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Identification of Neurons Containing Calcium-Permeable AMPA and Kainate Receptors Using Ca^{2+} Imaging

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Abstract

Calcium-permeable AMPA receptors (CP-AMPARs) and kainate receptors (CP-KARs) play crucial roles in synaptic plasticity and are implicated in various neurological processes. Current methods for identifying neurons expressing these receptors, such as electrophysiological recordings and immunostaining, have limitations in throughput or inability to distinguish functional receptors. This protocol describes a novel approach for the vital identification of neurons containing CP-AMPARs and CP-KARs using calcium imaging. The method involves loading neurons with Fura-2 AM, a calcium-sensitive fluorescent probe, KCl application to identify all neurons, and further addition of specific AMPAR agonists (e.g., 5-fluorowillardiine) in the presence of voltage-gated calcium channel blockers and NMDAR/KAR antagonists to identify CP-AMPAR-containing neurons. CP-KAR-containing neurons are identified using domoic acid applications in the presence and absence of NASPM (a CP-AMPAR antagonist). This technique offers several advantages over existing methods, including the ability to assess large neuronal populations simultaneously, distinguish between different receptor types, and provide functional information about CP-AMPAR and CP-KAR expression in living neurons, making it a valuable tool for studying synaptic plasticity and neurological disorders.

Key features

- The described protocol allows vital identification of neurons containing calcium-permeable AMPA (CP-AMPARs) and kainate receptors (CP-KARs).
- This approach can be combined with other methods, such as electrophysiological recordings or immunostaining.
- The method is fast, reproducible, and allows non-invasive simultaneous identification of numerous CP-AMPAR-/CP-KAR-containing neurons.
- The described protocol can be used for pharmacological screening of different drugs, including neuroprotectors, or investigation of features of CP-AMPAR-/CP-KAR-containing neurons in health and disease.

Keywords: Calcium-permeable AMPA receptors, Calcium-permeable kainate receptors, GABAergic neurons, Identification, Calcium imaging, Intracellular Ca^{2+} concentration

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Background

Glutamate is a primary excitatory neurotransmitter in the mammalian nervous system that activates various types of glutamate receptors, including AMPA receptors (AMPARs). A subset of AMPARs, known as calcium-permeable AMPA receptors (CP-AMPARs), play a crucial role in synaptic plasticity mechanisms such as long-term potentiation (LTP) and depression (LTD) [1,2]. In addition to Na^+ and K^+ conductance, these receptors are permeable for Ca^{2+} ions due to either the absence of the GluA2 subunit or the presence of the unedited GluA2 subunits [3].

Although CP-AMPARs are involved in normal brain functioning, they are also associated with various pathologies [4], including neurodegenerative diseases such as Alzheimer's [5,6] and Parkinson's diseases [7,8]. CP-AMPAR surface expression is dynamically regulated in response to changes in synaptic activity: while a weak stimulus promotes CP-AMPAR incorporation into the plasma membrane, stronger stimulation favors calcium-impermeable AMPARs [4]. This dynamic regulation underlies their importance in both Hebbian and non-Hebbian forms of plasticity across different brain regions.

Despite CP-AMPARs' significance in synaptic processes, our understanding of their role and the neurons expressing them remains poorly studied. This knowledge gap is partly due to the challenges associated with identifying CP-AMPAR-containing neurons. Current methods for detecting these receptors have notable limitations. Electrophysiological techniques, such as current-voltage relationship analysis and the use of polyamine antagonists, can identify synapses with CP-AMPARs and study their functions in individual neurons [9]. However, these approaches are labor-intensive and limited in their ability to assess large neuronal populations simultaneously. Additionally, some antagonists used in these studies may also affect kainate receptors, potentially confounding results [10]. Immunostaining methods, typically using anti-GluA2 antibodies, allow the evaluation of CP-AMPAR expression across larger groups of neurons [11,12]. However, this approach cannot distinguish between edited and unedited GluA2 subunits, which is crucial as unedited GluA2-containing AMPARs are also calcium-permeable. While most GluA2 subunits in the adult brain are edited, thus resulting in the formation of calcium-impermeable AMPARs, editing levels can change under certain pathological conditions, potentially leading to inaccurate conclusions about CP-AMPAR expression.

To address these limitations, we have developed a novel protocol based on vital fluorescent calcium imaging. This method offers several advantages over existing techniques:

1. It enables the identification of neurons expressing both GluA2-lacking CP-AMPARs and those containing unedited GluA2 subunits.
2. The approach visualizes neurons with a significant number of CP-AMPARs sufficient to induce detectable somatic calcium influx.
3. It facilitates the evaluation of changes in CP-AMPAR expression at the population level, allowing for the study of various experimental manipulations in models of brain pathologies.
4. The technique is less labor-intensive than electrophysiological recordings and provides more functional information than immunostaining alone.

Our calcium imaging-based protocol for identifying CP-AMPAR-containing neurons offers a valuable tool for advancing research in synaptic plasticity, neuronal development, and neuropathology. By bridging the gap between single-cell electrophysiology and population-level immunostaining, this method provides a more comprehensive understanding of CP-AMPAR distribution and function in the nervous system. We believe that it will shed light on the roles of these receptors in both physiological and pathological processes, opening new avenues for research into neurological disorders and potential therapeutic interventions.

Materials and reagents

Biological materials

1. Postnatal (P0-2) Wistar male rats (branch of the M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences)

Reagents

1. Poly(ethyleneimine) solution 50% w/v in water (Sigma-Aldrich, catalog number: P3143); stock and working solutions are stored at 4 °C

2. (±)-Verapamil hydrochloride (Sigma-Aldrich, catalog number: V4629); powder is stored at 4 °C, whereas aliquots are stored at -20 °C
3. Penicillin–streptomycin 100× solution (Sigma-Aldrich, catalog number: P4333); aliquots stored at -20 °C
4. L-Glutamine (Sigma-Aldrich, catalog number: G85402); powder is stored at 4 °C, whereas aliquots are stored at -20 °C
5. (S)-5-Fluorowillardiine (Sigma-Aldrich, catalog number: F2417); powder and aliquots are stored at -20 °C
6. Neurobasal-A medium (Life Technologies, catalog number: 10888022); stored at 4 °C.
7. 50× B27 supplement (Life Technologies, catalog number: 17504044); aliquots of stock solution (we recommend using 1 mL aliquots) are stored at -20 °C
8. Trypsin 2.5% (Life Technologies, catalog number: 15090046); stock solution and working solution aliquots are stored at -20 °C
9. Fura-2 AM (Molecular Probes, catalog number: F1221); undissolved dye and aliquots of the stock solution are stored at -20 °C; the stock solution should be bubbled with argon (recommended) or nitrogen and tightly sealed before freezing
10. Bicuculline (Cayman Chemical, catalog number: 11727); the powder is stored at 4 °C, whereas aliquots are stored at -20 °C
11. UBP310 (Tocris Bioscience, catalog number: 3621); powder and aliquots are stored at -20 °C
12. Domoic acid (Tocris Bioscience, catalog number: 0269); powder and aliquots are stored at -20 °C
13. NASPM trihydrochloride (Tocris Bioscience, catalog number: 2766); powder and aliquots are stored at -20 °C
14. ATPA [(RS)-2-Amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid] (Tocris Bioscience, catalog number: 1107); powder and aliquots are stored at -20 °C
15. D-AP5 (D-2-Amino-5-phosphopentanoic acid) (Alomone Labs, catalog number: D-145); powder and aliquots are stored at -20 °C
16. HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (AppliChem Panreac, catalog number: A3268); room temperature storage
17. Sodium chloride (NaCl) (USP, BP, Ph. Eur., JP) pure, pharma grade (AppliChem Panreac, catalog number: 141659); room temperature storage
18. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S5136); room temperature storage
19. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5156); room temperature storage
20. Potassium chloride (KCl) (USP, BP, Ph. Eur.) pharma grade (AppliChem Panreac, catalog number: 191494); room temperature storage
21. Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, catalog number: P5761); room temperature storage
22. D-(+)-Glucose (Sigma-Aldrich, catalog number: G7021); room temperature storage
23. EDTA [2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid] (AppliChem Panreac, catalog number: A5097); room temperature storage
24. Magnesium sulfate 7-hydrate (USP, BP, Ph. Eur.) pure, pharma grade ($MgSO_4·7H_2O$) (AppliChem Panreac, catalog number: 141404); room temperature storage
25. Calcium chloride ($CaCl_2$) 1 M in H_2O (Sigma-Aldrich, catalog number: 21115); store at 4 °C
26. Isoflurane (IsoNic) (Vetoquinol, 1,000 mg/g); store at 4 °C (the cap of the opened vial should be sealed with Parafilm M or another appropriate material)
27. Dimethyl sulfoxide (DMSO) (USP, BP, Ph. Eur.) (AppliChem Panreac, catalog number: 191954), store at room temperature in the dark
28. Trypan blue (Sigma-Aldrich, catalog number: 302643)

Solutions

1. Polyethyleneimine (PEI) solution (see Recipes)
2. Versene solution (see Recipes)
3. Hank's balanced salt solution (HBSS) (see Recipes)
4. Neuron-glial cell culture growth medium (see Recipes)
5. Trypan blue 0.4% (w/v) solution (see Recipes)

Recipes

1. Polyethyleneimine (PEI) solution 1 mg/mL

Stock polyethyleneimine solution (M_n ~60,000; M_w 750,000) is jelly-like. Weigh the stock solution in a glass beaker, applying the substance on the beaker walls using a spatula. After weighing, add the required volume of double-distilled

water and stir the solution for at least 30 min at room temperature using a magnetic stirrer. Then, filter the obtained PEI solution through a 0.22 μm membrane syringe filter for sterilization. Store the working solution at 4 °C and avoid freezing.

2. Versene solution

Reagent	Final concentration
NaCl	137 mM
KCl	2.7 mM
KH_2PO_4	2 mM
Na_2HPO_4	8 mM
EDTA	0.6 mM

After EDTA addition, the solution should be heated to 40–50 °C at constant stirring to dissolve this chelator. To obtain a sterile Versen solution, it should be filtered through a 0.22 μm sterile syringe filter after dilution of all components. Double-distilled water is used to prepare the Versene solution. The prepared solution can be stored at room temperature or 4 °C.

3. Hank's balanced salt solution

Reagent	Final concentration
NaCl	137 mM
KCl	3 mM
Na_2HPO_4	0.35 mM
KH_2PO_4	1.25 mM
$NaHCO_3$	4.2 mM
D-(+)-Glucose	10 mM
HEPES	10 mM
$MgSO_4 \cdot 7H_2O$	0.8 mM
$CaCl_2$	1.4 mM

Adjust pH to 7.35 using 10 M NaOH (the temperature of the solution during the pH adjustment must correspond to the temperature at which imaging experiments will be performed; in our studies, we perform all experiments at 28 °C). Since the neurons are sensitive to the pH of the extracellular solution, check the pH of HBSS before the experiments if HBSS is stored at 4 °C or more than one day at room temperature (slight alkalization is observed).

4. Neuron-glial cell culture growth medium

Reagent	Final concentration
Neurobasal A-medium	n/a
50× B27 supplement	2% (v/v)
L-glutamine	0.5 mM
100× Penicillin-streptomycin	1% (v/v)

L-glutamine is unstable in aqueous solutions. Add L-glutamine immediately before the cell culture preparation or use stable analogs. Due to the instability of some components, we recommended preparing small portions of neuron-glial cell culture growth medium (50–100 mL) and storing them at 4 °C for no more than 1 week.

5. Trypan blue 0.4% (w/v) solution

Trypan blue 0.4% working solution is prepared by dissolving the weighed powder in phosphate-buffered saline (PBS). PBS composition is listed below (pH 7.35). Before the application, the solution was filtered through a syringe filter with a pore size of 0.22 μm to remove undissolved aggregates.

Reagent	Final concentration
NaCl	137 mM
KCl	2.7 mM
KH_2PO_4	2 mM
Na_2HPO_4	8 mM

Laboratory supplies

1. Cell culture dishes 35 mm (SPL Lifesciences, catalog number: 11035)
2. Cell culture dishes 60 mm (SPL Lifesciences, catalog number: 11060)
3. Cell culture dishes 150 mm (SPL Lifesciences, catalog number: 10151)

4. Sterile microcentrifuge tube 1.5 mL (GenFollower, catalog number MCTB015)
5. Sterile centrifuge tubes 50 mL (GenFollower, catalog number B-2SCTB50YE)
6. Low-retention pipette tips 200 µL (GenFollower, catalog number E-FTR200-L-S)
7. Low-retention pipette tips 1000 µL (GenFollower, catalog number E-FTR1000-L-S)
8. Round cover glasses 25 mm (VWR, catalog number 630-2122)
9. Syringe filter 0.22 µm (Membrane Solutions, catalog number MS-SFPES025022SI)
10. Cover glasses 24 × 24 (Minimed, catalog number 12003316)

Equipment

1. Thermoshaker (Biosan, model: TS-100, catalog number BS-010120-AAI) with thermoblock SC-18/02 (Biosan, catalog number BS-010120-CK)
2. High-speed centrifuge (DLab, model: D2012, catalog number 9032002121)
3. Laminar flow hood (Lamsystems, model: BMB-II-"Laminar-S."-1,2 NEOTERIC, catalog number 2E-B.001-12)
4. Trinocular inverted microscope (Nikon, model: Eclipse TS100)
5. Stereo microscope (Leica Microsystems, model: EZ4D)
6. CO_2 incubator (N-Biotek, model: NB-203XL)
7. Epifluorescent inverted microscope (Leica Microsystems, DMI 6000B) with CCD-camera (Hammamatsu, model: 9100C) and illuminator (Leica Microsystems, model: EL6000)
8. Analytical scale (Ohaus, model: Explorer EX324)
9. pH meter (Mettler Tolledo, model: S20 SevenEasy)
10. Magnetic stirrer with a hot plate (Biosan, model: MSH-300)
11. Bidistiller (GFL, model: 2104)
12. Multi-output animal anesthesia machine (RWD Life Science, model: R550)
13. Drying and heating chamber with natural convection (Binder Inc., model: ED23)
14. Neubauer chamber (JVLAB, catalog number: 1103)
15. Instruments for tissue extraction and preparation: dental curved tweezers 150 mm (catalog number: 10-94), dental spatula (catalog number: 10-97), and medical blunt-pointed straight scissors (catalog number: 4-26) from Mozhaisk Medical Supplies Factory; curved scissors (catalog number: TH-06-041-11,3) and scalpel (catalog number: TC-02-051-15) from Tumbotino Medical Supplies Factory; sharp-pointed straight (catalog number: ST-14) and curved (catalog number: ST-15) tweezers from NAGARAKU, Shanghai Xijian Electronic Technology Co., Ltd.

Software and datasets

1. ImageJ v1.53s (https://imagej.net/ij/, May 2022)
2. OriginLab Pro 2016 version b9.3.226 (commercially available software, license required; other free software for plot creation can be used as an alternative, October 2015)

Procedure

A. Hippocampal cell culture preparation

1. Coat the round cover glasses with polyethyleneimine solution.
a. Sterilize the glasses in a dry heat sterilizer (or using a laboratory burner).
Note: Sterilization in a dry heat sterilizer should be performed for 2 h at a temperature of 150 °C. In the case of the laboratory burner, use tweezers and hold a cover glass in the flame for no more than 1 s each side. After that, hold for 10–20 s away from the flame to allow it to cool. If the cover glass is overheated, it can crack during cooling. Hence, sterilization with a dry heat sterilizer is preferable.
b. Coat sterile glasses with 1–2 mL of polyethyleneimine solution and leave it in a laminar hood for 1 h (fan should be turned off to prevent drying; on-board UV lamp can be turned on).
Note: You can simultaneously coat dozens of cover glasses placed in large sterile Petri dishes (≥120–150 mm in diameter)

or another appropriate bath. For these purposes, both dishes and lids can be used. Approximately 15–18 cover glasses (25 mm diameter) can be arranged in a 150 mm Petri dish.
c. Wash the glasses three times with sterile (boiled for 15 min and cooled to room temperature) double-distilled water and leave them for 12 h in a laminar hood to dry completely.
Note: If the Petri dishes are used for the arrangement of the cover glasses during PEI treatment, sterile water can be added to the dish during the washing procedures. The volume of water used for each wash step depends on the used bath but, to efficiently wash the cover glasses, the level of water in the bath has to be 3–5 mm higher than the surface of the cover glasses.
d. Place the PEI-treated dry glasses in the sterile 35 mm cell culture dishes.

2. Before the hippocampus extraction, prepare the sterile instruments (medical scissors, scalpel, dental spatula, and tweezers) and a small bath with ice.
3. Place in the bath the 60 mm cell culture dish with the Versene solution (8–10 mL) and two microcentrifuge tubes (one with 1 mL of neuron-glial cell culture growth medium and the second one with Versene solution).
4. To euthanize the animal, put it in the preconditioning (induction) chamber and fill the chamber with isoflurane (5%) or another appropriate inhaled anesthetic.
Note: The incubation time depends on the anesthesia used and the weight and strain of the used animal.
5. After incubation, take the euthanized animal from the chamber and thoroughly treat it with 70% ethanol (it is better to use a pulverizer for this purpose).
6. Then, using sharp medical scissors, quickly decapitate the animal.
7. Carefully cut the skull using sharp scissors (curved nail scissors are recommended for this procedure).
8. Extract the brain using a dental spatula and put it into the 60 mm cell culture dish with the ice-cold Versene solution.
9. Cut hemispheres along the line from the cerebellum to the olfactory bulb with a scalpel.
Note: It is better to use curve tweezers to hold the brain during making the cut and further procedures.
10. Remove the parts of the hippocampus from both hemispheres and put them into the 1.5 mL microcentrifuge tube with ice-cold Versene solution.
Note: We recommend using a dental spatula and tweezers to remove the hippocampus.
11. Cut the tissue in the tube into 0.5–2 mm fragments using sterile scissors.
12. Replace the Versene solution in the tube with 500 µL of 1% trypsin solution.
Note: To obtain the working trypsin solution, dilute the stock in Versene.
13. Incubate the hippocampal fragments with trypsin at 37 °C at constant stirring for 10 min (stirring rate of 500 rpm, 0.6× *g*).
Note: A laboratory shaker with a heater can be used for this purpose.
14. Remove the trypsin solution and wash the tissue fragments two times with 500 µL of cold neuron-glial cell culture growth medium to inactivate trypsin.
Note: Avoid the trituration of tissue fragments when replacing the medium in the tube. Pipette tips produced from plastic with low retention are recommended since the trypsinized tissue fragments are sticky.
15. Add 1 mL of room-temperature neuron-glial cell culture growth medium and gently triturate tissue fragments using a 1 mL pipette tip.
16. Wait for 30–60 s until the non-triturated fragments sediment; then, carefully remove them.
17. Carefully remove the non-sedimented film-like tissue fragments using a 200 µL pipette tip.
18. Centrifuge the obtained cell suspension at 2,000× *g* for 3 min at room temperature.
19. Remove the supernatant and resuspend the pellet to obtain a cell suspension with a cell quantity of 10^6–10^7 cells/mL.
Note: The cells can be counted manually with different counting chambers or automatically using cell counters. We used the Neubauer chamber for cell counting. For the staining, the "stock" cell suspension was diluted 10 times with neurobasal medium to expedite the counting procedure, and this dilution factor was considered in calculations of average cell quantity. Before counting, the diluted cell suspension was mixed in proportion 1:1 with 0.4% trypan blue solution (5 min staining).
20. Dilute the obtained suspension if necessary and drop the aliquots of the cell suspension on the PEI-treated round cover glasses. The optimal cell density is 30,000–50,000 cells/mm^2.
Note: To concentrate the cells in the restricted area of the cover glass, we use the sterile glass cylinders placed on the cover glasses lying in 35 mm cell culture dishes (Figure 1A). The height used in the study cylinders was 6 mm, and the internal diameter was 5 mm; the working volume ≈ 100 µL. The cylinders can be produced from glass serological pipettes, for instance. After cell attachment, the cylinders are carefully removed with the curved tweezers. Twenty-four hours after the preparation, the representative image of the hippocampal cell culture (Figure 1B) demonstrates the average cell density that can be achieved using glass cylinders.

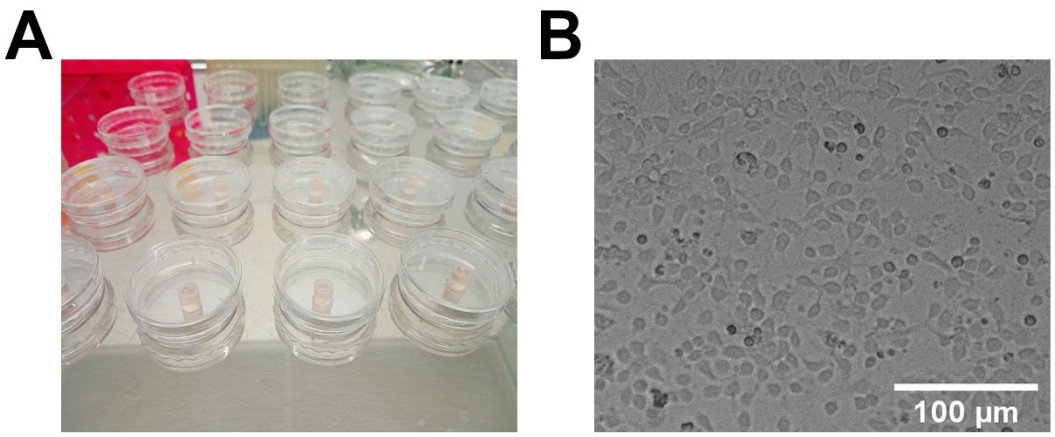

Figure 1. Hippocampal cell attachment. A. Glass cylinders used during cell culture preparation. B. Brightfield images of a representative cell culture 24 h after cell attachment.

21. Put the cell culture dishes with the cylinders in the CO_2 incubator for 30–60 min for cell attachment.
22. Remove the dishes with the cell cultures and add 2 mL of the neuron-glial cell culture growth medium (temperature ≈ 37 °C) in each cell culture dish. If the glass cylinders were used, carefully remove them (use curve tweezers) before the addition of the growth medium.
Note: The average passage of the hippocampal cell cultures obtained from one animal includes 12–14 cell cultures (cell culture dishes with the PEI-treated glasses covered with the hippocampal cells).
23. Cultivate the cultures in a CO_2 incubator at 37 °C in an atmosphere containing 5% CO_2 (humidity ≥ 95%) for 12–14 days and use in experiments.
Note: Frequent cell culture medium replacement affects the quality of neuronal networks in the cell culture. Replace 1/2 of the medium volume at 5–6 DIV (days in vitro) and keep the humidity in the incubator at ≥ 95% (this is important).

B. Staining of the cultures with Fura-2

1. Dissolve Fura-2 AM stock solution (1 mM in DMSO) to a working concentration of 3 µM and incubate the cell cultures with the working solution for 40 min at 28 °C in the dark.
Note: To decrease the consumption of Fura-2, the glasses with the cell cultures can be transferred from the cell culture dishes to their lids and covered with 200 µL of the working solution. This volume is enough for cell staining.
2. Remove the dishes with 12–14 DIV neuron-glial cell cultures from the CO_2 incubator, wash them twice with HBSS solution (2 mL per wash), wait for 10 min, and wash once again (2 mL).
Note: The pause between washes is required to allow the non-esterified probe to flow from the cells to the extracellular medium.
3. Mount the cover glass with the cell culture into the chamber for measurements.
4. Choose the area with the monolayer to record the signal from the maximal number of cells. The optimal ratio between magnification and the number of cells in the view field can be achieved with 10.0× and 20.0× objectives (Figure 2A).
5. Use a perfusion system providing a 10 mL/min flow rate to effectively and quickly replace the medium in the microscope chamber.

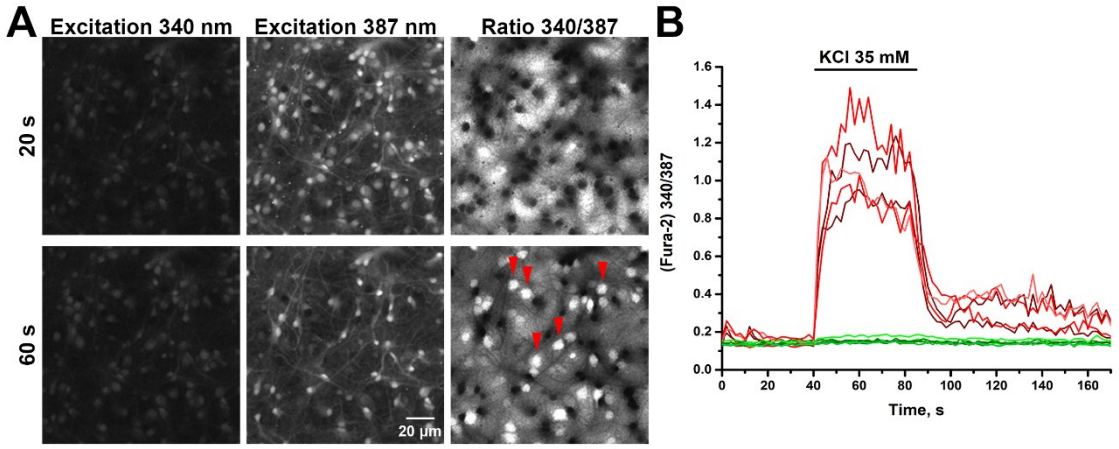

Figure 2. Fura-2 imaging. A. Representative image of Fura-2-stained rat hippocampal cell culture. Objective HC PL FLUOTAR 10.0 × 0.30 DRY with an additional 1.6 magnification lens. Red arrows show the neurons whose changes in intracellular Ca^{2+} concentration are shown in panel B. Images show the fluorescence of cells before (20 s) and during (60 s) the KCl application (see panel B). Excitation 340 nm: maximum of the excitation spectrum of Ca^{2+}-bound form of Fura-2; 387 nm: maximum of the excitation spectrum of Ca^{2+}-free form of Fura-2. B. Changes of intracellular Ca^{2+} concentration in cells of the neuron-glial culture in response to KCl (35 mM) application. Red curves correspond to neurons, while green curves correspond to glial cells.

C. Identification of neurons in hippocampal cell culture

1. To visualize the neurons stained with a Ca^{2+}-sensitive fluorescent probe (Fura-2 AM in this case), perform the application of 35 mM KCl.

Note: As shown in previous works, KCl application (35 mM) depolarizes the neurons and stimulates the release of neurotransmitters, particularly glutamate. Depolarization induced by KCl and activation of ionotropic glutamate receptors (AMPARs, NMDARs, and KARs) promotes the opening of voltage-gated Ca^{2+} channels (VGCC) [13,14], mediating Ca^{2+} inflow into the soma of neurons. These Ca^{2+} changes are detected with different Ca^{2+}-sensitive probes, such as Fura-2, Fluo-3, Fluo-4, or Fluo-8. Neurons quickly restore calcium homeostasis after KCl application. Therefore, this method of identification of all neurons in a view field can be used in routine calcium imaging experiments.

2. The cells responding with a sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to KCl application are neurons (Figure 2B).

Note: Using a combination of live cell fluorescent calcium imaging and post-vital immunostaining, we have previously demonstrated that cells responding to KCl application contain the neuronal marker, NeuN [15].

D. Identification of neurons containing Ca^{2+}-permeable AMPA receptors

1. To identify neurons containing the calcium-permeable AMPA receptors, perform the application of selective AMPAR agonist (5-fluorowillardiine, AMPA, etc.) in the presence of non-selective blocker of VGCC (verapamil or diltiazem, for example), antagonists of NMDA and kainate receptors [16].

Note: As shown in Figure 3A, $[Ca^{2+}]_i$ elevation in neurons in the presence of AMPAR agonists is mediated in most neurons by VGCC, NMDARs, and KARs. The sustained elevation in the presence of the appropriate antagonists/blockers remains only in a minor population of neurons.

2. The cells responding with sustained $[Ca^{2+}]_i$ elevation to the application of AMPAR agonists in the presence of VGCC blockers and NMDAR and KAR antagonists can be attributed to neurons containing calcium-permeable AMPA receptors (CP-AMPAR-neurons).

Note: This $[Ca^{2+}]_i$ elevation is sensitive to the antagonist of Ca^{2+}-permeable AMPARs, NASPM (Figure 3B).

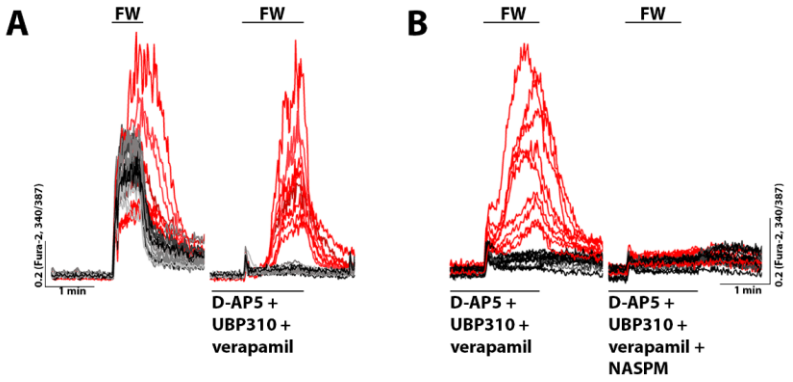

Figure 3. Identification of neurons containing CP-AMPARs. A. Responses of neurons in rat hippocampal neuron-glial cell cultures to the application of selective AMPAR agonist, 5-fluorowillardiine (FW, 500 nM) in the absence and presence of the blocker of voltage-gated calcium channels (verapamil, 300 μM) and NMDAR and KAR antagonists (D-AP5, 10 μM and UBP310, 10 μM, respectively). Red curves: neurons containing CP-AMPARs; grey curves: other neurons. B. Effect of NASPM (selective antagonist of CP-AMPARs, 30 μM) on the $[Ca^{2+}]_i$ elevation induced by AMPAR activation in neurons containing CP-AMPARs.

D. Identification of neurons containing Ca^{2+}-permeable KA receptors

1. To identify neurons containing the calcium-permeable KA receptors, perform two applications of KAR/AMPAR agonist, domoic acid (DoA, 200–500 nM), in the presence and absence of NASPM (30 μM).

Note: We have shown in our previous works that in rat hippocampal neuron-glial cultures, the neurons containing CP-AMPARs and CP-KARs are GABAergic [16,17], and their selective activation suppresses the activity of the innervated glutamatergic neurons. Due to insufficient GABA-mediated innervation, GABAergic neurons [16], including neurons containing CP-KARs, respond earlier to depolarizing stimuli. To eliminate the effects associated with GABA-mediated inhibition and the identification of neurons containing CP-KARs, DoA applications should be performed in the presence of GABA(A)R antagonists [the contribution of GABA(B)R is less pronounced in this case]. We used bicuculline (10 μM) for this purpose, but other GABA(A)R antagonists, such as gabazine or picrotoxin, can also be used. Notably, the application of GABA(A)R antagonists induces epileptiform activity manifested as oscillations of $[Ca^{2+}]_i$.

2. The neurons insensitive to NASPM (Figure 4, green curves) can be attributed to neurons containing calcium-permeable KARs.

Note: We have demonstrated that neurons containing CP-KARs and neurons containing CP-AMPARs also respond with $[Ca^{2+}]_i$ elevation to the application of selective agonist of GluK1/GluK3 containing KARs, ATPA (300–500 nM) [16]. Since ATPA demonstrates lower excitotoxicity than DoA or selective AMPAR agonists, ATPA-induced $[Ca^{2+}]_i$ elevations can be considered an additional marker of GABAergic neurons containing CP-KARs or CP-AMPARs.

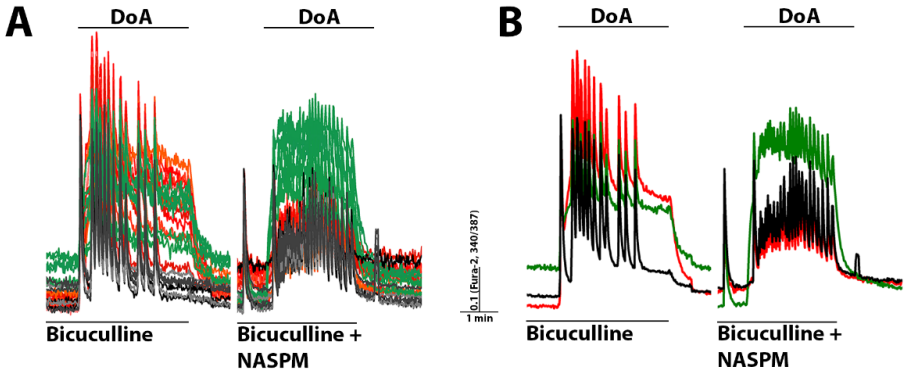

Figure 4. Identification of neurons containing CP-KARs. A. Responses of representative neurons to AMPAR/KAR antagonist, domoic acid (DoA 200 nM) in the presence or absence of NASPM (30 μM). Both DoA applications were performed in the presence of GABA(A)R antagonist, bicuculline (10 μM). Green curves correspond to neurons insensitive to NASPM and expressing CP-KARs. The neurons in which the amplitude of the sustained DoA-induced $[Ca^{2+}]_i$ elevation

decreases in the presence of NASPM (red curves) contain CP-AMPARs. Grey curves: other neurons without CP-AMPARs. B. Averaged responses of neurons from panel A.

Data analysis

1. The series of fluorescent images can be analyzed with free software ImageJ.
2. To subtract background noise, use the *Subtract Background* function in ImageJ (*Process* → *Subtract Background*). The variable parameter *Rolling ball radius* should be chosen in the range of 30–50 pixels (depending on magnification).
3. To calculate time-lapse changes in the fluorescence intensity of cells, place ROIs on the soma without covering processes to avoid capturing the background fluorescence (Figure 5).

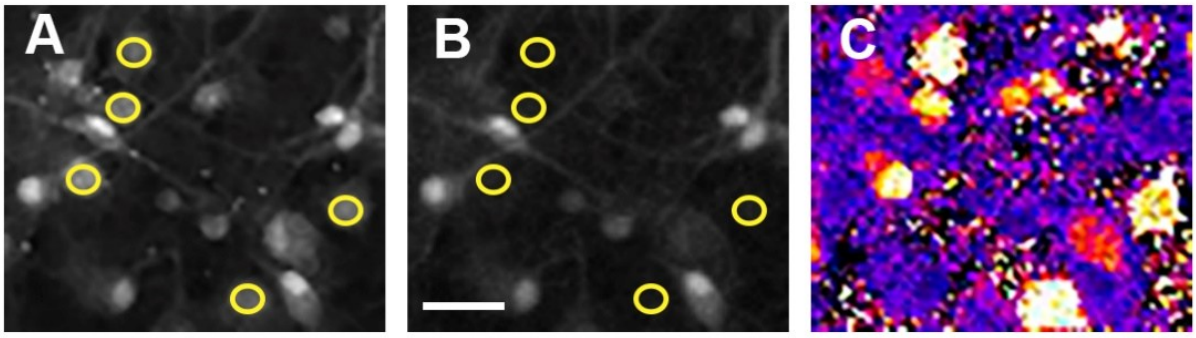

Figure 5. Analysis of representative Fura-2 images. A, B. Fura-2 fluorescence upon 387 nm excitation (Ca^{2+}-free form of the probe) before (A) and during (B) KCl application. C. 340/387 Fura-2 ratio during KCl application; "Fire" lookup table. Yellow circles demonstrate the regions of interest (ROIs) where the average signal intensity was measured. Scale bar 20 μm.

4. In the case of ratiometric probes, such as used Fura-2, the series of images corresponding to the channels with excitation 340 nm have to be divided by the appropriate series of images corresponding to the channel with excitation 387 nm (*Process* → *Image Calculator*; *32-bit* option should be chosen).
5. To calculate the mean Fura-2 340/387 ratio (reflects the changes in $[Ca^{2+}]_i$) for cells in a view field, perform the command in *ROI Manager More* → *Multi Measure*. Set *Mean Grey Value* as the calculated parameter (*Analyze* → *Set Measurements*).
6. Copy or export obtained results and draw plots using any appropriate software (e.g., OriginLab Pro).
7. In the case of non-ratiometric Ca^{2+}-sensitive fluorescent probes (Fluo-4 family or others), use similar algorithms excluding step 4.

Validation of protocol

This protocol or parts of it have been used and validated in the following research articles:

- Zinchenko et al. [17]. Properties of GABAergic Neurons Containing Calcium-Permeable Kainate and AMPA-Receptors. Life (Basel) (Figures 1–5).
- Gaidin et al. [16]. A novel approach for vital visualization and studying of neurons containing Ca2+ -permeable AMPA receptors. J Neurochem (Figures 1–6).
- Maiorov et al. [10]. Peculiarities of ion homeostasis in neurons containing calcium-permeable AMPA receptors. Arch Biochem Biophys (Figures 1–4).
- Zinchenko et al. [18]. Participation of calcium-permeable AMPA receptors in the regulation of epileptiform activity of hippocampal neurons. Front synaptic neurosci (Figure 3).

General notes and troubleshooting

General notes

1. The proposed methods can be used not only in cell cultures but also in brain slices.
2. Any Ca^{2+}-sensitive fluorescent cell-permeant probes can be used in the experiments.
3. The method allows combining with electrophysiological measurements or immunostaining.
4. The described approaches allow the expressed identification of many neurons in a view field.
5. Since permeability for Ca^{2+} is determined in the case of AMPARs and KARs not only by the subunit composition but also by Q/R editing of pre-mRNA encoding GluA2, GluK1, GluK2 subunits, we suppose that the described approach allows identification of receptors consisting of non-edited subunits.
6. We suppose that the suggested approaches allow the identification only of neurons containing a significant number of CP-AMPARs and CP-KARs. If the percentage of the calcium-permeable receptors in a neuron is low, the signals of the Ca^{2+}-sensitive probes can be negligible.

Troubleshooting

Problem 1: Low-amplitude response to DoA (or the absence of the response) or slow restoration of $[Ca^{2+}]_i$ after washout.
Possible cause: Inappropriate concentration of the agonist.
Solution: Change the concentration (the optimal ranges are listed above).

Problem 2: Absence of response to AMPAR agonists in the presence of NMDAR, KAR antagonists, and VGCC blockers.
Possible cause: Disturbances of the neuronal network formation and death of neurons at early stages of the cultivation.
Solution: Increase cell density during the culture preparation to improve the viability of neurons and promote synaptogenesis and maturation. Hold the humidity in a CO_2 incubator at $\geq$ 95% to prevent excessive evaporation from the cell culture dishes and shift the osmolarity of the neuron-glial culture medium.

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Competing interests

The authors declare no conflict of interest.

Ethical considerations

All animal procedures were approved by the Bioethics Committee of the Institute of Cell Biophysics (ICB) and carried out according to Act708n (August 23, 2010) of the Russian Federation National Ministry of Public Health, which states the rules of laboratory practice for the care and use of laboratory animals, and the Council Directive 2010/63 EU of the European Parliament on the protection of animals used for scientific purposes. ICB RAS Animal Facility provided the animals for experiments in accordance with the applications approved by the Commission on Biosafety and Bioethics of Institute of Cell Biophysics (Permission No. 6, 12 December 2017; Permission No. 2, 12 June 2020, Permission No. 3, 12 April 2021; Permission No. 4, 17 July 2021, Permission No. 3, February 12, 2022; Permission No. 4, 17 June 2022, Permission No. 3, March 12, 2023).

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