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An Upconversion Nanoparticle Enables Near Infrared-Optogenetic Manipulation of the Caenorhabditis elegans Motor Circuit

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Supporting Information

ABSTRACT: Near-infrared (NIR) light penetrates tissue deeply, but its application to motor behavior stimulation has been limited by the lack of known genetic NIR light-responsive sensors. We designed and synthesized a Yb³⁺/Er³⁺/Ca²⁺-based lanthanide-doped upconversion nanoparticle (UCNP) that effectively converts 808 nm NIR light to green light emission. This UCNP is compatible with Chrimson, a cation channel activated by green light; as such, it can be used in the optogenetic manipulation of the motor behaviors of Caenorhabditis elegans. We show that this UCNP effectively activates Chrimson-expressing, inhibitory GABAergic motor neurons,



leading to reduced action potential firing in the body wall muscle and resulting in locomotion inhibition. The UCNP also activates the excitatory glutamatergic DVC interneuron, leading to potentiated muscle action potential bursts and active reversal locomotion. Moreover, this UCNP exhibits negligible toxicity in neural development, growth, and reproduction, and the NIR energy required to elicit these behavioral and physiological responses does not activate the animal's temperature response. This study shows that UCNP provides a useful integrated optogenetic toolset, which may have wide applications in other experimental systems.

KEYWORDS: lanthanide upconversion nanoparticle, optogenetics, Caenorhabditis elegans, action potential, locomotion

he discovery of light-activated ion channels and pumps, such as Channelrhodopsin (ChR), Chrimson, Halorhodopsin (NpHR), and Guillardia θ anion channelrhodopsin (GtACR), has revolutionized neuroscientific research.¹⁻⁶ When a neuron is engineered to express lightactivatable channels and pumps, its activity becomes controllable by light. Through the selective expression of lightsensitive proteins in specific neuronal groups and the controlled delivery of light stimulation at high temporal and spatial resolutions, opto-stimulation can precisely target neurons for activation or inhibition in millisecond scale. This invention, called optogenetics, has become a powerful means

to decode the circuit mechanisms that underlie the physiological functions and pathological states of the brain.^{7,8}

One of the best-understood neural circuit principles is the rhythm generating circuit for motor behaviors. Motor circuits, which generate diverse forms of motor functions, consist of an interconnected network of motor neurons and premotor interneurons.9,10 Electrophysiological studies have revealed key properties of the motor circuit, such as the spontaneous

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oscillation of membrane potentials in the rhythm-generating network. Optogenetics, meanwhile, has emerged as a complementary tool for discerning the functions of specific neuronal groups in large motor circuits and their effects on behavior. For example, the optogenetic silencing of Shox2positive interneurons in the murine spinal cord by NpHR leads to an altered motor rhythm, without changing the patterned sequence and coordination of motor neuron activity.¹¹ These results reveal the nonessential but regulatory role of Shox2positive interneurons in the generation of motor rhythm.

A technical limitation for optogenetics is the lack of optical transparency of most living tissues. All currently developed genetic light sensors are activated by visible light, whose tissue penetration is limited due to tissues' high light absorption and scattering.⁴ 700–1700 nm near-infrared (NIR) light elicits reduced tissue auto fluorescence and scattering, resulting in deeper tissue penetration.^{12,13} In the absence of genetic NIR light sensors, NIR light nanotransducers, such as lanthanide upconversion nanoparticles (UCNPs), have been fabricated to convert NIR light into visible light emission to stimulate established optogenetic tools such as ChR2 (~470 nm) and NpHR (~580 nm).^{14–18} UCNPs display excellent optical properties including anti-Stokes shift and photostability,^{16,19} making them an outstanding alternative source for optogenetic stimulation.

The nematode Caenorhabditis elegans (C. elegans) is used extensively in the optogenetic manipulation of intact circuits to determine the neuronal basis of motor behaviors. Its small nervous system, amenability to genetic manipulation, and quantifiable motor parameters have made it a fast and mature experimental system to critically evaluate and optimize the in vivo effects and efficacy of optogenetic tools.²⁰⁻²⁴ Several recent studies of UCNP-mediated neuronal stimulation and inhibition have been reported.^{17,18,25–27} NIR-mediated locomotion behavior has been demonstrated in specific neuronal circuits; however, systematic, in-depth studies of this mediated behavior are rare. Previously, a 980 nm excited UCNP was reported to have the capacity to stimulate ChR2-expressing neurons, triggering reversal behaviors in C. elegans.²⁸ However, 980 nm irradiation may induce severe thermal damage to cells and tissues,^{29,30} and blue light emitted from the UCNP may trigger an intrinsic, lite-1-dependent photophobic response in C. elegans.³¹ Considering the high sensitivity of neuronal activity and stability to temperature changes,³² it is difficult to interpret whether the observed behavioral response is a direct consequence of the activation of ChR2-expressing neurons or an innate avoidance response to heat or blue light. Thus, it is critical to carefully assess the effects of short- and long-term UCNP application on animal physiology and to distinguish the underlying causes of animals' behavioral changes from thermally induced innate temperature responses. Moreover, the activation of ChR2 requires relatively high excitation power.^{5,33} The need for higher NIR irradiation power may increase the temperature of local tissue, while the higher concentration of UCNPs may induce biotoxicity. Considering that the generation of efficient upconverting emissions requires the sequential absorption of multiple NIR photons, it is worthwhile to develop UCNPs for neuronal circuit modulation that combine enhanced luminescence efficiency with sensitive optogenetic sensors.

We present here an Er-doped UCNP that allows for the sensitive and specific optogenetic regulation of *C. elegans* motor circuit activity (Scheme 1). The synthesized UCNPs are





"Schematic diagram showing a green emissive core-shell-shell NaGdF₄:Yb,Er,Ca @NaYbF₄:Gd,Ca@NaNdF₄:Gd,Ca nanoparticle (UCNP) illuminated by 808 nm NIR light to active Chrimson and change the locomotion behaviors. Chrimson, a green light-sensitive cation channel, was expressed in the GABAergic D-class motor neurons (D-MNs) and glutamatergic DVC interneuron. NIR illumination attenuated and potentiated the muscular action potential firing in the presence of UCNP, which induced the paralyzed and backward locomotion, respectively.

excited at 808 nm, a wavelength that exhibits minimal tissue absorption and maximal tissue penetration.^{34–36} We show that, upon illumination, the UCNPs activate a more sensitive optogenetic sensor Chrimson expressed by *C. elegans* motor neurons and interneurons. This activation elicits electrophysiological and behavioral changes that are specific to targeted neuronal activity manipulation. Importantly, the UCNPs have negligible biotoxicity upon activation, and their activation does not trigger the animal's innate temperature responses. Using this UCNP, we verify a key activator for the reversal motor circuit. This study provides a significantly improved tool for optogenetic manipulation that can be extended to *in vivo* studies in other nervous systems, such as deep brain tissues.

RESULTS AND DISCUSSION

Synthesis and Characterization of Core–Shell–Shell Green Emissive UCNPs. To generate effective upconverting nanoparticles excited at 808 nm, we adopted our previous configuration,^{36,37} wherein the sandwich-type NaGd- F_4 :Yb,Er,Ca@NaYbF₄:Gd,Ca@NaNdF₄:Gd,Ca nanoparticles were synthesized layer-by-layer through a modified thermal decomposition method (Figure 1A).

Transmission electron microscopy (TEM) images of the UCNPs' core (NaGdF4:Yb,Er,Ca), the core-shell (NaGd-F4:Yb,Er,Ca@NaYbF4:Gd,Ca), and the sandwich-type UCNPs revealed narrow size distribution and monodispersity, with a mean diameter of 14.72 \pm 0.57 nm, 16.07 \pm 0.81 nm, and 26.08 ± 1.34 nm, respectively (Figure 1B). The highresolution TEM (HR-TEM) images further revealed the crystalline nature of the nanoparticles with 0.52 nm d-spacing values for the hexagonal phase (Figure 1C). The X-ray diffraction (XRD) patterns of the nanoparticles match those of JCPDS no. 27-0699 (Figure 1D), which confirmed that the UCNPs are the hexagonal phase. The presence of all the above elements in UCNPs was demonstrated by energy dispersive Xray (EDX), which confirmed that two shells were grown on the core nanoparticle layer-by-layer (Figure S1). When illuminated by 808 nm NIR light, the UCNPs showed green and red emissions with peak values at 520/540 and 660 nm, respectively (Figure 2B). The intensity of green emissions



Figure 1. Design and synthesis of UCNPs. (A) Scheme of the synthetic process for this nanoparticle. (B) TEM images and size distribution of (i) core NaGdF₄:Yb,Tm,Ca; (ii) core-shell NaGdF₄:Yb,Tm,Ca@ NaYbF₄:Gd,Ca; (iii) core-shell-shell NaGdF₄:Yb,Tm,Ca@ NaYbF₄:Gd,Ca @NaNdF₄:Gd,Ca nanoparticles, respectively. Scale bar: 100 nm. (C) HR-TEM image of a single core-shell-shell NaGdF₄:Yb,Er,Ca@NaYbF₄:Gd,Ca@NaNdF₄:Gd,Ca nanoparticle. Scale bar: 5 nm. (D) XRD patterns of as-synthesized core (C), core-shell (C/S) and core-shell-shell (C/S/S) nanoparticles.

was significantly higher than that of red emissions; therefore, the overall visual output was green (Figure 2B).

To make the UCNPs biocompatible with *in vivo* experiments, we used PEGylated phospholipid (DSPE-PEG) to render nanoparticles hydrophilic (denoted as UCNPs@DSPE-PEG) (Figure 2A). The DSPE-PEG modification was confirmed using Fourier transform infrared (FTIR) spectroscopy (Figure 2D); two bands at 1738 and 1114 cm⁻¹ in the spectrum of UCNPs@DSPE-PEG were assigned to the stretching vibration of the carboxyl ester and the ether bond of PEG chains, respectively.

We then examined UCNPs@DSPE-PEG's water dispersity, water stability, and photostability. Dynamic light scattering (DLS) measurement indicated that UCNPs@DSPE-PEGs were well dispersed in water (Figure 2B), with a mean hydrodynamic diameter of 56.54 nm (Figure 2C). The diameter of dispersed UCNPs@DSPE-PEG showed no significant size change after 2 days in water, revealing the excellent intrinsic stability of these nanoparticles (Figure S2). The ζ potential of the UCNPs@DSPE-PEG in water was measured to be -4.05 mV. The upconversion spectrum of

UCNPs@DSPE-PEG in water was similar to that of the unmodified UCNPs in hexane (Figure 2B). The slightly decreased fluorescence intensity of UCNPs@DSPE-PEG in water likely reflected water molecules' quenching effect. Lastly, the emission of UCNPs@DSPE-PEG exhibited no photobleaching during 1 h of continuous laser illumination (Figure 2E). These results demonstrated that the UCNPs were properly modified to be water dispersible and stable.

Henceforth, hydrophilic UCNPs@DSPE-PEG, referred to as Er-UCNPs for simplicity, were used for all physiological experiments in the modulation of *C. elegans* motor circuits and behavior.

UCNPs Activate Inhibitory GABAergic Motor Neurons To Decrease Body Wall Muscle Action Potential Firing. We investigated the effectiveness of Er-UCNPs as an alternative light source for neuronal stimulation, using *C. elegans* strains that express Chrimson, a cation channel that can be activated by a broad spectrum from green to red light, in either motor neurons or interneurons. We first examined their effect using dissected neuromuscular junction electrophysiology preparations. Er-UCNPs (5 mg/mL) were supplied to the



Figure 2. Preparation and characterization of UCNPs@DSPE-PEG. (A) A schematic illustration showing the structure of UCNPs@DSPE-PEG. (B) Upconversion luminescence spectra of as-prepared oleic acid (OA)-coated UCNPs in hexane and UCNPs@DSPE-PEG in water (10 mg/mL) under excitation at 808 nm NIR (0.64 W/mm²) (insets are their corresponding photographs). (C) Hydrodynamic size distribution of OA-UCNPs in hexane and UCNPs@DSPE-PEG in water, determined by DLS. (D) FTIR spectrum of OA-UCNPs and UCNPs@DSPE-PEG. (E) Photostability of OA-UCNPs in hexane and UCNPs@DSPE-PEG in water, upon 1 h at 808 nm, 0.64 W/mm² CW laser excitation.

Sylgard base (Methods), where the *C. elegans* neuromuscular junction preparation was prepared. Whole-cell patch clamp recording of body wall muscles was carried out under temporally controlled NIR light illumination.

In the *C. elegans* motor circuit, the D-class motor neurons (D-MNs) are inhibitory: They release GABA to muscle arms, leading to the inhibition of action potential firing and to muscle relaxation.^{21,38–41} We examined the electrophysiological effect of 808 nm NIR illumination in a transgenic strain *hpIs593* that specifically expresses D-MNs with Chrimson in the presence of Er-UCNPs (Figure 3A, Figure S3). Under the current clamp configuration, we observed an instantaneous and sustained reduction of action potential frequency upon local application of NIR illumination when Er-UCNPs were added to the Sylgard (Figure 3B, C). The amplitude of action potentials was unchanged (Figure 3D). Without Er-UCNPs, neither the frequency nor the amplitude of muscle action potentials was affected by the same intensity of NIR illumination (Figure 3E-G), confirming the specificity of the UCNP-mediated effect.

We and others previously reported that in dissected preparations from animals that expressed ChR2 in their D-MNs, strong blue light stimulation led not only to the elimination of muscle action potentials but also to the hyperpolarization of muscle resting membrane potentials (RMPs).^{38,42} Under experimental conditions for this study, we did not observe the hyperpolarization of RMPs upon 808 nm NIR illumination (Figure 3B). This is likely caused by the differences in intensity of the stimulations applied to these preparations. Together, these results suggest that Er-UCNPs properly activate Chrimson expressed neurons.

UCNP-Mediated D-MN Activation Alters *C. elegans* **Locomotion.** We next determined whether UCNP-mediated D-MN activation is sufficient to alter intact *C. elegans* locomotion by assessing the extent of body bending, defined as the mean absolute curvature value along the *C. elegans* body (Figure 4A, B), and the instanenous velocity, defined as the midpoint displacement at 100 ms intervals (Figure 4C, D) (Methods).

The inhibition of action potential firing in body wall muscles, when all members of D-MNs are activated simultaneously, leads to muscle relaxation along the body.³⁸ Indeed, when animals that expressed Chrimson in their D-MNs were briefly exposed to 808 nm NIR illumination, an instantaneous decrease of bending along the entire body occurred, halting locomotion (Figure 4A–D and Supplementary Video S1). These animals' body bending and velocity were not changed when they were cultured with only Er-UCNPs but



Figure 3. Muscular action potentials were inhibited by NIR-UCNPs. (A) Schematic diagram of the electrophysiological experiment. Excited by 808 nm NIR light, Er-UCNPs emit green light to activate the light sensor Chrimson and excite GABAergic D-motor neurons. Excited D-motor neurons inhibit postsynaptic muscular action potential firing. (B, E) Representative muscular action potential firing before, during, and after illumination by NIR light (0.64 W/mm²) with (B) or without (E) Er-UCNPs (5 mg/mL), respectively. (C, D, F, G) Normalized action potential frequency and amplitude before, during, and after illumination by NIR light with (C, D) or without (F, G) Er-UCNPs, respectively. ** P < 0.01 against control (black bar: NIR -) by the Mann–Whitney U test, n.s. not significant by one-way ANOVA. $n \ge 6$ per group. Error bars, SEM.

did not receive NIR illumination (Figure 4A, UCNPs group), nor when they received NIR illumination but were not cultured with Er-UCNPs (Figure 4A, NIR group).

To ensure that these behavioral effects were not caused by the stimulation of other neurons, such as thermal sensory neurons (see below) or non-D type GABAergic neurons, we restricted the NIR illumination to the body, away from the head region where those neurons reside, throughout the behavioral experiments (Methods). We used a curvature heat map to illustrate rhythmic body undulation. In the UCNPs + NIR group, 808 nm NIR illumination led to reduced body curvature as well as reduced undulation frequency. By contrast, no obvious body curvature change was found in the NIR group (Figure S4), indicating the thermal effect of NIR light was not involved in the body bend regulation. We quantified the curvature value change in the posterior middle body (27th segment) at 8-16 s (Figure 4B). Accompanying the reduction of undulation cycle, the mean velocity was also reduced (Figure 4C, D). Decreased motor activity was also observed in a thrashing assay, wherein animals generated body bends in an aqueous M9 solution. NIR illumination led to a significantly reduced C-shape body bending frequency with a saturated Er-UCNP concentration of ~0.5 mg/mL (Figure S5 and Supplementary Video S2).

The Dosage Response of UCNPs. To ascertain the efficient concentration of UCNPs for D-MN activation, we tested the dose responses of UCNPs under the same conditions in advance. The average locomotion velocity of *C*.

elegans during 808 nm NIR illumination was comparable in 5 and 10 mg/mL concentrations of Er-UCNPs, suggesting a saturated efficiency for D-MN activation (Figure 4E). Importantly, a 0.25 mg/mL concentration of Er-UCNPs could reduce velocity almost by half. This reduction was mostly attributed to the strong upconversion efficiency of the core-shell-shell Er-UCNPs (Figures 2B and 4E and Figure S5C). Using saturated 5 mg/mL Er-UCNPs, we found that only 0.64 W/mm² NIR was the minimum illumination required to generate the maximal inhibition (\sim 72%) of *C. elegans*'s locomotion velocity (Figure 4F).

UCNPs Uptake by *C. elegans* Regulates Locomotion in Vivo. We further tested whether D-MNs could be activated when Er-UCNPs were ingested. After 48 h starvation, *hpIs593* animals were fed by *Escherichia coli* food with Er-UCNPs. Bright green emissions from Er-UCNPs under NIR illumination were progressively accumulated in the alimentary canal of animals, until a saturation level after about 12 h feeding (Figure 5A, B). In consistent with our previous *in vitro* observation, the ingested Er-UCNPs upon 808 nm NIR illumination also induced a significant decrease in the locomotion velocity of these animals (Figure 5C–E). Moreover, we observed that the ingested Er-UCNPs in the fed animals excreted gradually ($K_{off} \sim 2.6$ h) when transferred them to normal *E. coli* food without UCNPs, accompanying with reduced velocity inhibition as time elapse (Figure S6).

Confocal imaging was performed to ascertain the accurate location of the ingested Er-UCNPs *in vivo* (Methods). The



Figure 4. Body bending and locomotion activity inhibitions by NIR-UCNPs *in vitro*. (A) Example body postures of crawling transgenic animals that express Chrimson in GABAergic motor neurons. Control group: Animals without exposure to Er-UCNPs and NIR illumination. UCNPs group: Animals exposed to Er-UCNPs (5 mg/mL) but not NIR illumination. NIR group: Animals illuminated by NIR light (0.64 W/mm²) but not exposed to Er-UCNPs. UCNPs + NIR group: Animals exposed to both Er-UCNPs (5 mg/mL) and NIR illumination. Only animals in the UCNPs + NIR group showed obvious relaxation of body bends. Also see the Supplementary Video S1. (B) (Left panel) Representative body curvature kymographs from head to tail (upper panel) and regional (dish line in upper panel) body curvature (lower panel) for an animal under NIR illumination. The upper right arrow on the curvature color map indicates the forward locomotion. The horizontal arrow denotes the pause state of the animal. (Right panel) Quantification of regional body curvature before, during and after irradiation by NIR light in the presence of UCNPs. (C) (Left panel) An example velocity profile of an animal upon NIR illumination. (Right panel) Quantification of the mean velocity before, during, and after NIR light illumination (0.64 W/mm²) in the presence of UCNPs (5 mg/mL). (D) Quantification of velocity under different conditions of nontransgenic wild-type (N2) animals and transgenic animals that express Chrimson in GABAergic motor neurons. Green light: 63.7 μ W/mm². (E) Velocity of transgenic animals before, during, and after NIR light illumination in the presence of different concentrations of Er-UCNPs. (F) Velocity of transgenic animals in the presence of Er-UCNPs (5 mg/mL), during NIR illumination of varying intensity. *n* = 10 per group. Statistical analysis was performed with the Student's *t* test, *** *P* < 0.001 against control (black bar) in each group. Error bars, SEM.

green fluorescence from Er-UCNPs upon 808 nm irradiation was observed within the alimentary canal, while the red D-MNs was found in the ventral nerve cord (Figure 5F–H). This result suggests that the Er-UCNPs regulate behaviors probably indirectly from the alimentary canal without the need of diffusion into neurons. To confirm the assumption, we performed the transection *C. elegans* TEM imaging of these animals (Figure 5I). Indeed, the UCNPs were accumulated exclusively in the lumen of intestine (Figure 5J). No UCNPs were observed in the cytoplasm of intestine cell or other tissues, like the nerve cord where the neurons localized (Figure 5K).

Collectively, these results reveal that green emissions from the alimentary canal Er-UCNPs are sufficient for the activation of Chrimson in the D-class motor neurons to modulate the motor behaviors of *C. elegans*.

The Glutamatergic DVC Interneuron Initiates Reversal Locomotion by Activating Muscle Action Potential Bursting. To further investigate whether Er-UCNPs can be used to stimulate excitatory neurons and reveal the as-yet unknown physiological mechanism underlying motor behaviors, we generated a transgenic strain that expressed Chrimson in C. elegans DVC (hpIs622), an excitatory glutamatergic interneuron localized in the tail (Figure 6A).43 Previous research reported DVC's role in activating reversal, but reported responses were sparse.^{44,45} Moreover, the underlying neural mechanism is largely unknown. When we applied NIR to DVC in the presence of Er-UCNPs, these transgenic animals reliably initiated and sustained backward movements (Figure 6B, C and Supplementary Video S3). This effect was similar to that which we observed in specimens under direct green light illumination in the absence of Er-UCNPs (Figure 6D and Figure S7). Importantly, in situ whole-cell current clamps of body wall muscles revealed that 808 nm NIR illumination activated action potential bursts specifically in the presence of Er-UCNPs. By contrast, no obvious action potential change was found in the -UCNP group (Figure 6E, F). These results not only confirm the positive role of DVC interneuron activation in the initiation of reverse locomotion but also reveal the physiological basis underlying this behavioral response. Taken together, the above experiments demonstrate that the NIR illumination of Er-UCNPs with Chrimson is practicable in both inhibitory and excitatory neurons.



Figure 5. UCNPs uptake by *C. elegans* regulate locomotion. (A) UCNP fluorescent signals were observed in the bodies of transgenic animals (*hpIs593*) after different feeding times. Animals were fed by 5 mg/mL UCNPs. (B) Animals spent around 12 h to absorb the UCNPs to a saturated level. (C) An example velocity profile of a 12 h UCNPs (5 mg/mL) fed animal that expresses Chrimson in GABAergic D-MNs (*hpIs593*) before, during, and after NIR light illumination (0.64 W/mm²). (D) Quantification of the velocity under different conditions of NIR and UCNPs. n.s. not significant by one-way ANOVA, *** P < 0.001 against control group (no NIR, no UCNPs) by the Mann–Whitney U test. $n \ge 10$ per group. Error bars, SEM. (E) Average velocity of no UCNPs fed transgenic animals upon green light illumination (545 nm) at different power intensities. $n \ge 10$ per group. Error bars, SEM. (F) Confocal images of transgenic animals (*hpIs593*) fed with UCNPs (5 mg/mL, 12 h). Scale bar: 100 μ m. (G) (Upper panel) Localization of the UCNPs (green) and D-motor neurons (red). Arrows denote the UCNPs, scale bar: 100 μ m. (Lower panel) Relative location of UCNPs and a single D-motor neuron, star denotes the neuronal soma, scale bar: 5 μ m. (H) The spatial distribution of the two fluorescence signals demonstrated no obvious colocalization of UCNPs and D-motor neuron. (I) TEM image of a transection *C. elegans* fed with 5 mg/mL UCNPs for 18 h. The UCNPs were observed exclusively in the intestine

Figure 5. continued

lumen (blue loop). Yellow loop: intestine, pink loop: nerve cord. (J) Local zoom of the distribution of UCNPs and nerve cord. Scar bar: 1 μ m. The UCNPs were accumulated in the lumen of intestine. No UCNPs were observed in the cytoplasm of intestine cell or other cells. (K) No obvious UCNPs were found in the nerve cord, where the neuronal soma localized. Scar bar: 500 nm.



Figure 6. NIR-UCNPs lead to optogenetic activation of the DVC interneuron. (A) DVC neuron with long neurite extension to the nerve ring (NR) from the soma in the tail. A: anterior, D: dorsal. (B, C) Representative velocity plot of a crawling animal (B) and quantification of the mean velocities (C) before, during, and after NIR illumination in the presence of Er-UCNPs (5 mg/mL). $n \ge 10$ per group. Also see the Supplementary Video S3. (D) Quantification of velocity under different conditions of nontransgenic wild-type (N2) animals and transgenic animals that express Chrimson in DVC interneuron. Positive and negative values indicate the directionality of movements as forward and backward, respectively. Control: no NIR no UCNPs, UCNPs: 5 mg/mL, NIR: 0.64 W/mm², Green light: 63.7 μ W/mm². $n \ge 10$ per group. (E) Muscle action potential firing was significantly elevated by NIR illumination (0.64 W/mm²) in the presence of Er-UCNPs (5 mg/mL, + UCNPs), but not unchanged without Er-UCNPs (-UCNPs). (F) Quantification of the action potential frequency. $n \ge 8$ per group. Statistical analysis was performed with the *Student's t* test, * P < 0.05, *** P < 0.001 against control (black bar) in each group. Error bars, SEM.



Figure 7. Presynaptic development is normal in animals exposed to UCNPs and NIR illumination. (A) A representative image of the expression pattern of a presynaptic marker SNB-1/Synaptobrevin-1::GFP in GABAergic motor neurons. A: anterior, D: dorsal. Scar bar: 50 μ m. Individual presynaptic terminal is shown as a packet of fluorescent signals evenly distributed along the dorsal nerve cord; scale bar: 10 μ m. (B) (Left panel) A schematic of experimental conditions. Worms freely crawling on plates incubated without or with Er-UCNPs (5 mg/mL) and 808 nm NIR light (0.64 W/mm²). (Right panel) The distribution of SNB-1::GFP puncta of 10 individuals of the different groups; scale bar: 10 μ m. (C) Quantification of maximum fluorescence intensity (upper panel) and puncta density (lower panel) of SNB-1::GFP signals in animals without or with Er-UCNPs of SNB-1::GFP signals in animals under different incubation times with Er-UCNPs. n.s. not significant by one-way ANOVA. Error bars, SEM.

Deep Tissue Penetration of 808 nm NIR Illumination. NIR light exhibits deeper tissue penetration than visible light.^{36,37} To ascertain our UCNP-optogenetics efficiency in deep tissue, chicken breast with different thickness was used to block the interaction between Er-UCNP fed animals and 808 nm NIR illumination (Figure S8A). A gradual decay of NIR power intensity was observed upon increase of the tissue thickness (Figure S8B). The NIR inhibited body bending in a thickness-dependent manner and was still observed until 2.0 mm chicken breast was used (Figure S8C and Supplementary Video S4). These results confirmed that 808 nm NIR with Er-UCNPs exhibits good tissue penetration, which is crucial for *in vivo* applications in deep tissues of large animals, like mice brain.

The Thermal Effect of NIR Illumination Does Not Contribute to Behavioral Response. While NIR has the advantage of deeper tissue penetration than visible light, the thermal effect of NIR light remains a common concern. 808 and 980 nm are generally chosen as the excitation source for UCNPs, which is due to the absorption cross-section of the sensitizers, a key factor to synthesize highly emissive UCNPs. Yb³⁺ with the energy level in 980 nm is usually chosen as the sensitizer under 980 nm excitation. Nd³⁺, with 10-times higher absorption cross-section than that of Yb3+ at 808 nm, is an ideal element to construct of 808 nm light responsive nanoparticles.^{30,34,35} The water absorption of 808 nm is significantly lower than that of 980 nm, thus the 808 nm sensitive UCNPs avoid the overheating effect of the tissue,^{15,16,30,34} which might significantly improve the practical application of UCNP in bioapplication. In this work, we choose Nd³⁺ as the main sensitizers and Yb³⁺ relays the energy to the emitters (Figure S9). In such way, the synthesized Er-UCNPs exhibited higher emission intensity upon illumination by 808 nm NIR compare to that of 980 nm (Figure S10). Therefore, the velocity inhibition of Er-UCNPs by 808 nm NIR light illumination, under same illumination conditions (power density and duration), was stronger than that of by 980 nm NIR (Figure 4C, Figure S11).

To determine whether the thermal effect of 808 nm NIR light was involved in the observed locomotion inhibition, we first measured temperature variation on the surface of NGM plates during NIR illumination, where we performed the C. elegans behavior experiments (Methods). 808 nm NIR illumination induced negligible temperature increase; even under the extreme experiment condition of 5 min of 3.06 W/ mm² NIR illumination, only a moderate increase of the NGM surface temperature was observed from 21.2 to 23.2 °C (Figure S12A). 980 nm illumination, however, induced intense temperature increase from 21.2 to 54.1 °C under the same condition (Figure S12B). C. elegans has multiple thermosensory neurons, such as AFD, AWC, ASI, etc., which localize in the head and detect temperature fluctuations at this range.^{46,47} Animals lacking either the cyclic nucleotide-gated channels of TAX-4, TAX-2, or guanylate cyclases GCY-8 GCY-18 GCY-23 showed the athermotactic phenotype, in which the animals move almost randomly on a temperature gradient.⁴⁸⁻⁵⁰ In these mutant animals, we observed that 808 nm NIR illumination induced an inhibition of velocity similar to that which was observed in wild-type animals under the same experimental conditions (Figure S13), suggesting that the thermal sensitive proteins were not involved for the velocity inhibition induced by 808 nm NIR illumination. These data confirmed that the thermal effect generated by 808 nm NIR illumination was not involved in locomotion inhibition.

UCNPs Do Not Affect Neuronal Development. A key criterion for UCNPs' application is that their presence and ingestion should not affect the neuronal development and/or health of the animal. To determine whether the synthesized UCNPs and/or NIR illumination changed neuronal development, we visualized the synapse morphology of dorsal D-motor neurons using SNB-1::GFP, a synaptic marker.⁵¹ SNB-1::GFP markers exhibit punctate and evenly distributed signals along the nerve cord (Figure 7A). *C. elegans* cultured in the presence of Er-UCNPs and NIR illumination exhibited normal SNB-1::GFP morphology and distribution (Figure 7B, C). Importantly, the exposure to Er-UCNPs from 0.5 h up to 72 h did not affect synaptic morphology (Figure 7D, Figure S14). These data confirmed that synthesized Er-UCNPs did not affect neuronal development in *C. elegans*.

UCNPs Exhibit Negligible Long-Term Biotoxicity. To determine the long-term toxicity of UCNPs, we compared the

growth rate, fertility, and lifespan of C. elegans cultured with or without Er-UCNPs at a concentration of 5 mg/mL. Wild-type (N2) and transgenic wild-type (hpIs593) animals were examined under the same experimental conditions used in the motor behavior tests. The general health of animals was assessed by growth rate, quantified as the increase in body length across larval development (12, 24, 48, and 72 h after hatch). We found that the mean body length was not changed in either N2 or transgenic hpIs593 animals when incubated with Er-UCNPs (Figure S15A, B). The fertility of adults was examined by measuring the number of eggs. No differences were observed when animals were exposed to Er-UCNPs at varying concentrations (0.125, 0.25, 0.5, 2, 5 mg/mL) nor were they observed between N2 and transgenic hpIs593 animals (Figure S15C, D). When these animals were continually cultured with Er-UCNPs, the lifespan of adults showed a trend of concentration-dependent slight decrease (Figure S15E), in both N2 and hpIs593 animals. The slight decrease of the lifespan at high concentrations of Er-UCNPs was probably due to multiple reasons, including food osmosis, pH value, and mechanical resistance. These results demonstrate that Er-UCNPs' biotoxicity is negligible.

Taken all together, these results demonstrate that the 808 nm NIR illumination of Er-UCNPs with Chrimson provides a highly efficient, low-toxicity approach for optogenetic stimulation.

CONCLUSIONS

We here developed a NIR-mediated Chrimson optogenetic approach, relying on light transducer Er-UCNPs, to regulate *C. elegans* locomotion. Chrimson exhibits high light sensitivity from green light to red light. Green emissions from Er-UCNPs with low NIR illumination power density were sufficient to activate Chrimson expressed neurons. The efficiency of Er-UCNPs makes them feasible nanotransducers for optogenetic manipulation *in vivo*, especially for deep tissues. Thus, this study demonstrated an efficient upconversion-based optogenetics toolset for neuroscience. This efficient and *in vivo* validated optogenetic tool is ready to be tested by the research community.

METHODS

Materials and Reagents. Gadolinium acetate hydrate (99%), ytterbium acetate hydrate (99%), and calcium acetate hydrate (99%) were all purchased from Alfa Aesar. Erbium acetate hydrate (99%) and neodymium acetate hydrate (99%) were purchased from Sigma-Aldrich. 1-Octadecene (90%) and oleic acid (90%) were purchased from Aladdin. Sodium hydroxide (>98%), ammonium fluoride (98%), hexane (AR), ethanol (AR), and chloroform (AR) were all purchased from Sinopharm Chemical Reagent Co., Ltd. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) was purchased from Shanghai Ziqi Biotechnology Co., Ltd. All reagents were used as received without further purification.

Synthesis of NaGdF₄:Yb,Er,Ca@NaYbF₄:Gd,Ca@ NaNdF₄:Gd,Ca Upconversion Nanoparticles. Refer to the method described in our previous report, by which the core, coreshell, and core-shell-shell nanoparticles were synthesized.³⁶

Preparation of UCNPs@DSPE-PEG. To render the UCNPs biocompatible, DSPE-PEG2000 was used to modify the surface of UCNPs according to a previously reported method with slight modifications.⁵² Typically, a 5 mL UCNPs solution in chloroform (20 mg/mL) was added to a 5 mL DSPE-PEG2000 solution in chloroform (20 mg/mL) and stirred at room temperature (RT) for 24 h. The chloroform was slowly evaporated *via* vacuum, and the

residue was dissolved in 5 mL of water. After sonication, the solution was transferred to a microtube and centrifuged. The sediment was discarded to remove possible large aggregates. The resulting solution was further filtered through a 0.22 μ m membrane filter to remove some aggregates and then kept at 4 °C for later use.

Characterization. Transmission electron microscopy (TEM) images were recorded on a HITACHI HT7700 microscope operating at 120 kV. High-resolution TEM (HR-TEM) imaging and energydispersive X-ray (EDX) spectroscopy were done using a transmission electron microscope (Tecnai G2 F30, FEI) equipped with an X-ray energy dispersive spectrometer. The crystal structure of UCNPs was studied by X-ray diffraction (XRD) using a diffractometer (X'Pert PRO, PANalytical B. V., Almelo, Netherlands) using Cu K α radiation at $\lambda = 0.15406$ nm and operating at 40 kV and 40 mA. Each sample was recorded from 10° to 70° with a step size of 0.0131 and a step time of 9.945 s. To obtain the upconversion photoluminescence spectra, samples were dispersed in hexane/water (10 mg/mL) in a standard quartz cuvette at room temperature, and fluorescence spectra were acquired on a Fluoromax-Plus, Horiba Jobin Yvon spectrofluorometer with a commercial 808 nm NIR laser (Changchun Laser Optoelectronics Technology Co., Ltd. China). The hydrodynamic diameter of nanoparticles was determined by a dynamic light scattering instrument (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK) equipped with 4 mW He–Ne laser source ($\lambda = 633$ nm, scattering angle: 90°). FTIR spectra were acquired on Fourier transform infrared spectroscopy (VERTEX 70, Bruker Corp., Germany)

Temperature Measurement. The NGM plate covered with OP50 was irradiated with an 808 and 980 nm NIR for 5 min. The plate was then placed at the focal point (808 nm NIR: focal length 8 cm, focal spot diameter 1 mm; 980 nm NIR: 1.2 mm \times 1.8 mm collimating light) of the condenser collimator, which connected to the end of the fiber (φ 200 μ m). The temperature of the NGM plate at the focal point was measured by an infrared thermal imager (FLIR ES0, FLIR Systems, Inc., US). At least three NGM plates were repeatedly measured at the same laser power and were finally averaged.

Strain Maintenance. Strains were maintained as described.⁵³ All *C. elegans* were cultured on standard Nematode Growth Medium (NGM) plates seeded with OP50 and maintained at 20 °C incubators. Unless otherwise stated, the wild-type animal refers to the Bristol N2 strain. Unless otherwise stated, all transgenic strains that contain Chrimson (*hpIs593* and *hpIs622*) were cultured in darkness on OP50 NGM plates supplemented with 0.5 mM ATR.²¹ Transgenic lines used in this study include: ZM9276 *hpIs593* (*Pttr-39::*Chrimson::wCherry), ZM9297 *hpIs622* (*Pceh-63::*Chrimson::wCherry) *lin-15(n765)*, SGA40 *tax-4(ks11); hpIs593*, SGA57 *tax-2(p691); hpIs593*, SGA50 *gcy-23(nj37) gcy-8(oy44) gcy-18(nj38); hpIs593*, CZ333 *juIs1* (*Punc-25::*SNB-1::GFP). Other genetic mutants used for constructing transgenic lines and compound mutants were obtained from the Caenorhabditis Genetics Center; all were backcrossed at least four times against N2 prior to analyses.

NIR-UCNPs Optogenetic Locomotion Analysis. A single L4 stage C. elegans, maintained in standard culture conditions, was transferred to a 60 mm imaging plate seeded with a thin layer of OP50 with or without Er-UCNPs. One min after the transfer, a 2 min video of the crawling animal was recorded on a stereoscopic fluorescence microscope (Axio Zoom V16, Zeiss) equipped with a digital camera (acA2500-60um, Basler). The NGM plate was irradiated with an 808 nm laser coupled with a φ 200 μ m fiber with a condenser collimator (focal length 8 cm, focal spot diameter 1 mm). The NGM plate was placed at the focal point of the condenser collimator. The green light optogenetic assay used the same protocol but with a direct green laser source (545 nm, Laserwave). The final green light intensity on the NGM plate was around 63.7 μ W/mm², as measured by the optical power meter (PSL-PM02). All images were captured with a 1× objective (Apo, Zeiss) at 10 frames per second by Pylon 4 software. Data recorded on the same day were pooled and quantified. Postimaging analyses utilized WormLab 4.0 and in-house written MATLAB scripts. A Wormlab or homemade Matlab script was used to divide a virtual image of each C. elegans body into 33 segments. The

midpoint was used to track and calculate the animal's body length, velocity, and direction of movements between each frame (Figure 4, Figure S15). The angle between three joint points was used to calculate the curvature of the middle point loci. The curvature values for each body segment from the head to tail over time were plotted and were converted to a heat map of movements (Figure 4B, Figure S4).

Confocal Imaging. The strain juIs1 (Punc-25::SNB-1::GFP) of C. elegans, which expressed the synaptic protein SNB-1 to GABAergic Dclass motor neurons, was used to detect the potential toxicity of UCNPs and NIR to neuronal development. For UCNPs + NIR experiments, animals were cultured with UCNPs (5 mg/mL) and then were irradiated by 808 nm NIR (0.64 W/mm^2) 3 times every 5 s. After resting for 30 min, the entire synaptic fluorescence of the animal was recorded. The puncta from the dorsal middle body were then used to quantify fluorescence intensity and distribution (Figure 7A). Eggs, L2, L3, and L4 larva were picked into UCNPs plates to test the effects of UCNPs on neuronal synaptic development for various culture times (from 0.5 to 72 h). Fluorescence signals were captured from live L4 larva using a Plan-Apochromatic $20 \times$ (Figure 5A) or $60 \times$ (Figure 7) objective on a confocal microscope (FV3000, Olympus) in the same conditions. Worms were immobilized by 2.5 mM levamisole (Sigma-Aldrich) in M9 buffer. Straightened dorsal nerve cords were extracted from the raw images using the "straightened to line function" from ImageJ. Background fluorescence was subtracted by the average intensity in a region devoid of SNB-1::GFP puncta. Two main parameters were determined: maximal fluorescence intensity and puncta number. Maximal fluorescence intensity along the cord was calculated using the "plot profile" function of ImageJ. Synaptic puncta numbers were calculated using the "analyze particles" function, with an intensity threshold of 400.⁵⁴ For analyzing the spatial distribution of ingested Er-UCNPs, Zeiss-LSM780 confocal laser scanning microscopy (Oberkochen, Germany) with a femtosecond laser (Mai Tai HP, 690 nm-1040 nm) was used (Figure 5F-H).

High-Pressure Freezing and Freezing Substitution. Living worms were picked into a type A specimen carrier (200- μ m well) containing E. coli with 2% low-melting agarose as filler. Then they were placed in a treated type B carrier (flat) with hexadecane and closed. The samples were fast frozen with the HPF device (Leica HPM100, Germany) in -176 °C and were immersed into a freezing tube containing 2% osmium tetroxide in 98% acetone/2% water and placed into the freeze substitution (FS) device (Leica EMAFS, Germany) set to the following parameters: T1 = -90 °C for 72 h, S1 = 5 °C/h, T2 = -60 °C for 12 h, S2 = 5 °C/h, S3 = 10 °C/h, T3 = -20 °C for 8 h, then slowly warmed to 15 °C (5 °C/h). Following FS, samples were rinsed four times in 100% acetone, 15 min each, at RT. Next, they were stained in 1% uranyl acetate dissolved in acetone (centrifuged before use), for 2 h in the dark at RT. Then samples were rinsed three times in 100% acetone, 15 min each. After that, they were successively infiltrated in series mixture of an Embed812 resin (EMS, USA) and acetone (1:3, 1:1, 3:1) and rotated at RT for 5 h, 12 h, and overnight, respectively. Next, samples were changed into 100% resin, which was replaced four times over next 3 days, with constant rotation. Finally, we separated an intact worm using a stereoscope and embedded it in a mold with fresh resin and polymerized for 48 h at 60 °C

Ultrathin Sectioning. *C. elegans* embedded samples were trimmed and sliced into 50 nm sections with a diamond knife using a Leica ultramicrotome EM UC6 (Leica, Germany). The sections were collected on the EM grids coated with carbon film.

Transmission Electron Microscopy Imaging. TEM sample grids were imaged under a Spirit Transmission Electron Microscope (FEI Company, The Netherlands) operating at 100 kV.

Electrophysiology. Dissection and recording of *C. elegans* were carried out using same protocols described in previous reports.^{38,40,55} Briefly, 1 or 2 day-old hermaphrodite adults were glued (Histoacryl Blue, Braun) to a Sylgard-coated cover glass covered with bath solution (Sylgard 184, Dowcorning) under a stereoscopic microscope (M50, Leica). After clearing the viscera by suction through a glass pipet, the cuticle flap was turned and gently glued down using

WORMGLU (GluStitch Inc.) to expose the neuromuscular system. Body wall muscle cells were patched using 4–6 $M\Omega\text{-resistant}$ borosilicate pipettes (1B100F-4, World Precision Instruments). Pipettes were pulled by micropipette puller (P-1000, Sutter) and fire-polished by microforge MF-830 (Narishige). Action potentials were recorded in the whole-cell configuration by EPC9 amplifier (HEKA, Germany), using the pulse and processed with Igor 6 (WaveMetrics) and Clampfit 10 software (Axon Instruments, Molecular Devices). Action potentials were recorded at 0 pA. Data were digitized at 10-20 kHz and filtered at 2.6 kHz. The pipet solution contains (in mM): K-gluconate 115; KCl 25; CaCl₂ 0.1; MgCl₂ 5; BAPTA 1; HEPES 10; Na₂ATP 5; Na₂GTP 0.5; cAMP 0.5; cGMP 0.5, pH7.2 with KOH, ~320 mOsm. cAMP and cGMP were included to maintain the activity and longevity of the preparation. The bath solution consists of (in mM): NaCl 150; KCl 5; CaCl₂ 5; MgCl₂ 1; glucose 10; sucrose 5; HEPES 15, pH7.3 with NaOH, ~330 mOsm. Chemicals were obtained from Sigma unless stated otherwise. Experiments were performed at RT (20-22 °C). Optogenetic stimulation was performed with NIR light at 808 nm for hpIs593 and hpIs622. 10-20 s NIR exposure was used to evoke D-class motor neurons and DVC interneuron. The frequency and amplitude of action potentials were analyzed using Clampfit 10.

Thrashing Assay. Different concentrations of UCNPs mixed in M9 buffer were dropped (50 μ L each) in 12 well plates with NGM beforehand. One min after the animals (4–6 h post-early L4 stage) were transferred, thrashing behaviors were recorded by a digital camera (acA2500–60um, Basler). Images were captured at 35 fps with proper magnification (24×). For NIR irradiation experiments, each animal was exposed to 808 nm NIR for 3–5 s to reduce possible heating effects. The thrashing score measured the frequency of body bending for every 3 s.⁵⁶

Body Length Measurement. Eggs before hatch from N2 and *hpIs593* were picked to OP50 NGM plates without or with Er-UCNPs (5 mg/mL) for different periods of time (12, 24, 48, 72 h). The body length was recorded by a stereo fluorescence microscope (Axio Zoom V16, Zeiss) under $25 \times$ magnification. The body length was analyzed by WormLab 4.0 software. An example image of an L4 larva shows that the body length was measured from the head to tail along the central axis (Figure S15A).

Egg Laying Assay. Eggs before hatch from N2 and *hpIs593* were picked to OP50 NGM plates without or with different concentrations of Er-UCNPs (0.125, 0.25, 0.5, 2, 5 mg/mL). For each group, 10 eggs were put into one plate until they became young adults. After the first day of egg laying, they were transferred to fresh UCNPs-OP50 plates every 24 h to avoid food shortages. The number of laid eggs in each group was then counted manually. The total number of eggs was calculated by accumulating all data. Experiments were repeated three times for each group.²⁸

Lifespan Assay. Lifespan studies were performed on 60 mm NGM plates at 20 °C as previously described.³⁷ For each group, 10–12 L4 animals from N2 or *hpIs593* were picked and transferred every other day to fresh OP50 NGM plates without or with different concentrations of UCNPs (0.125, 0.25, 0.5, 2, 5 mg/mL). A young adult with 3-5 eggs in its body was described as adult day 0. Survival rate was scored every 1-2 days, and worms were censored if they crawled off the plate, hatched inside the NGM, or lost vulvar integrity during reproduction. Experiments were repeated 10 times for each group. *P* values were calculated using the log-rank (Kaplan–Meier) method.

Statistical Analysis. The Mann–Whitney *U* test, two-tailed Student's *t* test, and one-way ANOVA test were used to compare data sets. *P* < 0.05 was considered to be statistically significant (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001). Graphing and subsequent analysis were performed using Igor Pro (WaveMetrics), Clampfit (Molecular Devices), ImageJ (National Institutes of Health), Matlab (Math-Works), GraphPad Prism 5 (GraphPad Software Inc.), and Excel (Microsoft). For electrophysiology, behavior analysis, and fluorescence imaging, unless specified otherwise, each recording trace was obtained from a different animal; data were presented as the mean ± SEM.

ASSOCIATED CONTENT

S Supporting Information

and supplementary videos. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b09270.

EDX spectrum of UCNPs; DLS of UCNPs@DSPE-PEG; expression pattern of Chrimson; body bending without UCNPs; thrashing assay; elimination of uptake UCNPs in *C. elegans*; reversal initiation of DVC::Chrimson transgenic animals; tissue penetration of 808 nm NIR; energy-transfer process; upconversion luminescence spectra of UCNPs; locomotion inhibition by 980 nm NIR; thermal effect of NIR illumination; neuronal development, growth rate, fertility, and lifespan of *C. elegans* incubated with Er-UCNPs (PDF)

Video S1 (AVI) Video S2 (AVI) Video S3 (AVI) Video S4 (AVI)

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Notes

The authors declare no competing financial interest.

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