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Jeremiah Huang April 1, 2012

Adeno-associated Viral Vector Delivery of Clostridial Tetanus Toxin Light Chain for Clinical Use

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Abstract

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Clostridial bacterial toxins are gaining greater significance as therapeutic agents in the field of neuromodulation. The success of botulinum neurotoxin has encouraged further investigation into other Clostridial toxins, such as tetanus neurotoxin, as possible means of gene therapy. The inhibitory function of tetanus light chain can provide an avenue for neuromodulation for conditions characterized by aberrant neural firing. Viral vectors can be employed to induce stable LC expression as opposed to the commonly used botulinum treatment method, which requires repeated injections. Earlier studies have achieved effective synaptic inhibition using adenoviral (Ad) directed-LC expression. However, the desired effect was only temporary because of adenovirus's transient gene expression in the spinal cord. To overcome the short-lived adenoviral gene expression, adeno-associated viral (AAV) vector was evaluated for its ability to provide persistent LC expression following ipsilateral spinal cord injection of viral vector. As exemplified in previous studies, administration of Ad.LC resulted in an initial inhibition of motor function followed by recovery while AAV.LC induced sustained locomotor inhibition that lasted for thirty days post treatment. AAV.LC specifically inhibited sensorimotor activity to the ipsilateral hind limbs. Expression of LC selectively decreased expression of vesicle associated membrane protein 1 (VAMP1) compared to that of other proteins found in the soluble NSF attachment protein receptor (SNARE) complex. The control vectors, Ad.GFP and AAV.GFP, revealed no changes in motor function, which suggests inhibition was due to LC expression in motor neurons. Furthermore, no gross changes were observed in motor neuron density or structure in the spinal cord following administration of any of the four vectors. Results from the study prompt continued investigations into the therapeutic possibilities of delivering tetanus toxin LC to treat conditions involving atypical spinal neural transmission.

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1 Introduction

The field of medicine defines neuromodulators as substances that alter activity in the nervous system by either stimulating or inhibiting nerve impulses. Recently, neuromodulation has become an increasingly interesting field of science, emerging as a feasible treatment for acute and chronic pain syndromes, epilepsy, dystonia and spasticity, and movement disorders, all of which arise from aberrant neural activity. There are currently several methods of neuromodulation used in medicine today. When pharmacological drugs, such as opioids, fail due to built-up tolerance, other forms of neuromodulation must be employed. These include ablative surgery, implanted devices that stimulate or deliver drugs, and deep brain stimulation (DBS) (Bittar 2006). While effective, the aforementioned techniques have their flaws. Each approach is relatively invasive and costly. Lesioning, which causes loss of function by physically damaging tissue, is irreversible and fails to lend itself to fine-tuning. Implanted pumps and electrodes, which deliver medication directly to the target site or electrically stimulate the region of interest, respectively, may be prone to hardware malfunctions or complicated by infection, both of which will require repeated corrective surgeries. When performing DBS, a brain pacemaker is implanted and stimulates an area of the brain via electrical impulses to control neural firing. Despite the non-destructive and reversible nature of DBS, there is still an issue of specificity and infection associated with the procedure.

Research in neuromodulation has progressed, focusing on the molecular components of the nervous system in order to correct atypical neural activity. Affecting nerve impulses on a molecular scale requires modification of the genes that produce proteins involved in the neural activity pathways. Thus, gene therapy has been employed to manipulate gene expression so as to

alter various factors such as neurotransmitter release and re-uptake, ion channels, vesicle-docking protein, receptor binding, and second messengers (Fig. 1).

The overall goal of gene therapy is to deliver genes to bring about a therapeutic effect. A viral vector is used as the vehicle for gene-based therapeutics. The vector delivers a gene sequence of interest by carrying the gene and entering into a cell where it alters gene expression within the cell to bring about a therapeutic result (Walther & Stein 2000). Viral vector construction begins with identifying the viral sequences necessary for structural components of the virus particles, packaging of the viral genome into the particles, and delivery of the gene of interest, also known as the transgene, to the target cells. Then, dispensable genes are removed to decrease the virulence and immunogenicity of the virus (Pfeifer & Verma 2001). The end result is a viral vector consisting of only the viral genes necessary to deliver and express the transgene.

There are many factors that contribute to a successful viral vector. The ideal vector must be efficient and easily produced in order to provide enough infectious particles to reach the target cell. Toxicity to the target cells and eliciting an unwanted immune response should be avoided otherwise the cells may deteriorate or the vector may be removed. Persistence and regulated transgene expression are desired characteristics depending on the disease being treated. Cell specificity is sought after since an effective vector exclusively transduces certain cell types in order to treat the disease and prevent possible side-effects associated with off target effects. Being able to infect both dividing and non-dividing cells allows for greater treatment possibilities. The majority of cells in the central nervous system are non-dividing cells. Therefore, a vector capable of transducing non-dividing cells is optimal for gene therapy targeting the spinal cord. Lastly, site-specific integration is desired because otherwise there is

the possibility of integration into oncogenes, which results in mutagenesis (Pfeifer & Verma 2001).

Among the earliest viruses used as a vector is the family of RNA viruses known as Retroviridae. Retroviruses exercise the ability to reverse-transcribe and convert their genome from RNA to DNA with the help of the enzyme reverse transcriptase. They are a group of enveloped, double-stranded RNA viruses and can be categorized as simple or complex. Of all the retroviruses, the complex retrovirus known as lentivirus has been the most extensively studied and used in gene therapy. Retro- and lentiviral vectors have offered the ability to transduce non-dividing cells, such as CNS cells, and induce stable long-term expression of transgenes. Both of these characteristics make retro- and lentiviral vectors popular choices for delivery of transgenes into the CNS. The main disadvantage of these vectors is integrational mutagenesis, which has prevented these vectors from becoming highly translational.

One candidate emerged from the family Adenoviridae, and is known as adenovirus (Ad). Adenoviruses exhibit a non-enveloped icosahedral capsid approximately 60-90 nm in diameter. A single double-stranded linear DNA molecule about 36 kb long makes up the adenovirus genome. Among the fifty serotypes, the most extensively studied adenoviruses are Ad2 and Ad5, both of subgroup C (Pfeifer & Verma 2001). Currently, Ad5 is the most widely used serotype for gene delivery.

Wild type Ad elicits a strong immune response upon infection. Such high immunogenicity has been the obstacle that scientists have been working to overcome in order to construct an adenovirus-based vector. Initial attempts targeted the E1 gene, which is necessary for viral replication and activation of other Ad transcription units. Removal of E1 resulted in a greater carrying capacity; transgenes 4.7-4.9 kb in length could be inserted into the vector.

Additional deletions of the non-essential E3 region increased the cloning capacity to ~ 8 kb, thus permitting insertion and expression of most cDNAs (Pfeifer & Verma 2001). The first-generation vectors lacking the E1 gene still replicated in 293 cells, which express the remaining 11% of the Ad genome including the E1 region, and thus the vectors could be easily produced in these cells (Pfeifer & Verma 2001). In addition to easy production, the Ad vectors infected a wide range of dividing and non-dividing cells (Pfeifer & Verma 2001). Despite the initial promise of adenovirus, there are two main disadvantages. Preclinical and clinical studies have demonstrated that Ad exhibits transient expression of the transgene, approximately three weeks, thus diminishing its potential as a long-term solution for gene therapy. The second disadvantage is Ad's high immunogenicity, which contributes to the transient nature of Ad. Studies have shown that antibodies against Ad1, Ad2, and Ad5 exist in 40-60% of children because of an upper respiratory tract infection, which could lead to several immune responses resulting in elimination of the vector (Pfeifer & Verma 2001). The two drawbacks of Ad, the transient nature of gene expression and toxicity, are overcome in viral vectors based on Adeno-associated virus (AAV).

AAV come from the family Parvoviridae. The viral structure consists of a non-enveloped icosahedral capsid with an 18-26 nm diameter that surrounds a ~ 4.7 kb genome of single-stranded linear DNA. AAV genome is composed of two open reading frames, *rep* and *cap*, as well as inverted terminal repeats (ITRs) at both ends. The ITRs contain the origins of DNA replication and packaging signals and mediate chromosomal integration (Heilbronn & Weger 2010). The *rep* gene is required for viral DNA replication, regulation of gene expression, and integration into the host genome while the *cap* gene is responsible for producing capsid structural proteins. In the construction of AAV-based vectors, the *rep* and *cap* genes are replaced with the

transgene and promoters responsible for regulating transgene expression (Walther & Stein 2000). The only AAV element that remains in the vector are the ITRs because they are the only sequences required in *cis*; *cap* and *rep* are able to be delivered in *trans*. AAV has already been shown to be nonpathogenic, but splitting up the necessary genes for AAV replication and placing them in different plasmids has enabled AAV to be produced without live helper viruses, thus diminishing AAV vector immunogenicity even further.

There are currently twelve AAV serotypes that have been described, all derived from man and nonhuman primates (Heilbronn & Weger 2010). While AAV is similar to Ad in that it can transduce dividing and non-dividing cells, AAV surpasses Ad as it provides long-term, stable transgene expression. The best studied AAV serotype is AAV2, which has led to its use in the initial development of AAV vectors. An advantageous characteristic of both wild type AAV and AAV2 is the site-specific integration of viral DNA at locus q13.4 on human chromosome 19 (Walther & Stein 2000). However, AAV vectors are incapable of integrating at the specific site because *cap* and *rep* genes have been removed. Instead of integrating into the host genome, AAV vectors remain as episomes within the host cell nucleus. Since AAV does not elicit a significant immune response, AAV can exist outside the host genome because the vector will not be degraded as quickly and continue to express the therapeutic gene within the cell. The episomal state is especially effective for non-dividing cells such as muscle cells and neurons (Pfeifer & Verma 2001). AAV has emerged as an excellent candidate for human gene therapy because in addition to displaying a tropism for a variety of cell types, AAV overcomes the Ad's disadvantages by demonstrating low immunogenicity and persistent transgene expression.

Several disorders that lend themselves to neuromodulation, such as epilepsy, pain, and spasticity, require a means to inhibit neuronal firing so as to modulate neural activity. Recently,

clostridial neurotoxins (CNT) have been shown to have promise as therapeutic agents as a means of neuromodulation. Tetanus (TeNT) and seven serotypes (A-G) of botulinum neurotoxin (BoNT) constitute the species of bacterial neurotoxin in the Clostridial family. With the help of their zinc-dependent metalloprotease activity, the CNTs are able to selectively cleave one of three soluble NSF (N-ethylmaleimide sensitive fusion protein) attachment protein receptor (SNARE) proteins involved in forming a SNARE complex: synaptobrevin (VAMP), SNAP-25, and syntaxin (Montecucco & Schiavo 1994). Cleavage of a SNARE protein inhibits neurotransmitter release, thus causing either limp (BoNT) or spastic (TeNT) paralysis (Foster 2009). CNT are composed of a 100 kDa heavy chain (HC) and 50 kDa light chain (LC) linked by both non-covalent protein-protein interactions and single disulfide bond (Teng *et al* 2005). The HC is comprised of two functional domains responsible for neuronal penetration, retrograde transport within the axon, and transport of LC from endosomes into the cytoplasm (Eisel *et al* 1993). The LC contains the neurotoxin's zinc-dependent metalloprotease activity (Fig. 2). The most commonly used CNT in the clinic, botulinum neurotoxin (BoNT), has been successfully used for cosmetic procedures involved in activation of facial muscles as well as for several disorders that arise from an imbalance in neuromuscular activation (Schulte-Mattler 2008). While BoNT continues to find success in clinical settings, BoNT has drawbacks such as the fact that BoNT's transient nature requires repeated injections for prolonged effect, which leads to immune recognition and tolerance (Schulte-Mattler 2008). Thus, new possibilities have emerged for TeNT in the realm of neuromodulation.

Several of TeNT's characteristics make it an attractive option for neuromodulation. Neuronal specificity among the different CNTs results in varying potential for synaptic inhibition at different sites. BoNT primarily inhibits acetylcholine release at neuromuscular junctions of

peripheral cholinergic neurons, while TeNT specifically targets spinal cord interneurons and blocks glycine and gamma-amino butyric acid (GABA) release by cleaving synaptobrevin II (VAMP1) (Foster 2009). The specificity for the spinal cord and synaptic inhibitory nature of LC reveal TeNT's potential in gene-based neuromodulation.

In this study, we attempt to achieve long-term expression of TeNT LC using AAV vectors compared to the transient expression observed in adenovirus-directed transgene expression demonstrated in previous studies. We hypothesize that Ad.LC-GFP will exhibit an earlier and short-lived inhibition of motor function while AAV.LC-GFP will achieve sustained inhibition of motor function following direct spinal cord delivery. Prior studies utilizing Ad have shown robust yet temporary expression of LC in adult rats (Teng *et al* 2005). The brief transgene expression and high immunogenicity associated with Ad are overcome by AAV, which make it a safer and more effective vector for human clinical trials. The goal of the project is to gauge the potential of AAV.LC-GFP as a form of gene-based neuromodulation that could serve as a possible treatment for conditions characterized by atypical neural activity such as spasticity, spinal cord injury, psychiatric disease, epilepsy, and chronic pain.

2 Materials and Methods

2.1 Vector Construction

The Viral Vector Core Facility at Emory University designed the vector constructs: Ad.GFP (green fluorescent protein), AAV.GFP, Ad.LC-GFP, and AAV.LC-GFP. The following plasmids were used pACCMVpLpA(-)loxP.SSP and pAAV.CMV vectors for vector constructions. The 1496 bp gene sequence encoding the LC of tetanus toxin was cloned into either of the two vectors, all of which were driven by the cytomegalovirus (CMV) promoter. In order to measure gene expression, an internal ribosomal entry site (IRES)-GFP sequence was placed downstream of the LC gene for all vector constructs. Ad.GFP and AAV.GFP lacked the LC gene and served as control vectors. Ad.LC-GFP was produced through Cre-mediated in vitro recombination. AAV1 was the AAV serotype chosen. A standard triple-transfection calcium phosphate precipitation method using human embryonic kidney 293 cells was implemented to yield recombinant AAV1 vectors. The production plasmids were (i) pAAV.CMV.LC, (ii) rep2-cap1–modified AAV helper plasmid encoding the cap serotype 1, and (iii) an adenovirus type 5 helper plasmid (pAdhelper). Vectors were purified from clarified 293 cell lysates by sequential iodixanol gradient purification and ion exchange column chromatography with a linear NaCl salt gradient for particle elution. Vector production was performed at the Viral Vector Core at the University of North Carolina at Chapel Hill. Each of the vectors was produced at a titer of 4 x 10^{12} viral particles/mL. Vector genome titers were determined by quantitative polymerase chain reaction (qPCR).

2.2 Animal Use

The Emory University Institutional Animal Care and Use Committee (IACUC) approved all animal protocols. Surgery was performed on adult (8-12 week-old) Sprague Dawley rats (Charles River Laboratories; Wilmington, MA). Each rat was randomly assigned to one of three experimental groups and baseline motor function was assessed for one week prior to surgery.

2.3 Behavioral Testing

Baseline testing for motor function was done on each animal and for each test a week before surgery. Beginning three days after surgery, with the first post-operative day being the day following direct spinal cord injection surgery, behavioral testing continued in three day intervals. All assessments were carried out by a blinded observer. The tests administered to the animals evaluated the neuromuscular effects vector administration had on the animals gait, hind limb strength, and coordination.

Hindlimb locomotor function and gait were assessed using the Basso-Beattie-Bresnahan (BBB) scale for the locomotor function evaluation (Basso *et al.*, 1995). The BBB measure is a 21-point scale (21 = normal motor function, 0 = no observable hind limb movement) that assesses animal motor function ranging from a normal gait to complete paralysis and additional characteristics of progressive debilitation in between. Based on the BBB scale, the hip, knee, and ankle joint movements as well as balance and position of the trunk and tail were determined.

A modified version of the Tarlov motor scale provided an overall motor function score for each animal. The scale is as follows: 0, no voluntary movement (complete paraplegia); 1, perceptible movement at the joint; 2, good joint mobility but inability to stand; 3, ability to stand

and walk; and 4, complete recovery (Hirose *et al.*, 2000; Taoka *et al.*, 1997; Tarlov and Klinger 1954).

An automated grip strength meter (Columbus, Instruments; Columbus, OH) was used to evaluate individual hind limb strength. Animals were allowed to grasp a wire grid, which was connected to an electronic force transducer, with either the left or right hind limb. Once the animal establishes a firm grip, it was gently pulled back at a constant speed and strength away from the grid until the animal released its grasp. The mode of the force transducer was at "T Peak", which records the peak force exerted on the grid by the animal. During each session of testing, each rat underwent three sequential trials and the average grip strength of each rat for the session was recorded.

Animal balance and coordination was assessed using the Rotarod Performance test, which utilized a Rotarod (Columbus Instruments; Columbus, OH). The Rotarod is an apparatus that contains a rotating rod that increases speed at a set rate due to forced motor activity being applied. Beginning at an initial acceleration of 5 rpm/min, the Rotarod increased the acceleration by increments of 5 rpm at 60 s intervals. Animals were placed on the rotating rod and allowed to walk until they could no longer maintain their balance and coordination. Three sequential trials were done for each animal and the average time on the rod was recorded for each day.

2.4 Gene Delivery

In order to administer the viral vectors, direct injections of vector into the spinal cord were performed on the animals. The rats were anaesthetized (1.8% in O_2 0.5-0.6 L/min) and placed in a stereotactic frame, which was fixed to an isoflurane ventilation system (2% in O_2 0.8 L/min). A 2 cm midline incision was made over the lumbar lordosis using a #10 scalpel blade.

1% lidocaine was given to the paraspinous muscles on either side of the spinal cord for analgesia. A laminectomy was performed to expose the L2 region of the spinal cord. A 27 ½ G needle was used to remove the dura mater enveloping the spinal cord. A total volume of 5 µL containing 2 x 10^{10} viral particles was administered into the lumbar spinal cord parenchyma in two unilateral injections, 2.5 µL per injection, of Ad.LC-GFP, AAV.LC-GFP, Ad.GFP, or AAV.GFP. Injections were done by mounting a micropipette (~100 µm diameter) onto a Nanoject apparatus (Nanoject II Auto-Nanoliter Injector, Drummond Scientific; Broomall, PA). Micropipettes were advanced 1.3 mm into the spinal cord parenchyma to target the spinal cord ventral horn. Each injection was done 3 mm apart and 1 mm lateral to the midline in order to achieve widespread vector distribution. The Nanoject was set so that it gradually administered 50 nL boluses every ten seconds to minimize trauma. Once the injection was complete, the micropipette was kept in place for two minutes before it was retracted in order to prevent reflux. Both the paraspinous muscles and skin were sutured and the animal was given subcutaneous injections of buprenorphine (0.03 mg/kg) for analgesia. The animals were then allowed to recover overnight.

2.5 Perfusion

Animals were deeply anesthetized with an intraperitoneal injection of 100 mg/kg of sodium pentobarbital, which is a veterinary grade euthanizing agent. The animals were perfused transcardially with 0.9% saline followed by 4% PFA solution. The cords were first post-fixed in a cooled 4% PFA solution for twenty-four hours and then immersed for one week in a 30% sucrose solution to cryoprotect the tissue.

2.6 Immunohistochemistry

After perfusing the animals and extracting the spinal cords, cervical and lumbar segments of the cord were set in OCT compound (Adwin Scientific Tissue-Tek), frozen, sectioned at a thickness of 40 µm, and mounted onto glass slides. Sections were permeated and washed in a 0.1% Triton-PBS solution. Following the wash, the sections were blocked for one hour in a 10% normal goat or donkey serum solution diluted in PBS and incubated overnight at 37 ºC in primary antibody diluted in blocking solution. After the blocking, the sections were washed in PBS and incubated for one hour in an appropriate secondary antibody diluted in blocking solution. The primary antibody used was affinity-purified goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:100; Millipore; Billerica, MA). The secondary antibody used was Cy3 donkey anti-goat (1:500; Jackson ImmunoResearch Lab., Inc; West Grove, PA). Slides were mounted with either Vectashield alone or Vectashield containing DAPI (Vector Laboratories; Burlingame, CA). Photomicrographs were obtained using a Nikon DS-Fil color digital camera on a Nikon E400 microscope (Nikon Instruments; Melville, NY) and quantified with NIS-Elements software.

2.7 Motor Neuron Density Measurements

Quantification of motor neuron density was achieved by analyzing the sections of lumbar spinal cord as prepared in the *Immunohistochemistry* section. ChAT stains were performed to identify ChAT positive cells. Photomicrographs were obtained using a Nikon DS-Fil color digital camera on a Nikon E400 microscope (Nikon Instruments; Melville, NY). Images of three random cross sections of the lumbar spinal cord were taken from approximately the same region in each animal (n = 4 per group). Counts of ChAT positive cells both ipsilateral and contralateral

to the site of injection were obtained using NIS-Element BR software (v3.0, Nikon). Averages of the motor neuron counts within each animal and across each group were calculated. Quantification existed as percentages of ChAT positive cells on the ipsilateral and contralateral sides of the cord.

2.8 Western Blotting

Spinal cord lysates from the L1-L4 regions of the lumbar spinal cord of rats seven days post-treatment underwent Western blot analysis. Without perfusing the animals, the rats were euthanized with an overdose of anesthetics and the spinal cord was harvested and homogenized in lysis buffer (0.1 M NaCL, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, and 1 µg/mL aprotinin). The homogenates were centrifuged at 10,000 xg for ten minutes at 4°C. Bicinchoninic acid (BCA) assay (Pierce/ThermoScientific; Rockford, IL) was employed to determine the total protein concentration. Samples of 20-30 µg were loaded into a 12% polyacrylamide gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed. After electrophoresis of the gel, it was transferred to a nitrocellulose membrane. The membrane was then probed with primary antibodies: vesicle associated membrane protein 1 (VAMP1) polyclonal antibody (1: 50; Santa Cruz Biotechnology; Santa Cruz, CA) and Synaptosomal-associated protein 25 (SNAP-25) polyclonal antibody (1:100; Santa Cruz Biotechnology; Santa Cruz, CA). To show GFP expression an anti-GFP antibody (1:100; Millipore; Billerica, MA) was employed. The loading control for the gels was beta actin as anti-beta actin antibodies (dilution 1:1000; Millipore; Billerica, MA) were used. Following a wash in TRIS-buffered saline containing Tween 20, the gels were incubated with horseradish peroxidase-conjugated anti-goat IgG, anti-mouse, or anti-rabbit secondary antibodies (JacksonImmuno Research; West Grove, PA). An ECL Plus

detection method (GE Healthcare Life Sciences; Piscataway, NJ) was employed to develop the blots.

2.9 Statistical Analysis

Quantitative data were presented as mean ± SEM. Statistical tests included analysis of variance (ANOVA), T-test, and *post hoc* comparison and were accomplished using GraphPad PRISM software, Version 4 for Windows (GraphPad Software, Inc.; San Diego, CA). Probability values of $p<0.05$ were considered statistically significant.

3 Results

3.1 BBB

The BBB scale is an open field locomotor scale that tests locomotor function and assesses recovery following spinal cord injury. Each animal's hind limbs were individually assessed according to the BBB scale in order to evaluate the degree of motor deficit due to the injections of viral vectors. The scale ranged from normal locomotion (score 21) to complete paralysis (score 0) with numerous descriptions of abnormal gait (Appendix A). BBB baseline performance was normal prior to vector injection (score 21). By post-operative day seven, animals treated with both vectors expressing LC showed a decline in the mean values of BBB for the hind limb ipsilateral to the injection site: Ad.LC-GFP (10.80 ± 2.43) and AAV.LC-GFP (16.90 ± 2.43) (Fig. 3A). The ipsilateral BBB scores for Ad.GFP (20.00 ± 1.00) and AAV.GFP (20.40 ± 0.60) did not change significantly throughout the thirty day period. At day fifteen, motor function in Ad.LC-GFP animals improved (17.00 ± 2.00) and returned to normal by day thirty (20.40 ± 0.60) (Fig. 3A). In contrast, AAV.LC-GFP animals displayed sustained motor deficits fifteen and thirty days post injection, (16.30 ± 1.89) and (15.78 ± 2.15), respectively (Fig. 3A). Contralateral hind limb BBB scores did not deviate to any significant degree during the thirty day period for any of the four vectors (Fig. 3B).

3.2 Tarlov Motor Score

The Tarlov motor score is another grading system that evaluates the functionality of a rat's mobility. Unlike the BBB scale which provides an evaluation of individual limbs, the Tarlov motor score is an overall grade based on the motor function of all limbs; scale ranges from paralysis (score 0) to normal locomotion (score 5) (Appendix B). Tarlov motor scores

followed similar trends observed in the ipsilateral hind limb BBB scores of all four animal groups (Fig. 4). Ad.LC-GFP treated rats experienced lower motor scores by post-operation day seven (3.50 ± 0.42) and then saw an increase by day fifteen (4.33 ± 0.33), eventually reaching near baseline scores by day thirty (4.83 ± 0.16). The Tarlov motor score for AAV.LC-GFP consistently remained below baseline at all three time points analyzed (3.33 ± 0.33) while Ad.GFP (5.00 ± 0.00) and AAV.GFP (4.8 ± 0.20) exhibited little change.

3.3 Grip Strength

Grip strength measured the impact that LC expression in the spinal cord had on hind limb motor control and muscular strength both ipsilateral and contralateral to the site of injection (Fig. 5). Rats were allowed to grip a mesh grid attached to a Grip Strength Meter and pulled back until the animal released its grip. The maximal peak force was measured. Three trials were performed for each hind limb and the averages were taken for each test day. Ad.GFP (93.17 ± 5.15%) and AAV.GFP (101.41 ± 4.81%) injected animals maintained grip strength scores similar to baseline measurements. Ad.LC-GFP (34.49 ± 14.08% ($P < 0.05$)) and AAV.LC-GFP (52.17 ± 8.51% ($P < 0.001$)) treated animals exhibited considerable decrease in ipsilateral grip strength seven days post-operation (Fig. 5A). There were no changes observed in contralateral hind limb motor function for any of the groups: Ad.GFP (115 ± 4.34%), AAV.GFP (108.43 ± 4.03%), Ad.LC-GFP (102.30 ± 14.64%), and AAV.LC-GFP (99.67 ± 7.72%) (Fig. 5B). At approximately fifteen days following gene delivery, animals in the Ad.LC-GFP group showed a steady increase in grip strength (75.30 ± 14.84%) and no difference in percent change ($p=0.24$) compared to Ad.GFP (96.95 ± 6.18%) (Fig. 5A). At post-operative day fifteen, AAV.LC-GFP continued to demonstrate hind limb grip strength inhibition (29.17 ± 9.18% ($p < 0.05$) compared

to Ad.GFP injected controls. By day thirty, Ad.GFP (106.70 ± 4.48%) and Ad.LC-GFP (101.80 ± 9.21%; p = 0.89) groups had comparable grip strength scores to baseline in their ipsilateral hind limbs (Fig. 5A). Animals treated with AAV.LC-GFP continued to demonstrate impairment in ipsilateral hind limb motor function, maintaining grip strength scores significantly lower than baseline (37.53 ± 13.56% ($P < 0.05$)). Similarly to time point seven days post gene delivery, none of the animal groups experienced a significant change in the contralateral hind limb grip strength at fifteen and thirty days post injection.

3.4 Rotarod

The Rotarod test was used to determine the effects viral vector delivery had on balance and coordination. Animals were placed on a rotating rod that accelerated at specified intervals and allowed to walk. The time it took for rats to drop was recorded. Three trials were done for each animal and the times of each animal were averaged for each day. The measurements in the Rotarod test corresponded with the trends seen in BBB, Tarlov motor score, and grip strength. A comparison between Ad.LC-GFP and Ad.GFP treated animals showed significant difference in time values at seven days post gene transfer (Ad.LC-GFP = 50.84 ± 10.68% ($p <0.05$); Ad.GFP = 94.74 ± 2.60%)), but no significant difference at either fifteen or thirty days post treatment (Fig. 6). At each of the three time points analyzed, AAV.LC-GFP exhibited motor coordination deficits (day 7=54.40 ± 7.09%, day 15=70.53 ± 10.32%, day 30=74.15 ± 7.43%) whereas AAV.GFP showed little change in balance and coordination by day seven post-operative (102.33 ± 15.24%) (Fig. 6).

3.5 Immunohistochemistry

After perfusing and freezing the spinal cords of the rats, sections were taken and placed on glass slides for immunohistochemical purposes. ChAT stains were performed to display the location of motor neurons within the spinal cord sections. GFP expression indicated the presence of viral vectors because the IRES-GFP sequence was also present in each of the viral vector constructs. Co-localization of ChAT and GFP suggested that the viral vectors entered and transduced the motor neurons. The patterns depicted in motor function reflected the transduction of motor neurons. Photomicrographs of spinal cord seven days post vector administration displayed ipsilateral GFP expression among all groups (Fig. 7). By day thirty however, only animals injected with AAV.LC-GFP showed persistent GFP expression (Fig. 8). There appeared to be no evidence of toxicity to the tissue despite high transgene expression.

Additionally, the number of ChAT expressing motor neurons on the ipsilateral side compared to those on the contralateral side exhibited no change when assessed at both seven and thirty days post-operation (Fig. 9). The data was expressed as a percentage of the ChAT expression on the contralateral side.

3.6 Western Blotting

Western blot analysis was used to determine whether or not VAMP1 protein levels were affected following delivery of Ad.LC-GFP and AAV.LC-GFP. At day seven post-operative, animals were euthanized and their spinal cords were extracted, homogenized in a lysis buffer, and centrifuged. BCA assays were done on the lysates to determine protein concentration. Samples were pipetted into a 12% polyacrylamide gel and Western blot analysis was done. Antibodies for VAMP1, SNAP 25, GFP, and beta actin were used to determine the concentration

of each protein in the lysate samples. VAMP1 is selectively cleaved by TeNT LC and was the protein of interest. SNAP 25 is one of the proteins that forms the SNARE protein complex involved in vesicle docking and release, and was used to compare with VAMP1. Measuring GFP concentration served as an additional control to indicate the presence of the viral vectors in the samples. Beta actin served as the loading control. Seven days after vector delivery, animals injected with Ad.LC-GFP and AAV.LC-GFP exhibited a reduction in VAMP1 spinal cord protein expression while those that received Ad.GFP saw no change in VAMP1 expression (Fig. 10). Expression of the SNARE protein SNAP 25, which is cleaved by the commonly used therapeutic neurotoxin BoNT-A, did not vary among the different vectors. There were no differences in GFP or beta actin expression in animals treated with LC compared to control animals treated with Ad.GFP seven days following gene transfer (Fig. 10).

4 Discussion

In this study, we evaluated the effectiveness of AAV as a viral vector for delivering the tetanus toxin LC gene to the spinal cord of adult rats. Using Ad and AAV as viral vectors, LC was successfully expressed in motor neurons following direct spinal cord injections at the L2 region of the spinal cord. The unilateral direct injections of viral vectors performed in this study were able to achieve selective neuromuscular inhibition on the ipsilateral side. Co-localization of GFP and ChAT expression in motor neurons corresponded with the degree of motor inhibition for each vector group at day seven and day thirty post-operative. Even during the peak of LC transgene expression, there appeared to be no change in motor neuron density between ventral horns on the sides ipsilateral and contralateral to the site of injection. The data upheld previous findings that TeNT LC selectively cleaves VAMP1. Successful AAV-driven LC expression without destruction of motor neuron structure and density strengthens the possibilities of using AAV.LC as an alternative treatment for neural modulation.

Inhibition of motor function in the hind limbs demonstrated the persistent nature of the AAV vector. Over the course of thirty days, Ad.GFP and AAV.GFP groups exhibited motor deficits in neither ipsilateral nor contralateral hind limbs as exemplified by unvarying scores for all behavioral tests: BBB, Tarlov motor score, grip strength, and rotarod. Without the LC transgene, Ad and AAV were unable to cleave VAMP1, and thus kept the SNARE protein complex intact, which allowed for functional neurotransmitter release at synapses. Behavioral tests for Ad.LC-GFP injected animals demonstrated transient transgene expression as evidenced by a sharp decline in motor function after seven days post-operative, followed by a steady recovery period. Such a trend reflected the work of previous studies, which showed temporary expression of LC in adult rat spinal cord following Ad injections (Teng *et al* 2005). While motor

deficits in the hind limbs due to Ad-driven LC expression demonstrated the feasibility of delivering LC to specific regions of the spinal cord, well established disadvantages still remained. Thus to improve the therapeutic potential of the transgene LC, AAV was used. Data from BBB and grip strength showed ipsilateral hind limb motor impairment by day seven and scores continued to remain well below baseline scores throughout the testing period. Regardless of the vector used, the contralateral hind limbs exhibited no functional deficits, testing near baseline in BBB and grip strength assessments throughout. Both Rotarod and Tarlov motor score data corresponded with the trends seen in BBB and grip strength tests for all four viral vectors. Ad.LC-GFP and AAV.LC-GFP both induced selective inhibition of motor function to the ipsilateral hind limb due to successful delivery of LC, which prevented neurotransmitter release by disrupting the SNARE complex involved in vesicle exocytosis in presynaptic terminals. Unlike Ad.LC-GFP, AAV.LC-GFP achieved sustained motor function inhibition, thus demonstrating that AAV vector can be used for long-term regulation of neuronal function without the need for repeat transgene delivery.

Immunohistochemistry provided qualitative data that supported the trends seen in behavioral tests. Ad.GFP, AAV.GFP, Ad.LC-GFP, and AAV.LC-GFP all exhibited co-localization of GFP and ChAT expression seven days after vector delivery, which indicated successful delivery of the vectors to motor neurons; all the vectors expressed the GFP gene while ChAT confirmed that the expression was in motor neurons. Co-localization of GFP and ChAT in the ipsilateral ventral horns of Ad.LC-GFP and AAV.LC-GFP animals coincided with the onset of motor deficits at the same time point for animals in both vector groups. Thus, motor inhibition was due to LC activity in the motor neurons. At day thirty, GFP expression was only observed in AAV.LC-GFP, which correlated with behavioral data that showed that only animals

in the AAV.LC-GFP group continued to display impairments in motor function thirty days after surgery. GFP and ChAT staining revealed that both Ad and AAV are capable of delivering the LC transgene selectively to motor neurons at the injection site. GFP expression also validated the transient nature of Ad and displayed how AAV continued to exist in motor neurons up to thirty days post-treatment.

Histological results highlighted a lack of toxicity associated with delivery of any of the vectors. Tissue morphology was comparable among spinal cord tissue samples taken from all the animal groups; there was no obvious tissue damage to either the ipsilateral or contralateral ventral horns. Moreover, a comparison of ChAT expressing motor neurons in either ventral horn revealed that there was no significant difference in the number of motor neurons regardless of the viral vector injected. Therefore, none of the viral vectors compromised the integrity of cells within the spinal cord, thus indicating the relative safety of using AAV as a viral vector.

Western blot confirmed that the study's method of delivering LC was selective in its effects on SNARE proteins. Only the vectors carrying LC, Ad.LC-GFP and AAV.LC-GFP, exhibited reduced VAMP1 protein expression while Ad.GFP maintained normal expression. The expression of other proteins did not differ among the vectors. The decrease in VAMP1 supported prior findings that TeNT LC specifically targets VAMP1 as opposed to other SNARE proteins such as SNAP-25 (Eisel *et al* 1993). Even though wild type TeNT preferentially targets spinal interneurons, there exist many therapeutic possibilities of vector induced LC for regulating cellular activity in a number of cell types and tissues that possess VAMP1.

Successful clinical applications of BoNT for treating dystonia, strabismus, pain syndromes, and cosmetics have paved the way for the therapeutic possibilities of TeNT LC. A disadvantage of the method employed in the study is light chain's inability to undergo retrograde

transport without the help of its heavy chain. An effective means of targeting specific cells, such as spinal neurons, is by intraparenchymal injections at the site of interest. Issues of LC eliciting an immune response are minor considering that antibodies directed against the LC domain of tetanus neurotoxin are rarer than those against the heavy chain portion (Lin *et al* 1985). The lack of antibodies against TeNT LC upholds the relevancy of vector-driven LC expression to modulate neural activity in the CNS. While additional studies of AAV-driven LC expression may need to be done, preliminary evidence has brought AAV-driven LC expression to the forefront of gene therapy seeing that safe, long-term LC expression has been achieved following this study.

5 Conclusion

Experiments have shown the potential of AAV.LC as an inhibitory means of neuromodulation. Prior research has demonstrated that adenoviral vectors are capable of delivering and expressing the light chain of tetanus neurotoxin into the spinal cord tissue without adverse effects to cellular structure. Even though Ad.LC strongly expresses the transgene and induces loss of motor function, the effects are temporary; expression ends after seven days and the animals recover locomotor function. AAV.LC successfully overcomes the shortcomings of Ad.LC as it exhibits prolonged inhibition of hind limb movement while maintaining the health of motor neurons. Moreover, LC when inserted into Ad and AAV successfully reduces VAMP1 protein concentrations as illustrated by Western Blot. AAV.LC appears to be a viable form of gene-based neuromodulation for clinical applications.

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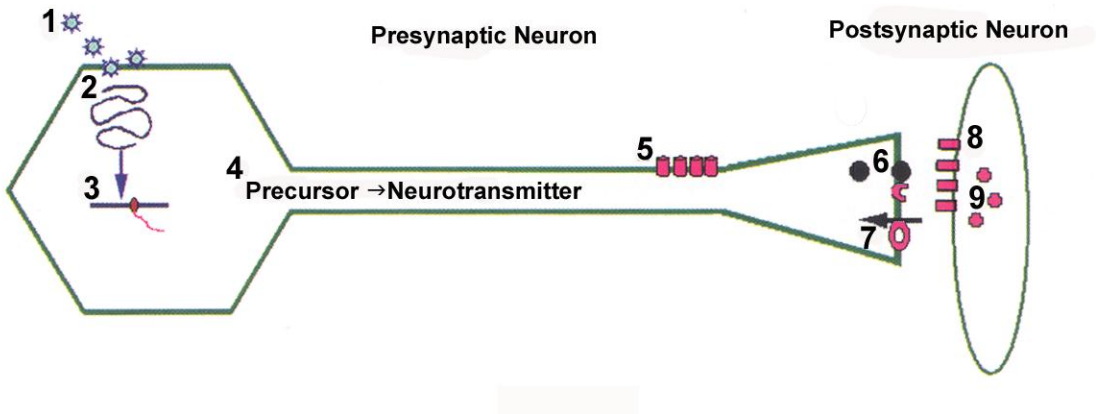

Figure 1. Possible biological targets of gene-based neuromodulation. A diagram depicting a presynaptic neuron and postsynaptic neuron as well as points of modulation of (1) neuronal vector uptake, (2) transgene transcription, (3) transgene translation, (4) neurotransmitter precursor or enzyme, (5) ion channel, (6) vesicle docking protein, (7) neurotransmitter re-uptake protein, (8) receptor, (9) second messengers.

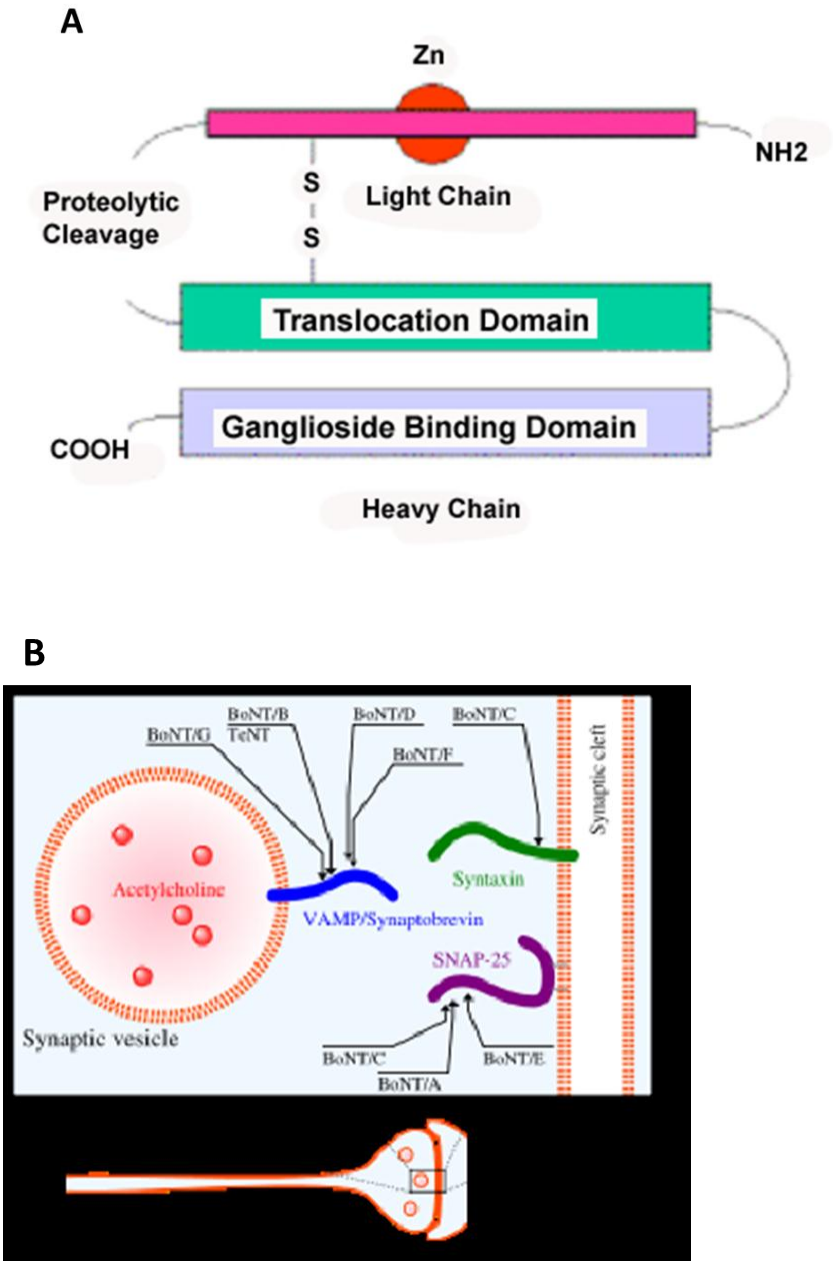

Figure 2. Tetanus toxin protein and protein selective cleavage. (A) Tetanus toxin protein with light chain (LC), which is responsible for cleaving VAMP1, and heavy chain (HC), which is responsible for neuronal binding and uptake. (B) Mode of inhibition exhibited by various CNTs; target of SNARE proteins at presynaptic terminal. TeNT selectively cleaves VAMP1/synaptobrevin II.

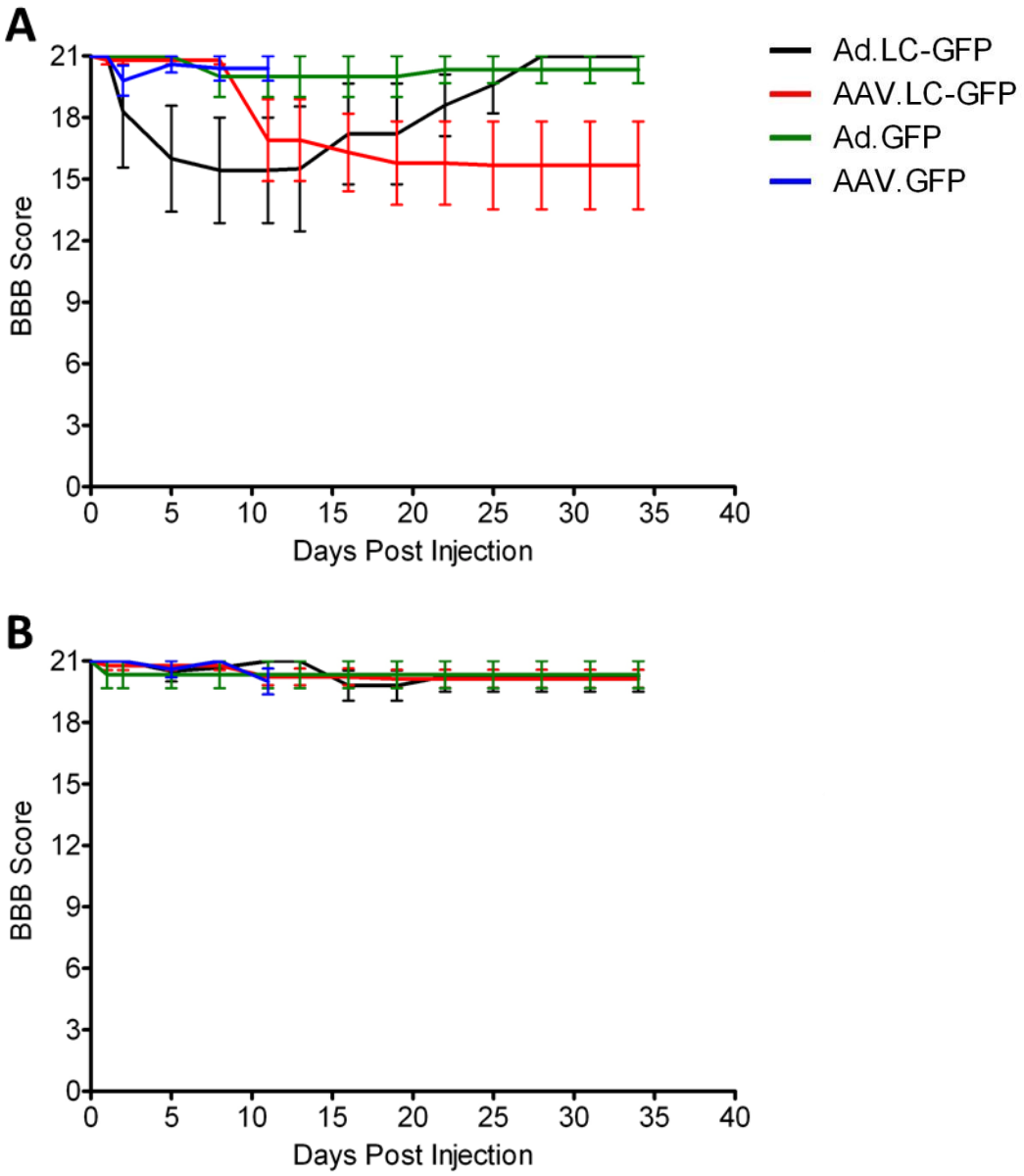

Figure 3. BBB scores of ipsilateral and contralateral hind limbs following viral vector administration. (A) Ipsilateral hind limb BBB scores did not fluctuate for Ad.GFP and AAV.GFP. Ad.LC-GFP exhibited a drop at day seven followed by a gradual recovery to a score close to a perfect 21. (B) Contralateral hind limb BBB scores showed little change across all four viral vectors.

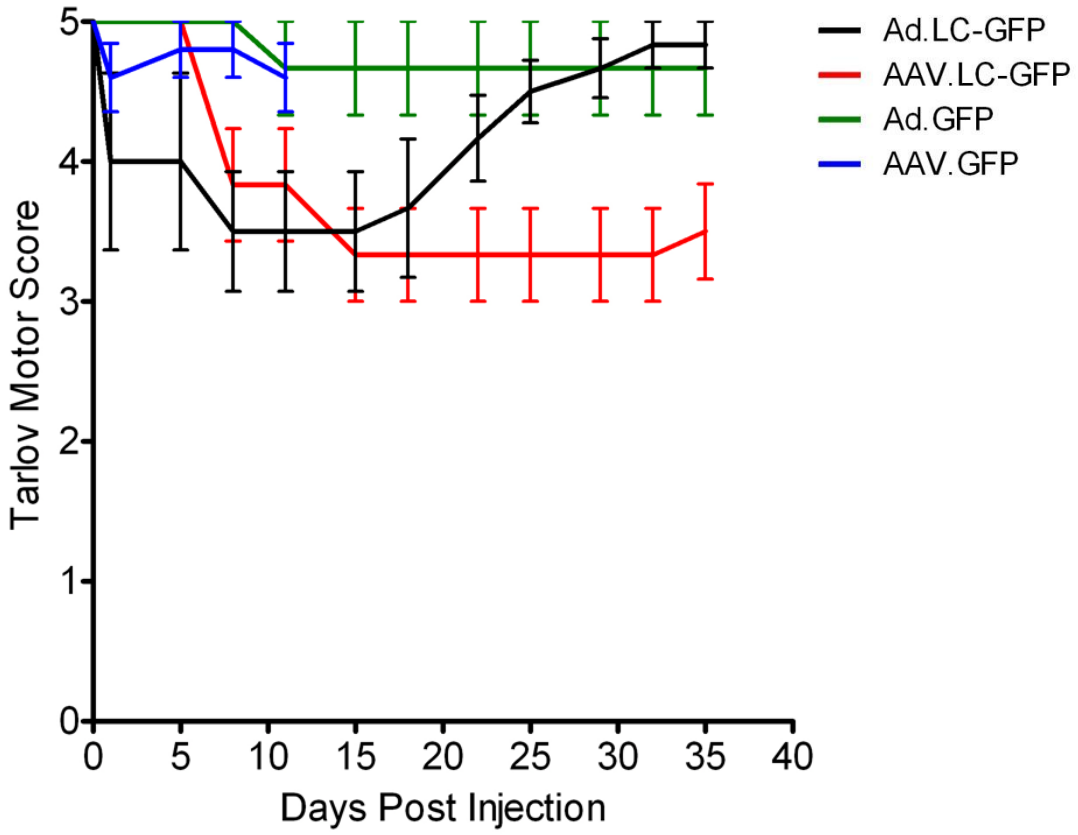

Figure 4. Modified Tarlov motor score following viral vector injection. Ad.GFP and AAV.GFP exhibited little change in modified Tarlov motor score. Both Ad.LC-GFP and AAV.LC-GFP demonstrated a reduction in initial overall scores by day seven. AAV.LC-GFP treated animals continued to show a decrease in score up to day thirty while Ad.LC-GFP treated animals steadily recovered to normal Tarlov motor scores.

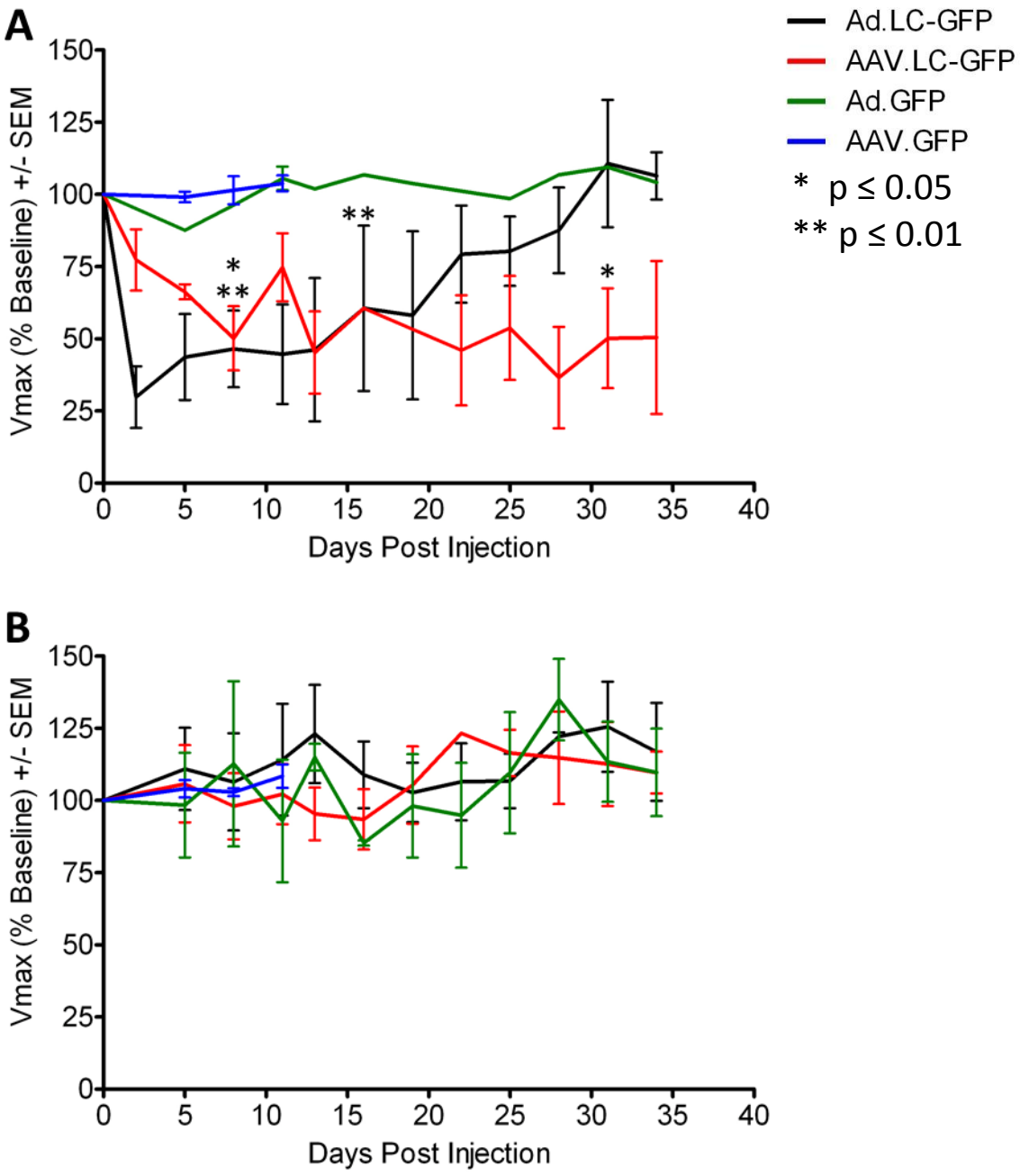

Figure 5. Grip strength measurements of ipsilateral and contralateral hind limbs represented as percentage of baseline values. (A) The ipsilateral grip strength of Ad.GFP and AAV.GFP maintained forces comparable to those of baseline. Ad.LC-GFP decreased considerably and then rose to near 100% baseline by day thirty. AAV.LC-GFP declined as well, but remained at a low percentage of the baseline values. (B) No change in grip strength occurred in the contralateral hind limb for any of the viral vector groups.

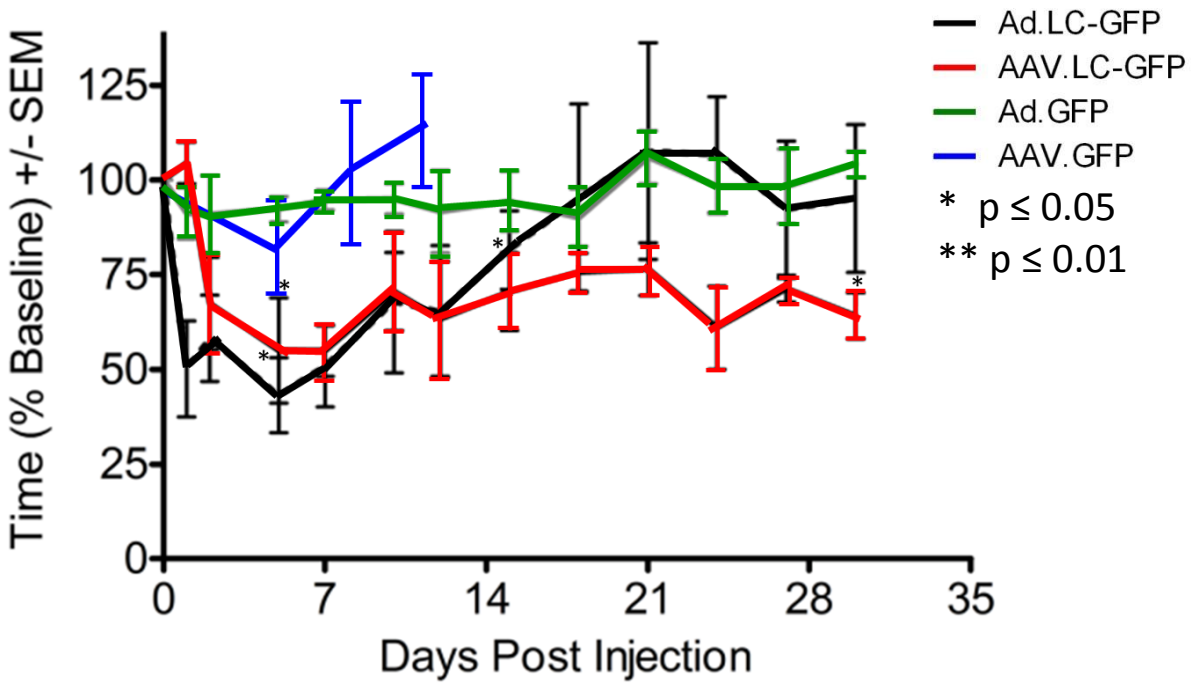

Figure 6. Rotarod measurements represented as percentages of baseline times after vector administration. Compared to Ad.GFP and AAV.GFP, Ad.LC-GFP and AAV.LC-GFP treated animals showed a sharp decline in time spent on the rotarod by day seven. The times for the Ad.LC-GFP group increased till the times were comparable to baseline. The AAV.LC-GFP group remained on the rotarod for a shorter period of time than baseline throughout the thirty days.

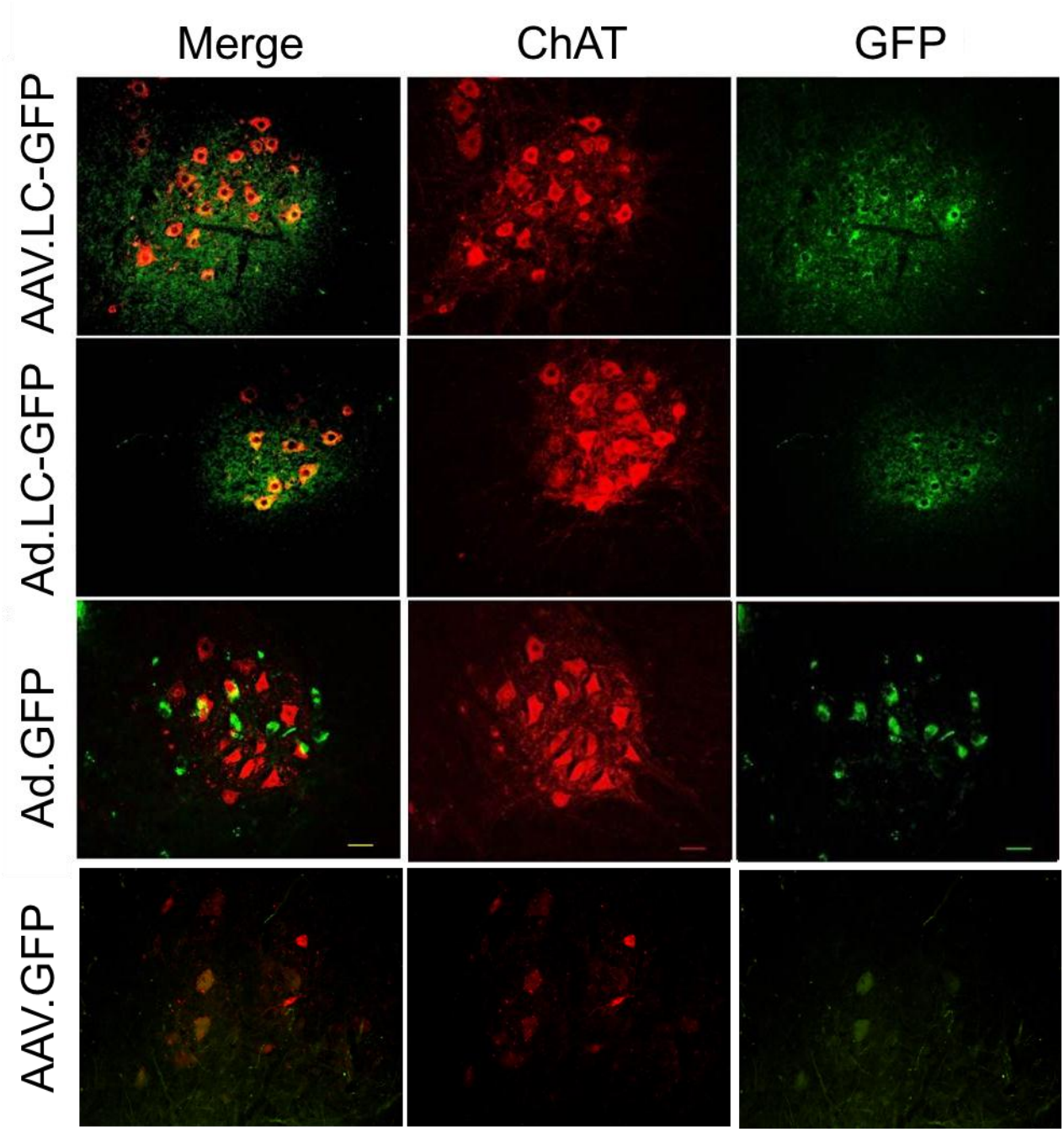

Figure 7. ChAT and GFP expression seven days following spinal cord LC gene transfer. Animals from all treatment groups showed abundant ChAT and GFP expression in the ventral horn. Scale bar = 100 µm.

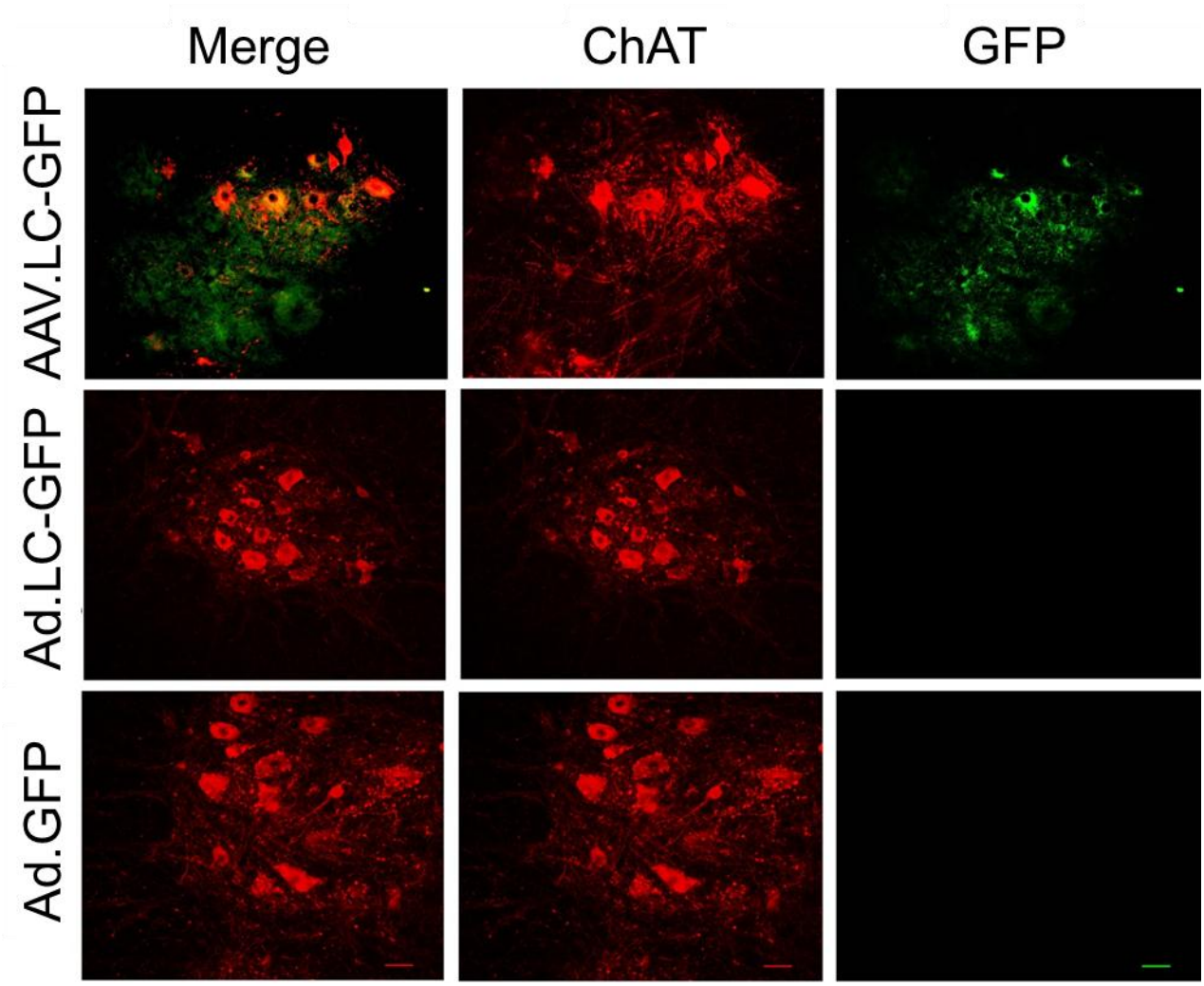

Figure 8. ChAT and GFP expression thirty days following spinal cord LC gene transfer. The only group that expressed GFP in the ventral horn at thirty days was AAV.LC-GFP. Scale bar = 100 μm.

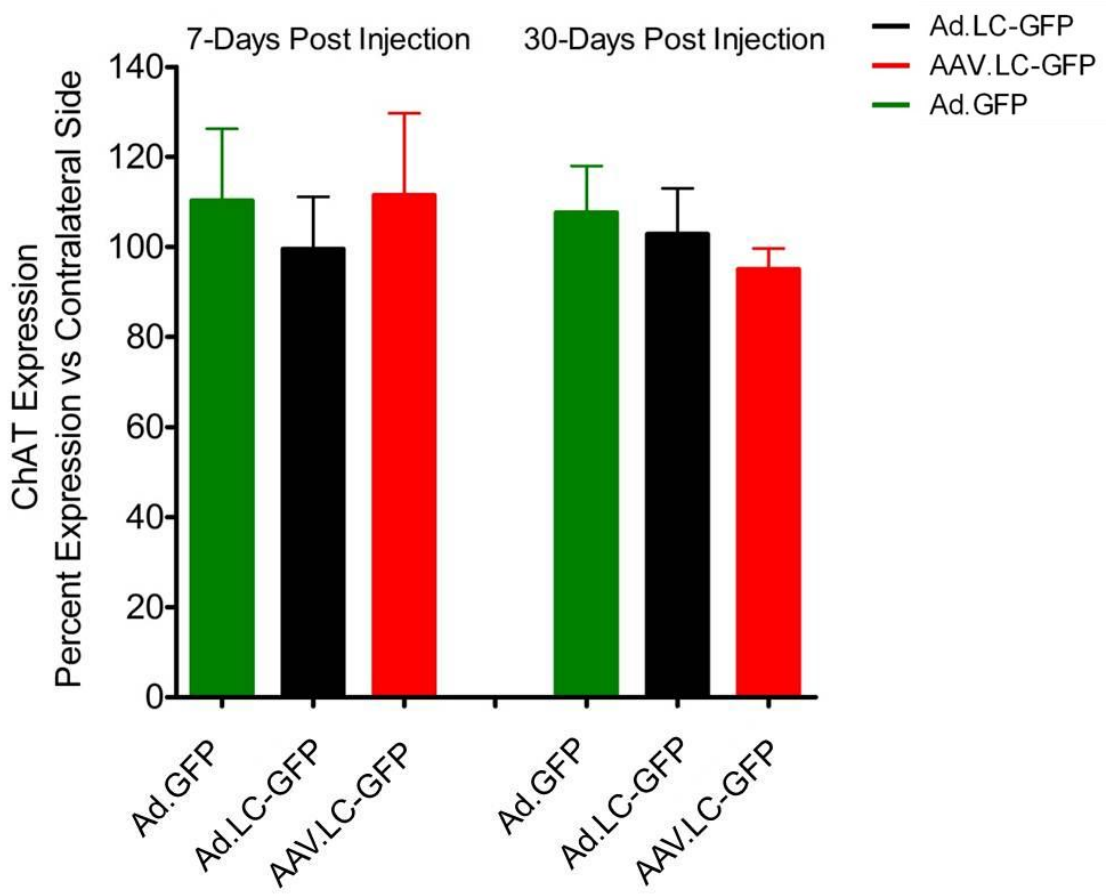

Figure 9. Percent comparison of ChAT expression between ipsilateral and contralateral hind limbs following spinal cord vector administration. There were no changes observed in the the number of ChAT-expressing cells in the ipsilateral ventral horn when compared to ChAT expressing motor neurons on the contralateral side when assessed at both 7 days and 30 days post treatment. Error bars represent SEM.

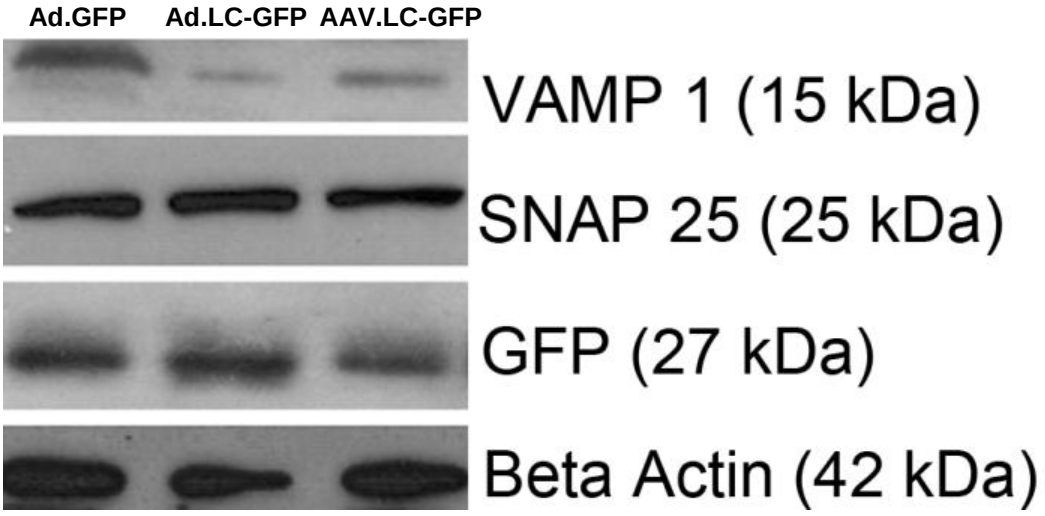

Figure 10. Western blots illustrating vector impact on VAMP1 protein expression. Western blots were performed using antibodies against VAMP1, SNAP-25, GFP (used to indicate transgene expression), and beta actin (loading control). Seven days after vector delivery, Ad.LC-GFP and AAV.LC-GFP injected rats showed a decrease in VAMP1 protein concentration while Ad.GFP did not show this decrease.

Appendix A. Basso Beattie Bresnahan Scale

BBB Score	Description
0	No observable hind limb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee.
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint.
3	Extensive movement of two joints.
4	Slight movement of all three joints of the HL.
5	Slight movement of two joints and extensive movement of the third.
6	Extensive movement of two joints and slight movement of the third.
7	Extensive movement of all three joints of the HL.
8	Sweeping with no weight support or plantar placement of the paw with no weight support.
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping.
10	Occasional weight supported plantar steps, no forelimb (FL)-HL coordination.
11	Frequent to consistent weight supported plantar steps and no FL-HL coordination.
12	Frequent to consistent weight supported plantar steps and occasional FL-HL coordination.
13	Frequent to consistent weight supported plantar steps and frequent FL-HL coordination.
14	Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping.
15	Consistent plantar stepping and consistent FL-HL coordination; and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact.
16	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off.
17	Consistent plantar stepping and and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off.
18	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off.
19	Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time.
20	Consistent plantar stepping and consistent coordinated gait; consisten toe clearance; predominant paw position is parallel at initial contact and lift off; tail consistently up; and trunk instability.
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up.

Appendix B. Tarlov Motor Score Flowchart