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Christina A. Krudy April 14th, 2010

Gene-based Neuromodulation in the Central Nervous System via an Inwardly Rectifying Potassium Channel

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Abstract

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By Christina A. Krudy

Neuromodulation therapies aim to correct imbalanced neural activity, which underlie diseases such as epilepsy, pain, and spasticity. Gene-based neuromodulation employs viral vectors to alter neural activity at a molecular level with greater specificity and reversibility. The inward rectifying potassium channel 2.1 (Kir2.1) has the potential to inhibit excessive neural activation. Kir2.1 acts to stabilize membrane potential below the activation potential of voltage gated sodium channels. Therefore, we hypothesized that with adenoviral-mediated delivery of Kir2.1 to the spinal cord, motor neurons would become stabilized below the level of action potential threshold and thus exhibit an inhibitory effect on neuromuscular function. Additionally, the Kir2.1 transgene was placed under an inducible promoter, Rheoswitch®, which becomes activated only in the presence of its orally administered ligand. *In vitro* analysis confirmed the ability of the Rheoswitch® promoter to effectively regulated Kir2.1 gene expression. Unilateral injections of the viral vector to lumbar spinal cord of rats demonstrated neuromuscular inhibition exclusively in rats that received the ligand. Histological analysis showed evidence of both Kir2.1 gene expression as well as motor neuron loss in rats that received ligand. Rats that did not receive ligand did not express the transgene, had no functional deficits, and contained normal numbers of motor neurons. Expression of Kir2.1 appears to be toxic to motor neurons. To compare vector expression in a different neuronal population, unilateral injections targeting the hippocampus were performed. Hippocampal neurons are known to endogenously express the Kir2.1 channel, whereas it is not normally found in motor neurons. However, the vector was unsuccessful at transducing hippocampal neurons at low volumes. Future experiments are needed to confirm the safety of this vector and whether Kir2.1 toxicity is exclusive to motor neurons.

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Introduction

The traditional neuroscience definition describes neuromodulators as a group of chemical agents in the nervous system that can either potentiate or inhibit a nervous impulse. Within the last two decades, the medical field has applied this concept to treat disorders such as epilepsy, pain, and spasticity that result from an imbalance in the activity of neural pathways. The application of medical neuromodulation involves the specific alteration of neuronal activity and is employed when pharmacological intervention is unsuccessful. Focal inhibition of neural activity has been proven effective in restoring neural function in such disorders (Krames 2002). Common techniques employed by neurosurgeons include focal lesioning, implanted drug pumps or electrodes, and deep brain stimulation (DBS) (Bittar 2006, Day 2000). However, there are significant limitations to these approaches. Lesions are not reversible or adjustable. Implanted hardware can be complicated by infection, device malfunction, and the need for repeated surgeries. While DBS is the most successful, it lacks specificity and its exact mechanisms are still unknown.

To improve the methods of neuromodulation, research is shifting focus to manipulating neural activity at a molecular level (Federici and Boulis 2007, Mata and Fink 2007). Gene-based neuromodulation seeks to employ different genes whose function could modify nervous impulses. There are several different mechanisms within neurons that offer themselves as potential targets to enhance or reduce electrical activity. These include altering ion channels, neurotransmitters, vesicle-docking proteins, re-uptake proteins, receptors, and second messengers. Genes encoding for proteins that are involved in these pathways could be therapeutic if expressed under the appropriate

circumstances.

Viral vectors allow for the ability to deliver novel genes to a cell population in an organism. By nature, a virus inserts its own genome into a cell and takes advantage of the host's cellular pathways in order to replicate (Thomas et al. 2003). Viral vectors exploit this behavior to express a new gene. Replication and pathogenic genes are replaced with the transgene of interest. A genetically reconstructed virus can carry a therapeutic gene that is then injected into a host cell for protein expression. A variety of viruses are used as vectors, including lentivirus, adenovirus, adeno-associated virus (AAV), and retrovirus. Viral vectors differ in cloning capacity, duration of gene expression, inflammatory response, potency of expression, and specificity of the cell type that is transduced. Over the past two decades, researchers have demonstrated the efficacy of different viral vectors in many organisms (Tomanin and Scarpa, 2004). Viral vectors are therefore a promising method for gene therapy applications. The use of viral vector based therapy offers many advantages over current methods of focal inhibition treatment. Viral gene therapy can provide molecular specificity that can manipulate specific pathways in the generation of an action potential. Furthermore, it eliminates destruction and permanent alteration of neural pathways and has the ability to be regulated.

The inwardly rectifying potassium channel 2.1 (Kir2.1) has presented itself as a promising candidate for translation in the field of neuromodulation. The Kir2.1 channel is a voltage gated ion channel that works to repolarize the cell membrane after approaching threshold or an action potential (Nichols and Lopatin 1997). The Kir2.1 channel has been identified as one of the strongest inward rectifiers of the 15 known mammalian inward rectifier potassium channels (Pruss 2005). It is expressed ubiquitously in cardiac and

neuronal brain cells, but is highly concentrated in the dentate gyrus of the hippocampus (Pruss 2005).

Characterization of the potassium channel first emerged in cardiology research. The Kir2.1 channel has been primarily studied with regards to mechanistic questions in cardiac function. Clinical research has found a reduction in inwardly rectifying potassium currents in patients with heart failure (Beuckelmann et al. 1993). Mice knockouts lacking the Kir2.1 gene were used to examine cerebral vasculature (Zaritsky et al. 2000). Although these mice die within 8-12 hours after birth, researchers found that the Kir2.1 channel was a necessary component for inward potassium currents in cerebral arterial myocytes (Zaritsky et al. 2000). Additionally, mutations in the Kir2.1gene, *KCNJ2*, are known to cause Andersen-Tawil syndrome, a heart disorder that is a form of long QT syndrome (Plaster et al. 2001). Long QT syndrome is a prolongation of cardiac action potentials, in this case due to a dysfunctional Kir2.1 channel that leads to a reduction in repolarizing K+ currents. Patients with Andersen-Tawil syndrome exhibit periodic paralysis and ventricular arrhythmias. Adenoviral-mediated delivery of Kir2.1 to ventricular myocytes was done in guinea pigs to determine the physiological effects of Kir2.1 over-expression (Miake et al. 2003). Electrophysiological results showed shortened action potential duration, hyperpolarized the resting membrane potential, and accelerated repolarization compared to control cells (Miake et al. 2003).

The electrophysiological properties of Kir2.1 have also been explored within neurons as well. *In vitro* experiments demonstrated the ability of adenoviral-mediated Kir2.1 expression to inhibit both induced and spontaneous electrical activity in cultured superior cervical ganglion neurons (Johns et al., 1999). In the same experiment, Johns et

al. were able to effectively reverse inhibition through administration of barium (Ba^{+2}), which selectively blocks Kir2.1 channels. Further *in vitro* experiments utilized over-expression of Kir2.1 in cultured neonatal hippocampal neurons to study plasticity and were able to successfully reduce excitability in these cells (Burrone et al. 2002, Hartman et al. 2006).

Subsequent *in vivo* and *in ovo* studies have continued to show the inhibitory effects of the Kir2.1 channel. Several studies have effectively delivered Kir2.1 to the central nervous system (Yu et al. 2004, Mizuno et al. 2007, Yoon et al. 2008, Favero et al. 2009, Howorth et al. 2009). Kir2.1 has been delivered via an avian replication-competent retroviral vector to chicks *in ovo* to demonstrate how inhibition of neuronal firing in development can prominently alter the electrical properties of developing motor neurons (Yoon et al. 2008). Neuronal differentiation and formation of neuromuscular structure is guided by spontaneous electrical activity in chicken embryos. Such activity generates limb movements, or kick, that are visibly seen during development. Kir2.1 expression in lumbar motor neurons was able to inhibit electrical activity and reduce the number of kicks compared to control groups (Yoon et al. 2008).

One *in vivo* study over-expressed Kir2.1 in muscle fibers of mice to determine its effect on activity dependent competition in the central nervous system (Favero et al. 2009). Normally, neonates are born with multiple axons innervating the same muscle fiber. Through excitatory competition between these axon terminals, elimination occurs and only one axon is left to innervate that muscle fiber. Using electroporation of DNA plasmids, they found that Kir2.1 inhibits synapse elimination by reducing the neurons ability to achieve an action potential (Favero et al. 2009). They also confirmed a more

negative resting membrane potential, consistent with the findings from Miake et al. (2003). Most recently, one group found that expressing Kir2.1 channels in pontospinal noradrenergic (NA) neurons caused increased sensitivity to thermal nocioception (Howorth et al. 2009). An adenovirus encoding for the Kir2.1 channel was injected into the lumbar dorsal horn where it was taken up by the axon terminals of NA neurons and retrogradely transported (Howorth et al. 2009).

In this study, we attempt to regulate neuromuscular function with adenoviral-mediate expression of Kir2.1 in lumbar spinal cord motor neurons. We hypothesize that over-expression of Kir2.1 should make it more difficult for a motor neuron to achieve an action potential, thereby inhibiting motor function. Our laboratory has developed an effective model to characterize viral vectors for gene-based neuromodulation, which involve intraparenchymal spinal cord injections and behavioral measurements (Teng et al., 2004). To show proof of principle for this experiment, we have chosen adenovirus for our viral vector. Although the adenovirus has a higher inflammatory response compared to AAV and lentivirus, it has one of the largest cloning capacities (up to ~7.5 kb), transduces all cells types (dividing and non-dividing) with 100% efficiency, and turns on expression quickly. For the purpose of this experiment, the adenovirus has been chosen to investigate the preliminary effects of Kir2.1 (Lowenstein et al. 2003, Boulis 1999).

Cell cultures assays were included to confirm the viability of our viral vector before moving into the animal model. Targeted injections of our viral vector to motor neurons in the lumbar spinal cords of rats would allow for a blind observer to assess whether neuromuscular function is impaired due to the silencing effect of Kir2.1. Since the Kir2.1 channel is not endogenously expressed in motor neurons, we deliver the same

vector to hippocampal neurons in the brain where Kir2.1 expression is well-documented (Pruss et al. 2005). Lastly, since regulation of viral vectors is desirable for clinical translation, Kir2.1 expression was placed under the control of an inducible promoter.

Materials and Methods

Vector Construction

Kir2.1 cDNA was fused with Green Fluorescence Protein (Kir-GFP). Originally, the expression of Kir-GFP was placed directly under the cytomegalovirus (CMV) promoter in the adenoviral vector. However, attempts to replicate this vector were unsuccessful because constitutive expression of the gene in producer cell lines had a toxic effect on the cells. Therefore, the Rheoswitch® Therapeutic System (RTS), produced by Intrexon Corporation (Blacksburg, Virginia), was inserted into the vector to achieve regulatory inducible expression of Kir-GFP. The expression cassette is depicted in Figure 1. The expression of Kir-GFP is under the GAL4 Response Element (RE) promoter. The GAL4 protein has a DNA binding region that recognizes and binds specifically to the Response Element to inhibit transcription of the gene sequence. The production of GAL4 is constitutively expressed under the CMV promoter. Thus, the constant presence of GAL4 binds to the GAL4 RE promoter region and blocks transcription of Kir-GFP. In the presence of the orally administered RheoChem™ induction ligand (RG-115819), RL binds to GAL4 and causes a conformational change that activates transcription of the Kir-GFP gene. In the absence of the induction ligand, expression of Kir-GFP does not occur. Toxicity and dosage studies have been completed and reported by Intrexon Corporation for the RheoChem™ induction ligand. The expression cassette was inserted into an

adenovirus and then purified and amplified in human embryonic kidney (HEK) 293 cells. The final titer of the vector was 1.1 x 10^{12} plaque forming units/milliliter (PFU/ml). PFU is a functional measurement of how many viral vectors will actually transduce a cell with the proper gene as opposed to vectors which are defective or do not reach their cell target.

Cell Culture Assay

SH-SY5Y is a human neuroblastoma cell line obtained from ATCC (Manassas, VA). The cells were grown in medium containing a 1:1 mixture of Eagle's Minimum Essential Medium (EMEM) and Ham's F12 Medium (90%), supplemented with 10% fetal bovine serum (FBS). To determine optimal multiplicity of infection (MOI), which is a calculation of how many vector particles transduce a single cell, a preliminary dilution series of 10^4, 10^3, 10^2, and 10^1 was done (3 wells per group). Cell cultures were treated at 50% confluence. Vector was added to fresh media with the addition of 0.1mM ligand. Plates were incubated at 37˚C for 24 hours before the vector was removed and fresh media with 2mM 5-fluoro-2'-deoxyuridine (FUDR, Sigma) was added to stop cell division. After another 48 hours, cell cultures were assessed for GFP expression and cell density.

Based on the findings of the series dilution, the optimal MOI dosage was used to transduce cells at 50% confluence. Each well received the same MOI of Ad.Rheo.Kir2.1-GFP. One group (n=6 wells) received 1ul of 01.mM RL for each media change while the other group (n=6) received the same media changes but not the ligand. Cells were incubated at 37° for 48 hours with the viral vector before media was renewed. After an additional 24 hours, cells were fixed in 4% PFA for 20 minutes and rinsed with PBS.

Cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) at 1:10,000 dilution and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Applied Sciences) staining was performed at 1:4 dilution for apoptotic cell death. Cells were analyzed under a fluorescent microscope (Leica) and images captured for cell counts with a Retiga EXi camera (Q Imaging). Five images were randomly taken from each well. Each DAPI positive/TUNEL positive cell was counted and averaged for both conditions.

Intraspinal Cord Delivery

All protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Surgery was performed on approximately 8-12 week old Sprague Dawley wild type rats from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with isoflurane (2% inhalation in oxygen) and placed in a stereotaxic frame (Stoelting). The paraspinous muscles were infused with 0.2ml of 1% lidocaine prior to incision to reduce muscleskeletal pain. A laminectomy was performed to expose the L1 region of the spinal cord. The dura mater was incised with a 27 ½ G needle followed by unilateral injections of Ad.Rheo.Kir2.1-GFP into the lumbar spinal cord parenchyma. A micropipette (approximately 100 μm diameter) was mounted onto an oocyte injector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific) and advanced 1.5mm to target the ventral horn region. In order to achieve widespread distribution, 2.5μl of vector at the highest titer were injected at sites approximately 3mm apart and 1mm lateral to the midline. A volume of 50nl (x50) was injected every 10 seconds and then left to diffuse for 1 minute. All 16 rats were injected with Ad.Rheo.Kir2.1-GFP and 8 rats were randomly chosen to receive the induction ligand. The ligand was administered orally on a daily basis (50 mg/kg) starting immediately after

the surgery and 6 days post-operatively for a total of 7 doses. Following suture, buprenorphine (0.1ml subcutaneous) was given to reduce pain and rats were placed on heating pads for 30 minutes. Rats were housed in the Emory University Division of Animal Resources and allowed water and food *ad libitum*.

Behavioral Analysis

For animals undergoing intraspinal cord (ISC) injections, baseline behavior measurements were performed on each rat the day prior to surgery in order to assess motor function. After 24 hours of rest following surgery, all rats underwent behavioral testing at post-operative days 1, 4, and 7. As stated above, rats assigned to the ligand group received daily doses for 7 days. In order to determine if there is a recovery of neuromuscular function after ligand administration is stopped, 3 animals randomly chosen from each group had additional behavioral testing on days 14 and 21. To clarify, rats that received behavior up to Day 7 were then sacrificed that day whereas the rats that received additional behavior were sacrificed on Day 21. Since complications from surgery including spinal cord injury manifest themselves within 24 hours, any rats showing impairments were eliminated from the study (Teng et al. 2005). Behavioral assays included grip strength and locomotor assessment. An automated grip strength meter (Columbus Instruments) measured hindlimb grip strength in Newtons. The meter is designed to record the peak force exerted by the animal's hindlimb in motion. The average of 3 trials was recorded for each hindlimb. Grip strength scores received for post-operative behavior analyses were normalized to pre-operative measurements. Locomotor assessment was determined using the Basso-Beattie-Bresnahan (BBB) scale (Basso et al. 1995). Based upon this scale, movements of the hip, knee, and ankle joints were

recorded and scored for the forelimbs and hindlimbs, as well as the balance and position of the trunk and tail. Scores on this scale range from absence of movement (score=0) to normal mobility and balance (score=21). Body weight was also monitored as a surgical safety precaution.

Histological Analysis of Spinal Cord Tissue

Upon reaching postoperative days 7 or 21 for the intraspinal cord group, rats were perfused with cold 0.9% saline followed by 4% paraformaldehyde. The lumbar spinal cord was dissected out, cryprotected overnight in 30% sucrose, frozen in OTC, and sectioned on a cryostat at 20 μm. To assess GFP expression in the spinal cord, a fluorescence camera attached to a Nikon Eclipse E400 microscope was used to capture images of the lumbar central canal and ventral horn.

The quantification of motor neuron density and size was performed on 20 μm thick sections of lumbar spinal cord stained with Cresyl Violet (Fisher Scientific) followed by 95% and 100% ethyl alcohol and mounted with Histoclear. The ventral horns of each sample were captured using a Nikon Microphot-FXA microscope and Spot RT color CCD camera. Three random cross sections of the lumbar spinal cord were taken from approximately the same region in each animal. The area of ventral horn neurons was measured with NIS-Elements BR software (v3.0, Nikon). Nissl positive cells with cell body area greater than 300 μm^2 were considered to be motor neurons. Neuron counts and size were averaged within the animal and across each group.

Statistical Analysis

Mean values of grip strength, BBB scores, motor neuron counts and size (± standard error of the mean; SEM) were calculated to make comparisons between groups. Statistically significance was determined using unpaired T tests.

Intrahippocampal Delivery

All protocols were approved by the Emory University IACUC. Surgery was performed on approximately 8-12 week old Sprague Dawley wild type rats from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with isoflurane (2% inhalation in oxygen) and placed in a stereotaxic frame (Kopf) designed to anatomically locate the hippocampus. A craniotomy was performed and a micropipette (approximately 100 μm diameter) was mounted onto an oocyte injector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific) and inserted into the hippocampus based on previously determined stereotaxic coordinates (4.0 mm anterior to bregma, 2.0 mm lateral, 3.2 mm ventral to dura) (Paxinos and Watson 2007). A volume of 69.0nl (x29) was injected every 15 seconds for a total volume of 2.0ul and then left to diffuse for 3 minutes. A total of 6 rats were unilaterally injected with Ad.Rheo.Kir2.1 and 3 rats were randomly chosen to receive the induction ligand. The ligand was administered orally on a daily basis (50 mg/kg) starting immediately after the surgery and 6 days post-operatively for a total of 7 doses. Following sutures, buprenorphine (0.1ml subcutaneous) was given immediately after surgery to reduce pain and rats were placed on heating pads for 30 minutes. Rats were housed in the Emory University Division of Animal Resources and allowed water and food *ad libitum*. Rats receiving hippocampal brain injections did not undergo behavioral testing but were monitored for recovery.

Histological Analysis of Brain Tissue

Upon reaching postoperative Day 7, rats were perfused with cold 0.9% saline followed by 4% paraformaldehyde. The entire brain was dissected out, cryprotected overnight in 30% sucrose, frozen in OTC, and sectioned on a cryostat at 40 μm. Slides were immunostained with the neuronal antibody NeuN (1:100, Millipore) and an AlexaFluor 594 secondary antibody (1:500, Invitrogen). GFP expression and positive NeuN cells were identified under the same fluorescent microscope and images were captured.

Results

Transfection of SHSY5Y Cell Culture

SHSY5Y cells were transduced with Ad.Rheo.Kir2.1-GFP at MOIs of 10^2, 10^3, and 10^4 (Figure 2). GFP expression was found only at MOIs of 10^3 and 10^4. However, there was an apparent decrease in cell density in cultures treated at 10^4 MOI. Cell densities at 10^3 and 10^2 were comparable. Optimal vector expression was taken to be at MOI of 10^3.

SHSY5Y cells were then treated with Ad.Rheo.Kir2.1-GFP at an MOI of 10^3 for 48 hours. The group that received the ligand (n=6) showed robust GFP expression 72 hours after exposure to the viral vector. No GFP expression was detected in the control group (n=6) (Figure 3). Cell nuclei and apoptosis were quantified with DAPI and TUNEL staining, respectively (Figure 3). No significant difference ($P>0.01$) in cell survival was determined between the ligand group (307 ± 61.1) and the control group (256.6 ± 50.4) (Figure 4). Normalized positive TUNEL cells count for the ligand group

(2.98 ± 1.73) and the control group (2.54 ± 0.38) had no significant statistical difference (Figure 4).

Ad.Rheo.Ki2.1-GFP Causes Neuromuscular Inhibition

Behavioral deficits were seen exclusively in the ligand-treated subjects (Figure 5). Behavioral tests at Day 1 post-operatively were notably similar to baseline measurements. A decrease in neuromuscular function in the hindlimb corresponding to the side of spinal cord injection (ipsilateral) was predominately noted at Day 4 and Day 7 for both grip strength measurements (Figure 5A) and BBB score (Figure 5C) which correlates to adenoviral activation and expression (Boulis et al, 1999). The normalized behavior for average grip strength measurements was statistically significant at Day 4 and Day 7 between the ligand-treated animals (0.358 ± 0.097, 0.424 ± 0.100) and the control group (0.977 ± 0.0520, 0.959 ± 0.017) (***=$p<0.001$). Little to no neuromuscular deficits were seen on the contralateral side of injection in the ligand-treated group as well as both hindlimbs in the control group (Figure 5B,5D). Animals that continued to Day 21 but stopped ligand administration at Day 7 showed a slight improvement closer to baseline behavior.

Ad.Rheo.Kir2.1-GFP Results in Cell Loss

Cresyl Violet staining of spinal cord tissue was done to assess motor neuron density in the lumbar ventral horn. Neuronal cells were notably absent from the ipsilateral side of the central canal (Figure 7B.1) and the ventral horn (Figure 7D.1) in the ligand-treated rats. At the central canal in ligand treated subjects, the contralateral side of injection displayed healthy cell populations and no GFP expression (Figure 7B.2).

Healthy motor neuron and interneuron cell populations were also seen in the control subjects at the central canal (Figure 7A.2) and ventral horn (Figure 7C.2).

Quantification of motor neurons stained with Cresyl Violet revealed a significant decrease in motor neuron population specific to ligand-treated subjects in the ipsilateral ventral horn (Figure 8A). Statistical significance was determined between the ligand treated group (9.54 ± 6.66) and the control group (21.17 ± 4.82) when comparing average number of motor neurons on the ipsilateral side (* = $p<0.05$). Normal density was found in both groups on the contralateral side (Figure 8A). The average cell body area of the surviving motor neurons in the ligand-treated subject was normal compared to the control group (Figure 8B).

In concurrence with the *in vitro* results, fluorescent microscopy revealed that GFP expression in the spinal cord was restricted to subjects that received ligand. GFP expression was noted in the ventral horn as well as areas stretching to the central canal but localized to the side of injection (ipsilateral) (Figure 9B). It was predominately found that intense levels of GFP corresponded to these areas that had a lack of cell bodies (Figure 9C.1). In sections of spinal cord adjacent to these areas for the ligand-treated subjects, a return of cell bodies is noted with less intense levels of GFP expression and some transduced motor neurons (Figure 9C.2).

Ad.Rheo.Kir2.1-GFP Expression in Hippocampus

Fluorescent microscopy revealed GFP expression in the hippocampus of only rats treated with ligand (Figure 10). GFP expression did not achieve as widespread of a

distribution as was seen in the spinal cord, but stayed within the path of the needle tract. NeuN antibody staining was done to identify neuronal cells in the hippocampus. NeuN positive staining was not found to be co-localized with GFP expression (Figure 11).

Discussion

Gene-based neuromodulation is the selective control of certain genes to regulate neuronal activity with the potential to be more efficient that current surgical techniques. In this study, we aimed to determine the efficacy of driving Kir2.1 expression in lumbar motor neurons to achieve regulated neuromuscular inhibition. Using an inducible adenoviral vector, we were able to successfully transduce cultured SHSY5Y cells with minimal evidence of apoptotic cells. The behavioral assays indicated that neuromuscular inhibition was exclusive to rats that received the ligand. The effects were predominately found in the ipsilateral hindlimb, which supports our ability to target the vector via stereotaxic injection. However, histological analysis showed motor neuron loss occurred in ligand subjects at the epicenter of injection. Diffusion of the vector from the epicenter to adjacent areas likely led to a gradient of lower vector concentrations. In these areas, we found many healthy motor neurons that were transduced. After the ligand was discontinued at Day 7, the rats followed for 3 weeks began to show neuromuscular recovery. The motor deficits seen can be attributed mostly to a loss of motor neuron cell bodies in the lumbar spinal cord. It is unclear to what extent that motor neurons transduced with Ad.Rheo.Kir2.1-GFP contributed to neuromuscular deficits. Since adenoviral vectors are known to mediate short-term gene expression (i.e. 1-2 weeks), recovery could have occurred because of down-regulation of Kir2.1 and/or compensatory sprouting by the remaining motor neurons (Brown 1984, Boulis et al. 1999). Rats that did

not receive ligand did not express the transgene, had no functional deficits, and contained normal numbers of motor neurons. Hippocampal brain injections were unsuccessful at transducing neuron populations that endogenously express the Kir2.1 protein channel. Our study also demonstrated the ability of RheoSwitch® to mediate inducible gene expression as well as the viral vector's efficiency to produce biologically active ion channels.

Post-operative behavior measurements were not significantly different from pre-operative baseline measurements for rats that did not received the ligand. Fluorescent microscopy did not detect any GFP expression. Additionally, motor neuron counts were similar between the ipsilateral and contralateral ventral horns. These results indicate that the surgical procedure is safe and that the RheoSwitch® tightly regulates gene expression. Since the control group still received the same viral vector but did not have protein production, the motor neuron cell death in ligand treated animals is a result of Kir2.1 protein expression rather than an immune response to the viral vector itself.

The histological results in Figure 9 led to speculate that our original concentration of viral vector injections was too high. As the highest concentrations of the viral vector diffused out to adjacent areas, motor neurons expressing GFP are found with a return of normal motor neuron counts. This suggested that if the viral vector concentration was diluted, motor neurons could be transduced without cell death. The original stock of viral vector was diluted ½, 1/10, and 1/1000 and unilateral spinal cord injections were repeated. At 1/1000, there was no detectable GFP expression and motor function was normal. A dilution of 1/10 showed minimal GFP expression but failed to produce any motor deficits. At ½ dilution, GFP expression occurred as well as motor deficits but

histology again showed motor neuron loss. Our efforts to dilute the original concentration to achieve gene expression with neuromuscular inhibition in the absence of motor neuron loss were unsuccessful.

We also attempted to replicate the findings of Yoon et al. (2008) in order to assure the quality of our vector. Spinal cord injections of Ad.Rheo.Kir2.1-GFP were performed in embryonic chicks at day 3 of development. However, administration of the ligand to turn on gene expression was a barrier. The ligand manufactured by Intrexon Co. is a white powered substance that is not water-soluble and would not easily cross the chick membrane without causing harm. Therefore, no gene expression of Kir2.1 occurred and we could not successfully duplicate the study.

The reason for Kir2.1 gene transfer toxicity in adult rat motor neurons could have many possible explanations. Successful experiments using Kir2.1 as previously mentioned were largely done in cell cultures and developing nervous systems (i.e. embryonic chicks and neonate mice). Viral vectors often work well *in vitro* but may fail to translate to an animal model. Plasticity that occurs during development of the nervous system to refine synaptic connectivity may play a role in how Kir2.1 expression is received. Howorth et al. (2009) is the only study to deliver Kir2.1 in adult rats. They reported successful transduction of pontospinal noradregnic (NA) neurons, which do not normally express the Kir.21 channel. However, they do not report on any viral vector uptake that may have occurred at the dorsal horn where they injected to retrogradely infect NA neurons.

Our study is the first to look at adenoviral-mediated Kir2.1 expression in the

lumbar spinal cord, specifically in adult rat motor neurons. Kir2.1 appears to be primarily toxic to motor neurons. Since Kir2.1 is not endogenously expressed in motor neurons, over-expression of this ion channel in a developed nervous system may alter the electrophysiology of motor neurons in a way that triggers apoptosis. Motor neurons are known to have unique physiological properties, which make them selectively vulnerable to neurological diseases such as amyotrophic lateral sclerosis (Boillee et al. 2006). A similar phenomenon may be occurring when Kir2.1 is over expressed in these channels. As noted in the spinal cord, a large amount of non-neuronal cells expressed GFP as well (Figure 9) since adenovirus by nature is a promiscuous virus and targets all cell types. It is possible that glial cells expressing Kir2.1-GFP may be having a bystander effect on surrounding neurons.

Experimental evidence for toxicity of Kir2.1 in motor neurons is inconclusive. Brain injections of our viral vector to the hippocampus did not result in any neuronal transduction. Based on the morphology of cells expressing GFP, it appears to be in astrocytes. Further attempts to transduce hippocampal neurons will be done with additional brain injections at high vector volumes. The rationale is that if Kir2.1 can be safely expressed in these neurons where it is normally found, it would eliminate the possibility that our vector is producing protein in extreme amounts. Excessive protein production in cells has been hypothesized to cause cell death unrelated to the function of the protein itself (Chalfie & Kain, 1998). It would also support our speculation that Kir2.1 toxicity is specific to motor neurons.

Conclusion

It is reported that Ad.Rheo.Kir2.1-GFP is a viable vector capable of regulated gene expression. *In vitro* and *in vivo* assays demonstrated the vectors ability to transduce cells. However, at high concentrations of Kir2.1, motor neuron cell death occurred. A direct explanation for this effect is inconclusive and will be explored with further experiments. Consequently, Kir2.1 does not seem suitable for clinical translation to the nervous system.

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Figure 1: Map of Ad-RheoSwitch-Kir Expression System. Kir2.1 cDNA was fused with GFP sequence (Kir-GFP). The expression of Kir2.1 was regulated by the RheoSwitch® promoter. Kir-GFP was under the control of GAL4 RE (response element) promoter. The expression of GAL4 was directed by the CMV promoter. In the presence of the orally administered RheoChem™ induction ligand (RL; RG-115819), RL forms a compound with GAL4 and activates Kir-GFP expression. In the absence of the induction ligand, translation of Kir2.1 does not occur.

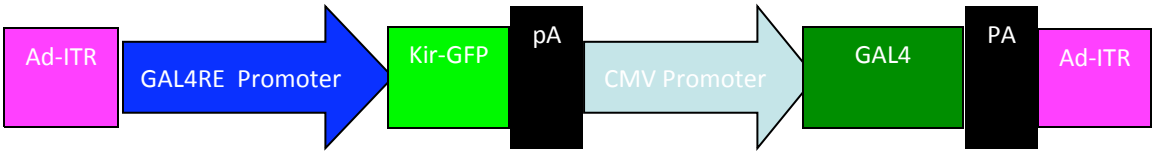

Figure 2: SHSY5Y cell cultures transfected with Ad.Rheo.Kir2.1 and given induction ligand to determine optimal multiplicity of infection. At 10^2 MOI, there is normal cell density (phase) but no GFP expression. 10^3 MOI exhibits both normal cell densities with GFP expression. 10^4 MOI shows a decrease in cell density but still has GFP expression.

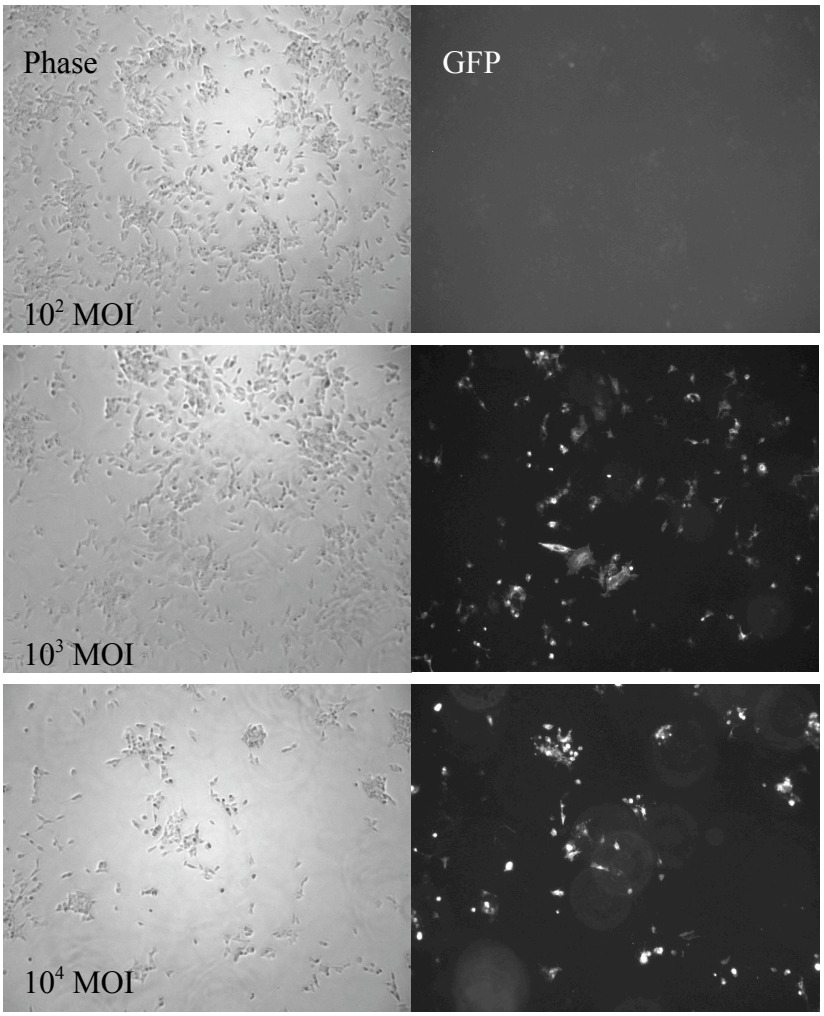

Figure 3: SHSY5Y cells treated at 10^3 MOI. GFP positive (green) cells are seen only in the ligand-treated group. DAPI positive cells are shown as blue. TUNEL positive cells are shown as red.

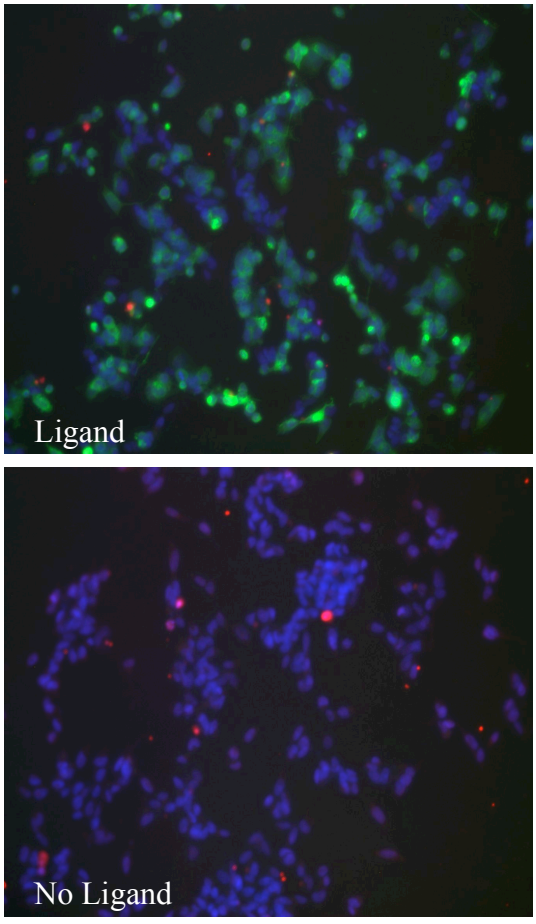

Figure 4: Average number of DAPI positive and TUNEL positive cells in SHSY5Y cell cultures treated at 10^3 MOI.

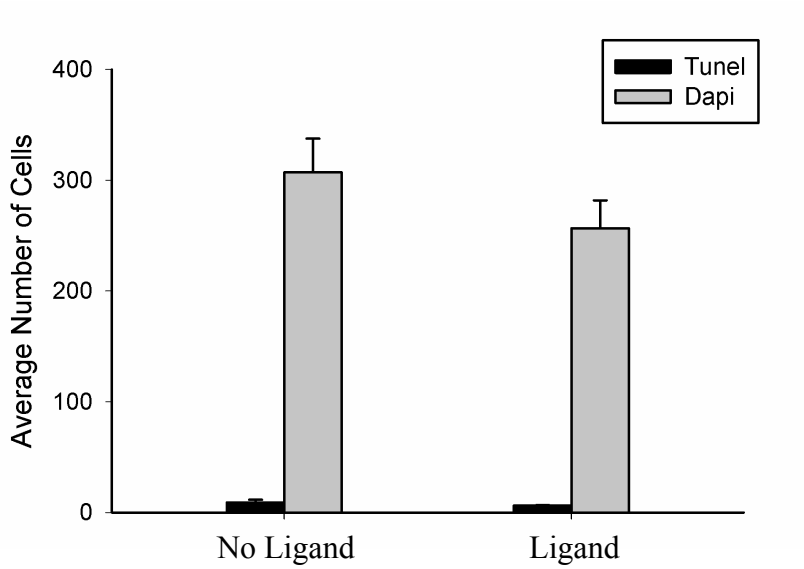

Figure 5: Hindlimb motor impairment. **A-B,** The mean (± SE) normalized hindlimb grip strength for the ipsilateral side (A) and the contralateral side (B). Note: N=8 for both No Ligand and Ligand groups days 0-7. N=3 for both groups at days 7-21. in No Ligand group for days 0-7, N=2 for days7-21. (**=P<0.0001)

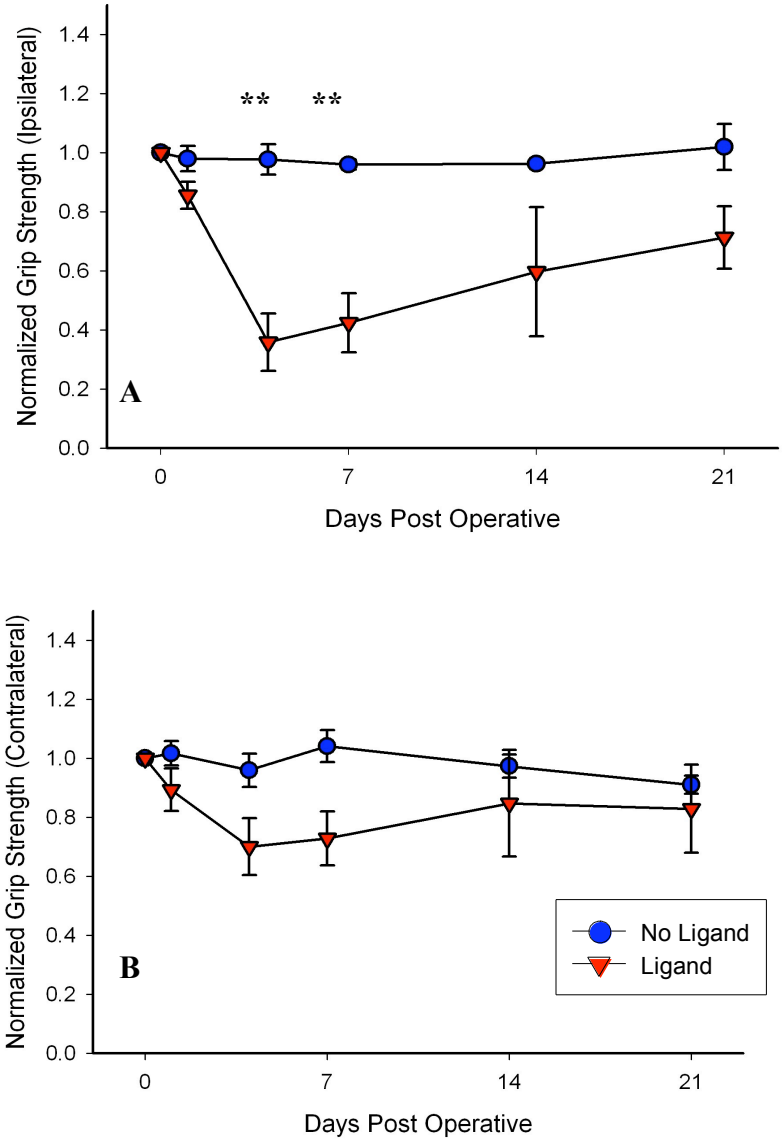

Figure 6: Hindlimb motor impairment **A-B,** The mean (± SE) BBB score for the ipsilateral side (A) and the contralateral side (B). Note: N=8 for both No Ligand and Ligand groups days 0-7. N=3 for both groups at days 7-21. in No Ligand group for days 0-7, N=2 for days7-21 (*=P<0.05).

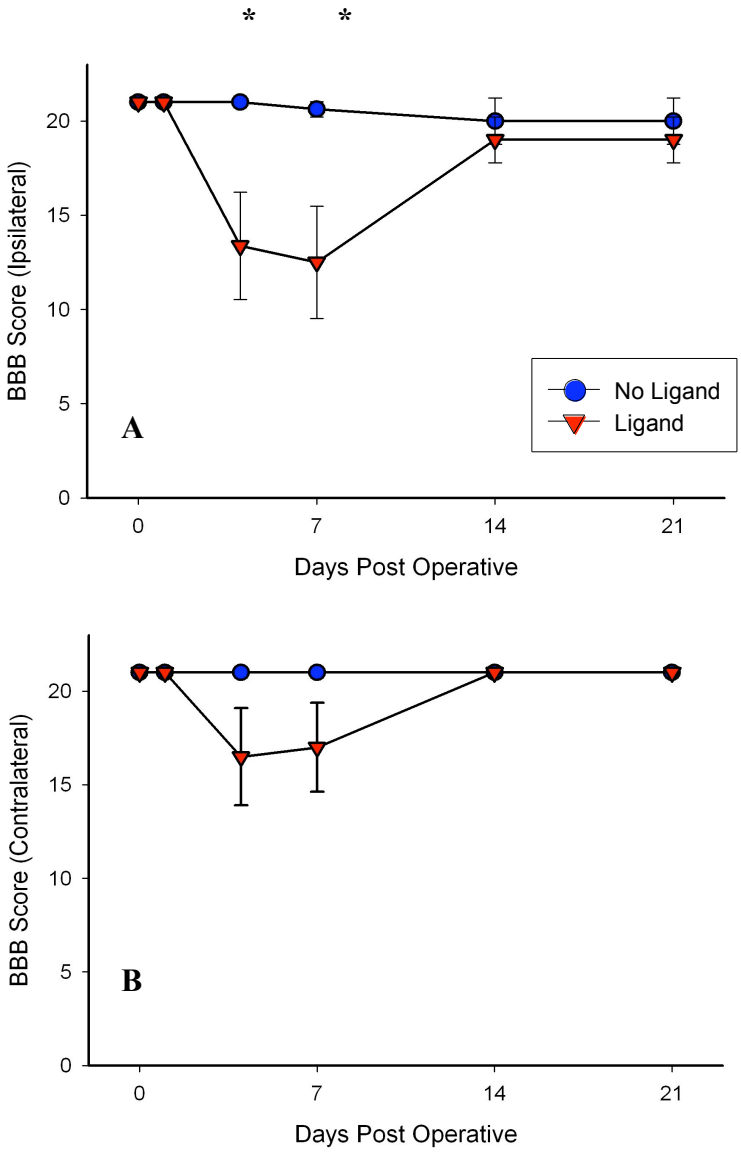

Figure 7: **A** Central canal of lumbar spinal cord in non-ligand treated animal. **B** Central canal of lumbar spinal cord in ligand-treated animal. **C** Ventral horn of non-ligand treated animal with Cresyl Violet/Nissl positive cells. **D** Ventral horn of ligand-treated animal lacking motor neurons.

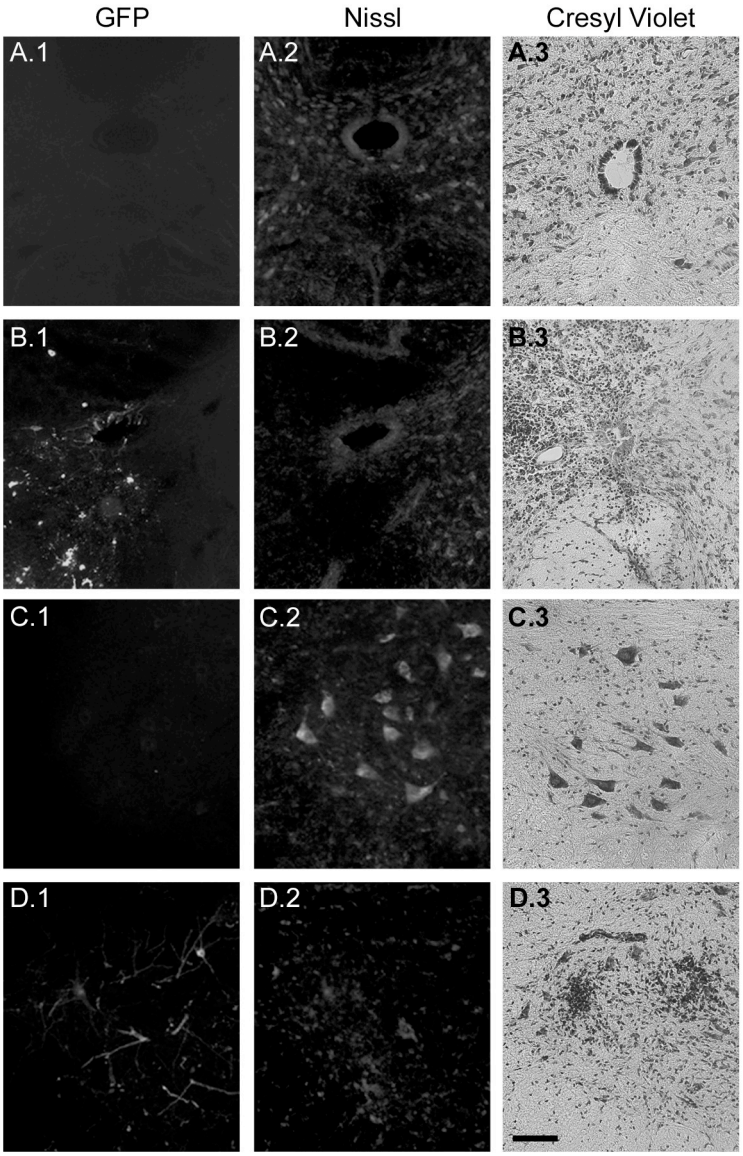

Figure 8: **Motor Neuron Density and Area.** Motor neurons were assessed in cresyl violet stained spinal cord sections from both ligand and no-ligand subjects. **A**, Ligand-treated subjects showed a significant decrease in cell density on the injected side as compared to their contralateral side as well as no ligand-treated rats. **B,** Mean motor neuron area was not different between groups. (*, P<0.05)

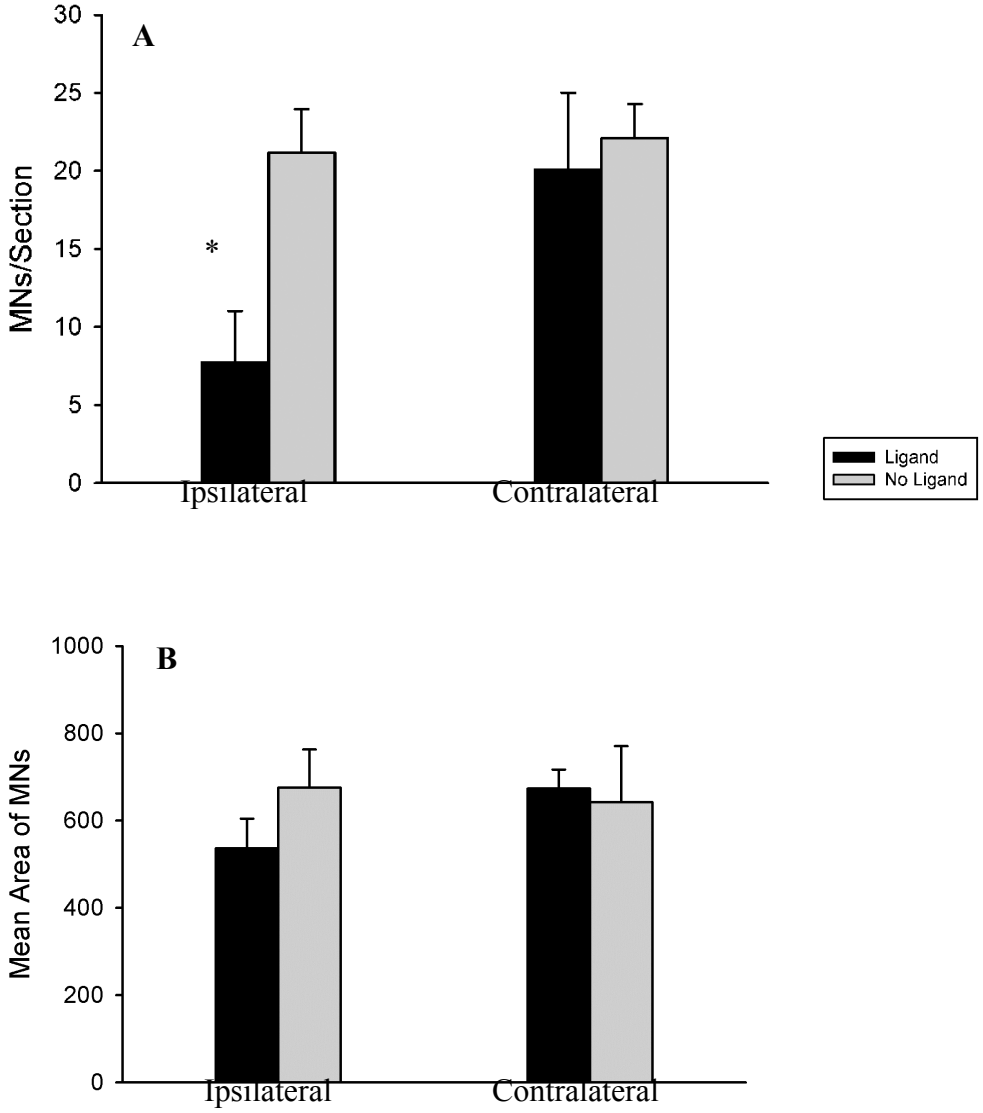

Figure 9: Fluorescent Nissl Staining and Kir-GFP Expression in Rat Spinal Cord. **A.** Nissl staining for ligand-treated subject at epicenter (A.1) and adjacent to epicenter (A.2) of injection. **B.** GFP expression was predominately found ipsilateral to the injection (left) side. GFP expression was more widespread at the injection epicenter (B.1) compared to adjacent spinal cord segments (B.2). **C.** Merged Nissl and GFP images show that GFP expression was found mostly in non-neuronal cells at the epicenter (C.1), but can be found in motor neurons in adjacent regions (C.2).

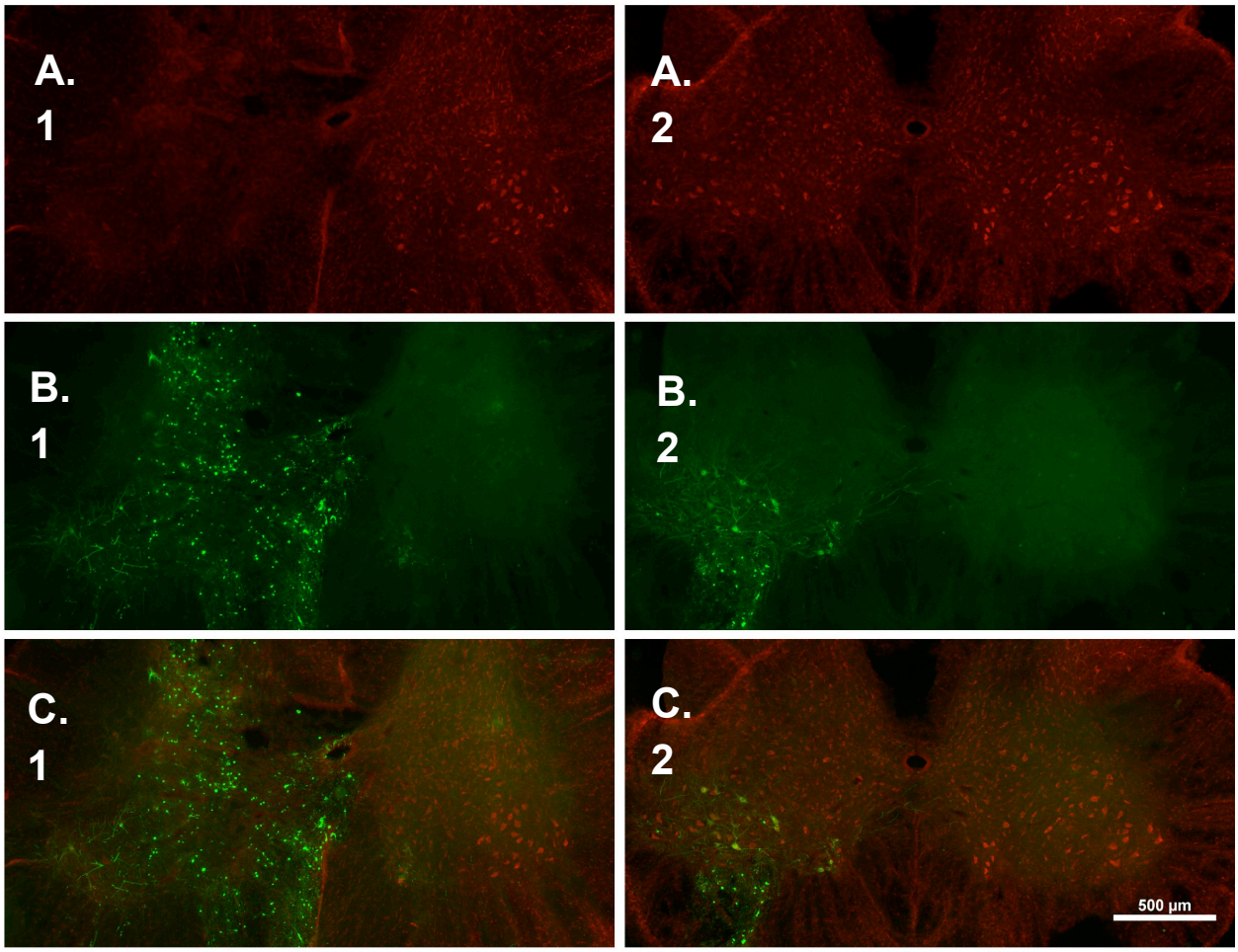

Figure 10: GFP expression in the hippocampus.

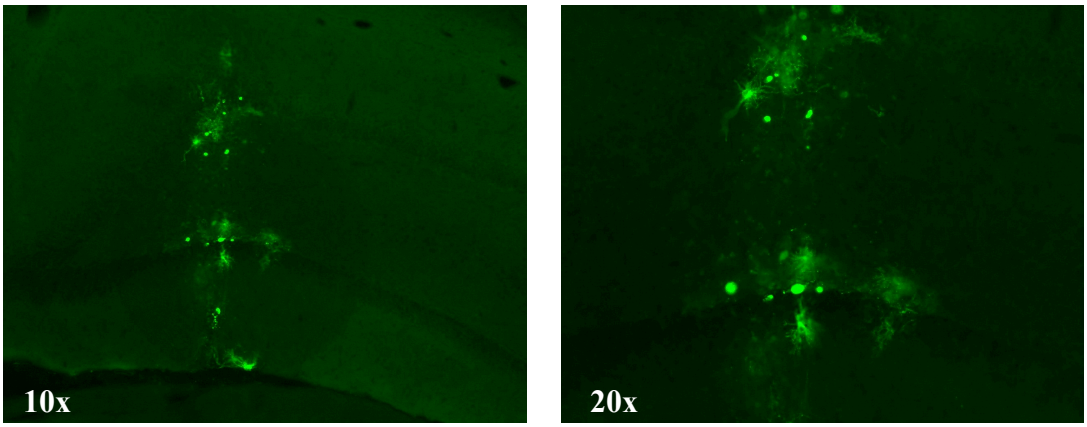

Figure 11: GFP expression with NeuN staining in the hippocampus.