Channelrhodopsins (ChRs) are used to optogenetically depolarize neurons. We engineered a variant of ChR, denoted red-activatable ChR (ReaChR), that is optimally excited with orange to red light (λ ~590–630 nm) and offers improved membrane trafficking, higher photocurrents and faster kinetics compared to existing red-shifted ChRs. Red light is less scattered by tissue and is absorbed less by blood than the blue to green wavelengths that are required by other ChR variants. We used ReaChR expressed in the vibrissa motor cortex to drive spiking and vibrissa motion in awake mice when excited with red light through intact skull. Precise vibrissa movements were evoked by expressing ReaChR in the facial motor nucleus in the brainstem and illumination with red light through the external auditory canal. Thus, ReaChR enables transcranial optical activation of neurons in deep brain structures without the need to surgically thin the skull, form a transcranial window or implant optical fibers.

ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation

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Channelrhodopsins (ChRs) are used to optogenetically depolarize neurons. We engineered a variant of ChR, denoted red-activatable ChR (ReaChR), that is optimally excited with orange to red light (λ ~590–630 nm) and offers improved membrane trafficking, higher photocurrents and faster kinetics compared to existing red-shifted ChRs. Red light is less scattered by tissue and is absorbed less by blood than the blue to green wavelengths that are required by other ChR variants. We used ReaChR expressed in the vibrissa motor cortex to drive spiking and vibrissa motion in awake mice when excited with red light through intact skull. Precise vibrissa movements were evoked by expressing ReaChR in the facial motor nucleus in the brainstem and illumination with red light through the external auditory canal. Thus, ReaChR enables transcranial optical activation of neurons in deep brain structures without the need to surgically thin the skull, form a transcranial window or implant optical fibers.

RESULTS

Development of a red-activatable ChR

The red-shifted ChR VChR1 (ref. 14), which has minimal trafficking to the membrane as well as poor expression in mammalian cells11,15, served as a template to engineer an efficient red light–activated ChR. To improve membrane trafficking, we examined the superior membrane trafficking of the variant ChIEF7, which gives almost exclusive plasma membrane expression with minimal cytosolic aggregation in mammalian cells without the need for additional trafficking signals, unlike many other ChR variants (Supplementary Fig. 1)15. We thus replaced the N terminus of VChR1 before the first transmembrane domain with the corresponding ChIEF sequence. The new construct, denoted C-VChR1, had considerably improved membrane trafficking15 (P < 0.001 for VChR1 compared to C-VChR1). To increase the expression level, we replaced transmembrane domain F of VChR1 with the corresponding VChR2 helix, a strategy that has been shown previously to increase the expression level of ChR in ChR1-ChR2 chimeras16. This new variant, named VCOMET for VChR optimized for membrane expression and trafficking, had a red-shifted response spectra similar to that of C-VChR1 or VChR15, with spectral peaks at 590 and 530 nm for the maximum and steady-state (also called plateau) responses, respectively (Supplementary Fig. 2b). VCOMET expressed strongly in mammalian cells and retained robust membrane trafficking (Supplementary Fig. 1). It also yielded greater photocurrent in HEK293 cells (39 ± 8 pA pF−1 (mean ± s.e.m.), n = 13) compared to C-VChR1 (6.9 ± 1.0 pA pF−1, n = 13; P < 0.01) and comparable photocurrents to mammalian codon–optimized ChEF
Figure 1  Basic properties of ReaChR compared to those of C-VChR1 (VChR1 with the ChIEF N terminus) and C1V1E122T. (a) Schematic design of ReaChR. ReaChR consists of the N terminus of the ChEF (or ChIEF) variant (ChEF/ChIEF), transmembrane domains A–E and G of VChR1, transmembrane domain F of VChR2 and the L171I point mutation. (b) Examples of C1V1E122T and ReaChR expression in HEK293 cells as visualized by the fluorescence of fused Citrine. Much of the ReaChR expression was at the plasma membrane, whereas C1V1E122T expression was more intracellular, with strong aggregation. Scale bar, 20 µm. (c) The relative plasma membrane expression level (left) and the ratio of plasma membrane to intracellular fluorescence (right) of VChR1 (n = 19), C-VChR1 (n = 29), C1V1 (n = 26), C1V1E122T (n = 16) and ReaChR (n = 21) as measured with Citrine fluorescence. AU, arbitrary units. (d) The mean photocurrent amplitudes of C-VChR1 (n = 13), C1V1 (n = 10), C1V1E122T (n = 10), VCOMET (n = 13) and ReaChR (n = 9) recorded from HEK293 cells. The current amplitudes were measured at the wavelengths that evoked the greatest response in each variant and were normalized to cell capacitance. The data in c and d are shown as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, Kruskal-Wallis test with post-hoc Dunn’s test performed on all pairs of variants (only significant differences detected with Dunn’s tests with VCOMET or ReaChR are shown); the statistical tests shown in d also include comparison to oChEF (n = 12) and oChIEF (n = 7). H = 31.63, k = 5, P < 0.0001 (c, left); H = 41.28, k = 5, P < 0.0001 (c, right); H = 48.02, k = 7, P < 0.0001 (d). (e) The responses of C-VChR1 (top), C1V1E122T (middle) and ReaChR (bottom) to 650, 630, 610, 590, 470 and 410 nm light of the same photon flux (5.1 × 10^{16} photon mm−2 s−1). (f) The spectra of the maximum response (left) and steady-state (plateau) response (right) of C-VChR1 (n = 7), C1V1E122T (n = 8) and ReaChR (n = 7). The responses were normalized to the maximum response of each cell. The gray regions highlight the response above 600 nm. The data in f are shown as the mean ± s.e.m.

(oChEF; 48 ± 8 pA pF−1, n = 12; Supplementary Fig. 2a) and mammalian codon-optimized ChIEF (oChIEF; 52 ± 7 pA pF−1, n = 7). VCOMET had a reversal potential (12 ± 2 mV (mean ± s.e.m.), n = 8) that was significantly higher than that of oChIEF (4 ± 1 mV, n = 5; P > 0.05) but not that of the calcium-translocating ChRs, CatChs\(^{11}\) (5 ± 1 mV, n = 6) in physiological saline (H = 10.39, k = 4, P = 0.0155 for comparisons between VCOMET, ChIEF, CatCh and ReaChR) and a reduced inward rectification similar to that of oChIEF (Supplementary Fig. 2c). Although VCOMET responded strongly to light above 600 nm (Supplementary Fig. 2b), the responses at these longer wavelengths were desensitizing and did not recover completely in the dark without reconditioning with 410 nm light (Supplementary Fig. 3). We thus sought to reduce the desensitization of VCOMET to light above 600 nm through known point mutations of ChRs. The ChETA mutation\(^{12}\) did not red shift or reduce the desensitization of VCOMET. The corresponding H134R mutation of ChR2 (ref. 18) slowed the channel kinetics and thus degraded the temporal fidelity of the Chr. One mutation, L171I (Fig. 1a), which corresponds to the same position of the ChIEF mutation\(^{7}\), increased the amplitude of the photoresponse at both 610 and 630 nm light through reduced desensitization while retaining the membrane trafficking and expression of VCOMET (Figs. 1 and 2) and Supplementary Fig. 4) and thus led to an increase in the steady-state (plateau) spectral peak at 630 nm (Fig. 1f). This formed ReaChR, the new ChR. ReaChR retained the reversal potential (7 ± 4 mV, n = 6), reduced inward rectification and photocurrent amplitude (34 ± 4 pA pF−1, n = 9) of VCOMET (Fig. 1b–d). The one limitation of ReaChR relative to C-VChR1 was the slower channel closure rate after termination of the light pulse: τ_{1/e} = 137 ± 7 ms (mean ± s.e.m. n = 11) for ReaChR compared to τ_{1/e} = 85 ± 4 ms (n = 9) for C-VChR1 (P < 0.05) (Fig. 2d and Table 1). Comparisons of red-shifted ChR variants in cell culture The Chr variant C1V1E122T has been shown to excite the neuronal membrane potential with 630 nm light that is pulsed for 50 ms\(^{11}\). To gauge the potential benefit of ReaChR over C1V1 and related constructs, we characterized the attributes of ReaChR in comparison to those of C1V1, C1V1E122T and VCOMET (Table 1). C1V1 and C1V1E122T both expressed strongly in HEK293 cells as visualized with Citrine\(^{13}\) fluorescence of the C-terminal fusion protein. However,
neither C1V1 nor C1V1E122T trafficked to the membrane as well as ReaChR or VCOMET in these cells, and they both showed high amounts of intracellular aggregation and strong cytosolic fluorescence (Fig. 1b,c). The mean photocurrent of C1V1 expressed in HEK293 cells (13 ± 3 pA pF⁻¹, n = 10) was smaller than those for VCOMET and ReaChR (Fig. 1d), possibly because of reduced efficiency in trafficking to the plasma membrane (Fig. 1c). Introduction of the E122T mutation to form C1V1E122T reduced the C1V1 photocurrent ~66% (4 ± 1 pA pF⁻¹, n = 10; Fig. 1d), although its action spectrum was red shifted, with peaks at 590 and 610 nm for the maximum and steady-state (plateau) responses, respectively (Fig. 1e,f). The channel kinetics of C1V1E122T were significantly slower than those of VCOMET and C-VChR, with a channel closure time constant of τ_c = 315 ± 26 ms (n = 8), although this value is similar to that of the parent construct, C1V1 (τ_c = 306 ± 10 ms, n = 6; Fig. 2d). The activation kinetics of C1V1E122T were also 1.5- to 3-fold slower than ReaChR and C-VChR at the same light intensities for 610 and 630 nm light (Fig. 2c). However, the photocurrent amplitudes of C-VChR, ReaChR and C1V1E122T showed similar functional forms in response to incident light intensity (Supplementary Fig. 4). With C-VChR, C1V1E122T and ReaChR, increasing the stimulation intensity of 610 nm light led to reduction of the steady-state (plateau) response amplitudes at higher light intensities (Fig. 2a and Supplementary Fig. 4). As ReaChR has improved membrane trafficking, greater photocurrents and faster kinetics compared to C1V1E122T, we propose that ReaChR is a robust means to stimulate neurons with red-orange to red light.

Comparison of ChR variants in cultured neurons

We compared the properties of ReaChR with those of other ChR variants in cultured neurons.20 ReaChR traffics to the membrane well and expresses strongly in neurons (n = 22), as we observed in HEK293 cells (Fig. 3a). The variants C1V1E122T (n = 13) and C1V1E122T with an additional trafficking sequence, denoted C1V1E122T-TS (n = 17),21,22, trafficked to the membrane as well as ReaChR in cultured neurons (Fig. 3a,b), which is in contrast to the relatively greater trafficking that we observed for ReaChR in HEK293 cells. However, the expression levels of C1V1E122T and C1V1E122T-TS were approximately 80–84% lower than those of ReaChR under identical conditions (Fig. 3a,b). We observed occasional cells with high expression levels of C1V1E122T and C1V1E122T-TS, although they occurred too infrequently to be of general utility.

In order to compare the photocurrent amplitudes of different ChR variants, we used a titer-matched lentivirus with a truncated human synapsin 1

### Table 1 Comparisons of the channel kinetics and properties of C-VChR1, VCOMET, ReaChR, C1V1 and C1V1E122T

| Variant      | Maximum Response spectra (nm) | Steady state response spectra (nm) | Mean membrane fluorescence (AU) | Mean ratio of membrane to cytosolic fluorescence | Mean photocurrent (pA pF⁻¹) | Channel on rate, n_1/q (ms), 610 nm at 7.6 mW mm⁻² | Channel on rate, n_1/q (ms), 630 nm at 7.6 mW mm⁻² | Channel off rate, n_1/q (ms) |
|--------------|--------------------------------|-----------------------------------|---------------------------------|-----------------------------------------------|----------------------------|------------------------------------------------|------------------------------------------------|-----------------------------|
| C-VChR1      | -570                           | -550                              | 58 ± 1                          | 0.98 ± 0.07                                   | 6.9 ± 1                    | 19.4 ± 0.8                                     | 49.4 ± 2.0                                     | 84.9 ± 3.9                  |
|              | (n = 29)                       | (n = 29)                          | (n = 29)                        | (n = 13)                                      | (n = 11)                   | (n = 11)                                       | (n = 11)                                       | (n = 11)                     |
| VCOMET       | -590                           | -530                              | ND                              | 1.10 ± 0.07                                   | 38.9 ± 7.8                 | ND                                             | ND                                             | 100.4 ± 6.6                 |
|              | (n = 26)                       | (n = 26)                          | (n = 26)                        | (n = 13)                                      | (n = 9)                    | (n = 9)                                         | (n = 9)                                         | (n = 9)                      |
| ReaChR       | -590                           | -630                              | 218 ± 29                        | 1.12 ± 0.07                                   | 33.5 ± 3.6                 | 20.7 ± 0.6                                     | 68.1 ± 4.2                                     | 137.2 ± 7.1                 |
|              | (n = 21)                       | (n = 21)                          | (n = 21)                        | (n = 9)                                       | (n = 9)                    | (n = 9)                                         | (n = 9)                                         | (n = 9)                      |
| C1V1         | ND                             | ND                                | 87 ± 17                         | 0.70 ± 0.06                                   | 13.0 ± 3.0                 | ND                                             | ND                                             | 306.3 ± 10.3                |
|              | (n = 26)                       | (n = 26)                          | (n = 26)                        | (n = 10)                                      | (n = 10)                   | (n = 10)                                        | (n = 10)                                        | (n = 10)                     |
| C1V1E122T    | -600                           | -610                              | 72 ± 11                         | 0.58 ± 0.06                                   | 4.4 ± 0.6                  | 41.5 ± 4.6                                     | 115.8 ± 11.1                     | 315.4 ± 26.0                |
|              | (n = 16)                       | (n = 16)                          | (n = 16)                        | (n = 11)                                      | (n = 7)                    | (n = 7)                                         | (n = 7)                                         | (n = 7)                      |

Electrophysiological characterizations were made under voltage-clamp recordings in HEK293 cells in which the membrane potential could be accurately clamped at -60 mV. Plasma membrane and intracellular fluorescence were measured in HEK293 cells transiently expressing ChR fused to Citrine imaged with a confocal laser-scanning microscope. ND, not determined; AU, arbitrary units.

*The value of VCOMET was acquired in a separate experiment with different pixel-dwelling time settings.

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**Figure 2** Kinetics of C-VChR1, C1V1E122T and ReaChR. (a) Representative responses of a C-VChR1–expressing (left), a C1V1E122T-expressing (middle) and a ReaChR-expressing (right) HEK293 cell to 0.08, 0.19, 0.34, 0.74, 1.93, 4.09, 7.65 and 11.75 mW mm⁻² of 610 nm light. (b) The responses of the same cells as in (a) to 630 nm light of varying intensities. (c) Channel onset time constants of C-VChR1 (n = 10), C1V1E122T (n = 6) and ReaChR (n = 10) to 610 nm (left) and 630 nm (right) light of different intensities. Data are shown as the mean ± s.e.m. (d) Channel closure time constants of C-VChR1 (n = 11), C1V1 (n = 6), C1V1E122T (n = 8), VCOMET (n = 9) and ReaChR (n = 11). *P < 0.05, **P < 0.01, ***P < 0.001, Kruskal-Wallis test with post-hoc Dunn’s multiple comparison tests on all pairs of variants (n = 36.76, k = 5, P < 0.0001). Data are shown as the mean ± s.e.m.
promoter (hSyn) to express enhanced yellow fluorescent protein–tagged human codon–optimized ChR2H134R (hChR2H134R-eYFP), oChIEF-Citrine, C1V1E122TS-Ts-Citrine and ReaChR-Citrine in cultured neurons. We selected cells for recording on the basis of their morphology under bright-field visualization. We did not use fluorescence signal–based selection or the selective α-CamKIIα promoter20 to avoid bias in the selection process. In all cases, ChRs were activated by 10 mW mm⁻² of light at the respective maximum response wavelengths. We recorded the photocurrents under voltage clamp. We observed that currents from ReaChR-Citrine–expressing cells (219 ± 50 pA, n = 16) were greater than those from hChR2H134R-expressing (37 ± 21 pA, n = 16) and C1V1E122Ts-expressing (23 ± 11 pA, n = 14) neurons but were non-significantly greater than those from oChIEF-expressing neurons (75 ± 31 pA, n = 17) (Fig. 3c). None of the ReaChR-expressing cells tested were unresponsive to light, whereas many hChR2H134R-expressing (n = 9) and C1V1E122Ts–expressing (n = 5) neurons had no detectable response to light.

To test the utility of ReaChR to induce suprathreshold depolarization in neurons, we recorded cells under a current clamp without blockers of voltage-gated channels. We injected current through the patch pipette to maintain the membrane potentials of all cells at −65 mV; we did not correct junction potentials. In ReaChR-expressing neurons, we were able to achieve suprathreshold depolarization with blue (470 nm), red-orange (617 nm) and red light (627 nm) in response to 1 s light pulses (Fig. 3d). The amount of depolarization and the spike-delayed time were dependent on wavelength, light intensity and expression level, in addition to the membrane properties of the neurons (cell type, membrane resistance and capacitance). Cells expressing ReaChR retained normal morphological appearances and physiological membrane properties (Supplementary Fig. 5), suggesting minimal toxic effects with expression. With 617 nm light, we were able to achieve 25–45 mV depolarization (mean, 35 mV) in the expressing cells (Fig. 3e) and trigger action potentials with a 7–40 ms delay time (mean, 15 ms; Fig. 3f) when stimulated at a high light intensity (n = 14). With 627 nm light, we were able to achieve 24–44 mV depolarization (mean, 34 mV; Fig. 3e) and spike delays of 7–35 ms (mean, 17 ms; Fig. 3f) (n = 14). Blue light at 470 nm was also effective in achieving suprathreshold depolarization of ReaChR-expressing cells, with depolarization of 30–40 mV (mean, 34 mV; Fig. 3e) and a spike delay of 4–19 ms (mean, 9 ms; Fig. 3f) (n = 8). In comparison, the maximum depolarization achieved in neurons expressing hChR2H134R was 22–32 mV (mean, 26 mV; Fig. 3e), and the spike delay ranged from 7 to 25 ms (mean, 11 ms) (Fig. 3f) (n = 8). In oChIEF-expressing neurons, the amount of depolarization was 8–38 mV (mean, 26 mV; Fig. 3e), and the spike delay ranged from 1.6 to 7 ms (mean, 8 ms; Fig. 3f) when stimulated with 470 nm light (n = 5).
Figure 4 The responses of neurons expressing the different ChR variants to a 10 Hz pulsed-light stimulation train. (a) Example recordings of neurons expressing the indicated ChR variants to 10 Hz of light stimulation at 10 mW mm\(^{-2}\) of 1 ms duration. Two ten-pulse trains 250 ms apart were used to simulate two bursting episodes. Currents were injected to sustain the resting membrane potential at ~65 mV. (b) The percentage of pulses resulting in action potentials of varying light intensities and pulse durations recorded from neurons expressing the different variants. (c) Percentage of pulses resulting in extra action potentials of varying light intensities and pulse durations of the different variants. For 627 nm stimulation of ReaChR in b and c, \(n = 5–12\) for each of the conditions. For 617 nm stimulation of ReaChR in b and c, \(n = 3–11\). For 470 nm stimulation of ReaChR in b and c, \(n = 4–8\). For stimulation of hChR2H134R in b and c, \(n = 7–8\). For the stimulation of oChIEF in b and c, \(n = 5–7\) (see also Supplementary Data Set 1). The data in b and c are shown as the mean ± s.e.m. (d, e) Summaries of the mean latencies and s.d. of the light-triggered action potentials of each pulse in ReaChR-, hChR2H134R- and oChIEF-expressing neurons to the indicated wavelengths, intensities and durations. Pulses that did not trigger action potentials were not included in the analysis. For 627 nm stimulation of ReaChR in d and e, 7 and 8 cells were tested, respectively. Each data point in d and e is the mean of 2–6 and 3–6 values, respectively. For 617 nm stimulation of ReaChR in d and e, 8 and 9 cells were tested, respectively. Each data point in d and e is the mean of 2–6 and 1–6 values, respectively. For 470 nm stimulation of ReaChR in d and e, 8 cells were tested, and each data point is the mean of 2–8 values. For the stimulation of hChR2H134R in d and e, 8 cells were tested, and each data point is the mean of 2–8 values. For the stimulation of oChIEF in d and e, 6 cells were tested, and each data point is the mean of 6 values (see also Supplementary Data Set 1). The data in d and e are shown as the mean ± s.d.

Pulsed illumination at 10 Hz was an effective trigger for spiking in ReaChR-expressing neurons at 470, 617 and 627 nm (Fig. 4a). Light pulses of weak intensity and short duration often lead to loss of temporal fidelity from insufficient depolarization (Fig. 4b). Light pulses of too high an intensity lead to extra action potentials and depolarization block as a result of insufficient repolarization between light pulses, which were the main reasons for the loss in temporal fidelity that we observed (Fig. 4c). In addition to stimulation intensity and pulse duration, cell membrane properties and the expression level of ReaChR also affect the ability to trigger action potentials in response to pulsed light. Thus, one specific stimulation protocol that achieved complete fidelity in one ReaChR-expressing cell, for example, 0.5 ms of 5 mW mm\(^{-2}\) of 617 nm light or 1 ms of 10 mW mm\(^{-2}\) of 627 nm light, could fail to trigger action potentials in another cell with different membrane properties and expression levels. We observed this same phenomenon with stimulation at 5 Hz (Supplementary Fig. 6).

With regard to the fidelity of spike generation in neurons with ChR variants other than ReaChR, we observed lower firing reliabilities with hChR2H134R-expressing cells in response to 470 nm light. This variant introduced a high amount of channel desensitization that led to subthreshold depolarization with light pulses toward the end of a pulse train (Fig. 4a). As the overall amount of depolarization that we observed with hChR2H134R was lower, extra spiking was rare. Cells expressing oChIEF had the highest temporal precision, as measured by the percentage of pulses achieving suprathreshold depolarization, the shortest spike delay and a variability in spike latency (Fig. 4b–e), and this higher fidelity was sustained at higher stimulation frequencies (Supplementary Fig. 6). Yet even with oChIEF, we observed...
**Figure 5** In vivo expression and utilization of ReaChR for cortical control. (a) Schematic of vM1 stimulation and recording. ReaChR was expressed in vM1 and visualized by Citrine fluorescence (green). Scale bar, 500 µm. (b) ReaChR-expressing neurons in vM1. Scale bar, 10 µm. (c) Photostimulated spikes in a ReaChR-expressing neuron recorded in vivo during anesthesia (recording depth, 410 µm). Single 2 ms pulses of 617 nm light (orange bar) evoked one or more spikes (indicated by asterisks; n = 12 pulses at 2 Hz). Arrows indicate low-amplitude artifacts associated with the light switching on and off. Vertical scale bar, 100 µV; horizontal scale bar, 2 ms. (e) Spike interval histogram and waveforms (inset) of the same unit demonstrating the unit as a single neuron. Vertical scale bar, 100 µV, horizontal scale bar, 0.5 ms. (f) A ReaChR-expressing neuron activated with 2 ms pulses of 617 nm light at 5, 10, 20 and 30 Hz (12 pulses). Top, voltage trace during a train of 12 pulses (vertical scale bar, 100 µV). Middle, rasters of spikes during six trains. Bottom, peristimulus time histogram of spikes across all trains. (g–i) Number of spikes per pulse (normalized to the maximum number of spikes evoked by the light pulses of the indicated duration; g) and probability of one or more evoked spikes (h) across a population of ReaChR-expressing neurons (n = 8) as a function of pulse duration (2–20 ms; pulse rate, 2 Hz). (j) Probability of one or more evoked spikes (h) and latency to the first spike (i) in neurons as a function of stimulus rate (1–30 Hz; pulse duration, 5 ms). (k) Schematic of in vivo activation of vM1 with ReaChR (green) through intact skin and bone of awake mice. Vibrissae movements were measured with high-speed video. (l) Traces of vibrissae movements in response to single 100 ms pulses (orange arrows and bar) of 617 nm light emitted by an LED placed 10 mm above the skin. Increasing values denote vibrissae protraction. Vertical scale bar, 40°; horizontal scale bar, 1 s. (m) Absolute movement amplitudes evoked by 100 ms pulses of 470, 617 and 655 nm light through intact skin in ReaChR-expressing mice (n = 3 mice, ten stimulus repetitions per condition per mouse). Additionally, three mice injected with viral vehicle solution (mock control) were stimulated through the skin with 617 nm light (black). (n) Boxplots of movements during the first second after vM1 photoactivation through the intact skull of mock-transduced (veh, black; n = 3 mice; 617 nm illumination) and ReaChR-expressing mice (n = 3 mice; 470–655 nm wavelengths). Vertical lines indicate the data range, boxes indicate the 25th to 75th percentile ranges, and the central lines mark the median. ***P < 0.001, one-sided Kolmogorov-Smirnov test. The light intensity used in a–n was 100 mW.

extra action potentials after strong depolarization in cells with high expression. The spike latency and variability were similar between ReaChR and hChR2H134R, with a higher variability in the responses to stimulation pulses present later in the pulse train.

**Excitation of ReaChR-expressing neurons in vivo**

As red light can penetrate mammalian tissues in vivo with less attenuation compared to blue or green light\(^1\), we used ReaChR to stimulate deep brain structures in vivo. We targeted two areas for in vivo stimulation: vM1, which is involved in the control and execution of vibrissa motion\(^2\), and the facial motor nucleus of the seventh cranial nerve, whose motoneurons innervate the muscles that are responsible for vibrissa movements. We incorporated ReaChR-Citrine into a recombinant adeno-associated virus (rAAV) with a hSyn promoter and injected it into either vM1 or the facial motor nucleus of individual mice.

We confirmed ReaChR expression in vM1 3 weeks after injection. We head fixed the mice and monitored vibrissae movements with high-speed video either during light anesthesia with 1.0–1.5% (vol/vol) isoflurane or while the mice were awake (Fig. 5). For electrophysiological recordings, we removed the bone over vM1, mapped the zone of infection with epifluorescence imaging of the cortical surface and positioned a 617 nm LED 10 mm above the surface of the brain (Fig. 5a,b). We used short pulses of light, 1–20 ms in duration, to activate ReaChR-expressing neurons (Fig. 5c) and evoke spikes, as recorded extracellularly (Fig. 5d–f). ReaChR-expressing vM1 neurons
were reliably photoactivated at short latencies and with low temporal jitter (Fig. 5d). The evoked activity of a representative cortical single unit is shown in Figure 5d–f. This unit, which probably expressed ReaChR, fired one or more spikes in response to 2 ms pulses with submillisecond jitter and a short, 2.4 ms spike latency. The unit was reliably activated with trains of light pulses that ranged from 5 to 30 Hz. Increasing the rate of optical activation to greater than 20 Hz resulted in a larger number of extra spikes per pulse (Fig. 5f). We observed a monotonic increase in the number of evoked spikes and in the spiking probability as a function of stimulus duration (n = 8 single units; Fig. 5g,h). The spiking probability was above 0.95 when the stimulus duration was 5 ms or longer (Fig. 5h). When the stimulus rate exceeded 10 Hz, the population response increased (P = 0.002, one-sided Kolmogorov-Smirnov test; Fig. 5i) and the latency to the first spike decreased (P = 0.01, one-sided Kolmogorov-Smirnov test; Fig. 5j); these effects probably result from the slow closing kinetics of ReaChR.

Consistent with in vivo neural activation of ReaChR-expressing neurons, we found that vibrissa movements could be elicited by LED illumination in awake mice (Fig. 5k–n). Removal of skin and bone was not required to evoke these movements, and placing the stimulating LEDs 10 mm above the fur line was sufficient in all cases (Fig. 5k). Movements were elicited with both blue (470 nm) and red (617 nm and 655 nm) illumination. Evoked movements were characteristic of those observed with electrical stimulation of vM1, showing both a rapid component and a sustained period, albeit with unpredictable duration, of rhythmic whisking that outlasted the stimulus (Fig. 5l,m).

Our in vivo cortical experiment demonstrated stronger evoked movement responses to 617 nm as compared to 470 nm light (P < 0.0001, one-sided Kolmogorov-Smirnov test; Fig. 5m,n), which is consistent with the larger responses to orange-red light that we observed in cultured HEK293 cells (Fig. 1f and Supplementary Fig. 7) and the greater attenuation of blue compared to red light in mammalian tissue (Fig. 5m). Because we were able to evoke movements with 470 nm light in ReaChR-expressing mice, we tested whether movement could be evoked non-invasively with a standard blue light–activated ChR variant. We thus expressed hChR2134R-eYFP in vM1 with an identical AAV vector and viral titer. Vibrissa movements could be evoked in mice expressing hChR2134R in vM1 with 470 nm light illumination (n = 3), but these movements were more infrequent, notably smaller.
and shorter lasting compared to the movements evoked with 617 nm light in ReaChR-expressing mice (Supplementary Fig. 7c–f).

The durations and amplitudes of the movements evoked by vM1 stimulation in ReaChR-expressing mice were unpredictable (Fig. 5i), mirroring the results obtained with electrical stimulation in awake mice but nonetheless complicating comparisons of different stimulation parameters. As a more stringent test of the sensitivity of ReaChR to different wavelengths in vivo, we targeted ReaChR to the mystacial motoneurons in the facial motor nucleus of the brainstem that directly control vibrissa movements (Fig. 6a, b). It has been shown previously that in deeply anaesthetized mice, direct electrical stimulation of these neurons can reliably drive vibrissa movements, producing a relatively noise-free and controlled behavioral output. In mice, the facial motor nucleus is located at a depth of up to 6 mm in the ventral part of the brainstem. We delivered light at 470, 530, 591, 617, 627 and 655 nm wavelengths in a non-invasive manner by placing an LED at the opening of the external auditory canal, thus illuminating the brainstem at a distance of 8–10 mm from the midline through intact tissue and bone (Fig. 6c).

The external ear canal provides a convenient anchor point for non-invasive LED placement, bypasses fur and provides an opportunity to limit photoactivation to one side of the brainstem. We typically flashed the LEDs for periods of 100 ms at 1 Hz with a 1–100 mW light output; we found no visual indications of any heat-related damage to the outer ear tissue. Activation of ReaChR-expressing neurons elicited highly reproducible and temporally precise vibrissa movements, which is consistent with the direct activation of motoneurons (Fig. 6, d, e and Supplementary Video 1). Although a topographic organization of the facial muscle representation within the facial motor nucleus has been documented, the exact distribution of all the motoneurons innervating muscles that are involved in vibrissa control is not known. Both protraction and retraction of the vibrissae are actively controlled by multiple extrinsic and intrinsic muscles.

We found no consistent pattern between the intrafacial motor nucleus locations of infected motoneurons and the evoked movement trajectories, and in several mice, both net protraction and retraction could be elicited by adjusting the light intensity appropriately (Fig. 6, d, e). As an example, Figure 6d shows evoked movements in a mouse in which light stimulation led to the retraction of all vibrissae independently of light intensity (10–100 mW light output, 100 ms pulses; Supplementary Video 2). Evoked retractions were three times larger when ReaChR was activated at 617 nm as compared to 470 nm (40° compared to 13°, respectively, at maximum amplitude). Similarly, light stimulation of a different mouse (Fig. 6e) produced sixfold larger movements at 617 nm compared to 470 nm (19° compared to 3°, respectively, at maximum amplitude). We observed this pattern of stronger activation at 617 nm compared to 470 nm in all ReaChR-expressing mice (Fig. 6f).

Vibrissa movements in ReaChR-expressing mice could be evoked across a broad range of wavelengths, which is consistent with our in vitro results. The largest movements were evoked at 617 nm, followed by 627 nm and 470 nm. Smaller vibrissa movements were also evoked with 530, 591 and 655 nm illumination (Fig. 6g). We confirmed that these movements resulted from photoactivation of ReaChR and not LED-related heating of neural tissue or visually evoked responses by the fact that we did not observe any movement in a cohort of mice expressing the blue light–activated hChR2H134R channel in the facial motor nucleus (n = 15; Fig. 6g and Supplementary Fig. 7g–l) and by recording single units in the brainstem of ReaChR-expressing mice that reliably responded with short latencies and submillisecond temporal jitter to light stimulation (Fig. 6h).

The variability in whisking between individual mice in response to illumination of the facial motor nucleus was great (Fig. 6f). This was probably caused by variations in the exact locations of ReaChR-expressing motoneurons within the facial motor nucleus, which is ~1 mm in width, and/or high expression levels in individual motoneurons that resulted in very low spiking thresholds. In principle, this variability could also be caused by direct stimulation of the facial motor nerve. We confirmed that motoneuron axons were labeled with ReaChR-Citrine and that vibrissa movements could also be evoked by direct illumination of the peripheral motor nerve through the skin of the lower cheek. These movements, however, were smaller and of shorter duration than those evoked through the ear (Supplementary Fig. 7a). In addition, movements were not evoked when stimulating through the ear contralateral to the side of ReaChR expression (617 nm, 100 mW light output), which shows that the lateralization of optogenetically evoked brainstem activity can be controlled with through-skull stimulation (Supplementary Fig. 7b).

### DISCUSSION

The optimal wavelength range for optical imaging into mammalian tissue is near infrared (600–1,300 nm), where light scattering decreases with increasing wavelength and absorption by endogenous chromophores is reduced, yet absorption by water is still negligible. This paves the way for deeper penetration of light into tissue with reduced attenuation. Optogenetic tools to manipulate neuronal activity should ideally be excited by near-infrared light. Current development of ChRs has produced many new variants that are maximally activated by blue and green light, such as ChR2H134R, ChETA, TC, SFOD156A, ChD, oChEF, oChIEF, CatCh and ChRGR (8, 10, 17, 18, 28, 29). Many of these variants have improved properties regarding kinetics, expression and amounts of desensitization compared with native ChR2. However, there has been little progress in the development of red-shifted ChRs.

In this study, we engineered two red-shifted ChR variants, VCOMET and ReaChR, that had strong membrane expression in mammalian cells. The ReaChR variant responded strongly to red light, with a secondary steady-state spectral peak at 630 nm in addition to a primary steady-state spectral peak at ~540 nm. The mechanism for this shift involves the L171I mutation in VCOMET. Past work attributed this position to the red shift of the response spectrum of VChR1 (Leu126) relative to ChR2 (Ile131), which implies that this L171I modification should lead to a blue shift in the response spectrum. However, this idea is based on the charge distribution of all-trans retinal in the binding pocket of bacteriorhodopsin in the dark state. In bacteriorhodopsin, some transient intermediate photocycle states have absorption spectra above 600 nm, whereas the initial activation of the protein has a λmax value of ~570 nm. It seems that the main effect of the L171I mutation in ReaChR is to extend the lifetime of the red-shifted transient states and reduce the desensitization of the transient state, leading to a greater photocurrent to red light. In support of this hypothesis, we found differential spectral peaks with the transient and steady-state responses of C-VChR1 and VCOMET, with the transient states having a λmax value of ~550–590 nm and the steady-state having a λmax value of ~530 nm (Fig. 1f and Supplementary Fig. 2b).

The initially described red-shifted ChR, VChR1, does not express well in mammalian cells and traffic poorly to the membrane, limiting its utility for in vivo applications. The recently published variant C1V1 and its derivatives have been reported to have improved expression, membrane trafficking and kinetic properties compared to VChR1 (ref. 11). However, in our tests, C1V1 and its variants were still limited in their membrane trafficking and expression compared to VCOMET and ReaChR, in addition to having slower channel kinetics.
(Figs. 1 and 2). The most red-shifted C1V1 derivative, C1V1E122T, has a spectral peak at ~600 nm and slower channel kinetics and limited expression compared with ReaChR (Figs. 1–3). The E122T mutation variant also has a reduced photocurrent, as has been previously reported, that may result from the position of this residue within the putative channel pore of ChR, as predicted from the crystal structure of ChERS. Thus, the reduction of C1V1E122T photocurrents and slow kinetics negate the spectral advantage that the E122T mutation provides.

In contrast, ReaChR is strongly expressed, consistently traffics to the membrane and can be used to reliably trigger temporally precise spiking. Although ReaChR has an improved spectral response to light at wavelengths greater than 600 nm, it still suffers slow kinetics in its channel closure rate compared to variants such as ChR2, ChIEF, ChD and ChETA. In the blue light–activated ChR variants, single point mutations that improve the channel kinetics also reduce the channel’s light sensitivity. The other known ChR mutation that increased the channel kinetics, E123T of ChR2, E162T of C1V1 and E163T of VCOMET, does so at the expense of reduced light sensitivity and reduced photocurrents. In the context of ReaChR, this E-to-t mutation increases the channel kinetics (off rate <30 ms) and surpasses C1V1E122T in terms of kinetics but blue shifts the spectral peak to 550 nm (Supplementary Fig. 8) and increases the desensitization of the channel. We did not find incorporation of this mutation useful in the current development of ReaChR. Important future goals in developing red-shifted ChRs should include improving light sensitivities and faster kinetics simultaneously and reducing the activation of these variants by light below 500 nm to allow for the independent manipulation of two populations of neurons with two different wavelengths of light.

We were able to exploit the spectral advantages of ReaChR to achieve efficient activation of expressing neurons through fur, skin and intact bone of adult mice with red-orange (617 nm), red (627 nm) and even far-red (655 nm) light (Figs. 5 and 6). This enables new in vivo applications of transcranial and deep-brain stimulation with optogenetics. Transcranial stimulation is essential for many chronic studies, as cranial windows can lead to activated microglia and astrocytes as part of an inflammatory response that in turn alters neuronal physiology and plasticity, as well as pial vasculature. Alternatives to our approach for non-invasive transcranial stimulation include clinically approved treatments for several neurological disorders and stroke, such as electroconvulsive therapy, repetitive transcranial magnetic stimulation and transcranial direct-current stimulation. These techniques do not, however, have the spatial and cellular specificity that can be achieved through optogenetics. Thus, optogenetic approaches using red-shifted ChRs, such as ReaChR, have the potential to improve the efficiency of transcranial stimulation to treat neurological disorders.

In the present study, we introduced the ChR ReaChR through injections of an engineered virus directly into the region of interest. In future work, it may be possible to label specific populations of neurons by retrograde transport of ReaChR from a known target. For example, pools of motoneurons in the brainstem or the spinal cord could be labeled by injection into specific muscles rather than into motor nuclei themselves. Such an approach would substantially reduce the invasiveness of an optogenetic strategy for future therapeutic use. Further, one could label opposing muscle groups with short- or long-wavelength ChRs to permit differential optical control of opposing muscle groups. The weak absorption of short wavelengths by ReaChR will not compromise this strategy if the anatomically deeper motor pools are labeled with ReaChR. ReaChR may also facilitate neurotrophic treatment and neuroprosthetic control through transcranial and possibly transvertebral stimulation.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Nucleotide sequences for VCOMET and ReaChR have been deposited in the NCBI GenBank nucleotide database under accession codes KF448070 and KF448069, respectively.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

J.Y.L. was funded by the Foundation of Research, Science and Technology New Zealand, and P.M.K. was supported by a long-term fellowship from the Human Frontier Science Program (HFSP). The project was supported by grants to R.Y.T. from the US National Institutes of Health (NS027177) and the Howard Hughes Medical Institute and grants to D.K. from the US National Institutes of Health (DA029706, OD006831 and NS058668). We thank P. Tsai for suggesting the name ReaChR. We thank K. Deisseroth (Stanford University) for the hChR2(334G)-eYFP construct, K. Svoboda (Howard Hughes Medical Institute Janelia Farm Research Campus) for the CAG promoter vector, E. Boyden (Massachusetts Institute of Technology) for the lentiviral vector, L. Tian (Howard Hughes Medical Institute Janelia Farm Research Campus) for the AAV2 vector and D. Troncoso (École Polytechnique Fédérale De Lausanne) for the pMPPX2 and pMDD2G lentivirus packaging vectors. AAV2-ReaChR-Citrine and pLenti-ReaChR-Citrine constructs can be requested from http://tsienlab.ucsd.edu/Samples.htm.

AUTHOR CONTRIBUTIONS

J.Y.L. designed and developed ReaChR and conducted and analyzed the experiments in HEK293 and neuron cultures. P.M.K. and A.M. conducted and analyzed the in vivo experiments. D.K. and R.Y.T. contributed to the design and analysis of the experiments. All authors contributed to the writing and discussions of the manuscripts.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Molecular cloning and transgene expression. ChR chimeras and point mutants were generated by overlapping PCR with Phusion PCR mix (New England Biolabs, Ipswich, MA, USA). Genes coding mammalian codon-optimized VChR1, VChR2 and ChIEF were synthesized separately according to the published peptide sequence (GenScript, Piscataway, NJ). The gene encoding hChR2H134R-eYFP was a generous gift from K. Deisseroth (Stanford University). The gene and peptide sequences of ReaChR and C1V1L1227 are shown in Supplementary Figures 9 and 10. Standard digestion and ligation techniques were used to insert the mutant into the expression vector. For experiments with HEK293 cells, the ChR variants were expressed with the pcDNA3.1 vector under the cytomegalovirus (CMV) promoter and fused to the YFP variant Citrine1145 with an in-frame XhoI restriction site (LeuGlu). Cells were transfected with Eugene HD (Roche, Basel, Switzerland). In the experiments measuring membrane expression of hChR2, ChIEF, VChR1, C-VChR1 and VCOMET in primary cultured neurons, ChR-Chrine fusions (with an in-frame Clal restriction site) were expressed under the CAG promoter with a WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) sequence inserted after the stop codon of the ChR-Citrine coding sequence (plasmid courtesy of L. Tian, Howard Hughes Medical Institute Janelia Farm Research Campus). In the experiments measuring membrane expression of C1V1L1227, C1V1L1227-TS and ReaChR, ChR-Chrine fusions (with an in-frame XhoI restriction site) were expressed under the hSyn promoter with a WPRE sequence inserted after the stop codon of the ChR-Chrine coding sequence in an AAV2 vector (plasmid courtesy of L. Tian, Howard Hughes Medical Institute Janelia Farm Research Campus). The resulting constructs (at the same DNA concentration) were electroporated into the neurons before plating (Lonza, Walkersville, MD). For the stimulation of cultured primary hippocampal neurons, red, light ReaChR-Citrine was introduced with recombinant lentivirus.

Lentivirus and rAAV production. The genes encoding hChR2H134R-eYFP, C1V1L1227-TS, Citrine, oChIEF-Citrine and ReaChR-Citrine were subcloned into a generation-two lentiviral construct with the hSyn promoter. The lentivirus was made according to the protocols at http://vectorcore.salk.edu/protocols.php with minor modifications. In brief, 293A cells (Life Technologies, Carlsbad, CA) were grown to 85% confluence, and transfer vectors containing ChR–fluorescent protein, pSPAX2 and pMD2.G (gifts from D. Trono, Ecole Polytechnique Fédérale De Lausanne) were transfected with calcium phosphate (Clontech, Mountainview, CA). Virus particles were harvested from serum-free medium and concentrated with a 20% sucrose cushion with ultracentrifugation. The titer of the lentivirus was estimated with the Lentivirus Rapid Quantitation Kit (Cell Biolabs Inc., San Diego, CA) in parallel just before each infection. The lentiviral vector was a gift from E. Boyden (Massachusetts Institute of Technology). rAAV with serotype 8 containing hChR2H134R-eYFP and ReaChR-Citrine was produced and purified according to the protocols at http://vectorcore.salk.edu/protocols.php by transfecting an AAV2 inverted terminal repeat (ITR) vector (gift from L. Tian, Howard Hughes Medical Institute Janelia Farm Research Campus) containing ReaChR-Citrine and the helper plasmids XX6-80 and XR8 (National Vector Biorepository) into 293A cells. rAAV2/8 (recombinant AAV serotype 8 packaged with AAV2 ITR) were released from the cells by freeze thawing and purified with iodixanol gradient purification. The virus was further concentrated using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) with a 50 kDa cutoff. The rAAV titer was measured by the Salk Vectorcore service with quantitative PCR and was estimated to be 3 × 1017 GC ml−1.

Measurement of relative efficiencies of ChR membrane expression. Relative levels of plasma membrane expression of ChR-Chrine fusions in HEK293 and cultured cortical neurons were measured by imaging on a Zeiss Live 5 Confocal microscope with Zen software (Thorwood, NY). Transfections and measurements of expression were performed with the same concentrations of DNA electroporated into the neurons. The transfected cells were imaged with the same setting for comparison. The measurements of expression were performed on all cells with detectable visible fluorescence and normal cell morphology non-discriminatively in the culture dish to ensure fair comparisons, typically starting from one end of the dish and moving to the other. Membrane expressions were measured by taking the mean fluorescence intensities of the membrane and the cytosol of a single in-focus imaging plane with ImageJ software. In the experiments in which ChR photocurrents were normalized to the membrane fluorescence, images were acquired with an electron multiplying charge-coupled device (EMCCD) camera (Photometric, Tucson, AZ) at a 512 × 512 pixel resolution with Slidebook (Intelligent Imaging Innovations, Inc., Denver, CO) in epifluorescence mode before electrophysiological recordings.

Cell culture, electrophysiological recordings and stimulation of cultured cells. Characterization of spectral responses, reversal potentials, kinetics and membrane trafficking of C-VChR1, C1V1, C1V1L1227, VCOMET, ReaChR, CatCh, oChIEF and oChEF were done with whole-cell patch-clamping on 293A cells 2 d after transfection. HEK293 cells, which have a smaller size, high membrane resistance and minimal expression of voltage-gated ion channels, were used for these characterizations to reduce the known voltage-clamping errors in neurons.39 All recordings were performed with an extracellular solution containing 118 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Na3-ATP and 0.15 mM Na2-GTP (pH 7.25, 310 mOsm) and an intracellular solution containing 110 mM Cs-methanesulfonate, 30 mM tetraethylammonium chloride, 10 mM ethylene glycol tetracetic acid (EGTA), 10 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 2 mM Mg-ATP and 0.15 mM Na2-GTP (pH 7.25, 285 mM). Site-directed mutagenesis at (670–75%) was used in the kinetics and reversal potential measurements in HEK293 cells. HEK293 cells with low membrane resistance (<200 MΩ) were often discarded from analysis and testing because of the inaccuracy of voltage clamping in these conditions. These cells are typically found in clusters. All chemicals were acquired from Sigma-Aldrich (St. Louis, MO). Electrophysiological characterization of ChR in neurons were performed on in vitro day 14–25 neurons extracted from postnatal day 2 neonatal Sprague-Dawley rat pups (both sexes were used, and the neurons were pooled together for the culture) after at least 9 d of infection with lentivirus. For the measurement of photocurrent amplitudes in neurons, the recordings were performed with Cs-methanesulfonate–based intracellular solution to block the potassium channel, and 1 µM tetrodotoxin and 100 µM Cd2+ were included in the extracellular solution to block voltage-gated sodium and calcium channels, respectively. Also included in the extracellular solution were 10 µM NBQX (6-nitro-2,3-dioxo-1,4-dihydrobenzo[f]quinoline-7-sulfonamide), 10 µM bicuculline and 20 µM 3-aminopropylindole (AMAP5). Recordings were performed in current-clamp mode with the current injected to sustain a −65 mV membrane potential at rest. In pulsed stimulation experiments, cells that did not reach suprathreshold depolarization with 20 ms of 50 mW µm−2 light pulses were excluded from the analysis. For experiments of neurons with 10 Hz stimulation under the current clamp, ReaChR–Chrine– and oChIEF–Citrine–expressing cells were selected on the basis of bright-field morphology without visualization with fluorescence. High membrane eYFP fluorescence was used to select hChR2H134R-eYFP cells for recordings, as most cells with medium to low levels of fluorescence did not reach the threshold when stimulated with light. Recordings were performed with the Axopatch 200B patch clamp amplifier (Molecular Devices, Union City, CA) and acquired through Digidata 1320 (Molecular Devices) to a personal computer (Dell, Austin, TX) running pClamp 9.2 or pClamp 10 software (Molecular Devices). Analyses were conducted with AxographX (Sydney, New South Wales, Australia). Junction potentials were not corrected. Most light stimulation was provided by a monochromator xenon light source (Polychrome IV, TILL Photonics, Victor, NY) with 15 nm half width.

doi:10.1038/nn.3502
The shuddering and intensity of the light were controlled with a custom-fitted 25 mm mechanical shutter (Vincent Associates, Rochester, NY) and a neutral density wheel (Thorlab, Newton, NJ). For the spectral response measurements and experiments in which the light intensity was not described, cells were stimulated with ~5.1 x 10^16 photon mm^-2 s^-1 across the light spectra. The photon flux of the monochromator at each wavelength was measured with a power meter and integrating sphere (Newport Corporation, Irvine, CA) placed on the objective, and the photon flux was corrected with the neutral density wheel to give the same flux. The response spectra of the ChRs were measured with a 1 s light pulse from 650 to 410 nm of the same photon flux at 20 nm intervals, with the maximum response taken as the maximal photocurrent obtained within the 1 s pulse at the described wavelength, and the steady-state response was measured between 0.95–1.00 s after the onset of the stimulation light pulse. For most experiments, 410 nm light was delivered 20 s after the initial stimulation to recondition the protein before the delivery of the next testing pulse 20 s later. Stimulation of neurons under the current clamp was conducted with 470 nm (SR-05-B0040), 617 nm (SR-05-H2070) or 627 nm (SR-05-D2050) LED illumination (Luxeonstar, Brantford, Ontario, Canada). The LEDs were connected to a computer-controlled current source (Mightex, Pleasanton, CA) coupled to a quartz fiber into the microscope.

Expression and stimulation of ChR-expressing neurons in vivo. Recombinant AAV2/8-hSyn-RecaChR-Citrine or AAV2/8-hSyn-hChR2H134R-eYFP virus was injected stereotaxically into the vibrissa motor cortex (vM1) or the facial motor nucleus in the brainstem of C57BL/6 female mice using a nanoliter injector (Nanoject II, Drummond, Broomall, PA) under isoflurane anesthesia (2% vol/vol). The injection coordinates for vM1 were 1 mm posterior, 1 mm lateral and 0.8 mm ventral, and the coordinates for the facial motor nucleus were 4.8–5.6 mm posterior, 1.2–1.4 mm medial and 5.3–6.0 mm ventral to the bregma. The skin was retracted to expose the injection coordinates, and a single 0.3 mm diameter hole was drilled for each injection site. Suspension of virus was injected in 50 nl aliquots every 5 min (total volume, 500 µl) in vM1 and in five 12 µl aliquots (total volume, 60 µl) every 5 min in the facial motor nucleus. The injection hole was filled with sterile saline and topped with a thin layer of antibiotic ointment after the injection pipette was withdrawn. The skin was sutured back in place, and the mouse was left to recover for the viral incubation period. After a waiting period of 4 weeks, the mice were prepared for in vivo experiments. The injections of virus were done on 3-month-old mice, and the tests were performed 3–6 weeks later. All mice were housed on a normal 12 h light, 12 h dark cycle in groups of between 3 and 5 mice per cage, and all mice were tested during the light cycle.

One day before the experiments, a single 10 mm incision was made perpendicularly to the midline and caudal to lambda, the skull was exposed, and a titanium alloy head bar was attached to the skull using cyanoacrylate gel and a layer of dental cement (C&B-Metabond, Parkell Inc., Edgewood, NY) during isoflurane anesthesia. The skin rostral to the lambda was not damaged, and the skull under it was neither exposed nor covered by any amount of cement, except for during the electrophysiological recordings in vM1, for which a new craniotomy was opened where the virus had been injected previously. Over the next several daysicyme were head fixed either when awake or during isoflurane anesthesia (0.5–2%), as indicated. RecaChR was activated in vivo by LEDs with wavelengths of 470 nm (LXML-PB01-0030), 530 nm (LXML-PM01-0100), 591 nm (LXM2-PL01-0000), 617 nm (LXM2-PH01-0070), 627 nm (LXM2-PD01-0050) and 655 nm (LXM3-PD01-0260), Luxeonstar, Brantford, Ontario, Canada) connected to a computer-controlled current source (Mightex, Pleasanton, CA).

Single units in vM1 and the facial motor nucleus were recorded extracellularly with 9–12 MΩ tungsten electrodes (UEWLCESML16G, FHC Inc, Bowdoin, ME), buffered, preamplified (Alpha Omega Co., Alpharetta, GA) and acquired onto a personal computer (PCIe-6361, National Instruments, Austin, TX) using custom-written software for Matlab (MathWorks, Natick, MA). Spikes were sorted using the Chronux library in Matlab (http://chronux.org). Vibrissa movements were monitored with high-speed video and tracked offline using the WhiskerTracker software (code available at https://github.com/pmnutten/whiskertracker). After in vivo experiments, the mice were perfused with 4% paraformaldehyde, the brains were extracted, and ReaChR expression was confirmed with epifluorescence imaging and localized histologically using a fluorescent Nissl counterstain (NeuroTrace N21479, Life Technologies, Carlsbad, CA). The bone overlying the injection sites was inspected after perfusions and was invariably found to have regrown entirely. In most mice, the bone that had regrown after viral injections appeared thickened. Only mice with ChR expression in the correct regions identified with postmortem histochemical analysis were included in the analysis of spiking and vibrissae movement. Although the experimenter was not blinded to group status, the measurement was automated, so blinding status would not affect the outcome of the experiment. All animal procedures, including the extraction of primary neurons, were approved by the UCSD Institutional Animal Care and Use Committee.

Statistical analyses. Comparisons of the properties of ChR variants in culture were done by nonparametric Kruskal-Wallis test with post-hoc Dunn’s multiple comparison tests between all possible pairs. Only the P values for the Kruskal-Wallis tests are shown. The statistical analyses were done with Graphpad Prism 5.0 (San Diego, CA). The comparisons in vivo were conducted with nonparametric one-sided Kolmogorov-Smirnov tests in Matlab. All values are presented as the mean ± s.e.m. All data in the graphs are presented as the mean ± s.e.m., with the exception of the 10 Hz stimulation experiments and spike latency analysis, in which the s.d. is shown to illustrate the variability. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications. Most analyses in cultured cells were summarized from multiple transfection or transduction experiments. The full results of the statistical analyses are available in Supplementary Data Set 1.