popular vector to use due to its lack of pathogenicity, long transgene expression, and availability of several serotypes (Daya and Berns, 2008). There are currently 12 human derived AAV serotypes (AAV 1-12) and hundreds of non-human serotypes. AAV2 is the most prevalent and widely used, as it was the first to be discovered. The differences in serotypes include the capsid structure, antigen diversity, and tissue tropisms. AAV has already been in phase I clinical trials for gene delivery in certain CNS diseases, such as Canavan's, Parkinson's, and Batten's disease (Asokan et al., 2012). The possibility of producing synthetic AAV strains and the continuing developments make AAV vectors an attractive vehicle for gene delivery.

Significant research has been done in the past decade in the realm of gene therapy targeting the DRG using different AAV vectors serotypes, especially for peripheral neuropathy. Cultured newborn mice DRGs and fetal human DRGs transduced with AAV2-GFP expressed sustained GFP expression throughout the 28-day transduction period (Fleming et al., 2001). Additionally, AAV2 transduction was studied in rats *in vivo* with different modes of delivery: subcutaneous, sciatic nerve, intrathecal, and direct injection to the DRG (Xu et al., 2003). Direct injection proved to have the most effective and longest expression, sustaining 6-8 months post-surgery. These studies, as well as others, establish the feasibility of AAV vectors in gene delivery to the DRG. However, it is important to determine which AAV serotype is the most effective. A study compared the efficacy of AAV serotypes 1, 2, 3, 4, 5, 6, and 8, in direct injection to the adult rat DRG, and found AAV 1, 5 and 6 to transduce the most cells (Mason et al., 2010). AAV5 expressed >90% co-localization with β-tubulin. Another study with direct injection to the lumbar DRG in adult rats used AAV6 and AAV8, and found that AAV6 had a higher rate of transduction (Yu et al., 2013). This was also due to the fact that they found

differences in which type of cells the vectors infected, as AAV6 transduced a full range of DRG neurons, terminals and pain fibers, while AAV8 was restricted to the larger neurons.

Following these initial studies determining which AAV serotype was most effective in the rat DRG; researchers also focus on using these vectors in the context of peripheral neuropathy. As discussed previously, a characteristic of chronic pain is the regeneration of pain fibers from lesioned DRG. Liu et al. discovered a way to detect axon regeneration using self-complementary AAV2 following an axonal lesion of the rat DRG (Liu et al., 2014). They injected AAV2 into the DRG neurons to anterogradely trace their axons in both normal and lesioned models, by acting as a self-replicating tracer. The implications of this study can be used to target new axonal growth without using tracers and immunohistochemistry, which can be time-consuming. Another study focused on the nociceptor TRPV1 (vanilloid receptor 1), by delivering a AAV9 vector expressing short-hairpin RNA (shRNA) intrathecally (Hirai et al., 2014). TRPV1 is one of the nociceptors that react to burning pain, and can be upregulated causing neuropathy. By administering an shRNA that suppresses TRPV1 expression, the researchers found a gradual decrease of thermal allodynia 10-28 days after treatment. Similarly, Fisher et al. delivered channel-binding domain 3 (CBD3) peptide that blocks voltage-gated Ca^{2+} channels using AAV6, injected directly into the rat DRG (Fischer et al., 2014). They saw a decrease in hyperalgesia and response to cold pain 6 weeks after treatment, indicating the therapeutic potential of gene therapy, although immunohistochemistry showed only ~30% transduction.

Further research is necessary in the area of discovering more therapeutic genes for pain relief, using DRG as the target, as well as translating the delivery method from small animals to human subjects. Other possible genes that may provide analgesia include a regulator of GTP

cyclohydrolase synthesis, which in turn regulates BH4, which is upregulated in the DRG following peripheral nerve injury (Tegeder et al., 2006). Another recently discovered glycoprotein that is upregulated is TSP4 (thrombospondin-4), which amplifies excitatory synaptic transmission among the DRG neurons (Pan et al., 2015). Discovery of more molecules and channels that cause sensitization or hyperexcitability of the sensory neurons is crucial to gene therapy development, as well as the correct AAV serotype for this use. Although local treatment of the PNS seems favorable, more information is needed before continuing to human trials despite the success in rodent models; such as, performance in other, larger animals, more evidence that AAV has low immunogenicity, if the vectors may be tumorigenic, and the translation of the delivery method to be minimally invasive for humans (Beutler, 2010). However, a new method of CT-mediated intraganglionic (IG) injection in pigs has been successful in delivering AAV1 using convection-enhanced delivery (CED) (Pleticha et al., 2014). CED uses bulk flow to deliver large volumes of the vector in a localized area, using a special catheter that narrows at the end. The researchers conclude that this method has high potential to be easily translated to humans, which will be more favorable than surgical injections to the DRG.

This study will utilize primary embryonic DRG in cell culture to determine which AAV serotype, 1, 2, 5, 6, or 8 expressing GFP, will transduce the sensory neurons and pain fibers with the most co-localization. Primary cell culture has the potential for high yield, being able to isolate neurons compared to postnatal rats (Melli and Hoke, 2009). It is also an adequate model for diabetic, HIV and chemotherapy induced neuropathies. β-tubulin will be used in immunohistochemistry to stain for nerve cells, soma, dendrites and axons in the DRG (Caceres et al., 1986). We will also stain for TRPM8 (transient receptor potential cation channel, subfamily

M, member 8), which is a nociceptor that responds to noxious cold stimuli, and is present in both C and Aδ pain fibers (Kobayashi et al., 2005). Lastly, IB4 (isolectin-b4) will be stained to distinguish the C-fibers exclusively (Fang et al., 2006). It is important to determine if the AAV serotypes have differences in targeting the normal sensory neurons compared to the pain fibers, which is the reason for the separate stains. Our goal in this pilot study is to determine the most effective AAV serotype in order to further investigate gene therapy for chronic pain relief.

Materials and Methods

DRG Harvesting and Extraction

Pregnant Sprague-Dawley female rats were ordered from Charles River one week before the surgery. At E16, the pregnant rat is sacrificed via isoflurane anesthesia (Med Vet International, RXISO-250) and intraperitoneal injection of Euthasol (DV Medical Supply, 100 mg/kg). The uterus with embryos was surgically removed and placed in a clean 150mm petri dish. One female rat usually produces 10-14 embryos. The pups were placed in Lebovitz-15 (L15, Gibco, 11415064-500ML) media for DRG dissection in the NuAire Horizontal Laminar Airflow Hood (NU-201) with an Olympus SZX9 microscope. The spinal cords were taken out of each embryo, and then the DRG were clipped off and moved to another dish. This part of the method was performed by Zach McEachin or Dr. Sukreet Raju , while the rest of the protocol was done by the honors student (Nawoo Kim).

Preparation of Plates

The night before the surgery, the 24-well plates (Corning Costar, 3527) were coated with rat tail collagen (Sigma, C7661-5MG). Sterile, 1 mL of 0.1 M acetic acid was added to 5 mg of collagen and swirled until fully dissolved. 1 mL of the solution was transferred to a 100 mL flask and diluted with 99 mL of distilled water, for a 1:100 dilution. The solution was sterile filtered with 0.22 μm membrane filter (Millex-GS, SLGSM33SS). Approximately 500 μL of the solution was spread in each well the night before surgery and dried overnight in the flow hood. The plates were washed (3x10minutes) with sterile phosphate-buffered saline (PBS) (Sigma, D1408) before adding cells.

Dissociation and Plating DRG

The DRGs were centrifuged at 800 rpm for 10 minutes. After removing the supernatant, 1 mL of 0.25% trypsin in Hanks Balanced Salt Solution (Sigma, T4049) was added, and the contents incubated at 37ºC for 30 min. The tube was centrifuged at 800 rpm for 5 min. and the supernatant was removed. Then 1 mL of AN2 medium that was prepared beforehand was added and the pellet pipetted up and down to dissociate the cells. The cells were counted to ensure a seeding density of 1-5E4 cells per well. The calculated amount of resuspended cells were added to each well and covered with AN2 media, and then incubated at 37ºC. The AN2 media was made with 410 mL of Earle's Minimum Essential Medium with L-glutamine (Gibco, 11090-081), 75 mL of 15% Calf Bovine Serum (Hyclone, SH30073.03), 15 mL of 20 w/v glucose (Sigma, G8270), 5 mL of 1% Penicillin-Streptomycin (Sigma, P4458), 10 µL of 10 ng/mL nerve growth factor-7S (Invitrogen, 13290-010) reconstituted in Bovine Serum Albumin (Fisher, BP 1605-100), 10 µL of 10 µM 5-Fluoro-2′-deoxyuridine (Sigma, F0503-100MG), and 10µL of 10 µM uridine (Sigma, U3003-5G).

Transduction

The AAV vectors have been produced by the Ohio Vector Core and express enhanced green fluorescent protein (eGFP) for later analysis. Three multiplicities of infection (MOI), or virus particles per cell, were tested: an MOI of 1E6, 1000, then 1E5. The MOI of 1E5 was determined to be used for the subsequent DRG transductions due to the effective GFP transduction compared to the other two MOIs. The necessary amount of AAV vector serotypes 1, 2, 5, 6, and 8 to have the respective MOI for each replicate were calculated for three wells per serotype. The vector titers were produced as follows: AAV 1 at 4.1E12 vp/mL, AAV 2 at 8.4E12 vp/mL, AAV 5 at 2.3E12 vp/mL, AAV 6 at 2.6E12 vp/mL, and AAV 8 at 4.0E12 vp/mL. They

were diluted in sterile PBS as necessary to have enough solution for reasonable pipetting amounts. The day after plating, the media was aspirated off the cells and 150 μL of the respective AAV vector and AN2 media solution was added to each well. The plates were incubated in 37°C and rocked every 15-20 minutes for 2-4 hours. Three wells were transduced per serotype, and three were left with no virus, totaling 18 wells per set of DRGs from one pregnant rat. The wells were topped off with AN2 media, incubated in 37°C, and fed every two days for one week. One week after transduction, the cells were washed with sterile PBS, then 4% paraformaldehyde (PFA) (Sigma, 252549) was added and incubated at 37°C for 30 min. Then the cells were washed with sterile PBS again and plates were wrapped in parafilm (Thomas Scientific, 7315D11) and aluminum foil, and stored at 4°C.

Immunohistochemistry

Post-fixation, immunohistochemistry was performed with the following primary antibodies: β-tubulin III (Sigma, T8328), anti TRPM8 (Abcam, ab109308) and anti IB4 (Vector Labs, B-1205). The cells were blocked for 30 min. in 5% donkey serum (Jackson, 017-000-001) diluted in PBS and stored overnight at 4°C with a 1:500 dilution of primary antibody diluted in blocking solution and incubated at 4°C overnight. The next day the cells were washed (3x10minutes) with PBS and incubated for one hour with the appropriate secondary antibody with a 1:500 dilution in blocking serum. Secondary antibodies were donkey anti-mouse (Jackson, 715-165-150), donkey anti-rabbit (Jackson, 711-165-152), or donkey anti-goat (Jackson, 705-165-147) IgG conjugated with Cy3. Each of the three wells per serotype was used for the different stains. The wells were washed with PBS and the plates kept in 4°C wrapped in

aluminum foil. Photomicrographs were obtained using a Leica DMIRE2 inverted microscope and Retiga Exi (QImaging) camera.

Cell Counting

The effectiveness of the transduction was quantified by counting the number of cells expressing GFP and either β-tubulin, TRPM8, or IB4 staining. Five random images of 20x magnification was taken of each well and counts of GFP positive and Cy3 positive were obtained. The percentages of GFP positive cells of the sensory neurons or pain fibers were calculated for each serotype with the equation: ((number of co-localized cells)/(number of [β-tubulin, TRPM8, and IB4] positive cells total)x100)%.

Data Analysis

Three sets of DRGs were used for analysis: DRG 1 with an MOI of 1E6, and DRG 3 and 5 with an MOI of 1E5. There were not enough replicates to determine statistical significance.

Results

MOI Comparisons

Among the MOI of 1000, 1E5, and 1E6 tested, the MOI of 1E5 showed reasonable levels of GFP expression for further analysis. The MOI of 1000 did not show any GFP expression but had β-tubulin, TRPM8, and IB4 expression (data not shown). The MOI of 1E6 and 1E5 both showed β-tubulin co-localized with GFP, but the MOI of 1E6 was determined to have too much GFP expression for proper analysis (Figure 1). The MOI of 1E5 had sufficient GFP expression for analysis, but was not too excessive to see differences among the AAV serotypes.

AAV Serotype Comparisons

Five pictures from each well were taken for β-tubulin, TRPM8, and IB4 with Cy3 fluorescence and GFP expression for each serotype. The Cy3 and GFP image were merged together to determine co-localization. For β-tubulin staining, all the serotypes had GFP expression as well as β-tubulin, but with observable differences (Figure 2). AAV2 expressed robust GFP transduction of cells with some that were not co-localized with β-tubulin. AAV6 and AAV8 expressed the most co-localization. Similarly for TRPM8, all serotypes transduced the cells expressing TRPM8 but with variability (Figure 3). AAV2 again showed GFP expression with some cells that were not co-localized with TRPM8; AAV5 and 8 had the most co-localization. Finally for IB4, all serotypes again were successful in transduction of cells expressing IB4 (Figure 4). Consistent with prior observations, AAV2 showed robust GFP expression with only some co-localization. AAV6 and AAV8 had the most co-localization.

Percentage Co-localization

The percent of co-localization with GFP positive cells were calculated among β-tubulin, TRPM8, and IB4 expressing cells of each serotype and for each DRG replicate (Figure 5). There was a large degree of variability in the percentage of GFP co-localization with β-tubulin, TRPM8, and IB4. For co-localization with β-tubulin (DRG 3 and 5), AAV 8 consistently showed the highest percentages for the two replicates of MOI of 1E5 (51.06% and 48.98%) followed by AAV6 (61.11% and 34.43%), whereas AAV 1 showed the lowest (28.77% and 21.88%). For co-localization with TRPM8, AAV 1 showed the lowest consistent percentages (44.07% and 30.43%), while AAV 8 showed the greatest variability between DRG 3 and 5 (71.58% and 23.44%), followed by AAV5 (60.20% and 34.62%). There were no cells present in the DRG 5 well stained with TRPM8. For co-localization with IB4, these cells showed the greatest variability between DRG 3 and 5. The lowest percentages were with AAV 1 (48.57% and 20.69%), while the highest percentages but with the greatest variability were with AAV 8 (94.44% and 20.00%), AAV6 (89.55% and 25.00%), and AAV5 (79.71% and 28.13%). There were not enough replicates for statistical analysis and too much variation for averaging the percentages.

Discussion

In this study, we determined the effectiveness of AAV serotypes 1, 2, 5, 6 and 8 in transducing the sensory neurons and pain fibers in primary embryonic DRGs. We saw that every serotype successfully transduced the cells of interest by analyzing co-localization of the specific stain to GFP expression, but to varying degrees. The co-localization seen with TRPM8 and IB4 indicates that the AAV vectors were successful in transducing the pain fibers. Difference between the success of the serotypes transducing either C or Aδ fibers could not be established. If in the case that the antibody stains did not recognize 100% of the cells, the phase (bright field) images were compared with the fluorescent images to determine correlation. From an observational standpoint, AAV1 showed the lowest percentage of co-localization among all three stains. AAV2 showed the most GFP positive cells that were not co-localized with β-tubulin, TRPM8, and IB4 expression, indicating that the serotype was effective in transducing other types of cells other than the sensory neurons and pain fibers. The serotype that showed the highest average co-localization was AAV8 among all of the stains, but no significance was established. For the different stains, IB4 (C-fibers) had a surprisingly high transduction rate for DRG 3 specifically, for AAV5, 6, and 8, but the lowest co-localization percentage for DRG 5. In fact, DRG 5 had the lowest co-localization among the three DRG extractions for all serotypes and stains even with the MOI of 1E5. An explanation for this phenomenon could be that during this particular process of plating the embryonic DRGs, we were only able to obtain enough cells to plate a density of 7.8E3 cells per well rather than the goal of 1-5E4. The low density of cells hinders cell growth in culture, and is hard to detect fluorescence with a low cell count.

Although one AAV serotype could not be determined to be the most effective, the results of this study have correlations among the broader literature. The results show that all of the AAV

serotypes were successful in transducing the sensory neurons and pain fibers, as shown in previous studies comparing the different serotypes (Mason et al., 2010). Although several groups used AAV2 in gene delivery to the DRG (Fleming et al., 2001; Xu et al., 2003; Liu et al., 2014), there is evidence that other serotypes, such as AAV5 (Mason et al., 2010) or AAV8 (Storek et al., 2008) may prove to have more robust transduction of the DRG. This is consistent with the present findings, as AAV8 showed the highest average co-localization percentages, and AAV5 performed better than AAV1 and AAV2. There is also literature that supports AAV8 and AAV5 as transducing mainly the large-diameter neurons in the DRG while AAV6 targets the small diameter neurons (Vulchanova et al., 2010; Yu et al., 2013). In this study we see similar patterns with β-tubulin, IB4, which stained for the small-diameter, C-fibers, and TRPM8 which stained for both large (Aδ) and small. AAV6 showed relatively high co-localization with DRG 1 compared to AAV5 and AAV8. Unfortunately the same conclusion could not be established for the other replicates, but it is an interesting trend. Overall the research comparing different AAV serotypes for DRG transduction support AAV5, AAV6 and AAV8 to be the most effective, which is consistent with the present data. Additionally, there may be discrepancy in which serotype is most effective depending on the method of delivery in vivo: if injected intrathecally, AAV8 outperformed AAV1 (Storek et al., 2008) while direct injection had AAV5 and AAV1 being more effective (Mason et al., 2010). Further comparisons between serotypes and delivery methods will be advantageous to the development of a gene therapy treatment for chronic pain.

Shortcomings

Although the different stains for sensory neurons and pain fibers and AAV transduction were successful, there were not enough replicates to conduct proper statistical analysis. Many

unfortunate circumstances lead to these shortcomings. First, there was the issue of time, as we had to select what MOI will allow for adequate transduction. Our first transduction (DRG 1) with MOI of 1E6 had a very high yield of 2.64E4 cells per well with successful growth, but as mentioned before, had too much GFP expression for appropriate analysis. The second set of DRGs had a successful yield and growth, with 1.75E4 cells per well, but with an MOI of 1000 that were too low for transduction. DRG 3 then had the MOI of 1E5 with 1.25E4 cells per well, and was determined to have the correct MOI for analysis. There was further difficulty in acquiring a sufficient number of cells in a subsequent extraction. There were also issues with the DRG dissociation process and risk of contamination with primary cell culture. For one of the replicates, DRG 4, after surgery on two pregnant rats and DRG extraction, the pellet after centrifugation that contained the cells were accidentally aspirated with the vacuum. The solution was still plated in case some cells were left, but no cell growth was seen. In addition to the cell dissociation and plating process, are the difficulties in cell growth from primary tissue and potential contamination. Primary cell culture has many sources of contamination, including the media, the cell's own flora, and the equipment, and this can happen at any step in the whole process (Vierck et al., 2000). Also, the time of the cells leaving the natural environment of the organism to the wells must be minimal to maximize cell growth. Attempts at obtaining two more replicates, DRG 6 and 7, were hindered by contamination of the AN2 media that was detected after it was placed on the cells. They were dislodged and washed away with PBS during fixation. Additionally, the lack of cell growth of DRG 5 may be due to the time it took for DRG extraction.

Future Directions

There are many applications and further experiments that can be performed after this initial study. To determine how many more replicates we would need for proper statistical analysis, we can perform an *a priori* power test. For this test, we would establish α (the probability of finding significance where there is none), the power (the probability of finding significance) and the effect size (quantitative measure of the strength of correlation or mean difference) to determine the needed sample size. After determining the number of replicates needed and performing the experiment, the one-way ANOVA test would be performed to establish with confidence which AAV serotype transduces the sensory neurons or pain fibers of the DRG most effectively. The success of future experiments can be ensured with better sanitation procedures, modified plating techniques, efficient surgical methods, and new methods of transduction. These changes may include pre-incubation of the wells, using poly-D-lysine coated coverslips, (Burkey et al. 2004). With more cell growth, AAV serotype comparison will be attainable, as we have established that they do transduce both sensory and pain fibers in the embryonic rat DRG. We will also be able to determine which serotype transduces the pain fibers as compared to the sensory neurons, adding another layer of analysis.

After determination of the right AAV serotype, a plausible next step will be to perform its efficacy in vivo. Direct injection in the DRG is a possibility, and has been performed in studies with success (Fisher et al., 2011; Mason et al., 2010; Yu et al., 2013). However, before *in vivo* studies, we are interested in testing a different delivery method using a biosynthetic hydrogel. Dr. Andrés Garcia from the Georgia Institute of Technology have worked together with Emory University to engineer a hydrogel which can be used as a vehicle for therapeutic agents, including virus vectors, that degrades in a controlled manner to release the contents over a longer

period. They have used this method to treat diabetes in mice, inserting insulin-producing cells inside the hydrogel placed near the small intestine (Phelps et al., 2013). Four weeks after the treatment, the mice had normal glucose levels. We want to use this hydrogel and insert the most effective AAV serotype to see if the delivery is successful in cultured rat DRGs before inserting it into the live rat.

The overall goal of this project is to express a gene of interest, to dampen the hyperexcitable DRG neurons or prevent certain growth factors of pain sensitization, using the AAV vector and determine if there is pain relief in animal models. The determination of the best AAV serotype is an important initial step in this process of utilizing the hydrogel and for future applications in gene therapy to the DRG.

References

Asokan A, Schaffer DV, Samulski RJ (2012) The AAV vector toolkit: poised at the clinical crossroads. Molecular therapy : the journal of the American Society of Gene Therapy 20:699-708.

Azhary H, Farooq MU, Bhanushali M, Majid A, Kassab MY (2010) Peripheral neuropathy: differential diagnosis and management. American family physician 81:887-892.

Beutler AS (2010) AAV provides an alternative for gene therapy of the peripheral sensory nervous system. Molecular therapy : the journal of the American Society of Gene Therapy 18:670-673.

Caceres A, Banker GA, Binder L (1986) Immunocytochemical localization of tubulin and microtubule-associated protein 2 during the development of hippocampal neurons in culture. The Journal of neuroscience : the official journal of the Society for Neuroscience 6:714-722.

Chung JM, Chung K (2002) Importance of hyperexcitability of DRG neurons in neuropathic pain. Pain practice : the official journal of World Institute of Pain 2:87-97.

Costigan M, Scholz J, Woolf CJ (2009) Neuropathic pain: a maladaptive response of the nervous system to damage. Annual review of neuroscience 32:1-32.

Daya S, Berns KI (2008) Gene therapy using adeno-associated virus vectors. Clinical microbiology reviews 21:583-593.

Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN (2006) Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:1281-1292.

Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC, Wallace MS (2007) Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain 132:237-251.

Fang X, Djouhri L, McMullan S, Berry C, Waxman SG, Okuse K, Lawson SN (2006) Intense isolectin-B4 binding in rat dorsal root ganglion neurons distinguishes C-fiber nociceptors with broad action potentials and high Nav1.9 expression. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7281-7292.

Finnerup NB, Sindrup SH, Jensen TS (2010) The evidence for pharmacological treatment of neuropathic pain. Pain 150:573-581.

Finnerup NB, Otto M, McQuay HJ, Jensen TS, Sindrup SH (2005) Algorithm for neuropathic pain treatment: an evidence based proposal. Pain 118:289-305.

Fischer G, Pan B, Vilceanu D, Hogan QH, Yu H (2014) Sustained relief of neuropathic pain by AAV-targeted expression of CBD3 peptide in rat dorsal root ganglion. Gene therapy 21:44-51.

Fischer G, Kostic S, Nakai H, Park F, Sapunar D, Yu H, Hogan Q (2011) Direct injection into the dorsal root ganglion: technical, behavioral, and histological observations. Journal of neuroscience methods 199:43-55.

Fleming J, Ginn SL, Weinberger RP, Trahair TN, Smythe JA, Alexander IE (2001) Adeno-associated virus and lentivirus vectors mediate efficient and sustained transduction of cultured mouse and human dorsal root ganglia sensory neurons. Human gene therapy 12:77-86.

Geurts JW, van Wijk RM, Wynne HJ, Hammink E, Buskens E, Lousberg R, Knape JT, Groen GJ (2003) Radiofrequency lesioning of dorsal root ganglia for chronic lumbosacral radicular pain: a randomised, double-blind, controlled trial. Lancet 361:21-26.

Gold MS, Weinreich D, Kim CS, Wang R, Treanor J, Porreca F, Lai J (2003) Redistribution of Na(V)1.8 in uninjured axons enables neuropathic pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:158-166.

Goss JR, Mata M, Goins WF, Wu HH, Glorioso JC, Fink DJ (2001) Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion. Gene therapy 8:551-556.

Hilaire C, Campo B, Andre S, Valmier J, Scamps F (2005) K(+) current regulates calcium-activated chloride current-induced after depolarization in axotomized sensory neurons. The European journal of neuroscience 22:1073-1080.

Hirai T, Enomoto M, Kaburagi H, Sotome S, Yoshida-Tanaka K, Ukegawa M, Kuwahara H, Yamamoto M, Tajiri M, Miyata H, Hirai Y, Tominaga M, Shinomiya K, Mizusawa H, Okawa A, Yokota T (2014) Intrathecal AAV serotype 9-mediated delivery of shRNA against TRPV1 attenuates thermal hyperalgesia in a mouse model of peripheral nerve injury. Molecular therapy : the journal of the American Society of Gene Therapy 22:409-419.

Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, Noguchi K (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. The Journal of comparative neurology 493:596-606.

Lee DH, Chang L, Sorkin LS, Chaplan SR (2005) Hyperpolarization-activated, cation-nonselective, cyclic nucleotide-modulated channel blockade alleviates mechanical

allodynia and suppresses ectopic discharge in spinal nerve ligated rats. The journal of pain : official journal of the American Pain Society 6:417-424.

Lee JY, Fink DJ, Mata M (2006) Vector-mediated gene transfer to express inhibitory neurotransmitters in dorsal root ganglion reduces pain in a rodent model of lumbar radiculopathy. Spine 31:1555-1558.

Liu CN, Devor M, Waxman SG, Kocsis JD (2002) Subthreshold oscillations induced by spinal nerve injury in dissociated muscle and cutaneous afferents of mouse DRG. Journal of neurophysiology 87:2009-2017.

Liu Y, Keefe K, Tang X, Lin S, Smith GM (2014) Use of self-complementary adeno-associated virus serotype 2 as a tracer for labeling axons: implications for axon regeneration. PloS one 9:e87447.

Mailis A, Taenzer P (2012) Evidence-based guideline for neuropathic pain interventional treatments: spinal cord stimulation, intravenous infusions, epidural injections and nerve blocks. Pain research & management : the journal of the Canadian Pain Society = journal de la societe canadienne pour le traitement de la douleur 17:150-158.

Mason MR, Ehlert EM, Eggers R, Pool CW, Hermening S, Huseinovic A, Timmermans E, Blits B, Verhaagen J (2010) Comparison of AAV serotypes for gene delivery to dorsal root ganglion neurons. Molecular therapy : the journal of the American Society of Gene Therapy 18:715-724.

Melli G, Hoke A (2009) Dorsal Root Ganglia Sensory Neuronal Cultures: a tool for drug discovery for peripheral neuropathies. Expert opinion on drug discovery 4:1035-1045.

Meyerson BA (2001) Neurosurgical approaches to pain treatment. Acta anaesthesiologica Scandinavica 45:1108-1113.

North RB, Kidd DH, Campbell JN, Long DM (1991) Dorsal root ganglionectomy for failed back surgery syndrome: a 5-year follow-up study. Journal of neurosurgery 74:236-242.

Pan B, Yu H, Park J, Yu YP, Luo ZD, Hogan QH (2015) Painful nerve injury upregulates thrombospondin-4 expression in dorsal root ganglia. Journal of neuroscience research 93:443-453.

Phelps EA, Headen DM, Taylor WR, Thule PM, Garcia AJ (2013) Vasculogenic bio-synthetic hydrogel for enhancement of pancreatic islet engraftment and function in type 1 diabetes. Biomaterials 34:4602-4611.

Pleticha J, Maus TP, Christner JA, Marsh MP, Lee KH, Hooten WM, Beutler AS (2014) Minimally invasive convection-enhanced delivery of biologics into dorsal root ganglia: validation in the pig model and prospective modeling in humans. Technical note. Journal of neurosurgery 121:851-858.

Sapunar D, Kostic S, Banozic A, Puljak L (2012) Dorsal root ganglion - a potential new therapeutic target for neuropathic pain. Journal of pain research 5:31-38.

Storek B, Reinhardt M, Wang C, Janssen WG, Harder NM, Banck MS, Morrison JH, Beutler AS (2008) Sensory neuron targeting by self-complementary AAV8 via lumbar puncture for chronic pain. Proceedings of the National Academy of Sciences of the United States of America 105:1055-1060.

Tegeder I et al. (2006) GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence. Nature medicine 12:1269-1277.

Vierck JL, Byrne K, Mir PS, Dodson MV (2000) Ten commandments for preventing contamination of primary cell cultures. Methods in cell science : an official journal of the Society for In Vitro Biology 22:33-41.

Vulchanova L, Schuster DJ, Belur LR, Riedl MS, Podetz-Pedersen KM, Kitto KF, Wilcox GL, McIvor RS, Fairbanks CA (2010) Differential adeno-associated virus mediated gene transfer to sensory neurons following intrathecal delivery by direct lumbar puncture. Molecular pain 6:31.

Wolter T (2014) Spinal cord stimulation for neuropathic pain: current perspectives. Journal of pain research 7:651-663.

Woolf CJ, Ma Q (2007) Nociceptors--noxious stimulus detectors. Neuron 55:353-364.

Xu Y, Gu Y, Wu P, Li GW, Huang LY (2003) Efficiencies of transgene expression in nociceptive neurons through different routes of delivery of adeno-associated viral vectors. Human gene therapy 14:897-906.

Yu H, Fischer G, Ferhatovic L, Fan F, Light AR, Weihrauch D, Sapunar D, Nakai H, Park F, Hogan QH (2013) Intraganglionic AAV6 results in efficient and long-term gene transfer to peripheral sensory nervous system in adult rats. PloS one 8:e61266.

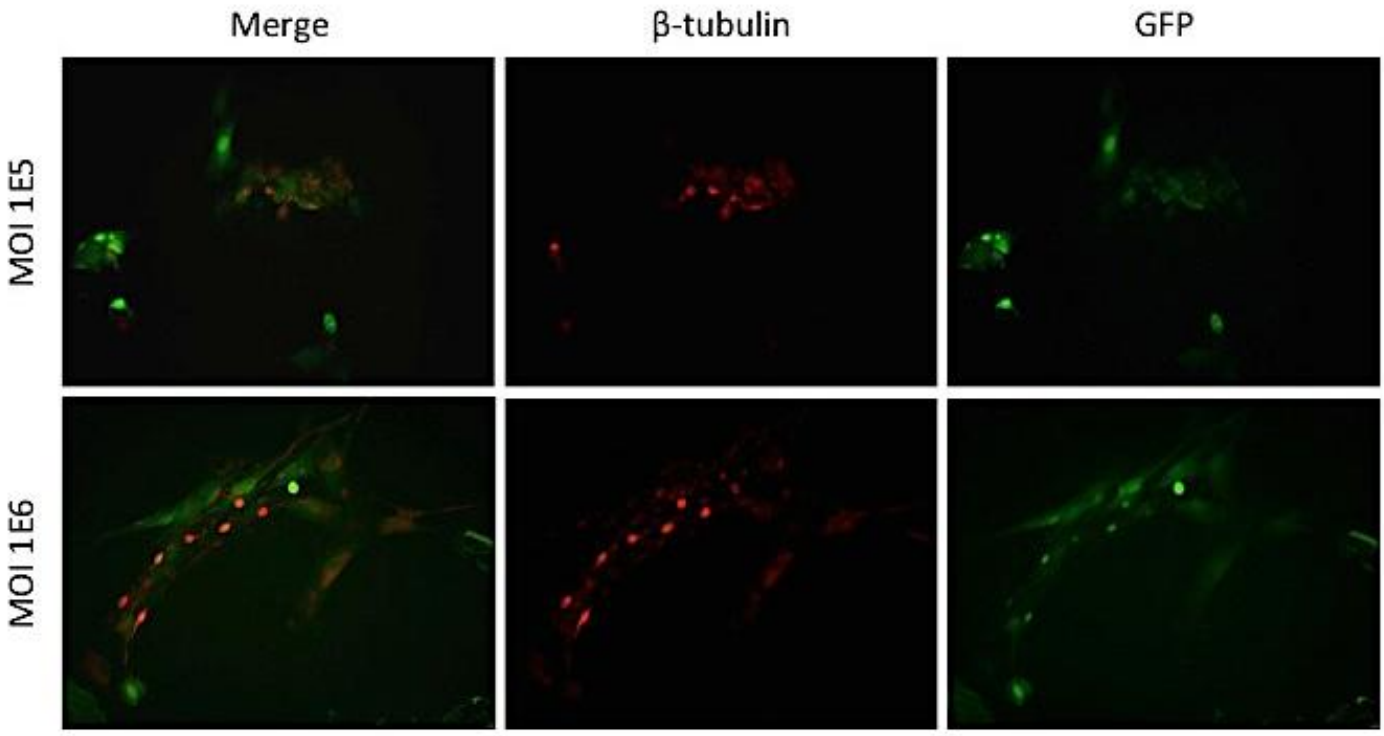

Figure 1. Comparison of AAV 1 DRG transduction with an MOI of 1E5 and 1E6. Both MOIs examined showed β-tubulin and GFP expression. Magnification = x20

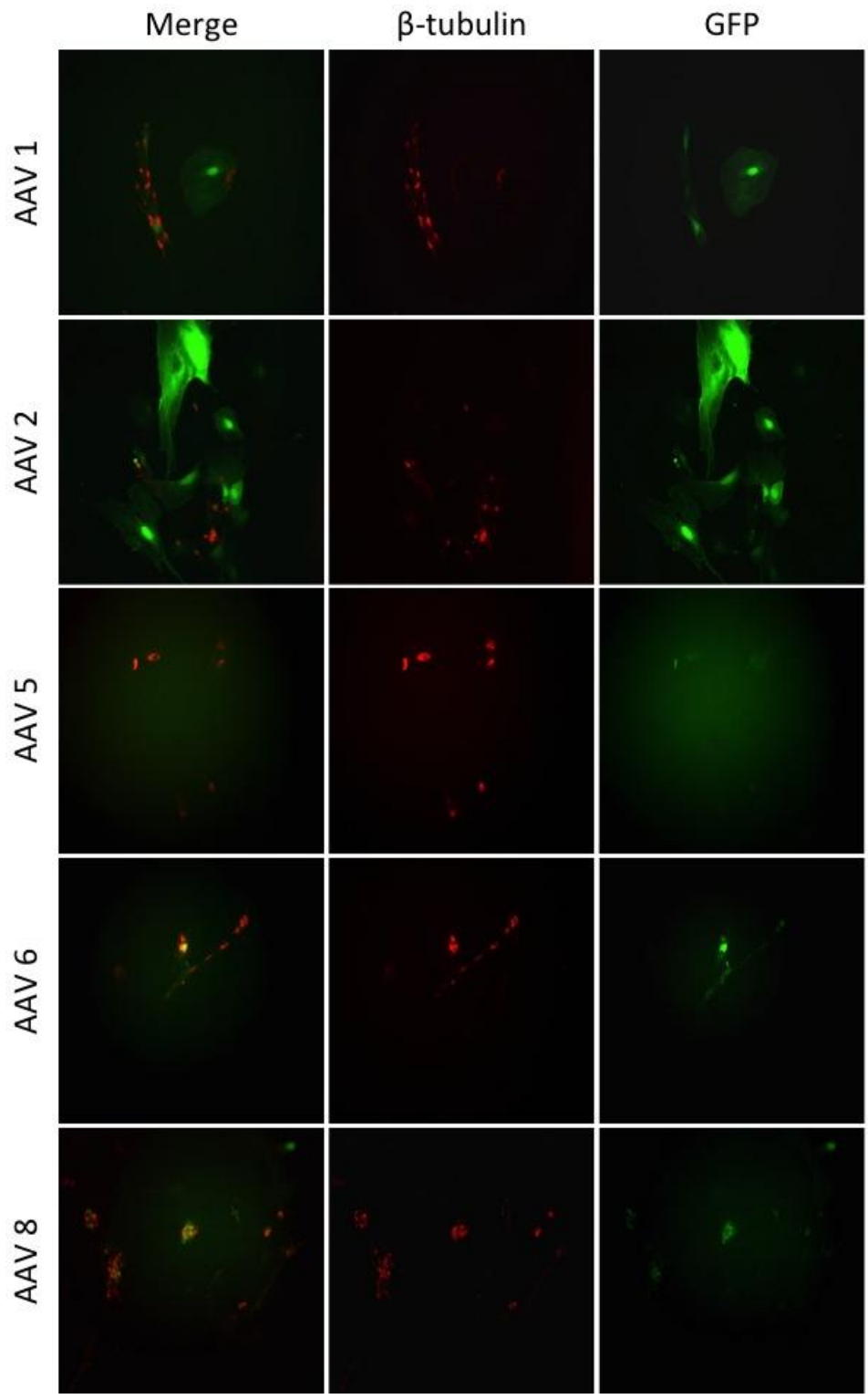

Figure 2. Comparison of β-tubulin and GFP co-localization following AAV transduction. AAV 2 expressed robust GFP transduction that was not co-localized with β-tubulin; AAV 6 and 8 had the most co-localization. Magnification = x20

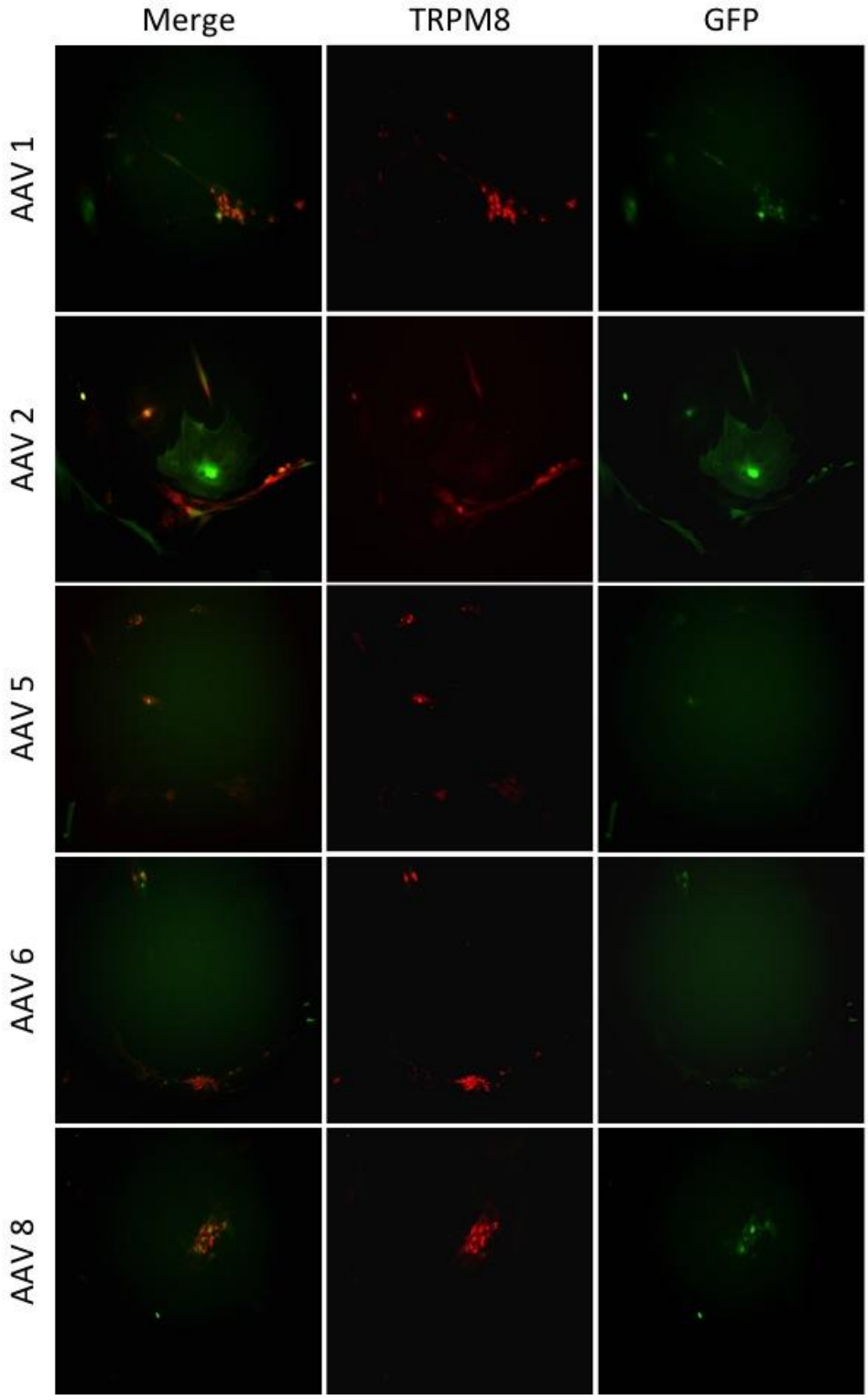

Figure 3. Comparison of TRPM8 and GFP co-localization following AAV transduction. AAV 2 had robust GFP transduction that was not co-localized with TRPM8; AAV 5 and 8 displayed the most co-localization. Magnification = x20

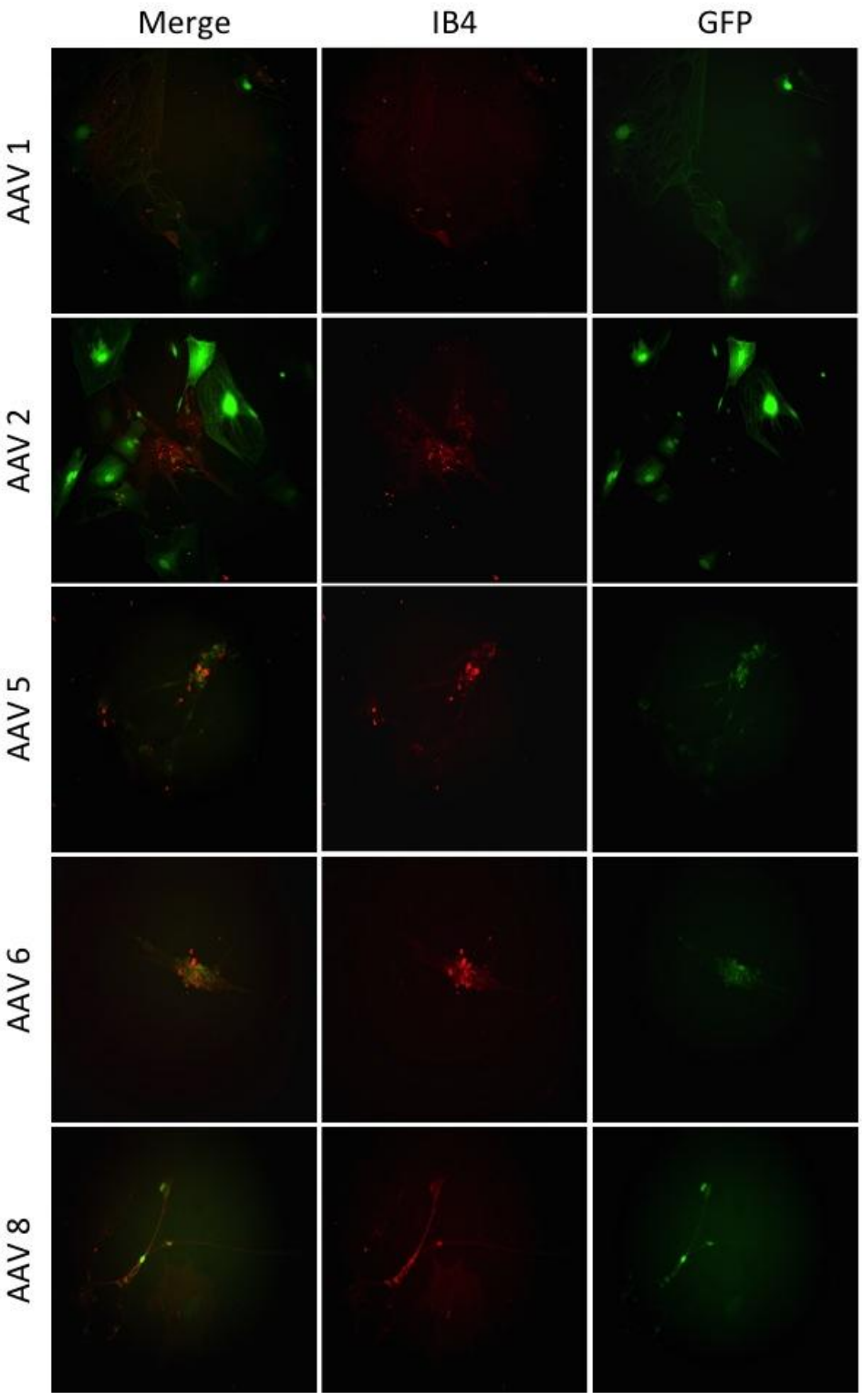

Figure 4. Comparison of IB4 and GFP co-localization following AAV transduction. Consistent with the prior results, AAV 2 had robust GFP transduction that were not co-localized with IB4; AAV 6 and 8 showed the most co-localization. Magnification = x20

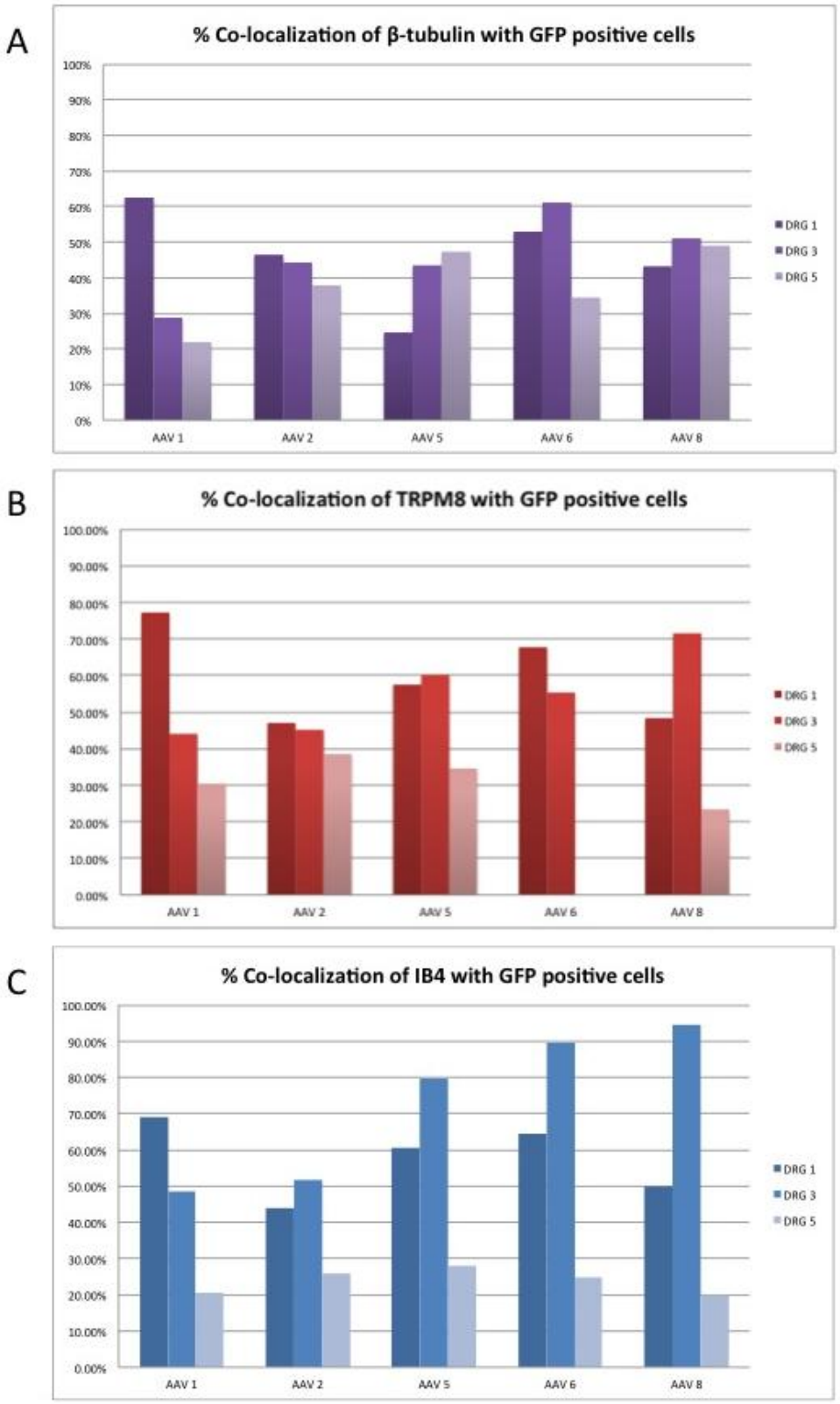

Figure 5. Comparison of average percent co-localization of β-tubulin, TRPM8, and IB4 expression with GFP positive cells. DRG 1 was transduced with an MOI of 1E6 and DRG 3 and 5 with an MOI of 1E5. Each percentage was from an average of 5 separate pictures from the same well. (A) Percent of β-tubulin co-localized with GFP was mostly under 60% (B) Percent of TRPM8 co-localized with GFP varied greatly between the different replicates. There were no cells detected for the well transduced with AAV 6 in DRG 5 (C) Percent of co-localization with IB4 showed great variation among DRG 3 and DRG 5, with >70% for DRG 3 with AAV 5, 6 and 8.