Bidirectional Optogenetic Modulations of Peripheral Sensory Nerve Activity: Induction vs. Suppression through Channelrhodopsin and Halorhodopsin

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Abstract

In this study, we investigated the potential of optogenetics for modulating activity of peripheral sensory nerves, particularly tactile and proprioceptive afferents, which are vital for movement control. Using adeno-associated virus serotype 9 vector, we selectively transduced channelrhodopsin (ChR2) and halorhodopsin (eNpHR3.0) into large-diameter sciatic nerve afferents of rats. Diverging from conventional dorsal root ganglion (DRG) approaches, we applied optical stimulation at the distal portion of the afferent nerve. The intensity of optical stimulation varied to modulate the extent of induction and suppression of afferent activity. Then, the effect of optical stimulation was determined by the activity recorded in the dorsal root of the same afferents. Our findings show successful induction and suppression of activity in large-diameter afferents via optical stimulation. By increasing the intensity of blue (for ChR2) and yellow (for eNpHR3.0) light stimulation, the activity of fast-conducting afferent fibers was preferentially evoked or inhibited in an intensity-dependent manner. These data indicate that the activity of large-diameter afferents can systematically be regulated by optogenetics. The present innovative methodology for manipulating specific sensory modalities at the nerve level offers a targeted and accessible alternative to DRG stimulation, expanding the therapeutic scope of optogenetics for treating sensory disorders.

Keywords: adeno-associated virus, peripheral nerve, dorsal root ganglion neuron, touch,

proprioception

Introduction

Peripheral sensory nerve activity is integral in conveying sensory experiences to the central nervous system (CNS). Dysregulation of the peripheral sensory nerves, manifesting hyper- or hypoactivation, is a hallmark of various sensory disorders (1, 2). Conventional treatments for regulating peripheral nerve activity, such as pharmacological and electrical interventions (3−6), often lack specificity and may introduce adverse effects (4, 7, 8). The advent of optogenetics – a technique that manipulates neural activity with light-sensitive proteins – offers an alternative solution with unprecedented temporal and spatial precision that has already been established in the CNS (9−15).

In the present study, we attempted the extension of this optogenetic technique to peripheral sensory nerves, specifically targeting large-diameter afferents associated with tactile and proprioceptive sensations. The dorsal root ganglion (DRG) is the standard site for neural modulation (16−19), but is encumbered by its anatomical complexity and procedural risks (20, 21). By contrast, peripheral nerves are more accessible targets with reduced complexity, as evidenced by successful optogenetic manipulation of motor neuron axons (22−25). The heterogeneity of peripheral sensory nerves, comprising afferents of varying modalities and diameters (26−29), introduces specific challenges for optogenetic application. Our previous work demonstrated the efficacy of adeno-associated virus serotype 9 (AAV9) vector for gene transduction into DRG neurons linked to fast-conducting primary afferent

58 fibers (19, 30). In this study, we used the AAV9 vector to target selectively large-diameter
59 sensory fibers in the sciatic nerve of rats transduced with a light-activated ion channel,
60 channelrhodopsin (ChR2), and a light-driven chloride pump, halorhodopsin (eNpHR3.0). We
61 found that optical stimulation of the sciatic nerve evoked or suppressed action potentials in its
62 afferent fibers, with the magnitude of effect directly proportional to the light intensity. This
63 suggests the feasibility of optogenetic approaches to fine regulation of sensory signals
64 transmitted through peripheral nerve fibers.
65

66 **Results**

67 **Transduction of ChR2 and eNpHR3.0 Using AAV9 Vector**

68 We successfully transduced ChR2 (*n* = 7) or eNpHR3.0 (*n* = 10) into DRG neurons by intra-
69 nerve injection of AAV9 vector (Fig. 1A and B). Using an acute experimental setup, we
70 evaluated the effect of optical stimuli on the distal portion of the sciatic nerve (Fig. 1C and
71 E).

72

73 **Optogenetic Induction and Suppression of Sensory Afferent Activity**

74 In rats transduced with ChR2, the effect of blue light was examined by recording the
75 conducting afferent volleys at the dorsal root (Fig. 1C). For rats transduced with eNpHR3.0,
76 the nerve was pre-activated by electrical stimulation, and the effect of yellow light applied
77 proximally to the stimulation site was then assessed at the dorsal root (Fig. 1E).
78 Representative results are shown in Figure 1D and F. Specifically, in the ChR2-transduced
79 rats, 470-nm light produced long-lasting volleys in the dorsal root, while 593-nm light had no
80 modulatory effect (Fig. 1D). In the eNpHR3.0-transduced rats, the volley size evoked by
81 electrical stimulation (Fig. 1F, gray line) was significantly diminished by optical stimulation
82 at 593 nm (Fig. 1F, black line). We found comparable induction or significant suppression of
83 action potentials in all ChR2- (*n* = 7) and eNpHR3.0-transduced rats (*n* = 10; see the "Further
84 Characterization of eNpHR3.0-Mediated Suppression" section for details). These findings

demonstrated that light application could effectively evoke action potentials in sensory fibers of the ChR2-transduced rats and suppress sensory fiber activity in the eNpHR3.0-transduced rats.

Modulations of Sensory Afferent Activity Dependent on Optical Stimulation Intensity

Next, we examined the impact of varying optical stimulation intensity on sensory afferent activity. In the ChR2-transduced rats, increasing the intensity of blue light from 20.5 to 51.6 mW resulted in a gradual increase in the amplitude of the recruited volley (Fig. 2A and B; significant main effect of light power analyzed by Friedman's test, $\chi^2_{(6,60)} = 63.9$, $p < 0.0001$). In eNpHR3.0-transduced rats, on the other hand, increasing the power of yellow light (≥ 8.7 mW) gradually decreased the amplitude of the electrically evoked volley (Fig. 2D and E; significant main effect of light power analyzed by Friedman's test, $\chi^2_{(9,81)} = 73.8$, $p < 0.0001$). These observations confirmed that optical stimulation could regulate the amplitude of recruited volleys by tuning the stimulation intensity. In addition, increasing the light power resulted in a shorter onset latency of the recruited volley in the ChR2-transduced rats (Fig. 2C) and a longer onset latency of the electrically evoked volley in the eNpHR3.0-transduced rats (Fig. 2F, latency suppression). The overall findings indicate that sensory afferent nerve activity can be finely controlled by adjusting optical stimulation parameters.

Selective Modulation of Large-diameter Afferent Activity

Next, we examined the afferent selectivity of optogenetic modulation, given the tropism of AAV9 vector for fast-conducting, large-diameter afferents (19, 30). In the ChR2-transduced rats, the lower power of optical stimulation exclusively recruited early-onset volleys, while its higher power additionally recruited late-onset volleys as well (Fig. 3A). Specifically, early-onset volleys were not observed at 11.4 mW irradiation, but became apparent at around 20.5 mW irradiation (Fig. 3B). By increasing the light power, late-onset volleys were first observed at around 64.7 mW (Fig. 3B). This outcome aligned with the expected large-diameter selectivity of AAV9 vector. We further compared the maximal amplitude between the early-onset (1.5–4 ms after stimulus onset; Fig.3A, red shading) and the late-onset (5–10 ms after stimulus onset; Fig.3A, blue shading) volleys. The earlier range corresponded with a conduction velocity of 15–45 m/s, which well fitted within the expected range for fast-conducting, large-diameter Aα/β fibers (> 14 m/s) (31). The results showed that the amplitude was larger in volleys with shorter latencies (Fig. 3C; Wilcoxon signed-rank test, $p = 0.016$). Therefore, we concluded that large-diameter afferents, potentially Aα/β fibers, were preferential targets for optogenetic induction and modulation in the ChR2-transduced rats.

For the eNpHR3.0-transduced rats, we also confirmed preferential regulation of large-diameter afferents. We increased the intensity of electrical stimulation to determine how optical stimulation with the same parameters suppressed the size of electrically evoked

volleys. We found a marked effect on volleys evoked by a stimulus current just above the recruitment threshold (1T in Fig. 3D). Notably, these lowest-threshold volleys were eliminated (100% amplitude inhibition) by optical stimulation in five of the 10 animals. By contrast, the optical stimulation did not eliminate the volley evoked at 2T, and the suppressive effect was less marked at 5T. We quantified the relationship between the suppressive effect and the electrical current in this animal (Fig. 3E). As reported earlier (32, 33), without optical stimulation, the volley size first increased rapidly and then slowly as a function of electrical current. With optical stimulation, however, this relationship changed from a non-linear to a more linear manner, and the size of the volley linearly increased as a function of current. This suggests the possibility that the suppressive effect may be afferent selective. For example, optogenetic suppression was more effective in the volley evoked at 2T (57%) than 5T (6%; ratio of gray line to black line in Fig. 3E). We calculated the efficacy of inhibition of the volley amplitude in all animals (Fig. 3F) and found that it was indeed larger for volleys evoked by a lower stimulus current. Maximal efficacy was obtained at around 1T and then decreased quickly, though it was still obvious until at 2T. The inhibitory effect was not statistically significant (Fig. 3F; one-tailed Wilcoxon signed-rank test, p = 0.063 without Holm–Bonferroni correction) despite that the stimulation intensity was increased to five times as much as the threshold level. We also found a comparable change in the onset latency of volleys. Indeed, the efficacy to generate a late-onset latency was

142 decreased in volleys evoked by a higher stimulus current (Fig. 3G and H). These findings
143 showed that optogenetic suppression had a greater effect on volleys evoked by lower-
144 intensity electrical stimulation, suggesting preferential suppression of large-diameter afferent
145 fibers. The overall results indicate that large-diameter sensory afferent activity can selectively
146 be evoked and suppressed by optogenetics.
147

148 **Correlation Between Efficacy of Gene Expression and Extent of Optogenetic**
149 **Modulation**

150 We examined the relationship between the gene expression levels and the optogenetic
151 modulation effects in both the ChR2- and the eNpHR3.0-transduced rats (Fig. 4). An example
152 in Figure 4A shows recruited volleys and yellow fluorescent protein (YFP) expression in
153 DRG neurons of two ChR2-transduced rats with varying expression levels. One animal
154 exhibited larger efficacy in optical stimulation and stronger YFP signal intensity. We found a
155 significant correlation coefficient between the maximal magnitude of recruited volleys and
156 the YFP signal intensity (Fig. 4B; $r = 0.99$, $p < 0.0001$). A similar comparison was done in the
157 eNpHR3.0-transduced rats (Fig. 4C). Again, a significant correlation coefficient was found
158 between the amplitude inhibition percentage and the YFP signal intensity (Fig. 4D; $r = 0.88$,
159 $p = 0.001$). It should be noted here that these significant correlations were maintained when
160 an animal with notably high YFP expression was excluded (ChR2: $r = 0.82$, $p = 0.047$;

161 eNpHR3.0: $r = 0.68$, $p = 0.043$). The results indicate the importance of consistent expression
162 efficacy across animals for reproducible optogenetic modulation of sensory afferent activity.
163 Overall, our findings lead to similar conclusions in both of the ChR2- and the
164 eNpHR3.0-transduced rats regarding the stimulation intensity-dependent modulation of large-
165 diameter sensory afferent fibers and the correlation between the optogenetic effects and the
166 gene expression efficacy. However, a notable difference was also observed between the two
167 rat models, as described below (see the "DRG vs. Nerve: Comparative Analysis of Light
168 Irradiation Sites" section).
169
170 **Further Characterization of eNpHR3.0-Mediated Suppression**
171 Since this is the first study to inhibit action potential conduction at the peripheral nerve level,
172 we further characterized the optogenetic suppression to determine a stable and reversible
173 manner for inhibiting action potential conduction. The drop in the peak-to-peak amplitude of
174 electrically evoked volleys occurred immediately after light irradiation (Fig. 5A, "Light") and
175 returned to baseline levels ("Baseline") promptly after stopping light irradiation (Fig. 5A,
176 "Recovery"). Significant differences were found between the "Baseline" and the "Light"
177 session in a representative animal (Fig. 5B; one-way analysis of variance [ANOVA], $F_{(2,57)} =$
178 4365, $p < 0.0001$; Tukey's post hoc test, $p < 0.0001$), and all animals (Fig. 5C; Friedman's
179 test, $\chi^2_{(2,18)} = 15.2$, $p < 0.0001$, Dunn's post hoc test, $p = 0.001$). No difference was seen

between the “Baseline” and the “Recovery” session ($p = 0.61$ in the representative animal and $p = 0.99$ in all animals), suggesting that light irradiation causes no side effects. In addition, there was no significant correlation between the elapsed time of light irradiation and the peak-to-peak amplitude in the “Light” session (Fig. 5A, $r = -0.073$, $p = 0.76$), indicating no time trend in this session. Furthermore, the onset latency of the volley in the “Light” session was significantly longer than that in the “Baseline” session in a representative animal (Fig. 5D; one-way ANOVA, $F_{(2,57)} = 742.3$, $p < 0.0001$; Tukey’s post hoc test, $p < 0.0001$), and all animals (Fig. 5E; Friedman’s test, $\chi^2_{(2,18)} = 10.6$, $p = 0.002$, Dunn’s post hoc test, $p = 0.011$). No difference was found between the “Baseline” and the “Recovery” session ($p = 0.79$ in the representative animal and $p = 0.99$ in all animals), suggesting the reversible nature of latency inhibition. These results indicate that optical stimulation can stably and reversibly inhibit action potential conduction of afferent fibers.

DRG vs. Nerve: Comparative Analysis of Light Irradiation Sites

Building on previous studies that reported the optogenetic modulation of sensory afferent activity through light irradiation toward DRG neurons (7, 16−19), we next aimed to compare the efficacy of modulation between optical stimulation of DRG neurons and peripheral nerve fibers.

In the ChR2-transduced rats, we found that nerve-evoked volleys exhibited smaller

199 amplitudes compared with DRG-evoked volleys (Fig. 6A–C). Since we applied the same
200 intensity of optical stimulation in both conditions, this indicates that induction of sensory
201 afferent activity by nerve irradiation is less effective than by classical DRG stimulation (Fig.
202 6C; Wilcoxon signed-rank test, $p = 0.016$).

203 Contrastingly, in the eNpHR3.0-transduced rats, suppression of afferent fiber
204 activity through nerve irradiation displayed the superior efficacy to DRG irradiation. This
205 was demonstrated by more pronounced suppression of sensory afferent activity upon optical
206 stimulation of the nerve compared with the DRG stimulation (Fig. 6D and E; Wilcoxon
207 signed-rank test, $p = 0.031$).

208 These findings collectively suggest that while optical stimulation of sensory
209 afferent fibers and DRG are capable of both inducing and suppressing their activity (as shown
210 in Figs. 1–4), its effectiveness varies depending on the irradiation site. Specifically, in the
211 eNpHR3.0-transduced rats, nerve irradiation exhibited more prominent suppression of
212 activity compared with the classical DRG irradiation approach. Conversely, in the ChR2-
213 transduced rats, the nerve application proved to be less effective in inducing sensory afferent
214 activity. Such an efficacy difference highlights the significance of selecting the appropriate
215 irradiation site to achieve the desired outcome in optogenetic modulation of sensory afferents.
216 Notably, for induction of sensory afferent activity, the DRG may be a preferable choice,
217 despite the increased complexity and surgical challenges associated with access thereto. This

consideration is critical when weighing the trade-off between the efficacy and the procedural complexity in optogenetic manipulation of sensory pathways.

Discussion

There are three points of novelty in the present study. First, we have succeeded in inducing and suppressing peripheral sensory nerve activity by optical stimulation. Second, the activity of fast-conducting, large-diameter afferent fibers was preferentially evoked or suppressed in a stimulation intensity-dependent manner. Third, the efficacy of optical stimulation varied depending on the irradiation site. In particular, the peripheral nerve was the preferred target for optogenetic suppression.

Optogenetic Control of Peripheral Nerve Activity

In our study, we have found that optical stimulation can recruit primary afferent activity, with their amplitude and onset latency modulated in an intensity-dependent manner. A previous study reported that optical stimulation activated opsins expressed in axons at the node of Ranvier and generated action potentials (34). When the estimated conduction velocity of optically evoked volleys was investigated in the present study, it was consistently within the range of fast-conducting, large-diameter Aα/β fibers (> 14 m/s) (31). This implies the induction of Aα/β fiber activity by optical stimulation. In addition, our data confirmed

237 recruitment of high-threshold late component volleys upon optical stimulation at high
238 intensity with longer light-pulse durations, suggesting an off-target effect of optical
239 stimulation (19). Nevertheless, the amplitude for late component volleys was usually smaller
240 than for early component volleys (Fig. 3B and C). Thus, we can conclude that the optical
241 stimulation in this study preferentially induces the activity of Aα/β fibers.

242 We also effectively demonstrated optogenetic suppression of axonal action
243 potentials which were beforehand activated at the time of stimulation. When the action
244 potentials were generated by electrical stimulation near the threshold current, we observed
245 almost complete elimination of electrically evoked volleys. Electrical nerve stimulation is
246 known to recruit action potentials in accordance with axonal diameter, as axons with larger
247 diameters offer lower electrical resistance, making them more susceptible to depolarization in
248 response to smaller input signals (35, 36). The earliest component of the recorded volleys,
249 known as the "Aα" wave, reflects action potentials generated by group I muscle afferent
250 fibers, which possess the largest axonal diameter among various categories of primary
251 afferents (33, 37−39). Consequently, the complete elimination of volleys by optogenetic
252 suppression in this study (Fig. 3D) indicates the superior effectiveness of optical stimulation
253 in suppressing the activity of group I afferents. We also found the efficient optogenetic
254 suppression of axonal action potentials primarily evoked by electrical stimulation at
255 intensities twice as much as their recruitment threshold or below. It is well established that at

256 such stimulation intensities, activities of both group I and II afferents are introduced, whereas
257 finer afferents (like those of group III) are not activated (32). Therefore, we suggest that
258 optogenetic manipulation in this study indeed inhibits the activity of large-diameter primary
259 afferents, such as group I and II fibers.

260 Until recently, optogenetic suppression of primary afferent activity has exclusively
261 been demonstrated by targeting small-diameter DRG neurons that are responsible for
262 transmitting nociceptive signals (7, 17, 18, 40−42), with limited attention given to large-
263 diameter neurons. In an effort to fill this gap, we previously attempted to transduce genes by
264 intra-nerve injection of AAV9 vector (19, 30), known for its affinity toward medium-to-large-
265 sized DRG neurons that are involved in conveying mechanoreceptive and proprioceptive
266 signals (43). Here, we have further extended our approach using the AAV9 vector to achieve
267 not only excitation (19), but also inhibition of large-diameter primary afferents including
268 group I and II fibers.

269

270 **DRG vs. Nerve**

271 Theoretically, there are four possible locations for light irradiation to activate opsins
272 expressed in the peripheral sensory system: peripheral tissue, spinal cord, DRG, and
273 peripheral nerve (5, 44). Among these, targeting the peripheral tissue has limitations. Light
274 penetration is restricted to skin tissue when it is irradiated from the body surface (24), thereby

275 confining the modulatory effect to skin receptors. The impact on deep primary afferent
276 terminals, such as muscle spindles and Golgi tendon organs, is either impossible or
277 incomplete. Similarly, targeting the spinal cord (from its surface) is inefficient due to the
278 highly myelinated dorsal white matter which makes the light scattered (45, 46). Light
279 intensity is likely to be significantly diminished before reaching the intermediate layer of the
280 spinal cord where group I fibers terminate. In the present study, we targeted the remaining
281 two candidates, DRG and peripheral nerve, and revealed that targeting the DRG yielded a
282 superior efficacy for optogenetic induction of large-diameter primary sensory afferents,
283 whereas targeting the peripheral nerve had the advantage of their suppression.

284 These observed differences in the effectiveness of optical stimulation between the
285 DRG and the peripheral nerve suggest the involvement of distinct mechanisms. In the
286 peripheral nerve, light irradiation activates opsins expressed in axons and changes the intra-
287 axonal potential (34). By contrast, irradiation toward the DRG leads to activation of opsins
288 expressed not only in axons, but also in somata. It was our expectation that the effect of light
289 irradiation might be amplified by addition of the somal effect. This is because DRG neurons
290 have active ion channels in their somata and can generate action potentials (47, 48), although
291 these action potentials are generally believed to be rare. Also, hyperpolarization of the somata
292 can influence the membrane potential at the T-junction of DRG, where axonal bifurcation
293 occurs within DRG. Hyperpolarization at the T-junction may interfere with action potential

propagation due to the possible impedance mismatch (49, 50). It seemed surprising that we obtained the expected result on optogenetic induction but the opposite result on optogenetic suppression, indicating a lower efficacy of DRG irradiation. Here, we propose two potential explanations for this outcome. First, the anticipated contribution of T-junction hyperpolarization may have been less influential than axonal hyperpolarization. Second, the effect of axonal hyperpolarization due to DRG irradiation may have been minor compared to nerve irradiation, stemming from structural differences between the two. Specifically, somata and satellite cells are located predominantly in the dorsomedial region of DRG, while passing fibers are more evenly distributed throughout the rest of the ganglion (51). Such a location of somata and satellite cells may result in scattering of the light when irradiated from the DRG surface, diminishing its intensity by the time it reaches the axons.

The superior efficiency of optical stimulation targeting the nerve, compared with targeting the DRG, is desirable for *in vivo* application of this technique. In the past, various chronic optical stimulation tools have been developed, such as nerve cuffs with LED or optical fibers (52−54), LED directly placed on the peripheral nerve (42), and even transdermal light irradiation to the peripheral nerve (24, 55). Conversely, methods for chronic optical DRG stimulation are limited *in vivo* (44) and are highly invasive, posing a risk of damaging DRG neurons during exposure at the stimulation site (20). Optical stimulation,

312 especially for optogenetic suppression, at the peripheral nerve level opens new experimental
313 possibilities of studying the involvement of sensory afferents in a variety of motor behaviors.
314

315 **Clinical implications**

316 Damage to the CNS in the case of stroke and neurodegenerative diseases, including spinal
317 muscular atrophy (SMA), lead to maladaptive changes in the periphery. In stroke, one
318 common complication is spasticity, which results from hyperexcitability of the spinal reflex
319 (56, 57). While it is assumed that reducing proprioceptive input to the spinal cord could help
320 prevent the onset of spasticity, there has been no clinical treatment to date that offers selective
321 and reversible manipulation of proprioceptive afferents. Similarly, SMA is characterized by
322 motor neuron death and muscle atrophy, with reduced neuronal activity in spinal
323 sensorimotor circuits as a secondary change (58, 59). Increasing proprioceptive input to the
324 spinal cord may compensate for motor impairments in patients with SMA (60). In the present
325 study, we have shown the feasibility of selective and reversible induction and suppression of
326 large-diameter primary sensory afferent fiber activity using optogenetics. This novel
327 technique has a significant potential for advancing clinical research works on and therapeutic
328 approaches to conditioning spinal sensorimotor circuits, thereby offering new possibilities of
329 targeted interventions against CNS damage caused by stroke and neurodegenerative
330 disorders.

Methods

Ethical approval

All surgery was performed according to the institutional guidelines for animal experiments and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were approved by the experimental animal committee of the National Institute of Neuroscience (approval number 2022020).

Experimental animals

Seventeen male Slc:Wistar rats (4 weeks old, 70–90 g) obtained from Japan SLC, Inc. (Shizuoka, Japan) were used. All animals were housed under a normal light/dark cycle (12 h:12 h), with the light on from 08:00 h until 20:00 h, in a temperature-controlled environment with food and water available ad libitum.

Production of viral vectors

AAV9-hSyn-ChR2(H134R)/EYFP (2.5×10^{13} genome copies/mL) (19) and AAV9-CMV-eNpHR3.0/EYFP (1.8×10^{13} genome copies/mL) were produced using the helper-free triple transfection procedure and purified by cesium chloride gradient. Viral titer was determined by quantitative PCR using Taq-Man technology (Life Technologies, Gaithersburg, MD,

350 USA). Vector purity was assessed by 4–12% sodium dodecyl sulfate–polyacrylamide gel
351 electrophoresis and fluorescent staining (Oriole, Bio-Rad, Hercules, CA, USA). A transfer
352 plasmid (pAAV-CMV-eNpHR3.0-EYFP-WPRE) was constructed by inserting the
353 eNpHR3.0-EYFP fragment with a Woodchuck Hepatitis virus (WHV) posttranscriptional
354 regulatory element (WPRE) sequence into an AAV backbone plasmid (pAAV-CMV,
355 Stratagene, La Jolla, CA, USA).
356
357 *Vector injections*
358 ChR2 (n = 7) or eNpHR3.0 (n = 10) were transduced into DRG neurons by retrograde
359 transport following a sciatic nerve AAV injection (Fig. 1A). The protocol for AAV injection
360 was based on previous studies (19, 30). Briefly, animals were anesthetized by intraperitoneal
361 injection of medetomidine/midazolam/butorphanol anesthesia (0.2, 2.0, and 2.5 mg/kg,
362 respectively). Adequate depth of anesthesia was monitored frequently by monitoring pupil
363 size and flexion reflex to the paw pinch. After anesthesia, the animals were fixed in a prone
364 position, and an incision was made in the left gluteal muscle. The sciatic nerve was identified
365 below the biceps femoris muscle and isolated approximately 20 mm from the surrounding
366 tissue. A 31-gauge needle tube was inserted 10 mm into the isolated nerve. The needle tube
367 was connected by polyethylene tubing (JT- 10, EICOM, Kyoto, Japan) to a Hamilton syringe
368 (1702RN, GL Science, Tokyo, Japan) that was mounted into a microinjection pump

369 (LEGATO 180, KD Scientific, Holliston, MA, USA). The tubing, syringe, and needle tube
370 were filled with an electrically insulating stable fluorocarbon-based fluid (Fluorinert, 3M, St
371 Paul, MN, USA). Then 1% Fast Green (0.6 μL) was added to the viral vector solution to
372 visualize the injected solution. After insertion, the needle tube was left in place for 5 minutes
373 to allow sealing of the tissue around the needle tip. After, 3 μL of viral vector solution was
374 injected at a rate of 0.6 μL/min. The needle tube was removed at 10 minutes after the end of
375 the injection to ensure absorption of the solution. Two separate injections were performed
376 into the common peroneal and tibial branches of the sciatic nerve to ensure that the nerve was
377 filled uniformly (in total, 6 μL of viral vector solution was injected). Finally, the wound was
378 closed with a non-absorbable suture, and the animals were allowed to recover at 37°C.
379
380 *Acute electrophysiological experiments*
381 To test whether ChR2 and eNpHR3.0 could be used to recruit and inhibit activities of large-
382 diameter somatosensory afferents *in vivo*, an acute electrophysiological experiment was
383 performed 4–8 weeks after AAV injection. First, animals were anesthetized by intraperitoneal
384 injection of medetomidine/midazolam/butorphanol anesthesia (0.2, 2.0, and 2.5 mg/kg,
385 respectively). After a tracheotomy, anesthesia was maintained by inhalation of 0.5–1.0%
386 isoflurane throughout surgery. Electrocardiogram, oxygen saturation levels, and rectal
387 temperature were monitored and maintained within a physiological range.

388 To expose the lumbar spinal cord, a laminectomy was performed from the 1st
389 lumbar to the 1st sacral vertebrae. Then, the upper thoracic spine as well as the base of the
390 tail were immobilized using clamps. The dura was opened with a sharp needle, and the 4th
391 and 5th lumbar dorsal roots were separated from the other roots. For electrical and optical
392 stimulation, the left sciatic nerve was exposed and isolated from the surrounding tissue. The
393 skin and muscle around the exposed roots and nerves were raised and tied to form a pool,
394 which was filled with mineral oil to protect the exposed roots and nerves from drying out.
395 After surgery, anesthesia was maintained by thiopental sodium (10 mg/kg), and rocuronium
396 bromide (10 mg/kg) was administered intravenously to achieve neuromuscular blockade.
397 To apply electrical stimulation, a bipolar cuff electrode was mounted on the
398 isolated sciatic nerve. Stimulus current was generated using an isolator (SS 102L, Nihon
399 Kohden, Tokyo, Japan) coupled to an electrical stimulator (SEN7203, Nihon Kohden) with
400 various stimulus intensities (1–10-times threshold) at a fixed pulse width (100 μs). A
401 multimode optical fiber (960 μm in diameter, 0.63 numerical aperture; Doric Lenses, Quebec,
402 Canada) connected to a light source (LISER_LED470, Doric Lenses) was placed 10 mm
403 proximal to the cuff electrode to ensure the stimulus site was not directly irradiated (Fig. 1E).
404 To record stimulus-evoked volleys, a bipolar hook electrode was mounted on the
405 isolated dorsal root at 15–25 mm proximal to DRG. Recorded potentials were amplified (×
406 1000) and band-pass filtered (15–10 kHz) using an amplifier (MEG-6108M, Nihon Kohden),

407 and digitized at 50 kHz using an analog/digital converter (Digidata 1550, Molecular Devices,
408 LLC, San Jose, CA, USA). The average of 20 responses, consisting of a 10 ms pre-stimulus
409 baseline period followed by a 100 ms post-stimulus period, was obtained. Then, the peak-to-
410 peak amplitude and latency between onset of the stimulus and beginning of the response were
411 calculated. The beginning of the response was defined as a time that exceeded the value of
412 the mean ± 3× standard deviations of the background signal recorded 10 ms before the
413 stimulation.

414 To confirm the effect of optical facilitation, optical stimulation of blue light at 470
415 nm was applied to the sciatic nerve. The stimulation was applied 20-times with a fixed
416 stimulus duration (1 ms) at an interval of 0.5 s (2 Hz). To test whether the effect of optical
417 stimulation was caused by activation of ChR2, irradiation at a different wavelength (593 nm,
418 1 ms duration) was also performed. To characterize the response properties of optical
419 facilitation, intensity–response characteristics of the irradiated light power were examined by
420 changing the intensity of light power (11.4–127.2 mW) at a fixed stimulus duration (1 ms).
421 To test whether optical stimulation activated large-diameter afferents selectively, intensity–
422 response characteristics with longer light pulse duration (10 ms) were further examined
423 because longer and stronger optical stimulation can recruit high-threshold non-target fibers
424 (19). The light power was measured at the tip of the optical fiber using a power meter
425 (PM100D, Thorlabs, Newton, New Jersey, USA). To facilitate comparison across animals,

426 volley amplitudes were normalized to the maximum amplitude of electrically evoked volleys
427 (10-times threshold intensity).

428 To confirm the effect of optical inhibition, three experimental sessions were
429 performed: a baseline session ("Baseline", light-off condition), a session during irradiation of
430 yellow light at 593 nm ("Light", light-on condition), and a session immediately following
431 light deactivation ("Recovery", light-off condition). Each session consisted of 20 electrical
432 stimuli at an interval of 0.5 s (2 Hz) and proceeded consequently without a break. Yellow
433 light was continuously irradiated for 10 s in only the "Light" session. To facilitate
434 comparison across animals, volley amplitudes in the "Light" and "Recovery" sessions were
435 normalized against the averaged amplitude in the preceding "Baseline" session. Considering
436 reported side effects of light irradiation, such as heating (14, 61−63) and light-off rebound
437 effects (64), the reversibility of light irradiation was confirmed by comparing "Baseline" and
438 "Recovery" sessions. Because prolonged light irradiation can decrease the efficacy of
439 inhibition over time (63, 65, 66), the temporal stationarity of optical inhibition was confirmed
440 by calculating the correlation between the elapsed time of light irradiation for each stimulus
441 and the peak-to-peak amplitude of each volley in the "Light" session.

442 To characterize the response properties of optical inhibition, three sessions were
443 repeated under various conditions. First, intensity–response characteristics of irradiated light
444 power were examined by changing the light power intensity (8.7–107.3 mW) at a fixed

445 electrical stimulation (1.5-times threshold intensity). Then, intensity–response characteristics
446 of electrical stimulation were examined by changing the intensity of electrical stimulation (1–
447 5-times threshold) at a fixed light power (107.3 mW). The percentage of amplitude inhibition
448 was calculated as the difference in normalized amplitude between "Baseline" and "Light"
449 sessions. The latency difference was also calculated as the difference in latency between
450 "Baseline" and "Light" sessions and compared in each condition.

451 At the end of the experiments, small electrolytic lesions were made in the dorsal
452 root and sciatic nerve using a 40 μA direct current stimulation of the recording and
453 stimulating bipolar hook electrodes for 40 s. Then, the animals were deeply anesthetized by
454 intravenous injection of thiopental sodium (20 mg/kg) and perfused transcardially with
455 phosphate-buffered saline (pH 7.4), followed by 300 mL of 10% neutral buffered
456 formaldehyde. After perfusion, the lumbar region of the spinal cord together with the DRG
457 and sciatic nerve were removed.

458

459 *Histological quantification*

460 To check expression of ChR2 and eNpHR3.0, the fluorescent signal intensity of YFP was
461 examined. Fluorescent images of entire DRG neurons were acquired using an inverted
462 microscope (BZ-X710, Keyence, Osaka, Japan) using fixed detector gain values without
463 sectioning. To calculate the fluorescent intensity, the images were converted to grayscale, and

then mean gray values within the region of interest were determined manually.

Statistical analyses

The Wilcoxon signed-rank test was performed for all two group comparisons. For group analysis, Friedman's test was performed with light power or recording sessions ("Baseline", "Light", and "Recovery" sessions) as a within-subject factor. One-way ANOVA was performed with three recording sessions only for single animal analysis in the optical inhibition experiment. Post hoc analyses were performed using Tukey's or Dunn's tests for multiple comparisons. When testing intensity–response characteristics of electrical stimulation in the optical inhibition experiment, one-tailed Wilcoxon signed-rank test with Holm–Bonferroni correction was performed to compare the percentage of amplitude inhibition in each stimulus intensity to the hypothetical value (0% inhibition). For all correlation analyses, Pearson's correlation coefficient was calculated. The level of significance was set at $\alpha = 0.05$. All electrophysiological data were analyzed using MATLAB 2021b (MathWorks, Natick, MA, USA), all fluorescent images were analyzed using OpenCV 4.6.0 with python 3.8, and all statistical tests were performed using Prism 10 (GraphPad Software, San Diego, CA, USA).

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Author contributions:

A.K. and K.S. designed the study. K.I. and M.T. developed the viral vectors. A.K. performed the surgery and electrophysiological experiments. A.K. analyzed the data. A.K. and K.S. wrote a draft of the manuscript. All authors approved the final version of the manuscript.

Declaration of interests: The authors declare they have no competing interests.

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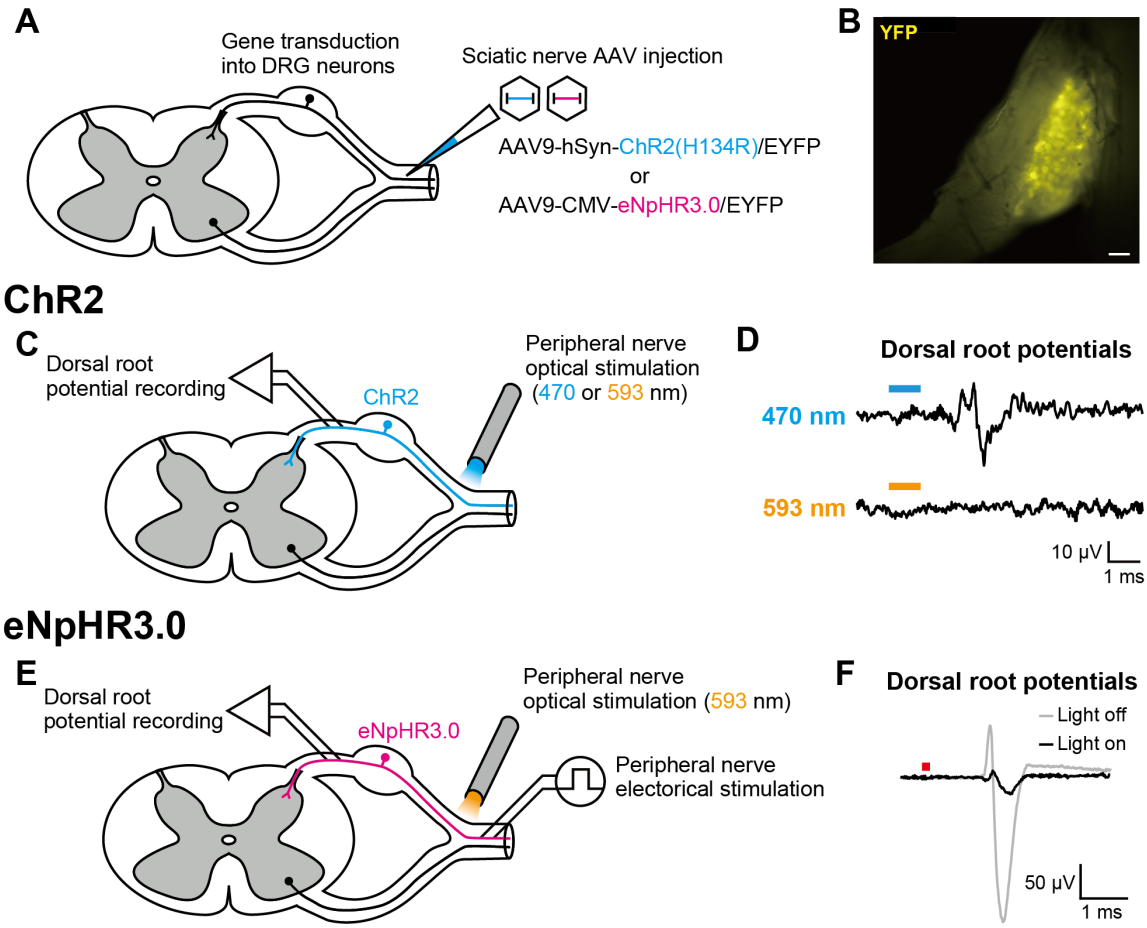

683

684 **Figure 1. Optogenetic manipulation of sensory afferent activity by adeno-associated**

685 **virus serotype 9 (AAV9)-mediated gene transduction into dorsal root ganglion (DRG)**

686 **neurons and optical stimulation of nerve fibers.** (A) Schema of AAV injection. Retrograde

687 transport via sciatic nerve injection enabled gene transduction into DRG neurons. (B)

688 Expression of YFP in the 5th lumbar DRG neurons. Scale bar: 200 μm. (C, D) Results on

689 channelrhodopsin (ChR2) transduction. (C) Schema of the acute *in vivo* electrophysiological

690 experiment. (D) Examples of dorsal root potentials evoked by light stimulation with different

691 wavelengths. Waveforms represent the stimulus-triggered averaging of 20 responses. The bar

692 above each waveform indicates the timing of light irradiation. (E, F) Results on

693 halorhodopsin (eNpHR3.0) transduction. **(**E) Schema of the acute *in vivo* electrophysiological

694 experiment. (F) Examples of electrically evoked volleys with (black line) and without (gray

695 line) light irradiation. Waveforms represent the stimulus-triggered averaging of 20 responses.

696 The red square above the waveforms indicates the timing of electrical stimulation.

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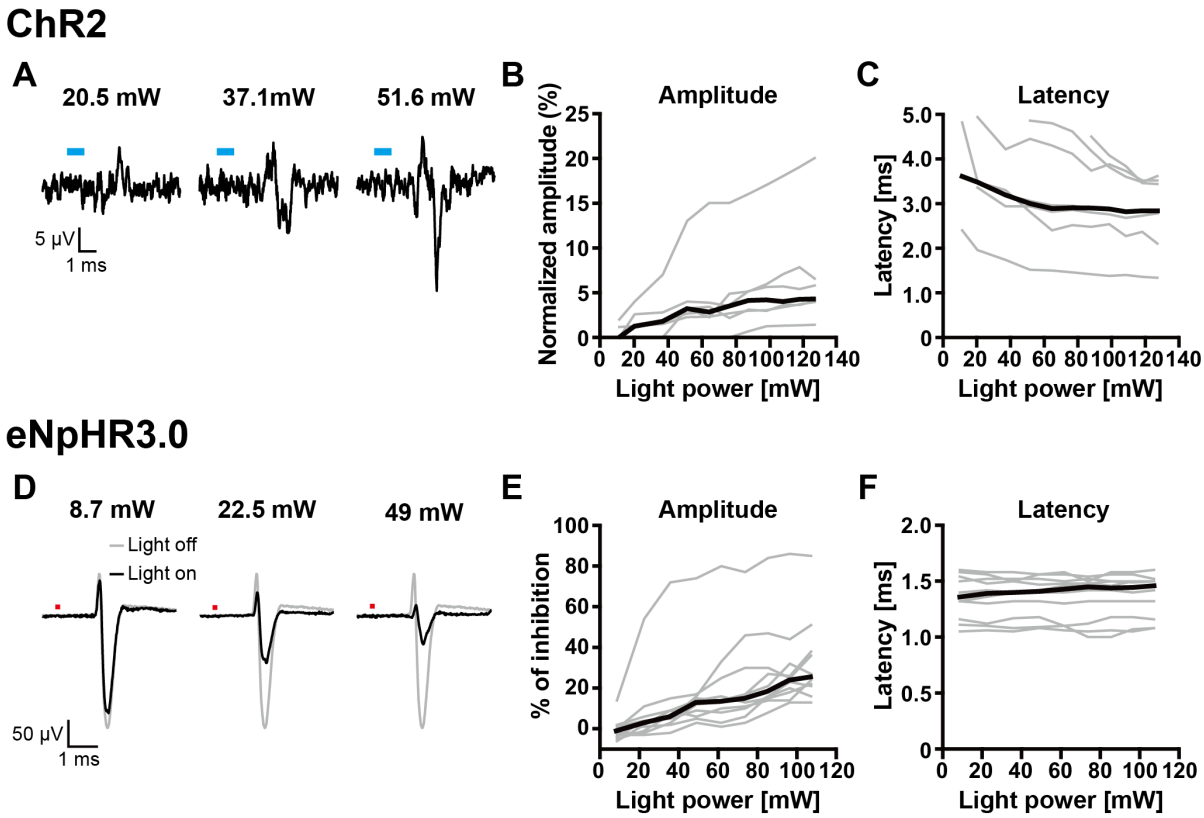

Figure 2. Effect of light power on optogenetic manipulation. (A−C) Results on channelrhodopsin (ChR2) transduction. (A) Examples of recruited volleys using different irradiated light powers. Waveforms represent the stimulus-triggered averaging of 20 responses. Blue bars above each waveform indicates the timing of light irradiation. (B, C) Changes in the amplitude and latency of recruited volleys as a function of light power. Each gray line represents one animal (n = 7), and thick black lines represent the median value of all animals. (D−F) Results on halorhodopsin (eNpHR3.0) transduction. (D) Examples of electrically evoked volleys using different irradiated light powers. Waveforms represent the stimulus-triggered averaging of 20 responses. Black lines indicate electrically evoked volleys with light irradiation and gray lines indicate volleys without irradiation. Red squares above

709 each waveform indicates the timing of electrical stimulation. (E, F) Changes in the

710 percentage of amplitude inhibition and latency of electrically evoked volleys as a function of

711 light power. Each gray line represents one animal (n = 10), and thick black lines represent the

712 median value of all animals.

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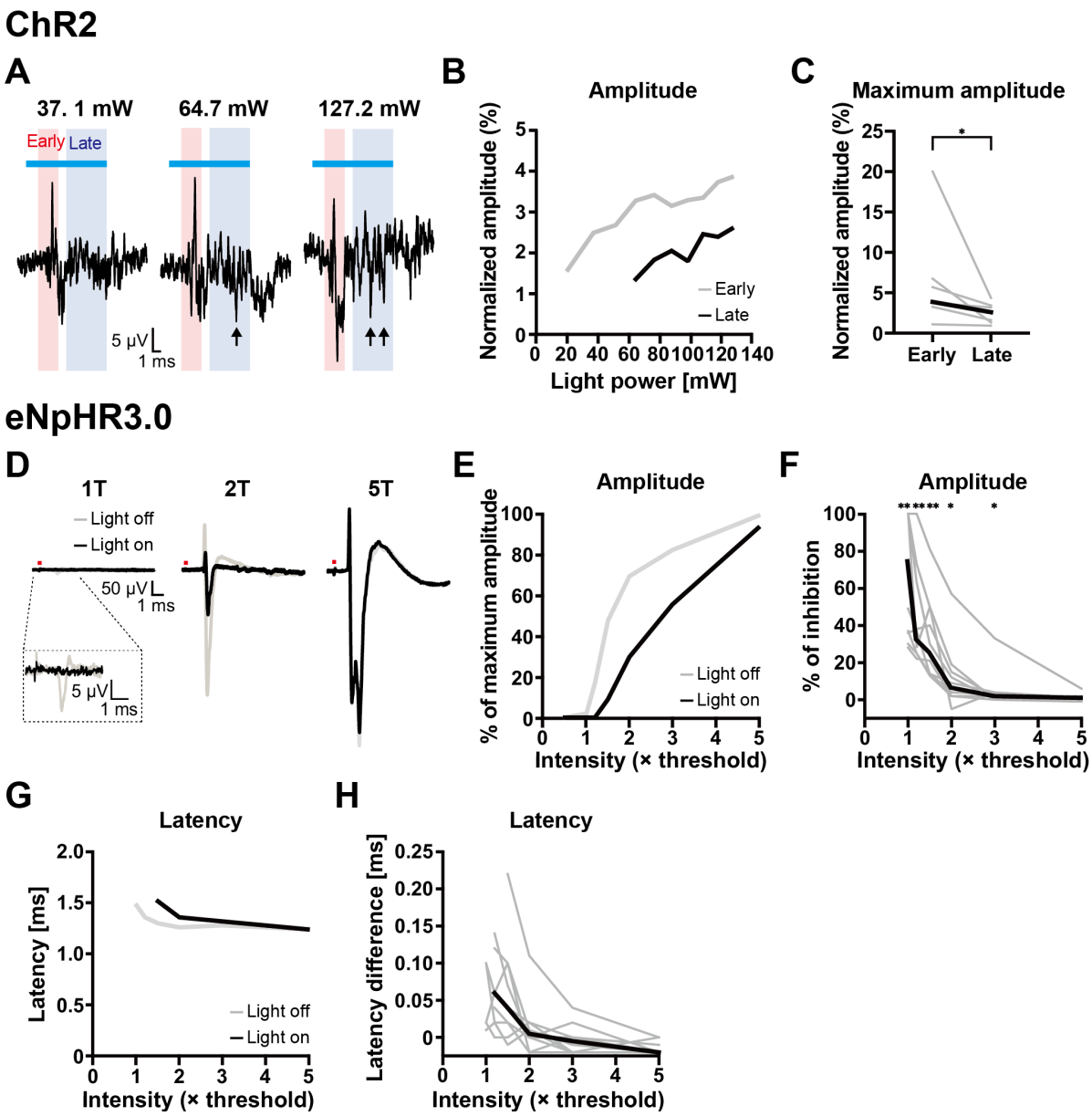

Figure 3 Large-diameter afferent preference. (A–C) Results of channelrhodopsin (ChR2) transduction. (A) Examples of recruited volleys using long light pulse durations. Waveforms represent the stimulus-triggered averaging of 20 responses. Red shading indicates the range of early-onset volleys (1.5–4 ms after stimulus onset), and blue shading indicates the range of late-onset volleys (5–10 ms after stimulus onset). Blue bars above the waveforms indicate the timing of light irradiation. Black arrows indicate individual peaks of late-onset volleys. (B)

721 Changes in normalized amplitude of early- and late-onset volleys as a function of light power.
722 The gray line indicates the early-onset component, and the black line indicates the late-onset
723 component. Note that each line is plotted only at the light power at which the volley was
724 observed. (C) Maximum normalized amplitude of early- and late-onset volleys evoked by
725 long light pulse duration. Each gray line represents one animal (n = 7), and thick black lines
726 represent the median value of all animals. *$p < 0.05$ (Wilcoxon signed-rank test). (D–H)
727 Results of halorhodopsin (eNpHR3.0) transduction. (D) Examples of electrically evoked
728 volleys using different intensities of electrical stimulation. Waveforms represent the stimulus-
729 triggered averaging of 20 responses. Black lines indicate electrically evoked volleys using
730 light irradiation and gray lines indicate volleys without irradiation. Red squares above each
731 waveform indicates the timing of electrical stimulation. (E) Example of intensity–response
732 curves of amplitudes with and without light irradiation from one animal. (F) Changes in the
733 percentage of amplitude inhibition as a function of electrical stimulation intensity. Each gray
734 line represents one animal (n = 10), and thick black lines represent the median value of all
735 animals. *$p < 0.05$; **$p < 0.01$ (one-tailed Wilcoxon signed-rank test with Holm–Bonferroni
736 correction compared to 0%). (G) Example of intensity–response curves of latency with and
737 without light irradiation from one animal. (H) Difference in latency as a function of electrical
738 stimulation intensity. Each gray line represents one animal (n = 10), and thick black lines
739 represent the median value of all animals.

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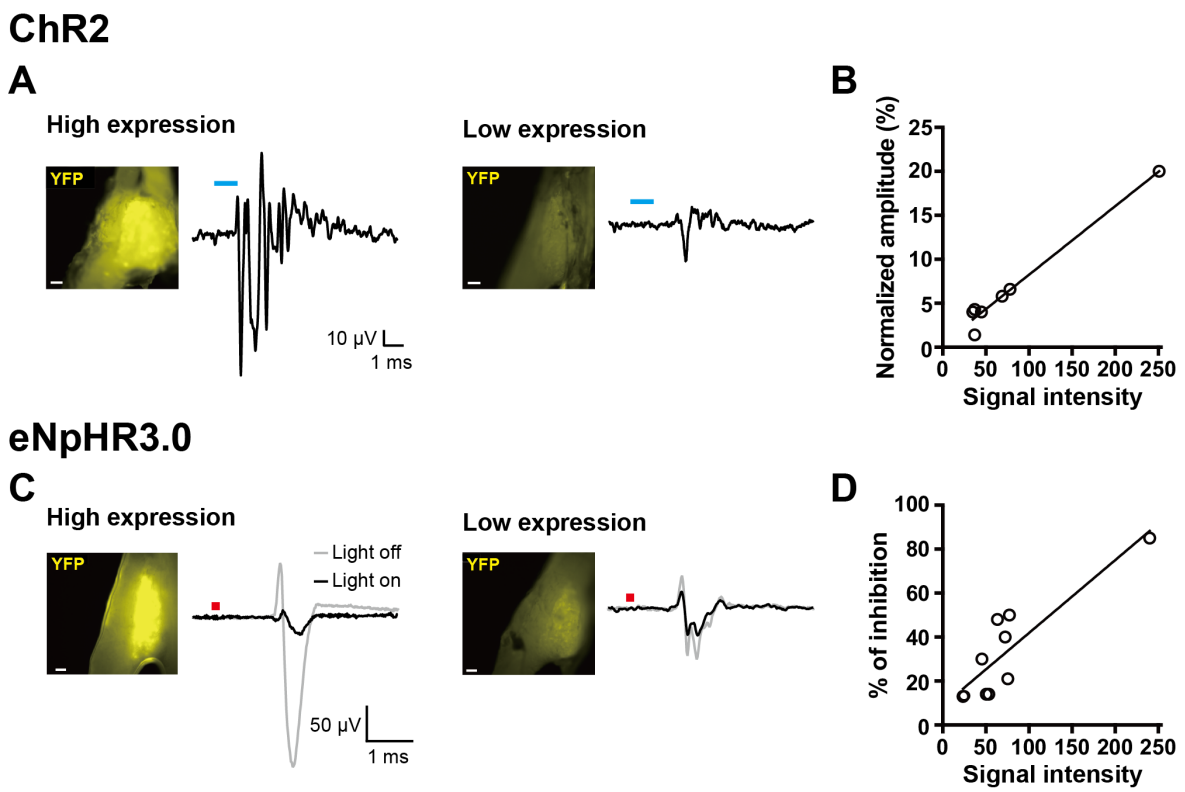

Figure 4. Correlation between optical efficacy and YFP intensity. (A, B) Results on channelrhodopsin (ChR2) transduction. (A) Examples of recruited volleys and YFP expression in dorsal root ganglion (DRG) neurons for two animals with different expression levels. Waveforms represent the stimulus-triggered averaging of 20 responses. Blue bars above each waveform indicates the timing of light irradiation. Scale bar: 200 μm. (B) Scatter plots and linear correlation between the normalized amplitude and YFP signal intensity of all animals (n = 7). (C, D) Results on halorhodopsin (eNpHR3.0) transduction. (C) Examples of electrically evoked volleys with (black line) and without (gray line) light irradiation and YFP expression in DRG neurons for two animals with different expression levels. Waveforms represent stimulus-triggered averaging of 20 responses. Red squares above the waveforms

752 indicate the timing of electrical stimulation. Scale bar: 200 μm. (D) Scatter plots and linear

753 correlation between the amplitude inhibition percentage and YFP signal intensity of all

754 animals (n = 10).

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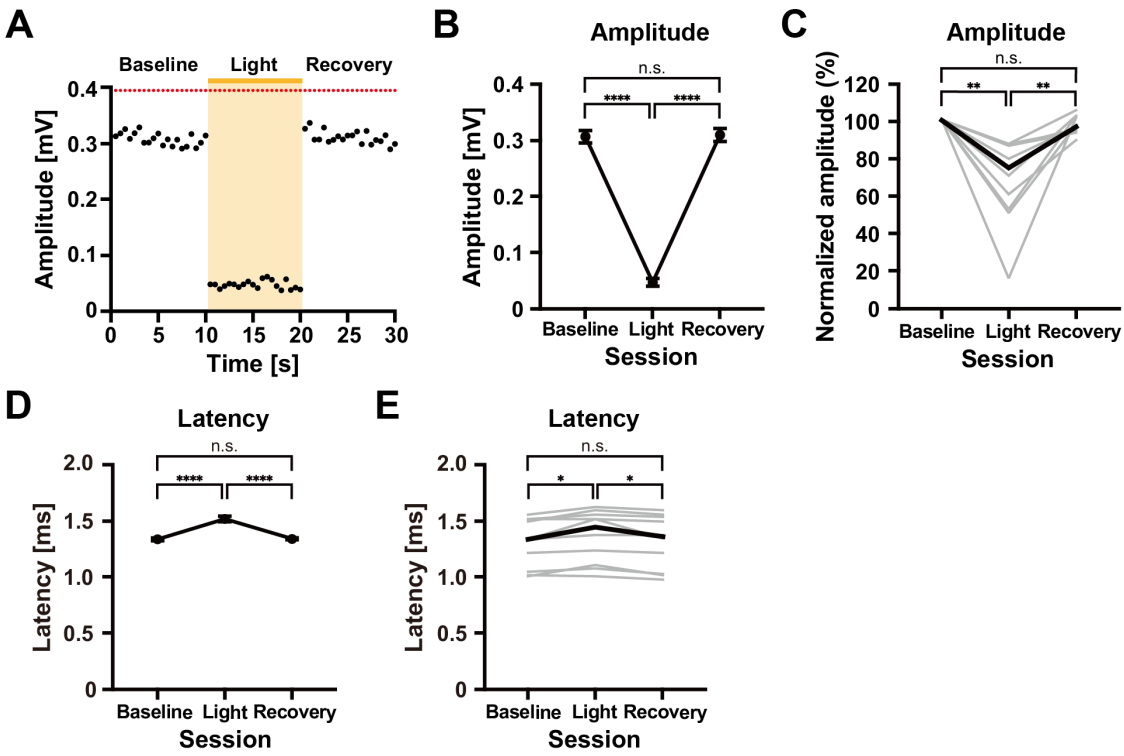

Figure 5. Characterization of optogenetic suppression. (A) Time course of peak-to-peak amplitudes of electrically evoked volleys. Red dots indicate the timing of electrical stimulation (2 Hz). The orange line and orange shaded area indicate the timing of light irradiation (10 s). (B, C) Effect of optical inhibition on the amplitude from a representative animal (B) and for all animals (C). For representative animal, data are represented mean ± standard deviation. For all animals, amplitudes in the "Light" and "Recovery" sessions were normalized by the average amplitude in the preceding "Baseline" session. Each gray line represents one animal (n = 10), and thick black lines represent the median value of all animals. (D, E) Effect of optical inhibition on latency from a representative animal (D) and for all animals (E). For representative animal, data are represented mean ± standard

767 deviation. For all animals, each gray line represents one animal (n = 10), and thick black lines

768 represent the median value of all animals. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

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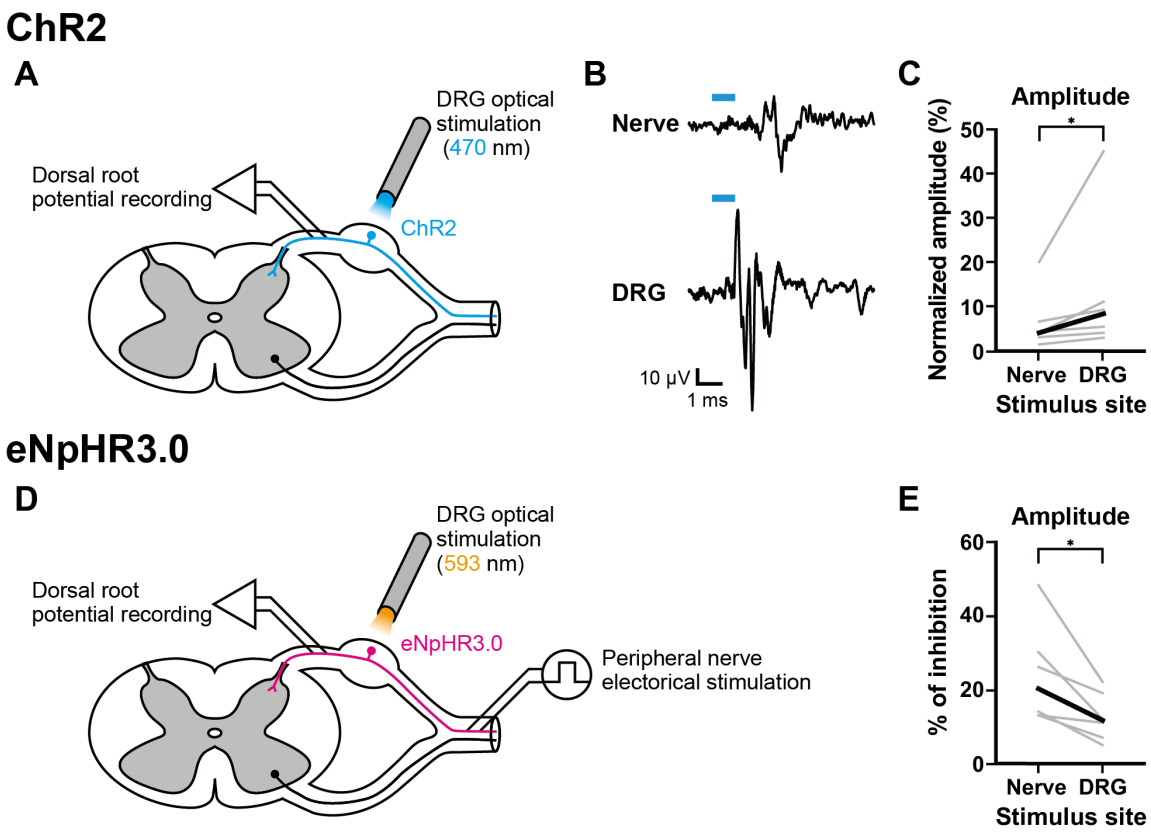

Figure 6. Sites and effects of optical stimulation. (A–C) Results on channelrhodopsin (ChR2) transduction. (A) Schema of the acute *in vivo* electrophysiological experiment. (B) Examples of recruited volleys by optical stimulation to the sciatic nerve and dorsal root ganglion (DRG). Waveforms represent the stimulus-triggered averaging of 20 responses. Blue bars above the waveforms indicate the timing of light irradiation. (C) The effect of the stimulus site on the amplitude. Each gray line represents one animal (n = 7), and thick black lines represent the median value of all animals. (D, E) Results on halorhodopsin (eNpHR3.0) transduction. (D) Schema of the acute *in vivo* electrophysiological experiment. (E) The effect of the stimulus site on the percentage of amplitude inhibition. Each gray line represents one animal (n = 6), and thick black lines represent the median value of all animals. *$p < 0.05$.