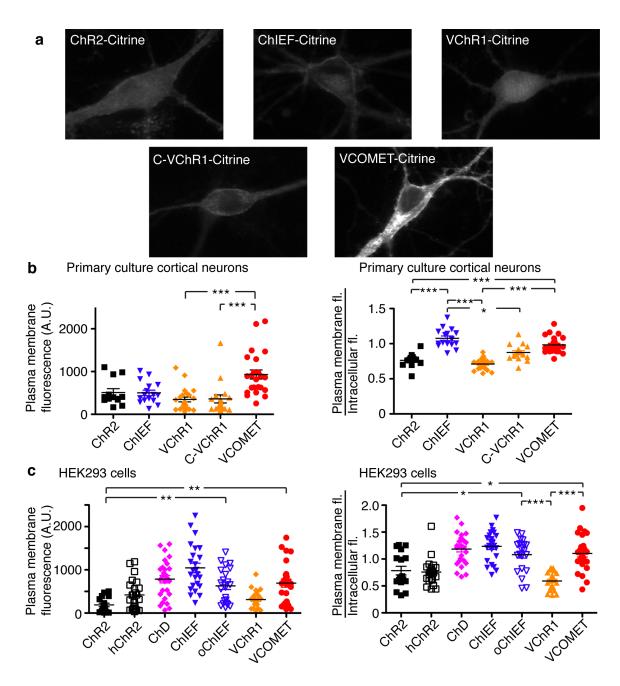
Supplementary Information

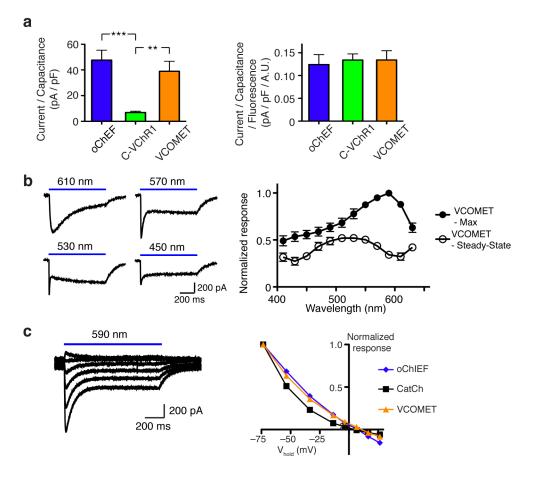
ReaChR: A red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation

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



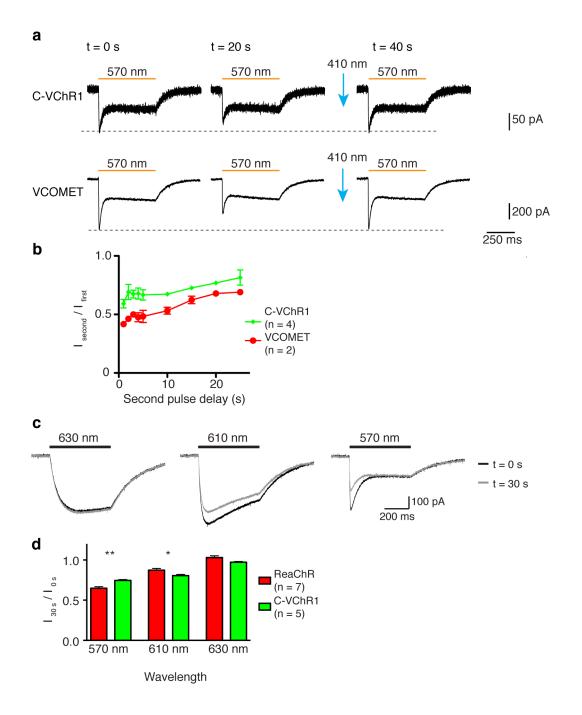
Supplementary Figure 1. Comparison of membrane trafficking and expression level of channelrhodopsin variants in primary cultured cortical neurons and HEK293 cells. (a) Representative fluorescent images of ChR2-Citrine, ChIEF-Citrine, VChR1-Citrine and VCOMET-Citrine expressed in primary cortical cultured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

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 \pm S.E.M.



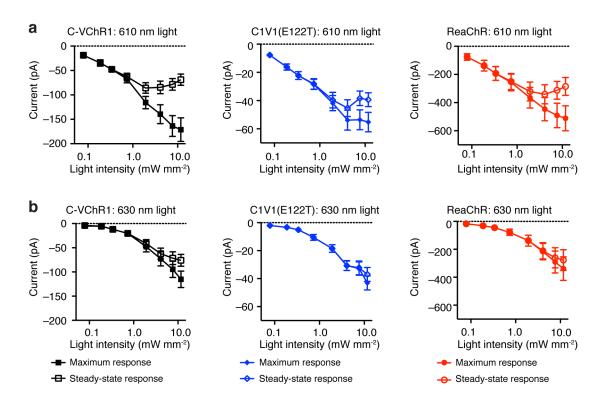
Supplementary Figure 2. Properties of VCOMET. (a) Comparison of mean photocurrent amplitudes of mammalian codon-optimized ChEF (oChEF), C-VChR1 and VCOMET without (left) and with (right) adjustment to membrane fluorescence recorded in HEK293 cells. **(b)** Representative electrophysiological traces of 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 \pm S.E.M. ** indicates 0.01 level of significance and *** indicates 0.001

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

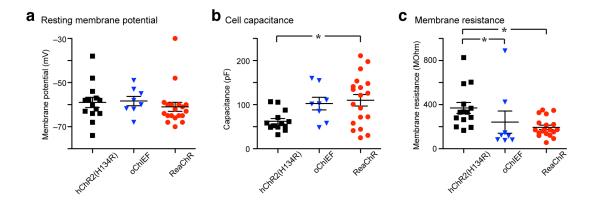


Supplementary Figure 3. The recovery of VCOMET, C-VChR1 and ReaChR after desensitization / inactivation. (a) With 2 repetitive stimulations 20 seconds apart, the desensitized transient component of C-VChR1 and VCOMET failed to recover completely in the dark without re-activation with 410 nm light. (b) 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

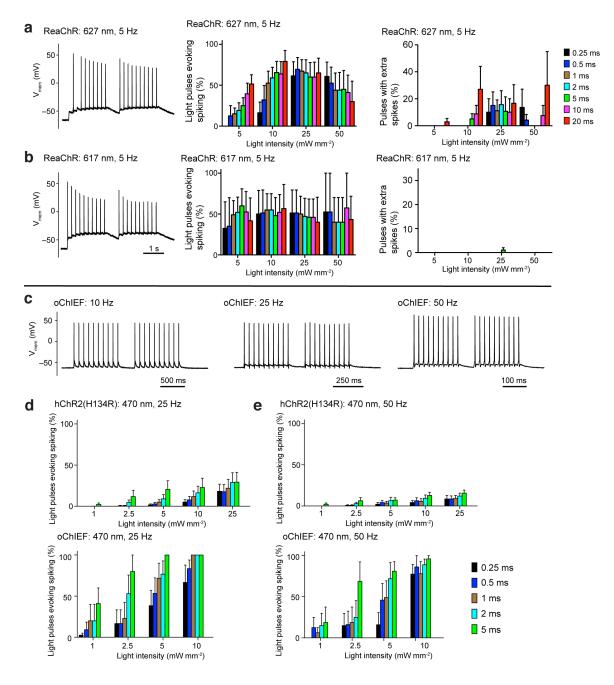
ReaChR to 750 ms light pulses 30 s apart at the indicated wavelength. With 570 and 610 nm stimulation but not with 630 nm, there were desensitized components that did not recovery fully. (d) Summary graph of the experiment shown in (c). Unpaired Student's t-tests (2-tailed) were used to compare the recovery 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 \pm S.E.M.



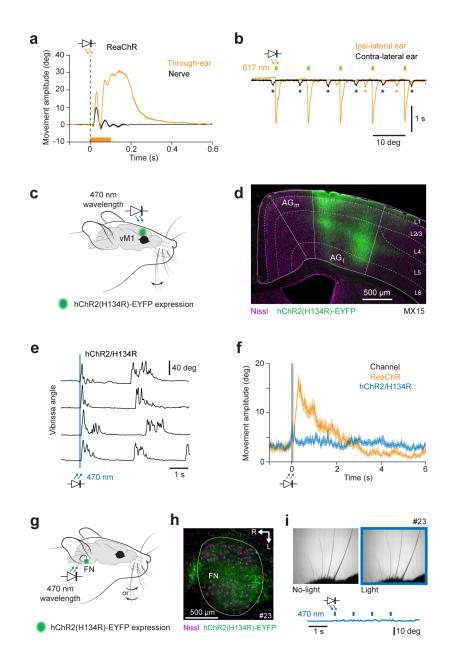
Supplementary Figure 4. The light-intensity-photocurrent relationships of C-VChR1, C1V1(E122T) and ReaChR. (a) The summary of light intensity-photocurrent amplitude relationships of C-VChR1 (left), C1V1(E122T) (middle) and ReaChR (right) to 610 nm light. (b) The summary of light intensity-photocurrent 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 \pm S.E.M.



Supplementary Figure 5. The membrane properties of ReaChR-expressing primary cultured hippocampal neurons compared to hChR2(H134R) and oChIEFexpressing neurons. The mean resting membrane potential (a) is not significantly different between the three groups. The mean cell capacitance (b) of the ReaChRexpressing 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 \pm S.E.M.

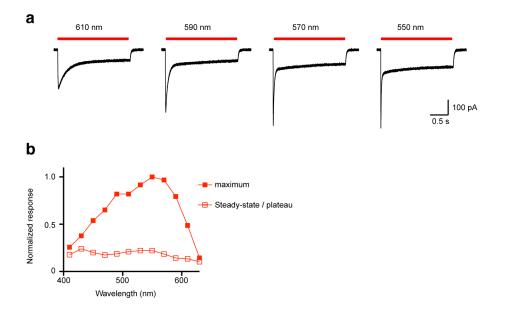


Supplementary Figure 6. The fidelity of ReaChR, hChR2(H134R) and oChIEFexpressing neurons to different frequencies of light stimulation. (a) The response of ReaChR-expressing cultured neurons to 627 nm light at 5 Hz (n = 2 - 9). An example of the electrophysiological recording of ReaChR-expressing neuron to 10 mW/mm² 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 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/mm² 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/mm² 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 \pm S.E.M.



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

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).



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.

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

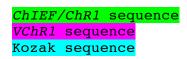


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841 AGCCCGTATGGCAGCGCGATTGGCCATAGCATTCTGGATCTGATTGCGAAGAACATGTGG 90 301 G V L G N Y L R V K I H E H I L Y G D 32 901 GGCGTGCTGGGCAACTATCTGCGCGTGAAAAATTCATGAACATATTCTGCTGTATGGCGAT 96 321 I R K K Q K I T I A G Q E M E V 34 961 ATTCGCAAAAAAACAGAAAATTACCATTGCGGGCCCAGGAAATGGAAGTGGAAACCCTGGTG 10 341 A E E D K Y E S 350					TGG								CTG				GAG						
301GVLGNYLRVKIHEHILLYGD32901GGCGTGCTGGGCAACTATCTGCGCGTGAAAATTCATGAACATATTCTGCTGTATGGCGAT96321IRKKQKITIAGQEMEVETLV34961ATTCGCAAAAAAACAGAAAATTACCATTGCGGGCCAGGAAATGGAAAGTGGAAACCCTGGTG10341AEEDKYES350			~	-	T m a m				_							ц	T v mm						
901 GGCGTGCTGGGCAACTATCTGCGCGTGAAAATTCATGAACATATTCTGCTGTATGGCGAT 96 321 I R K Q K I T I A G Q E M E V E I N 34 961 ATTCGCAAAAAAACAGAAAATTACCATTGCGGGCCAGGAAATGGAAGTGGAAACCCTGGTG 10 341 A E E D K Y E S 350																							
321 I R K Q K I I A G Q E V E I V 34 961 ATTCGCAAAAAACAGAAAATTACCATTGCGGGGCCAGGAAATGGAAGTGGAAACCCTGGTG 10 341 A E E D K Y E S 350			~										_		_		_			-	-	-	
961 ATTCGCAAAAAACAGAAAATTACCATTGCGGGCCAGGAAATGGAAGTGGAAACCCTGGTG 10 341 <mark>A E E E D K Y E S S</mark> 350																							
341 <mark>A E E D K Y E S S</mark> 350			-				2						_	~	_				_	_			
			_												UNA.	AIG	JAA	919	JAA	ACC	0100	тG	тU
	1						_		_		~			-									

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

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



Glu 123 Thr

ACC			S	R	R	Р	W	\mathbf{L}	\mathbf{L}	Α	L	Α	L	Α	V	Α	\mathbf{L}	Α	Α	G
	ATG	STG.	AGC	AGA	AGA	CCC	TGG	CTG	СТС	GCC	СТС	GCC	CTG	GCC	GTG	GCC	СТС	GCC	GCC	GGC
21	S	Α	G	А	S	т	G	S	D	Α	т	V	Р	V	Α	т	Q	D	G	Р
61	AGCO	SCC	GGC	GCC	AGC	ACC	GGC	AGC	GAC	GCC	ACC	GTG	CCC	GTG	GCC	ACC	CAG	GAC	GGC	CCC
41	D	Y	V	F	Н	R	А	Н	Е	R	М	L	F	Q	т	S	Y	т	L	Е
121	GAC	CAC	GTG	TTC	CAC	AGA	GCC	CAC	GAG	AGA	ATC	CTG	TTC	CAG	ACC	AGC	TAC	ACC	CTG	GAG
61	N	Ν	G	S	V	Ι	С	Ι	Р	N	N	G	Q	С	F	С	L	А	W	L
181	AACA	AAC	GGC	AGC	GTG	ATC	TGC	ATC	CCC	AAC	AAC	GGC	CAG	TGC	TTC	TGC	CTG	GCC	TGG	CTG
81	K	S	N	G	т	N	А	Е	Κ	L	Α	Α	N	Ι	L	Q	W	Ι	т	F
241	AAGA	AGT.	AAC	GGC	ACC	AAC	GCC	GAG	AAG	СТС	GCC	GCC	AAC	ATC	CTG	CAC	TGG	ATC	ACC	гтc
101	A	\mathbf{L}_{-}	S	Α	L	С	\mathbf{L}	М	F	Y	G	Y	Q	т	W	Κ	S	Т	С	G
	GCC	CTG.	AGC	GCC	CTG	TGC	CTG	ATG	TTC	TAC	GGC	TAC	CAG	ACC	TGG	AAG	SAGT	ACC	TGC	GGC
121	W	Е	т	Ι	Y	V	Α	т	Ι	Е	М	Ι	Κ	F	Ι	Ι	Е	Y	F	H
361	TGGC	SAG	acc	ATC	TAC	GTG	GCC	ACC	ATC	GAG	ATC	SATC	AAG	TTC	ATC	ATA	GAG	TAC	TTC	CAC
141	Е	F	D	Е	Р	Α	V	Ι	Y	S	S	Ν	G	Ν	Κ	Т	V	W	L	R
	GAG	TC	GAC	GAG	CCC			ATC	TAC	AGC	AGC	AAC	GGC	AAC	AAG		GTG	TGG		AGA
161	Y	Α	Е	W	L	L	Т	С	Р	V	L	L	Ι	Н	L	S	Ν	L	Т	G
481	TACO										CTO			CAT		AGC		CTG		GGC
181	L	Κ	D	D	Y	S	Κ	R	Т	М	G	L	L	V	S	D	V	G	С	Ι
	CTG																			
201	V	W	G	Α	Т	S	Α	М	С	Т	G	W	Т	Κ	Ι	L	F	F	L	Ι
	GTG																			
221	S	L	S	Y	G	М	Y	Т	Y	F	Н	A	A	Κ	V	Y	I	Е	Α	F
	AGCO																			
241	H	Т	V	P	K	G	I	C	R	E	L	V	R	V	M	A	W	Т	F	F
721																				
261	V	A	W	G	M	F	P	V	L	F		L	G	T	E	G	F	G	H	I
781																				
281	S	Р	Y	G	S	A	I	G	H	S	I	L	D	L	Ι	A	K	N	M	W
	AGCO																			
301	G	V	L	G	N	Y	L	R	V	K	I	H	E	H	I	L	L	Y	G	D
901																				
321	I	R	K	K	Q	K	I	Т	I	A	G	Q	E	M	E	V	E	Т	L	V
	ATTO												GAA	ATG	GAA	GTG	GAA	ACC	CTG	ЗТG
341	A	Е	E	E	D	K	Y TAC	E	S	S	35	-								

* Both *ChIEF/ChR1* and *VChR1* sequences are mammalian-codon optimized

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.