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Anna Ecanow March 23, 2022

Bioluminescent Optogenetics to Enhance Recovery from Peripheral Nerve Injury

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Abstract

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Functional recovery from peripheral nerve injury (PNI) is poor. Experimental therapies increasing injured neuronal activity are effective in accelerating axon growth and improving outcomes. Bioluminescent optogenetics (BL-OG) is a novel approach to increasing injured neuron activity. BL-OG uses luminopsins – light sensing ion channels (opsins) fused with light-emitting luciferase – which generate bioluminescence when exposed to a suitable substrate, such as coelenterazine (CTZ). Injured neurons expressing an excitatory luminopsin could be activated by BL-OG to promote axon regeneration. We hypothesized that induction of BL-OG after PNI would result in an increased number of motor and sensory neurons that have regenerated, relative to controls. Sciatic nerves of mice were injected unilaterally with an adeno-associated viral vector encoding either an excitatory luminopsin (eLMO3) or a mutated form (R115A) that can generate bioluminescence but not activate neurons. After waiting two weeks for retrograde viral transport and viral transduction, injected sciatic nerves were cut and repaired, and mice were then treated with a single dose of CTZ. Four weeks later, different retrograde fluorescent tracers were injected into gastrocnemius and tibialis anterior muscles to mark motor and sensory neurons innervating them. Counts of neurons were made from histological sections of spinal cords and DRGs and included those that were only retrogradely labeled and those that were both retrogradely labeled and contained green fluorescent protein (GFP), indicating presence of the luminopsin. The number of retrogradely labeled motor and sensory neurons in mice expressing eLMO3 was significantly greater than the number in mice expressing the R115A luminopsin. However, only a small proportion of labeled cells contained GFP, indicative of luminopsin expression. Treatments using CTZ to induce BL-OG enhanced axon regeneration are promising but illuminating the exact mechanism will require further investigation.

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Acknowledgements

I would like to thank Dr. Arthur English for his invaluable guidance and mentorship throughout preparing this thesis, as well as the entirety of my experience in his laboratory. I would also like to thank Dr. Robin Isaacson for performing the surgeries, and Dr. Isaacson and Dr. Dario Carrasco for their continuing support and training. Thank you to Dr. Ken Burglund for supplying the viral vectors. Finally, I would like to thank my committee members for their time and for their participation in the evaluation of my thesis.

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Background/Introduction

Peripheral nerve injuries (PNIs) are a prevalent clinical issue, impacting the quality of life of those who are injured and providing significant economic burden for those affected (Bekelis et al., 2015). Estimates suggest that two million individuals in the United States alone are affected by some form of nerve injury (NIH Publication No. 18-NS-4853), most commonly resulting from high-speed trauma. Other age-associated etiologies include falls in the elderly and birth related injuries in pediatric populations (Bekelis et al., 2015). Despite the prevalence of PNIs, only ~10% of patients experience some degree of recovery. This leaves many individuals permanently disabled, with irreversible symptoms such as impaired motor function, loss of sensation, and often pain (Scholz et al., 2009).

The extent to which functional recovery is not attained is often attributed to the fact that axon regeneration is slow and inefficient (English et al., 2014). Two factors that can greatly affect the capacity of nerves to regenerate are distance and time. If there is too great a distance between the injury site and the targets of the injured axons in the periphery, or if there is a delay prior to surgical repair of a nerve, regenerative capacity decreases (Gordon et al., 2011). As both of these scenarios are common in humans, recovery from PNIs remains poor. Thus, novel treatments directed at enhancing axon regeneration are required to improve the outcomes for those with PNIs.

Based on the results of existing preclinical research, such treatments are possible. Two experimental treatments, brief low frequency (20 Hz) electrical stimulation and moderate exercise, are especially effective in accelerating axon growth and improving functional recovery (Jaiswal et al., 2020). Increasing the activity of injured neurons is both necessary and sufficient to improve axon regeneration (Ward et al., 2016, 2018). Although these treatments are effective,

there are barriers to their clinical use. Such barriers include delays prior to surgery, as well as co-morbidities that prevent their application (Gordon and English, 2016). Additionally, not all nerve injured patients are able to be stimulated or enrolled in an exercise program. An alternative to these two successful activity-dependent experimental therapies is needed.

Bioluminescent optogenetics (BL-OG) could be such an alternative. BL-OG uses luminopsins – light sensing ion channels (opsins) fused with light-emitting luciferase (Figure 1). When exposed to an appropriate substrate (in this case coelenterazine, CTZ), bioluminescence is generated by the luciferase component and sensed by the opsin component. In this way injured neurons that express an excitatory luminopsin could be activated directly in order to promote axon regeneration.

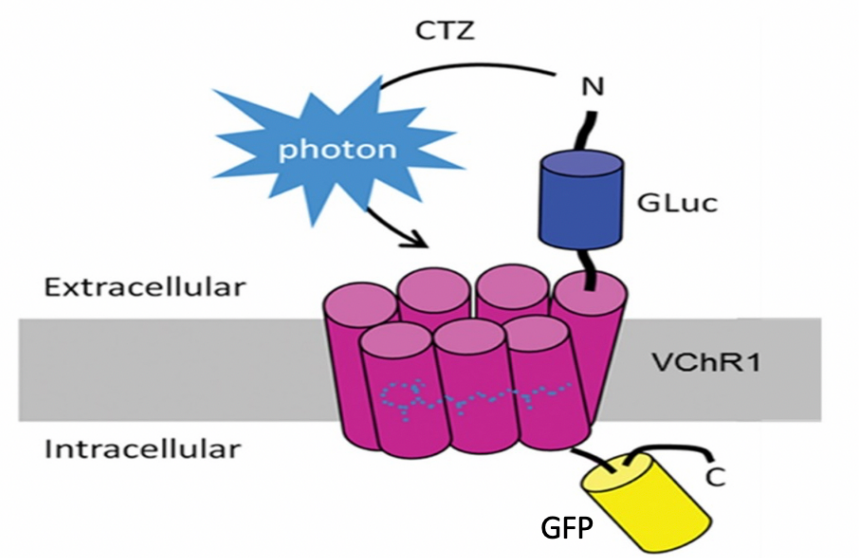

Figure 1. Diagram of the excitatory luminopsin (eLMO3), which contains luciferase enzyme (Gluc) fused to light sensitive channel-rhodopsin (VChR1). When treated with a luciferase substrate, coelenterazine (CTZ), bioluminescence is produced and the cation channel of VChR1 is opened, exciting the neuron. The luminopsin also contains a green fluorescent protein (GFP) for visualization. (English et al., 2021)

This project aimed to evaluate BL-OG enhanced regeneration of injured axons in a peripheral nerve by determining the number of motor and sensory neurons whose axons successfully reinnervated a target muscle in BL-OG treated and control mice. In doing so, we sought to examine the feasibility of inducing luminopsin expression in neurons with axons in a peripheral nerve using a viral vector. In prior studies, transgenic mice engineered to express an excitatory luminopsin (eLMO3) exclusively in neurons were treated once with CTZ after sciatic nerve transection and repair. More motoneurons regenerated axons and successfully reinnervated target muscles in animals treated with a fully functioning luminopsin than in wild type controls and those treated with a mutated (R115A) luminopsin (English et al., 2021). Thus, the aim of this project was to evaluate if similar results could be achieved by BL-OG when using a viral vector to induce luminopsin expression in the injured neurons and if a similar effect is seen in sensory neurons. The central hypothesis of this study was that BL-OG treatment after a peripheral nerve injury will result in the successful regeneration of axons of more motor and sensory neurons than controls.

Methods

Animals & Surgeries – To evaluate the feasibility of expressing an excitatory luminopsin using a viral vector, 12 intact C57B6 mice (8 female, 4 male) ranging from 6-13 weeks of age were used. Mice were anesthetized using isoflurane, and the right sciatic nerves of the mice were injected above the branching point with 1-2 µL of an adeno-associated (AAV2/9) viral vector encoding either an excitatory luminopsin (eLMO3) (1.2x10^14 vg/mL) or a luminopsin with a mutated opsin component (R115A) (3.5x10^14 vg/mL) (Figure 2).

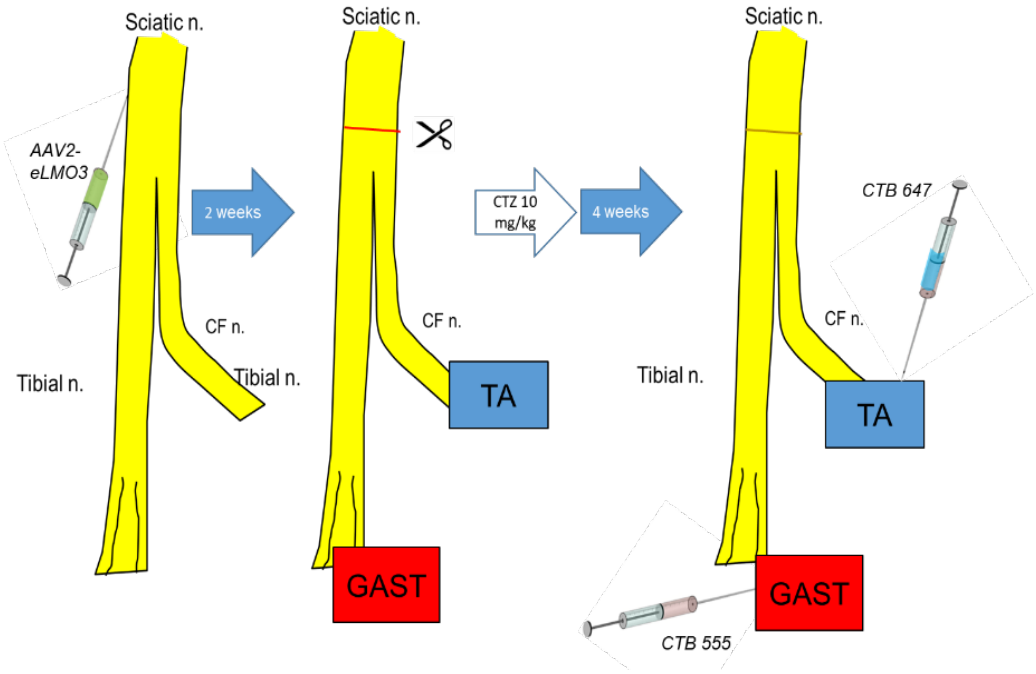

Figure 2. Design of injury, repair, and BL-OG treatment for feasibility testing of BL-OG.

This mutated luminopsin contained a single amino acid perturbation (arginine to alanine at location 115, R115A) in the VChR1 component. In this mutant, the luciferase component of the luminopsin could generate bioluminescence in the presence of CTZ but the channelrhodopsin component could not respond to that light and activate neurons. The use of this R115A luminopsin allowed us to ensure that any effect can be attributed to neuronal activity, rather than other components of the BL-OG system (English et al., 2021). The use of this mutant also acts as

a control for any effect of CTZ alone. CTZ has been shown to have antioxidant properties so that its presence might affect regeneration (Dubuisson et al., 2005). Even though CTZ treatment alone did not enhance axon regeneration in transgenic mice (English et al, 2021), it is important to control for this possibility. The construct in both vectors also contained a sequence for expression of green fluorescent protein (GFP) for visualization.

After waiting two weeks for retrograde viral transport and transduction of spinal motoneurons, injected sciatic nerves were cut and repaired (Akhter et al., 2019), with the contralateral sciatic nerve remaining as an intact control. Immediately after repair, the nerve was injected with a single dose of CTZ (10 mg/Kg, i.p.). In transgenic mice expressing the same luminopsin constructs, an injection of this dose of CTZ did not increase spontaneous neuromuscular activity, which would be associated with increased motoneuron firing, but it did lower the threshold for reflex excitation of luminopsin-expressing motoneurons suggesting an increase in their excitability (English et al., 2021). This change in excitability reached a peak 45 minutes after CTZ injection and returned to baseline in three hours (English et al., 2021).

Four weeks after nerve repair and CTZ treatment, different retrograde fluorescent tracers were injected into the gastrocnemius and tibialis anterior muscles. Two microliters of a 1% solution of the beta subunit of cholera toxin, conjugated to Alexafluor 555 (CTB555) were injected into each head of the gastrocnemius muscle (GAST) (4 µL total), and 2 µL of a similar reagent, but conjugated to Alexafluor 657 (CTB647) were injected into the tibialis anterior muscle (TA). These tracers were used to mark motor and sensory neurons re-innervating the injected muscles. Mice were euthanized and perfused five days after retrograde tracer injections.

Tissue Processing & Immunohistochemistry – Following euthanasia, lumbar spinal cords, as well as right and left L4 and L5 DRGs were collected from each animal and preserved in sucrose solution. Spinal cords and DRGs were serially sectioned on a cryostat at 50 µm and 30 µm thickness, respectively, and mounted on glass microscope slides. Spinal cord tissue was reacted

with antibodies to GFP to amplify visualization of the eLMO3 construct. Slides were incubated with blocking solution containing 0.3% Triton X-100 and 10% normal goat serum for 1 hour before being incubated with primary antibody (rabbit anti-GFP, 1:500) overnight. Tissue was then washed three times with PBS before being incubated with secondary antibody (goat anti-rabbit Alexafluor 488, 1:300) for 1 hour, before being washed three more times.

Imaging – Images of spinal cords were captured using a Leica DM6000 microscope, a Hamamatsu camera, and HCImage software. Images of DRG sections were captured using a Keyence BZ-X microscope. Motoneurons that were retrogradely labeled, indicating that their axons had successfully regenerated and reinnervated the gastrocnemius or tibialis anterior muscle, were counted and their sizes measured. Motoneurons were counted as retrogradely labeled if the label filled the cell body and proximal dendrites, with a visible nuclear shadow, as well as intensity above background level. Note was made of those that contained both GFP (indicating presence of the luminopsin) and a retrograde label. Counts of DRG neurons, with the same criteria for inclusion, were conducted as well to determine the effect that BL-OG had on the regeneration of muscle sensory axons. Both motor and sensory neuron soma sizes were measured using ImageJ software to determine if successfully regenerating axons have a different size distribution than those typically found in intact animals.

Results

BL-OG Enhances Regeneration of Motoneurons

The number of retrogradely labeled motoneurons in mice injected with the vector encoding the functioning eLMO3 and treated with CTZ was greater than the number found in similarly treated mice injected with the R115A luminopsin (Figure 3b). In cells which reinnervated the gastrocnemius, this difference was significant (ANOVA, $F_{2,21} = 7.282$, $p < 0.01$). While there was an increased number of cells reinnervating the tibialis anterior in nerves injected with eLMO3, it was not significantly different from those injected with the R115A luminopsin (ANOVA, $F_{2, 20} = 2.921$, $p > 0.05$). Additionally, in both muscles, there was no significant difference between the number of regenerated neurons from injured nerves treated with eLMO3 and those from intact controls. This indicates that not only did BL-OG have a positive effect on axon regeneration but suggests near complete reinnervation when treated with BL-OG. A small number of cells contained both retrograde tracers, indicating they had innervated both GAST and TA. There was no significant difference in this number between groups, suggesting that BL-OG treatment did not affect this double innervation.

As the motoneuron soma size measurements were not normally distributed, a non-parametric test (Kruskal-Wallis ANOVA) was performed in order to determine if any significant differences between groups were present. There were no such differences between groups in motoneurons reinnervating GAST, while cells innervating the TA were significantly smaller in nerves injected with the R115A luminopsin than in intact controls and those injected with eLMO3 ($H(5) = 25.03$, $p < .001$) (Figure 3c). There were no significant differences between the median size of neurons innervating GAST across all groups. However, when the number of the largest GAST motoneurons (>500 square microns) in the different groups was considered, significantly fewer were found in animals treated with the R115A luminopsin (ANOVA, $F_{2,18} =$

3.555, $p < 0.05$). No significant difference was found in the number of motoneurons with successfully regenerating axons in this size grouping between intact and eLMO3 groups. This finding, along with similar observations in TA, suggests that BL-OG treatment may selectively stimulate the regeneration of axons of these larger cells.

While, based on these results, BL-OG has a positive effect on axon regeneration after injury, only a small proportion of labeled cells were also immunoreactive for eLMO3, indicative of luminopsin expression (Figure 3d.). Thus, treatment using CTZ that induces BL-OG did enhance motor axon regeneration, but the exact mechanism remains unclear.

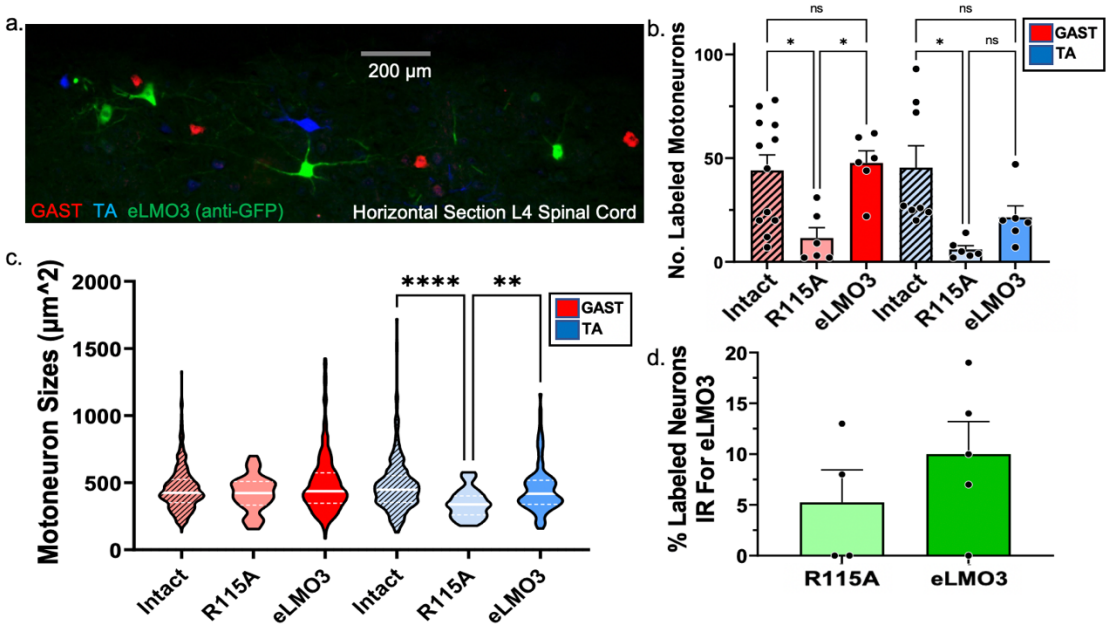

Figure 3. BL-OG enhances regeneration of motoneurons a. Fluorescence microscope image of horizontal L4 section of spinal cord from animal treated with BL-OG. Cells labeled red (CTB555) reinnervated the gastrocnemius, blue cells (CTB647) the tibialis anterior, and green cells contain the eLMO3 virus. b. Mean (+SEM) counts of labeled motoneurons reinnervating the two muscles from the intact left side and animals treated with either the mutant or the excitatory luminopsin. c. Violin plots of soma sizes of labeled motoneurons from each muscle, as well as all cells which were immunoreactive for eLMO3, indicating presence of the virus. Solid line denotes median, dashed lines denote quartiles. d. Percent of labeled motoneurons that *also* are immunoreactive for the eLMO3 virus from animals treated with either the mutant or excitatory luminopsin. * = $p < .05$, ** = $p < .01$, **** = $p < .0001$.

BL-OG Enhances Regeneration of Sensory Neurons

In counts of retrogradely labeled neurons from L4 and L5 DRGs, the number of cells reinnervating the gastrocnemius was significantly greater in animals injected with the virus encoding eLMO3 than in those injected with the virus encoding the R115A luminopsin

(ANOVA, $F_{5,19} = 24.30$, $p < 0.0001$) (Figure 4b). Counts of sensory neurons which reinnervated the tibialis anterior were very low across all groups. As this included the intact side, it may be that these low counts are due to difficulties in the injection or visualization of the tracer. A small number of neurons contained both retrograde tracers, with no significant differences in the number between groups. The distribution of cell sizes was consistent throughout all groups (Figure 4c), with no significant differences between the medians of any of the groups. Similar to the motoneurons, eLMO3 does not affect the size distribution of reinnervating sensory neurons. Interestingly, in the DRGs, as many as half of all labeled sensory neurons also contained GFP, indicating the presence of a luminopsin construct (Figure 4d).

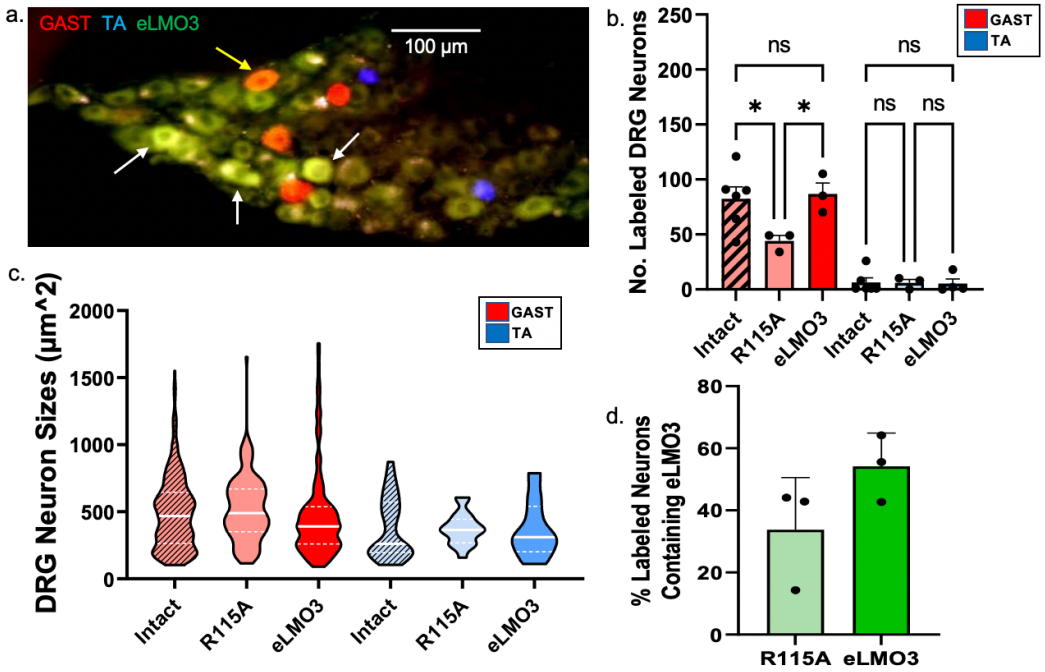

Figure 4. BL-OG enhances regeneration of sensory neurons a. Fluorescence microscope image of L4 DRG from animal treated with BL-OG. Cells labeled red (CTB555) reinnervated the gastrocnemius, blue cells (CTB647) the tibialis anterior, and green cells contain the eLMO3 virus (indicated by white arrows) Yellow arrow indicates double-labeled cell. b. Mean (+SEM) counts of labeled sensory neurons reinnervating the two muscles from the intact left side and animals treated with either the mutant or the excitatory luminopsin. c. Violin plots of soma sizes of labeled sensory neurons from each muscle, as well as all cells which contained eLMO3. Solid lines denote median, dashed lines denote quartiles. d. Percent of labeled motoneurons that *also* contain the eLMO3 virus from animals treated with either the mutant or excitatory luminopsin. * = $p < .05$

Discussion

Peripheral nerve injuries remain a prevalent problem with poor functional recovery, leaving many individuals with permanent symptoms and disabilities (Scholz et al., 2009). As such, the development of novel treatments aimed at enhancing the slow and inefficient process of axon regeneration could improve outcomes for those affected (English et al., 2014). Increasing the activity of injured neurons has been shown to be effective in accomplishing this enhanced effect (Ward et al., 2016, 2018). As previously developed activity-dependent therapies have barriers to their application, BL-OG could be an alternative which directly stimulates axons in order to enhance axon regeneration. In this study, we aimed to investigate the feasibility of inducing BL-OG via viral vector injection prior to a PNI.

The main finding of this study was an increased number of neurons whose axons successfully regenerated and reinnervated their muscle targets in animals expressing the fully functioning eLMO3 as compared to those expressing the R115A luminopsin. This promising result was comparable between both motor and sensory neuron populations. The increased number of motoneurons seen here was consistent with the results of previous studies investigating the use of BL-OG in transgenic mice (English et al., 2021). However, this is the first study to investigate effectiveness of BL-OG treatments using the viral induction of luminopsin in WT mice, as well as its effects on the participation of both motor and sensory neurons. Furthermore, across all cell populations studied, there was no significant difference between the cell counts from the intact side, and the injured nerves injected with the viral vector encoding the excitatory luminopsin. This suggests a near complete reinnervation by both sensory and motor neurons in injured nerves treated with BL-OG. Based on these results, BL-OG remains a promising potential therapy for enhancing axon regeneration.

The inclusion of cell body measurements in this study provided new information on the sizes of regenerating neurons. Motoneurons can be divided into two subgroups, alpha and gamma, which have different roles in innervating extrafusal and intrafusal muscle fibers, respectively. As such, alpha motoneurons are predominately responsible for force-generation, while gamma motoneurons modulate the sensitivity of muscle spindles to stretch. These cell populations can also be distinguished by size, with 360 μm^2 as the cutoff between their size distributions (Friese et al., 2009). By this definition, very few, if any, of the neurons studied here would be classified as gamma motoneurons. This suggests that, perhaps, the labeling method employed here could not detect them. The size difference between eLMO3 and R115A mice that was observed here was the absence of large (>500 μm^2) motoneurons in the R115A group. As these motoneurons are recruited only when large forces are required, their presence in the eLMO3 group indicates an increase in function resulting from a greater capacity for force-generation.

As there were no significant differences between median sizes throughout each group of sensory neurons, BL-OG seemingly does not alter the size distribution of the cell bodies of regenerating axons in this population. In addition, when looking at the distribution of sensory neurons across three size groupings (<300 μm^2, 300-700 μm^2, and >700 μm^2) (Ruscheweyh et al., 2007), there were no differences noted between the animals injected with the virus encoding the excitatory luminopsin and those injected with the virus encoding the R115A luminopsin.

While the increased number of regenerated motor and sensory axons is promising, we are unable to attribute the positive effect specifically to luminopsin expression, as it proved difficult to visualize the GFP marker attached to either luminopsin construct. If increased neuronal excitability induced by BL-OG was the driver of enhanced regeneration, it might be expected that most, if not all, retrogradely labeled cells would also express the luminopsin

(indicated by GFP). In the sensory axon population, about 30-50% of labeled DRG neurons also visibly contained the GFP marker, which could be a proportion sufficient to promote axon regeneration. However, only a small proportion of motoneurons whose axons had regenerated also contained the GFP marker for the luminopsin. Thus, attributing the considerable difference in motor axon regeneration seen between the eLMO3 and R115A groups entirely to BL-OG might be difficult. It is possible that the luminopsin was expressed by many motoneurons, but we were simply unable to visualize it. Other methods, such as PCR or further amplifying the GFP signal, could be employed to evaluate the extent to which the virally induced luminopsin is expressed. Another possible reason for the difficulty in visualizing the luminopsin is that it became distributed throughout the extensive dendritic membranes found in motoneurons, making it more difficult to visualize. The extent of its expression was much greater in dorsal root ganglion cells, which do not have the elaborate dendritic arbor that motoneurons possess. Finally, it is possible that, while only a small number of motoneurons might express the luminopsin, their activity might be sufficient to increase the activity of other cells around them, leading to enhanced regeneration. Future studies should be aimed at investigating these possible explanations for the sparse GFP expression in motoneurons.

Overall, BL-OG enhanced the regeneration of the axons of motor and sensory neurons after peripheral nerve injury. The use of a viral vector in WT animals in this study moved the use of the therapy closer towards potential clinical applications, and the inclusion of sensory neurons and regenerating cell soma sizes provided valuable new information on the action of BL-OG. Further investigation into clinically relevant applications, such as administering BL-OG after injury, are still necessary. The use of BL-OG in enhancing regeneration after peripheral nerve injury remains a promising avenue through which those affected could regain function.

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