Central targeting of channelrodopsin2 by motif of potassium channel Kv2.1 can be altered due to overexpression of construct

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Abstract

Subcellular targeting of opsins in optogenetics provides new possibilities for investigating the function of nerve cells. One of the widely used motifs for central targeting of opsins is the motif of potential-dependent potassium channel Kv2.1. We have expressed construct CHR2-Venus-Kv2.1 in the layer 2/3 pyramidal neurons of the murine cerebral cortex by means of *in utero* electroporation. It was found that, although the majority of neurons expressing CHR2-Venus-Kv2.1 demonstrated mainly central localization of fluorescence in the soma, proximal dendrites and axon, there was also significant population of neurons with disruption of the "correct" targeting resulting in the fluorescent protein distributed uniformly throughout the entire cell surface. We have suggested that observed mislocalization was caused by overexpression of the construct. Indeed, a decrease in the plasmid concentration during the *in utero* electroporation procedure resulted in almost complete absence of neurons with altered targeting. Thus, the possibility of "incorrect" targeting of CHR2 by the potassium channel motif Kv2.1 should be taken into account when using this construct in optogenetic experiments.

Keywords: optogenetics, channelrhodopsin2, subcellular targeting, potassium channel Kv2.1, mislocalization

Introduction

Rapid development of optogenetic methods, observed in the last 15 years, has led to significant progress in cellular neuroscience. Advances in optogenetic research largely depend on the emergence of new molecular tools - light-activated proteins which are able to control neuronal activity. One of the trends in development of optogenetic tools is engineering of light-activated proteins with targeted localization into various subcellular compartments of neurons, which opens new possibilities for studying function of the nerve cell. To ensure expected targeting of opsin, fragments of different protein molecules that have specific localization in the neuron are used. One of these motifs, successfully used for the central targeting of opsins, is the motif of voltage-gated potassium channel Kv2.1. This specific subtype of potential-dependent potassium channels is expressed predominantly in the dendro-somatic compartment of neurons [1]. It was also demonstrated that 26-amino acid fragment of the Kv2.1 channel is capable of localizing fused fluorescent protein in the soma and proximal dendrites of cultured hippocampal neurons [2]. Later, this motif was used in several optogenetic research papers to target different opsins. For example, central localization of channelrhodopsin2 made it possible to provide selective stimulation of neurons by means of two-photon excitation for mapping of cortical networks [3]. Central localization of CHR2 in this case allowed avoiding simultaneous activation of several neurons which often happens when opsin is distributed uniformly throughout the whole cell surface [4]. Also, the central location of chloride conducting opsin GtACR2 prevented some of the negative consequences of its uniform distribution along the neuronal tree [5]. In addition, subcellular opsin targeting was used in such a promising approach as creating ON-OFF receptive fields in ganglion cells for optogenetic retinal

prosthetics [6]. However, in [7] the motif of potassium channel Kv2.1 failed to provide central localization of high-photocurrent channelrhodopsin CoChR. In the current study, we have studied in detail specific features of channelrhodopsin2 targeting with motif of potassium channel Kv2.1 in neocortical slices of mice transfected by *in utero* electroporation. Our experiments   demonstrated conditions leading to inaccurate dendro-somatic localization of a product containing a proximal restriction and clustering signal of Kv2.1.

Methods

All experimental procedures used in this study were approved by Institute of Higher Nervous Activity and Neurophysiology RAS Ethical Committee.

Plasmid construction

Channelrhodophsin2 plasmid (pcDNA3.1hChR2-EYFP) was a gift from Karl Diesseroth (Stanford University, Stanford, CA), and was subcloned into the pCAG plasmid and used before [8]. Fragment of potassium   channel   Kv2.1 (QSQPILNTKEMAPQSKPPEELEMSSMPSPVAPLPARTEGVIDMRSMSSIDSFISCATDFPEATRF) was amplified from cDNA and inserted at the 5' end of Venus [2].

In utero electroporation

In utero electroporation was performed as previously described elsewhere [9, 10]. Briefly, pregnant ICR mice at E14-E15 day of embryonic development were anesthetized for the surgery with isoflurane. Plasmids were injected into the left lateral ventricle of the embryonic brain in a volume of 0.5-1 microliters and concentration of 1 or 0.35 µg/µl (see results). Electroporation was performed with the electroporator NEPA21 Type II (Nepa Gene, Japan) using a planar platinum electrodes applied to the head of the embryo through the uterine wall. Five 35V 50ms voltage steps with 1 s interval were used for electroporation.

Preparation of acute brain slices and electrophysiology

Acute brain slices and whole-cell recordings were performed as previously described using conventional methods at the age P21–30 [11]. Micro-mirror array based spatial illuminator Polygon400 (Mightex, USA) equipped with light-emitting diode (LED) with output power 400 mW and peak emission wavelength of 470 nm was used for optical stimulation of CHR2-expressing neurons.

Morphological analysis

Mice at the age of 1.5 months were anesthetized and perfused intracardially with phosphate buffered saline (PBS) followed by 2% paraformaldehyde in PBS. Brains were dissected and fixed overnight in 2% paraformaldehyde. Coronal slices of the somato-sensory cortex of the left brain hemisphere (50µm) were cut using a vibratome (VT1000S, Leica) and mounted in AquaPolyMount. Fluorescent images were obtained with Zeiss Axioplan epifluorescence microscope (Zeiss, Germany) equipped with CCD camera (Thorlabs, USA). Filter set for yellow fluorescent protein was used: excitation BP500/20, dichroic FT515, emission BP535/30. Transfected neurons were photographed at different focal planes in 1 µm steps and then images were combined to z-stack using the ImajeJ software (NIH, USA). To eliminate uneven background fluorescence we created lowpass-filtered copies of the images using Gaussian filter (blur radius adjusted to remove all morphological details of the neurons, same

value set for all images in a z-stack) and subtracted them from the original images. Finally, the z-stack was collapsed into a two-dimensional image. To reduce experimenter bias in selection of the cells for analysis, all well-visible non-overlapping neurons were numbered. Some neurons were located close to the surface of the slice with their processes directed towards the surface and being cut off during slice preparation. These cells were excluded from the analysis. After that, we selected 100 neurons among the numbered cells based on the pseudo-random number generator sequence. More details of morphological analysis see in "Results" section.

Results

In utero transfection of the mouse embryos on E14-E15 day of prenatal development with the plasmid vector pCAG-CHR2-Venus-Kv2.1 resulted in pronounced expression of the construct in pyramidal neurons of the layer 2/3 of the neocortex at least up to the age 1.5 months. The functional activity of channelrhodopsin2 was confirmed by electrophysiological experiments with intracellular recording of the activity of transfected neurons by the whole cell patch clamp method at age P21-P30. It was shown that illumination of neurons with light with a wavelength of 470 nm causes a pronounced inward transmembrane current in the voltage clamp mode and generation of action potentials in the current clamp (data not shown). Visual examination of transfected neurons revealed that in the majority of the cells fluorescence was localized mainly in the soma, proximal dendrites and the axon of the neuron (Fig. 1A). In the body and the proximal dendrites of transfected cells fluorescence had pronounced membrane localization. However, in the same slice there were cells with fluorescence traced far along the apical and basal dendrites (Fig. 1B). In order to systematically study the localization of fusion protein CHR2-Venus-Kv2.1 a detailed morphometric analysis of transfected neurons in fixed brain sections was carried out. Neurons were captured with CCD camera mounted on an epifluorescence microscope and the brightness of the dendrite membrane was measured over its entire visible length. 1.5 μm width ROIs were manually drawn along the dendrite membrane: one on the left and another on the right side of the dendrite. Similarly, 1.5 μm thick ROI was drawn along the somatic membrane of the neuron (Fig. 1C, D). Fluorescence intensity profiles were calculated along both dendritic ROIs and then averaged to reduce the effect of artifacts. Fluorescence in dendrite was normalized to the average fluorescence intensity of the somatic ROI. For the subsequent analysis points on the apical dendrite located in 10 μm steps from the body of the cell were selected, from 10 to 100 μm. For each point, a 2 μm segment was selected on the fluorescence intensity profile, and the fluorescence values inside this short selection were averaged (for example, the fluorescence value for a 10-μm point is an average intensity from 9 to 11 μm from the soma, Fig.1 E). As a control, we used pCAG-CHR2-Venus plasmid with non-targeted channelrhodopsin2, which was applied to cortical neurons of layers 2/3 by *in utero* electroporation in the same way as the experimental plasmid. All in all 10 slices from four animals from two litters were analyzed in Kv2.1-targeted group and 8 slices from 3 animals from one litter in control group. Figure 2A shows the normalized values of fluorescence along apical dendrite in control neurons and in the neurons expressing pCAG-CHR2-Venus-Kv2.1. The average intensity of Venus fluorescence at all points along the first 100 microns of the apical dendrite in neurons expressing channelrhodopsin2 targeted by the potassium channel Kv2.1 motif was significantly lower than in control neurons. The difference was significant at all measured points ($p < 0.001$, Kruskal-Wallis one way analysis of variance on ranks with Bonferoni correction). However, in 14% of the analyzed neurons expressing pCAG-CHR2-Venus-Kv2.1, the fluorescence was traced beyond 100 μm from the cell body, i.e. central targeting of channelrhodopsin2 by Kv2.1 was disrupted. To estimate the number of cells with "incorrect" targeting pattern, we measured maximal visible length of the apical dendrites for

all analyzed cells. The distributions of the visible lengths of dendrites for neurons expressing non-targeted channelrhodopsin2 and pCAG-CHR2-Venus-Kv2.1 are shown in Fig. 2 B (blue and red histograms). It is clearly seen that the vast majority of the neurons expressing control construct pCAG-CHR2-Venus had at least 100 µm long   apical dendrites (all cells in which the fluorescence was traced more than 100 µm from the soma fell into the category >= 100). The distribution of the visible lengths of the apical dendrites in the neurons expressing pCAG-CHR2-Venus-Kv2.1 had a pronounced bi-modal shape. While the most of the cells demonstrated an average length of the visible process about 30 µm, there was quite large group of neurons in which the mode of distribution of the dendrite lengths corresponded to 100 µm (arrow on Fig.2B). We hypothesized that the over-expression of the product, which leads to "depletion" of the targeting ability of Kv2.1, can cause the wrong fusion protein targeting, and then it spreads unaddressed over the entire surface of the cell. To test this assumption, we performed neuronal transfection by *in utero* electroporation using a lowered plasmid concentration (0.35 µg/µl instead of 1 µg/µl). Visual analysis of brain sections of mice transfected with pCAG-CHR2-Venus-Kv2.1 at low concentration showed that the neurons with the fluorescence observed at any significant distance from the soma were nearly absent. Subsequently, these cells were subjected to the morphometric analysis described above. As seen from Fig. 2A (green dots), the fluorescence intensity along the apical dendrite of the neurons transfected with a low concentration of pCAG-CHR2-Venus-Kv2.1 decreases steeper than in cells from animals transfected with the same plasmid at high concentration. However, we did not observe significant differences between these two groups except of the point 20µm (p<0.001 Kruskal-Wallis one way analysis of variance on ranks with Bonferroni correction, Dunn's post hoc p< 0.01), which is obviously due to abrupt decrease of the number of the cells with dendrites traceable that far from soma (indicated by the numbers near each point in Fig. 2A) in the group with a low plasmid concentration. And the distribution of the visible length of the processes (Fig 2B, green) shows a virtually complete absence of neurons in which Venus fluorescence was discernible at a distance of 40 or more microns from the soma. Moreover, the average length of the visible part of the process differed significantly between all three groups: 140 ± 6.9 µm for neurons expressing non-targeted CHR2, 54.3 ± 4.1 µm for cells transfected with plasmid pCAG-CHR2-Venus-Kv2.1 at a concentration of 1 µg/µl and 21.6 ± 1.8 µm in neurons transfected with the same plasmid at a concentration of 0.35µg/µl (p<0.001 Kruskal-Wallis One Way Analysis of Variance on Ranks, p <0.05 for all combinations, Tukey post hoc) (Fig.2C).

Discussion

        We have confirmed that targeting motif of the potassium channel Kv2.1 is able to provide the localization of CHR2 mainly to the soma, the proximal areas of the dendrites and the axon of the nerve cell. However, under certain conditions, correct targeting can be violated and the construct CHR2-Venus-Kv2.1 can be distributed throughout the neuron's dendrite tree. This happens, most likely, due to overexpression of the protein product. Indeed, threefold lowering CHR2-Venus-Kv2.1 plasmid concentration during *in utero* electroporation led to an almost complete disappearance of neurons with "incorrect targeting" in which fluorescence was spread uniformly through the whole dendritic tree. For the first time, the use of potassium channel Kv2.1 fragment as a target motif was proposed by Lim and co-authors [2]. In this paper, it was shown that Kv2.1 motif linked with GFP is able to target fluorescence protein to the somatic region of cultured hippocampal neurons. It is interesting to note that the authors also analyzed the effect of overexpression on Kv2.1 motif targeting abilities. Their general conclusion was that overexpression of HA-Kv2.1 did not alter the distribution in comparison with endogenous Kv2.1 and therefore overexpression did not saturate the localization machinery. However, it was shown that even then 48 hours after transfection 93% cells had a correct central localization, 96 hours after

transfection fraction of such cells dropped to 80%. Therefore, their data demonstrated a clear tendency to mislocalization with an increase of transfection time (i.e., overexpression), although the authors used the CMV promoter, which is weaker compared to the CAG that was used in our study.

It is interesting that in cells transfected with the high concentration of pCAG-CHR2-Venus-Kv2.1 the distribution of the visible lengths of the dendrites had a pronounced bimodal shape (Fig 2B, red bars). Most neurons had visible length of apical dendrite of 20-30 µm, as well as virtually all neurons transfected with pCAG-CHR2-Venus-Kv2.1 at low concentration. Amount of the neurons with intermediate process length (60-70 µm) was small (gap in the distribution) and there was another significant population of cells in which fluorescence was evenly distributed throughout the dendritic tree (arrow on Fig 2B, red bars). One may speculate that there is a certain threshold of localization machinery saturation and when it is exceeded in some cells, the CHR2-Venus-Kv2.1 structure is distributed unaddressedly over all the processes of the neuron. It explains relatively small amount of transfected neurons with intermediate visible dendrite length.

Motif of potential-dependent potassium channel Kv2.1 was used in several works for targeting of opsins to central area of the cell. This approach was implemented to improve spatial resolution in the optical stimulation of neurons (usually with the use of two-photon excitation) when mapping cortical network connections [3] and during re-creation of the central ON-component of the receptive field of ganglion cell [6]. None of these studies reported any issues with central targeting of opsins. This may be due to several reasons. First, it is possible that there was no systematic analysis of the CHR2-Kv2.1 fusion distribution in the cited papers. In principle, even under conditions of overexpression of the CHR2-Kv2.1 construct in our study, the average length of visible parts of the neuron dendrites was significantly and reliably lower comparative to that measured in control neurons expressing non-targeted CHR2 (Fig 2C). Thus, representation of only average data does not allow revealing the presence of individual neurons, in which the CHR2-Kv2.1 structure is mislocalized and distributed evenly throughout the cell. The second reason for the discrepancy between our results and previously published data may be the use of different transfection method (*in utero* electroporation instead of viral transduction) and stronger universal CAG promoters. In most of the cited works, a synapsin or CamKII promoters were used.

Thus, the possibility of incorrect targeting of the construction carrying the potassium channel Kv2.1 motif as a result of its overexpression must be taken into account when using this targeting motif in optogenetic experiments. One possible approach allowing avoiding the problem may be to reduce the plasmid concentration during the transfection procedure or use less potent promoters than the CAG promoter used in our study.

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FIGURE LEGENDS

Fig. 1 Neurons transfected *in utero* with CHR2-Venus-Kv2.1. Distribution of fluorescence and its quantification. **A** Microphotograph of pyramidal neurons expressing CHR2-Venus-Kv2.1 with "correct" localization. Animals were transfected *in utero* with pCAG- CHR2-Venus-Kv2.1 plasmid in concentration 1µg/µl. Fluorescence is distributed along soma, proximal parts of dendrites and an axon. Scale bar: 20 µm in A and B. **B** In the same slice there were cells in which fluorescence was uniformly spread along whole dendritic tree. **C, D, E** Quantification of the fluorescence pattern distribution. **D** ROIs for somatic and dendritic fluorescence. Scale bar: 10 µm in C and D. **E** Fluorescence profile along dendritic ROI normalized by somatic fluorescence level for the neuron shown in C and D. Fluorescence intensities along red sections of the curve were averaged in order to get values for points 10, 20, 30 and 40 µm from the soma (red circles).

Fig. 2 Distribution of the fluorescence in neurons transfected *in utero* with pCAG- CHR2-Venus-Kv2.1 plasmid in low and high concentration. **A** Normalized fluorescence intensity along apical dendrite in

neurons transfected with control non-targeted CHR2 (blue circles), pCAG-CHR2-Venus-Kv2.1 plasmid in concentration 1 µg/µl (red circles) and with pCAG-CHR2-Venus-Kv2.1 in lowered concentration 0.35 µg/µl (green circles). Significant difference is indicated by vertical lines with asterisks (*** - p<0.001, ** - p<0.01 Bonferroni-corrected Kruskal-Wallis one way analysis of variance). Numbers near each circle indicate amount of neurons, averaged for this point. Mean values ± SEM are presented. **B** Distributions of visible length of apical dendrites in neurons transfected *in utero*. Color code is the same as in A. All neurons with visible length of dendrites exceeding 100 µm fall into the bar 100-110 µm. Arrow points on the second mode of distribution in the group with high plasmid concentration. **C** Box-and-whiskers plot showing visible length of the apical dendrite in neurons from three experimental groups described above. Pairwise difference between all groups was significant (p<0.001 Kruskal-Wallis One Way Analysis of Variance on Ranks, p <0.05 for all combinations Tukey post hoc). Top and bottom edges of the box indicate the 75th and 25th percentiles, respectively. Top and bottom whiskers indicate maximal and minimal values correspondingly with outliners excluded. Thick black horizontal line denotes median. Outliers are plotted as circles.
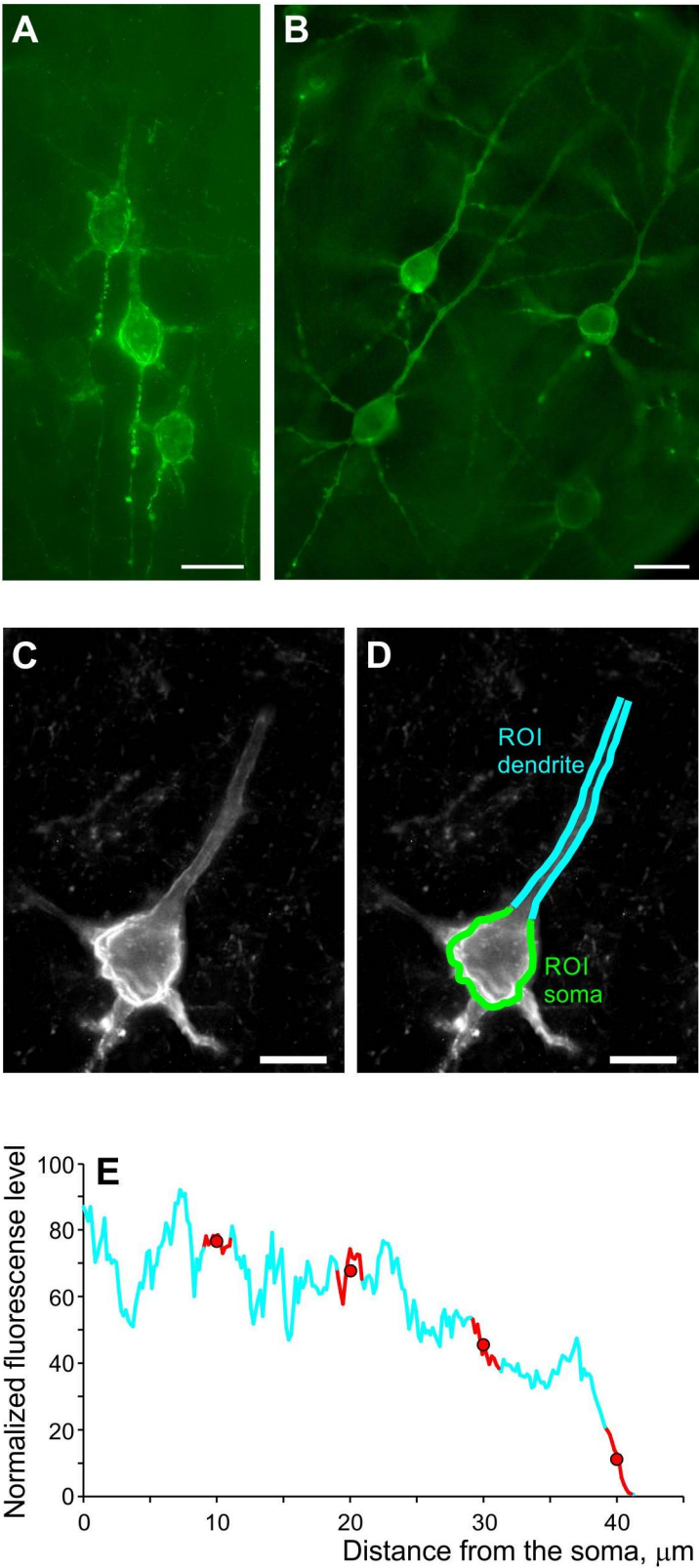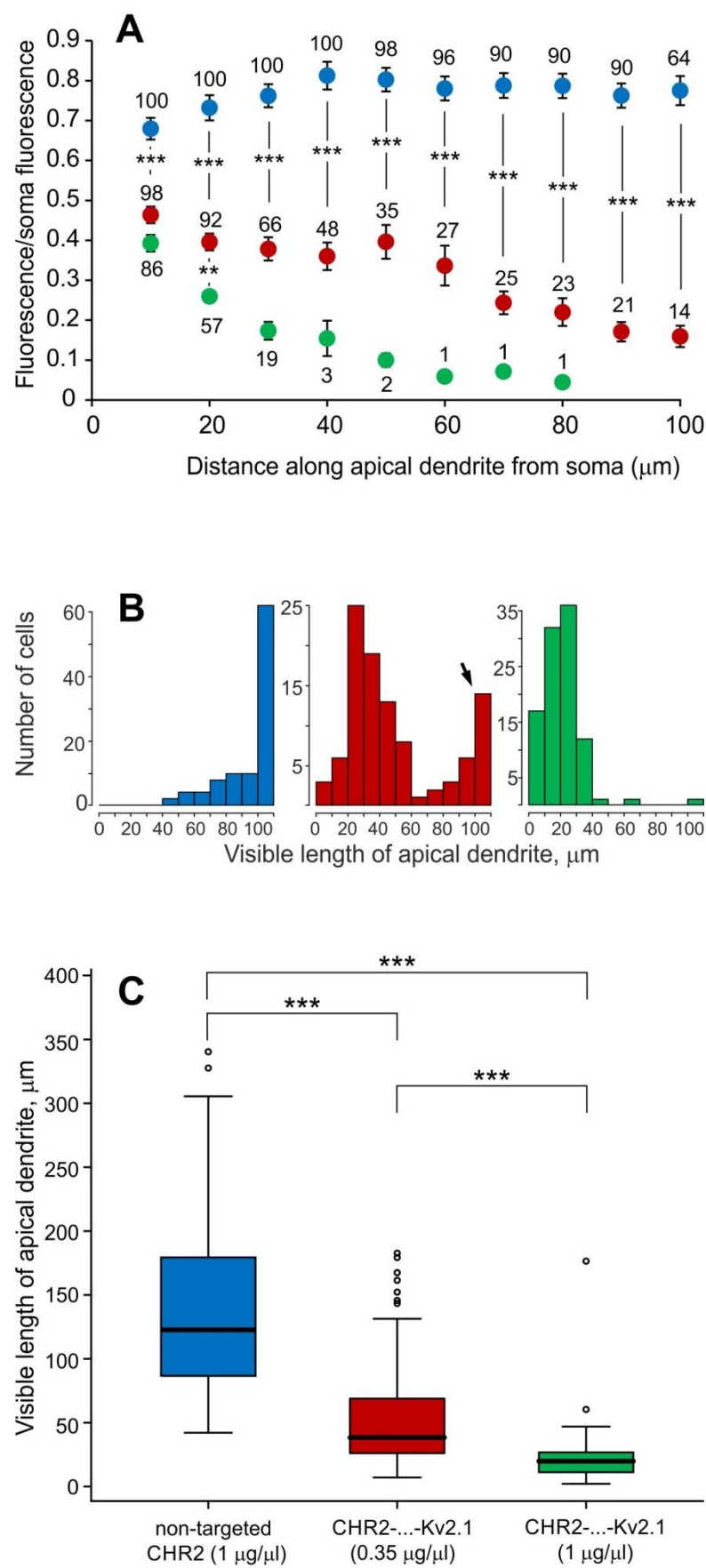
Fig. 1

Fig. 2