1 **Long title: Reversible temporally-specific inhibition of muscle using a light-activated**

2 **chloride channel**

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4 **Short title: Optogenetic inhibition of muscle**

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Abstract

Achieving reversible, temporally specific inhibition of motor neurons has the potential to revolutionize treatment of disorders marked by muscle hyperactivity. Current treatment strategies are inadequate – surgical interventions are irreversible and pharmaceutical interventions have off-target effects. Optogenetic strategies for inhibiting muscle activity have theoretical promise; however, trafficking and expression problems have prevented translatable optogenetic suppression of muscle activity. Here, we exploit recent innovations in opsin engineering to demonstrate virally mediated, temporally specific optogenetic inhibition of motor neurons and muscle activity *in vivo*. We show that intra-muscular injection of adeno-associated virus serotype 6 can drive expression of the inhibitory channelrhodopsin mutant iC++ in both immunodeficient and wild-type mice. Illumination of the sciatic nerve in wild-type mice resulted in 64.8% inhibition of evoked twitch force. Optical excitation during tetanic stimulation in wild-type mice resulted in 59.1% inhibition at 10 Hz and 55.4% at 25 Hz. The extent of optogenetic inhibition was titratable, and ranged from 0% to 78.4% as illumination intensity was changed from 1 mW to 20 mW/mm². These results could have therapeutic applicability to disorders such as spasticity, hypertonia and urinary incontinence, and provide a new tool to neuroscientists and muscle physiologists wishing to reversibly inhibit motor neuron activity *in vivo*.

Introduction

Disorders of the neuromuscular system can compromise activities of daily living. While varying widely in their causes, many of these disorders have excessive muscle activity as a common symptom. This excessive activity adversely affects quality of life in different ways.

40   Spasticity, for example, results in involuntary muscle contractions that interfere with precise

41   movement and locomotion[1, 2]. In overactive bladder syndrome, excessive activation of the

42   detrusor muscles of the bladder results in unwanted micturition[3, 4].

43   Surgical and pharmacological interventions to reduce muscle hyperactivity have important

44   limitations. Surgical approaches include division of nerves through rhizotomy or peripheral

45   neurectomy, which is irreversible and may cause unwanted results[5, 6]. Intrathecally

46   administered baclofen is commonly used to diminish spasticity but does not inhibit specific

47   muscles, and has implant associated morbidity[7]. Intramuscular injection of botulinum toxin

48   has only transient effects, and is complicated by redosing induced tolerance[8]. Off-label use

49   of medications such as diazepam and riluzole may offer some relief; however, they have

50   unreliable efficacy and side effects such as fatigue, dizziness, depression and cognitive

51   dullness[9, 10].

52   Optogenetic techniques enable temporally precise, reversible control of neuronal activity[11].

53   However, optogenetic strategies to excite neural activity have been more tractable than

54   optogenetic silencing[12-16]. In our experience, this has proven particularly true in control

55   of neuromuscular circuits[17]. Our laboratory, and others, have previously reported

56   optogenetic *excitation* of muscle activity *in vivo*, using a transgenic approach and using viral or

57   cell-transfer models that have some translational resonance[18, 19]. While we have been able

58   to achieve optogenetic inhibition of muscle activity using a transgenic mouse line[18], the

59   critical step of optogenetically inhibiting muscle through a translationally relevant approach

60   has, until now, remained intractable. Here, we exploit recent innovations in opsin

61   engineering[20] to show that the improved inhibitory opsin mutant, iC++, when virally

62   delivered to peripheral motor neurons, enables inhibition of muscle activity *in vivo*.

Materials and Methods

Animal test subjects

All animal procedures were approved by the Administrative Panel on Laboratory Animal

Care at Stanford University, in accordance with the "Guide for the Care and Use of

Laboratory Animals" from the National Institutes of Health.

Viral vectors

AAV6 expressing iC++ (AAV6-iC++) was produced at the University of North Carolina

Vector Core Facility. The expression cassette comprised iC++ fused to yellow fluorescent

protein (eYFP) under control of the human synapsin promoter. Genomic DNA was

packaged into the AAV6 capsid using helper plasmids. A single viral batch was used with a

dot-blot hybridization titer of 1.4×10^{13} v/ml.

Intra-muscular viral injections

Healthy juvenile female C57BL/6 and *Foxn1^{NU}* (Nu/J) mice, aged 4 weeks (average weight

23.0 g), were obtained from Jackson Laboratories (Bar Harbor, Maine) and kept on a 12:12

light:dark cycle. Mice were anesthetized using isoflurane (1-3%) and the gastrocnemius was

exposed. Using a syringe pump (World Precision Instruments, Sarasota, FL) fitted with a

Hamilton syringe (Hamilton, Reno, NV), 12 μl of AAV6-hSyn-iC++-eYFP was injected into

the medial and lateral left gastrocnemius across 4 sites, using a 35G needle, at a rate of 1.5

μl/min. The skin was then sutured closed and mice were allowed to recover. One week prior

to euthanasia, using cervical dislocation under isoflurane, mice were injected with 12 μl of

4% Fluorogold (Santa Cruz Biotechnology, Dallas, TX) in the virus-injected muscle, using

the procedures described previously[17, 18].

Histology

86   Spinal cord and sciatic nerve, were dissected, post-fixed (4°C overnight), and transferred to

87   30% sucrose in PBS (4°C until sectioning). Tissues were embedded in Tissue-Tek OCT

88   compound (Sakura, Holland) and sectioned on a Leica CM3050 cryostat (Leica, Buffalo

89   Grove, IL). Spinal cords were cut in 40 μm longitudinal sections and 20 μm cross sections

90   and mounted onto glass slides. Slides were stained and mounted in PVA-DABCO (Sigma-

91   Aldrich, St. Louis, MO). Sciatic nerves were processed in the same manner with 20 μm

92   section thickness. The percentage of Fluorogold positive motor neurons expressing iC++

93   was quantified in 13 out of 40 sections.

In vivo measurements

95   Nu/J and wild-type mice were anesthetized with isoflurane (1‑3%) and kept warm on an

96   electrical heating pad (37°C). The hindlimb was shaved, and the sciatic nerve exposed, and

97   the Achilles tendon isolated. The calcaneus was cut, and a small bone piece at the tendon

98   was attached through a light-weight, rigid hook to a force transducer (0.3 mN resolution;

99   300C-LR; Aurora Scientific, Aurora, Ontario). Electromyography (EMG) electrodes from

100   stainless steel, tetrafluoroethylene-coated wires (790700; A-M Systems, Carlsborg,

101   Washington) were inserted into the medial gastrocnemius muscle belly and muscle–tendon

102   junction with a ground electrode at the forelimb wrist.

103   A custom-built electrical cuff (Microprobes, Gaithersburg, MD) was placed around the

104   sciatic nerve and motor nerves were electrically stimulated (Powerlab 16/35, ADinstruments,

105   Colorado Springs, CO). Supramaximal stimulation was determined for each mouse by

106   incrementally increasing voltage until twitch force amplitude plateaued. Twitch stimulation

107   was 2 ms pulses at 1 Hz for 60-90 s at 50% supramaximal twitch force. Tetanic stimulation

108   was 2 ms pulse width at 10 and 25 Hz for 60-90 s at approximately 50% of supramaximal

109   twitch force. Rest periods were 120 s between trials.

5

Illumination

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111   Continuous illumination at the nerve was from a blue laser (473 nm, OEM Laser Systems,

112   East Lansing MI) via a multimode optical fiber (365 μm diameter, 0.37 numerical aperture,

113   Thorlabs, Newton NJ). Light power density was calculated as the power at the fiber tip

114   measured with a power meter (PM100, Thorlabs) divided by the light spot area of 1 mm^2

115   illuminating the nerve (light spot diameter of approximately 1.1 mm). Experiments with

116   Nu/J and wild-type mice involved 2 to 4 hours of repeated electrical and optical stimulation

117   (up to 75 trials per mouse). We observed no localized nerve damage and no systematic non-

118   optogenetic change in force or EMG during this time. Each trial included consecutively 10 s

119   pre-light, 30 s with light, and 20 s post-light twitches or 10 s pre-light, 70 s with light, and 20

120   s post-light twitches. The start, end, frequency, and pulse duration of the light within each

121   trial was controlled using Powerlab 16/35 (ADinstruments, Colorado Springs, CO). To

122   achieve optical inhibition, we illuminated axons in the nerve at the electrical stimulation cuff.

Data analysis

123

124   Force and EMG data were recorded at a sampling frequency of 1 kHz using Powerlab 16/35

125   and analyzed using both Labchart 7.0 (ADinstruments, Colorado Springs, CO) and custom

126   scripts written in MATLAB (Mathworks, Natick, MA). Passive force amplitude (muscle

127   force with no activation) was measured for each trial prior to electrical stimulation of the

128   nerve. Analyses were done on the active (total - passive) force.

129   Electrically evoked forces were calculated at three time-points: 'pre-light', during 'light', and

130   'recovery'. As electrically evoked forces were observed to decay in an approximately

131   exponential manner, the extent of optogenetically evoked inhibition was calculated based on

132   mean evoked forces during the final 20% of optical stimulation (at which point inhibition

133 intensity had stabilized. Depending on the duration of illumination, the time-period included

134 varied from 2–24 seconds. For consistency, 'pre-light' and 'recovery' forces were also

135 calculated as the mean forces evoked during the final 20% of each time-period.

136 Time-constants were determined for inhibition 'onset' by fitting observed force peaks to an

137 exponential function of the form $f(t) = a \cdot e^{-t/\tau_{on}} + b$.

Statistics

139 Calculations were performed separately for each stimulation trial, and the inhibition

140 parameters for all trials from a given mouse were then averaged together to determine the

141 parameters for that mouse. Group data means and standard errors were then calculated as

142 based on trial-averaged mouse results. P-values were calculated using the paired or unpaired

143 Student's t-test, as appropriate. Significance thresholds were set at $P < 0.05$ (*), $P < 0.01$ (**),

144 and $P < 0.001$ (***). Effect sizes were calculated using the Hedges g metric, through the

145 Measures of Effect Size Toolbox[21]. All grouped data are normalized and shown as mean $\pm$

146 s.e.m.

Results

148 We began by conducting a broad-spectrum scan of adeno-associated viruses and inhibitory

149 opsins to test whether different combinations of virus, opsin, dose and delivery route could

150 produce sufficient opsin expression to enable silencing of muscle activity *in vivo*. Surprisingly,

151 despite testing 23 such virus-opsin combinations *in vivo* (S1 Table), we were unable to

152 achieve consistent, robust optogenetic inhibition of electrically evoked muscle twitches.

153 Histological examination of the nerves and spinal cords of these mice indicated that the

154   principal reason was a lack of sufficient expression and trafficking of the opsin transgene in

155   primary motor neurons.

156   To overcome this, we adopted two new experimental approaches. First, because we had

157   previously observed a strong immune response to peripheral adeno-associated virus

158   administration[22], consistent with reports from clinical trials[23], we reasoned that

159   performing our experiments in immunocompromised mice would be useful to validate our

160   viral expression strategy. Second, because we had previously observed that

161   channelrhodopsin-2 could be virally expressed with high efficiency in primary motor

162   neurons[17], we reasoned that the recently developed inhibitory chloride-conducting

163   channelrhodopsin mutant, iC++, may exhibit high transduction efficiency, and may

164   therefore enable functional inhibition *in vivo.*

165   We assayed immunocompromised mice 5 weeks following injection of AAV6-hSyn-iC++-

166   eYFP into the gastrocnemius muscle. In stark contrast to our previous inhibitory

167   experiments, we observed that iC++ trafficked throughout the primary motor neuron with

168   high efficacy (Fig 1a). We performed Fluorogold injections into the virally injected muscle to

169   quantify the percentage of the motor pool transduced, and observed it to be ~38% (Fig 1a,

170   top, *n* = 2).

171   We then turned to functional assays of motor neuron inhibition. Following experimental

172   methods described previously[18], we placed an electrical cuff around the sciatic nerve, and

173   illuminated the region of the nerve that received electrical stimulation (constant light: 20

174   mW/mm^2, 473 nm; electrical stimulation: 1 Hz, 2 ms), while simultaneously measuring force

175   output and electromyographical activity (EMG) in the targeted muscle (S1 Fig). We observed

176   that blue light (473 nm) illumination in iC++ injected mice was sufficient to block force

8

177   generation in the targeted muscle, with concomitant reduction in the EMG waveform (Fig

178   1b). Importantly, optogenetically evoked inhibition was completely reversible, with force

179   output returning to 122.6 ± 1.8 % (n = 12) of baseline pre-light force (Fig 1b,c). Across 12

180   mice, iC++ inhibition during electrically evoked twitch force was robust; at 1 Hz we

181   observed an average inhibition of 79.9 ± 3.2% compared to pre-light baseline force (P =

182    $8.10e^{-09}$, effect size = 4.6, Fig 1c). Interestingly, in contrast with previous reports of light-

183   mediated inhibition in transgenic Thy1-NpHR2.0 mice[18], optogenetic inhibition and

184   recovery with iC++ was not instantaneous, and followed a consistently exponential onset

185   pattern (τ_{on} = 4.08s ± 0.49, R^2 = 0.955, P = 0.006, Fig 1b). Inhibition could be sustained

186   over a wide range of durations, lasting for as long as 70s, with no noticeable deleterious

187   effects on post-light force production (Fig 1d,e). We did not observe any reduction in

188   magnitude of inhibition over the course of the illumination period. The degree of

189   optogenetic inhibition could be titrated over a range of 78.4% to 0% by changing

190   illumination intensity from 20 mW/mm^2 to 1 mW/mm^2 (n = 1, Fig 1f), respectively. We

191   observed that this intensity-dependence was a function of the animal's light-sensitivity, and

192   varied with the animals' peak achievable inhibition. We categorized 4 animals, 2 high-

193   responding (dark green) and 2 low-responding (light green) that best displayed this variation

194   (n = 4, Fig 1g).

195

196   **Fig 1. Reversible optogenetic inhibition of twitch force in muscle.** a, top. Longitudinal

197   section of lumbar spinal cord 5 weeks post intramuscular injection of iC++ (eYFP, green)

198   and one week post fluorogold (FG, red) injection. Arrowheads denote co-localization of

199   eYFP with fluorogold, representing an average of 38.1% of the total motor neuron

200   population over 2 animals expressing iC++. Scale bar 100 μm. a, lower. Confocal image of a

201   cross-section of the sciatic nerve showing motor nerve axons expressing eYFP from the

202   fusion protein of iC++-eYFP (green) 5 weeks following intramuscular injection. Blue, DAPI,

203   scale bar 100 μm. Right panel shows inset drawn in left panel. Scale bar 50 μm b. An

204   example force trace is shown, top, with corresponding EMG, bottom. Electrical stimulation

205   of the sciatic nerve of Nu/J mice (2ms pulse duration) was applied at 1Hz to produce a 50%

206   maximal force for the duration of the 60s trial. Compared to pre-light force, we observed a

207   79.9 ± 3.2% average decrease in force occurring under constant blue light (473nm)

208   illumination (20mW/mm^2) for 30s, as indicated by blue bars (P = 8.1e^{-09}, effect size = 4.56,

209   n = 12). The mean time constant (τ onset) of the inhibition was calculated to be 4.08s ± 0.48

210   (r^2 = 09.6 ± 0.006). c. Summary data from 12 animals representing the average inhibition at

211   1Hz electrically evoked force. d. Representative force trace of chronic inhibition (> 50s), top

212   and corresponding EMG, bottom, with blue bars representing constant illumination of

213   sciatic nerve. e. Summary bar graph of mean chronic force inhibition showing 75.4 ± 7.5%

214   optical inhibition (P = 0.007, effect size = 2.50, n = 4) with a recovery of 133.2 ± 11.2% of

215   pre-light force. All grouped data are normalized and shown as mean ± s.e.m. f. Twitch force

216   inhibition was dependent on the density of illumination. Summary data of force traces at

217   50% maximum force (2 ms electrical pulse duration at 1 Hz) in response to varying light

218   densities of blue light (20, 15, 10, 5, 1 mW/mm^2, n=1), with maximum force inhibition

219   occurring at 20mW/mm^2. g. Example normalized plot of 4 mice (2 high-responding, dark

220   green, and 2 low-responding, light green) representing the difference in light evoked

221   inhibition over changes in light densities. Optically elicited force inhibition was shown to

222   vary over the individual mice tested.

223

224   We next examined whether iC++ could suppress higher frequencies of electrically evoked

225   muscle activity (10 Hz, 2 ms; 25 Hz, 2 ms). We observed significant optogenetic silencing of

226   muscle force production during electrical stimulation at 10 Hz, with the force envelope

227   decaying an average of 79.5 ± 9.2% (P= 0.009, effect size = 0.096, n = 4) of the immediate

228   pre-light force levels (Fig 2a,b). At 25 Hz contraction, due to electrical stimulation-evoked

229   fatigue, muscle force after illumination did not completely recover to peak pre-light levels;

230   however, it did reach 66.9 ± 3.6% (n = 5) average recovery of initial pre-light force

231   measurements (Fig 2c,d) following a 71.3 ± 11.4% (P = 0.001, effect size = 1.8) optical

232   inhibition.

233

234   **Fig 2. iC++ mediated tetanic force inhibition at 10 Hz and 25 Hz**. a. Example force

235   (top) and corresponding EMG (bottom) tetanic trace elicited by 60 s of 2 ms electrical

236   stimulation at 10Hz with 30 s of iC++ mediated inhibition using 473 nm constant light (blue

237   bars). b. 10 Hz summary data show an average optogenetic elicited inhibition of 79.5 ± 9.2%

238   compared to pre-light levels with a 94.7 ± 10.4% recovery (P = 0.009, effect size = 0.957, n

239   = 4). c. Representative trace of tetanic force (top) and EMG (bottom) generated by 2ms

240   electrical stimulation at 25 Hz for 60 s. d. Summary data showing a mean inhibition of 71.3

241   ± 11.4% compared to pre-light levels (P = 0.001, effect size = 1.89, n=5). Average post-light

242   force recovery was 66.9 ± 3.6% of baseline. All grouped data are normalized and shown as

243   mean ± s.e.m.

244

245   Our initial experiments were performed in immunocompromised mice. In the clinical setting,

246   gene therapy is often performed with simultaneous immunosuppression[23]; however, as this

247   poses significant side-effects[24], we examined if we could achieve virally-mediated

248   optogenetic inhibition in wild-type mice. We injected AAV6-hSyn-iC++-eYFP into the

249   gastrocnemius muscle of 4 week old wild-type mice and, in contrast with our previous

250   efforts with other opsin-virus cocktails, we observed robust expression in primary motor

251   neurons in these mice 5 weeks post-injection (Fig 3a). Opsin expression patterns were

252   broadly similar to those seen in immunocompromised mice.

253   We repeated all previously described *in vivo* experiments, achieving a substantially similar

254   degree of inhibition (Fig 3b-d). We obtained significant optogenetic inhibition at 1 Hz twitch

255   force with high efficacy (average suppression of 64.8 ± 4.3%, P = 0.0004, effect size = 2.33,

256   *n* = 9, Fig 3b) and were able to inhibit tetanic force production in wild-type mice (Fig 3c,d).

257   The extent of inhibition achievable in wild-type mice (gray) was comparable to that observed

258   in immunocompromised mice (green, Fig 3e).

259

260   **Fig 3. Optical inhibition of electrically evoked muscle force in wild type animals**. a.

261   Confocal image of sciatic nerve cross-section in wild type mouse expressing eYFP (top, left,

262   green) and FG (top, right, blue) in motor axons 5 weeks post injection. FG was injected one-

263   week prior to sacrifice. a, bottom. A merge of eYFP and FG expressing fiber. Scale bar 100

264   µm. b. Average iC++ mediated force inhibition over 9 wild type animals at 1 Hz was 64.8 ±

265   4.3% with a mean recovery of 93.5 ± 1.6% (P = 0.0004, effect size = 2.33, τ onset = 7.4s ±

266   0.8, r^2 = 0.92 ±0.01). c,d. Wild type muscle inhibition from 10 and 25 Hz recordings,

267   respectively, was similar to nude mice data. c. At 10 Hz evoked force, average inhibition

268   observed was 59.1 ± 10.9% with a 93.0 ± 8.3% recovery of pre-light force (P = 0.005, effect

269   size = 1.35, n= 6). d. The mean electrically evoked force at 25 Hz was optically suppressed

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270   by 55.4 ± 8.5% and exhibited a 65.7 ± 13.9% recovery (P = 0.012, effect size = 3.76, n= 4).

271   e. Scatter plot representing the individual distribution of mean force inhibition across nude

272   (green, average = 79.9 ± 3.2%, P = 8.1e^{-09}, n=12) and wild type animals (gray, average =

273   64.8 ± 4.3%, P = 0.0004, n=9) at 1 Hz. All grouped data are shown as mean ± s.e.m.

274

Discussion

276   Here, we report light-mediated inhibition of peripheral motor neurons in nontransgenic mice.

277   This proved exceptionally difficult to achieve (S1 Table). Clinical translation of optogenetic

278   methods to inhibit muscle will require several additional innovations. Chief among these will

279   be the development of improved viral vectors with greater transduction efficacy and reduced

280   immunogenic profile. Additional improvements in inhibitory opsin photocurrents are also

281   likely to be of use[25].

282   A benefit of the optogenetic approach described here is that it allows inhibition of motor

283   neurons through illumination of the peripheral axon, without requiring illumination of the

284   soma within the spinal cord. In eventual clinical translation, it is possible to imagine

285   illuminating peripheral axons through the use of a wirelessly powered nerve cuff[26]. Key

286   remaining hurdles to translation include establishing inhibitory control of motor neurons in

287   species other than mouse and demonstrating optogenetic silencing of muscle during animal

288   locomotion. Additional translational targets are likely to include the urinary system in

289   disorders such as urinary incontinence and detrusor-sphincter dyssynergia. Clinical

290   modulation here would involve injecting the relevant muscles, such as the external urethral

291   sphincter, with retrogradely transducing AAVs, and then illuminating the transduced efferent

292   nerves to induce micturition on command[27-29].

293   The use of a novel light-activated chloride mediated inhibitory channel may also be of

294   interest in cardiac resynchronization therapy. To date, optogenetic inhibition of

295   cardiomyoctes has been limited to cultured rat cardiomyocte cells transfected with the

296   inhibitory proton pumps, Arch3[30] or Arch-T[31]. Although suppression of electrical

297   activity was observed, no *in vivo* aspects of function were tested. The light-activated chloride

298   channel, iC++, may prove useful in inhibition of cardiac muscle, *in vivo,* to achieve a tunable,

299   temporally precise inhibition for cardiac abnormalities such as tachycardia or arrhythmias.

300   The successful virally mediated optogenetic inhibition of efferent motor pathways we

301   describe here completes a quartet of proof-of-concept experiments for peripheral

302   optogenetic neuromodulation – it is now possible to use translationally relevant approaches

303   to optogenetically *excite* as well as *inhibit* both *afferent* sensory neurons and *efferent* motor

304   neurons. Translation of this neuromodulation quartet into the clinic will require careful

305   choice of initial disease targets, improvements to the viral vectors used, and greater

306   understanding of the consequences of long-term opsin expression.

Acknowledgements

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417

Supporting Information

419 **S1 Table. Selected virally mediated attempts.** Efficient transfection and function of an

420 inhibitory opsin was difficult. Over 23 different AAV serotype, promoter, opsin, and

421 fluorescent tag combinations were used before AAV6-hSyn-iC++-eYFP was chosen as the

422 most effective for peripheral motor neuron inhibition. Adeno-associated virus (AAV),

423 recombinant (r), self-complementary (sc).

424 **S1 Fig. Schematic of experimental force setup.** Motor axons of the sciatic nerve were

425 stimulated electrically with a stimulation cuff placed around the proximal sciatic nerve of an

426 anaesthetized mouse. The sciatic nerve was then illuminated with a blue laser light (473 nm)

427 at the cuff. Fine-wire EMG electrodes recorded electrical activity of the medial

428 gastrocnemius and a force transducer attached at the Achilles tendon recorded the
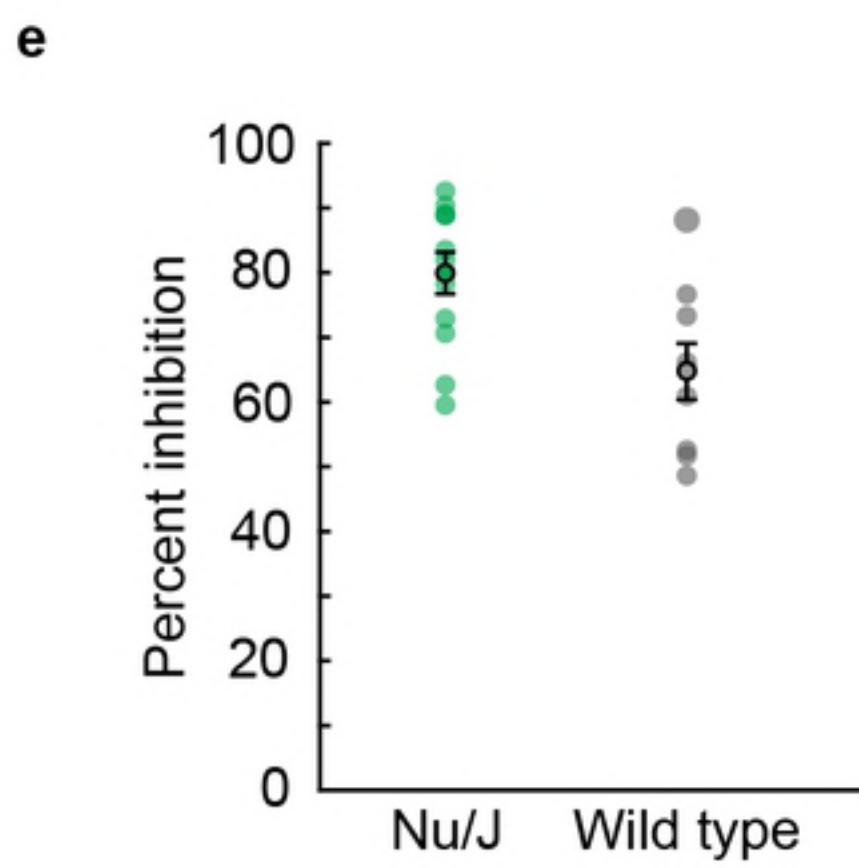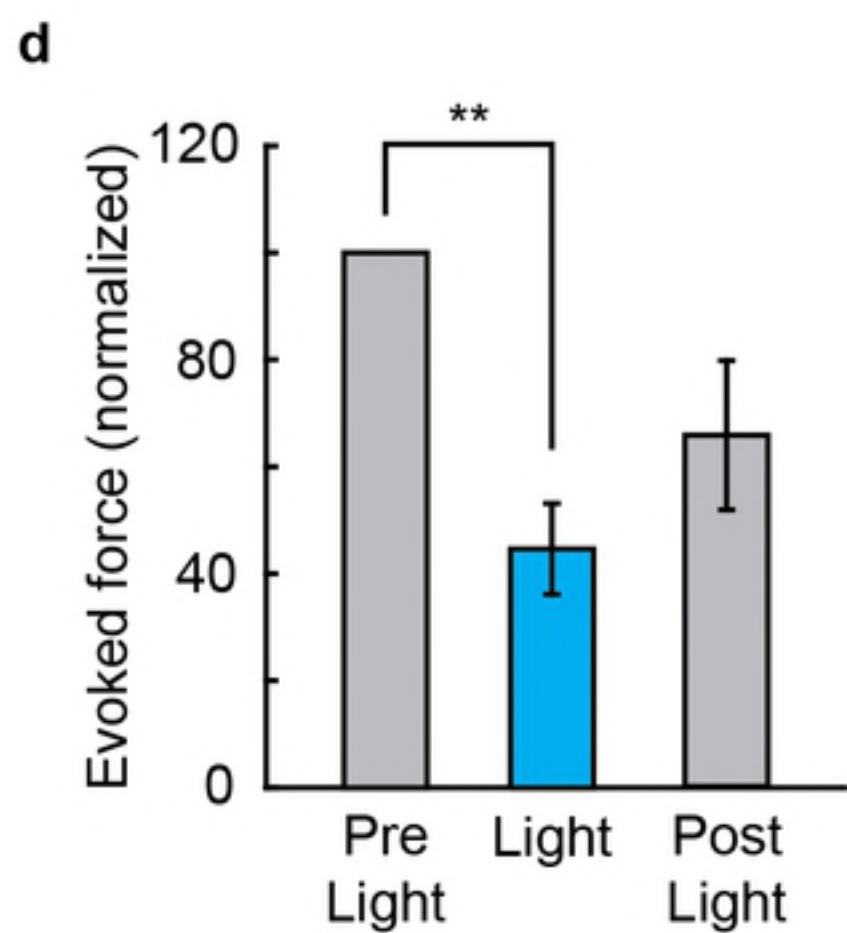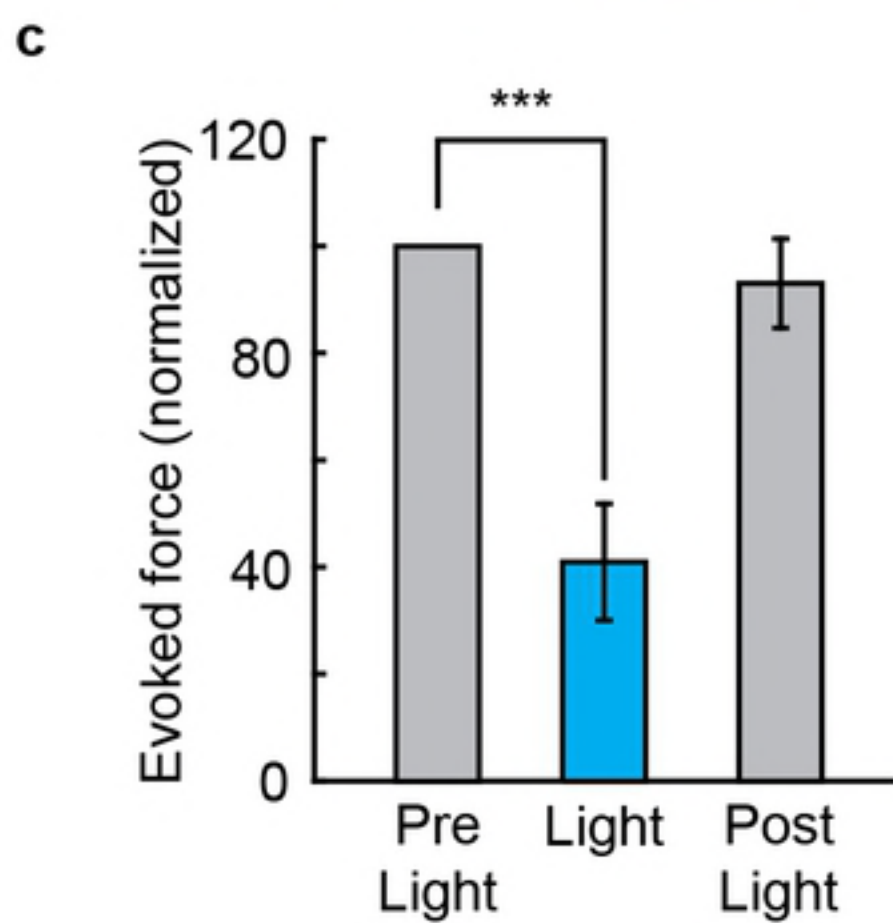
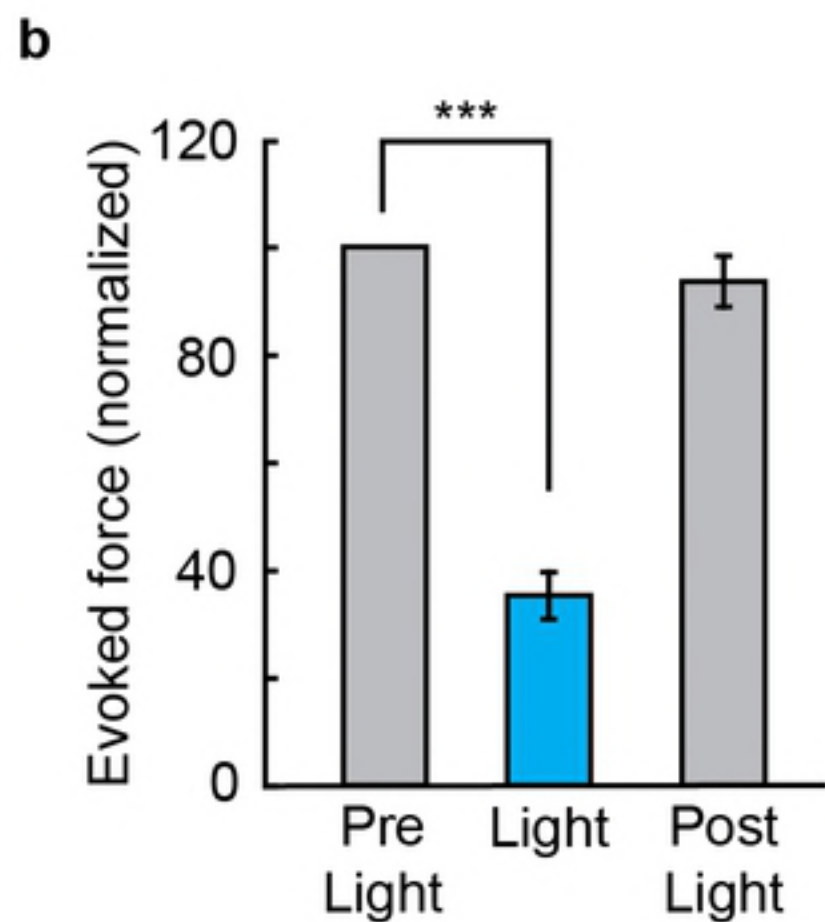429 contractile force of the lower limb muscles.

Figure 3

Figure 1

a

FG
eYFP

DAPI
eYFP

b

c

d

e

f

1 mW/mm²
5 mW/mm²
10 mW/mm²
15 mW/mm²
20 mW/mm²

g

Low-responding mouse
High-responding mouse

Figure 2