Machine learning-guided channelrhodopsin engineering enables minimally invasive optogenetics

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We engineered light-gated channelrhodopsins (ChRs) whose current strength and light sensitivity enable minimally invasive neuronal circuit interrogation. Current ChR tools applied to the mammalian brain require intracranial surgery for transgene delivery and implantation of fiber-optic cables to produce light-dependent activation of a small volume of tissue. To facilitate expansive optogenetics without the need for invasive implants, our engineering approach leverages the substantial literature of ChR variants to train statistical models for the design of high-performance ChRs. With Gaussian process models trained on a limited experimental set of 102 functionally characterized ChRs, we designed high-photocurrent ChRs with high light sensitivity. Three of these, ChRger1–3, enable optogenetic activation of the nervous system via systemic transgene delivery. ChRger2 enables light-induced neuronal excitation without fiber-optic implantation; that is, this opsin enables transcranial optogenetics.

ChRs are light-gated ion channels found in photosynthetic algae. Transgenic expression of ChRs in the brain enables light-dependent neuronal activation. These channels are widely applied as tools in neuroscience research; however, limitations of available ChRs prohibit a number of optogenetic applications. These channels have broad activation spectra in the visible range and require high-intensity light for activation (~1 mW mm⁻²). As ChRs are naturally low-conductance channels, 10⁻⁵–10⁻⁶ functional ChRs need to be expressed in the plasma membrane of a neuron to produce sufficient light-dependent depolarization and to induce neuronal activation. In the mouse brain, ChRs require ~1–15 mW light delivered ~100 μm from the target cell population to reliably activate action potentials. This confines light-dependent activation to a small volume of brain tissue (~1 mm³). Enabling optogenetics for large brain volumes without the need to implant invasive optical fibers for light delivery would be highly desirable.

Engineering ChRs to overcome limits in conductance and light sensitivity and extend the reach of optogenetic experiments requires overcoming three major challenges. First, rhodopsins are trans-membrane proteins that are inherently difficult to engineer because the sequence and structural determinants of membrane protein expression and plasma membrane localization are highly constrained and poorly understood. Second, because properties of interest for neuroscience applications are assayed using low-throughput techniques, such as patch-clamp electrophysiology, engineering by directed evolution is not feasible. And third, in vivo applications require retention and optimization of multiple properties; for example, localization in mammalian cells while simultaneously tuning kinetics, photocurrents and spectral properties.

Diverse ChRs have been published, including variants discovered from nature, variants engineered through recombination and mutagenesis, as well as variants resulting from rational design. Studies of these coupled with structural information and molecular dynamic simulations have established some understanding of the mechanics and sequence features important for specific ChR properties. Despite this, it is still not possible to predict functional properties of uncharacterized ChR sequences.

Our approach has been to leverage the substantial literature of ChRs to train statistical models that enable design of highly functional ChRs. These models take as their input the sequence and structural information for a given ChR variant and then predict its functional properties. The models use training data to learn how sequence and structural elements map to ChR function. Once known, that mapping can be used to predict the behavior of untested ChR variants.

Our trained models accurately predict the properties of untested ChR sequences. We used these models to engineer 30 ‘designer’ ChR variants with specific combinations of desired properties, a number of which have unprecedented photocurrent strength and light sensitivity. We characterized these low-light-sensitive, high-photocurrent ChRs for applications in the mammalian brain and demonstrate their potential for minimally invasive activation of populations of neurons in the brain enabled by systemic transgene delivery with the engineered adeno-associated virus (AAV), rAAV-PHP.8 (ref. 19). This work demonstrates how a machine learning-guided approach can enable engineering of proteins that have been challenging to engineer.

Results

Functional characterization of ChR variants for machine learning. In previous work, we explored structure-guided recombination of three highly functional ChR parents (CsChrimmR, CsChrimR, C1C2 (ref. 22)) by designing two ten-block recombination libraries with a theoretical size of ~120,000 (that is, 2×3¹⁰) chimeric variants. These recombination libraries are a rich source of functionally diverse sequences.

References
we generated 102 ChR variants selected from these recombination libraries. Of these, 76 were previously characterized for photocurrent properties (with patch-clamp electrophysiology) and 26 we characterized here. Together, these 102 ChR recombination variants provide the primary dataset used for model training. We supplemented this dataset with data from other published sources, including 19 ChR variants from nature, 14 single-mutant ChR variants and 28 recombination variants from other libraries (Supplementary Data 1). As the data produced by other laboratories were not collected under the same experimental conditions as data collected in our hands, they cannot be used for comparison for absolute ChR properties (that is, photocurrent strength); however, these data do provide useful binary information on whether a sequence variant is functional or not. Thus, we used published data from other sources when training binary classification models for ChR function.

Because our goal was to optimize photocurrent strength, wavelength sensitivity and off-kinetics, we used these measured properties to train machine-learning models (Fig. 1a). Enhancing ChR photocurrent strength would enable reliable neuronal activation even under low-light conditions. Different off-rates can be useful for specific applications: fast off-kinetics enable high-frequency optical stimulation, slow off-kinetics are correlated with increased light sensitivity and very slow off-kinetics can be used for constant depolarization (step-function opsins). In addition to opsins functional properties, it is also necessary to optimize or maintain plasma membrane localization, a prerequisite for ChR function.

Training Gaussian process (GP) classification and regression models. Using the ChR sequence and structure as well as functional data as inputs, we trained GP classification and regression models (Fig. 1). GP models successfully predicted thermostability, substrate binding affinity and kinetics for several soluble enzymes, and ChR membrane localization. For a detailed description of the GP model architecture used for protein engineering see refs. 25, 26. Briefly, these models infer predictive values for uncharacterized sequences from training examples by assuming that similar inputs (ChR sequence variants) will have similar outputs (photocurrent properties). To quantify the relatedness of inputs (ChR sequence variants), we compared both sequence and structure. ChR sequence information is encoded in the amino acid sequence. For structural comparisons, we convert the three-dimensional crystal-structural information into a ‘contact map’ that is convenient for modeling. Two residues are considered to be in contact and potentially important for structural and functional integrity if they have any nonhydrogen atoms within 4.5 Å in the C1C2 crystal structure (3UG9.pdb). We defined the sequence and structural similarity between two variants by aligning them and counting the number of positions and contacts at which they are identical.

We trained a binary classification model to predict whether a ChR sequence will be functional using all 102 training sequences from the recombination library (Supplementary Data 2) as well as data from 61 variants published by others (Supplementary Data 1). We then used this trained classification model to predict whether uncharacterized ChR sequence variants were functional (Fig. 1b). To test prediction accuracy, we performed 20-fold cross-validation on the training dataset and achieved an area under the receiver operator curve of 0.78, indicating good predictive power (Supplementary Table 1).

Next, we trained three regression models, one for each of the ChR photocurrent properties of interest: photocurrent strength, wavelength sensitivity of photocurrents and off-kinetics (Fig. 1c). Once trained, we used these models to predict photocurrent properties of untested ChRs sequence variants. To test prediction accuracy, we performed 20-fold cross-validation on the training dataset and observed high correlation between predicted and measured properties for all models (Pearson correlation coefficient (R) between 0.77 and 0.9; Supplementary Tables 1–2). Models built using contact maps from either the ChR2 crystal structure or C1Chrmisson crystal structure perform as well as models built with a contact map from the C1C2 structure (Supplementary Table 3 and Supplementary Fig. 1c,d), even though these maps share only 82% and 89% of their contacts with the C1C2 map, respectively (Supplementary Fig. 1a,b).

Selection of designer ChRs using trained models. To select ‘designer’ ChRs (that is, ChRs predicted to have a useful combination of properties), we used a tiered approach (Fig. 1d). First, we eliminated all ChR sequences predicted to not localize to the plasma membrane or predicted to be nonfunctional. To do this, we used classification models of ChR localization and function to predict the probability of localization and function for each ChR sequence in the 120,000-variant recombination library. Not surprisingly, most ChR variants were predicted to not localize and not function. To focus on ChR variants predicted to localize and function, we set a threshold for the product of the predicted probabilities of localization and function (Fig. 1b); any ChR sequence above that threshold would be considered for the next tier of the process. We selected a conservative threshold of 0.4.

The training data made clear that the higher the mutation distance from one of the three parents, the less likely it was that a sequence would be functional; however, we expect that more diverse sequences would also have the more diverse functional properties. To explore diverse sequences predicted to function, we selected 22 ChR variants that passed the 0.4 threshold and were multi-blockswap sequences containing on average 70 mutations from the closest parent. We synthesized these 22 sequences, expressed them in HEK cells and measured their photocurrent properties with patch-clamp electrophysiology. Of the tested sequences 59% were functional (Fig. 1e), compared with 38% of the multi-block-swap sequences randomly selected (that is, not selected by the model) and having comparable average mutation level. This validates the classification model’s ability to make useful predictions about uncharacterized functional sequences, even for sequences that are very distant from those previously tested. We updated the models by including data from these 22 sequences for future rounds of predictions.

From the 120,000-variant recombination library, 1,161 chimeric sequence variants passed the 0.4 predicted localization and function threshold (Fig. 1). For the second tier of the selection process, we used the three regression models trained on all functional variants collected up to this point to predict the photocurrent strength, wavelength sensitivity of photocurrents and off-kinetics for each of these 1,161 ChR sequence variants (Supplementary Data 3). We selected 28 designer ChRs predicted to be highly functional with different combinations of properties, including those predicted to have the highest photocurrent strength, most red-shifted or blue-shifted activation wavelengths and off-kinetics from very fast to very slow (Supplementary Figs. 2 and 3).

We synthesized genes encoding the 28 selected designer ChR variants, expressed them in HEK cells and characterized them for their photocurrent properties with patch-clamp electrophysiology (Figs. 1 and 2). All 28 designer ChRs selected using the updated classification model above the 0.4 threshold both localize and function. For each of the designer ChR variants, the measured photocurrent properties correlated well with the model predictions (R > 0.9 for all models) (Fig. 1f and Supplementary Table 1), demonstrating the power of this data-driven predictive method for engineering designer ChRs. As a negative control, we selected two ChR variant sequences from the recombination library that the model predicted would be nonfunctional (ChR_29_10 and ChR_30_10). These sequences resulted from a single-block swap from two of the most highly functional ChR recombination variants tested. As predicted, these sequences were nonfunctional (Fig. 2b), which shows that
ChR functionality can be attenuated by incorporating even minimal diversity at certain positions.

**Sequence and structural determinants of ChR functional properties.** We used L1-regularized linear regression models to identify a limited set of residues and structural contacts that strongly influence ChR photocurrent strength, spectral properties and off-kinetics (Supplementary Fig. 4a). We can assess the relative importance of each of these sequence and structural features by weighting their contributions using L2-regularized linear regression models approximate the fitness landscape for each property of interest (insets show hypothetical fitness landscapes). A machine-learning pipeline to identify 28 top ChR variants. Measurements of training set and model-predicted ChR photocurrent properties (n = 3–8 cells per variant; Supplementary Data 2). Each gray-colored point is a ChR variant. Training set data are shaded in blue. Mean number of mutations for each set is below the plots.

Fig. 1 | Machine learning-guided optimization of ChRs. a, ChR properties used for training classification and regression models. b, The classification models’ predicted probabilities of function and localization. A threshold for the product of the predicted probabilities (pp) of 0.4 to eliminate nonfunctional ChRs is indicated. c, Regression model predictions versus measured photocurrent property on the training set data (20-fold cross-validation). Regression models approximate the fitness landscape for each property of interest (insets show hypothetical fitness landscapes). d, Machine-learning pipeline to identify 28 top ChR variants. e, Measurements of training set and model-predicted ChR photocurrent properties (n = 3–8 cells per variant; Supplementary Data 2). Each gray-colored point is a ChR variant. Training set data are shaded in blue. Mean number of mutations for each set is below the plots. f, Regression model predictions versus measured photocurrent property for each of the 28 designer ChRs (that is, the test set).
CheRiff and C1C2 but not in CsChrimR, which has a cysteine at that position. D195 is also part of a contact with L192 that contributes strongly to photocurrent strength (Supplementary Fig. 4).

A number of contacts proximal to retinal contribute strongly to photocurrent strength. For example, the most heavily weighted contact includes A295 (from CsChrimR), which is adjacent to the...
conserved lysine residue that covalently links retinal (Supplementary Fig. 4). This position is a serine in both C1C2 and CheRiff.

Machine-guided search identifies ChRs with a range of useful functional properties. We assessed photocurrent amplitude, wavelength sensitivity and off-kinetics of the designer ChRs and the three parental ChRs (Fig. 2). In addition to the 28 regression model-predicted ChRs, we also assessed the two top-performing ChRs from the classification models’ predictions (ChR_9_4 and ChR_25_9), for a total of 30 model-predicted highly functional ChRs as well as the two negative control ChRs (ChR_29_10, ChR_30_10). Of the 30 model-predicted ChRs, we found 12 variants with at least two times higher blue-light-activated photocurrents than the top-performing parent (CsChrimR) (Fig. 2b). Three variants exhibit at least 1.7 times higher green-light-activated photocurrents than CsChrimR. Eight variants have larger red-light-activated photocurrents when compared with the blue-light-activated parents (CheRiff and C1C2), though none outperform CsChrimR. Both ChR variants predicted to be nonfunctional by the models produce <30 pA currents.

Designer ChRs’ off-kinetics span three orders of magnitude (τ off = 10 ms to >10 s) (Fig. 2c). This range is quite remarkable given that all designer ChRs are built from sequence blocks of three parents that have similar off-kinetics (τ off = 30–50 ms). We found that five designer ChRs have faster off-kinetics than the fastest parent, while 16 have more than five times slower off-kinetics. The two fastest variants, ChR_3_10 and ChR_21_10, exhibit τ off = 13 ± 0.9 ms and 12 ± 0.4 ms, respectively (mean ± s.e.m.). Four ChRs have particularly slow off-kinetics with τ off > 1 s, including ChR_15_10, ChR_6_10 and ChR_13_10 (τ off = 4.3 ± 0.1 s, 8.0 ± 0.5 s and 17 ± 7 s, respectively). Two ChRs with very large photocurrents, ChR_25_9 and ChR_11_10, exhibit τ off = 220 ± 10 ms and 330 ± 30 ms, respectively.

Three designer ChRs exhibit interesting spectral properties (Fig. 2d and Supplementary Fig. 5). ChR_28_10’s red-shifted spectrum matches that of CsChrimR, demonstrating that incorporating sequence elements from blue-shifted ChRs into CsChrimR can still generate a red-shifted activation spectrum. ChR_11_10 has a broad activation spectrum relative to the parental spectra, with similar steady-state current strength from 400 to 546 nm light and strong currents (700 ± 100 pA) when activated with 567 nm light. ChR_25_9, on the other hand, exhibits a narrow activation spectrum relative to the parental spectra, with a peak at 481 nm light.

We assessed the light sensitivity of select designer ChRs. Compared with CsChrimR, CheRiff and C1C2, the designer ChRs have at least nine times larger currents at the lowest intensity of light tested (10^−2 mW mm^−2), larger currents at all intensities of light tested and minimal decrease in photocurrent magnitude over the range of intensities tested (10^−2–10^−1 mW mm^−2), suggesting that photocurrents were saturated at these intensities and would only attenuate at much lower light intensities (Fig. 2e). These select designer ChRs are expressed at levels similar to the CsChrimR parent (the highest-expressing parent), indicating that the improved photocurrent strength of these ChRs is not solely due to improved expression (Supplementary Figs. 6 and 7).

We compared three of the top designer ChRs (ChR_9_4, ChR_25_9 and ChR_11_10) with ChR2(H134R) (ref. 64), an enhanced photocurrent single mutant of ChR2 commonly used for in vivo optogenetics, and CoChR (from Chloromonas oogama)43, reported to be one of the highest-conducting ChRs activated by blue light11 (Supplementary Fig. 8). The selected designer ChRs produce three to six times larger photocurrents than ChR2(H134R) when exposed to high-intensity (2.2 mW mm^−2) 481 nm light and 10–18 times larger photocurrents than ChR2(H134R) when exposed to low-intensity (6.5 × 10^−2 mW mm^−2) 481 nm light (Supplementary Fig. 8d–g). Although CoChR produced peak currents of similar magnitude to the designer ChRs, CoChR decays to a much lower steady-state level (Supplementary Fig. 8d,e), with the designer ChRs producing two to three times larger steady-state photocurrents than CoChR when exposed to high-intensity light and three to four times larger steady-state photocurrents than CoChR when exposed to low-intensity light (Supplementary Fig. 8f,g and Supplementary Table 4). The increased low-light sensitivity of these select designer ChRs is likely due in part to their relatively slow off-kinetics, leading to the increased accumulation of the open state under low-light conditions44.

Validation of designer ChRs for neuroscience applications. For further validation we selected three of the top high-conductance ChRs, ChR_9_4, ChR_25_9 and ChR_11_10, and renamed them ChRger1, ChRger2 and ChRger3, respectively, for channelrhodopsin Gaussian process-engineered recombinant opsin (Fig. 3 and Supplementary Fig. 9). When expressed in cultured neurons, the ChRgers display robust membrane localization and expression throughout the neuron soma and neurites (Fig. 3b). The ChRgers outperform both CoChR and ChR2(H134R) in photocurrent strength with low-intensity light in neuronal cultures (Fig. 3c). The designer ChRgers require one to two orders of magnitude lower light intensity than CoChR and ChR2(H134R) for neuronal activation (Fig. 3d and Supplementary Fig. 8h).

Next, we performed direct intracranial injections into the mouse prefrontal cortex (PFC) of rAAV-PHP.eB packaging either ChRger1–3 or ChR2(H134R) under the hSyn promoter (Supplementary Table 5). After ~3–5 weeks of expression, we measured light sensitivity in ChR-expressing neurons in acute brain slices. We observed greater light sensitivity for the ChRgers compared with ChR2(H134R) (Fig. 3g,h). The ChRgers exhibit >200 pA photocurrent at 10^−2 mW mm^−2, while at the equivalent irradiance ChR2(H134R) exhibits undetectable photocurrents. The ChRgers reach >1,000 pA photocurrents with ~10^−2 mW mm^−2 light, a four-fold improvement over ChR2(H134R)’s irradiance-matched photocurrents (Fig. 3g). Our characterization of ChR2(H134R)’s light sensitivity and photocurrent strength is consistent with previous results from other laboratories22.

Designer ChRs and systemic AAVs enable minimally invasive optogenetic excitation. We investigated whether these light-sensitive, high-photocurrent ChRs could provide optogenetic activation coupled with minimally invasive gene delivery. Previous reports of noninvasive optogenetics relied on invasive intracranial virus delivery, which results in many copies of virus per cell and thus very high expression levels of the injected construct29. The AAV capsid rAAV-PHP.eB (ref. 9) produces broad transduction throughout the central nervous system with a single minimally invasive intravenous injection in the adult mouse30,31. Use of rAAV-PHP.eB for optogenetic applications has been limited, however, by the low multiplicity of infection with systemically delivered viral vectors, resulting in insufficient opsin expression and light-evoked currents to control neuronal firing with commonly used channels (for example, ChR2).

We hypothesized that the ChRgers could overcome this limitation and allow large-volume optogenetic excitation following systemic transgene delivery. We systemically delivered rAAV-PHP.eB packaging ChRger1–3, CoChR or ChR2(H134R) under the hSyn promoter and observed broad expression throughout the brain (Fig. 3i). Using cell-attached recordings in acute brain slices, we measured the fraction of opsin-expressing cells with sufficient opsin-mediated currents for light-induced firing (Fig. 3j). Only 4% of ChR2(H134R)-expressing neurons produced light-induced firing, while 77% of CoChR-expressing neurons, 89% of ChRger1-expressing neurons and 100% of ChRger2- or ChRger3-expressing neurons produced light-induced activity. With systemic delivery, we observed superior light sensitivity of ChRgers compared with CoChR in both photocurrent strength (Fig. 3k) and spike
Fig. 3 | ChRger variants in cultured neurons and in acute brain slices outperform ChR2(H134R) and CoChR. a, ChRs were cloned into an AAV vector with either the hSyn or CamKIIα promoter and packaged into rAAV-PHP.eB for expression in culture and in vivo. b, Cultured neurons expressing ChRgers and ChR2(H134R) under the hSyn promoter (repeated independently six times per construct with similar results). c, Peak and steady-state photocurrent with low-intensity (8 × 10⁻³ mW mm⁻²) and moderate-intensity (0.8 mW mm⁻²) light in cultured neurons (ChR2, n = 16 cells; CoChR, n = 17 cells; ChRger1, n = 9 cells; ChRger2, n = 24 cells; ChRger3, n = 9 cells). d, Spike fidelity with varying-intensity light for 5 ms light-pulse width at 2 Hz stimulation (ChRger1, n = 6 cells; ChRger2, n = 6 cells; CoChR, n = 7 cells; ChR2, n = 7 cells). e, Spike fidelity with varying stimulation frequency with 2 ms light-pulse width in cultured neurons (ChRger1, n = 9 cells; ChRger2, n = 12 cells; ChRger3, n = 7 cells; ChR2, n = 8 cells). f, Representative voltage traces of ChRgers and ChR2(H134R) with a 300 ms light pulse at varying light irradiances in acute brain slice after direct injection of rAAV-PHP.eB-packaged hSyn-ChRger2 or hSyn-ChR2(H134R) resulted in broad expression throughout the cortex (5 × 10¹¹ VG per animal). g, Photocurrent strength with varying light irradiances in acute brain slice after direct injection of rAAV-PHP.eB-packaged hSyn-ChR construits into the PFC (ChRger1, n = 11 cells; ChRger2, n = 11 cells; ChRger3, n = 11 cells; ChR2, n = 9 cells) or after systemic delivery of CamKIIa-ChRger2 (ChRger2, n = 6 cells; 5 × 10⁹ VG per animal). h, Representative current traces of ChRgers and ChR2(H134R) with a 300 ms light pulse at varying light irradiances in acute brain slice after direct injection. i, Systemic delivery of rAAV-PHP.eB-packaged hSyn-ChRger2 or hSyn-ChR2(H134R) resulted in broad expression throughout the cortex (5 × 10¹⁰ VG per animal; repeated independently five times per construct with similar results). j, The fraction of light-excitable neurons in the PFC after systemic delivery of hSyn-ChRs measured by cell-attached recording in acute slice targeting only neurons expressing the eYFP marker (1 × 10¹⁰ VG per animal). k, l, Peak (solid line) and steady-state (dashed line) photocurrent strength (k) and spike fidelity (l) with varying light irradiances in acute brain slice after systemic delivery (1 × 10⁹ VG per animal) of hSyn-ChRger2 (n = 13 cells) and hSyn-CoChR (n = 14 cells) (recorded in PFC neurons). m, Spike fidelity with varying stimulation frequency in acute brain slice after systemic delivery (1 × 10⁹ VG per animal) of hSyn-ChR, n = 15 cells; ChRger1, n = 9 cells; ChRger2, n = 5 cells; ChRger3, n = 8 cells) with 1 mW mm⁻² light intensity. n, Representative voltage traces with blue-light-driven (1 mW mm⁻²) spiking at the indicated frequencies. VG, viral genomes. Plotted data are mean ± s.e.m.

These results demonstrate the need for light-sensitive and high-photocurrent opsin for applications where systemic delivery is desired.
We systematically delivered rAAV-PHP.eB packaging ChRger1–3 under the CaMKIIa promoter. With systemic delivery of ChRger2, we observed photocurrent strength similar to results observed after direct injection into the PFC in acute brain slices (Fig. 3g). When expressed in pyramidal neurons in the cortex, ChRger2 and ChRger3 enabled robust optically induced firing at rates between 2 and 10 Hz, although spike fidelity was reduced at higher frequency stimulation (Fig. 3m,n). ChRger2 performed best with higher frequency stimulation while ChRger1 performed worst. CoChR has better spike fidelity than the ChRgers at higher frequency stimulation (20–40 Hz) (Fig. 3m).

We next evaluated the optogenetic efficiency of ChRger2 using optogenetic intracranial self-stimulation of dopaminergic neurons of the ventral tegmental area (VTA) [32]. We systematically delivered rAAV-PHP.eB packaging a double-floxed inverted open reading frame (DIO) containing either ChRger2 or ChR2(H134R) into Dat-Cre mice (Fig. 4a,b and Supplementary Table 5). At 3 weeks after systemic delivery and stereotaxic implantation of fiber-optic cannulas above the VTA, we placed mice in an operant box and conditioned them to trigger a burst of 447 nm laser stimulation via nose poke. Animals expressing ChRger2 displayed robust optogenetic self-stimulation in a frequency-dependent and laser power-dependent manner. Higher frequencies (up to 20 Hz) and higher light power (up to 10 mW) promoted greater maximum operant response rates (Fig. 4c). Conversely, laser stimulation failed to reinforce operant responding in ChR2(H134R)-expressing animals (Fig. 4c); these results were consistent with results in acute slice where the light-induced currents of ChR2(H134R) are too weak at the low copy number produced by systemic delivery for robust neuronal activation.

To determine whether ChRger2 would enable both minimally invasive transgene delivery and minimally invasive optical excitation, we assayed directional control of locomotion in freely moving animals by optogenetic stimulation of the right secondary motor cortex (M2) [33]. In this assay, unilateral stimulation of M2 disrupts motor function in the contralateral lower extremities, causing mice to turn away from the stimulation side. We systematically administered rAAV-PHP.eB packaging either ChRger2 or ChR2(H134R) under a CaMKIIa promoter for transgene expression in excitatory pyramidal neurons in the cortex (Fig. 4d and Supplementary Table 5). We observed broad expression throughout the cortex for both ChRger2- and ChR2(H134R)-injected animals.
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(FIG. 2b AND Supplementary Fig. 11). We secured a fiber-optic cannula guide to the surface of the thinned skull above M2 without puncturing the dura and therefore leaving the brain intact (FIG. 2b), which we consider to be minimally invasive. Despite the presence of the optically scattering calvarial bone, stimulation with 20mW 447 nm light-induced left-turning behavior in animals expressing ChR2 but not in animals expressing ChR2(H134R) (FIG. 4f and Supplementary Videos 1 and 2). Left-turning behavior terminated on conclusion of optical stimulation (Supplementary Video 1). Behavioral effects were seen at powers as low as 10mW. To ensure that the turning behavior was not due to visual stimuli or heating caused by the stimulation laser, we repeated treadmill experiments using 671 nm light, which is outside the excitation spectrum of both opsins. Furthermore, 20mW 671 nm light failed to induce turning in both ChR2 and ChR2(H124R). Overall, these experiments demonstrate that ChR2 is compatible with systemic gene delivery and can enable minimally invasive optogenetic excitation.

Discussion

We demonstrated a data-driven approach to engineering ChRs properties that enables efficient discovery of highly functional ChR variants based on data from relatively few variants. In this approach we approximate the ChR fitness landscape for a set of ~120,000 chimeric ChRs and use it to efficiently search sequence space and select top-performing variants for a given property. By first eliminating the vast majority of nonfunctional sequences, we can focus on local peaks scattered throughout the landscape. Then, using regression models, we predict which sequences lie on the fitness peaks.

Machine learning provides a platform for simultaneous optimization of multiple ChR properties that follow engineering specifications. Application of this machine-learning pipeline (limited data collection from diverse sequences, model training and validation, and prediction and testing of new sequences) has great potential to optimize other neuroscience tools; for example, anion-conducting ChRs, calcium sensors, voltage sensors, and AAVs.

We designed high-performance ChRs (ChR1–3) with unprecedented light sensitivity and validated ChR1’s application for in vivo optogenetics. The high-photon-photosensitivity of these ChRs overcome the limitation of low per-cell copy number after systemic delivery. Systemically delivered ChRs have great potential for optogenetic activation of neuronal populations that are difficult to access surgically (for example, DRG, nodose ganglia, sympathetic chain ganglia and cardiac ganglia) or are widely distributed (for example, the enteric nervous system). Coupling ChRs with recently reported upconversion nanoparticles may allow for noninvasive optogenetics in deep brain areas with systemic transgene delivery and tissue-penetrating near-infrared light for neuronal excitation. ChR2 enabled neuronal excitation with high temporal precision without invasive intracranial surgery for virus delivery or fiber-optic implantation for superficial brain areas, extending what is currently possible for optogenetics experiments.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41592-019-0583-8.

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Author contributions

C.N.B., K.K.Y., V.G. and F.H.A. conceptualized the project. C.N.B. coordinated all experiments and data analysis. C.N.B. and K.K.Y. built machine-learning models. C.N.B. performed construct design and cloning. C.N.B. and E.D.M. performed AAV production. E.D.M. prepared cultured neurons. C.N.B. and J.E.R. conducted electrophysiology. C.N.B. and J.E.R. performed injections. J.E.R. performed fiber cannula implants and behavioral experiments. C.N.B. performed all data analysis. C.N.B. wrote the manuscript with input and editing from all authors. V.G. supervised optogenetics/electrophysiology, and F.H.A. supervised the protein engineering.

Competing interests

A provisional patent application (CIT File No.: CT-8092-P) has been filed by Caltech based on these results. C.N.B., K.K.Y, V.G. and F.H.A. are inventors on this provisional patent.

Additional information

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Methods

Construct design and cloning. The design, construction and characterization of the recombination library of chimeras are described in detail by Bedbrook et al.1. The ten-block contiguous and ten-block noncontiguous recombination libraries were designed and built using SCHEMA recombination. Software packages for calculating SCHEMA energies are openly available at cheme.che.caltech.edu/groups/haa/Software.htm. Each chimeric ChR variant in these libraries is composed of blocks of sequence from the parent ChR (CChrimR (ref. 1), C12 (ref. 1) and CheRiff (ref. 2)), including chimeras with single-block swaps (chimeras consisting of nine blocks of one parent and a single block from one of the other two parents) and multi-block-swap chimera sequences.

Selected ChR variant genes were inserted into a constant vector backbone (pFCK from Addgene plasmid no. 51693 (ref. 22)) with a Golgi export trafficking signal sequence (KSRITSEGEYIPLDQIDINV)5 and fluorescent protein (mKate). In all backbones, each ChR was inserted with a trafficking backbone (Addgene plasmid no. 26973), a pAAV-CamKIIa vector backbone (Addgene plasmid no. 80698) and a pAAV-CAG-DIO vector backbone (Addgene plasmid no. 104052). In all backbones, each ChR was inserted with a trafficking signal sequence and fluorescent protein (eYFP).

HEK293T cell and primary neuronal cultures. The culturing and characterization of ChRs in HEK cells is described by Bedbrook et al.1. Briefly, HEK cells were cultivated at 37 °C, 5% CO2 in DMEM containing 10% (vol/vol) fetal bovine serum, 1% sodium bicarbonate and 1% sodium pyruvate. HEK cells were transfected with purified ChR variant DNA using FuGENE 6 reagent (Promega). Software packages for open-source packages in the Python programming language to perform baseline adjustments, find the peak and steady-state inward currents, perform exponential fits of photocurrent decay for off kinetics, and quantify spike fidelity. Only neurons with an uncompensated series resistance between 5 and 25 MΩ, Rsh > 90 MΩ and holding current > −150 pA (holding at −60 mV) were included in data analysis (Supplementary Fig. 10). The photocurrent amplitude was not adjusted for expression level since both expression and correlation contribute to the variance observed. The currents of expression with photocurrent strength for all ChR variants tested are included in Supplementary Fig. 6 and 7.

On light exposure, ChRs open and reach a peak inward current and then desensitize reaching a lower steady-state current. As metrics of photocurrent strength, peak and steady-state photocurrent were used (Fig. 1b). As a metric for the ChR wavelength sensitivity of activation spectrum (that is, spectral properties), the normalized current strength induced by exposure to green light (546 nm) was used (Fig. 1a), which easily differentiates blue-shifted ChRs (peak activation: −450–480 nm) and red-shifted ChRs (peak activation: −520–650 nm). Two parameters were used to characterize ChR off kinetics: the time to reach 50% of the light-activated current after a 0.5 s light pulse and the photocurrent decay rate (τoff) after a 1 ms light exposure (Fig. 1a).

AAV production and purification. Production of recombinant AAV-PHP-eB packaging pAAV-hSyn-X-Ts-eYFP-WPRE, pAAV-CAG-DIO-[X-Ts-eYFP]-WPRE and pAAV-CaMKIIa-X-Ts-eYFP-WPRE (X = ChR12934, ChR1, ChR1r1 or ChR2r2) were performed using pAAV-1.2-CMV-Promega (Promega) with the methodology described by Liddelow et al.23 and Challis et al.24. Briefly, triple transfection of HEK293T cells (ATCC) was performed using polyethylenimine. Viral particles were harvested from the media and cells. Virus was then purified over iodixanol (Optiprep, Sigma; D1556) step gradients (15%, 25%, 40% and 60%). Viruses were concentrated and formulated in PBS. Virus was tested for virus titer, and viral titers are represented as millireplicas per nanoliter (mRPL). In vivo, the virus was delivered intracranially using 25 gauge microinjections/needle (inner diameter: 0.75 mm, length: 20 mm).

Intravenous injections, stereotactic injections and cannula implantation. Intravenous administration of rAAV vectors was performed by injecting the virus into the retro-orbital sinus at viral titers indicated in the text. There were no observable toxic or after effects with the dosages and time points with the viruses administered at the dosage levels. Intracranial injection of virus into the brain was performed using stereotactic coordinates from a previous report of properties of cultured hippocampal neurons10 and FPECRII antibodies in slice (ref. 1) (Supplementary Fig. 10). For cell culture experiments, the experimenter was blinded to the identity of the ChR being tested but not to the fluorescence level of the cells. For acute slice recordings, the experimenter was not blinded to the identity of the ChR.
The skull was thinned—40–50% with a standard drill-to create a level surface for the fiber–skull interface. Light was delivered from either a 447 nm or 671 nm laser (Changchun New Industries Model with PSU-H-LED) via mono-fiber-optic patch cable(s) (Doric Lenses, MFP_400/430/1100:0.48_3m_FC-ZB1_25) coupled to the fiber-optic cannula(e). Fiber-optic cannulae were secured to the skull with Metabond (Parkell, SKU 3936) and dental cement.

Analysis of behavioral experiments was performed using the open-source MATLAB program OptiMouse39 to track mouse nose, body and tail position while the mouse was running on the treadmill. Optogenetic intracranial self-stimulation was performed using a mouse modular test chamber (Lafayette Instruments, Model 80015NS) outfitted with an infrared nose port (Model 80167TM).

GP modeling. Both the GP regression and classification modeling methods applied in this paper are based on work detailed in refs. 44–46. For modeling, all sequences were aligned using multiple sequence alignment by log expectation (MUSCLE) (https://www.ebi.ac.uk/Tools/msa/muscle/). For modeling, aligned sequences were truncated to match the length of the C1C2 sequence, eliminating N- and C-terminal fragments with poor alignment quality due to high sequence diversity (Supplementary Data 1 and 2). Structural encodings (that is, the contact map) use the C1C2 crystal structure (3UG9.pdb) and assume that ChR chimeras share the contact architecture observed in the C1C2 crystal structure. Models built using structural encodings built from the ChR2 structure (6EID.pdb) and the C1Chrismion structure (5ZIIH.pdb) performed as well as models using the C1C2 structure (Supplementary Fig. 1c,d). The models are robust to differences in contact maps because they use both sequence and structural information, which is somewhat redundant.

For a given ChR, the contact map is simply a list of contacting amino acids with their positions. For example, a contact between alanine at position 134 and methionine at position 1 of the amino acid sequence would be encoded by (A134), (M1)). Both sequence and structural information were one-hot encoded. Regression models for ChR properties were trained to predict the log-ratio of the measured properties. All training data were normalized to have mean of zero and standard deviation of 1.

GP regression and classification models require kernel functions that measure the similarity between protein sequences. Learning involves optimizing the form of the kernel and its hyperparameters (Supplementary Table 2). The Matérn kernel was found to be optimal for all ChR properties (Supplementary Table 1).

For classification model training, all 102 functionally characterized ChR variants from our recombination libraries (Supplementary Data 2) were used as well as data from 61 sequence variants published by others (Supplementary Data 1). The model was then updated with data collected from the 22 additional ChR recombination variants with high sequence diversity (~70 mutations from the closest parent) and predicted to be functional (Fig. 1d). For training the regression models, all 102 functionally characterized training sequences (Supplementary Data 2) were initially used and then the models were updated with data collected from the 22 additional ChR variants (Fig. 1d).

GP regression. In regression, the goal is to infer the value of an unknown function f(x) at a novel point x; given observations y at inputs X. Assuming that the outputs are subject to independent and identically distributed Gaussian noise with variance $\sigma^2_y$, the posterior distribution of $f = f(X)$ for GP regression is Gaussian with mean

$$\tilde{f} = K^{-1}_f(K + \sigma^2_yI)^{-1}y$$

and variance

$$\nu = K(K + \sigma^2_yI)^{-1}k $$

where $I$ is the identity matrix. $K$ is the symmetric, square covariance matrix for the training set: $K_{ij} = k(x_i, x_j)$ for $x_i$ and $x_j$ in the training set. $k$ is the vector of covariances between the novel input and each input in the training set, and $k_0 = k(x, X)$. The hyperparameters in the kernel functions and the noise hyperparameter $\sigma$ were determined by maximizing the log marginal likelihood:

$$\log p(y | X) = -\frac{1}{2}y^TK_y^{-1}y - \frac{1}{2}\log |K_y + \sigma^2_yI| - \frac{n}{2}\log 2\pi$$

where $n$ is the dimensionality of the inputs. Regression was implemented using open-source packages in the SciPy ecosystem.

GP classification. In binary classification, instead of continuous outputs y, the outputs are class labels $y_i \in \{+1, -1\}$, and the goal is to use the training data to make probabilistic predictions $\pi(x_i) = p[y_i = +1|x_i]$. We used Laplaces method to approximate the posterior distribution. Hyperparameters in the kernels are found by maximizing the marginal likelihood. Classification was implemented using open-source packages in the SciPy ecosystem.

GP kernels for modeling proteins. GP regression and classification models require kernel functions that measure the similarity between protein sequences. A protein sequence $s$ of length $L$ is defined by the amino acid present at each location. This can be encoded as a binary feature vector $x_i$ that indicates the presence or absence of each amino acid at each position resulting in a vector of length 20L (for 20 possible amino acids). Likewise, the protein’s structure can be represented as a residue–residue contact map. The contact map can be encoded as a binary feature vector $x_i$ that indicates the presence or absence of each possible contacting pair. Both the sequence and structure feature vectors were used by concatenating them to form a sequence–structure feature vector.

Three types of kernel functions $k(x, s)$ were considered: polynomial kernels, squared exponential kernels and Matérn kernels. These different forms represent possible functions for the protein’s fitness landscape. The polynomial kernel with degree $d$ is defined as:

$$k(x, X) = \left(\sum_{i=1}^{d}x^i\right)^d$$

where $x$ is a protein sequence and $s$ its corresponding encoding and $\sigma_0$ and $\sigma_p$ are hyperparameters. We considered polynomial kernels with $d = 3$. The squared exponential kernel is defined as:

$$k(x, x') = \sigma_0^2 \exp(-\frac{||x - x'||^2}{2\ell^2})$$

where $\ell$ is a length scale and $\sigma_0$ and $\sigma_p$ are hyperparameters and $|1|_1$ is the L2 norm. Finally, the Matérn kernel with $\nu = \frac{1}{2}$ is defined as:

$$k(x, x') = \left(1 + \sqrt{\frac{3}{2}} \frac{||x - x'||}{\ell} \right) \frac{1}{2} \frac{1}{\ell} \exp\left(-\frac{\sqrt{3}}{2} \frac{||x - x'||}{\ell}\right)$$

where $l$ is once again a hyperparameter.

L1 regression feature identification and weighting. L1 regression was used to identify residues and contacts in the ChR structure most important for each ChR functional property of interest. First, residues and contacts that covary were identified using the concatenated sequence and structure binary feature vector for each of the training set ChR variants. Each set of covarying residues and contacts was combined into a single feature. L1 linear regression was used to select the features that contribute most to each ChR functional property of interest. The level of regularization was chosen by maximizing the log marginal likelihood of the GP regression model trained on the features selected at that level of regularization. We then performed Bayesian ridge regression on the selected features using the default settings in scikit-learn. Residues and contacts with the largest absolute Bayesian ridge linear regression weights were plotted onto the C1C2 structure (Supplementary Fig. 4). For feature identification and weighting, models were trained on both the training set and also the test set of 28 ChR sequences predicted to have useful combinations of diverse properties.

Statistical analysis. Plotting and statistical analysis were done in Python 2.7 and 3.6 and GraphPad Prism 7.01. For statistical comparisons, we first performed a D’Agostino and Pearson normality test. If the $P$ value of a D’Agostino and Pearson normality test was < 0.05, the nonparametric Kruskal–Wallis test with Dunn’s multiple comparisons post hoc test was used. If the data passed the normality test, a one-way analysis of variance was used.

Accession codes. GenBank: ChRger1, MN340983; ChRger2, MN340984; ChRger3, MN340985.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files. Source data for classification model training are provided in Supplementary Data 1 and 2. Source data for regression model training are provided in Supplementary Data 2. DNA constructs for the ChRger variants are deposited for distribution at Addgene (http://www.addgene.org, plasmid numbers 127237–44).

Code availability

Code used to train classification and regression models can be found at https://github.com/FHALab/channels.
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☐ Clearly defined error bars
☐ State explicitly what error bars represent (e.g. SD, SE, CI)

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Software and code

Policy information about availability of computer code

Data collection
Software to acquire confocal images with the Zeiss LSM 880 was Zen (version 2.3 from Zeiss). Software to acquire epifluorescence HEK cell images with the Andor Neo 5.5 sCOMS camera was Micro-Manager Open Source Microscopy Software version 1.4. Software for electrophysiology was pClamp [version 10.4 from Molecular Devices].

Data analysis
Gaussian Process regression and classification was implemented by custom code with open-source packages in the SciPy ecosystem (0.19.1) in the Python programming language. Electrophysiology data were analyzed using custom data-processing scripts written using open-source packages in the Python programming language to do baseline adjustments, find the peak and steady state inward currents, perform monoeponential fits of photocurrent decay for off-kinetic properties, and quantify spike fidelity. Animal behavior was analyzed using open-source OptiMouse software (version 3.0, available at: https://github.com/yorambenshaul/optimouse) using Matlab. Plotting and statistical analysis were done in Python 2.7 and 3.6 and GraphPad Prism 7.03.

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The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files. Source data for classification model training are provided in Dataset 1 and Dataset 2. Source data for regression model training are provided in Dataset 2.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical methods were used to predetermine sample size. We followed the standard sample sizes used for ChR comparisons in culture and in vivo.

Data exclusions

No data were excluded.

Replication

Each ChR variant was characterized by measurements made from many HEK cells. The ChRgers were characterized in multiple vector backbones. The ChRgers were characterized in HEK cells, neurons in culture, neurons slice, and in animals. In culture, multiple cells were recorded per transfection/transduction and multiple replicates of transfection or transduction were performed. In slice experiments, multiple animals were used and multiple cells measured per slice. In in vivo experiments, multiple animals were used. All attempts at replication were successful.

Randomization

Mice were randomly assigned to groups before injection and fiber placement.

Blinding

For all experiments in HEK cells authors were blind to the identity of the ChR being tested as all ChRs were given code names and the sequence identity of each ChR was only checked after data analysis. No blinding was performed for behavioral experiments because no human subjective qualitative metrics were assessed or reported.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study

- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

n/a Involved in the study

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

Green fluorescent protein (GFP) antibody from aves labs inc. catalog # GFP-1020. lot # GFP879484. Antibody staining was done at a 1:1000 dilution.

http://www.aveslab.com/products/epitope-tag-and-gfp-antibodies/anti-gfp-green-fluorescent-protein-antibodies-2/

Validation

Quality control of the GFP antibody was done by aves labs: antibodies were analyzed by western blot analysis (1:5000 dilution) and immunohistochemistry (1:500 dilution) using transgenic mice expressing the GFP gene product. Western blots were
Eukaryotic cell lines

Cell line source(s) | human embryonic kidney cells containing the SV40 T-antigen: HEK293T cells ATCC® CRL-3216
Authentication | Cell line used was not authenticated by us
Mycoplasma contamination | Cell line was not tested for mycoplasma contamination
Commonly misidentified lines (See ITLCG register) | This study did not use misidentified cell lines

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals | Dat-Cre C57Bl/6J mice (006660) and wild-type C57Bl/6J mice (000664) were purchased from Jackson Laboratory. Mice were injected at ages between 2-4 months and behavior was performed in animals between 3-5 months. Both males and females were used for experiments.
Wild animals | The study did not involve wild animals
Field-collected samples | The study did not involve field-collected samples