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Gene-based neuromodulation for sustained botulinum toxin expression in the treatment of muscle spasticity: a proof of principle study

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Methods: Adenoviral vectors encoding different botulinum toxin/A domains were injected into rat spinal cords at 4×10^4 transforming units to test their neural inhibitory effects: light chain, light chain and receptor binding domain, and light chain and translocation domain. To assess for dosage dependency, vectors were also tested at 4×10^5 and 4×10^6 transforming units. Pre-surgical baseline and post-operative motor function was measured by Basso-Beattie-Bresnahan open-field locomotor scoring, grip strength, and rotarod performance. After 3-weeks of postoperative behavioral testing, rats were euthanized, and spinal cords were immunostained and counted for neuronal nuclei to assess cytotoxicity from transgene expression. Prior to injection, vectors were tested *in vitro* in HEK293 cells to control for differences in transduction efficiency.

Results: Animals injected with adenoviral vectors containing genes for the light chain and receptor binding domain displayed the most consistent and sustained deficits in open-field assessment, grip strength, and rotarod performance. Rats injected with adenovirus containing light chain showed spontaneous recovery at 16 days post-injection, coinciding with the cessation of adenoviral gene expression. Spinal cord neuron counts revealed no signs of cytotoxicity between treatment groups and controls at injection titers of 4×10^4 and 4×10^5 but not at 4×10^6 transforming units. *In vitro* testing showed no difference in transduction efficiency between vectors and there were no differences in neuron density compared to controls.

Conclusion: These results suggest that vector-mediated gene transfer of botulinum toxin light chain and receptor-binding domain is most effective at synaptic inhibition that is not cytotoxic. This gene-based approach at focal neuromodulation may hold future applications in the treatment of neurological disease and the study of neural circuitry. Furthermore, these data suggest that the heavy chain may play a role in the characteristic persistence of botulinum neurotoxin/A inactivation of synaptic function that is still unexplained.

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Introduction.

Current approaches at neuromodulation

The peripheral and central nervous systems are composed of complex neuronal circuits involving intricate interactions between inhibitory, excitatory, and modulatory neurons. Disruption at any juncture of these fine-tuned interconnections can result in a wide variety of neurological and psychiatric diseases. Thus, several approaches aimed at inhibiting the function of excessively active neurons have been developed including surgical lesions, electrical stimulation, and a myriad of drugs. Surgical procedures, though still frequently used for the treatment of epilepsy, suffer from their irreversibility and have largely given way to electrical stimulation[1]. However, treatment mechanisms like deep brain stimulation (DBS) are limited by the need for surgically implanted hardware which is vulnerable to infection, malfunction, and periodical battery replacement. In addition, both approaches can result in significant mental and physical side-effects due to a lack of spatio-temporal specificity[2, 3]. Newly developed designer receptors exclusively activated by designer drugs (DREADDs) offers a promising approach to cell-specific, reversible neuromodulation[4]. However, the difficulty of designing drugs that can potently modulate specific neuronal circuits when taken at tolerable concentrations may be a potential limitation, and, as with any pharmaceutical approach to treatment, bring up the question of patient compliance. Thus, further development of therapies aimed at modulating neuronal activity at the cellular level is needed.

Disease-related muscle spasticity and current approaches to management

Spasticity is a functional, neurological disorder resulting from the hyperexcitability of alpha motor neurons in the spinal cord. Patients experience exaggerated, deep tendon reflexes that result in sudden, uncontrollable muscle movements or rigidity. Currently, spasticity affects over 12 million people worldwide with a majority of cases associated with other neurological disorders like cerebral palsy, multiple sclerosis, central nervous system injury, stroke, and amyotrophic lateral sclerosis (ALS)[5, 6].

The management of spasticity can include multiple approaches, often applied in concert. Current treatments include oral medications, surgery, and injection of botulinum neurotoxin (BoNT).[7] Oral medications (e.g. baclofen, diazepam, progabide) are first-line treatments but there is limited evidence to their effectiveness and they are almost always associated with side effects[8-10]. Pharmacologic management of spasticity is further complicated by the fact that sudden cessation of treatment, even of an apparently ineffective drug, can cause severe adverse effects and a rebound increase in spasticity[11, 12]. In addition to pharmacological approaches, different surgical approaches have been used to treat spasticity with varying degrees of success. The most common surgical options are either orthopedic procedures (tendon-release operations)[13] or neurosurgical procedures (ablative and non-ablative operations). Indeed, the therapeutic efficacy of ablative procedures can range from variable to poor and with various surgical complications including: sensory loss, flaccid paralysis, and permanent neurological deficits[14]. Currently, the most frequent non-ablative procedure is the implantation of intrathecal baclofen (ITB) pumps[15]. Although effective in improving postoperative spasticity, ITB pumps are still highly susceptible to surgery-related morbidity and mechanical pump failure[16].

Botulinum Toxin

The injection of botulinum neurotoxin (BoNT) has proven to be an effective therapy for spasticity when used in small doses and is currently the standard-of-care treatment. Produced by the anaerobic bacteria *Clostridium botulinum,* BoNT is the most potent neurotoxin known to man. There are seven BoNT serotypes (BoNT/A – BoNT/G) with BoNT/A being the most potent and having the most clinical applicability[17]. However, BoNT therapy requires repeated injections every 3-6 months which can be costly, painful, and inconvenient. In addition, repeated exposure to BoNT from injections may result in antibody formation and subsequent treatment failure. For this reason, increasingly higher doses of BoNT are required for therapeutic effect[18, 19]. Thus, while BoNT injection is the current standard of care, it continues to be a suboptimal management paradigm. However, focused BoNT expression through vector-mediated gene transfer may provide an alternative method of neural inhibition in the treatment of muscle spasticity and other applications.

BoNT/A is composed of two protein domains, a light chain (LC) and heavy chain (HC), linked by a disulfide bond. The LC contains the catalytic domain which is critical for BoNT's proteolytic mechanism and blockade of Acetylcholine (Ach) exocytosis. The HC consists of the receptor binding (RBD) and translocation domains (TD) important for LC cell-entry and translocation[20]. The general consensus is that BoNT exerts its neurotoxic effect in a four-step mechanism: (1) the binding of BoNT to peripheral nerve endings of acetylcholinergic neurons, (2) clathrin-mediated endocytosis mediated by RBD binding to SV2 receptors[21], (3) TD facilitated translocation of the LC into the cytosol[22] and (4) proteolytic cleavage of SNAP-25 at Gln^{197}-Arg^{198} residues preventing synaptic vesicle binding[23, 24]. Literature has also suggested that the RBD and TD of the heavy chain contribute to the catalytic activity of the LC by mediating protein folding

and transport[25, 26]. Thus, without the RBD and TD, inadvertent release of LC from neurons cannot affect nearby cells as the LC does not have the capacity to enter neurons or exit from clathrin-coated vesicles if it is endocytosed. Still, the exact contribution of the HC to the catalytic activity of the LC and the persistence of LC-induced synaptic inhibition remains controversial.

Gene-based neuromodulation

Gene-based neuromodulation is a subfield of gene therapy, which focuses on altering the genetic make-up of neurons and/or glia to treat or cure neurological and psychiatric diseases. For spasticity, it may be possible to create sustained toxin expression in motor neurons through vector-mediated BoNT gene transfer, alleviating the need for repeated injections of BoNT and potentially mitigating the antibody response. In addition, cell-specific targeting using nature's most potent neural inhibitor has vast implications for the treatment of neurological disorders and the elucidation of neural circuitry. The present study tests the hypothesis that adenoviral transfer of BoNT transgenes into the rat spinal cord will result in non-cytotoxic, focal neural inhibition and concomitant sensorimotor dysfunction. We compared the effectiveness of three combinations of BoNT transgenes encoding the LC, LC and RBD, and LC and TD protein domains at three different viral titers: 4×10^4 transforming units (TU), 4×10^5 TU, and 4×10^6 TU. To test the effect of adenoviral injections containing genes for BoNT LC (Ad.LC), LC and RBD (Ad.LC.RBD) and LC and TD (Ad.LC.TD) *in vivo*, the viruses were unilaterally injected into the rat lumbar spinal cord. Behavioral tests indicated differences between Ad.LC, Ad.LC.RBD, and Ad.LC.TD injected rats with pre-injection baseline and control rats injected with adenovirus containing green-fluorescent protein (GFP). These differences were isolated to the injected limb indicating the anatomically discrete effects of BoNT gene transfer. Comparison between treatment groups

revealed that Ad.LC.RBD injected at 4×10^5 TU was the most effective at inducing long-term sensorimotor dysfunction. Interestingly, Ad.LC injected animals displayed behavioral recovery at 3 weeks following surgery corresponding with the accepted timeline for transient adenoviral gene expression. This indicated that spinal motor neurons tolerated BoNT gene expression which was later confirmed by non-significant differences in neuron density between treatment and control animals at all injection titers. In addition, infection of HEK293 cells *in vitro* confirmed that viral vectors containing different transgene fragments were operating at equal efficiency. Contrarily, Ad.LC.RBD and Ad.LC.TD did not show behavioral recovery despite no signs of neuron loss, thus signifying that these protein domains may play a role in the persistence of BoNT/A's effects. We conclude that BoNT LC gene transfer into spinal motor neurons disrupts synaptic communication and induces sensorimotor dysfunction that is facilitated and lengthened by BoNT RBD co-expression. This form of focal, gene-based neural inhibition has a range of clinical and research applications and may lead to future discoveries on the cooperative effects of the LC and HC in BoNT synaptic inhibition.

Materials and Methods.

Recombinant adenovirus production

Three synthetic genes encoding the BoNT/A LC, LC and TD, and LC and RBD were each cloned into an adenovirus vector (pACCMVpLpA(-)loxP-SSP). For identification of gene expression *in vitro* and *in vivo*¸ IRES-GFP sequences were also cloned into vectors downstream from BoNT transgenes. The resulting vectors were named pAd.BoNT4 (LC), pAd.BoNT5 (LC and TD), and pAd.BoNT6 (LC and RBD). These plasmids were then used to create recombinant adenoviral vectors: Ad.LC, Ad.LC.TD, and AD.LC.RBD (Vector Core, University of Michigan, Ann Arbor, MI). An adenoviral vector encoding an enhanced GFP sequence served as a control (Vector Biolabs, Malven, PA).

Confirmation of vector transduction *in vitro*

Differentiated HEK293 cells cultured on a 6 well plate were infected at 50% confluency with adenoviral vectors at a multiplicity of infection (MOI) of 5. Four wells were infected with Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP each and one well was given PBS as a negative control. Infected cells were incubated at 37 C for 48 hours after which cells were imaged using a Leica DM IRB inverted research microscope (Leica Microsystems Inc., Buffalo Grove, IL). Brightfield and fluorescence images at 488 nm were taken at 10x magnification at 3 random sites per well. Brightfield and fluorescence images were overlayed using ImageJ, an image processing program. Image overlay displayed areas of circular bright green fluorescence overlapping adherent HEK293 cell bodies with corresponding projections (**Figure 1B**). The number of GFP+ HEK293 cells and total number of cells were counted and the percent infected HEK293 cells was calculated aand

expressed as measure of each vector's transduction efficiency. Mean proportion of infected HEK293 cells by each vector was compared with a one-way ANOVA and post-hoc Tukey HSD for multiple comparisons.

Rat lumbar hemi-laminectomy surgery and Ad.BoNT injections

All experimental protocols were reviewed and approved by the Emory University Institutional Animal Care and Use Committee and experiments strictly followed guidelines outlined in the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences Press, Washington, DC, 1996). Adult male Sprague-Dawley rats (Charles River Laboratories, _____) weighing 200-400 g were randomly assigned to four groups receiving either Ad.LC, Ad.LC.TD, Ad.LC.RBD, or Ad.GFP injections. Six days prior to surgery, rats were acclimated for 3 days on a Rotamex-5 Rota Rod (Columbus Instruments, San Diego, CA) and grip strength meter (Columbus Instruments, San Diego, CA) and baselined for 3 consecutive days on BBB, rotarod, and grip strength assessments. Values taken on the last day of pre-operative assessment were considered a rat's baseline motor function. Immediately prior to surgery, rats were anesthetized with isoflurane (3% in O2) and placed in a stereotaxic frame (Stoetling Co., Wood Dale, IL) to restrict movement. The last pair of false ribs, which articulates with the spinal column at the intersection of the T13-L1 vertebrae, was palpated to approximate the incision site. The area was groomed with electrical clippers and sterilized with betadine and ethanol swabs. A Leica MZT5 dissecting microscope (Leica Microsystems Inc., Buffalo Grove, IL) was used for all surgical procedures and a 3 cm midline incision was made over the lumbar lordosis. The L2 spinous process (L3-L4 of the underlying spinal cord[27]) was identified and the right lamina removed (**Fig. 1A**). Great care was taken to not damage the underlying dura or spinal cord. Spinal cord injections were

then performed using an nanofil syringe (World Precision Instruments, Inc., Sarasota, FL) and microinjector (World Precision Instruments, Inc., Sarasota, FL) and micromanipulator (Stoetling Co., Wood Dale, IL). Microinjectors were positioned over the posterior median sulcus, identified as the spinal cord midline, and advanced through the meninges to 1 mm right of midline and a depth of 2mm to target the anterior horn. Injections were performed gradually over a 7-minute period to prevent reflux of virus and trauma from injection. After the first injection, the needle was removed from the cord, advanced 1 mm caudally and another injection was performed in the same manner. Each injection composed of 5 μL of virus for a total of 10 μL over two injection sites. The first group received injections of Ad.LC (n=5, titer 4×10^4 TU/mL). The second group received injections of Ad.LC.TD (n=6, titer 4×10^4 TU/mL). The third group was injected with Ad.LC.RBD (n=7, titer 4×10^4 TU/mL). Control rats received injections of Ad.GFP (n=3, titer 1×10^4 TU/mL). This treatment regimen was repeated for four more groups at a titer 1 order of magnitude higher (n=3, 7, 7, 5 for Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP respectively) and then again with four additional groups at a titer 2 orders of magnitude higher than the original injections (n=7, 7, 5, 6 for Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP respectively). Rats were single-housed for 3 days post-surgery and monitored for any immediate behavioral deficit. As adenoviral gene expression is delayed in the spinal cord[28], rats displaying hindlimb deficit during this monitoring period were assumed to have spinal cord injury from surgery and excluded (two rats).

Animal behavioral assays

Rats were allowed a week to recover upon which behavioral testing resumed on the 7th post-operative day, which was presumed to be the time point for optimal adenoviral gene expression[28]. Behavioral testing continued until the 22nd post-operative day allowing us to

effectively visualize the onset of BoNT action[29] as well as the decline of adenoviral gene expression[29]. An investigator blinded to the treatment conditions asses animal motor function every 3 days from the 7th to the 22nd post-operative day using the BBB locomotor scale[30]. Originally developed to assess locomotor function in studies on spinal cord injury (SCI) in rats, the BBB assessment employs qualitative observations to quantify the effect of injury or treatment on the degree of locomotor dysfunction on a 21-point scale (key criteria include: 21 = normal motor function, 14 = rotated predominant paw position, 8 = absence of weight support, 4 = slight movement of all three joints of the hindlimb, 0 = no observable hindlimb movement). Due to its high reproducibility and sensitivity, the scale has since been adapted for use in studies on motor neuron disease as well[31-33].

The second assay of motor function tested hindlimb grip strength using an automated grip strength meter. This method has been shown to be a reliable and valid method to test motor function in studies on neuromuscular disease and SCI[34-36]. The instrument uses an electronic precision force gauge that assesses the peak amount of force exerted by an animal when it grasps a specially designed pull bar assembly. Animals were positioned by the investigator with their abdomen facing the force gauge and an individual hindpaw resting on the pull bar (**Fig. 2**). After the investigator confirmed the rat was gripping the pull bar, the animals were slowly pulled back until it released the bar and readings were obtained in Newtons from the device's digital force gauge. Each rat was tested on five sequential assessments for both hindlimbs and the average force generated was considered. Grip strength function was assessed in the injected and non-injected hindlimbs as a percentage of baseline values. In previous unpublished findings, we've observed gradual increases in grip strength in normal rats corresponding to an increase in body weight, which we believe may be contributed to a growth in muscle mass over time. To control for this,

grip strength deficit was calculated as the difference in percent baseline values for the injected and noninjected sides.

A third assessment of motor function tested the maximum speed rats were able to reach on a Rotamex Rota Rod (Fig. 2B) Rotarod performance is one of the most commonly used measurements to test motor function in rodent models for a variety of neurological disorders and conditions[37-39]. Compared to the two behavioral assays listed previously, rotarod testing offers a more investigator-independent method to assess sensorimotor function on all limbs as well as balance and coordination[40]. Generally, there are 2 testing paradigms for rotarod: (1) rodents can be tested at a set speed between trials and the latency to fall can be recorded, or (2) the RPM can be gradually accelerated and the maximum (V_{max}) rotations per minute (RPM) reached can be recorded, with the latter design testing muscle fatigue in addition to balance and coordination[41].

We chose to test our rats on an accelerating rod and record the V_{max} as this was the method employed in previous studies on BoNT[42] and because BoNT injections have been shown to increase rates of muscle fatigue in addition weakness[42]. During the acclimation period, rats were trained to remain on a rod rotating at 5 RPM and accelerating by 5 RPM every minute. Any rats that repeatedly fell were placed back on until they were able to stay on for at least 3 minutes. Latency to fall and V_{max} was digitally recorded by the instrument for each animal. For pre-operative baseline and post-operative testing, the average V_{max} was recorded for 3 sequential assessments.

Confirmation of transgene expression in the rat spinal cord

At 22 days after Ad.BoNT or Ad.GFP injection, rats were euthanized with 0.5 mL of intraperitoneal pentobarbital and underwent transcardiac perfusion with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Lumbar spinal cords were dissected, fixed overnight in 4% PFA, transferred to a 30% sucrose solution, and stored at 4° C until sectioning. Spinal cords were cryosectioned serially at 40 μm using a Leica CM 1850 cryostat and mounted on superfrost plus microscope slides (ThermoFisher Scientific, Waltham, MA). Tissue was blocked with 10% Normal Goat in PBS (Jackson ImmunoResearch, West Grove, PA, USA) stained with a biotin-conjugated Mouse anti-NeuN biotin (neuronal nuclei) primary antibody (Millipore, Temecula, CA) and a secondary streptavidin Alexa Fluor 555^{TM} (Invitrogen, Waltham, MA). Slides were mounted and coverslipped using Vectashield Mounting Medium with DAPI counterstain (Vector Laboratories, Burlingame, CA) and sealed with nailpolish. GFP and NeuN expression was detected under a fluorescent microscope and representative images were taken with a Leica SP8 Multiphoton confocal microscope. Transduced motor neurons expressing GFP were identified by established morphological standards. Tissue sections were compared for differences in GFP expression between Ad.LC, Ad.LC.RBD, Ad.LC.TD, and Ad.GFP injected animals and histological differences indicative of SCI.

Neuron counts for Ad.BoNT injected animals and comparison with controls

40 μm spinal cord sections of experimental animals were stained with antibodies against NeuN (see above). Neuron counts were performed to investigate concerns about cytotoxicity from BoNT transgene expression and adenoviral infection at higher titers[29, 43]. Prior to cryosectioning, extreme care was taken to mark and preserve spinal cord orientation during dissection for later identification of injected (right) and non-injected (left) sides during sectioning, staining, and

micrscopy. Whole serial images of spinal cord were taken using a Nikon Eclipse E400 fluorescent microscope and NIS-Elements imaging software at 4x magnification. Images were opened using the ImageJ image processing software and automated contrast and brightness enhancement were performed. Region of interest (ROI) selection and cropping was conducted for the right ventral horn of each slice and subsequent thresholding and particle counting were performed **(Fig. 3)**. The neuron count for each slice was recorded and averaged to yield an average neuron count per ROI for each animal. Mean counts were stastically compared between Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP injected animals at injection titers of 10^4, 10^5, and 10^6 TU.

Statistical analysis

Quantification of Ad.BoNT and Ad. GFP infected HEK293 cells, mean neuron counts of rat spinal cords after gene transfer, and rat behavioral data (BBB score, grip strength, rotarod V_{max}) was compared using IBM SPSS Software. The mean percentage of infected HEK293 cells was compared between vectors using a one-way ANOVA with post-hoc Tukey's HSD multiple comparison procedure. Mean neurons density 22 days after Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP was compared between treatment groups and between injection titers using the same statistical tests.

Analysis of behavioral data employed a within-subjects design to compare post-operative scores with baseline as well as a between-subject comparison of treatment groups. On each post-operative testing day, each animal was tested on rotarod and the V_{max} was recorded and calculated as percent baseline. Then animals were placed in an open-field container and assigned a BBB score. Finally, grip strength was measured, calculated as percent baseline, and grip strength deficit was represented as the difference in values between the injected and non-injected sides. Pains were

taken to maintain the order of testing for each behavioral assay to prevent possible data confounding from muscle fatigue or rat stress. A two-way mixed ANOVA was performed to investigate whether there was a significant interaction between vector-mediated gene transfer and time on BBB score, grip strength, and V_{max} reached on rotarod. A one-way repeated measures ANOVA was used to determine a statistically significant difference in motor performance from baseline over the 2-week testing period. Time points included pre-surgery (D-0) and 6 days post-surgery (D-7, 10, 13, 16, 19, 22). Finally, one-way ANOVA tests followed by Tukey HSD post-hoc comparisons were conducted to determine if there were differences sensorimotor dysfunction between treatment groups and controls at specific time points. P-values were considered significant if they were less than an alpha value of 0.05.

Results.

BoNT transgene expression from AdLC, AdLC.TD, and AdLC.RBD vectors

Three separate gene combinations encoding the clostridium botulinum type A LC, LC and TD, and LC and RBD were cloned into individual adenoviral vectors and gene expression was controlled under a human cytomegalovirus (CMV) promoter (Table 1). To track transgene expression *in vitro* and *in vivo*, a GFP sequence was also cloned downstream from the CMV-BoNT transgene sequences (Fig. 1a). Three stocks of first-generation adenovirus were generated from these vectors through Cre-mediated *in vitro* recombination between the vector plasmid pAd.BoNT4/5/6 and the 3' adenoviral sequence. To confirm transgene expression and compare transduction efficiency between adenoviral vectors, HEK 293 cells in 6-well plates were infected with AdLC, AdLC.TD, and Ad.LC.RBD viruses at similar confluency and an MOI of 5. Plates were then imaged for GFP fluorescence and the transduction efficiency was recorded as percent infected cells **(Fig. 1b, c)**. All adenoviral vectors had similar mean rates of infection, approximately 63%, and One-way ANOVA statistics confirmed a non-significant difference in transduction efficiency between vectors (P=.919, F=0.086, df= 2).

Spinal cord expression of BoNT transgenes results in loss of sensorimotor function

To test the efficacy of BoNT transgene expression in neuromuscular inhibition and determine the optimal transgene combination for disrupting synaptic transmission, rat lumbar spinal cords were injected unilaterally with Ad.LC, AD.LC.TD, Ad.LC.RBD, and an Ad.GFP control at 4×10^4 transforming units (TU). Animals injected with adenoviral vectors containing BoNT transgenes gradually developed unilateral hindlimb weakness characterized by deficits in balance, weight

support, and grip strength. Rats injected with Ad.GFP displayed preserved function in both hindlimbs. Rats were acclimated for five days on all behavioral assays and baselined on the sixth day, one-day prior to surgery. Locomotor function was assessed using the Basso Beattie Bresnahan (BBB) locomotor rating and all animals displayed a baseline (D-0) score of 21 reflecting full hind-limb function. Any rats that showed immediate dysfunction post-surgery were excluded for spinal cord injury from injection. After a 7-day recovery period, BBB testing resumed for every 3 days over a two-week time-course. At the end of the two-week testing period (D-22), Ad.LC.RBD and Ad.LC injected animals presented with mean BBB scores of 10.86 ± 2.03 (± s.d.) and 9 ± 0 respectively (**Fig. 2A)**. A score of 9 on the BBB scale reflects weight support in stance (i.e. when stationary) only while a score of 10 reflects occasional weight supported steps with an absence of forelimb-hindlimb coordination. Milder deficits were displayed in animals injected with Ad.LC.TD at D-22 who presented with mean scores of 16 ± 2.89 reflecting frequent toe-dragging and rotation of the hindpaw during locomotion. Ad.GFP injected animals showed minimal deficit with means scores of 20.71 ± 0.7 at the end of the testing period. Two-way ANOVA statistics indicated a highly significant interaction between adenoviral gene transfer and time on BBB score decrease ($P < 0.0001$, $F = 11.325$, $df = 12$). One-way repeated measures ANOVA showed a significant change in BBB score over-time in Ad.LC.RBD ($P<0.0001$, $F=18.861$, $df =6$), Ad.LC ($P=0.004$, $F=24.67$, $df=1$), and Ad.LC.TD ($P=0.014$, $F=6.422$, $df=2$) with only animals injected with Ad.LC.RBD showing sustained motor deficit until post-operative day 22 ($P=0.0004$). In addition, pairwise comparisons to baseline revealed that Ad.LC injected animals did not present with significantly lower BBB scores on day 7 and day 10, while Ad.LC.TD injected animals did not display significantly different scores on days 10, 13, and 22. However, a repeated measures ANOVA could not be used for all timepoints due to mean scores on certain days having a standard

error of 0. Thus, a non-parametric sign test was also used to compare scores to baseline which further confirmed previous significance findings and showed a significant deficit in Ad.LC.TD injected animals on day 19. In contrast, both ANOVA and sign statistics demonstrated that Ad.GFP injected animals showed no change in locomotor function.

In conjunction with BBB measurement, grip strength assessment was also employed to test sensorimotor function in a more quantifiable and sensitive manner. Grip strength measures the maximum pulling force a rat can exert when gripping a metal bar connected to a monitoring device as a researcher pulls it away. In addition, by testing both hindlimbs, grip strength measurements offers the advantage of measuring hindlimb strength on the injected side in comparison to the contralateral limb. Grip strength testing followed the same regimen as BBB assessments and mean measurements at baseline for the right hindlimb were 490 ± 106, 502 ± 65, 608 ± 52, and 671 ± 40 N for Ad.LC.RBD, Ad.LC, Ad.LC.TD, and Ad.GFP respectively. Similar to BBB scores, Ad.LC.RBD injected animals displayed significantly lower grip strength force at all timepoints compared to baseline ($P=0.004$, $F=6.56$, $df=3$). On the first post-operative testing day (D-7), grip strength measurements had fallen to -42.47% of baseline values reaching a maximum deficit of -59.1% at post-operative day 19. Animals injected with Ad.LC showed similarly significant deficits compared to baseline ($P=0.008$, $F=8.15$, $df=2$) reaching a maximum deficit on post-operative day 13 at -53.39% of baseline value. However, from that point animals began to recover, reaching non-significance and near baseline values on post-operative day 22 ($P=0.3$) – similar to previous findings by Teng et al. and mimicking the time course for adenoviral gene expression. Although not significantly different from baseline, Ad.LC.TD injected animals showed a similar trend in grip strength deficit to the Ad.LC.RBD group. Thus, we conclude that spinal cord adenoviral BoNT gene transfer disrupts sensorimotor function.

Ad.LC.RBD gene expression results in the most significant and consistent sensorimotor deficit across behavioral assays

To compare the effect of Ad.LC, Ad.LC.RBD, and Ad.LC.TD spinal cord injection on sensorimotor function, BBB and grip strength values for each group was compared to GFP controls at specific time points using one-way ANOVA statistics followed by Tukey post-hoc tests for between-group comparisons (**Table 2**). Throughout the 2-week testing period, Ad.LC.RBD injected animals displayed the most consistent deficits between grip strength and BBB assessment in comparison to GFP controls. Animals injected with Ad.LC.RBD showed significantly lower mean scores compared to GFP controls for 4 out of the 7 post-operative BBB assessments and significantly larger deficit in grip strength on 5 out of the 7 post-operative testing days. Ad.LC injected animals displayed less deficits, only showing significantly more grip strength deficit than GFP controls on 2 out of 7 testing days and lower BBB scores on 5 out of the 7 testing days. Ad.LC.TD injections had the least effect on sensorimotor function with animals only displaying significantly lower BBB scores than GFP control on the last post-operative assessment day and significantly larger deficits in grip strength on 4 out of the 7 time points. We therefore conclude that LC.RBD spinal cord expression leads to the greatest deterioration of hind-limb sensorimotor function and is the optimal gene combination for BoNT gene-based neuromodulation.

Ad.LC.RBD neuroinhibition is optimal at 4×10^5 TU

Previously, Teng et al. had shown that LC expression dependent deterioration of hindlimb function can be highly titer dependent. Having confirmed LC.RBD as the optimal BoNT transgene combination for functional neuroinhibition, it was then necessary to compare Ad.LC.RBD effects at different dilutions. Thus, the effect of Ad.LC.RBD spinal cord injection on sensorimotor

function was tested at 4×10^5 TU and 4×10^6 TU. Previously literature has shown that nervous system injection of adenovirus at 10^6 TU and higher can lead to prevalent neuroinflammation and cytotoxicity. Therefore, to monitor for titer-dependent, adenoviral-induced inflammation sensorimotor function of Ad.GFP injected control animals was monitored at corresponding titers as well. In addition to BBB and grip strength tests, the rotarod assay of motor performance, which measures the maximum rotational velocity (V_{max}) rats can run on a spinning cylinder, was used as a more objective and investigator-independent assessment of hindlimb motor function.

Mixed ANOVA statistics revealed a significant interaction between treatment dilution and postsurgical day of testing on BBB score (p=.04, F=1.93, df=12). At all dilutions scores on BBB for Ad.LC.RBD injected animals significantly decreased over time post-injection (P<0.01) and were significantly lower than the scores of animals injected with report virus(P<.01). **(Fig. 3A**). Deficits in grip strength followed a similar trend, with Ad.LC.RBD injected animals showing significant deficits compared to baseline over-time at all viral titers (p<0.05) (**Fig.3B**). However, only animals injected with Ad.LC.RBD at 4×10^4 TU and 4×10^5 TU showed significantly greater deficits in hindlimb grip strength than GFP controls at similar titrations (p=.001). Interestingly, animals injected with Ad.GFP at 1×10^6 TU showed significantly greater deficit than animals injected with Ad.LC.RBD (p=.047), supporting our previous suspicions about titer-dependent cytotoxicity which would later be confirmed with neuron counts. Finally, when assesed on rotarod, only animals injected with 4×10^5 TU of Ad.LC.RBD showed a significant decrease in rotarod V_{max} after spinal cord adenoviral injection (**Fig. 3C)**. At the end of the testing period (D-22), Ad.LC.RBD injected animals were only capable of reaching 66% of their original V_{max} baseline prior to surgery. Yet, mixed ANOVA statistics indicated neither a significant interaction between Ad.LC.RBD injection titer and rotarod performance over time (p=.09) nor was there a significant

difference between animals injected with 4×10^5 TU of Ad.LC.RBD and animals injected with 1×10^5 TU of Ad.GFP (p.075). However, it should be noted that virus was only injected unilaterally into the lumbar spinal cord and rotarod is a measurement of holistic limb function. Thus, it was believed that a mean deficit to 66% of baseline V_{max}, although not statistically significant, adequately reflects unilateral loss of sensorimotor function with rats being able to sufficiently compensate with their contralateral limb. These results seemingly indicate that Ad.LC.RBD injections at 4×10^5 TU results in a deterioration of hindlimb motor function that is comparable, if not greater than, Ad.LC.RBD injections at 4×10^4 TU.

BONT transgenes are expressed in the right, ventral horn of the rat spinal cord

Immunofluorescence analysis confirmed the expression of BoNT trasngenes in the form of GFP positive fluorescence in all animals. At 22 days after surgery, injected spinal cords displayed weak but clearly apparent green fluorescence reflecting the transient expression profile of adenoviral vectors **(Fig. 4A)**. Similar to previous experiments performed in cell culture, gene expression did not appear to differ between Ad.LC, Ad.LC.TD, Ad.LC.RBD, and Ad.GFP controls. Gene expression was largely restricted to the grey matter of the anterior horn of the right lumbar spinal cord in concordance with unilateral injections. The majority of GFP expression occurred adjacent to the injection site or needle tract. Higher power imaging at 20x magnification revealed that a large proportion of transduced neurons displayed a motor neuron (MN) morphology (**Fig. 4B)**. Observational assessment of GFP fluorescence between animals did not reveal any discrepancies in gene expression resulting from Ad.LC, Ad.LC.RBD, or Ad.LC.TD injection reflecting results found *in vitro*.

Ad.LC, Ad.LC.RBD, and Ad.LC.TD gene transfer does not cause significant cell death

For the future use of adenoviral-mediated BoNT gene expression in the treatment of nervous system disorders to be safe and effective, it is necessary that BoNT gene expression disrupt synaptic transmission without causing neuronal death. However, motor deficits displayed in injected animals could be attributed to either cause. Thus, to confirm the validity of behavioral results and to further evaluate the clinical practicality of Ad.BoNT gene-based neuromodulation, the cytotoxicity of Ad-BoNT injections and gene expression was investigated. The cytotoxicity of Ad.BoNT gene expression was measured in animals injected with Ad.LC, Ad.LC.RBD, and Ad.LC.TD and compared to Ad.GFP controls using immunofluorescent staining for neuronal nuclei (NeuN), subsequent quantification of cell density by automated cell counting. Neuron density was evaluated strictly in the right, ventral horn where GFP expression was greatest and cell density was compared between groups using a one-way ANOVA with Tukey post-hoc pairwise comparison. Neuron density between Ad.LC, Ad.LC.RBD, Ad.LC.TD, and Ad.GFP injected animals were not significantly different at 10^4 ($p=0.534$), 10^5 ($p=.323$), or 10^6 ($p=0.264$) TU.

Adenovirus spinal cord injections at 1×10^6 TU results in cell death and sensorimotor dysfunction

According to prior gene therapy studies, injections of high concentration of adenovirus into nervous tissue can result in prevalent neuroinflammation and cell death. Literature suggests at a concentration of 10^6 TU and higher, adenoviral injections can result in the production of inflammatory cytokines, increased concentration of reactive astrocytes, and decreased neuronal density[44]. Thus, to further characterize adenoviral BoNT gene transfer in functional neuroinhibition for clinical and research use we compared the sensorimotor function of animals

injected with Ad.GFP at 1×10^4, 1×10^5, and 1×10^6 TU as well as spinal cord NeuN counts at the end of the testing period. Animals injected with Ad.GFP at 1×10^6 TU displayed gradually greater deficits in grip strength over time ((p=.045), a trend that was not seen in animals injected at 1×10^4 and 1×10^5 TU (**Fig. 5A**). In addition, it was previously shown that sensorimotor deficit resulting from Ad.LC gene transfer at 1×10^4 TU spontaneously resolves by post-operative day 22 (**Fig. 5B)**. At a concentration of 1×10^5, Ad.LC injected animals display a similar trend, reaching significant post-operative deficit by post-operative day 16 (p=.004) but returning to normal values at day 22. However, at 1×10^6 TU, this spontaneous recovery for Ad.LC injected animals disappears with animals showing persistent behavioral deficit till the end of the testing period (p=.001). However, realizing that grip strength assessment carries inherent subjectivity and that the variability in motor deficit between animals was quite high, we deferred to NeuN counts for further confirmation. Cell counts revealed that the mean number of cells for animals injected with Ad.GFP at 1×10^6 TU was 105.95 ± 30.35 neurons per ventral horn, significantly lower than a mean of 156.99 ± 27.26 neurons per ventral horn for animals injected with Ad.GFP at 1×10^5. Thus, in agreement with previous scientific findings, we conclude that adenoviral gene expression induces neuronal death at viral concentrations greater than or equal to 1×10^6 TU. These observations support the use of

Ad.BoNT vectors for gene-base neuromodulation at concentrations not exceeding 1×10^5 TU in future research and clinical applications.

Discussion.

BoNT and other methods of focal neuroinhibition have achieved exceedingly prevalent roles in the treatment of neurological disorders in the clinic as well as the elucidation of neural circuitry in the laboratory. However, though effective in small doses, clinical BoNT injections are rendered sub-optimal by a need for repeated injections[17], concerns about immunogenicity[18, 19], toxin diffusion resulting in adverse effects[19, 45-47], and limited applicability to the PNS due to CNS toxicity[43] . Here we demonstrate not only the feasibility of vector-mediated BoNT gene transfer in inhibiting synaptic function but also the optimal transgene combinations and viral titer for future Ad.BoNT gene-based neuromodulation. Finally, our study replicates results shown previously in Teng et al.[48] , that neurons tolerate clostridium LC gene expression with functional deficit arising from LC expression alone and not neuronal death.

Neuromodulation and has swiftly become an indispensable treatment mechanism in the fields of functional neurosurgery and clinical neurology in the past several decades. Surgical resection has been a long practiced technique in the management of neurological disorders like epilepsy[49], while electrical stimulation, like DBS, has stirred a revival in the treatment of movement, pain, and neuropsychiatric disorders[50, 51]. However, surgical lesion techniques have largely fallen out of practice due to the irreversibility of the procedure and low spatio-temporal targeting[52]. Alternatively, DBS suffers from the need for an implanted electrode which leaves open the possibility of post-operative infection and also is unable to distinguish between specific cell types.[53] Optogenetics may provide a solution through vector-mediated expression of light sensitive ion channels and photostimuation to modulate cell-specific activity, but clinical application has been limited in the face of a variety of translational challenges[54]. Indeed, the need for a potent,

cell-targeted neuromodulatory therapy with a well understood physiological mechanism remains unfulfilled. Our current study demonstrates that the unilateral injection of BoNT trasngenes into the spinal cord results in focal neuroinhibition and anatomically discrete sensorimotor dysfunction. Animals receiving spinal cord injections of adenovirus containing BoNT transgenes at 4×10^5 displayed significant deficits on a variety of behavioral assays when compared to pre-operative baseline and control animals. Moreover, a bilateral comparison of grip strength deficit demonstrated that deterioration of motor function occurred only on the injected side. Unilateral gene expression was further confirmed through histological imaging of spinal cords showing focal BoNT transgene expression in the right anterior horn. Higher resolution confirmed vivid green fluorescence in the nuclei and cell body of pyramidal motor neurons without any significant changes neuron density between Ad.LC., Ad.LC.RBD, Ad.LC.TD, and Ad.GFP injected animals. Finally, long-term assessment of grip strength deficit in Ad.LC injected animals showed functional recovery back to baseline three weeks after surgery reflective of transient adenoviral gene expression[29]. Thus, we have established that gene-based neuromodulation employing the use of BoNT transgenes is a non-toxic, effective method of focal neuroinhibition.

Our study was aimed at proving the principle of using gene therapy as an approach for neuromodulation. We selected adenovirus as our viral vector of choice for its high transduction efficiency[55] and transient expression profile[56]. This allowed us to better visualize any behavioral changes resulting from Ad.BoNT gene expression including functional recovery coinciding with the timeline for adenoviral gene expression. Still, a myriad of cell-specific promoters[57, 58], capsid engineering techniques[59], and viral vectors[60] available exponentially increases the potential and flexibility of Ad.BoNT applications in both clinical and preclinical settings. For example, adeno-associated virus (AAV) would be an ideal vector for BoNT gene transfer for neurological disorders

in which conventional BoNT injections are the standard, such as spasticity. Due to its dependence on a helper virus to replicate and execute lytic infection, the risks for unwanted viral infection are highly reduced[61]. Furthermore, AAV1, AAV2, and AAV5 viral capsids display strong neuronal tropism for the targeting of discrete populations of spinal motor neurons[62]. This, combined with the use of neuron specific promoters like synapsin[63] and AAV's significantly longer transgene expression[64], may allow for BoNT gene transfer in focused neural inhibition of both the PNS and CNS without adverse toxin diffusion or a need for repeated injections.

In addition to the current study on BoNT gene transfer therapy, conventional BoNT itself has long been employed as the gold-standard of care for a variety of neurological disorders[65, 66]. As a result of its prevalent clinical applications as well as studies on the clostridium botulinum bacteria and botulin poisoning, the neurophysiological mechanism of BoNT action is extremely well studied[67]. Of relevance to the current study is research conducted on the cooperative actions of the LC, RBD, and TD on BoNT serotype A (BoNT/A) enzymatic activity. The RBD determines cellular specificity by binding to surface protein receptor SV2, resulting in receptor-mediated endocytosis[21, 68]. The TD is then believed to serve as transmembrane pore for the LC to exit from the endocytic vesicle and initiate proteolysis of SNAP-25 in the SNARE complex. [20, 22, 69]. Distal to the neuromuscular junction, the RBD has also been implicated in the transport of BoNT across gut epithelial cells lining the mucosal surface in food born botulism[20, 70]. BoNT transport across the intestinal lumen was found to be mediated by clathrin dependent transport initiated by the RBD. The role of the RBD in BoNT transport has also been identified at the site of BoNT injection. Two preeminent studies conducted by Wiegand et al. and Haber displayed the retrograde transport of radiolabeled BONT from the injection site in the gastrocnemius to the corresponding spinal cord level[71, 72]. From then on, a wealth of studies has been conducted on the retrograde and anterograde

transport of locally injected BoNT. In these cases, the RBD again carries the minimum determinants necessary for long-range fast-axonal transport of BoNT within neurons - although mechanisms and exact cellular substrates involved remain to be elucidated [25, 73, 74]. Here, we demonstrate that Ad.LC.RBD injections resulted in more consistent and significant neural inhibition and concomitant sensorimotor deficit in the injected limb than Ad.LC or Ad.LC.TD. In vitro assays showed no significant differences in transduction between adenoviral vectors and histological comparisons showed no observable difference in transgene expression in spinal cords of animal injecteds with Ad.LC, Ad.LC.RBD, and Ad.LC.TD. Differences in cytotoxicity between vectors was considered but quantification of neuron density between animals revealed no significantly difference quickly dispelling this concern. Upon reflection, we believe our findings agree with previous research on the mechanisms of action and transport of BoNT in neurons. Part of our rational stems from the fact that vector-mediated BoNT gene transfer naturally circumvents several key steps in BoNT's natural mechanism of action such as receptor-mediated endotyosis, formation of a transmembrane pore, and LC escape into the intracellular space. In Ad.BoNT gene expression, all toxin components will be transcribed and translated at and near the nucleus of infected neurons. Thus, differences in sensorimotor function after Ad.LC, Ad.LC.RBD, and Ad.LC.TD injection must arise from either difference in catalytic activity of the LC or differences in the rates of transport of BoNT proteins from the cell body to the axon terminal. Indeed, literature on the role of the RBD in BoNT toxicity point to the latter hypothesis, but further testing is necessary.

The functional recovery seen in Ad.LC injected animals coinciding with adenoviral gene expression is an interesting phenomenon. While this may point to a reduction in intracellular LC concentration due to a decline in gene expression, the effects of clinical BoNT treatment persist

for months, much longer than the average turnover rate for cellular proteins[75]. In addition, any indication of functional recovery was absent in Ad.LC.RBD and Ad.LC.TD injected animals. An explanation regarding this discrepancy is critical in understanding the neurophysiological mechanisms to inform future applicability of focused, gene-based BoNT neural inhibition. The long-lasting inhibition of synaptic communication is a characteristic feature of BoNT/A therapy and stands in stark contrast with BoNT/E exposure, with the effects of the latter being rather short-lived despite sharing the same substrate, SNAP-25[76]. A current theory contends that because BoNT/A and BoNT/E cleave SNAP-25 at different sites[77] the differing proteolytic fragments of SNAP-25 may contribute to effect duration by disrupting normal function at the pre-synaptic membrane[78, 79]. Still, these propositions remain heavily controversial and would fail to explain the differing effect duration between domain combinations of the same BoNT serotype. Alternatively, researchers have proposed that the longevity of BoNT/A intoxication derives from the persistence of LC presence in the cytosol and resistance to enzymatic breakdown. Tsai et al. has shown that BoNT/E LC, in comparison to BoNT/A LC, is much more susceptible to ubiquitin dependent proteolysis (UDP) due to increased interactions with the TRAF2 ubiquitin signaling protein[80]. It is currently unclear what contributions, if any, the BoNT/A HC has to LC UDP resistance. A potential role the BoNT/A HC may having in conferring resistance to enzymatic breakdown is its role in cell entry, LC translocation, and intracellular trafficking. Shoemaker et al. hypothesizes that differing cell entry or endosomal release processes between toxin subtypes domains could lead to differences in LC trafficking to its site of action or exposure to cytosolic degradation pathways[81]. Unique trafficking of BoNT/A LC could potentially allow it to reach pre-syanptic SNARE complexes faster and through protected environments that results in less damage to the LC protease. We hypothesize that this may be due to interdomain interactions between the LC

and fragments of the RBD and TD that serve to chaperone and protect the LC from enzymatic breakdown. This hypothesis would certainly explain the discrepancies in apparent functional of Ad.LC injected animals between behavioral tests. Functional recovery was most apparent in the distal muscles implicated in grip strength as opposed to more proximal muscles involved in BBB scoring. Difficulty with LC transport down long axons through hazardous cellular compartments to distal muscles could explain the isolated functional recovery seen on grip strength as adenoviral gene expression declined. Ultimately, it is currently unclear the neurophysiological mechanisms contributing to the persistent behavioral effects seen in Ad.LC.RBD and Ad.LC.TD injected animals and further investigation is needed.

Finally, adenovirus is one of the most common viral vectors employed in gene therapy and is considered the most efficient gene delivery system among the current selection of viral vectors[82]. However, a drawback of adenoviral gene transfer is that the capsid can induce a potent inflammatory response which may reduce the viruses efficiency in transducing cells and even result in neurodegeneration[44, 83, 84]. A strong immune response is most often seen at viral concentrations at or above 10^5 or 10^6 TU with degenerative neuron morphologies appearing at higher concentrations[85-87]. In our study, we observed a significant reduction in mean neuron density (-32%) in the injected anterior horns of control animals injected with Ad.GFP at 1×10^6 TU but not at 1×10^4 or 1×10^5 TU. This coincided with the appearance of sensorimotor dysfunction in control animals – approximately a 48% decline in grip strength - as well as the elimination of delayed functional recovery in Ad.LC injected animals at the magnitude of concentration. For reference, in patients with amyotrophic lateral sclerosis, a 50% decline in motor unit density roughly corresponded to a 20% decline in grip strength[88]. Thus, in concordance with previous literature, we conclude that any behavioral deficits seen with Ad.BoNT injections at concentrations

higher than 1×10^6 TU are not a result increased synaptic inhibition but rather rampant neuroinflammation and neuron death. Therefore, vector bioactivity and immunogenicity should be comprehensively reviewed for future clinical or research applications of vector-mediated BoNT gene transfer.

In addition, it is important to underscore that BoNT/A LC is inert prior to entering target neurons expressing SV2 receptors on cell membrane. Cell entry, as described above, is dependent on binding and translocation from the endocytotic vesicle mediated by the RBD and TD respectively. An absence of any of the components of this tri-modular toxin will effectively preclude the LC from exiting or entering any cells that were not originally transduced by viral vectors. This prevents vector-mediated BoNT gene transfer from having any other adverse physiological effects and posing a risk to animal health. Correspondingly, no deleterious side effects were observed in our experimental animals.

In summary, we have shown that Ad.LC.RBD mediated gene transfer can result in long-term, focal neural inhibition and concomitant sensorimotor deficit without cytotoxicity. The use of gene therapy as an approach for BoNT therapy provides several advantages over conventional intramuscular injection. Sustained gene expression can provide extended therapeutic relief thereby eliminating the need for repeated injection and cell-specific targeting can prevent toxin diffusion and adverse effects. Furthermore, selective BoNT/A LC.RBD gene delivery now allows for the use of nature's most potent neural inhibitor for the treatment of neurological disorders and the elucidation of neural circuitry in the CNS.

Tables and Figures

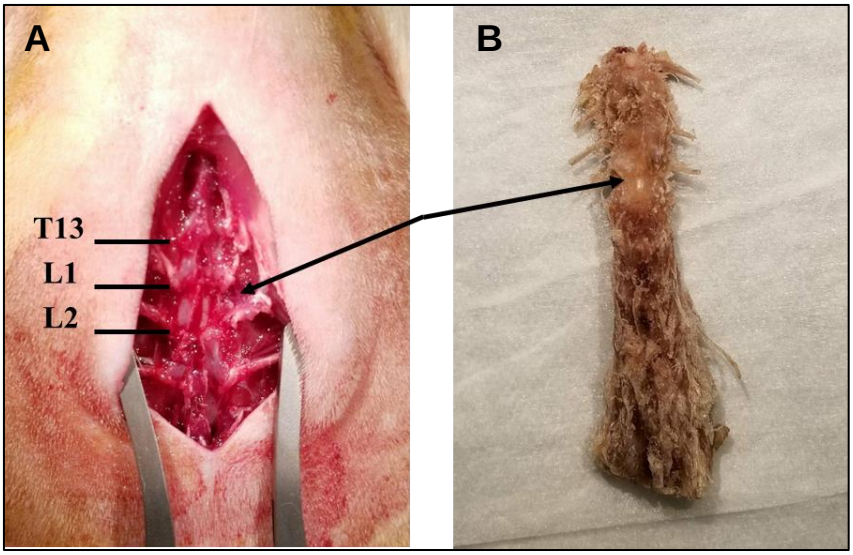


Figure 1: Images showing midline incision with corresponding vertebral levels and laminectomy site. **(A)** Identification of T13, L1, and L2 vertebra and laminectomy show underlying L3-L4 cord. **(B)** Identification of laminectomy site showing underlying cord (white) after transcardiac perfusion and dissection.

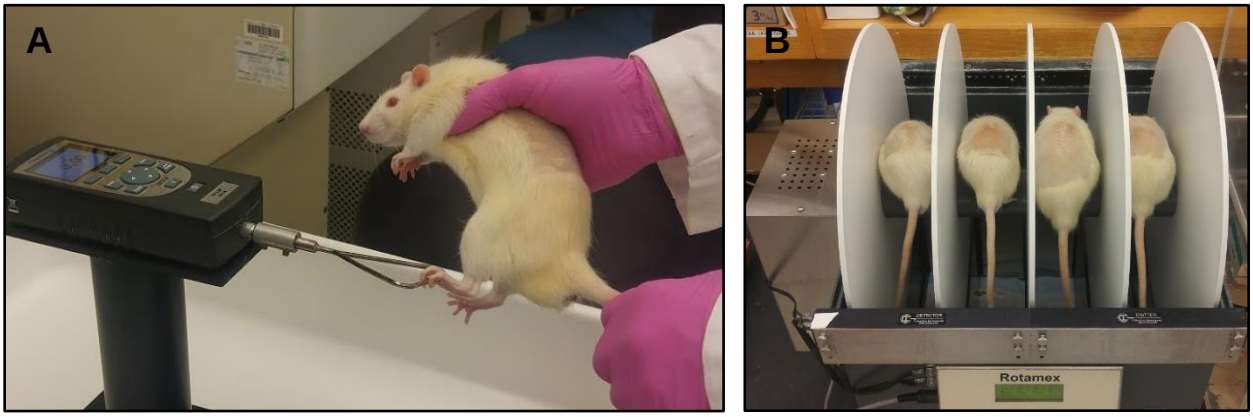


Figure 2: Grip strength and rotarod assays to test sensorimotor function. **(A)** A demonstration of rat handling for individual hind paw grip strength tests. **(B)** Rotarod tests rats balance and coordination as they run on a spinning dowel at increasing speeds.

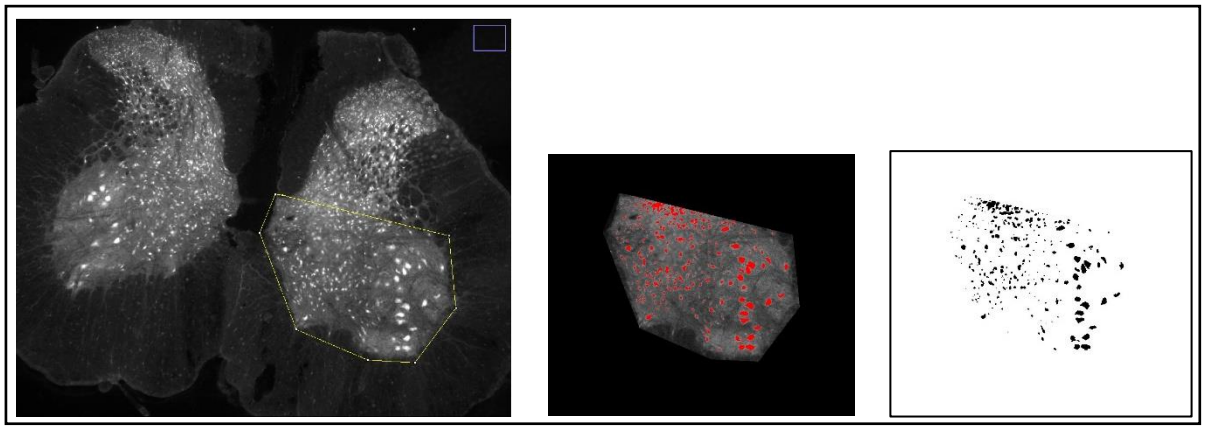


Figure 3: ROI selection, thresholding and particle analysis procedure used to quantify NeuN staining density in the right anterior horn of rat spinal cords.

Vector	Plasmid	Encoded Amino Acids	Toxin Domain
pAd.BoNT4	pACCMVpLpA(-)loxP-SSP	Met1-Thr439	LC
pAd.BoNT5	pACCMVpLpA(-)loxP-SSP	Met1-Ile870	LC-TD
pAd.BoNT6	pACCMVpLpA(-)loxP-SSP	Met1-Thr439-Ile870-end	LC-RBD

Table 1

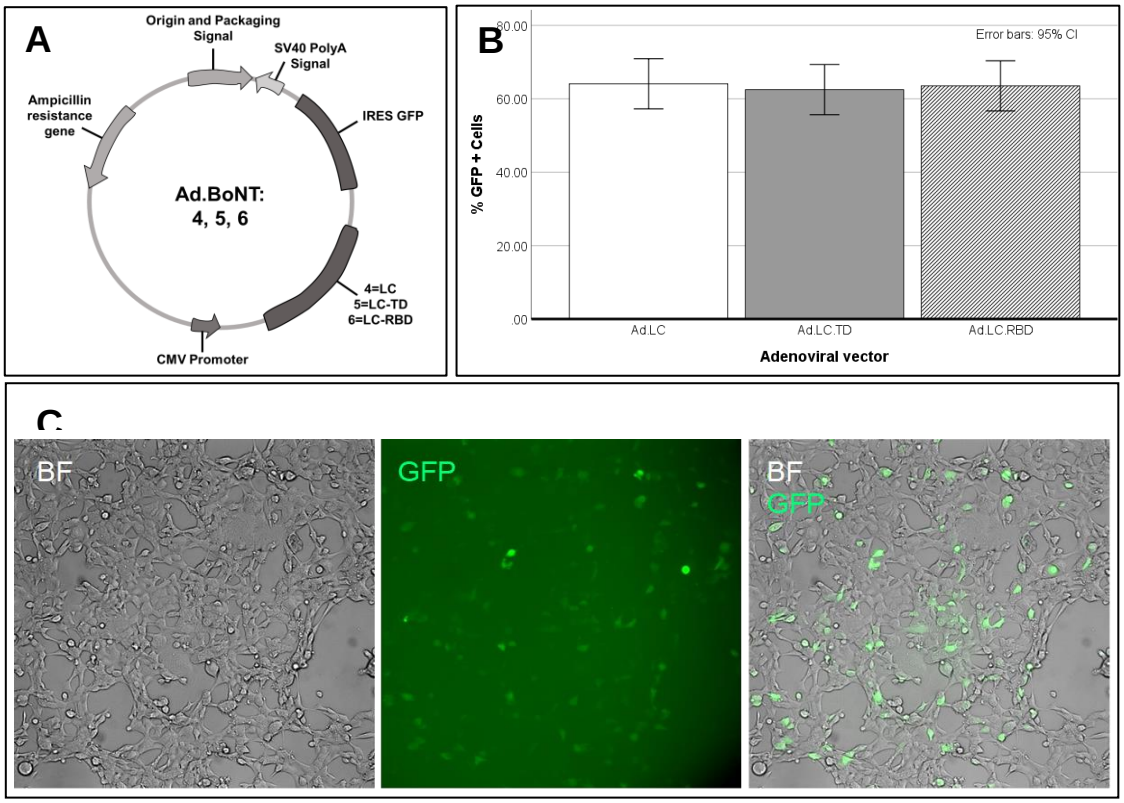


Figure 4: Plasmid map and adenoviral vector gene expression in HEK 293 cells. **(A)** A schematic diagram of the shuttle plasmid engineered to produce AdLC, AdLC.TD, and Ad.LC.RBD adenoviral vectors. **(B)** Adenoviral vectors were tested *in vitro*. HEK 293 cells, grown to 50-60% confluency in 6-well plates, were infected with Ad.LC, Ad.LC.TD, Ad.LC.RBD, or an Ad.GFP control at an MOI of 5. After 48 hours, cells were imaged for GFP expression. **(C)** Adenoviral vectors had nearly identical transduction efficiencies.

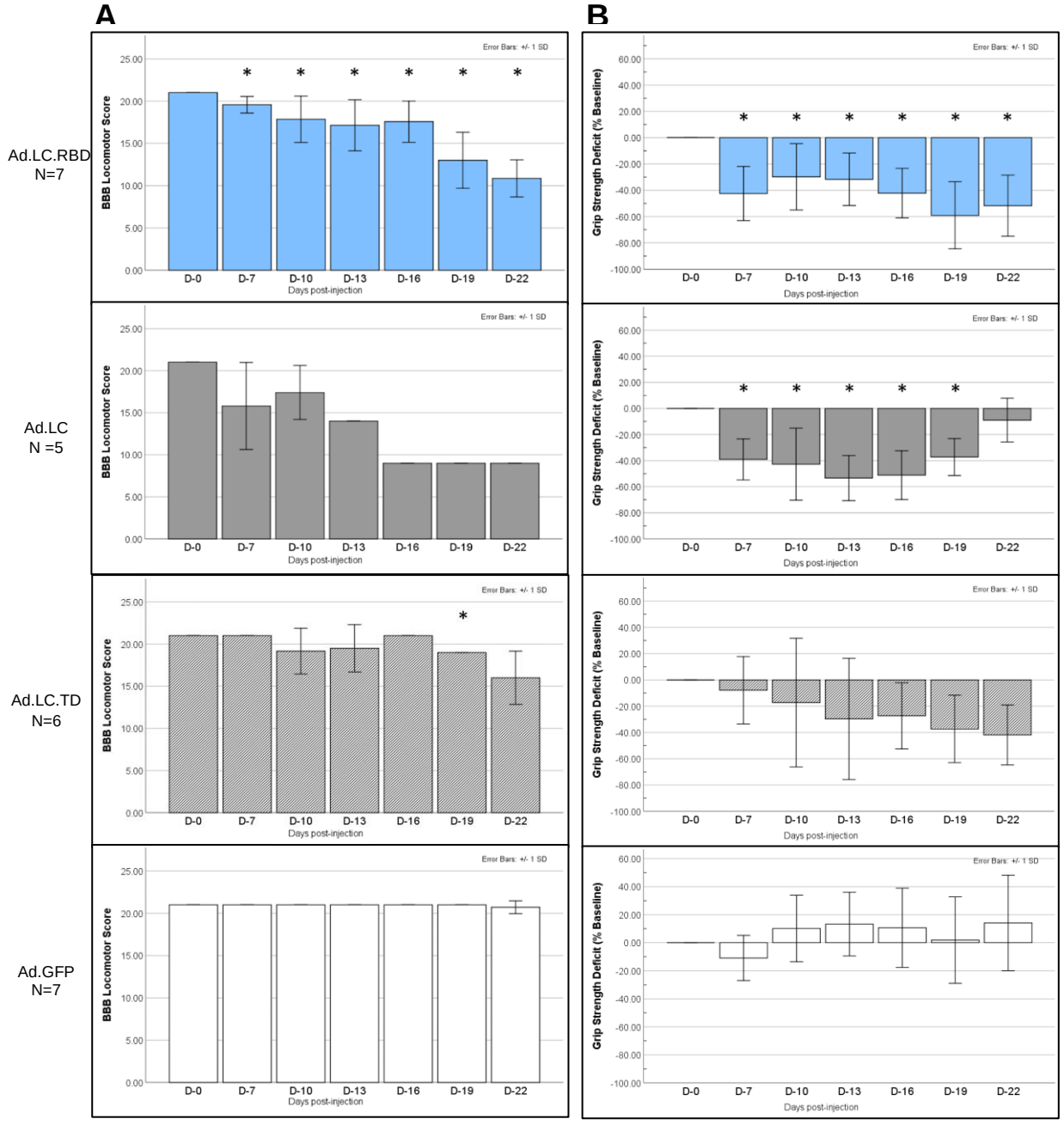


Figure 5: Ad.BoNT gene transfer into the spinal cord results in sensorimotor deficit. **(A)** Rats injected with 4×10^4 TU of Ad.LC.RBD, Ad.LC, and Ad.LC.TD show trends toward motor dysfunction compared to baseline as measured by BBB score with Ad.LC.RBD injected animals displaying the most statistically significant and sustained deficits. **(B)** Hind-limb right sided grip strength derived by comparing percent baseline difference in treated and untreated sides. Ad.LC.RBD, Ad.LC, and Ad.LC.TD injected animals display gradual increase in grip strength deficit reflecting hindlimb sensorimotor dysfunction. Ad.LC.RBD injected animals again show significant sustained motor deficit throughout the 22-day testing period while animals injected with Ad.LC spontaneously show full recovery 22 days after surgery corresponding to the time course for adenoviral gene expression.

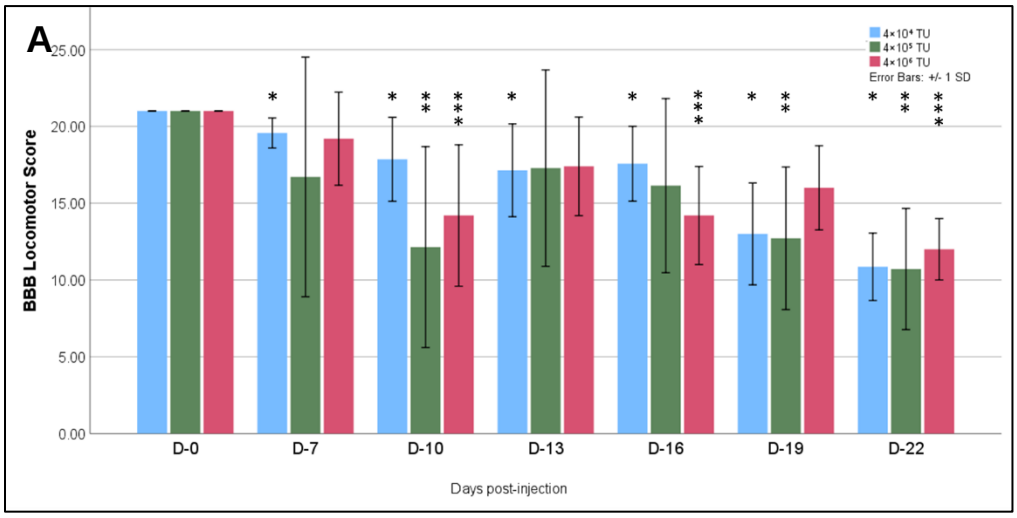
BBB (GFP reference)

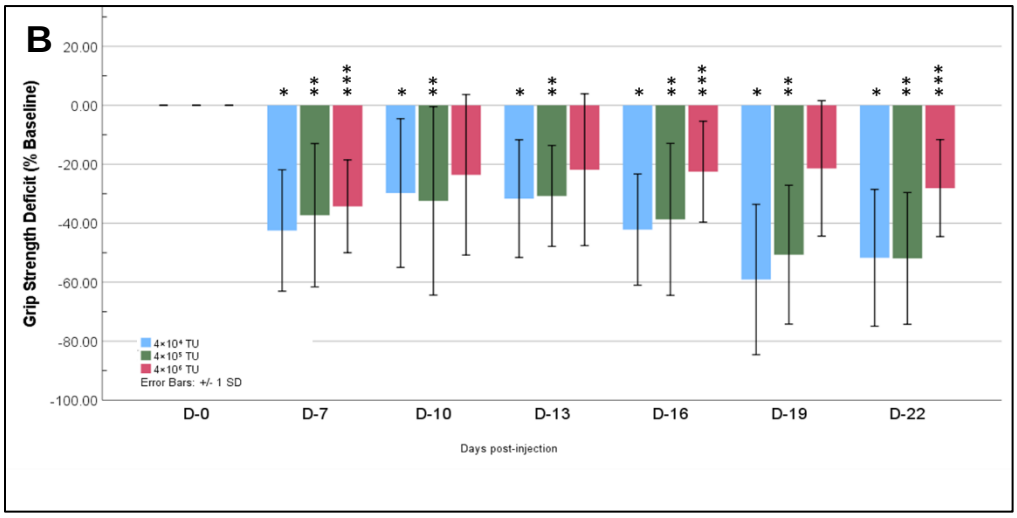
Day / Group	D-0	D-7	D-10	D-13	D-16	D-19	D-22
Ad.LC.RBD	p=1.00	p=0.661	p=.102	p=.013	p<.001	p<.001	p<.001
Ad.LC	p=1.00	p=0.005	p=.082	p<.001	p<.001	p<.001	p<.001
Ad.LC.TD	p=1.00	p=0.536	p=.590	p=1.00	p=.986	p=.210	p=.002

Grip strength (GFP reference)

Day / Group	D-0	D-7	D-10	D-13	D-16	D-19	D-22
Ad.LC.RBD	p=1.00	p=0.035	p=.132	p=.037	p=.002	p=.001	p<.001
Ad.LC	p=1.00	p=0.106	p=.051	p=.004	p=.001	p=.071	p=.439
Ad.LC.TD	p=1.00	p=0.993	p=.447	p=.061	p=.038	p=.053	p=.004

Table 2


A
BBB Locomotor Score
25.00
20.00
15.00
10.00
5.00
0.00
4×10⁴ TU
4×10⁵ TU
4×10⁶ TU
Error Bars: +/- 1 SD
D-0
D-7
D-10
D-13
D-16
D-19
D-22
Days post-injection


B
Grip Strength Deficit (% Baseline)
20.00
0.00
-20.00
-40.00
-60.00
-80.00
-100.00
4×10⁴ TU
4×10⁵ TU
4×10⁶ TU
Error Bars: +/- 1 SD
D-0
D-7
D-10
D-13
D-16
D-19
D-22
Days post-injection

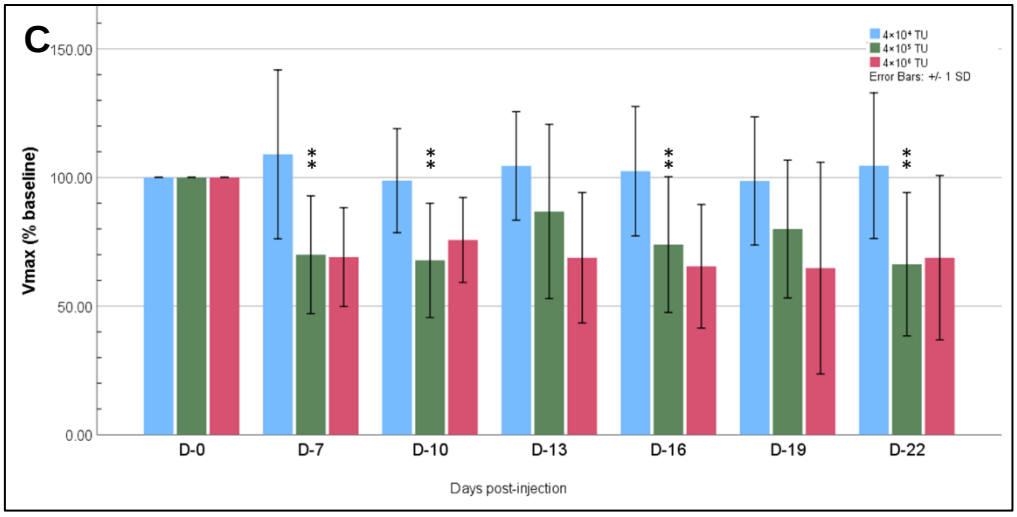

C
Vmax (% baseline)
150.00
100.00
50.00
0.00
4×10⁴ TU
4×10⁵ TU
4×10⁶ TU
Error Bars: +/- 1 SD
D-0
D-7
D-10
D-13
D-16
D-19
D-22
Days post-injection

Figure 6: A comparison of rat hindlimb sensorimotor dysfunction induced by Ad.LC.RBD gene delivery at 4×10^4, 4×10^5, and 4×10^6 TU. All indications of significance are in reference to baseline values. **(A)** Ad.LC.RBD injections do not induce a titer-dependent deficit in locomotor function assessed by BBB scoring. **(B)** Ad.LC.RBD injections do not induce a titer-dependent reduction in grip strength. **(C)** Ad.LC.RBD spinal cord injections at 4×10^5 TU results in significantly decreased rotarod V_{max} compared to baseline values but not at 4×10^4 or 4×10^6 TU.

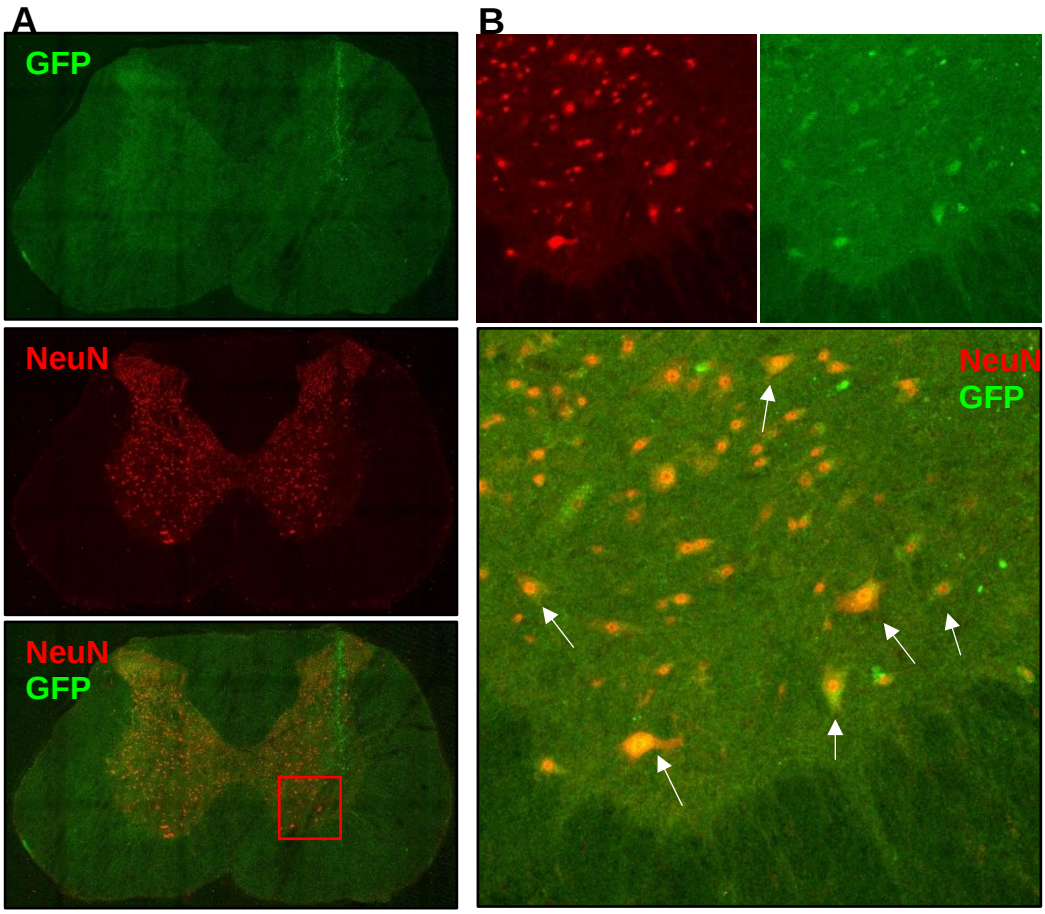


Figure. 7: Confocal images showing adenoviral gene expression with needle tract at the site of rat lumbar spinal cord injection. (A) Representative composite of tile images taken at 10x show unilateral GFP expression along a needle tract injecting into the right anterior horn. Images were taken 22 days after adenoviral injection; this animal was injected with Ad.LC.TD (4×10^5 TU). (B)

20x magnification reveals prevalent coexpression of GFP and NeuN (neuronal nuclei) fluorescence in pyramidal alpha motor neurons identified by their distinct morphology.

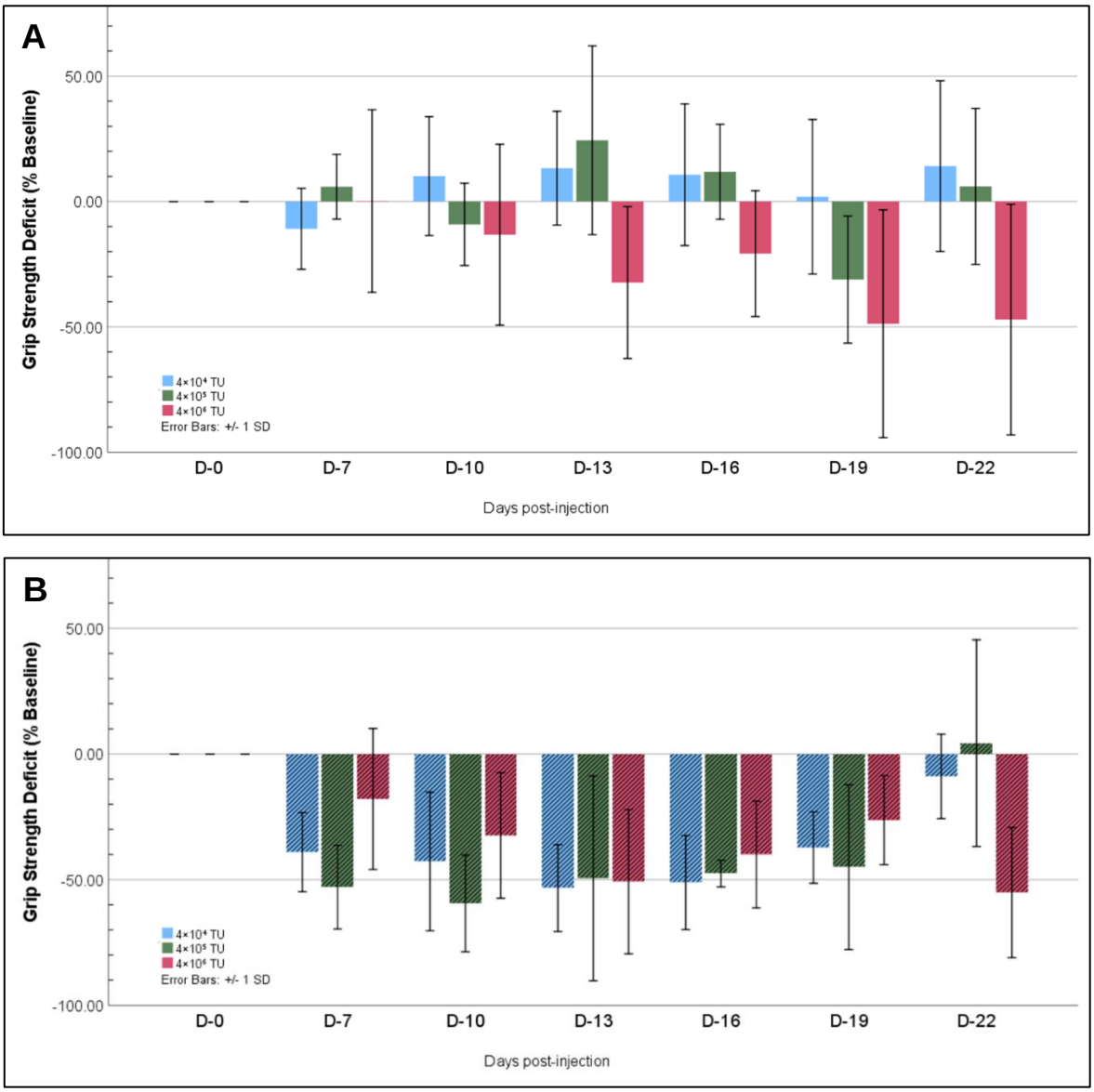


Figure 8: Trends in grip strength deficit after Ad.GFP and Ad.LC injection at different titrations. **(A)** Post-operative grip strength values in Ad.GFP injected animals at 1×10^4, 1×10^5, and 1×10^6 TU. Animals that received spinal cord injections at 1×10^6 TU displayed a trend towards increased grip strength deficit not seen at lower titer injections. **(B)** Post-operative grip strength values in

Ad.LC injected animals at 4×10^4, 4×10^5, and 4×10^6 TU. Animals that received Ad.LC injections at 4×10^4 and 4×10^5 TU display spontaneous recovery back to baseline approximately 19 days after surgery in concordance with the decline of adenoviral gene expression. This functional restoration is absent in animals injected with Ad.LC at 4×10^6 TU.

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