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OPTICAL INHIBITION OF MOTOR NERVE AND MUSCLE ACTIVITY IN VIVO

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ABSTRACT: Introduction: There is no therapeutic approach that provides precise and rapidly reversible inhibition of motor nerve and muscle activity for treatment of spastic hypertonia.

Methods: We used optogenetics to demonstrate precise and rapidly reversible light-mediated inhibition of motor nerve and muscle activity in vivo in transgenic Thy1::eNpHR2.0 mice.

Results: We found optical inhibition of motor nerve and muscle activity to be effective at all muscle force amplitudes and determined that muscle activity can be modulated by changing light pulse duration and light power density.

Conclusions: This demonstration of optical inhibition of motor nerves is an important advancement toward novel optogenetics-based therapies for spastic hypertonia.

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The upper motor neuron syndrome includes a constellation of symptoms caused by lesions affecting upper motor neuron pathways. These lesions, which occur with cerebral palsy, stroke, and other cerebrospinal injuries, frequently give rise to abnormal activity in lower motor neurons and involuntary muscle contractions through mechanisms that remain unclear. Spastic hypertonia, a major feature of the upper motor neuron syndrome, is characterized by exaggerated muscle activity in response to muscle stretch. Individuals with spastic hypertonia may be unable to perform voluntary movements, such as walking, and experience significant disability and reduced quality of life.

Current approaches to inhibit motor nerve and muscle activity for treatment of spastic hypertonia have important limitations. Oral medications such as dantrolene and tizanidine have poor specificity and side effects, including sedation, weakness, and withdrawal. Selective dorsal rhizotomy reduces spasticity through irreversible damage of the nerve. Intramuscular injection of botulinum toxin diminishes spasticity by blocking release of acetylcholine at the neuromuscular junction for a period of several months, but its use is limited by drug tolerance and muscle weakness. To date, no clinical treatment provides precise and rapidly reversible inhibition of motor nerve and muscle activity to treat spastic hypertonia. Thus, a novel means for precise, reversible inhibition is a long-sought goal of neuroscience, bioengineering, and clinical research.

Optogenetics employs light-sensitive proteins encoded by microbial opsin genes to facilitate optical excitation or inhibition of metazoan neurons. This method capitalizes on the precision of gene targeting of opsins to specific cell types and on the speed of optical interrogation. Two opsins, channelrhodopsin-2 (ChR2) and halorhodopsin (NpHR), have been the most widely used tools for optical deconstruction of neural circuits. When illuminated with blue light, ChR2 allows sodium ion influx into neurons causing membrane depolarization and excitation of action potentials. In contrast, NpHR, when illuminated with yellow light, pumps chloride ions into neurons, hyperpolarizing the membrane and inhibiting action potentials. Optical excitation and inhibition have been demonstrated in mammalian brain slices and in brains of living mice. In the peripheral nervous system, ChR2-mediated optical excitation of motor nerve axons in the mouse sciatic nerve was used to achieve orderly recruitment of motor units of lower limb muscles. In addition, cardiomyocytes in the mouse and rat and skeletal myocytes in the developing chick embryo have been excited with ChR2 and blue light. There have been no reports of optical inhibition of motor nerve and muscle activity in the peripheral nervous system.

The goal of this work was to determine whether NpHR could be used in the peripheral nervous system to inhibit motor nerve and muscle activity...
with light. We first sought to determine whether enhanced NpHR (eNpHR2.0) is expressed in axons of the sciatic nerve of Thy1::eNpHR2.0 mice. We next sought to demonstrate optical inhibition of muscle force and electromyographic activity and measure the time precision of inhibition. We examined inhibition over the range of electrically stimulated muscle force and characterized the dependence of inhibition on light power density.

**METHODS**

**Histology and Expression Analysis.** All animal procedures were approved by the institutional animal care and use committee at Stanford University, in accordance with the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health. Healthy adult male and female Thy1::eNpHR2.0 and wild-type C57BL/6 mice, ages 8–14 weeks (average mass 26 g), were studied. Thy1::eNpHR2.0 (line 2) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). eNpHR2.0 is a modified version of NpHR that has improved membrane localization and expression along neuronal processes. The latter feature is especially relevant for controlling motor nerves, which have axons that span great distances from cell somata in the spinal cord to muscles in the periphery.

We performed histology and expression analysis of the sciatic nerve to determine whether transgenic Thy1::eNpHR2.0 mice express eNpHR2.0 fused to enhanced yellow fluorescent protein (EYFP) in the peripheral nervous system. We perfused animals with 4% paraformaldehyde and prepared transverse 20-μm-thick nerve cryosections. For expression analysis, nerves from transgenic and control mice were processed on the same slide. Histology was performed against laminin, a basement membrane marker, as this protein is highly expressed in the peripheral nervous system and serves as a counterstain for axons. We labeled sections with a primary antibody against laminin (1:200; AL-4; Abcam, Cambridge, Massachusetts) and a secondary antibody, Cy3-conjugated donkey anti-rat (1:1000; Jackson Laboratories), using standard histological protocols. Scans in the EYFP channel were taken at 40× magnification and 2× zoom at a low gain (600 PMT) and high offset (1%) such that no fibers were saturated. Representative raw images are shown in Figure S1 (refer to Supplementary Material available online). eNpHR2.0 expression was sufficiently high so that all axons in nerves from transgenic mice were brighter than the background levels observed in axons from wild-type mice. ImageJ software was used to quantify mean fluorescence intensity and diameter of individual fibers.

**In Vivo Measurements.** We performed a series of experiments to determine whether electrically stimulated motor nerve and muscle activity could be inhibited by illumination of the sciatic nerve in anesthetized Thy1::eNpHR2.0 and wild-type mice. Mice were anesthetized with isoflurane (1–3%) and kept warm on an electrical heating pad (37°C). The hindlimb was shaved, and the sciatic nerve was exposed. The triceps surae muscle group of the lower limb was exposed, and the Achilles tendon was isolated. The calcaneus was cut, and a small bone piece at the tendon was attached through a lightweight, rigid hook to a force transducer (0.3-mN resolution; 300CLR; Aurora Scientific, Aurora, Ontario). Electromyography (EMG) electrodes made from stainless steel, tetrafluoroethylene-coated wires (50 μm; 790700; A-M Systems, Carlsborg, Washington) were inserted into the medial gastrocnemius muscle belly and muscle–tendon junction with a ground electrode at the forelimb wrist.

To initiate motor nerve activity we placed a custom-built stimulation cuff around the sciatic nerve and applied electrical stimulation (Figs. 1 and S2). The sciatic nerve includes motor and sensory axons; however, only motor activity was investigated here. Electrically stimulated motor axons propagate action potentials to the lower limb muscles to cause contractile and electrical muscle activity. Contractile activity was recorded by the force transducer, and electrical activity was recorded by the EMG electrodes. To achieve
optical inhibition, we illuminated axons in the nerve 1–3 mm distal to the electrical stimulation cuff with green laser light.

**Electrical Stimulation.** Motor nerves were electrically stimulated (S48 Stimulator; Grass Technologies, West Warwick, Rhode Island) through the stimulation cuff. Supramaximal stimulation was determined for each mouse by incrementally increasing voltage until twitch force amplitude plateaued. Stimulation voltage was alternately increased and decreased for subsequent trials to avoid possible temporal effects. Twitch stimulation was 0.1-ms pulses at 1 Hz for 20 s. Tetanic stimulation was 0.1-ms pulses at 100 Hz for 3 s at approximately 50% of supramaximal twitch force. Rest periods were 20 s and 5 min between trials of twitch and tetanus, respectively.

**Illumination.** Illumination was from a diode-pumped solid-state yellow-green laser (561 nm; CrystaLaser, Reno, Nevada) via a multimode optical fiber (400-μm diameter, 0.37 numerical aperture; Thorlabs, Newton, New Jersey). The 561-nm wavelength is within 70% of the eNpHR2.0 activation peak of 589 nm. Light power density was calculated as the power at the fiber tip measured with a power meter (PM100; Thorlabs) divided by the light spot area of 1.0 mm² illuminating the nerve (diameter of approximately 1.1 mm). The internodal length of C57BL/6 mouse sciatic nerve is approximately 0.63 mm. Therefore, we expect that this light spot illuminated, on average, 1 or 2 nodes of Ranvier per axon. The angle of light incidence at the nerve surface was insufficient to cause total internal reflection, assuming the nerve refractive index is similar to that of brain. We therefore expected that light would propagate transversely through the nerve with minimal longitudinal spreading. Average temperature increase at the nerve (1.4°C) during a 4-s pulse with 6.0 mW/mm² measured with a thermocouple (Omega, Stamford, Connecticut) was below the average temperature increase (2.1°C) related to hyperthermia-induced changes in rat hippocampal neurons after 10 min of heating (Table S1). Experiments with Thy1::eNpHR2.0 and wild-type mice involved 3–6 h of repeated electrical stimulation and illumination (up to 200 trials per mouse). We observed no localized nerve damage and no systematic change in force or EMG during this time.

**Data Recording and Analysis.** Force and EMG data were recorded at 10 kHz (PCI-6251; National Instruments, Austin, Texas) and analyzed using MATLAB (The Mathworks, Natick, Massachusetts). Passive force (muscle force with no activation) was measured for each trial prior to electrical stimulation of the nerve. Analyses were done on the active (total − passive) force. Average peak twitch force with light off (Fav_off) and light on (Fav_on) were calculated for each trial of 20 twitches. Percent inhibition was (Fav_off − Fav_on) / (Fav_on) * 100. Average tetanic force was calculated with light off (1.1–1.9 s of trial duration, Fav_off) and light on (2.1–2.9 s of trial duration, Fav_on). Data that were collected in the 100-ms durations after light onset and light offset were excluded from calculations due to the rapid changes in force amplitude. EMG was bandpass filtered (50–300 Hz) and full-wave rectified, and percent inhibition was calculated using the same method as was used for force. EMG amplitudes were normalized to the twitch and tetanus maximum EMG amplitudes observed in that mouse.

We quantified the time precision of inhibition by measuring the time durations between light onset and inhibition of tetanic force and between light offset and reversal of inhibition. Inhibition was defined to begin when force amplitude decreased ≥2 standard deviations below the mean force prior to illumination, and reversal was defined to occur when force amplitude increased ≥2 standard deviations above the mean force during illumination.

**Statistics.** Standard error of the mean (SEM) was calculated from the variance of the mean calculated for each mouse. P values were calculated for unequal sample size and unequal variance using a Welch t-test (two-sided, z < 0.01) with degrees of freedom calculated using the Welch–Satterthwaite equation.

**RESULTS**

**Optical Inhibition of Motor Nerve and Muscle Activity in Thy1::eNpHR2.0 Mice.** Thy1::eNpHR2.0 mice express eNpHR2.0-EYFP at high functional levels throughout the brain, and we found that these mice also express eNpHR2.0-EYFP in the peripheral nervous system. Confocal microscopy of EYFP fluorescence revealed high levels of eNpHR2.0 expression within nearly all axons (>99%) of the sciatic nerve (Fig. 2A). eNpHR2.0-EYFP appeared localized to neuronal membranes (Fig. 2B). However, we observed variability in the level of expression across the neuronal population (Fig. 2B and Fig. S1). No correlation was found between axon diameter and level of expression (r² = 0.11).

We observed robust and reversible inhibition of electrically stimulated twitch and tetanic muscle force and EMG by illuminating the sciatic nerve with pulses of green light (Fig. 3A and B). Twitch force and EMG amplitudes were inhibited during 4-s light pulses and returned to prior magnitudes upon cessation of each light pulse. Representative traces show >90% inhibition of twitch force and EMG. Representative traces show 48% inhibition...
of tetanic force and 77% inhibition of EMG during a 1-s light pulse. Thus, optical inhibition of motor nerve activity is possible and causes substantial and reversible inhibition of electrically stimulated muscle activity.

We measured the times between onset and offset of the light pulse and inhibition of tetanic force to quantify the time precision of optical inhibition. Inhibition of tetanic force occurred 65 ± 18 ms after light onset, and reversal of inhibition occurred 38 ± 5 ms (mean ± SEM) after light offset (n = 4 mice, 30 trials).

Inhibition over the Range of Electrically Stimulated Force. We quantified inhibition of twitch force and EMG amplitudes in Thy1::eNpHR2.0 mice and wild-type mice (Fig. 4A and B) at force amplitudes of approximately 50% of supramaximal force. Illumination of the sciatic nerve with a light power density of 6.0 mW/mm² was sufficient to cause inhibition in Thy1::eNpHR2.0 mice (mean ± SEM: 83 ± 3% and 84 ± 4% for force and EMG, respectively), indicating a significant difference from wild-type mice (P = 0.0000059 and 0.000087 for force and EMG, respectively).

Without illumination, increasing stimulation voltage increased twitch force amplitudes to generate a characteristic electrically stimulated motor unit recruitment curve. During illumination, we observed 81% inhibition of the plateau region of the recruitment curve. The representative curves demonstrate inhibition exceeding 74% at all amplitudes of twitch force (Fig. 4C).

To further establish the relationship of inhibition to force amplitude we determined percent inhibition over the range of electrically stimulated twitch forces in Thy1::eNpHR2.0 and wild-type mice (Fig. 4D). We found effective inhibition at all
amplitudes of twitch force with an average percent inhibition of 80 ± 6%. Illumination did not cause inhibition in wild-type mice (average percent inhibition = 1 ± 4%). This relationship suggests that optical inhibition may effectively reduce both chronic, low-level muscle activity and brief bursts of high muscle activity.

Relationship between Optical Inhibition and Light Power Density. We found that the degree of inhibition of twitch force was directly related to light power density. As illumination was increased incrementally up to 1.2 mW/mm², twitch force inhibition increased to 66 ± 2% (Fig. 5A). The average slope in this region was 57% per mW/mm². As illumination was further increased to 14.6 mW/mm², inhibition increased more modestly to 89 ± 1%, with an average slope of 1% per mW/mm². In the Supplementary Material, we provide a video demonstration of increasing inhibition of twitch force with increasing light power density (Video S1 and Fig. S3).

Tetanic forces were also modulated with illumination. As illumination was increased incrementally up to 1.2 mW/mm², inhibition increased to 47 ± 5% (Fig. 5B). The average slope in this region was 34% per mW/mm². As illumination was further increased to 14.6 mW/mm², inhibition increased to 88 ± 7%, with an average slope of 2% per mW/mm².

DISCUSSION

In this study we have demonstrated NpHR-mediated optical inhibition of motor nerve and muscle activity in the peripheral nervous system. We found that illumination of the sciatic nerve with 561 nm laser light causes robust and reversible inhibition of motor nerve activity and significantly reduces muscle force and EMG in Thy1::eNpHR2.0 mice. The data show that eNpHR2.0 is capable of intercepting spikes in the axon, optical inhibition is effective at all amplitudes of electrically stimulated twitch force, and light power density modulates the degree of inhibition. The results demonstrate the potential of NpHR as a powerful tool for inhibiting motor nerve and muscle activity with light. Combined with optical excitation of muscle via illumination of ChR2-expressing motor nerve axons, our method for optical inhibition of muscle activity creates a powerful toolbox for temporally precise control of peripheral nerves and muscles with light.

Greater than 1 mW/mm² is required to activate eNpHR2.0, and light power density is reduced due to scattering and absorption when light is transmitted through rat and mouse cortex. Our results in mouse sciatic nerve suggest that increasing light power density at the nerve surface activates eNpHR2.0 through greater depths of the nerve cross-section. Percent inhibition increased most dramatically as light power density became sufficient to activate eNpHR2.0 throughout the nerve cross-section. Thus, we identified changing the light power density as a means to modulate the degree of inhibition of motor nerve and muscle activity. Further advancement of optical inhibition in the peripheral nervous system will require characterization of this relationship for nerves with different diameters and comparing various means to deliver light to the nerve.

Optogenetic approaches have facilitated the deconstruction of numerous neural circuits in the brain and may allow us to gain understanding of...
neural circuits in the peripheral nervous system and the diseases associated with them. Peripheral nerves can be excited with electrical stimulation and inhibited with high-frequency alternating current, but these electrical methods have limited ability to control activity in selected nerve pools. Although these results were obtained in transgenic mice in which nearly all nerves were sensitive to light, optogenetic methods enable activation and inhibition of selected nerves by utilizing viral targeting of opsins to these nerves. For example, optogenetics enabled systematic targeting of different elements of the Parkinson disease circuit. Selective optical stimulation in layer V of motor cortex was found to ameliorate parkinsonian symptoms, elucidating underlying mechanisms of therapy with deep brain stimulation. Future work that enables cellular-level optogenetic control of specific motor nerves may allow dissection of the neural circuits involved in spasticity and provide a novel means to elucidate the mechanisms of spasticity.

Development of optogenetics-based therapies will require selective, virus-mediated gene delivery of NpHR or other opsins to non-transgenic motor nerves in humans. Selective virus-mediated gene delivery of fluorescent protein to distinct motor neuron pools has been achieved in rodents and nonhuman primates by retrograde transport of adeno-associated virus (AAV) particles injected intramuscularly. Gene delivery of NpHR has shown high and well-tolerated neuron-specific expression in nonhuman primates, and clinical trials of gene delivery using a variety of AAV serotypes in humans are underway. Future advances in gene-delivery techniques could achieve selective delivery of NpHR to nontransgenic motor neurons in humans. Demonstration of long-term safety and efficacy in combination with advances in light delivery devices may enable clinical translation of robust, reversible, and selective optical inhibition in humans to treat spastic hypertonia.

Although there are many challenges associated with the translation of this technique to humans, the demonstration of optical inhibition of motor nerve and muscle activity in transgenic mice provides a first step toward novel optogenetics-based therapies for spastic hypertonia associated with upper motor neuron syndrome and hypertonicity associated with a wide range of other diseases.

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