Supplementary Information

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

John Y. Lin, Per Magne Knutsen, Arnaud Muller, David Kleinfeld and Roger Y. Tsien

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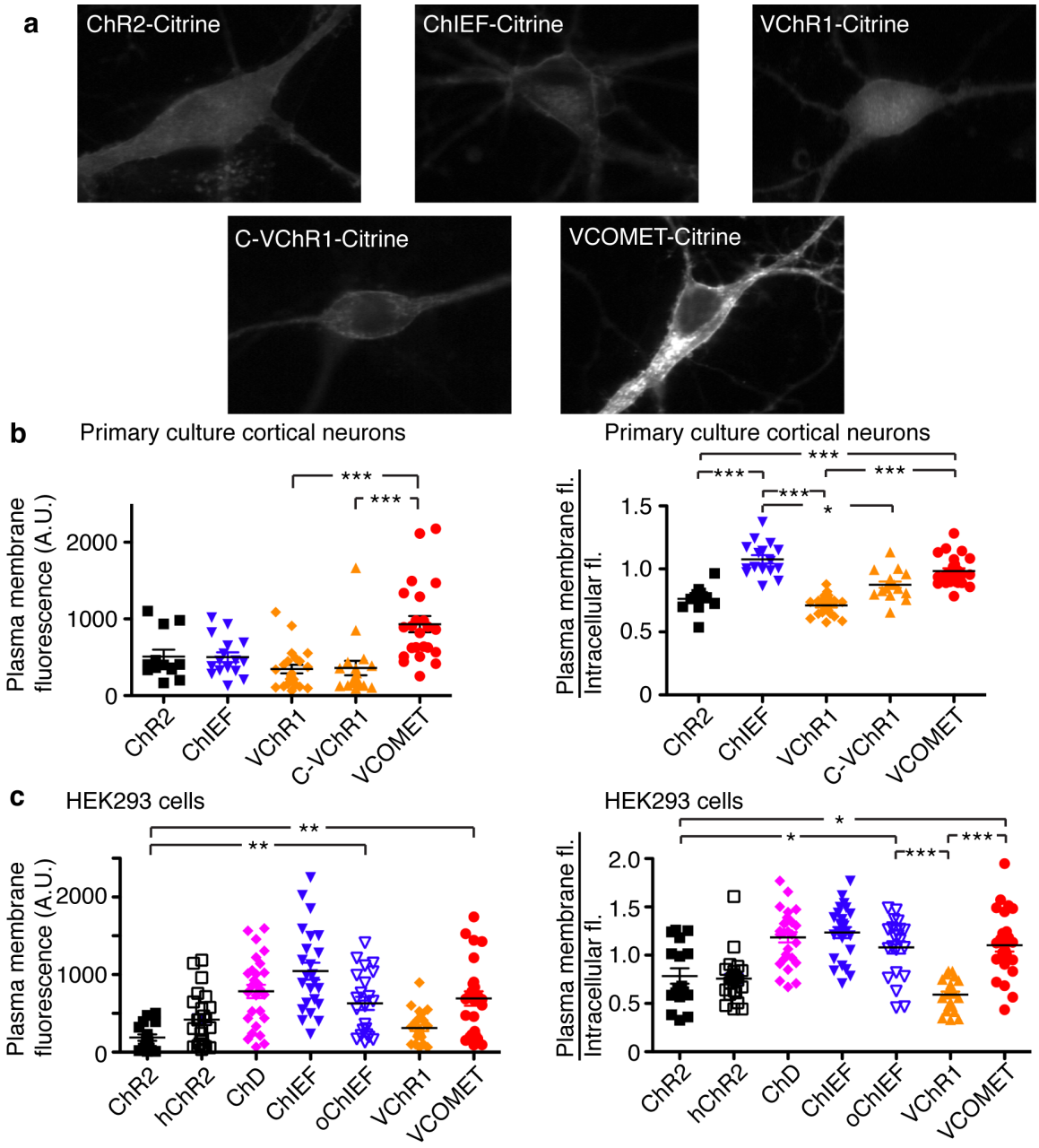

Supplementary Figure 1. Comparison of membrane trafficking and expression level of channelrhodopsin variants in primary cultured cortical neurons and HEK293 cells. (a) Representative fluorescent images of ChR2-Citrine, ChIEF-Citrine, VChR1-Citrine, C-VChR1-Citrine and VCOMET-Citrine expressed in primary cortical cultured neurons. The *ChR2* and *ChIEF* coding sequences are not mammalian codon-optimized. **(b)** Quantification of the mean plasma membrane fluorescence (left) and plasma

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membrane / cytosol fluorescence ratio (right) of the different variants in primary cultured cortical neurons. **(c)** Identical analysis of the different variants in HEK293 cells. * indicates 0.05 level of significance, ** indicates 0.01 level of significance and *** indicates 0.001 level of significance. Statistical tests were conducted with Kruskal-Wallis test with post-hoc Dunn's multiple comparison tests between all possible pairs. For **(b)**, $H = 30.58$, $k = 5$, $P < 0.0001$ for the left panel and $H = 63.90$, $k = 5$, $P < 0.0001$ for the right panel. For **(c)**, $H = 53.94$, $k = 7$, $P < 0.0001$ for the left panel and $H = 70.78$, $k = 7$, $P < 0.0001$ for the right panel. In **(b)**, only significant differences compared to ChIEF and VCOMET from Dunn's tests were indicated. In **(c)**, only significant differences compared to oChIEF and VCOMET from Dunn's tests were indicated on the graphs. Graphs are shown as mean ± S.E.M.

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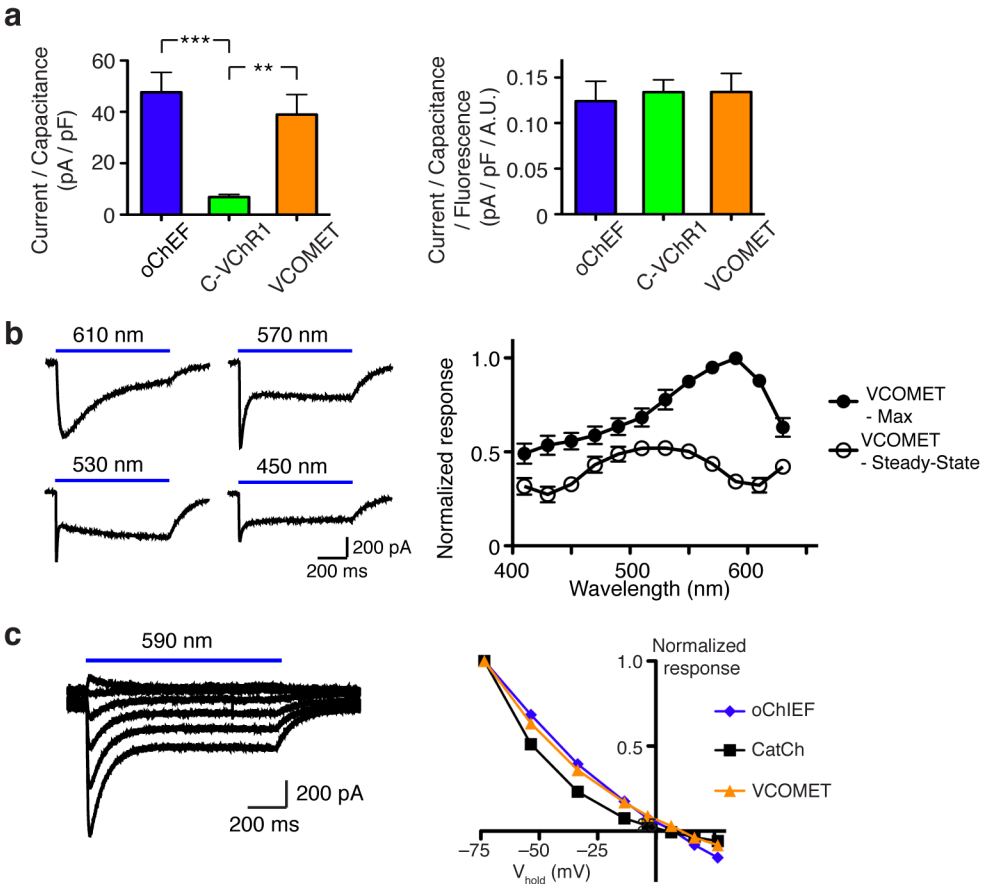

Supplementary Figure 2. Properties of VCOMET. (a) Comparison of mean photocurrent amplitudes of mammalian codon-optimized ChEF (oChEF), C-VChR1 and VCOMET without (left) and with (right) adjustment to membrane fluorescence recorded in HEK293 cells. **(b)** Representative electrophysiological traces of VCOMET to light stimulation of different wavelength (left) and the response spectrum (right). **(c)** Representative electrophysiological traces of VCOMET in response to 570 nm light at different holding potentials in voltage-clamp (left) and the I-V relationship of mammalian codon-optimized ChIEF (oChIEF), CatCh and VCOMET (right). Photocurrent amplitudes for VCOMET, C-VChR1, oChEF, oChIEF, ReaChR, C1V1 and C1V1(E122T) were compared with Kruskal-Wallis test followed by Dunn's multiple comparison tests for all possible pairs ($H = 48.02$, $k = 7$, $P < 0.0001$). Photocurrent amplitudes normalized to membrane fluorescence for oChEF, C-VChR1 and VCOMET were compared with Kruskal-Wallis test ($H = 0.9482$, $k = 3$, $P = 0.6225$). Graphs are shown as mean ± S.E.M. ** indicates 0.01 level of significance and *** indicates 0.001

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level of significance. For right panel of **(a)**, the n for oChEF, C-VChR1 and VCOMET are 12, 8 and 10, respectively.

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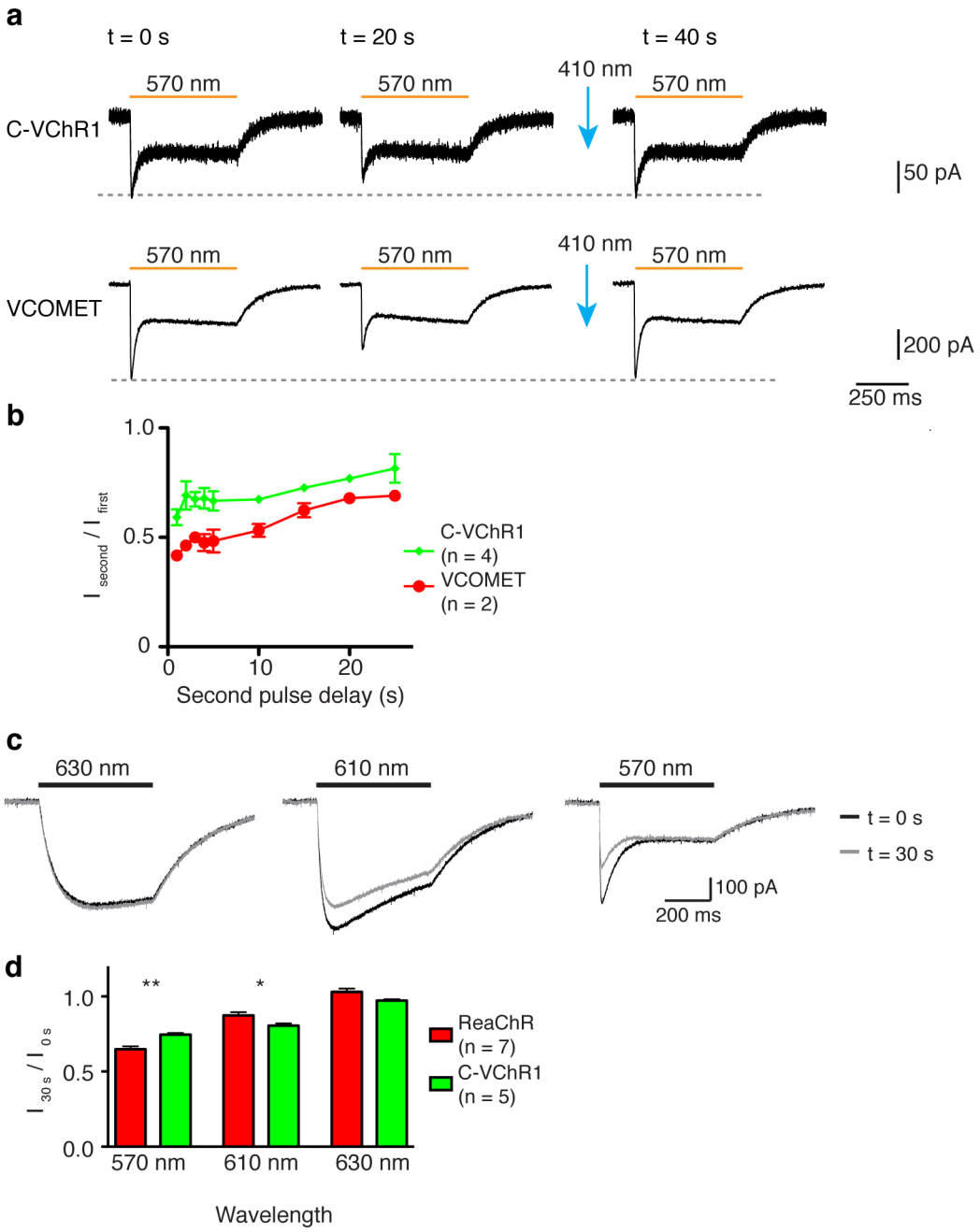

Supplementary Figure 3. The recovery of VCOMET, C-VChR1 and ReaChR after desensitization / inactivation. (a) With 2 repetitive stimulations 20 seconds apart, the desensitized transient component of C-VChR1 and VCOMET failed to recover completely in the dark without re-activation with 410 nm light. **(b)** The recovery of the desensitized transient peak response with various second pulse delay, both C-VChR1 and VCOMET failed to achieve 100% recovery after 25 seconds. **(c)** The responses of

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ReaChR to 750 ms light pulses 30 s apart at the indicated wavelength. With 570 and 610 nm stimulation but not with 630 nm, there were desensitized components that did not recovery fully. **(d)** Summary graph of the experiment shown in **(c)**. Unpaired Student's t-tests (2-tailed) were used to compare the recovery of photo-responses of C-VChR1 and ReaChR at 570, 610 and 630 nm. For 570 nm stimulation, $t(11) = 4.24$ and $P = 0.0014$, for 610 nm stimulation, $t(11) = 2.381$, $P = 0.0364$ and for 630 nm stimulation, $t(10) = 2.068$, $P = 0.0655$. Graph in **(d)** is shown as mean ± S.E.M.

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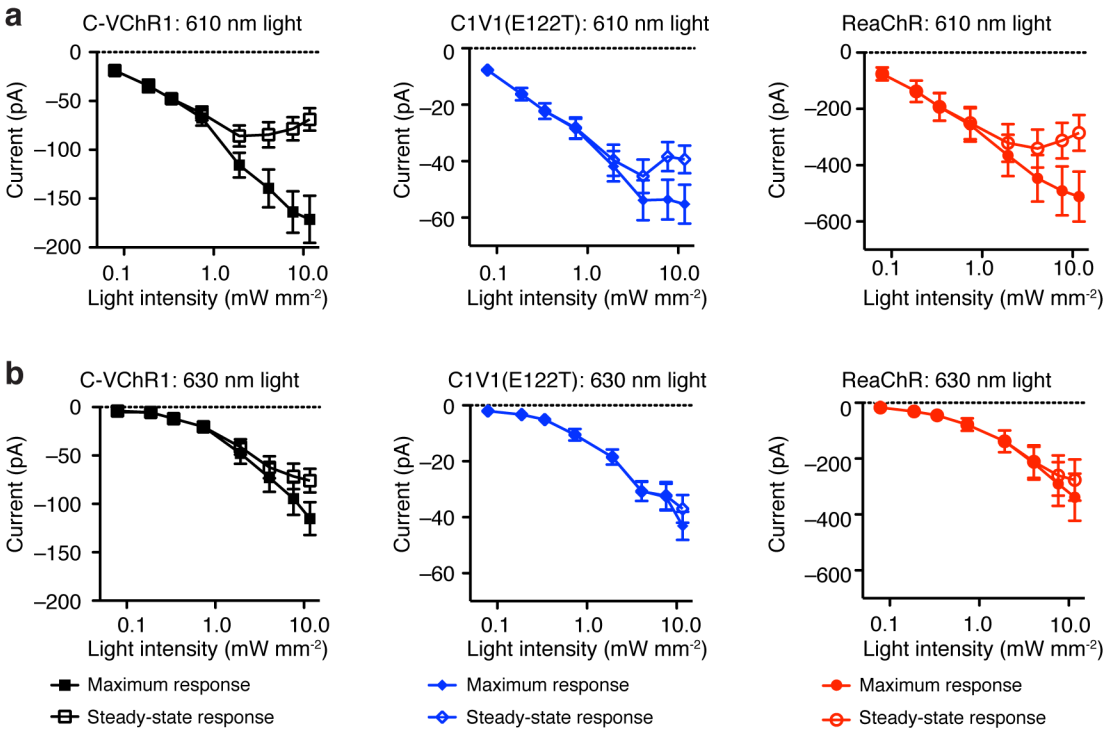

Supplementary Figure 4. The light-intensity-photocurrent relationships of C-VChR1, C1V1(E122T) and ReaChR. (a) The summary of light intensity-photocurrent amplitude relationships of C-VChR1 (left), C1V1(E122T) (middle) and ReaChR (right) to 610 nm light. **(b)** The summary of light intensity-photocurrent relationships of C-VChR1 (left), C1V1(E122T) (middle) and ReaChR (right) to 630 nm light. n = 10 for C-VChR1, n = 6 for C1V1(E122T) and n = 6 for ReaChR. Graphs are shown as mean ± S.E.M.

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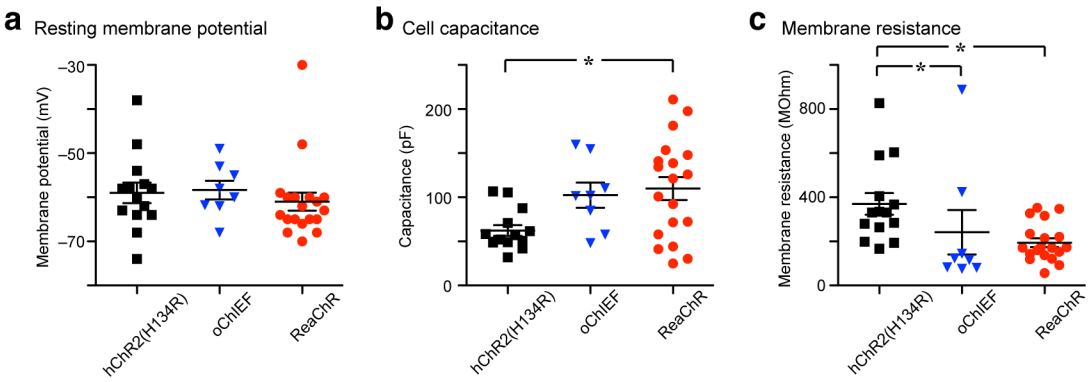

Supplementary Figure 5. The membrane properties of ReaChR-expressing primary cultured hippocampal neurons compared to hChR2(H134R) and oChIEF-expressing neurons. The mean resting membrane potential **(a)** is not significantly different between the three groups. The mean cell capacitance **(b)** of the ReaChR-expressing neuron is significantly greater than hChR2(H134R)-expressing neurons, indicating the expressing cells are bigger in size. The mean membrane resistance **(c)** of ReaChR-expressing neurons are lower than hChR2(H134R)-expressing neurons, consistent with greater cell size as measured with capacitance. The hChR2(H134R)-expressing cells are chosen based on the fluorescence of the expression, whereas ReaChR-expressing neurons are chosen based on healthy morphology under bright field visualization. The cells expressing a high level of hChR2(H134R) typically had smaller size, whereas the larger cells only expressed a low level of hChR2(H134R). Statistical tests were conducted with Kruskal-Wallis test with post-hoc Dunn's multiple comparison tests between all possible pairs. For **(a)**, H = 3.539, k = 3, $P = 0.1704$. For **(b)**, H = 6.823, k = 3, $P = 0.033$. For **(c)**, H = 11.47, k = 3, $P = 0.0032$. * indicates 0.05 level of significance. Graphs are shown as mean ± S.E.M.

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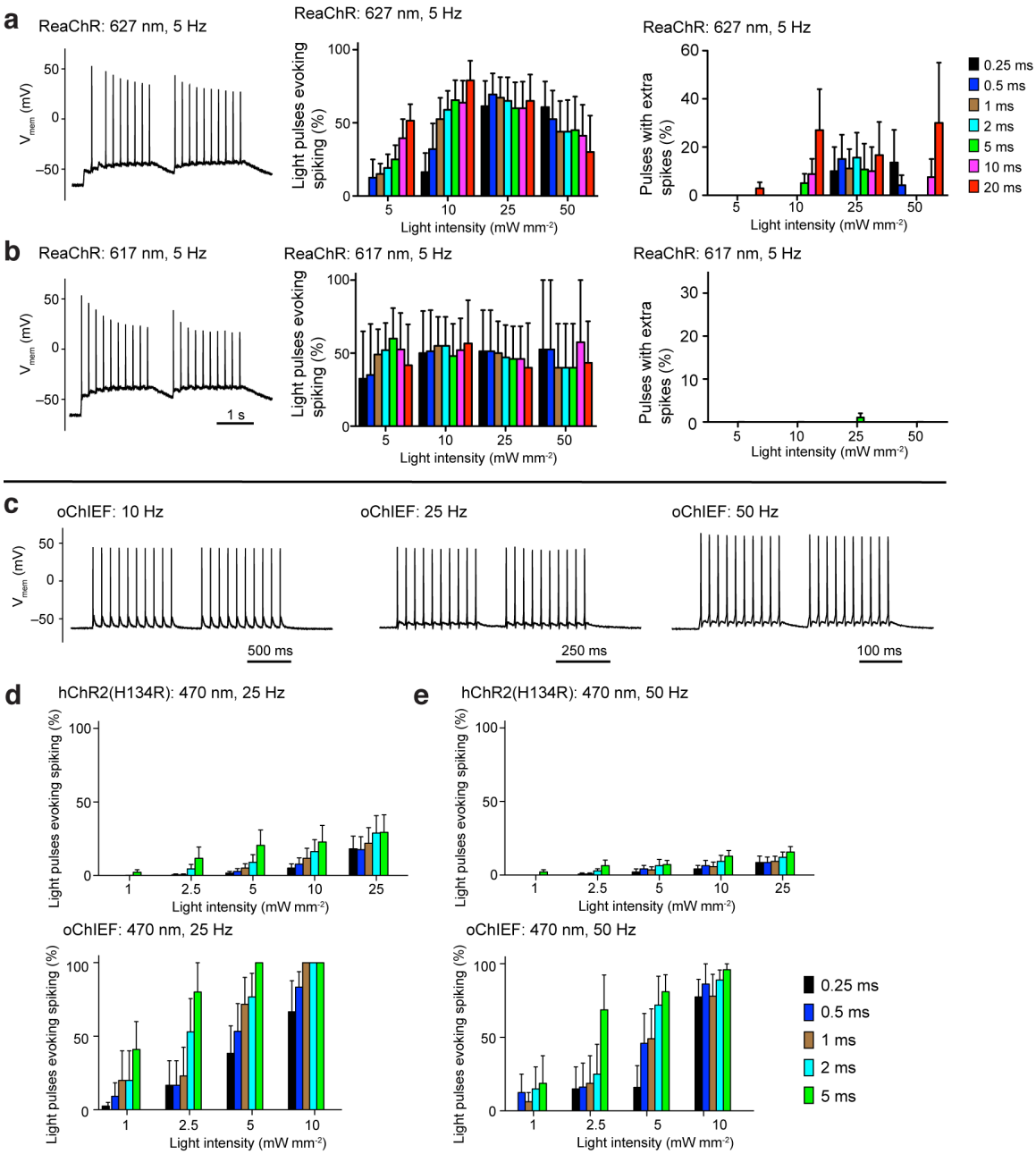

Supplementary Figure 6. The fidelity of ReaChR, hChR2(H134R) and oChIEF-expressing neurons to different frequencies of light stimulation. (a) The response of ReaChR-expressing cultured neurons to 627 nm light at 5 Hz (n = 2 - 9). An example of the electrophysiological recording of ReaChR-expressing neuron to 10 mW/mm^2 627 nm light stimulation of 10 ms pulse duration at 5 Hz (left). The graphs summarize the percentages of light pulses resulting in successful action potentials (middle) and the percentages of pulses resulting in extra action potential (right). **(b)** The response of

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ReaChR-expressing cultured neurons to 617 nm light at 5 Hz (n = 2 - 6) as in **(a)**. The electrophysiological recording shows the response of a ReaChR-expressing neuron to 10 mW/mm^2 617 nm light stimulation at 10 ms pulse duration (left) **(c)** Examples of oChIEF-expressing cultured neurons to 10, 25 and 50 Hz of light pulse stimulation. The stimulation light was 470 nm at 10 mW/mm^2 with 1 ms pulse duration for all three frequencies. **(d)** The percentage of light pulses resulting in light-triggered action potentials in neurons expressing hChR2(H134R)(n = 6) (top) and oChIEF (n = 6) (bottom) to 25 Hz blue light stimulation. **(e)** The percentage of light pulses resulting in light-triggered action potentials in neurons expressing hChR2(H134R)(n = 7) (top) and oChIEF (n = 5) (bottom) to 50 Hz blue light stimulation. No extra spikes were detected in any of the conditions tested. Graphs are shown as mean ± S.E.M.

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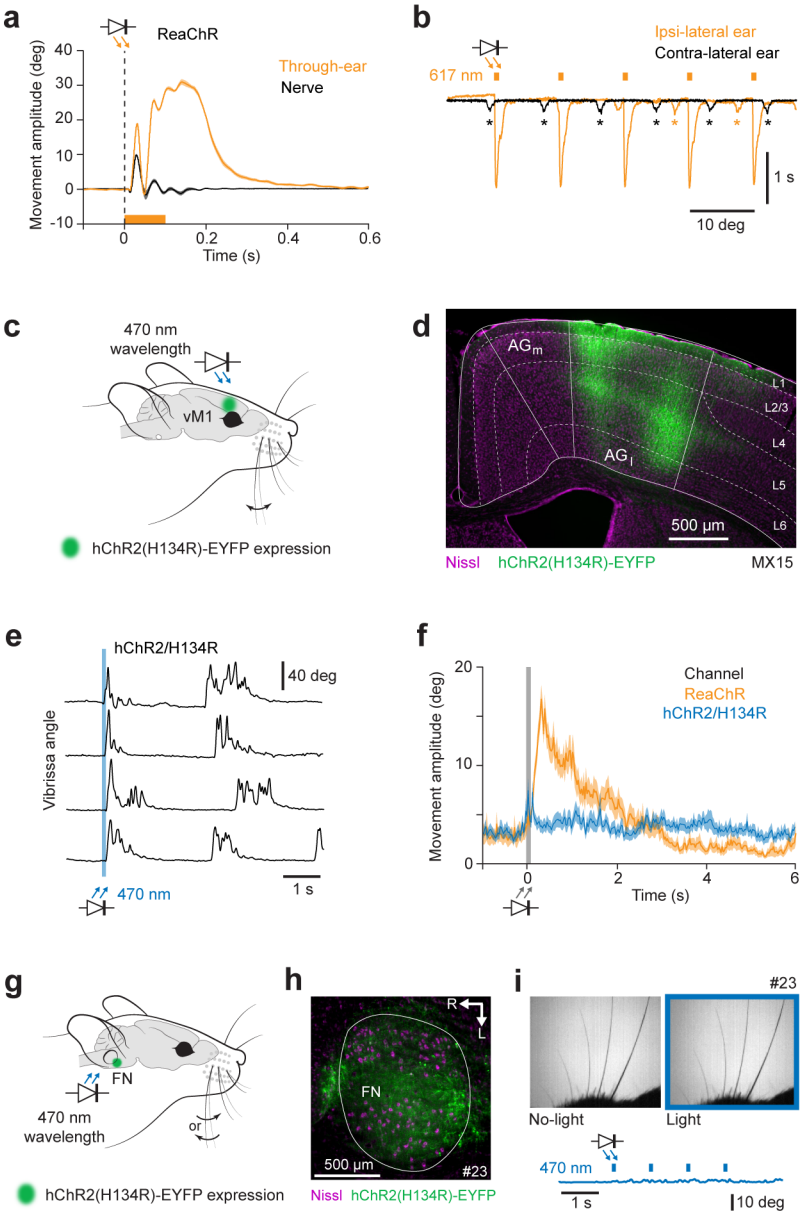

Supplementary Figure 7. Peripheral nerve activation *in vivo* and comparison of ReaChR and hChR2(H134R) expressing mice. (a) Comparison of through-ear (orange) and direct stimulation of the peripheral facial motor nerve (cranial VII; black) in an isoflurane anesthetized mouse. **(b)** Photo-activation of ReaChR expressing neurons in FN by stimulating through the ipsi- (orange) or contra-lateral (black) ear relative to the side of ReaChR expression in an isoflurane anesthetized mouse. Individual light pulses are indicated by bars (617 nm, 100 mW light output). Asterisk (*) indicates movements

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correlated with breathing. **(c)** Schematic of through-skull photo-activation of hChR2(H134R) expressing neurons in vM1 of the awake, head-fixed mouse. Whisker movements were monitored with high-speed video. **(d)** Coronal section through the medial (AGm) and lateral (AGl) agranular motor cortex (vM1) of an rAAV infected mouse shows hChR2(H134R)-EYFP (green) expression. Neurons were counterstained with a fluorescent Nissl-substance marker (NeuroTrace; magenta). Scale bar: 500 μm. **(e)** Traces of evoked whisker movements in response to single, 100 ms pulses (blue arrows and bar) of 470 nm light emitted by a LED placed 10 mm above the skin overlying vM1 cortex of an hChR2(H134R) expressing mouse. Increasing values denote protraction of the vibrissae. Scale bars: 40 deg and 1 s. **(f)** Average absolute movement amplitudes evoked by 100 ms pulses of 470 nm (blue) or 617 nm (orange) light through the intact skin overlying vM1 in hChR2(H134R) (n = 3 mice) or ReaChR (n = 3 mice) expressing mice, respectively (10 stimulus repetitions per condition/mouse). **(g)** Schematic of hChR2(H134R)-EYFP expressing motoneurons in the facial nucleus (FN) of awake mice illuminated through the ear by placing LEDs in the opening of the external auditory canal. Vibrissae movements were recorded with high-speed video. **(h)** Horizontal section through FN showing hChR2(H134R)-EYFP expressing neurons and associated processes (mostly motoneurons axons traversing FN and surrounding regions; green) and cell bodies counter-labelled with a fluorescent Nissl marker (NeuroTrace; magenta). **(i)** Example of absence of evoked whisker movements when stimulating a mouse expressing hChR2(H134R) in FN motoneurons. **Top:** Video frames show the whiskers in the reference retracted position (No-light) and at 100 ms following photo-activation (Light). **Bottom:** Traces of whisker position during attempted photo-activation with 470 nm light (blue).

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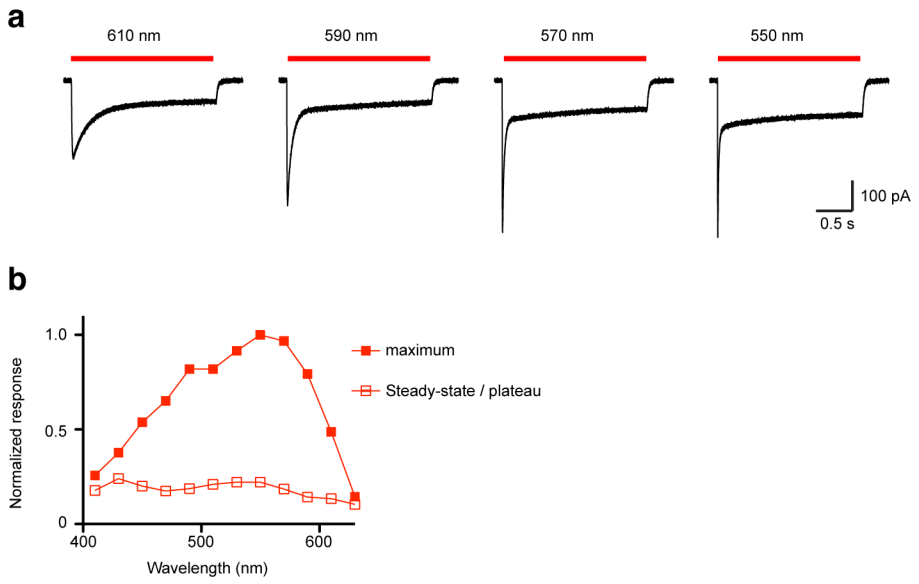

Supplementary Figure 8. The photocurrent responses of ReaChR + E163T. (a) The representative recordings of ReaChR + E163T to different wavelengths of light of the same intensity. **(b)** The response spectrum of ReaChR + E163T.

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Supplementary Figure 9. The nucleotide and amino acid sequence of ReaChR

ChIEF/ChR1 sequence
VChR1 sequence
VChR2 sequence
Kozak sequence

Leu 171 Ile: CTG -> ATT

```
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     21  S  A  G  A  S  T  G  S  D  A  T  V  P  V  A  T  Q  D  G  P  40
     61 AGCGCCGGCGCCAGCACCGGCAGCGACGCCACCGTGCCCGTGGCCACCCAGGACGGCCCC 120
     41  D  Y  V  F  H  R  A  H  E  R  M  L  F  Q  T  S  Y  T  L  E  60
    121 GACTACGTGTTCCACAGAGCCCACGAGAGAATGCTGTTCCAGACCAGCTACACCCTGGAG 180
     61  N  N  G  S  V  I  C  I  P  N  N  G  Q  C  F  C  L  A  W  L  80
    181 AACAACGGCAGCGTGATCTGCATCCCCAACAACGGCCAGTGCTTCTGCCTGGCCTGGCTG 240
     81  K  S  N  G  T  N  A  E  K  L  A  A  N  I  L  Q  W  V  V  F  100
    241 AAGAGTAACGGCACCAACGCCGAGAAGCTGGCCGCCAACATCCTGCAGTGGGTGGTGTTT 300
    101  A  L  S  V  A  C  L  G  W  Y  A  Y  Q  A  W  R  A  T  C  G  120
    301 GCGCTGAGCGTGGCGTGCCTGGGCTGGTATGCGTATCAGGCGTGGCGCGCGACCTGCGGC 360
    121  W  E  E  V  Y  V  A  L  I  E  M  M  K  S  I  I  E  A  F  H  140
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    141  E  F  D  S  P  A  T  L  W  L  S  S  G  N  G  V  V  W  M  R  160
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    161  Y  G  E  W  L  L  T  C  P  V  I  L  I  H  L  S  N  L  T  G  180
    481 TATGGCGAATGGCTGCTGACCTGCCCCGTGATTCTGATTcatCTGAGCAACCTGACCGGC 540
    181  L  K  D  D  Y  S  K  R  T  M  G  L  L  V  S  D  V  G  C  I  200
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    201  V  W  G  A  T  S  A  M  C  T  G  W  T  K  I  L  F  F  L  I  220
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    221  S  L  S  Y  G  M  Y  T  Y  F  H  A  A  K  V  Y  I  E  A  F  240
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    241  H  T  V  P  K  G  L  C  R  Q  L  V  R  A  M  A  W  L  F  F  260
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    261  V  S  W  G  M  F  P  V  L  F  L  L  G  P  E  G  F  G  H  I  280
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    321  I  R  K  K  Q  K  I  T  I  A  G  Q  E  M  E  V  E  T  L  V  340
    961 ATTCGCAAAAAACAGAAAATTACCATTGCGGGCCAGGAAATGGAAGTGGAAACCCTGGTG 1020
    341  A  E  E  E  D  K  Y  E  S  S  350
   1021 GCGGAAGAAGAAGATAAGTACGAGAGCAGC 1050
```

- The *ChIEF/ChR1*, *VChR1* and *VChR2* sequences are mammalian-codon optimized.

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Supplementary Figure 10. The nucleotide and amino acid sequence of C1V1(E122T)

ChIEF/ChR1 sequence
VChR1 sequence
Kozak sequence

Glu 123 Thr

```
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     61 AGCGCCGGCGCCAGCACCGGCAGCGACGCCACCGTGCCCGTGGCCACCCAGGACGGCCCC 120
     41  D  Y  V  F  H  R  A  H  E  R  M  L  F  Q  T  S  Y  T  L  E  60
    121 GACTACGTGTTCCACAGAGCCCACGAGAGAATGCTGTTCCAGACCAGCTACACCCTGGAG 180
     61  N  N  G  S  V  I  C  I  P  N  N  G  Q  C  F  C  L  A  W  L  80
    181 AACAACGGCAGCGTGATCTGCATCCCCAACAACGGCCAGTGCTTCTGCCTGGCCTGGCTG 240
     81  K  S  N  G  T  N  A  E  K  L  A  A  N  I  L  Q  W  I  T  F  100
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   1021 GCGGAAGAAGAAGATAAGTACGAGAGCAGC 1050
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* Both *ChIEF/ChR1* and *VChR1* sequences are mammalian-codon optimized

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Supplementary video legends

Supplementary Video 1: SupplVideo_01_Protraction.mov
Example of whisker protraction evoked by inter-aural stimulation of a mouse expressing ReaChR in FN motoneurons. Movements were captured with high-speed video (800 fps), during isoflurane anesthesia, and tracked offline. FN was stimulated by placing a 617 nm LED at the opening of the external auditory canal (100 mW light output; red vertical bars; see Methods). Upon photo-stimulation the whiskers promptly protracted and remained protracted for the duration of the light pulse (100 msec). The whiskers then retracted to their initial reference position.

Supplementary Video 2: SupplVideo_02_Retraction.mov
Example of whisker retraction evoked by inter-aural stimulation of a mouse expressing ReaChR in FN motoneurons. Movements were captured with high-speed video (800 fps), and whisker position and angle tracked offline. As the mouse recovered from isoflurane anesthesia, the whiskers slowly protracted and remained in a protracted position for approximately one minute. During this time window, ReaChR expressing neurons in FN were stimulated by inter-aural illumination with a 617 nm LED (100 mW light output; red vertical bars). Upon photo-activation, the whiskers promptly retracted and remained retracted for the duration of the light pulse (100 msec). As the light turned off, the whiskers then returned to their initial protracted position.

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