

# Cationic Channelrhodopsin from the Alga *Platymonas subcordiformis* as a Promising Optogenetic Tool

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**Abstract**—The progress in optogenetics largely depends on the development of light-activated proteins as new molecular tools. Using cultured hippocampal neurons, we compared the properties of two light-activated cation channels – classical channelrhodopsin-2 from *Chlamydomonas reinhardtii* (*CrChR2*) and recently described channelrhodopsin isolated from the alga *Platymonas subcordiformis* (*PsChR2*). *PsChR2* ensured generation of action potentials by neurons when activated by the pulsed light stimulation with the frequencies up to 40-50 Hz, while the upper limit for *CrChR2* was 20-30 Hz. An important advantage of *PsChR2* compared to classical channelrhodopsin *CrChR2* is the blue shift of its excitation spectrum, which opens the possibility for its application in all-optical electrophysiology experiments that require the separation of the maxima of the spectra of channelrhodopsins used for the stimulation of neurons and the maxima of the excitation spectra of various red fluorescent probes. We compared the response (generation of action potentials) of neurons expressing *CrChR2* and *PsChR2* to light stimuli at 530 and 550 nm commonly used for the excitation of red fluorescent probes. The 530-nm light was significantly (3.7 times) less efficient in the activation of neurons expressing *PsChR2* vs. *CrChR2*-expressing neurons. The light at 550 nm, even at the maximal used intensity, failed to stimulate neurons expressing either of the studied opsins. This indicates that the *PsChR2* channelrhodopsin from the alga *P. subcordiformis* is a promising optogenetic tool, both in terms of its frequency characteristics and possibility of its application for neuronal stimulation with a short-wavelength (blue, 470 nm) light accompanied by simultaneous recording of various physiological processes using fluorescent probes.

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## INTRODUCTION

Microbial rhodopsins (light-sensitive retinal-binding proteins) are the most versatile and widely used optogenetic tools [1]. When expressed exogenously in mammalian cells, they retain their activity as light-gated ion channels or pumps [2]. In particular, these proteins allow minimally invasive regulation of neuronal activity with a

high temporal and spatial resolution both *in vitro* [3, 4] and *in vivo* [5].

Channelrhodopsin-2 cloned from the unicellular alga *Chlamydomonas reinhardtii* (*CrChR2*) [2], is the most extensively used microbial rhodopsin in optogenetics. *CrChR2* requires no additional components to function and has a relatively fast kinetics, high membrane affinity, and virtually no side effects. In many cases, however, this is not sufficient. The development of optogenetics has resulted in the creation of specialized mutants and different variants of channelrhodopsins [6, 7] with accelerated kinetics, reduced desensitization, and modified excitation spectra. Of particular interest are newly

*Abbreviations:* *CrChR2*, channelrhodopsin from *Chlamydomonas reinhardtii*; LED, light-emitting diode; *PsChR2*, channelrhodopsin from *Platymonas subcordiformis*.

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discovered microbial opsins, which quite often are all but ready-made tools suitable for solving particular methodological challenges.

Recently, Govorunova et al. [8] described a new channelrhodopsin isolated from another chlorophyte alga, *Platymonas subcordiformis* (hereafter referred to as *PsChR2*). It differs from *CrChR2* in two important parameters, namely, faster recovery of the peak photocurrent amplitude and a short-wavelength shift of the action spectrum relative to the spectrum of *CrChR2* [8]. Therefore, *PsChR2* could be a potential optogenetic tool in the all-optical electrophysiology experiments, in which visible light is used for both stimulation of neurons and optical recording with fluorescent probes [9]. An essential requirement for such experiments is that the excitation spectra of the opsin and the fluorescent probe either do not overlap or overlap only slightly so that the probe excitation wavelength will not interfere with the physiological activity of the neuron.

Here, we studied the functional properties of *PsChR2* expressed in the primary culture of hippocampal neurons vs. the properties of *CrChR2*-expressing cells and found that *PsChR2* provided significantly better response frequency characteristics in the range of 20–50 Hz compared to *CrChR2*. The 530-nm light commonly used to excite fluorescent probes, activated both *CrChR2*-expressing and *PsChR2*-expressing neurons, while the longer-wavelength 550-nm light caused almost no response in the neurons expressing either of the studied opsins. These facts should be taken into account when planning optogenetic experiments.

## MATERIALS AND METHODS

**Adeno-associated serotype 2 viral vectors** AAV2-CAG-*PsChR2*-Venus (titer,  $5.5 \times 10^9$  genomes/ $\mu$ l) and AAV2-CAG-*CrChR2*-Venus ( $7.2 \times 10^9$  genomes/ $\mu$ l) were synthesized at the Federal Center of Brain Research and Neurotechnologies of the Federal Medical Biological Agency of Russia.

**Primary neuronal cultures** were prepared by the standard technique [10] from the hippocampus of newborn ICR mice on postnatal days 0–2. The hippocampi were extracted in ice-cold Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) containing 15 mM HEPES (Sigma-Aldrich, USA). The tissue was then minced, transferred to 0.08% (mass/vol) trypsin solution in DMEM, and digested for 15 min at 37°C. After centrifugation, the trypsin-containing supernatant was replaced with the incubation medium (see below), and the cells were repetitively triturated. The produced cell suspension was plated onto 12-mm coverslips coated with poly-D-lysine (Sigma-Aldrich). The neurons were cultured in a CO<sub>2</sub> incubator in Neurobasal™-A medium supplemented with B27™ and GlutaMAX™ according to the manufac-

turer's recommendations (Thermo Fisher Scientific) at 37°C in 5% CO<sub>2</sub> at a relative humidity of 95%.

**Viral transduction of cell cultures** was performed on days 5–6 *in vitro*. The neurons were incubated with 0.1% virus solution (vol/vol) in the incubation medium for 24 h. After 24 h, the incubation medium was replaced with the fresh medium.

**Neuronal whole-cell recordings** were performed on days 14–20 *in vitro* at room temperature by the patch-clamp method in the current- or voltage-clamp modes using a Multiclamp™ 700B amplifier and a CV-7B headstage (Molecular Devices, LLC, USA). The data were digitized with Axon™ Digidata® 1550A (Molecular Devices) at 10 kHz, filtered (Bessel filter with 10-kHz cutoff frequency), and recorded with the pCLAMP™ 10 software (Molecular Devices).

The recording bath was mounted on a stage of an Olympus BX51WI epifluorescence microscope (Olympus Corporation, Japan) and filled with the glucose-containing HEPES-buffered Hanks' Balanced Salt Solution (10 mM glucose, 10 mM HEPES, 139 mM NaCl, 5.3 mM KCl, 1.26 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.5 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 4.16 mM NaHCO<sub>3</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.4 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, pH 7.4). Borosilicate patch pipettes were filled with the recording solution designed to mimic the intracellular ion content (140 mM potassium gluconate, 10 mM HEPES, 5 mM KCl, 10 mM phosphocreatine, 4 mM MgATP and 0.3 mM Na<sub>2</sub>GTP, pH 7.31). The resistance of the patch pipettes was 4–7 M $\Omega$ .

Opsin-expressing pyramidal neurons were detected by the fluorescence of the Venus fluorescent protein. Light stimulation was provided with M470L2 ( $\lambda_{\text{max}}$ , 470 nm), M530L3 ( $\lambda_{\text{max}}$ , 530 nm) (Thorlabs, USA), and pE-100 ( $\lambda_{\text{max}}$ , 550 nm) (CoolLED, United Kingdom) light-emitting diodes (LEDs) through the microscope objective.

The illumination intensity under the objective at various LED driving currents was measured with a PM16-130 power meter (Thorlabs) and the data were used to plot the calibration curves that were then used to set the LED driver current in order to achieve the required light stimulus power.

During the experiments, the incident light beam was centered on the neuronal soma. The diameter of the beam was set to the minimally possible (~100  $\mu$ m), with the closed microscope field diaphragm, to reduce the off-target stimulation of the neighboring transfected neurons.

The data were analyzed with the pCLAMP™ 10 package and Python software [11]. Neurons with the resting membrane potential above –40 mV and the cells unable to consistently generate action potentials upon injection of the depolarizing current were excluded from further analysis.

**For statistical analysis**, we used the Mann–Whitney U test, Welch's *t*-test, and Student's *t*-test (the last two, for normally distributed data). The Shapiro–Wilk

test was used to assess the normality of the distribution. The data are shown as mean  $\pm$  standard error of mean unless indicated otherwise.

## RESULTS

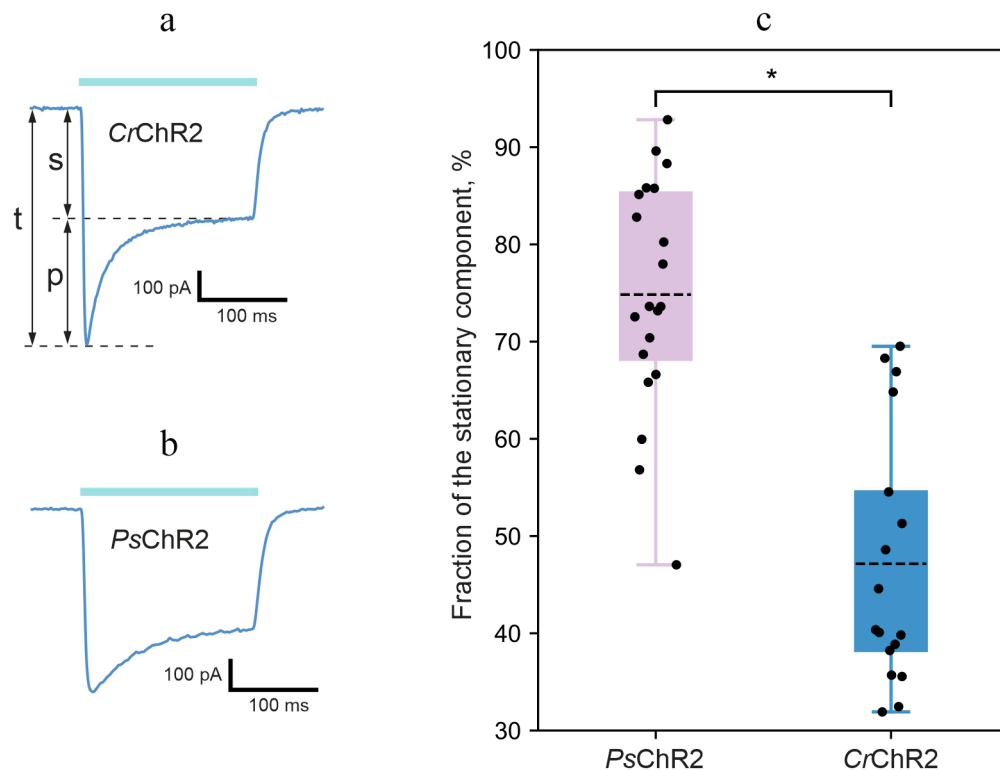
High expression levels of the fluorescent proteins in neurons and in a small number of glial cells were achieved with both viral vectors. All identified fluorescent neurons depolarized in response to a 470-nm light stimulus. The absorption maximum of *PsChR2* is 445 nm [8]. However, we thought it was important to compare the responses of the two opsins to the 470-nm light stimulation, since this wavelength is typical for the default light sources of epifluorescence microscopes commonly used in electrophysiological setups in optogenetic experiments *in vitro*.

In order to compensate for a possible variability in the opsin expression in different cells, the intensity of the light stimulus in the first series of experiments was adjusted for each individual neuron as the maximum intensity that did not induce generation of action potentials.

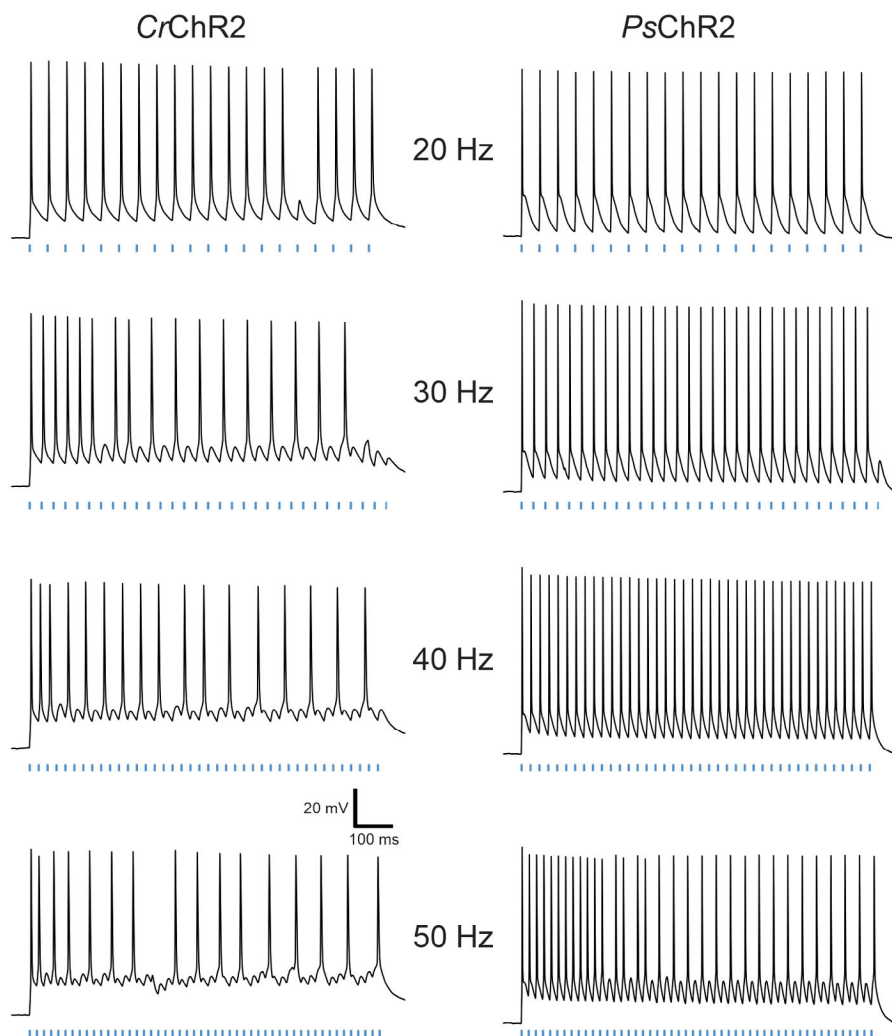
In this series of experiments, the inward light-induced current caused by the activation of channelrhodopsins was recorded in the voltage clamp mode. The average intensity of the light stimuli was  $0.20 \pm 0.16 \mu\text{W}/\text{mm}^2$  for *PsChR2* ( $n = 19$ ) and  $0.25 \pm 0.13 \mu\text{W}/\text{mm}^2$  for *CrChR2*

( $n = 16$ ) ( $p = 0.34$ , Student's *t*-test). A typical shape of the photocurrent induced by the light stimulation of channelrhodopsin-expressing neurons is shown in Fig. 1, a and b. The response included a fast peak component and a slow stationary component. The maximal amplitudes of the stationary component measured upon continuous illumination were close in the two experimental groups [ $320 \pm 30$  pA for *PsChR2* ( $n = 19$ ) and  $300 \pm 40$  pA for *CrChR2* ( $n = 16$ )], with no statistically significant difference between them ( $p = 0.73$ , Student's *t*-test). At the same time, the peak response values were significantly higher in the *CrChR2*-expressing neurons compared to the *PsChR2*-expressing cells ( $620 \pm 80$  pA vs.  $420 \pm 40$  pA, respectively;  $p < 0.05$ , Student's *t*-test). Among other factors, this difference was due to the different ratios between the peak and stationary components for the two channelrhodopsins (Fig. 1, a and b). The contribution of the stationary component to the overall response was substantially and significantly higher in the *PsChR2*-expressing neurons than in the *CrChR2*-expressing neurons ( $75 \pm 3\%$  vs.  $47 \pm 3\%$ , respectively;  $p < 0.001$ , Student's *t*-test) (Fig. 1c). It should be noted that the mean resting membrane potentials did not differ between the two groups ( $-59 \pm 7$  mV for *PsChR2* and  $-62 \pm 8$  mV for *CrChR2*;  $p = 0.34$ , Student's *t*-test).

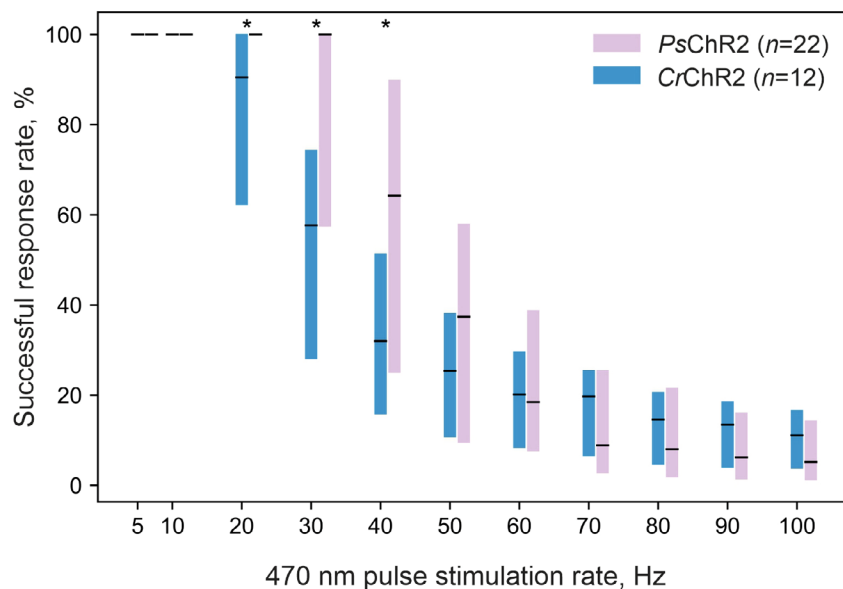
**Pulsed light stimulation.** Previously, it was shown that in channelrhodopsin-expressing human embryonic



**Fig. 1.** Kinetics of the light-induced transmembrane currents in cultured hippocampal neurons expressing *CrChR2* or *PsChR2*. a and b) Representative intracellular recordings of the photocurrents elicited by a 200-ms light stimulus at 470 nm (represented by horizontal blue bar) in channelrhodopsin-expressing neurons. Designations: s, stationary component of the response; p, peak component; t, overall response. c) Fraction of the stationary component in the total response (\*  $p < 0.001$ , statistically significant difference; Student's *t*-test).



**Fig. 2.** Action potentials evoked in cultured *CrChR2*-expressing and *PsChR2*-expressing neurons by the stimulation with short (3-ms) light pulses (represented with blue vertical bars) delivered at different rates (20 to 50 Hz).



**Fig. 3.** The ratio of the number of action potentials elicited by the pulsed light stimulation to the total number of light stimuli. Shaded boxes without whiskers, interquartile ranges; horizontal lines, medians; asterisks, statistically significant difference between the distributions according to the Mann–Whitney U test ( $p < 0.05$ ). When stimulated at 5 and 10 Hz, all neurons in both groups reached 100% successful response rate.

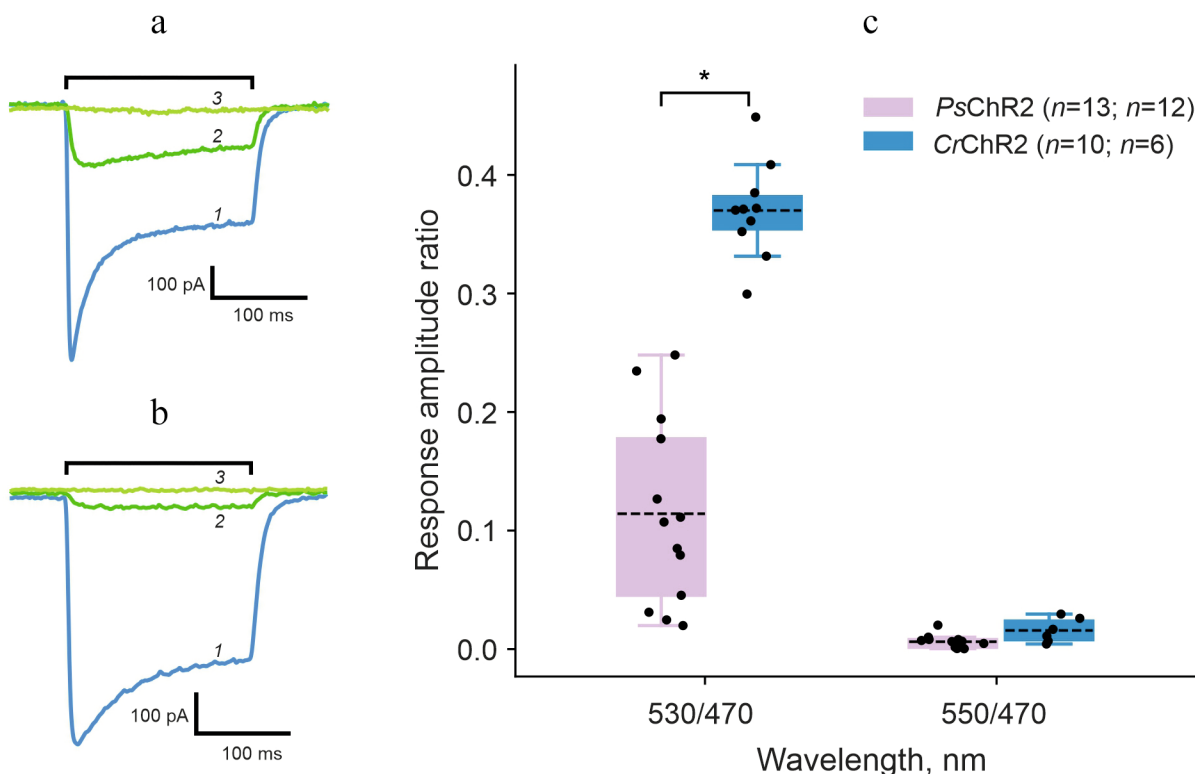
kidney HEK293 cells stimulated with two successive light stimuli, the peak response evoked by the second stimulus restored much better in the *PsChR2*-expressing cells than in the *CrChR2*-expressing cells across a wide range of stimulation frequencies [8]. In this regard, we decided to characterize the maximum frequencies of the action potentials induced by the pulsed light stimulation of *PsChR2*-expressing and *CrChR2*-expressing cultured hippocampal neurons. The light intensity in these experiments was set to be two times higher than the threshold value for the action potential generation (two rheobases).

In both experimental groups, neurons consistently generated action potentials when stimulated with short (3-ms) light pulses at a rate of 5–20 Hz (Fig. 2). The number of missed action potentials in the responses increased as the light pulse rate increased, but in the *PsChR2*-expressing neurons, this effect was observed at higher pulse rates (Fig. 2). The frequency characteristics of the average response had an S-shape (Fig. 3). In the middle frequency range (20–40 Hz), the proportion of successful action potentials in the *PsChR2*-expressing neurons was significantly higher than in the *CrChR2*-expressing neurons ( $p < 0.05$ ; Mann–Whitney U test).

**Responses evoked by green light stimulation.** An important feature of *PsChR2* is the shift of its excitation spectrum to the short-wavelength region relatively to

the spectrum of *CrChR2*, which makes *PsChR2* a more suitable candidate for the experiments using light of different wavelengths for the stimulation and recording of the neuronal activity. Therefore, we decided to test if the *PsChR2*-expressing and *CrChR2*-expressing neurons differ in their response to green (530 and 550 nm) light, which is commonly used for imaging of red fluorescent proteins, and which, to make the above experiments possible, should cause minimal (ideally, none) activation of channelrhodopsins. Similar to the previous series of experiments, the intensity of 470-nm light was adjusted individually for each neuron and set as the maximum intensity that did not evoke action potentials. The same intensity was then used for the stimulation at 530 and 550 nm. The photocurrents elicited by 200-ms light flashes were recorded in the voltage clamp mode.

We found that the 530/470 amplitude ratio in the *PsChR2*-expressing cells was 3.7 times lower than in the *CrChR2*-expressing neurons ( $0.10 \pm 0.08$  vs.  $0.37 \pm 0.04$ , respectively;  $p < 0.01$ ; Welch's  $t$ -test) (Fig. 4). In this series of experiments, the peak amplitude was not measured due to its almost complete absence upon the green light stimulation. The neuronal response induced by the 550-nm light of the same intensity was extremely small in both groups (*PsChR2*,  $0.006 \pm 0.005$ ; *CrChR2*,  $0.014 \pm 0.009$ ;  $p = 0.076$ ; Welch's  $t$ -test). Both results were consistent



**Fig. 4.** Transmembrane currents induced in *CrChR2*-expressing and *PsChR2*-expressing cultured hippocampal neurons by 470-, 530-, and 550-nm light stimuli of equal intensity. a and b) Representative intracellular recordings of the photocurrents in channelrhodopsin-expressing neurons evoked by a 200-ms light flash (indicated with black bracket) at (1) 470, (2) 530, and (3) 550 nm. c) Ratios between the amplitudes of the stationary responses evoked by 530 and 550 nm stimuli and the stationary response evoked by a 470-nm stimulus; \*  $p < 0.01$ , statistically significant difference, Welch's  $t$ -test.



with the previous measurements of the *CrChR2* and *PsChR2* action spectra in HEK293 cells [8]. It is important to note that at a sufficiently high 530-nm light intensity ( $\sim 4\text{--}5\ \mu\text{W}/\text{mm}^2$ ), the generation of action potentials was still possible in both groups of neurons, while the 550-nm light even at the maximum intensity available in our setup failed to produce the suprathreshold activation of neurons.

**All-*trans* retinal application did not affect the activity of channelrhodopsins.** The original paper [8], in which the properties of *PsChR2* were described for the first time, reported that culturing *PsChR2*-expressing neurons in the medium with an increased all-*trans* retinal concentration led to a significant increase in the light-induced current amplitude. In addition to  $0.5\ \mu\text{M}$  retinyl acetate (default component of the standard culture medium), the authors supplemented the medium with all-*trans* retinal to the final concentration of  $0.4\ \mu\text{M}$  [8]. In this regard, we decided to check whether the light response of channelrhodopsin-expressing neurons will be enhanced by adding retinal directly to the recording chamber during the electrophysiological experiment, since microbial rhodopsins are known to quickly capture and covalently bind retinal from the external medium [12].

The neuronal responses to the 200-ms light stimuli at 470 nm were recorded before the addition of  $0.4\ \mu\text{M}$  all-*trans* retinal, as well as 20 min after its addition. We found that the application of retinal did not significantly affect the responses of neurons. If one takes the peak amplitude of the response to the 470-nm stimulus as one, then 20 min after all-*trans* retinal application, the peak response was  $0.93 \pm 0.05$  in the *CrChR2*-expressing neurons and  $0.87 \pm 0.05$  in the *PsChR2*-expressing cells. The slow stationary response component was  $0.86 \pm 0.06$  and  $0.82 \pm 0.06$ , respectively (all differences were statistically insignificant, Student's *t*-test).

## DISCUSSION

*PsChR2* is presumably one of at least three types of *P. subcordiformis* phototaxis receptors. As shown previously, its primary structure and properties are similar to those of other channelrhodopsins from green algae [8]. However, the above-described electrophysiological properties of *PsChR2* distinguish it from others proteins. Therefore, we chose to study *PsChR2* as a potential tool for optogenetic experiments. Despite the similarity to the classical channelrhodopsin *CrChR2*, *PsChR2* consistently provided a higher action potential rate upon the pulsed light stimulation. One of the explanations for this feature might be the reduced desensitization of *PsChR2* during illumination, which had been previously shown by Govorunova et al. [8] and was manifested in our experiments as a smaller fraction of the fast component in the overall re-

sponse to light. At the same time, it should be taken into account that cultured neurons are not completely mature, since their source is the brain of newborn animals, and for a number of reasons they never develop fully. As a result, it is difficult for them to generate action potentials at frequencies above 50 Hz. Therefore, 20-50 Hz can be considered as their productive operating range, within which *PsChR2* has a tangible advantage over *CrChR2*. Light excitation of neurons at these frequencies is the key element in the experiments on the long-term synaptic plasticity induction, as well as in the studies on the features and mechanisms of information encoding and transmission between neurons.

Light illumination in our study was provided with a standard LED with 470-nm emission maximum and 40-nm bandwidth, which is routinely used to activate *CrChR2* in optogenetic experiments. At the same time, the maximum of the *PsChR2* action spectrum measured in HEK293 cells was at  $\sim 445\ \text{nm}$  [8]. This may explain the fact that the amplitude of the light-induced currents in the *PsChR2*-expressing neurons in our experiments was almost the same as in the *CrChR2*-expressing cells. It has been previously demonstrated that when stimulated at 440 nm, *PsChR2* generated a greater photocurrent than *CrChR2* stimulated with 470-nm light [8]. Yet we showed that successful activation of *PsChR2* with a standard 470-nm LED still allowed to achieve the generation of action potentials with high frequencies.

As demonstrated previously in *Xenopus laevis* oocytes, the channelopsin of *C. reinhardtii* is more resistant to degradation when bound to its all-*trans* retinal chromophore [13]. Prolonged incubation of cells in the medium with the elevated retinal content increased the fluorescence of the fused tag and the photocurrent amplitude. It remained unknown whether this effect or similar effects of another mechanism would manifest themselves upon a shorter (tens of minutes) incubation. However, we did not detect any significant effect of retinal application on the activity of *PsChR2* or *CrChR2* expressed in cultured hippocampal neurons.

One of the important features of the new *PsChR2* channelrhodopsin is the left (blue) shift of its action spectrum relatively to the spectrum of classical *CrChR2*. This offers opportunities for using *PsChR2* in all-optical electrophysiology experiments, in which the neuronal activity of a specimen is stimulated and monitored using the light at different wavelengths. Specifically, it is feasible to stimulate channelrhodopsin-expressing cultured neurons with 470-nm light and simultaneously detect the neurotransmitter release from synaptic vesicles with pHluorin-like red fluorescent probes. pHluorin is incorporated into synaptic vesicles and, upon vesicle release, changes its fluorescence intensity due to the pH difference between the contents of the vesicle and the extracellular medium [14]. Another version of a possible all-optical electrophysiology experiment involves optogenetic stim-

ulation of neurons and simultaneous imaging of the intracellular calcium levels in the cell using red fluorescent calcium indicators, such as R-GECO. To verify if the studied channelrhodopsins can be used in such experiments, we compared the responses of *CrChR2*-expressing and *PsChR2*-expressing neurons to illumination with 530-nm and 550-nm LEDs most commonly used to excite red fluorescent proteins. The amplitude of the photocurrent induced in the *PsChR2*-expressing neurons at 530 nm was significantly smaller than in the *CrChR2*-expressing cells. However, stimulation with the high-intensity 530-nm light still elicited action potentials in the *PsChR2*-expressing neurons, though the action potential firing rate was predictably lower than in the *CrChR2*-containing cells. In view of this, we studied the neuronal response to the 550-nm light and found that the light of this wavelength, even at the maximum used intensity, failed to induce action potentials in the neurons expressing either of the studied opsins. Therefore, 550 nm is a preferable wavelength for the excitation of the mentioned red fluorescent probes.

### CONCLUSIONS

Here we showed that the cationic channelrhodopsin from the unicellular alga *P. subcordiformis* is functionally superior to channelrhodopsin-2 from *C. reinhardtii* commonly used in optogenetics. First, *PsChR2* guarantees higher light stimulation frequencies of neurons; second, it is more suitable for the experiments involving simultaneous light stimulation of transfected neurons and optical detection of their responses with red fluorescent probes. In this case, however, the fluorescence of the red probes should be excited at 550 nm.

**Contributions.** A. Y. Malyshev and M. A. Ostrovsky conceived and guided the experiments; G. R. Smirnova and L. E. Petrovskaya carried out the molecular biology experiments; O. S. Idzhilova and D. A. Kolotova carried out the electrophysiological experiments; A. Y. Malyshev, O. S. Idzhilova, and M. A. Ostrovsky wrote and edited the manuscript.

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**Ethics declarations.** The authors declare no conflict of interest. All experimental procedures involving animals complied with the ethical standards of IHNA RAS and the approved legal acts of the Russian Federation and international organizations.

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