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Prolonged functional optical sensitivity in non-human primate motor nerves following cyclosporine-based immunosuppression and rAAV$_2$-retro mediated expression of ChR2

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Abstract— **Peripheral optogenetic stimulation of motor activity offers enticing advantages over traditional functional electrical stimulation for the purposes of reanimating paralyzed muscles. When facilitated by intramuscular injection of viral gene therapy constructs, however, the process of transducing light sensitive ion channels along motor nerves faces several challenges including uptake of the virus at the neuromuscular junction as well as evasion of both virus and expressed gene products from the immune system. These hurdles to successful peripheral motor gene therapy are often amplified when attempting to translate these techniques to non-human primates. In this study, we examined the efficacy of a systemic immunosuppression regimen and use of a designer adeno-associated virus in prolonging functional opsin expression in targeted peripheral nerves of a macaque. Using a regimen of daily cyclosporine and either an intramuscular or intraneural injection of an rAAV2-retro based vector, we observed functional nerve expression of ChR2 via EMG activity locked to optical stimulation of a targeted nerve for up to 24 weeks post-injection. Throughout this experiment, we observed a gross timeline of expression including an initial increase of ChR2 expression over 9-13 weeks followed by an eventual decline after cessation of the immunosuppression regimen. These results suggest a potential strategy for successful translation of peripheral motor gene therapy to human subjects.**

I. Introduction

The field of optogenetics has gradually expanded into the realm of peripheral motor control over the past five or more years. In this application, viral, stem cell, or transgenic methods are used to express light-sensitive ion channels (i.e. "opsins") in the axonal membranes of peripheral motor nerves, making the nerve sensitive to specific wavelengths of light. Optical stimulation of nerves labeled with opsins such as channelrhodopsin-2 (ChR2) has been shown to hold several potential advantages over traditional electrical stimulation of muscle activity for prosthetic and rehabilitation applications including a natural recruitment of muscle fibers, reduced fatigue with prolonged stimulation, and muscle specific stimulation from a proximal nerve location [1].

While this gene therapy holds promising potential for prosthetic applications, the two methods likely suitable for human translation (viral or stem cell mediated) have been largely limited to demonstration in rodent models [2], [3]. While our group has recently shown successful transduction and optical stimulation of non-human primate motor nerves, the duration of expression and functional sensitivity has been limited and presents a short window for study of this process [4],[unpublished data]. Viral mediated expression of fluorescent proteins in non-human primate brain has been reported to induce a strong immune response to the expressed gene products [5], offering a possible explanation as well as potential immunosuppression solutions [6], [7] for these observed shortened periods of expression.

In addition to consideration of the immune response, adeno-associated virus serotype 6 (AAV6) has been the primary vector utilized in rodent and non-human primate studies alike due to its demonstrated aptitude for retrograde transport from muscle to the motor neuron cell bodies in the spinal cord [2], [8]. However, an AAV variant specifically selected through directed evolution for its ability to retrogradely transfect neurons (e.g. rAAV2-retro) has shown efficacy in transducing neurons in the brain [9]. Whether this capacity for retrograde transport extends to the long axonal distances of the peripheral nervous system, and more specifically to the required virus uptake at the neuromuscular junction following intramuscular injection, has not been examined to date.

In the current study, we look to address these gaps as we present preliminary data examining whether the use of systemic immunosuppression and an AAV construct designed for retrograde transport may improve the timeline of opsin expression in peripheral nerves for muscle stimulation following intramuscular and intraneural virus injections. Prolonging the duration of opsin expression and efficiency of virus transduction will play a critical role in improving the feasibility of peripheral motor gene therapy as a viable clinical therapy.

II. Methods

A. Animal Subjects

One male rhesus macaque (*Macaca mulatta*) weighing approximately 5.2 kg was used in these experiments. All animal procedures were approved and conducted in

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accordance with the University of Pittsburgh's Institutional Animal Care and Use Committee.

B. Viral Vector

An AAV vector utilizing the retro capsid (rAAV2-retro) [9] and expressing ChR2 (H134R) and GFP under the Synapsin promoter was packaged and purified by Addgene (Cambridge, MA) at a titer of 7.4 x $x10^{12}$ gc/mL. Just prior to injection, 200 µL of viral stock was mixed with 1000 µL of hypertonic saline, resulting in an injected viral titer of 1.23 $x10^{12}$ gc/mL.

C. Immunosuppression

One week prior to the injection procedure, the monkey was started on a regimen of oral cyclosporine at 10 mg/kg once daily. The monkey remained on this regimen through the injection procedure and initial evaluation surgeries up until the evaluation surgery at 19 weeks post-injection. After this time point, no immunosuppression medications were administered for the remainder of the 24 week experimental timeline.

D. Virus Injection Surgery

The rAAV2-retro-hSyn-ChR2-GFP construct was injected into each leg, targeting the Tibialis Anterior (TA) muscle and corresponding Deep Peroneal (DP) nerve. Stimulating muscle injections were performed in the right TA muscle as described previously to localize areas of high neuromuscular junction density [4] with a total of 1 mL of virus solution injected over 5 sites.

In the left leg, virus solution was injected directly into the DP nerve. Following exposure of the TA muscle, the lateral muscle insertion of the biceps femoris was split using blunt dissection to provide access to the DP nerve. The injection needle was inserted into the nerve at a shallow angle, and 50 µL of virus solution was injected into the nerve. This process was repeated over 4 sites around the circumference of the nerve for a total injection volume of 200 µL.

E. Functional Evaluation of Opsin Expression

Following the injection surgery, optical sensitivity of each targeted DP nerve and corresponding activity of the TA muscle was first evaluated at 6 weeks post-injection. During an evaluation experiment, the DP nerve of each leg was exposed and tested under isoflurane anesthesia. The targeted nerve was optically stimulated using a 400 µm diameter core multimode fiber (ThorLabs, Newton, NJ) connected to a 150 mW, 472 nm fiber-coupled laser (LaserGlow Technologies, Toronto, Ontario). Maximum laser output at the fiber tip was typically around 110 mW. EMG responses to optical nerve stimulation were recorded using a pair of the same 30 gauge monopolar injectable needles (Technomed, Netherlands) used for injection inserted into the muscle belly of TA. A metal hub needle inserted through the skin edge was used as a ground electrode. To identify sensitive zones of optical sensitivity, the fiber tip was swept along the exposed length of the nerve as well as around its circumference. After identifying a suitable location for stimulation, if any, the fiber tip was positioned and held in place 2-3 cm from the target zone using a micropositioner. Laser power, optical pulse width, and optical pulse train frequency were varied within a session to assess the optical response characteristics of a targeted nerve. After evaluation, retracted muscle and skin were closed and sutured in layers. Similar evaluation experiments were performed every 3-4 weeks until a final evaluation experiment followed by terminal perfusion at 24 weeks.

III. Results

A. Timeline of Functional Opsin Expression

Table 1. shows the timeline of opsin expression as assessed by EMG activity elicited by optical stimulation of the target nerve, while Fig.1 shows representative EMG waveforms elicited from the right TA muscle at selected time points. As seen from these data, EMG responses to optical stimulation of either nerve were not observed until 9 weeks post-injection when optical stimulation of the left DP nerve (nerve-injected leg) evoked EMG activity. Muscle contractions were not visible at this time. EMG responses to optical stimuli emerged in the right leg when probed at 13 weeks. Visible muscle contractions did not appear until 16 weeks post-injection, and were only observed in the right leg (muscle-injected leg) at any point during the entire experimental timeline. Muscle contractions were visible through the skin, but were not forceful enough to produce functional movement (dorsiflexion) at the ankle.

While the monkey was on the immunosuppression regimen, optically-driven EMG activity was present in both legs after 9 and 13 weeks for the left and right DP nerves, and visible muscle contractions induced by optical stimulation were present in the right TA muscle, including immediately after stopping the cyclosporine regimen at week 19 post-injection. After removing immunosuppression, the right DP nerve maintained comparable magnitudes of EMG activity and TA muscle contraction in response to optical stimulation at the 22 week checkpoint. However, no responses to optical stimulation of the right DP nerve were observed at the final 24 week terminal experiment. The left DP nerve was not tested at the 22 week check due to unrelated anesthesia concerns, but did exhibit similar EMG responses with no visible contractions at the final check as during weeks 9-19.

B. Spatial Sensitivity to Optical Stimulation Varies Over Time

During early checkpoints in which a given nerve first

TABLE 1. Summary Results of Expression Timeline

Week Post-Injection	*Cyclosporine*	*EMG Response*		*Visible Contractions*	
		Right	*Left*	*Right*	*Left*
6	Y	NR	NR	N	N
9	Y	NR	0.5 mV	N	N
13	Y	0.09 mV	0.18 mV	N	N
16	Y	0.77 mV	0.04 mV	Y	N
19*	Y*	0.97 mV	0.05 mV	Y	N
22	N	0.48 mV	NT**	Y	NT**
24	N	NR	0.5 mV	N	N

NR – No Response. N- No. Y – Yes. EMG Responses indicate peak-to-peak twitch response.
* Last dose of cyclosporine administered day prior to 19 week experiment.
** NT – Not tested. Experiment shortened due to anesthesia concerns.

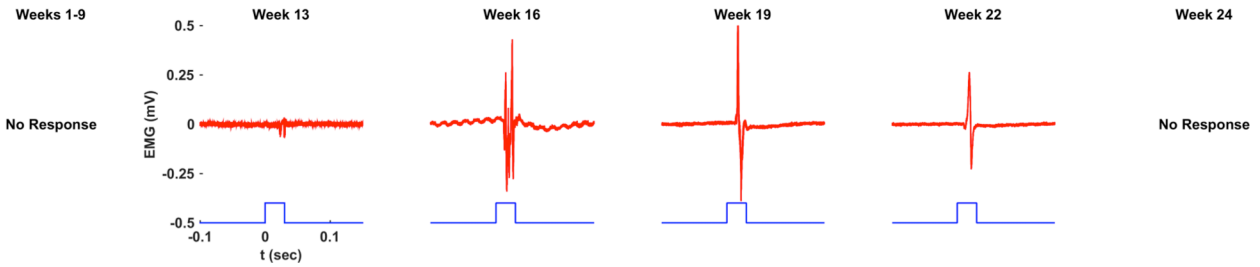

Figure 1. Timeline of EMG sensitivity to optical nerve stimulation. Red traces show individual EMG responses of the right TA muscle to single pulses of blue light (blue trace) applied to the right DP nerve. Optical trains consisted of 10-40 ms wide pulses at 0.5-1 Hz. The functional expression in the right DP nerve appears to grow and decay over the 24 week period. Cyclosporine-based immunosuppression was stopped at the 19 week time point. The left DP nerve and TA muscle showed a similar timeline but with EMG responses observed at weeks 9 and 24.

demonstrated optically-driven EMG activity, nerve sensitivity to optical stimulation was usually restricted to a small segment of nerve and required spatial scanning of the nerve with the tip of the fiber optic cable to identify such a segment. Fig. 2a displays EMG responses of the left TA muscle while scanning the left DP nerve with optical stimulation and highlights the sparsity of sensitivity along the nerve, while Fig. 2b demonstrates consistent stimulus-locked responses of the same nerve at a later time point with the fiber tip maintained in position over a sensitive portion of nerve for comparison. Similarly, at week 13, optical sensitivity of the right DP nerve was limited to a single 5-10 mm segment of nerve out of an approximately 5-8 cm length of the nerve exposed for stimulation. Even after identifying a potential sensitive zone during early checkpoints, the nerve often had to be positioned or stretched in a specific manner before EMG activity could be elicited, suggesting that weak opsin expression may have been shielded from stimulation by excess tissue depth, connective tissue, vessels, etc. At future checkpoints in which the strength of EMG activity and muscle contractions had grown (e.g. weeks 16-22 for the right DP nerve), optical sensitivity was much more uniformly distributed along the exposed length of nerve. However, at the end of the experimental timeline after

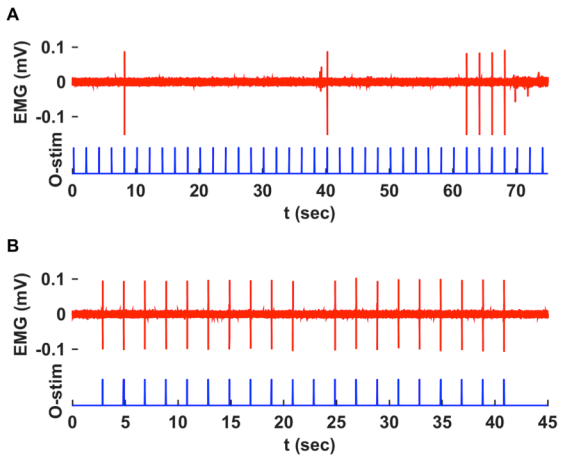

Figure 2. **Spatial sensitivity of Optical-EMG coupling.** A) EMG responses (red) of the left TA muscle to optical stimulation of the left DP nerve (blue) at week 13 post-injection as the optical fiber is slowly scanned along a length of the DP nerve. Note the long stretches of unresponsiveness of the muscle compared with EMG spikes time-locked to optical stimulus (~ t = 8, 40, and 60-70 seconds). B) Similar setup as (A) at later point of session but with optical fiber stationary over a sensitive portion of nerve. EMG responses are consistently coupled to optical responses except for one stimulus at ~ t = 23 sec.

immunosuppression had been stopped, sensitivity of the left DP nerve had contracted to a small section of nerve while we could not find any sensitive segments of the right DP nerve even after dissecting the nerve's trajectory deep into the muscle as well as proximally into the sciatic nerve region.

C. *Optical Sensitivity Frequency Response Varies Over Time*

Fig. 3 displays the EMG responses of the left TA muscle to optical stimulation of the left DP nerve at varying frequencies at two experimental time points (weeks 19 and 22). As seen from Fig. 3A, EMG responses were able to track optical stimulus trains only at low frequencies at week 19, with EMG responses disappearing after 1 second for a 7.5 Hz optical pulse train and sooner for higher frequencies. This falloff in EMG-optical coupling is lower than previously observed roll-off frequencies between 16-20 Hz [4]. However, in the same nerve week 3 weeks later (week 22), the frequency response had improved such that EMG-coupling was still highly consistent at 20 Hz. The right DP nerve exhibited similar variability in the frequency response of EMG-optical stimulus coupling, showing a frequency response similar to Fig. 3b early on and decaying to an inability to track stimuli above 2 Hz at the end of the experimental timeline.

IV. Discussion

A. *Prolonged Expression with Immunosuppression*

Based on our own data and the existing literature, 24 weeks (post-virus injection) is the longest demonstration of optically stimulated muscle activity in a non-human primate following viral transduction of peripheral motor nerves with light sensitive opsins as well as similar studies in rodents [4], [10]. From the timeline of optical sensitivity of targeted nerves and that of immunosuppression, we hypothesize that the immunosuppression regimen described in this study is at least partly responsible for the prolonged expression. While it is possible that individual variation in expression and immune response characteristics could account for the dramatically extended period of expression compared to prior studies, the coincident timing of functional expression decline following cessation of immunosuppression argues in favor of the regimen's role. However, it is also clear that the 19 weeks of immunosuppression was not long enough to clear a critical window after which the expressed gene products would not be recognized as foreign. Studies of AAV-mediated expression of canine dystrophin in a canine muscular dystrophy model showed prolonged transgene

expression following cessation of similar transient immunosuppression [7], [11]. These studies noted that expression degraded relatively quickly after stopping immunosuppression when a human dystrophin gene was used instead of canine, suggesting modification of the expressed gene products may be necessary to further extend expression beyond the immunosuppression period.

B. *Utility of rAAV2-retro Viral Construct*

The retrograde transportation utility of rAAV2-retro has generally been limited to study of circuits in the central nervous system, whether following brain [9] or spinal cord injection [12]. The injection of this AAV serotype in the periphery in this study, however, is a novel extension of its retrograde capabilities to transducing the peripheral nervous system. Whereas most previous studies examining transduction of peripheral motor nerves have utilized the retrograde transport propensity of AAV6 [2], [4], [10], [13], the success of rAAV2-retro in this study adds another potential candidate for further consideration and characterization in the periphery.

C. *Temporal variability in functional expression*

Through the course of this study, we were able to formulate a gross timeline of ChR2 expression following AAV injection. Functional evidence of the degree of expression included varying magnitudes of EMG responses to optical stimulation and the presence or absence of visible muscle contractions. In addition, the frequency response of EMG-optical stimulus coupling within a given nerve also varied considerably over the course of the experiment. This suggests that the frequency response of EMG responses to peripheral optical stimulation is not only a function of the kinetics of the expressed opsin, but may also depend on other factors including the number of axons actively labelled and the expression density along individual axons.

V. Conclusions

This study begins to address a critical component for the success of viral gene therapy in the peripheral nervous system, although further work is needed both to validate and expand upon these results. Although design of the viral vehicle for gene delivery is ultimately responsible for initial nerve transduction in the periphery, development and concomitant administration of immunosuppressive regimens will likely be at least as important to maintaining transgene expression at levels and durations useful for clinical therapy.

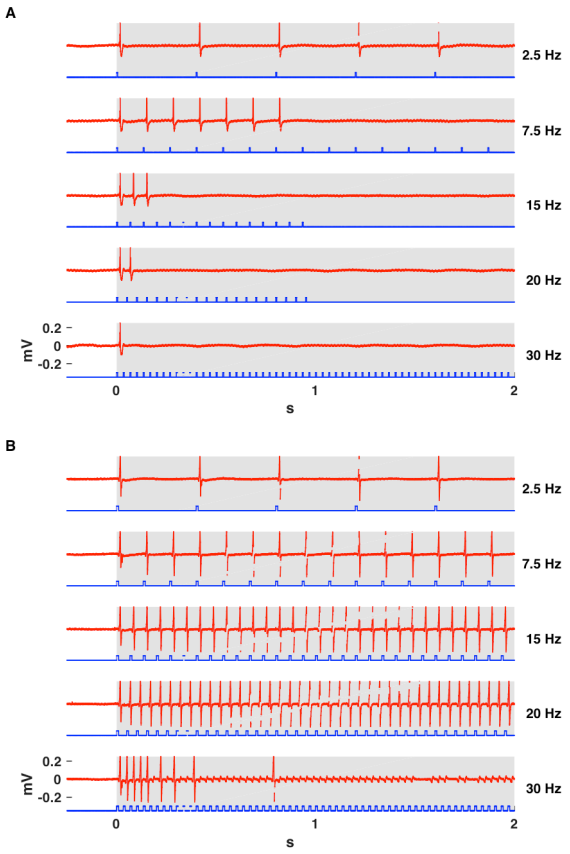

Figure 3. Frequency response of EMG-optical stimuli coupling varies with time. EMG responses to two second trains of optical stimulation of the left DP nerve at increasing pulse frequencies at A) 19 weeks and B) 22 weeks.

References

[1] M. E. Llewellyn, K. R. Thompson, K. Deisseroth, and S. L. Delp, "Orderly recruitment of motor units under optical control in vivo," *Nat. Med.*, vol. 16, no. 10, pp. 1161–1165, Oct. 2010.

[2] C. Towne, K. L. Montgomery, S. M. Iyer, K. Deisseroth, and S. L. Delp, "Optogenetic Control of Targeted Peripheral Axons in Freely Moving Animals," *PLoS ONE*, vol. 8, no. 8, p. e72691, Aug. 2013.

[3] J. B. Bryson *et al.*, "Optical Control of Muscle Function by Transplantation of Stem Cell–Derived Motor Neurons in Mice," *Science*, vol. 344, no. 6179, pp. 94–97, 2014.

[4] J. J. Williams, A. M. Watson, A. L. Vazquez, and A. B. Schwartz, "Viral-mediated optogenetic stimulation of peripheral motor nerves in non-human primates," *bioRxiv*, p. 261925, Feb. 2018.

[5] L. Samaranch *et al.*, "AAV9-mediated Expression of a Non-self Protein in Nonhuman Primate Central Nervous System Triggers Widespread Neuroinflammation Driven by Antigen-presenting Cell Transduction," *Mol. Ther.*, vol. 22, no. 2, pp. 329–337, Feb. 2014.

[6] H. Jiang *et al.*, "Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy," *Blood*, vol. 108, no. 10, pp. 3321–3328, Nov. 2006.

[7] J.-H. Shin, Y. Yue, A. Srivastava, B. Smith, Y. Lai, and D. Duan, "A Simplified Immune Suppression Scheme Leads to Persistent Micro-dystrophin Expression in Duchenne Muscular Dystrophy Dogs," *Hum. Gene Ther.*, vol. 23, no. 2, pp. 202–209, Feb. 2012.

[8] C. Towne, M. Pertin, A. T. Beggah, P. Aebischer, and I. Decosterd, "Recombinant adeno-associated virus serotype 6 (rAAV2/6)-mediated gene transfer to nociceptive neurons through different routes of delivery," *Mol. Pain*, vol. 5, no. 52, 2009.

[9] D. G. R. Tervo *et al.*, "A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons," *Neuron*, vol. 92, no. 2, pp. 372–382, Oct. 2016.

[10] B. E. Maimon *et al.*, "Transdermal optogenetic peripheral nerve stimulation," *J. Neural Eng.*, vol. 14, no. 3, p. 034002, Jun. 2017.

[11] Z. Wang *et al.*, "Successful Regional Delivery and Long-term Expression of a Dystrophin Gene in Canine Muscular Dystrophy: A Preclinical Model for Human Therapies," *Mol. Ther.*, vol. 20, no. 8, pp. 1501–1507, Aug. 2012.

[12] Z. Wang, B. Maunze, Y. Wang, P. Tsoulfas, and M. G. Blackmore, "Global connectivity and function of descending spinal input revealed by 3D microscopy and retrograde transduction," *J. Neurosci.*, vol. 1196–18, Oct. 2018.

[13] C. Towne, B. L. Schneider, D. Kieran, D. E. Redmond, and P. Aebischer, "Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6," *Gene Ther.*, vol. 17, no. 1, pp. 141–146, Sep. 2009.