Neuroscience Letters 687 (2018) 153–157

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Thermogenetic stimulation of single neocortical pyramidal neurons transfected with TRPV1-L channels

Matvey Roshchin[a], Yulia G. Ermakova[b], Aleksandr A. Lanin[c], Artem S. Chebotarev[c], Ilya V. Kelmanson[b], Pavel M. Balaban[a], Aleksei M. Zheltikov[c], Vsevolod V. Belousov[b], Evgeny S. Nikitin[a,*]

[a] Institute of Higher Nervous Activity and Neurophysiology RAS, Moscow 117485, Russia
[b] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 17997, Russia
[c] Physics Department, International Laser Center, M.V. Lomonosov Moscow State University, Moscow 119992, Russia

ARTICLE INFO

Keywords:
Thermogenetics
Neurostimulation
Neocortex
Patch clamp
Pyramidal neuron

ABSTRACT

Thermogenetics is a promising innovative neurostimulation technique, which enables robust activation of single neurons using thermosensitive cation channels and IR stimulation. The main advantage of IR stimulation compared to conventional visible light optogenetics is the depth of penetration (up to millimeters). Due to physiological limitations, thermogenetic molecular tools for mammalian brain stimulation remain poorly developed. Here, we tested the possibility of employment of this new technique for stimulation of neocortical neurons. The method is based on activation gating of TRPV1-L channels selectively expressed in specific cells. Pyramidal neurons of layer 2/3 of neocortex were transfected at an embryonic stage using a pCAG expression vector and electroporation *in utero*. Depolarization and spiking responses of TRPV1L + pyramidal neurons to IR radiation were recorded electrophysiologically in acute brain slices of adult animals with help of confocal visualization. As TRPV1L-expressing neurons are not sensitive to visible light, there were no limitations of the use of this technique with conventional fluorescence imaging. Our experiments demonstrated that the TRPV1-L + pyramidal neurons preserve their electrical excitability in acute brain slices, while IR radiation can be successfully used to induce single neuronal depolarization and spiking at near physiological temperatures. Obtained results provide important information for adaptation of thermogenetic technology to mammalian brain studies *in vivo*.

1. Introduction

Optogenetic tools offer the possibility to manipulate, in a temporally precise fashion, the electrical activity of specific neurons with brief pulses of light. Thermogenetics is a promising innovative neurostimulation technique, which enables robust activation of neurons using IR-sensitive transient receptor potential (TRP) cation channels. However, a broader application of this technique in mammalian neuroscience is hindered by lack of effective experimental protocols that can be applied to living brains.

Currently, genetically encoded bacterial channelrhodopsins gated by visible light (such as ChR2) are the most commonly used optogenetic probes for single neuron stimulation. On the other hand, usage of optostimulation with visible light *in vivo* causes complications and imposes additional experimental requirements such as two photon excitation [12] or light guide implantation into deeper cortical structures [10]. Thus, the use of conventional optogenetics *in vivo* is strongly limited by light spectra, which is not a limiting factor in the case of channels activated by IR radiation due to its high ability to penetrate live tissue to sufficient depth.

TRP channels are about three orders of magnitude more conductive than ChR channels [15]. Heat-activated TRP channels have been used for thermogenetic activation of cultured mammalian cells [7,9,14,16]. Thermal neurostimulation has been already successfully employed in studies in zebrafish [5], as well as in mice [13]. Importantly, IR-activated TRP channels have been used *in vivo* to control behaviors of Drosophila [11] and zebrafish [7]. Previous studies demonstrated electrical activity evoked in neurons of mammalian cell cultures or cold-blooded animals with thermosensitive channels expressed in temperature-controlled conditions only. So far, mammalian neurons transfected with a thermogenetic probe have not been shown to display normal electrical activity if developed at self-regulating temperature of

* Corresponding author.
E-mail address: nikitin@ihna.ru (E.S. Nikitin).

https://doi.org/10.1016/j.neulet.2018.09.038
Received 30 January 2018; Received in revised form 18 September 2018; Accepted 20 September 2018
Available online 26 September 2018
0304-3940/

a live mammalian brain (37–38 °C in rodents). This information would be crucial for *in vivo* studies as the threshold of a potential thermogenetic probe should fit into a physiological range of temperatures in order to avoid IR-induced heat shock response, which can emerge in the brain even after a ten second exposure to 43 °C [17]. On the other hand, the probe should not activate neurons spontaneously in normal physiological conditions to avoid abnormal firing patterns or excitotoxicity. Because of these restrictions, thermogenetic molecular tools for mammalian brain stimulation remain poorly developed so far. Here, we report a novel technique of thermogenetic stimulation of single pyramidal neurons of neocortical brain slices with the IR-activated TRPV1-L channel, a genetically expressed isoform of TRPV (vanilloid) channel family.

2. Methods

2.1. In utero electroporation

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Department of Humanitarian Expertise and Bioethics of Russian Academy of Sciences. *In utero* electroporation was performed as previously described elsewhere [2]. Briefly, mice (C57BL/6 or ICR) were anesthetized for the surgery with isofluran. The TRPV1-L channel and a red fluorescent protein tdTomato separated by a P2A sequence were expressed under control of the CAG promoter. Presence of P2A resulted in the co-translational 'cleavage' between the TRPV1-L and tdTomato genes. Expression was driven by pCAG-TRPV1L-P2A-tdTomato plasmid (see sequence in Supplement) injected into the left lateral ventricle of the brain of embryos in pregnant mouse at the 15th day of embryo development in a volume of 0.5–1 microliters and concentration of 1.4 mg*ml–1 (details in [2]). The threshold of activation of the TRPV1-L channel, a long isoform of vampire bat (*Desmodus rotundus*) thermoreceptive channel TRPV1 is about 40 °C [8]. Electroporation was performed with the electroporator NEPA21 Type II (Nepa Gene, Japan) using planar platinum electrodes applied to the head of the embryo through the uterus wall (Fig. 1B). Five 50 ms steps (+35 V with 1 s intervals) were used for electroporation.

2.2. Patch-clamp recording

Coronar slices of parietal cortex (300–350 μm) were cut at ice-cold temperature with a vibratome (VT1200S, Leica, Germany), incubated in dissection solution (in mM: 80 NaCl, 25 NaHCO3, 24 glucose, 133 sucrose, 1.3 KCl, 1.1 NaH_2PO_4, 3.3 $MgCl_2$; all from Sigma, USA) for 15 min at 34 °C, and then stored at room temperature. For experimental recording, slices were placed into a chamber continuously perfused with blocker-free ACSF (in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose; details in [1]). Patch pipettes (5–6 MΩ) were filled with intracellular patch solution (in mM): 132 K-Gluconate, 20 KCl, 4 Mg-ATP, 0.3 Na_2GTP, 10 Na-Phosphocreatine, 10 HEPES, pH 7.25–7.3 (all from Sigma). Neurons were patched at near physiological ambient temperature (34 °C) and visualized under DIC infrared optics. Membrane capacitance of each cell was compensated with hardware after establishing a whole-cell configuration. Membrane potential was recorded in whole-cell current clamp mode with an amplifier Axoclamp 2B (output bandwidth: 10 KHz) and acquired at 20 KHz with the ADC board DigiData 1440 A under control of Clampex 10 software (all from Molecular Devices, Sunnyvale, CA, USA).

2.3. Confocal imaging

Live cell imaging was performed with an LSM 5 Live confocal microscope (Zeiss, Germany) equipped with a chromatically corrected water immersion lens (Plan Apochromat IR DIC 63 × 1.0 NA, Zeiss). For imaging and patch-clamp recording, we selected tdTomato-positive pyramidal neurons in layer 2/3. Neurons were filled with a morphological tracer Alexa Fluor 488 (200 μM) by passive diffusion from the patch pipette for ~20 min. We imaged neurons using 488 nm laser/505 L P emission filter (Alexa) and 532 nm laser/550 L P emission filter (tdTomato).

2.4. Photostimulation of neurons

Neurons were stimulated with heat induced by a pulsed output of a fibre laser (IPG Photonics) delivering continuous-wave IR radiation at a wavelength of 1050 nm with an average power up to 10 W A mechanical shutter (SHB05, Thorlabs) was synchronized with the ADC board DigiData 1440 A to provide 30 ms pulses. The energy of pulses provided by the ytterbium laser was 60–210 mJ. To achieve shorter pulse durations (up to 10 ms), we employed trains of IR pulses delivered with a diode-pumped solid-state laser (1342 nm) with a tunable pulse-train duration and pulse-train repetition rate (Fig. 1; more details in Fig. S1 and [7]). IR lasers were coupled to an optical fiber with a core diameter of 50 μm and a numerical aperture of 0.22. The tip of the fiber was positioned at a distance of 0.3 mm from a target neuron to deliver IR radiation to an area with the neuronal soma in the middle of it (area diameter ~60 μm). IR pulses delivered to the preparation were continuously monitored with a photodiode and recorded with the ADC board.

2.5. Statistical analysis

Histograms show means ± SEM. For statistical analysis, ANOVA followed by post hoc tests were used.

3. Results

For thermogenetic stimulation of neocortical neurons, we engineered the plasmid TRPV1-L-P2A-tdTomato to co-transfect the cells with TRPV1-L channel and tdTomato, a fluorescent morphological

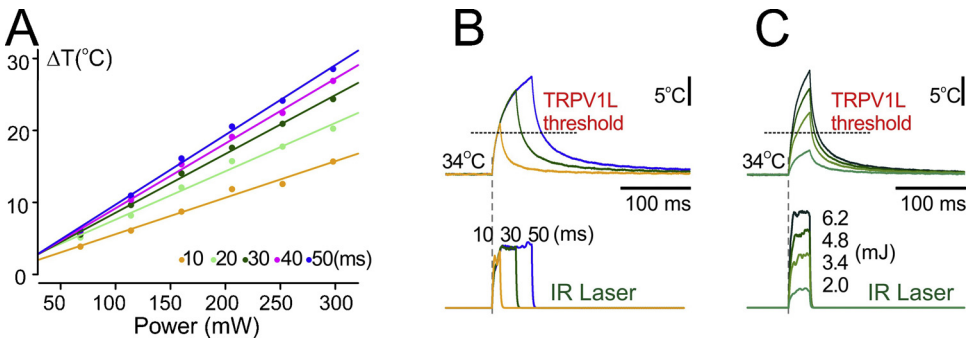

Fig. 1. Temperature calibration of local heating induced with the 1342-nm laser in acute brain slices. (A) Temperature calibration by temperature-dependent change in the current through a micropipette: circles show experimental data and lines are the best linear fit. Pulse durations of 10 ms, 20 ms, 30 ms, 40 ms, and 50 ms are color-coded. Temperature shifts are plotted against measured laser powers. (B) Dynamics of local temperature changes induced by 10 ms, 30 ms, and 50 ms pulses (energy: 1.4 mJ, 4.1 mJ, and 6.8 mJ respectively). Bottom traces show laser intensity as a function of time. (C) Dynamics of local temperature changes induced by 30-s long pulses of 2 mJ, 3.4 mJ, 4.8 mJ, and 6.8 mJ energy. Bottom traces show laser intensity as a function of time. More details in [7].

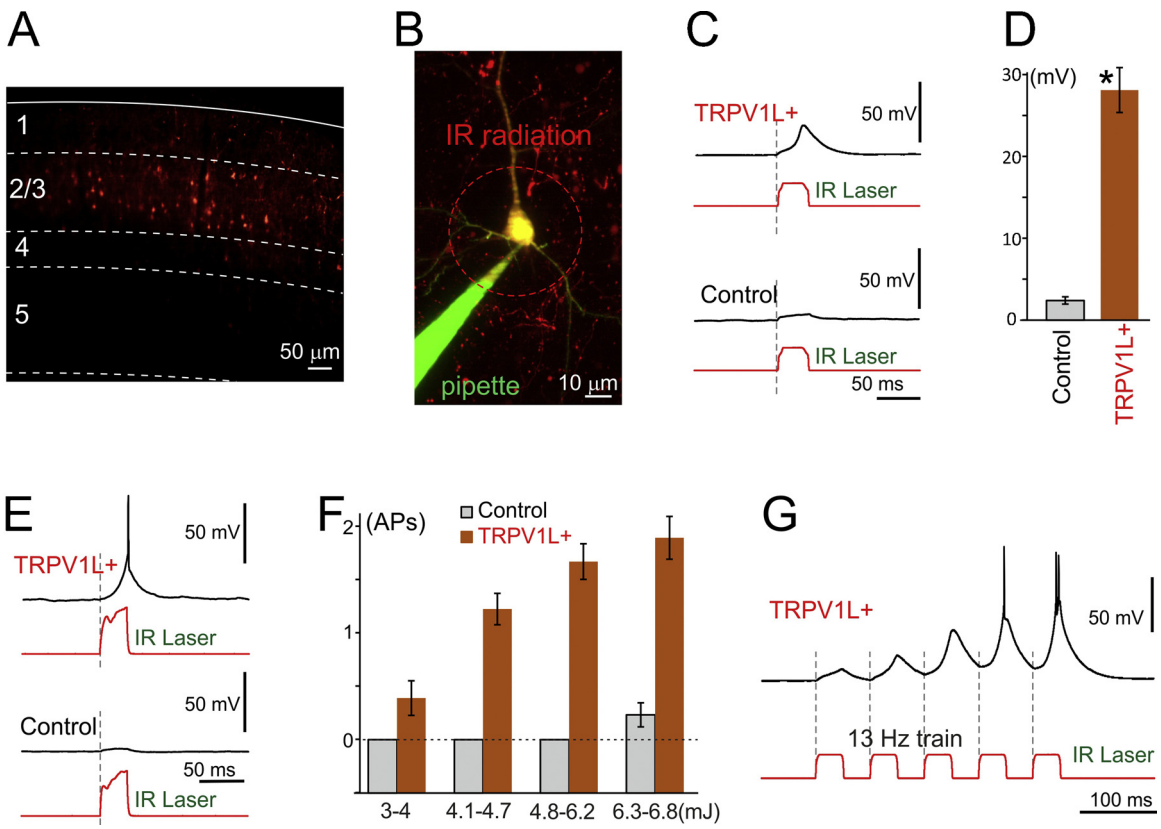

Fig. 2. Confocal imaging and whole-cell patch clamp recording of TRPV1L+ L2/3 neocortical pyramidal neurons. (A) TRPV1L expression in pyramidal neurons of layer 2/3 of neocortex was visualized with co-expressed tdTomato, a red fluorescent marker protein. (B) Green Alexa Fluor 488 dye was added to patch solution to make sure a TRPV1L+ neuron was patched. The dotted circle shows the IR-radiated area. (C) Subthreshold voltage responses in TRPV1L+ neurons (top traces) *vs* control neurons (bottom traces) to IR radiation pulses (duration: 30 ms; energy: 60 mJ; 1050 nm) applied to the area shown in B. (D) Summary histograms of response amplitudes reveal depolarization driven by TRPV1L activation in TRPV1L+ neurons compared to control TRPV1L- neurons. *$p < 0.01$, ANOVA, n = 5, 5 (from 2, 2 animals). (E) Example of spiking response of a TRPV1L+ L2/3 pyramidal neuron to threshold stimulus compared to control stimulation of a non-transfected neuron (duration: 30 ms; energy: 4.1 mJ; 1342 nm). (F) Summary diagram of spiking responses of TRPV1L+ *vs* control neurons to laser pulse powers of 3–4 mJ, 4.1–4.7 mJ, 4.8–6.2 mJ, and 6.3–6.8 mJ; duration: 30 ms; 1342 nm). Dotted line shows a "no spiking" baseline. (G) Summation of subthreshold voltage responses induced by a regular train of pulses leads to spiking (train 5 x 30 ms; single pulse energy: 60 mJ; 1050 nm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

marker. The threshold of activation of the TRPV1-L channel, a long isoform of vampire bat (*Desmodus rotundus*) thermoreceptive channel TRPV1 is about 40 °C [8]. Thus, TRPV1-L channels are unlikely to activate at normal physiological temperatures of rodent neocortex (38–39 °C; [6]). Mice were transfected *in utero* at E15 to achieve transfection of L2/3 pyramidal cells. It resulted in a sparse Golgi-like expression pattern in the adult (28–40 d.o.) neocortex, while tdTomato expression was normally restricted to the neocortical layer 2/3 (Fig. 2A). We used red fluorescence of tdTomato to detect TRPV1-L transfected cells in neocortical slices. After establishing a whole cell configuration, we stained the neurons with an additional green dye Alexa Fluor 488 for 10 ~ 20 min to make sure a TRPV1-L+ neuron was patched (Fig. 2B). The resting membrane potentials of TRPV1-L + neurons were not significantly different compared to control TRPV1-L- neurons (TRPV1-L+: -72.7.4 ± 1.9 mV, control: -74.9 ± 1.7 mV; n = 14, 16 cells from 7, 7 animals; ANOVA, Tukey's Test, P = 0.91; values not corrected for liquid junction potential). Likewise, neither there was difference in input resistance (TRPV1-L+: 81.4 ± 6.1 MΩ, control: -71.0 ± 2.5 MΩ; n = 14, 16 cells from 7, 7 animals; ANOVA, Tukey's Test, P = 0.11) nor in AP threshold (TRPV1-L+: -33 ± 1.9 mV, control: -30 ± 1.2 mV; n = 9, 11 cells from 5, 5 animals; ANOVA, Tukey's Test, P = 0.12) between TRPV1-L+ and TRPV1-L- neurons.

To test the ability of the TRPV1-L channel to evoke membrane depolarization, we recorded responses of neocortical pyramidal neurons to isolated 30-millisecond pulses of IR radiation applied to the soma of neuron, as well as to the proximal parts of axon and dendrites (Fig. 2B). Laser pulses with a central wavelength of 1050 nm and energy of 60 mJ were found to evoke subthreshold voltage responses in layer 2/3 pyramidal neurons (Fig. 2C). Statistical comparison revealed significantly higher depolarization in TRPV1L+ neurons (28.4 ± 2.7 mV) compared to control TRPV1L- neurons (2.4 ± 0.5 mV; n = 5, 5 cells from 2, 2 animals; ANOVA, Tukey's Test, $p < 0.01$; Fig. 2D). These results demonstrate the ability of TRPV1L channels to induce strong membrane depolarization (tens of millivolts) quite quickly, within tens of milliseconds.

To test the ability of the TRPV1-L channels to induce action potentials, we applied IR pulses of increased energy using a 1342 nm laser (Fig. 2E). This laser was able to evoke neuronal activity with relatively smaller energy pulses due to better absorbance by brain tissue. Although low energy (3–4 mJ) pulses usually failed to induce action potentials, threshold energies (4.2–4.7 mJ) induced at least one action potential in > 80% of trials (Fig. 2F). IR stimulation with higher (4.8–6.8 mJ) energies consistently induced 1–3 action potentials in each of the recorded neurons (n = 12 neurons from 5 animals; Fig. 2F). Control TRPV1L- neurons did not display any spiking to 3–6.2 mJ energies; while 2 of 9 neurons (from 5 animals) occasionally produced single APs to highest energy pulses of 6.3–6.8 mJ (Fig. 3F). Similar results were obtained using a 1050 nm laser (Fig. S2). Thus, there was a window of energies (*i.e.* 4.1–6.2, mJ, 1342 nm; Fig. 2F), where IR-

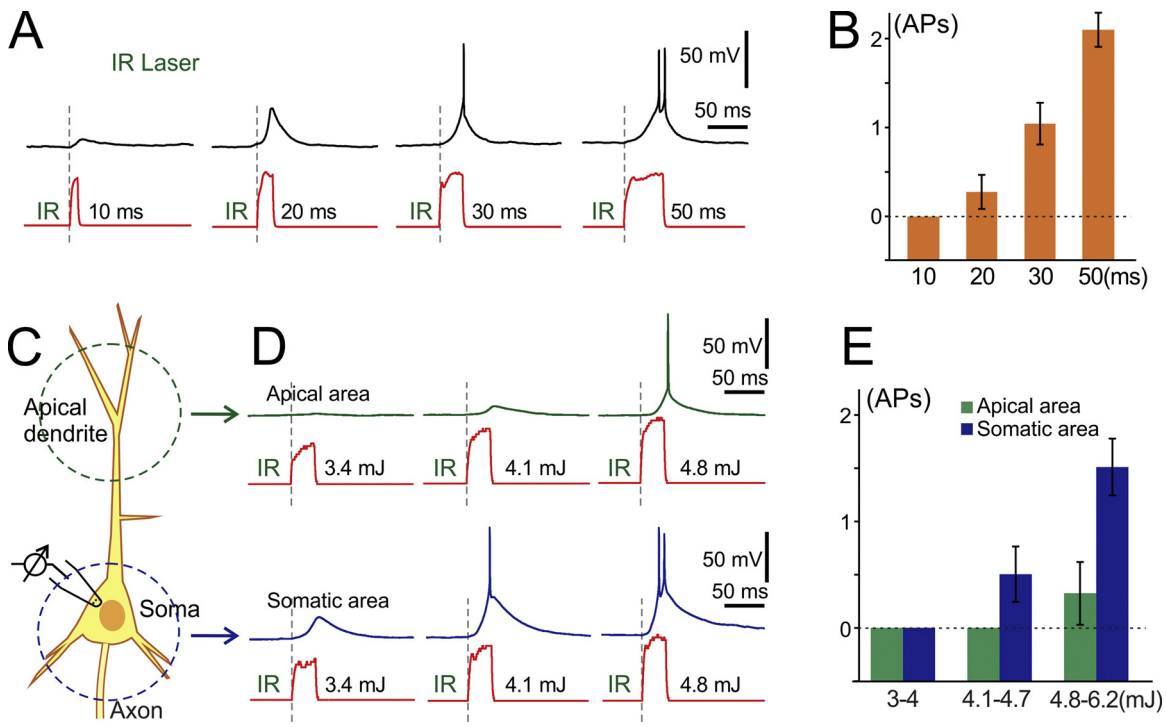

Fig. 3. Depolarizing responses of TRPV1L+ L2/3 pyramidal neurons to IR stimulation. (A) Example of responses of a neuron stimulated with increasing laser pulse durations (10 ms, 20 ms, 30 ms, 50 ms; energy: 1.4 mJ, 2.8 mJ, 4.1 mJ, and 6.8 mJ respectively; 1342 nm). (B) Summary diagram of spiking responses to different durations of laser pulses (5 cells, 3 animals). Dotted line shows a "no spiking" baseline. (C, D) Comparison of responses of a neuron stimulated with increasing energies of laser pulse (3.4–4.8 mJ; pulse duration: 30 ms) at somatic area compared to apical area (shown in C). (E) Summary diagram of spiking responses of neurons to different laser energies applied to somatic area *vs* apical area (3 cells, 3 animals; pulse duration: 30 ms; 1342 nm). Dotted line shows a "no spiking" baseline.

driven activation of TRPV1-L channels produced spiking in TRPV1L + neurons only.

Next, we used a train stimulation with five consecutive subthreshold (90 mJ, 1050 nm) 30 ms pulses separated with 50 ms intervals to reveal putative summation of depolarization induced by TRPV1L channel activation (Fig. 3G). This experiment showed that temporal summation of repetitive depolarizing pulses of subthreshold energy leads to reaching the threshold of action potential generation after several cycles of stimulation (n = 4 cells from 2 animals).

To test how variable duration of IR pulses affects neuronal responses of the TRPV1L+ neurons, we applied 10 ms, 20 ms, 30 ms, and 50 ms pulses of fixed laser power (135 mW, 1342 nm; Fig. 3A). 10 ms pulses did not produce any spiking (Fig. 3B), whereas 30 ms and 50 ms pulses induced APs in > 90% of trials (5 cells from 3 animals). Thus, longer laser pulses are more likely to induce spiking due to temporal summation of IR-driven activation of TRPV1L channels.

Next, we characterized local specificity of IR stimulation of TRPV1L + neurons. As we employed a relatively large laser spot in this study (size ~60 μm), only a comparison between somatic area (soma + proximal axon and dendrites) and apical area (distal apical tuft, Fig. 3C) was possible. Stimulation of somatic area induced spiking at lower powers compared to stimulation of the apical area of the same cell (Fig. 3D and 3E; 3 cells from 3 animals).

4. Discussion

In this study, we demonstrated the ability of the IR-activated thermosensitive cation channel TRPV1-L, expressed by means of *in utero* electroporation, to induce subthreshold depolarization and action potentials in L2/3 neocortical pyramidal neurons of acute brain slices *ex vivo*. Using the IR laser, we targeted a small (~60 μm) area of the slice, which could stimulate only few neurons. The area apparently included the axonal initial segment, a site of action potential generation in pyramidal cells [13]. Taking into account a sporadic expression pattern of TRPV1L, it is very likely that the IR stimulation excited only one neuron in the slice.

To the best of our knowledge, successful thermogenetic stimulation of a neocortical pyramidal neuron with a genetically expressed TRP channel and IR radiation reported in this work is the first experiment of this kind so far. Even more importantly, we demonstrated that TRPV1-L + neurons preserved their electrical excitability in a live mammalian brain. Our work provides an important step towards the adaptation of thermogenetic technology to mammalian brain studies *in vivo*. Compared to conventional optogenetics, IR stimulation does not normally interfere with visual experience of the animal and provides much better depth of penetration due to longer wavelengths of radiation employed.

In a recent study, Chen et al. [4] demonstrated that wireless magnetic stimulation of the ventral tegmental area using magnetic nanoparticles is able to induce immediate-early gene expression in TRPV1+ neurons. In those experiments, the TRPV1 receptor was used as a thermogenetic channel delivered to the brain using viral injection. However, the activation threshold for the TRPV1 channel was about 43 °C [3]. Heating to such a high temperature can be damaging to the brain depending on exposure time [17]. The activation threshold of the TRPV1-L isoform used in this work is ~40 °C [8]. Normally, the temperature of neocortex in awake rodents remains within the 37–38 °C range [6], which is below the activation threshold of TRPV1-L.

5. Conclusions

We introduce the TRPV1-L channel as a thermogenetic tool that activates single neurons in the mammalian brain. The TRPV1-L channel can be expressed in mammalian pyramidal neurons by embryonic electroporation and gated by IR radiation in acute brain slices at near physiological temperatures. The animals with induced TRPV1-L

expression can be potentially used in behavioral experiments with thermogenetic stimulation *in vivo*.

Acknowledgements

The study was supported by Programs of the Russian Academy of Sciences, RFBR grants (17-00-00212, 17-00-00214, 17-00-00216, 16-32-60163, 16-29-11799, 17-04-01690), RSF grant18-75-10112 (electroporation, electrophysiology, imaging), RSF grant17-14-01086 (cloning).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2018.09.038.

References

[1] N. Aseyev, M. Roshchin, V.N. Ierusalimsky, P.M. Balaban, E.S. Nikitin, Biolistic delivery of voltage-sensitive dyes for fast recording of membrane potential changes in individual neurons in rat brain slices, J. Neurosci. Methods 212 (2013) 17–27.
[2] J. Bai, R.L. Ramos, J.B. Ackman, A.M. Thomas, R.V. Lee, J.J. LoTurco, RNAi reveals doublecortin is required for radial migration in rat neocortex, Nat. Neurosci. 6 (2003) 1277–1283.
[3] M.J. Caterina, T.A. Rosen, M. Tominaga, A.J. Brake, D. Julius, A capsaicin-receptor homologue with a high threshold for noxious heat, Nature 398 (1999) 436–441.
[4] R. Chen, G. Romero, M.G. Christiansen, A. Mohr, P. Anikeeva, Wireless magnetothermal deep brain stimulation, Science 347 (2015) 1477–1480.
[5] S. Chen, C.N. Chiu, K.L. McArthur, J.R. Fetcho, D.A. Prober, TRP channel mediated neuronal activation and ablation in freely behaving zebrafish, Nat. Methods 13 (2016) 147–150.
[6] S. DeBow, F. Colbourne, Brain temperature measurement and regulation in awake and freely moving rodents, Methods 30 (2003) 167–171.
[7] Y.G. Ermakova, A.A. Lanin, I.V. Fedotov, M. Roshchin, I.V. Kelmanson, D. Kulik, Y.A. Bogdanova, A.G. Shokhina, D.S. Bilan, D.B. Staroverov, P.M. Balaban, A.B. Fedotov, D.A. Sidorov-Biryukov, E.S. Nikitin, A.M. Zheltikov, V.V. Belousov, Thermogenetic neurostimulation with single-cell resolution, Nat. Commun. 8 (2017) 15362.
[8] E.O. Gracheva, J.F. Cordero-Morales, J.A. González-Carcacía, N.T. Ingolia, C. Manno, C.I. Aranguren, J.S. Weissman, D. Julius, Ganglion-specific splicing of TRPV1 underlies infrared sensation in vampire bats, Nature 476 (2011) 88–91.
[9] H. Huang, S. Delikanli, H. Zeng, D.M. Ferkey, A. Pralle, Remote control of ion channels and neurons through magnetic-field heating of nanoparticles, Nat. Nanotechnol. 5 (2010) 602–606.
[10] X. Liu, S. Ramirez, P.T. Pang, C.B. Puryear, A. Govindarajan, K. Deisseroth, S. Tonegawa, Optogenetic stimulation of a hippocampal engram activates fear memory recall, Nature 484 (2012) 381–385.
[11] S.R. Pulver, S.L. Pashkovski, N.J. Hornstein, P.A. Garrity, L.C. Griffith, Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae, J. Neurophysiol. 101 (2009) 3075–3088.
[12] R. Prakash, O. Yizhar, B. Grewe, C. Ramakrishnan, N. Wang, I. Goshen, A.M. Packer, D.S. Peterka, R. Yuste, M.J. Schnitzer, K. Deisseroth, Two-photon optogenetic toolbox for fast inhibition, excitation and bistable modulation, Nat. Methods 9 (2012) 1171–1179.
[13] M.A. Popovic, A.J. Foust, D.A. McCormick, D. Zecevic, The spatio-temporal characteristics of action potential initiation in layer 5 pyramidal neurons: a voltage imaging study, J. Physiology 589 (2011) 4167–4187.
[14] M.G. Shapiro, K. Homma, S. Villarreal, C.-P. Richter, F. Bezanilla, Infrared light excites cells by changing their electrical capacitance, Nat. Commun. 3 (2012) 736.
[15] M. Tominaga, M.J. Caterina, Thermosensation and pain, J. Neurobiol. 61 (2004) 3–12.
[16] J. Yao, B. Liu, F. Qin, Rapid temperature jump by infrared diode laser irradiation for patch-clamp studies, Biophys. J. 96 (2009) 3611–3619.
[17] P.S. Yarmolenko, E.J. Moon, C. Landon, A. Manzoor, D.W. Hochman, B.L. Viglianti, M.W. Dewhirst, Thresholds for thermal damage to normal tissues: An update, Int. J. Hyperthermia 27 (2011) 320–343.