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Dexter Chase Allen April 15, 2015

Comparing Genetic Transduction between VSV-G and Rabies-G Lentiviral Pseudotypes

by

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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

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Abstract

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In an effort to optimize gene therapy to the spinal cord, this study compares the neuronal tropism of the VSV-G (vesicular stomatitis virus) lentiviral pseudotype with that of the rabies-G lentiviral pseudotype. To this end, these pseudotyped vectors, which will express Green Fluorescent Protein (GFP), will be injected into the lumbar spinal cord of the *Sprague Dawley* rat. Three weeks post-injection, rats will be sacrificed and their lumbar spinal cords will be sectioned. Immunohistochemistry will be performed to determine where the spread of GFP expression occurs in the spinal cord. Co-staining will be performed with Choline Acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP) to determine what cell types are transduced by the two lentiviral pseudotypes. We found that direct injection of VSV-G lentiviral pseudotype exhibited significantly greater longitudinal spread in the lumbar spinal cord than direct injection of Rab-G lentiviral pseudotype. Additionally, we found transduction from the Rab-G lentiviral pseudotype to be localized within cells and transduction from the VSV-G lentiviral pseudotype to be localized within axons. These results suggest a lentiviral pseudotype that would optimally deliver therapeutic genetic material in the treatment of motor neuron related diseases, such as Amyotrophic Lateral Sclerosis (ALS), Spinal Muscular Atrophy (SMA), and spasticity.

Acknowledgements

I would like to thank Drs. Boulis, Cafferty, and Frenzel for their time and energy in presiding as my thesis committee. I would also like to thank Dr. Deirdre O'Connor, for her tireless patience, mentorship, and teaching over the past two years. I have taken it upon myself to continue to cultivate the inspiration she has given me and to work and develop diligently as a medical scientist.

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Introduction

Current treatment for diseases affecting motor neurons begs for optimization and restrategizing. One such improvement has taken the form of viral-mediated gene therapy, whereby viral vectors transfer genes encoding therapeutic factors into aberrant motor neurons. The optimal transgene therapy can replace lost or dysfunctional genes in the cell, confer upon the cell protection, modulate neurotransmission, and foster neuroregeneration. In these ways, gene therapy has the potential to target and treat diseases such as Amyotrophic Lateral Sclerosis (ALS), Spinal Muscular Atrophy (SMA), and spasticity. ALS is a devastating progressive degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. It is a late onset disease course that typically lasts from 2-5 years (Williams and Windebank, 1991). Currently, ALS treatment is largely palliative with the administration of riluzole, currently the only Food and Drug Administration (FDA) approved treatment. It modestly prolongs median survival by around two to three months in ALS patients (Miller RG, *et al*., 2012). Intraspinal cord delivery of insulin-like growth factor (IGF-I) mediated by adeno-associated virus (AAV) 2 showed neuroprotective effects in a rat model of familial ALS (Franz CK *et al.*, 2009). Studies such as these have increased interest in not only how gene therapy may delay the progress of ALS but also into how vectors can be optimized for delivery of transgenes. Novel treatment of SMA, another motor neuron related disease, also involves exploring viral-mediated delivery. SMA is an autosomal recessive degenerative disease of spinal cord motor neurons that results in muscle atrophy. It often causes death in infants before the age of 2 years old and slowly progressing muscle weakness in individuals with milder

forms of the disease (d'Ydewalle C & Sumner CJ, 2015). Fortunately, intrathecal delivery of Survival Motor Neuron 1 (SMN1) mediated by AAV9 produced significant improvements in SMA mouse models (Passini, 2014). A more recent study found that a single, direct injection into the cerebrospinal fluid (CSF) of AAV9 vector carrying the human SMN gene significantly increases animal survival (Meyer, *et al*., 2015). Spasticity, another motor neuron disease that gene therapy can benefit, is a condition characterized by hypertonia, clonus, and spasms, caused by aberrantly firing upper motor neurons. Current treatment options for spasticity include oral administration of Baclofen, Tizanidine, and Botulinum toxin and intrathecal administration of Baclofen. (Rekand, 2010). However, novel delivery methods are being sought after to augment the efficacies of these drugs. Additionally, intrathecal baclofen therapy is associated with infection, cerebrospinal fluid leak, and device complications (Motta & Antonello 2014). Ablation is also performed, but with the consequence of permanent damage to implicated neurons, resulting in loss of function. An alternative therapy method was demonstrated when adenoviral (Ad)-mediated clostridial light chain delivery into the lumbar spinal cord of *Sprague Dawley* rats led to synaptic inhibition and loss of motor function in the injected side (Teng Q, *et al.,* 2005), thus paving the way for further investigation into gene therapy for spasticity.

The emergence of gene therapy in treating ALS, SMA, and spasticity has underscored not only the search for neurotrophic and neuromodulatory genes that can be delivered to treat implicated motor neurons, but also the exploration for vectors that can optimally deliver such genes. To this end, a variety of different gene therapy systems have been explored. These include viral vectors, naked DNA and cationic liposomes (Li

& Huang, 2000), cells manipulated with therapeutic genes (Li & Huang, 2000), and hybrid synthetic-viral vectors (Kaneda, 1999). Viral vectors are of particular interest because of their gene transfer efficiency and tropism specificity.

Adenoviral vectors (Ad), adeno-associated vectors (AAV), herpes simplex virus-based vectors (HSV), and lentiviral vectors (LV) have been investigated for their transduction efficiencies in neural tissue. HSV has some considerable advantages as a CNS-oriented viral vector. It is highly infectious, has a large genome (152Kb) (enabling it to transfer large transgenes), and can remain latent in sensory neuronal nuclei for the host's lifetime while remaining asymptomatic (leading to its application in pain models) (Burton, *et al*., 2002; Latchman, 1994). However, HSV's lytic replication causes encephalitis, a major source of concern for clinical trials (Latchman, 1994). Current work is investigating surmounting HSV safety concerns so that HSV's efficient transduction profile can be leveraged. For example, Miyagawa *et al*. rendered a HSV vector that can efficiently transduce nonneuronal cells without causing cytotoxicity (Miyagawa, *et al*., 2015).

Adeno-associated viral (AAV) vectors have a relatively ideal suite of characteristics. They are nonpathogenic, transduce both dividing and non-dividing cells, can provide stable and long-term gene expression, and can be produced at clinically relevant titers (Snyder, *et al*., 2010). Since it results in prolonged gene expression without damaging surrounding tissue, AAV is a choice vector for the therapy of a variety of neurodegenerative diseases, such as Parkinson's disease and ALS (Oh, *et al*., 2015; Dirren, *et al*., 2015). Furthermore, variation in AAV serotypes can confer different tropisms. The differing capsid protein composition of AAV serotypes gives the viral

vector specific direction for certain cell types. For example, it has been found that the AAV4 serotype has the propensity to transduce astrocytes and ependymal cells and the AAV5 serotype specifically transduces astrocytes and neurons (Liu, *et al*., 2005; Davidson, *et al*., 2000). There are also differences in transducing efficiency and bioavailability among the different AAV serotypes. AAV1 and AAV6 serotypes, for example, transduce neurons more efficiently and with greater bioavailability than AAV2 (Towne, *et al*., 2008). Furthermore, different delivery methods can augment the transduction efficiency and bioavailability of one serotype over another. Delivering AAV serotypes intravascularly and/or intraventricularly in neonate or adult mice demonstrated AAV8 and AAV9 transduction to be more efficient than that of AAV1 and AAV2 (Duque, *et al*., 2009; Foust KD, *et al*., 2009; Broekman, *et al*., 2006). A disadvantage of AAV, however, is that it has a small cloning capacity (Nanou & Azzouz, 2009).

Lentivirus is, also, a vector system whose advantages considerably outweigh its disadvantages. The domain of lentiviruses includes the primate subtypes human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), and the nonprimate subtypes equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), and feline immunodeficiency virus (FIV). Lentivirus can transduce non-dividing cells with high efficiency (Nanou & Azzouz, 2009). It has long-term gene expression as it stably inserts into the host gemone, which is also a concern as it poses a risk of insertional mutagenesis (Rothe, *et al*., 2015). Like adeno-associated viruses, the tropism of lentiviruses can be altered through pseudotyping, in which various glycoproteins are altered onto the lentiviral coat. HIV vectors pseudotyped with vesicular stomatitis virus (VSV-G), for example, allows for vector production in higher quantities

and for a broader tissue tropism than HIV alone (Naldini, *et al*., 1996). Additionally, pseudotyping with rabies-G resulted in retrograde transport to neurons from distal administration sites via EIAV-mediated delivery (Mazarkis, *et al*., 2001). Not much work, however, has investigated their relative transduction efficacies, nor the gray matter and white matter distribution, between lentiviral VSV-G and lentiviral rabies-G pseudotypes. Armed with more of an understanding of the distribution and transduction capacities of these two pseudotypes, we may be able to propose a way of optimizing the lentiviral system in its application in treating motor neuron-related conditions. This study proposes to compare transduction patterns and efficiencies of lentiviral VSV-G and lentiviral rabies-G pseudotypes resulting from direct parenchymal injection into the *Sprague Dawley* rat lumbar spinal cord. This study's aim is also to test for the safety in performing parenchymal injections of VSV-G and Rab-G lentiviral pseudotypes.

Methods

Vector Construction

Human immunodeficiency virus (HIV)-derived lentirival vectors were produced by Dr. Jakob Reiser, Food and Drug Administration (FDA). 293T cells were grown and then transfected with a cocktail of pNL-EGFP/CMV/WPREΔU3 lentiviral plasmid, pCD/NL-BH*ΔΔΔ packaging plasmid, and VSV-G-encoding pLTR-G plasmid to yield VSV-G lentiviral pseudotype. To yield rabies-G lentiviral pseudotype, 293T cells were transfected with a cocktail consisting of pNL-EGFP/CMV/WPREΔU3 lentiviral vector plasmid, pCD/NL-BH*ΔΔΔ packaging plasmid, and rabies-encoding plasmid. Lentiviral

concentration was performed by one of the following methods: ultracentrifugation, anion exchange chromatography using Mustang Q cartridges, or lentiviral vector precipitation using PEG6000. Lentiviral vectors were titrated by flow cytometry. Replication-competent lentivirus (RCL) monitoring was performed by analyzing p24 levels with a p24 ELISA kit (Kutner, et. al, 2009).

Animals

Male rats (*Sprague Dawley*) (63-67 days old) were acquired from Charles River Laboratories. All animals were handled according to Emory University (Atlanta, GA) Institutional Animal Care and Use Committee (IACUC) approved protocols.

Gene Delivery

Sprague Dawley rats were anesthetized with isoflurane at 5% for one minute, followed by successive 1% down-titrations every one minute until unconscious. Rats were placed in the prone position and fixed to a stereotactic frame attached to an isoflurane ventilation system where they received 2% isoflurane for the duration of the surgery. A 3 cm incision was made above the L2 vertebrae and 1% lidocaine was injected into the paraspinous muscles for analgesia. An incision was made on either side of the spinous process and the paraspinous muscles were retracted. A single laminectomy at the L2 level were performed and two 0.4 µL (8.85E9 TU units/mL) injections of either VSV-G or rabies-G lentiviral pseudotype were made 1.3 mm into the parenchyma 3 mm apart from each other and 1 mm lateral to the midline of the spinal cord. Paraspinous muscles

and skin were sutured using 4-0 Vicryl. Pre-and post-surgery, 0.3 mL injections of buprenorphine 0.05mg/kg (McKesson) were adminstered to reduce pain.

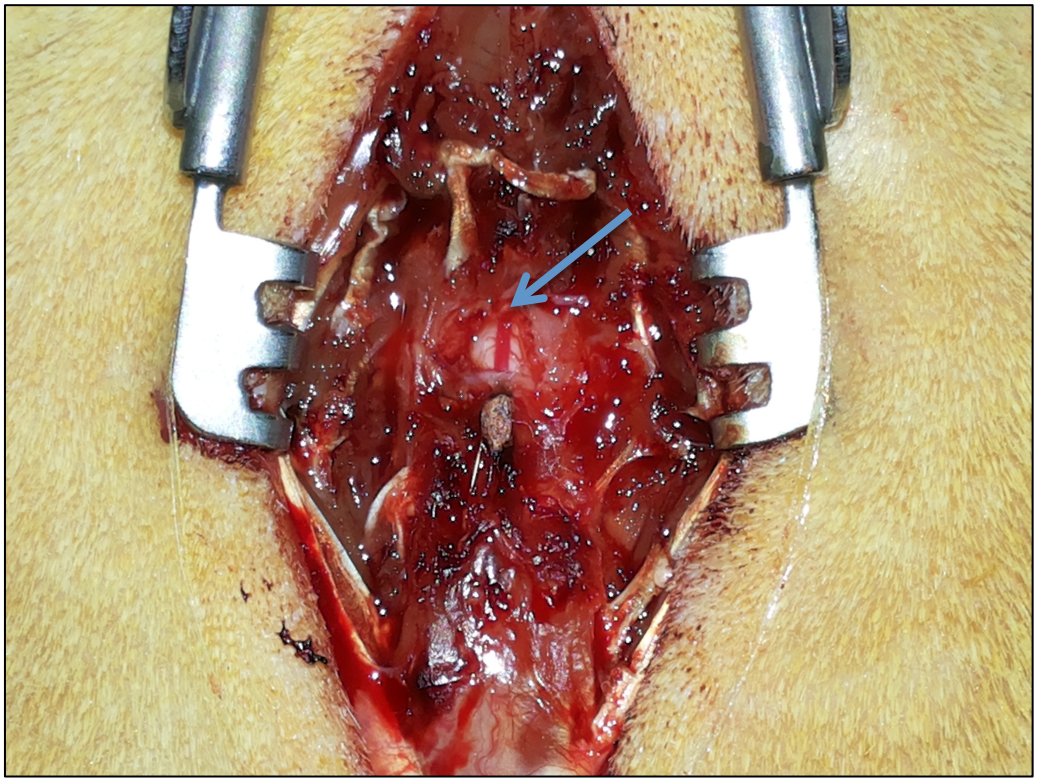


Figure 1 ***Successful Sprague Dawley L2 Laminectomy.*** Arrow points to the exposed central vessel, which was used as a reference point in performing injections. The central vessel runs dorsally along the midline of the spinal cord.

Perfusion

Rats were rendered unconscious with 5% isoflurane. An overdose of pentobarbital sodium and phenytoin sodium (Euthasol, Med Vet International) was delivered intraperitoneally. Rats were transcardially perfused with saline (0.09%) and then with 4% paraformaldehyde. Lumbar spinal cord was post-fixed in 4% paraformaldehyde for 24 hours and then fixed in 30% sucrose for 5-7 days. Fixed lumbar spinal cords were embedded in OCT compound (Tissue-Tek).

Immunohistochemistry

OCT-embedded lumbar spinal cords were cryosectioned into 40 μm thick coronal sections in sets, mounted onto glass slides, and rehydrated in PBS. Sections were stained for Green fluorescent protein (GFP), which entailed the following process. Sections were incubated in blocking buffer (5% normal goat serum, diluted in 0.1% Triton-PBS) for 30 minutes at room temperature. The primary anti-body applied was rabbit anti-GFP (1:500; Millipore; Merck KGaA, Darmstadt, Germany) in 2% normal goat serum (0.1% Triton-PBS) for two nights at 4^{o}C. Sections were washed 3x10 minute in 0.1% Triton-PBS. The secondary antibody, goat anti-rabbit (1:250; Jackson Immunoresearch; West Grove, PA), was applied for two hours at room temperature. Sections were washed 3x10 minute in PBST, and mounted with Vectashield Mounting Medium with Dapi (Vector Laboratories; Burlingame, CA). Sections from the same animals and with the same lumbar spinal cord representation as the GFP-stained sections were stained for choline acetyltransferase (ChAT), which entailed the following process. Sections were incubated in blocking buffer (5% normal donkey serum, diluted in PBS) for one hour. The primary antibody applied was affinity-purified goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:100; Millipore; Billerica, MA) for one night at 37°C. Sections were washed 3x10 minute with PBS. The secondary antibody, donkey anti-goat IgG conjugated with Alexa Fluor (1:100; Invitrogen; Carlsbad, CA), was applied for two hours. Sections were washed for 3x10 minute with PBS, and mount with VECTASHIELD Mounting Medium with Dapi (Vector Laboratories; Burlingame, CA).

Motor Neuron Counting

Using a Nikon Microphot-FXA microscope and a Spot RT color CCD camera, the ventral horns were photographed at 10x magnification. Photomicrographs were taken with the fluorescein isothiocyanate (FITC) excitation and emission filter.. In this manner, three lumbar sections of each slide were randomly chosen and photographed. Utilizing NIS-Element BR software, the areas of every ChAT positive motor neuron in the ventral horns were measured. Every motor neuron equal to or greater than 300μm^2 was counted. Motor neuron density comparisons were made between the ventral horns of the side of injection (ipsilateral) and the ventral horns of the opposite side (contralateral) by calculating the amount of motor neurons on the ipsilateral side as a percentage of the amount of motor neurons on the contralateral side. For each animal, these percentages were reported as the average of all corresponding sections.

Green Fluorescent Protein (GFP) Expression Analysis

Using a Nikon Microphot-FXA microscope and Spot RT color CCD camera, the incidence of GFP expression in rat lumbar spinal cord sections was determined and photomicrographs of the sections were taken. Sections were analyzed at 4x and 10x magnifications with the tetramethylrhodamine isothiocyanate (TRITC) excitation and emission filter. Presence of GFP expression in the lumbar sections was recorded on a representational diagram. Missing lumbar sections were also noted. For each animal, the number of lumbar sections that exhibited GFP expression was multiplied by 240μm to yield caudal GFP spread from the lumbar spine's anterior beginning. The sum of the amount of missing lumbar sections for each animal plus the amount of lumbar sections

that exhibited GFP expression was multiplied by 240μm to yield the possible extent of GFP expression for those animals missing lumbar sections. Averages were taken of each animals' caudal GFP spread according to lentiviral pseudotype cohort to yield ranges of GFP spread.

Results

Statistical Analysis

Quantitative data were reported as mean ± SD % for motor neuron density analysis and as mean μm for GFP expression analysis. The T-Test was performed as a statistical test for these two analyses using Microsoft Excel for Mac 2011, Version 14.4.7.; Redmond, Washington). Probability values of $p<0.05$ were considered to be statistically significant.

Surgeries Performed

Surgeries were preformed on 8 animals in total. One animal from the VSV-G lentiviral pseudotype cohort was excluded from the study because the injection tract in its lumbar spinal cord seemed to have been completely over the midline (Figure 2). This left the study with three animals remaining in the VSV-G lentiviral pseudotype cohort and four animals in the Rab-G lentiviral pseudotype cohort. As for the rest of these animals, injections did not seem to be made deep enough neither/nor were they medial enough to reach the ventral horns (Figures 6 & 7A). For other animals, injections were ambiguous (Figures 7B & 7C).

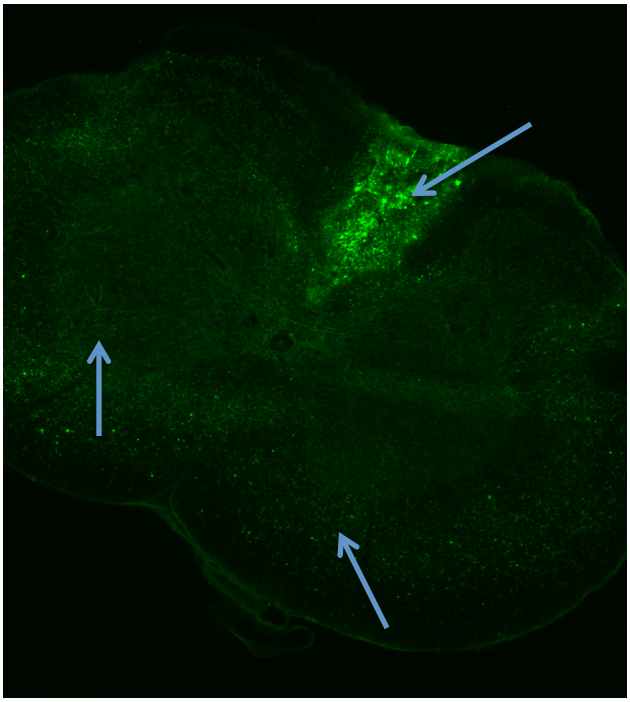
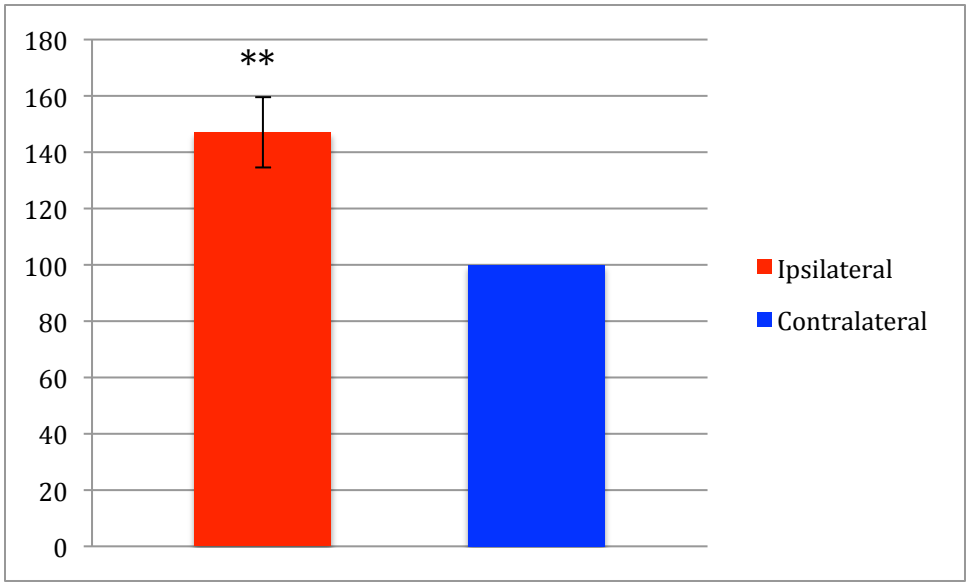


Figure 2 ***Photomicrograph showing a lumbar spinal cord cross section of the excluded animal from the VSV-G lentiviral cohort.*** GFP expression can be seen seemingly directly above the dorsal midline (top arrow). Bottom arrows point to the ventral horns. **FITC 10x**

Motor Neuron Density

A comparison of motor neuron distribution in the ventral horn of the lumbar spinal cord between the side of vector injection (ipsilateral) and the opposite side (contralateral) for the VSV-G lentiviral pseudotype showed the average motor neuron density of the ipsilateral side to be 147.13 ± 67.49% of that of the contralateral side. This was significantly higher than the contralateral side ($p<0.001$) (Figure 2A). For the rabies-G lentiviral pseudotype, the same comparison showed the average motor neuron density of the ipsilateral side to be 118.20 ± 74.69% of that of the contralateral side, which was not significantly different from the contralateral side ($p= 0.146897$) (Figure 2B). This indicates that the injection did not cause damage or loss of motor neurons.

A



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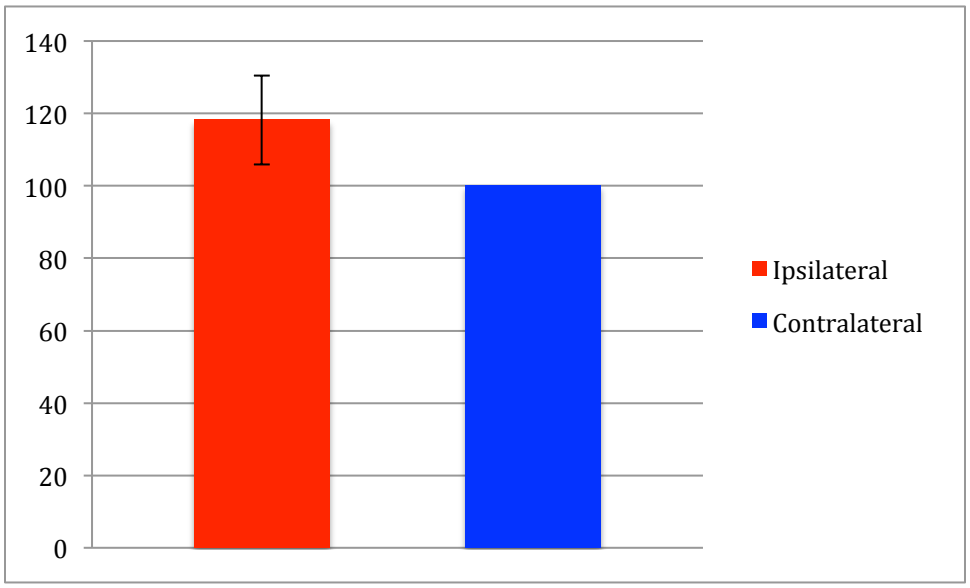
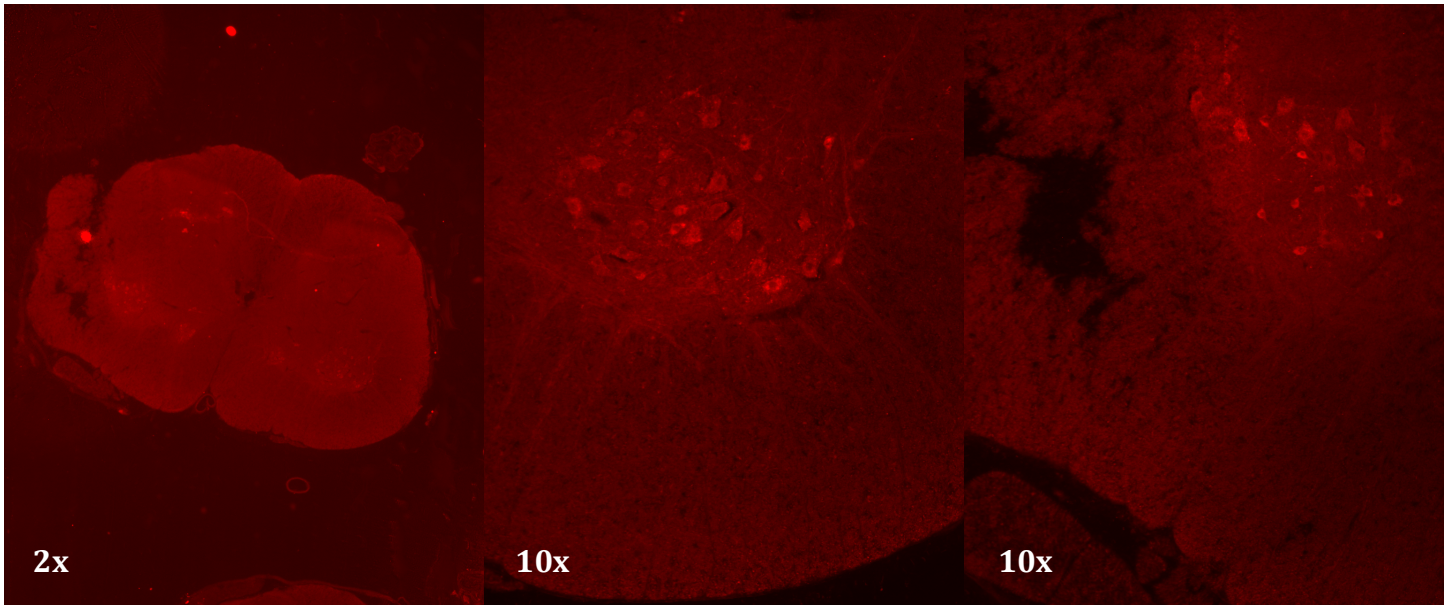


Figure 3 ***Ipsilateral motor neuron density following A. VSV-G and B. Rab-G lentiviral pseudotype injections.*** A. The density of motor neurons in the ventral horn of the side injected was significantly higher than that of the side that received no injection ($p<0.001$). B. The density of motor neurons in the ventral horn of the side injected was not significantly higher than that of the side that received no injection. ($p=0.0146897$)

A



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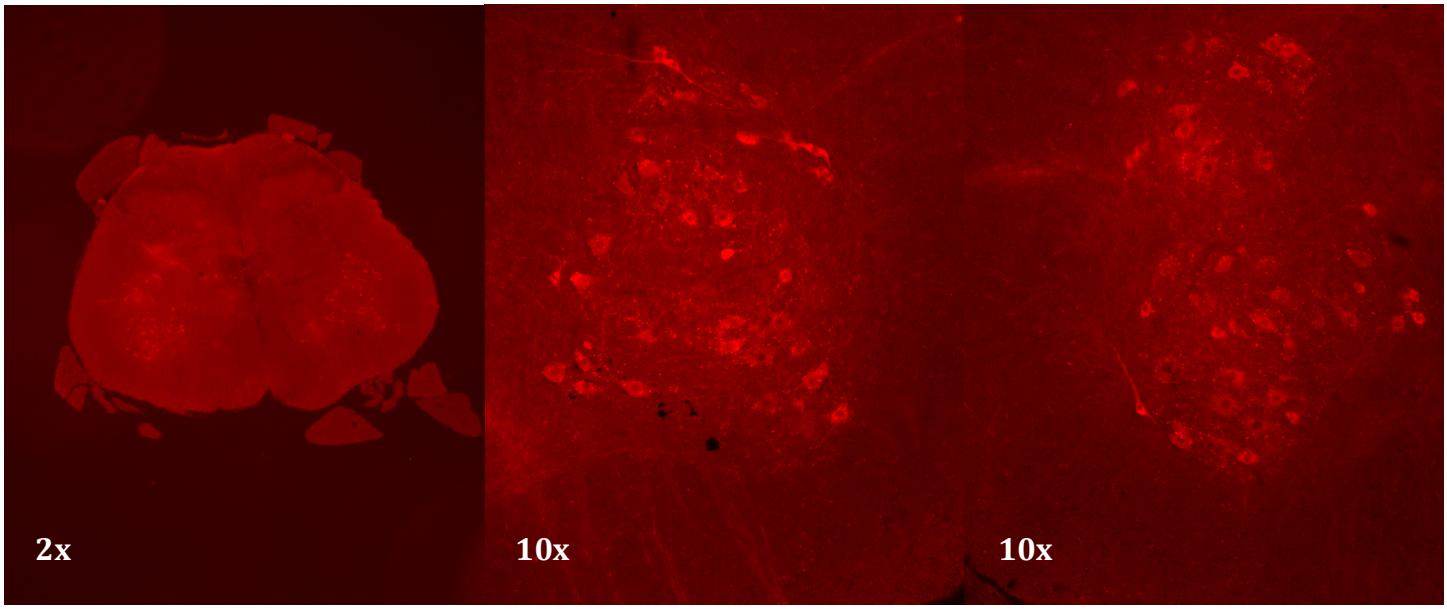


Figure 4 ***Conservation of motor neuron density following*** **(A)** ***rab-G and*** **(B)** ***VSV-G lentiviral pseudotype injection.*** When comparing the left ventral horn (middle) with the right ventral horn (right) in both cohorts, motor neuron density is approximately equal. A whole cross-section of the lumbar spinal cord for both cohorts is shown (left). **TRITC 2x, 10x, 10x**

Green Fluorescent Protein (GFP) Expression Analysis

Longitudinal spread of GFP expression was calculated for both VSV-G and Rab-G lentiviral pseudotypes based on the first sections of each set cut for each animal. The Rab-G lentiviral pseudotype cohort exhibited a mean longitudinal GFP spread of 8220 µm and the VSV-G lentiviral pseudotype cohort exhibited a mean longitudinal GFP spread of 12960 µm. The p value between the two cohorts was calculated to be 0.04691 (Table 1). Therefore, the difference in longitudinal GFP spread between the VSV-G and Rab-G lentiviral pseudotype cohorts was significant.

Table 1. Longitudinal GFP Spread in the Lumbar Spinal Cord

	Rab-G (µm)	VSV-G (µm)
Animal #1	8640	12960
Animal #2	4080	12960
Animal #3	9360	12960
Animal #4	10800	
Mean	8220	12960

p value	0.04691

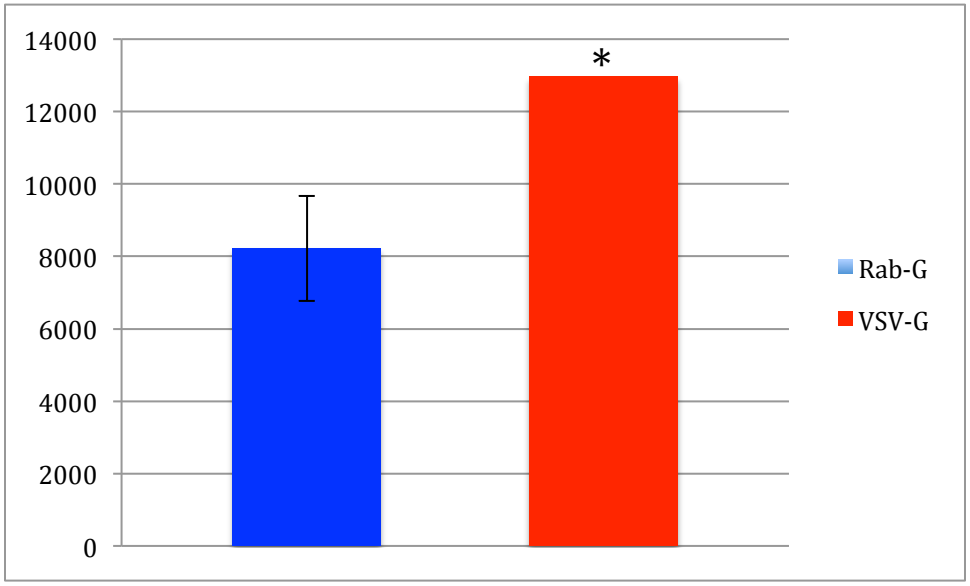
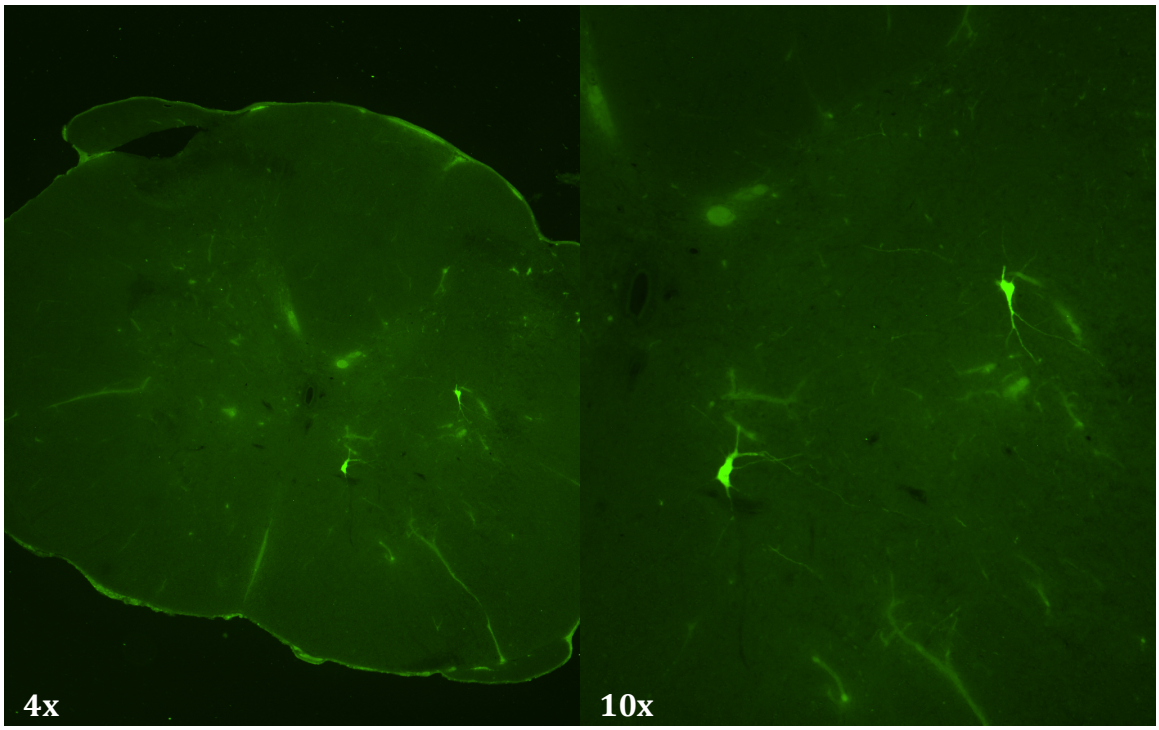


Figure 5 ***Longitudinal GFP expression following VSV-G and Rab-G lentiviral pseudotype injections.*** Longitudinal GFP expression in the lumbar spinal cord was significantly higher in rats injected with VSV-G lentiviral pseudotype than in rats injected with Rab-G lentiviral pseudotype.

A



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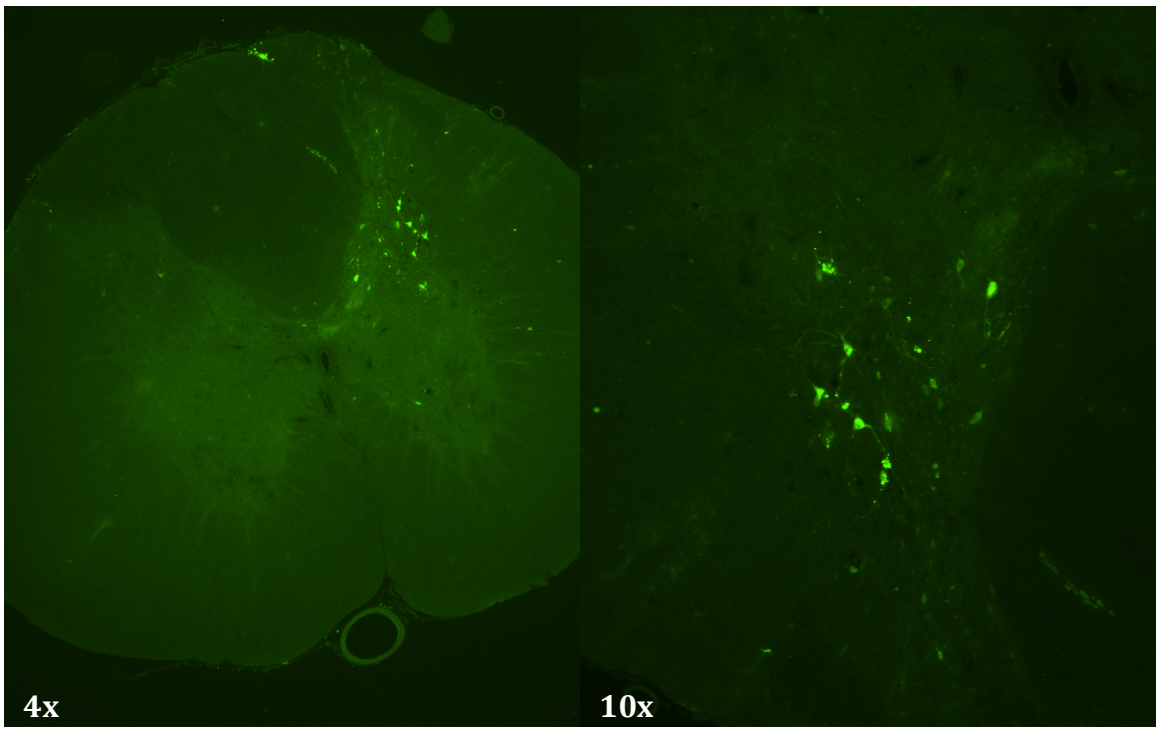

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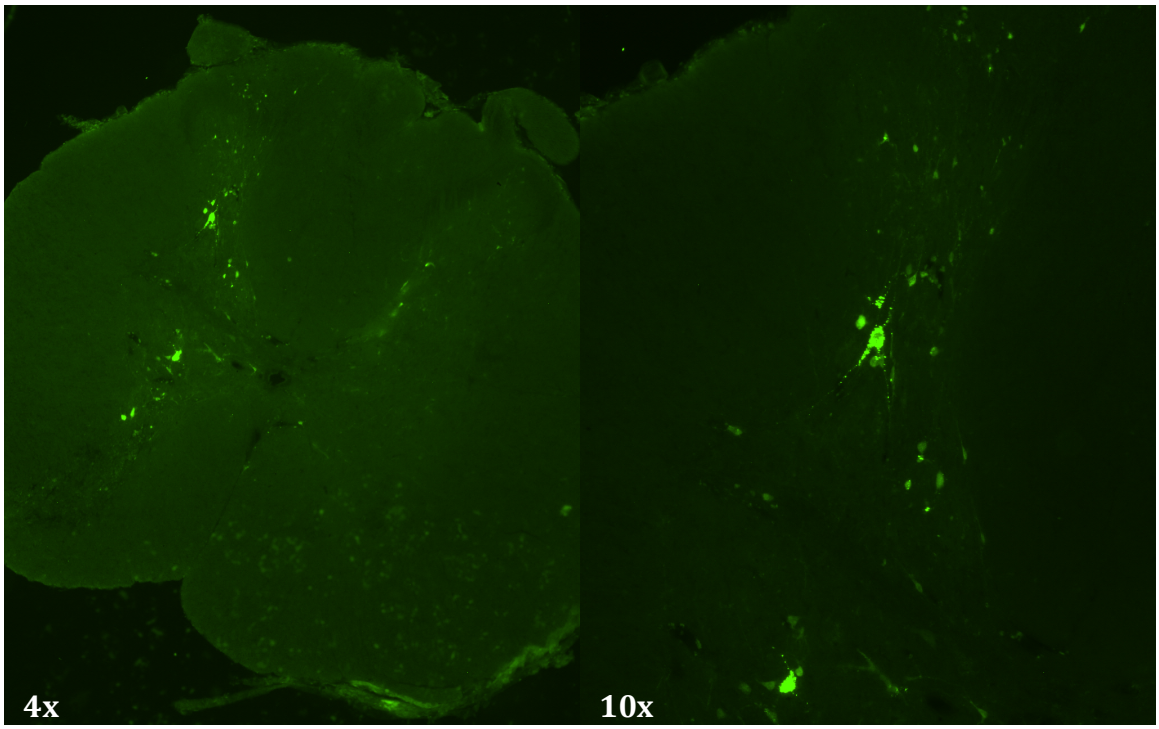

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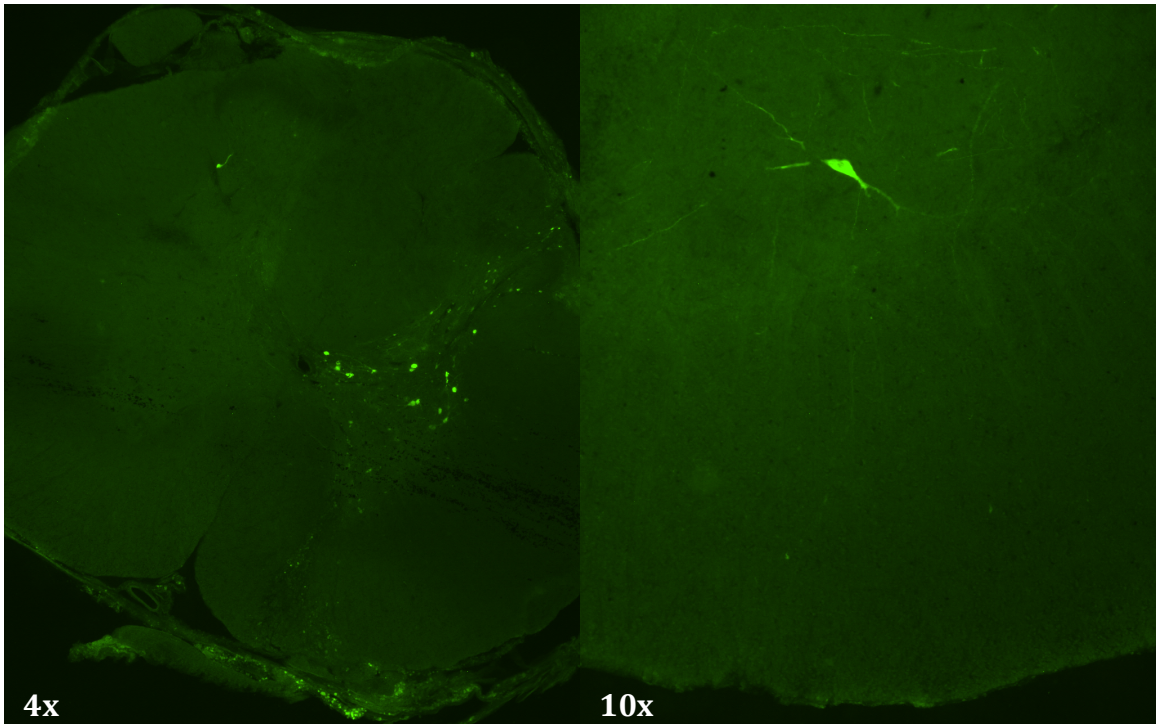
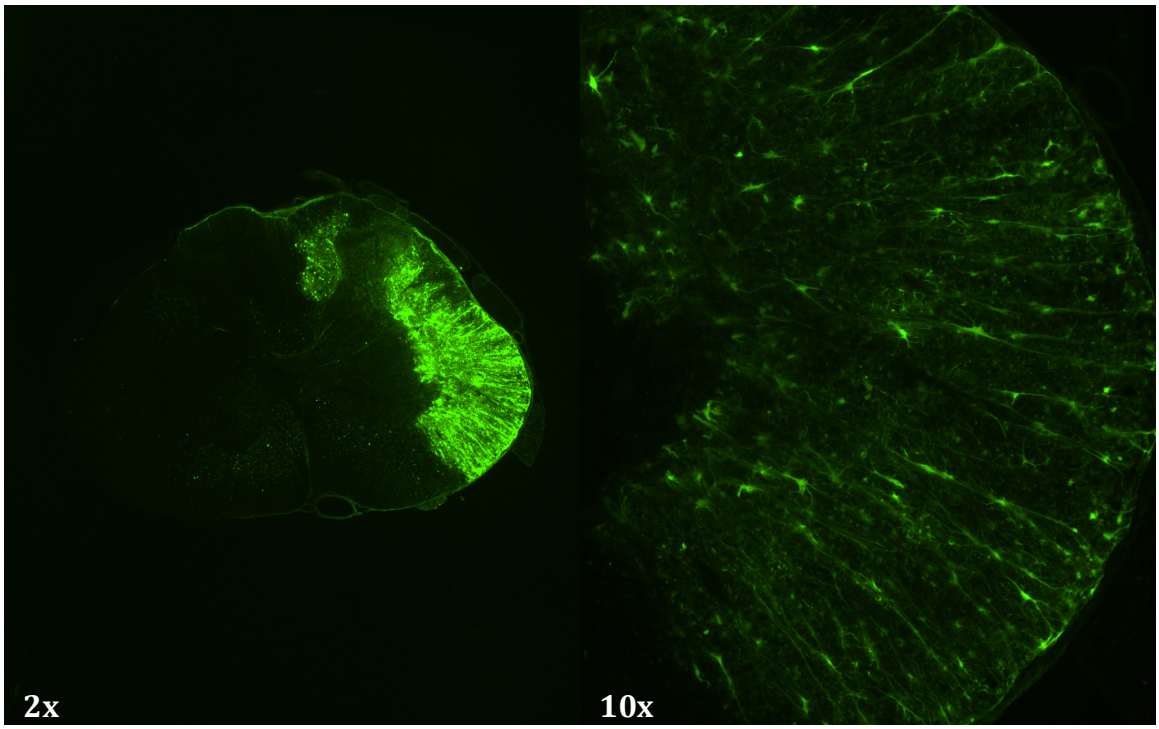


Figure 6 ***Characteristic GFP expression in all four animals A, B, C, D of the Rab-G lentiviral pseudotype cohort.*** Microscopy demonstrates that GFP expression is largely localized in neurons and that injections were made too dorsally, away from the targeted ventral horns. **FITC 4x, 10x**

A



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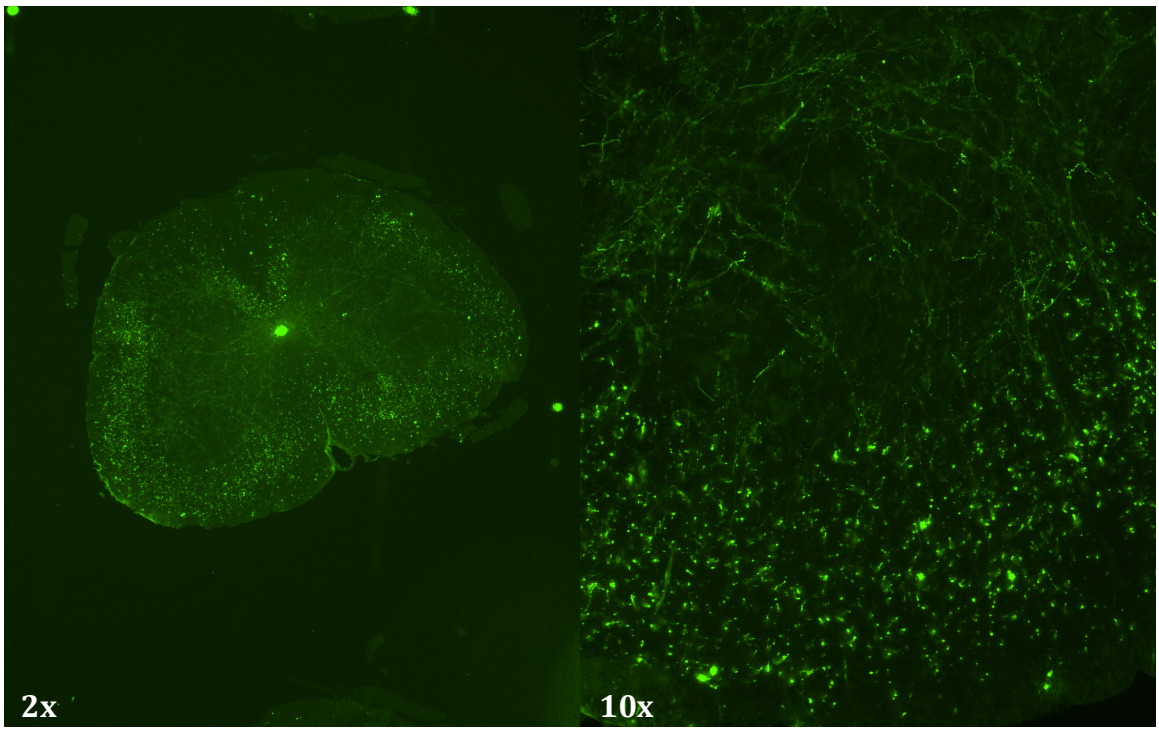

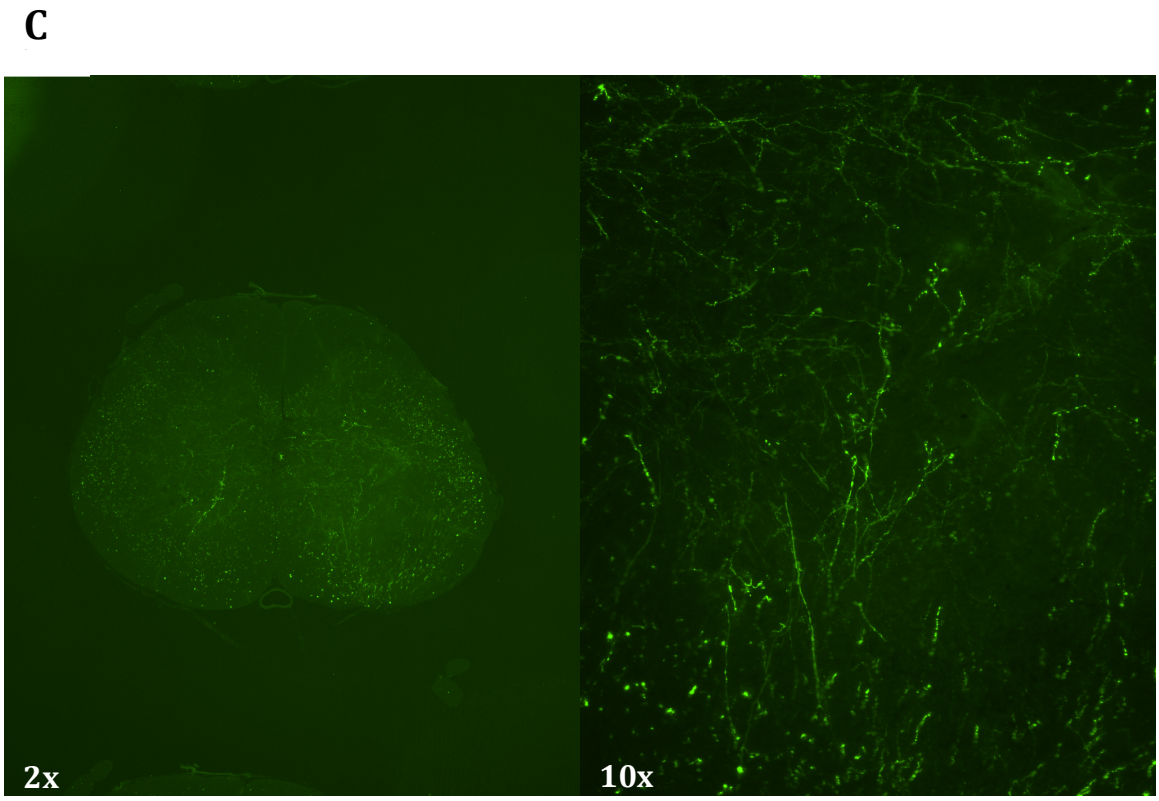

Figure 7 ***Characteristic GFP expression in all three animals A, B, C of the VSV-G lentiviral pseudotype cohort.*** Microscopy demonstrates that GFP is expressed in axons. A. Intense GFP expression is localized in white matter lateral to the left ventral horn. B. & C. GFP expressed diffusely in extensive axonal networks. **FITC 2x & 10x**

Colocalization

An attempt to demonstrate colocalization of ChAT and GFP expressions failed.

Discussion

The aim of this project was to compare the tropism and transduction efficiencies of the VSV-G and rabies-G lentiviral pseudotypes and to test the safety of their parenchymal injections in the *Sprague Dawley* rat. Safety was tested by performing motor neuron density analyses, in which motor neuron density was compared between

both ventral horns in the lumbar spinal cord, following direct injection of either VSV-G or rabies-G lentiviral pseudotype. Even though there was a significant difference in average motor neuron density between the ipsilateral and contralateral sides ($p<0.001$) resulting from direct injection of VSV-G lentiviral pseudotype, the difference resulted from a higher average motor neuron density on the ipsilateral side ($147.13 \pm 67.49\%$ of that of the contralateral side) (Figure 2A). In other words, there was no loss in motor neurons resulting from direct injection of VSV-G lentiviral pseudotype. As for the motor neuron densities between the ipsilateral and contralateral ventral horns resulting from direct injection of Rab-G lentiviral pseudotype, analysis showed that there was no significant difference between the two ($p=0.146897$) (Figure 2B). Motor neuron density analysis demonstrated motor neurons to be more or less conserved as the result of direct injection of either VSV-G or Rab-G lentiviral pseudotypes. This suggests direct injection of both pseudotypes to be safe. The validity of this suggested safety, however, was limited by our failure to make injections deep enough to reach the ventral horns. Therefore, the suggested safety that our motor neuron density analysis finds may or may not be associated with direct injection of VSV-G and Rab-G lentiviral pseudotypes into the ventral horns. Future experiments would perform motor neuron density analysis of ventral horn motor neurons resulting from accurate and precise injections of pseudotypes into the ventral horn in order to gather a clearer and stronger correlation between direct injection of pseudotypes and its safety. Future investigations would also analyze the bilateral conservation of other cell types. It has been demonstrated that intranasal administration of wild-type VSV-G caused 50% to 90% of infected rodents to die (Muik, *et al*., 2012; Reiss, *et al*., 1998) from encephalitis caused by neuronal necrosis (Preble, *et*

al., 1980). In fact, lesions from VSV-G related necrosis were found in the lumbosacral region of the spinal cord (Bi, *et al*., 1995; Christian, *et al*., 1996). It has also been shown that, following intranasal administration, VSV-G spreads caudally from the olfactory bulb to infect ependymal cells (Ireland & Reiss, 2006), astrocytes, and microglia (Chauhan, *et al*., 2010). In light of WT VSV-G's neurovirulence and characteristic spread following via intranasal administration, performing cell density analyses on other cell types, such as ependymal cells, astrocytes, and microglia, would further suggest the safety of direct VSV-G lentiviral vector injection. In light of intranasally administered WT VSV-G's propensity for causing encephalitis, testing for immunogenic responses to injection of VSV-G lentiviral vector, like assaying CD4+ and CD8+ T cell levels, would also further suggest the safety of direct VSV-G lentiviral vector injection.

Rab-G and VSV-G lentiviral pseudotype transduction efficiencies were compared by assaying longitudinal GFP spread resulting from direct injection of either pseudotype. Immunohistochemistry and microscopy revealed the average longitudinal GFP spread resulting from direct injection of VSV-G lentiviral pseudotype to be significantly higher than that of the Rab-G lentiviral pseudotype ($p=0.04691$) (Table 1, Figure 5). A substantial component of this study's discovery lies in the finding that VSV-G lentiviral pseudotype effects a more longitudinally extensive genetic transduction than the Rab-G lentiviral pseudotype. Immunohistochemistry and microscopy also found differences in the anatomical localization of the two pseudotypes' transductions. GFP expression resulting from direct injection of Rab-G lentiviral pseudotype seemed to be largely localized within cell bodies (Figure 6). It is known that rabies-G lentiviral vectors have a tropism for motor neurons (Frederici, *et al*., 2009; Tanase, *et al*. 2004). Our microscopy

demonstrates that injection of Rab-G lentiviral pseudotype in regions dorsal to the ventral horns results in GFP expression in cells other than motor neurons (as motor neurons are located in the ventral horns). Future experiments would immunohistochemically discriminate the cell types and colocalization between GFP expression, resulting from accurate and precise injection of Rab-G lentiviral vector into the ventral horn, and cell type morphology would be searched for. In this way, we would be able to determine relative levels of GFP expression, if any, in different cell types like glia, interneurons, and motor neurons. Direct injection of VSV-G lentiviral pseudotype caused GFP expression to be localized in axons (Figure 7). Despite it being known that VSV-G has a neurotropism for ependymal cells (Ireland & Reiss, 2006), astrocytes, and microglia (Chauhan, *et al*., 2010), the morphology of GFP expression did not seem to be that of cell bodies but of axons. To get a better sense of Rab-G lentiviral pseudotype's transduction pattern in and around motor neurons, future experiments would have to accurately and precisely place injections directly into the ventral horn. To better understand the Rab-G lentiviral pseudotype's transduction profile inside and/or outside of motor neurons, interneurons, and glia, future experiments will also have to immunohistochemically discriminate such cells and search for colocalization between cell type and GFP expression.

Future experiments will also have to make certain that both titers, which comprise the total volume of vector injected into each animal, are injected exactly at the L2 level of the lumbar cord (or at some other constant point on the lumbar cord) with a fixed distance in between the two of them. A limitation of this study was that injections were not assuredly performed at L2 of the lumbar spinal cord. This presents an issue in

the way that the longitudinal GFP spread that was assayed among all the animals may not have had the same starting points (injection sites) on the lumbar cord. This has the potential of truncating or elongating measurement of the actual longitudinal GFP spread in the animals. Furthermore, the two titers that were injected in each animal were not done so with a fixed distance in between the two. This also presents an issue as measured longitudinal GFP expression has the potential of being truncated or elongated based on how close to or far away from each other titers are injected. Therefore, future experiments will make certain that both titers are injected exactly at the L2 level of the lumbar cord (or at some other constant point on the lumbar cord) with a fixed distance in between the two of them.

Conclusion

This study demonstrated that longitudinal GFP spread resulting from direct injection of VSV-G lentiviral vector was significantly greater than that resulting from direct injection of Rab-G lentiviral vector. It also showed the transduction of Rab-G lentiviral vector to be localized within cell bodies, whereas it showed the transduction of VSV-G lentiviral vector to be localized within axons. Motor neuron density analysis suggested the safety of Rab-G lentiviral vector and VSV-G lentiviral vector direct injections. The validity of these results, however, was compromised by a failure to inject deep enough into the ventral horns. Also, further cell type immunohistochemical discrimination needs to be performed along with a search for colocalization between GFP expression and cell type. Nonetheless, the results suggest that the VSV-G lentiviral pseudotype to have the more effective transduction capacity compared to the Rab-G

lentiviral pseudotype. The specific transduction localizations of these two vectors, however, needs consideration in the context of gene therapy for motor neuron-related diseases. Although this study suggests that VSV-G lentiviral pseudotype a more effective transduction capacity, its transduction is not directly localized within cell bodies like it is resulting from Rab-G lentiviral pseudotype. This may have bearing on which pseudotype is deemed more appropriate for application in gene therapy for motor neuron diseases regardless of which one has the more effective transduction capacity.

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