IPSC-Derived Human Neurons with GCaMP6s Expression Allow In Vitro Study of Neurophysiological Responses to Neurochemicals

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Abstract

The study of human neurons and their interaction with neurochemicals is difficult due to the inability to collect primary biomaterial. However, recent advances in the cultivation of human stem cells, methods for their neuronal differentiation and chimeric fluorescent calcium indicators have allowed the creation of model systems in vitro. In this paper we report on the development of a method to obtain human neurons with the GCaMP6s calcium indicator, based on a human iPSC line with the TetON–NGN2 transgene complex. The protocol we developed allows us quickly, conveniently and efficiently obtain significant amounts of human neurons suitable for the study of various neurochemicals and their effects on specific neurophysiological activity, which can be easily registered using fluorescence microscopy. In the neurons we obtained, glutamate (Glu) induces rises in $[Ca^{2+}]_{i}$ which are caused by ionotropic receptors for Glu, predominantly of the NMDA-type. Taken together, these facts allow us to consider the model we have created to be a useful and successful development of this technology.

Keywords

IPSC · Neurons · TetON–NGN2 · GCaMP6s · Neurochemicals · Glutamate

Introduction

The study of human neurons is difficult due to tight restrictions on obtaining primary biomaterial. However, the discovery of induced pluripotent stem cells (iPSCs) has made it possible to obtain human neurons in vitro. Such models have formed a powerful platform for studying the molecular, morphological and physiological properties of human neurons. In addition, the use of iPSCs derived from readily available human skin cells has enabled us to obtain autologous neurons from different patient-carriers of various genetic neurodegenerative diseases. The approach can also be useful for the development of individual patient-specific therapeutic strategies for various pathologies.

There are various protocols for the differentiation of iPSCs into neural cells, followed by their in vitro cultivation. The traditional approach to generate neurons from iPSCs is the protocol of neuron differentiation through the transient stage of neural stem cells (NSC) by using small molecules and various growth factors that trigger and/or inhibit certain molecular cascades [1]. The most widely used protocol is the dual SMAD inhibition method, via inhibition of bone morphogenetic protein (BMP) and transforming growth factor β (TGF-β) signal pathways [2]. However, this protocol...
requires a long time (3 to 12 weeks), while further differentiation of the NSC yields a heterogeneous neuronal culture, which, in addition to different populations of neurons, also includes neural progenitors, glial cells, etc. A fundamentally different approach is the direct differentiation of pluripotent stem cells into neurons using forced overexpression of Neurogenin-2 (NGN2) in the iPSCs. NGN2 is a pro-neural transcription factor that is responsible for the initiation of their differentiation into neurons in vertebrates [3–5]. This method has allowed us to obtain homogeneous neuron cultures capable of synaptic function starting from the 7th day of differentiation [6, 7].

At the same time, the development of fluorescent calcium indicator proteins, has made it possible to obtain systems for the detection of specific physiological in vivo and in vitro neuronal activity using fluorescence microscopy [8–10]. In cells, calcium is involved in a huge number of different physiological processes, in particular, among other things, in neurons, calcium triggers the release of the neurotransmitters that mediate their functional activity [11, 12]. By visualizing the calcium signal, it is possible to detect membrane depolarization, and the presence and activity of various calcium channels in the neurons [13, 14].

Calcium indicators are divided into two main types: chemical indicators and genetically encoded calcium indicators (GECI). GECIs are chimeric proteins that are formed by fusing one or two fluorescent proteins with a calcium-binding protein. In contrast to chemical indicators, GECI have higher sensitivity and, in addition, the possibility of creating transgenes expressing a calcium indicator, which allows their use both in vitro and in vivo by detecting calcium signals [15, 16].

To date, several families of GECIs have been developed, differing in sensitivity, kinetics, fluorophore type, and other parameters. The choice of a calcium indicator is determined according to the tasks [10, 17].

GCaMP indicators are based on the green fluorescent protein GFP and calmodulin. To date, there are eight generations of GCaMPs with various modifications and improvements. The very first version, GCaMP1, was a compound of cp149EGFP, calmodulin, and calmodulin-binding peptide M13. However, such an indicator had insufficient brightness at 37 °C [18]. Further attempts at improvements led to the development of stable, bright, rapidly kinetic, and highly sensitive indicators that allowed the detection of even single calcium bursts.

One of the most popular modifications is the GCaMP6, developed in 2013. GCaMP6 have three modifications: GCaMP6s, 6m, 6f (for s—slow, m—medium, and f—fast calcium signal response kinetics) [8]. In 2019, the next generation of GCaMP—GCaMP7—was developed. Members of GCaMP7 have retained the structure and basic characteristics of GCaMP, but they have higher sensitivity compared to GCaMP6. Individual modifications of GCaMP7 (s, f, c, b) feature improved brightness and kinetic properties adapted for specific applications [9]. And as early as 2020, variants of GCaMP8 were announced.

Nevertheless, GCaMP6 indicators remain quite sought-after calcium indicators in routine work whose tasks are not characterized by increased complexity [15].

A disadvantage of all calcium indicators based on calmodulin, including all GCaMP representatives, is the possibility of interaction of calmodulin with cell proteins. An article [19] showed that some transgenic mouse lines exhibit aberrant neuronal activity, which is probably related to GCaMP6 expression. Nevertheless, GCaMP6 has no toxic effect on NSCs and neuronal cultures and has been successfully used in long-term experiments with calcium activity imaging [20].

Combining recent advances in this field in our work, we have developed a system for the rapid, convenient, and efficient production of human neuronal cultures based on human iPSC lines containing elements of the TetON–NGN2 system and the fluorescent calcium indicator GCaMP6s. Based on our original differentiation protocol, these neuronal cultures can survive for long periods (up to several months) and, with which, in vitro fluorescence microscopy offers the opportunity to record specific neurophysiological activity in response to various neurochemicals.

**Experimental Procedure**

**iPSC Culture**

A culture of induced pluripotent stem cells (iPSCs), iP5-KYOU, has been obtained at the Shinya Yamanaka laboratory (Kyoto University, Japan) by the retroviral reprogramming of adult female skin fibroblasts. The iP5-KYOU cell line was purchased from the ATCC cell bank (KYOU-DXR0109B, ATCC® ACS-1023™). For all pluripotent stem cell passaging, we used ACCUTASE™ cell detachment solution (Stem Cell Technologies) and Rockinhibitor Y-27632 (5 μM; Abcam), the plastic surface being pre-coated with BD-Matrigel™ solution (1/40 in DMEM/F12) (BD Bioscience). The cells were cultured in mTeSR™1 medium (Stem Cell Technologies) at 37°C in a CO2-incubator with 5% CO2 and 100% humidity.

**Generation of GCaMP6s Expressing iPSCs**

We used the Sleeping Beauty transposon transgene system consisting of the plasmid pCMV(CAT)T7-SB100 containing the Sleeping Beauty transposase gene and a specially designed pT2/HB vector with the CAG-GCaMP6s cassette. The pCMV(CAT)T7-SB100 was a gift from Zsuzsanna...
Izsvak (Addgene plasmid # 34879; http://n2t.net/eddige:34879; RRID:Addgene_34879). The pT2/HB was a gift from Perry Hackett (Addgene plasmid # 26557; http://n2t.net/eddige:26557; RRID:Addgene_26557). Cloning of the CAG-GCaMP6s cassette into the pT2/HB vector was performed at Evrogen company (evrogen.ru, Moscow, Russia).

Delivery of the genetic constructs was carried out using electroporation on a BioRad Gene Pulser X Cell device. Program—three pulses, with a height (voltage) of 155 V, a width (pulse time) of 5 ms, a delay between pulses of 0.1 s, with the optimal concentration of the plasmid DNA mix stock being 0.5–1.5 μg/μl. In each electroporation, we used approximately 5 μg each of both vectors pCMV(CAT) T7-SB100, and pT2/HB-CAG-GCaMP6s. The cells were resuspended in 400 μl of Electroporation buffer (BioRad). After electroporation, the iPSCs were plated on BD-Matrigel™-coated plates in mTeSR™1 medium supplemented with Rock-inhibitor. At the last stage, several iPSC clones with maximal GCaMP6s expression were selected.

Flow Cytometry and Sorting

After electroporation, two subsequent cell sortings were performed: 48 h and 7 days post transfection. The cell sorting was carried out on a BioRad S3e Cell Sorter with the ProSort™ software, on which a population of cells with green fluorescence was selected (GFP+). After each sorting, the cell culture was seeded in a 6-cm Petri dish coated with BD-Matrigel™ in mTeSR™1 medium supplemented with Rock-inhibitor. At the last stage, several iPSC clones with maximal GCaMP6s expression were selected.

Generation of TetON–NGN2 Expressing iPSCs.

Lentivirus Preparation and Lentiviral Transduction

To obtain iPSCs expressing the TetON system and the NGN2 gene, we used rTA-N144 and TRET-hNgn2-UBC-PuRo plasmids obtained from the Addgene repository. The rTA-N144 was a gift from Andrew Yoo (Addgene plasmid # 66810; http://n2t.net/eddige:66810; RRID:Addgene_66810). The pLV_TRET_hNgn2_UBC_PuRo was a gift from Ron Weiss (Addgene plasmid # 61474; http://n2t.net/eddige:61474; RRID:Addgene_61474). The lentiviruses were produced in HEK293T cells (10⁶ cells in 6-cm Petri dish) by co-transfection with three helper plasmids (pLP1, pLP2, pVSVG), in concentrations 4 μg of lentiviral vector DNA and pLP1—4 μg, pLP2—2 μg, pVSVG—1 μg, and packaged using Lipofectamine2000 reagent (Invitrogen) according to the protocol proposed by the manufacturer, in serum-free OPTI-MEM medium. The HEK293T cells were transfected for up to 4 h, then the medium was aspirated and 4 ml of complete growth medium was added. Media with lentiviruses complexes were harvested 48 h after transfection, centrifuged (100 g, 5 min at RT) and sterilized through a 0.45 μm filter. The collected supernatant containing the packaged lentiviruses was poured onto iPSC-GCaMP6s cells, mixing in a ratio of 2 ml of supernatant + 2 ml of complete growth medium, mTeSR™1. Polybrene was added at a final concentration of 5 μg/ml. The next day, the medium was changed to complete growth medium, mTeSR™1 containing 5 μM Rock-inhibitor. After transduction and until the beginning of differentiation, the iPSC cells expressing GCaMP6s and TetON–NGN2 were cultured on a medium supplemented with selective antibiotics—Puromycin (0.5 μg/ml; Sigma) and Hygromycin B (50 μg/ml; Serva). For better selection, the iPSC–GCaMP6s–NGN2 cells were cloned by limiting dilution, followed by selection of the most suitable clones.

Neural Differentiation of iPSCs

On day 0, iPSCs expressing GCaMP6s and TetON–NGN2 were plated on BD-Matrigel™ solution (1/40 in DMEM/F12)-coated 35 mm culture dishes at 3 × 10⁴ cells/cm² in mTeSR™1 medium supplemented with Rock-inhibitor Y-27632 (5 μM; Abcam) and mouse laminin (0.5 μg/ml; Corning). Doxycycline (1 μg/ml; Sigma) was added from day 0 to day 5 to induce NGN2 transgene expression. The medium was changed daily over a period of 6 days. To stop proliferation of undifferentiated iPSCs, cytosine arabinoside (Ara-C) (0.1 μg/ml; Sigma) was added to the culture medium on days 2 and 3. Small areas (diameter ~10 to 15 mm) of new culture dishes (or 14 mm diameter on 35 mm glass bottom dishes) were pre-coated with poly-d-lysine (Gibco) diluted in DPBS, for 1 h at 37 °C. The areas were washed three times with sterile DPBS, dried, uncovered, in a laminar hood for 2 h and coated with BD-Matrigel™ solution (1/40 in DMEM/F12).

On day 4 differentiating cultures were treated with ACC UTASE™ cell detachment solution (Stem Cell Technologies), washed in DPBS (PanEco) and plated as dissociated cells in drops on the pre-coated areas of new petri dishes (cells from 35 mm Petri dish were seeded in two drops (~200 μl) in mixed N2B27 (Neurobasal medium (Gibco), DMEM/F12 (PanEco), GlutaMax (1 mM; Gibco), sodium pyruvate (1 mM; Gibco), PenStrep (50 μg/ml; Gibco), β-Mercaptoethanol (0.1 mM; Sigma-Aldrich), N2-supplement (100x; Capricorn), B27-supplement (50x; Capricorn) and mTeSR™1 (1:1) supplemented with human BDNF (10 ng/ml; Petrotech), NGF (20 ng/ml; Petrotech), Rock-inhibitor Y-27632 (5 μM), mouse laminin (0.5 μg/ml) and doxycycline (2 μg/ml). On day 5 the medium was changed to N2B27 medium supplemented with human BDNF (10 ng/ml; Petrotech), NGF (20 ng/ml; Petrotech), Rock-inhibitor Y-27632 (5 μM), mouse laminin (0.5 μg/ml) and...
doxycycline (1 μg/ml). After day 7, half the culture medium was changed, twice a week.

**Immunocytochemical Staining**

Before staining, the cells were washed with PBS and fixed for 15 min in 4% paraformaldehyde at room temperature (22–24 °C). Then the cultures were gently washed in PBS three times (5 min at room temperature), incubated with the primary antibodies in blocking solution (PBS with 10% FBS and 0.1% Tryton-X-100) overnight (16–18 h) at +4 °C, washed 3 times again and incubated with the corresponding secondary antibodies (Alexa Fluor 546 goat anti-mouse IgG or Alexa Fluor 660 goat anti-rabbit IgG) (diluted 1:1000 in the blocking solution, Molecular Probes) for 1 h at 37 °C. Then the cell nuclei were contrasted with DAPI (1 mg/ml in PBS). The images were obtained with a fluorescence microscope (EVOS FL AUTO, Life Technologies) (Table 1).

**Quantitative RT-PCR Analysis**

Cells were harvested using Accutase (Stem Cell Technologies) and collected by centrifugation. Total RNA was extracted using an RNeasy Mini Kit (Cat# 74106, Qiagen). Up to 2*10⁶ cells were lysed in 350 μl of RLT buffer, then 300 μl of 70% ethanol was added. 700 μl of the lysate was transferred to a spin column. Then the spin column was washed with 350 μl of RW1 buffer and 30 units of DNase I were added (RNase free DNase Set (Cat# 79254, Qiagen). The spin column was incubated at room temperature for 15 min, then it was washed with 350 μl of RW1 buffer. After this the spin column was washed twice with 500 μl of RPE buffer and then the total RNA was eluted from the column with 30 μl of RNase-free water. The total RNA concentration was measured using an Implen P360 nanodrop system. 1 μg of total RNA was used to perform the cDNA synthesis. The first strand cDNA was synthesized using a MMLV Reverse Transcriptase kit (Cat# SK022, Evrogen) with oligo(dT)-primers. The reaction mix also contained 20 μmol of each nucleotide, 40 μmol of DTT, 100 units of MMLV Reverse Transcriptase and 30 nmol of oligo-dT primer. Reverse transcription was performed for 1 h at 37 °C and 10 min at 70 °C to stop the reaction. qRT-PCR was performed using the Bio-Rad CFX96 PCR System (Bio-Rad). The temperature profile was (1) 95 °C for 10 min, (2) 40 cycles of 95 °C for 15 s and 60 °C for 1 min, (3) melt curve analysis between 60 and 95 °C. HS-SYBR + ROX master mix (Cat#PK149L, Evrogen) was used to prepare the reaction mixtures. The quantitative RT-PCR analyses were performed in two independent biological and three technical replicates. The housekeeping genes PSMB4, REEP5 were used for normalization. The ΔΔC method was used in further calculations. The primers used in this work are listed in Table 2.

**Ca²⁺ Imaging**

[Ca²⁺], measurements were carried out using the “endogenous” Ca²⁺ sensor, GCaMP6s, or using the fluorescent Ca²⁺ indicator Fura-2 (ThermoFisher, USA). A fluorescent indicator SBFI (ThermoFisher) was used to measure

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**Table 1** List of antibodies used in this study

| Antibody | Source     | Catalog # | Species | Dilution |
|----------|------------|-----------|---------|----------|
| Nestin   | Millipore  | Mab5326   | Mouse   | 1:200    |
| NSE      | Dako       | M0873     | Mouse   | 1:200    |
| S100b    | Abcam      | Ab7852    | Mouse   | 1:200    |
| TuJ1     | Millipore  | Mab1637   | Mouse   | 1:200    |
| GFAP     | Millipore  | 04–1031   | Rabbit  | 1:200    |
| hNCAM    | Abcam      | Ab75813   | Rabbit  | 1:200    |
| NeuN     | Abcam      | Ab104225  | Rabbit  | 1:200    |
| SYN      | Abcam      | Ab8       | Rabbit  | 1:200    |
| SYP      | Abcam      | Ab32127   | Rabbit  | 1:200    |

**Table 2** List of primer sequences used in this study

| Gene   | Sequence 5′–3′ | Amplicon size |
|--------|----------------|---------------|
| 1 TUBB3 | FW CCAGACGACGCTATG | 152           |
|        | RV AAGACGACGAGGCA GC |               |
| 2 TH    | FW GCTCCAAGCAGACG | 90            |
|        | GTA CVTAAACGATACTCTCGA |           |
| 3 MAP2  | FW CTCAGACGCTATG | 95            |
|        | RV CATGGGCTTTCGAAAG |           |
| 4 NES   | FW CAAACGGAGCGAGGTC | 164           |
|        | RV GCTCTACATCTCTCTCTTCTTGTA |       |
| 5 MAPT  | FW TTTGGTTGTTGTTAGA | 73            |
|        | RV TATGCG CCAGGTCGTTGAAAGAT | |
| 6 PAX6  | FW AGTCGCGCTCCATCTTGC | 81            |
|        | RV CGCTTGTATGTATGTGT |           |
| 7 ASCL1 | FW ATCTTACAGGTCGGG | 232           |
|        | RV TTGGGCCCTTGATCTCAC |         |
| 8 BRN2  | FW GGGGGAAAACCTAGACC TT | 401          |
|        | RV GTCACACCTAGTTCACTGTG |       |
| 9 SOX1  | FW AAATCTGAGGACGACGC | 94            |
|        | RV AACCACGGTCGTTGTCAGC |          |
| 10 PSMB4| FW CATTCCCAGCTCCGGATT | 116          |
|        | RV CGAACAATACGGCGAGGAC |         |
| 11 EMC7 | FW AAGAGGTAGCTAGGCC GT | 92            |
|        | RV GTTGGCTTACACGCGTTTCCA |         |

[Ca²⁺], measurements were carried out using the “endogenous” Ca²⁺ sensor, GCaMP6s, or using the fluorescent Ca²⁺ indicator Fura-2 (ThermoFisher, USA). A fluorescent indicator SBFI (ThermoFisher) was used to measure
[Na⁺], The indicators were introduced into the cells in the form of acetoxymethyl esters (Fura-2/AM; 2 μM and SBFI/AM, 8 μM) for 50–60 min at 37 °C. Stock solutions of the AM esters in DMSO were pre-mixed with the nonionic detergent, Pluronic F-127 (0.02%; Molecular Probes, Oregon), to facilitate penetration of the esters into the cells and then added to the incubation medium at the concentrations indicated above.

The measurements were carried out at 26–29 °C in a buffer containing (mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 20 HEPES, 5 d-glucose; the pH 7.4 was adjusted with 1 M NaOH. The nominally the calcium-free solutions contained 2 mM MgCl₂ and 0.1 mM EGTA instead of CaCl₂. To examine the effects of Glu, receptor inhibitors and other agents the previous solutions in each dish with cells were replaced with new solutions (4 × 1 ml) for 40 s. Buffers with Glu and inhibitors of NMDA- and AMPA-type ionotropic glutamate receptors were prepared on the same day they were used.

Measurements of changes in [Ca²⁺] and [Na⁺] were performed using an image analysis system based on a Nikon Ti2 microscope (Japan), an LED-based illumination system (PE-340-fura, CoolLED, USA), a triple-band beam-splitter mirror DM468/526/596, a set of excitation filters 340 ± 13 and 387 ± 6 nm for Fura-2 and SBFI, and 442 ± 21 nm for GCaMP6s. The emissions from the Fura-2, SBFI, and GCaMP6s were recorded at 544 ± 12 nm. The light filters and cube were manufactured by Semrock (Thorlabs, USA). Exposure times were 200–500 ms, with the LED radiation intensity in the range of 5–20% of the maximum value.

All agents, except for the calcium and sodium indicators and Pluronic, were purchased from Sigma (USA).

The recording, storage and processing of the obtained information was carried out using the NIS software supplied with the described image analysis system. The plotting of changes and statistical analysis were performed using Prism 8.1. Differences with p < 0.05 in paired t-tests were considered significant.

Statistical Analysis

The gene expression analysis by quantitative RT-PCR experiments were performed in the format of two independent biological replicates for each point, and for PCR procedure itself, we staged three technical replicates for each point. The calculation of the separation coefficient (p-value) was made using the Mann–Whitney test. All experiments on the functional analysis of the obtained neurons (Ca²⁺ and Na⁺ measurements) were performed in two independent biological replicates. The number of measured neurons (technical replicates) in each experiment varied from 21 to 93.

Results

Obtaining of the iPSC–KYOU–GCaMP6s–TetON–NGN2 Line

During the first stage, we created a human iPSC line stably expressing the fluorescent calcium indicator GCaMP6s (iPSC–KYOU–GCaMP6s) (Fig. 1A). For this purpose, we applied the transgenesis method using the Sleeping Beauty system, sequential cell sorting, and cloning by limiting dilution. This line had typical iPSC morphology, and weak green fluorescence (Fig. 1B).

To enable rapid one-step differentiation of the iPSCs into neurons, the iPSC–KYOU–GCaMP6s line was transduced using lentiviral delivery of the proneural gene transgenes NGN2 and TetON transactivation system under doxycycline-activated promoter. The new iPSC–KYOU–GCaMP6s–TetON–NGN2 line was cloned by limiting dilution followed by the selection of clones that differentiated efficiently in response to doxycycline addition. Also, for additional selection, and to avoid the accidental silencing of transgenes, the line was maintained on selective antibiotics.

Adding doxycycline led to an immediate start of iPSC differentiation in the neural direction (Fig. 1B). By the 2nd day of incubation with doxycycline we could already observe the process of transformation of iPSCs into neuron-like cells with outgrowths. The addition of AraC on the 2nd and 3rd days of differentiation (DD) allowed us to effectively get rid of the remaining proliferating iPSCs (Suppl. Fig. 1).

Neuronal outgrowths do not adhere well to Matrigel and easily detach from the substrate when the culture medium is changed. Therefore, in our modification of the protocol, we transplanted differentiating cultures at the 4th DD to poly-ν-lysine and Matrigel double-coated plastic wells, and mouse laminin was added to the medium. The neurons had better adhesion to this substrate and did not detach from it (Suppl. Fig. 1). Under these conditions, the neurons remained viable for 2 months without any evident changes in morphology.

In the obtained cultures, we observed bipolar and multipolar neuronal morphology, with branching dendrites and very long axons. All neurites adhered tightly to the substrate and formed peculiar patterns. The somas of some neurons were enlarged and/or clustered (Suppl. Fig. 2).

Gene expression analysis by quantitative RT-PCR showed a significant reactivation of the neural markers TUBB3, TH, MAP2, NES, MAPT, PAX6, ASCL1, BRN2 and SOX1 in the derived neurons compared to the original culture of iPSCs (Fig. 2B).
After 21 DD, the obtained cultures were stained for the neuron markers Tuj1, Synaptophysin, Synapsin, NeuN, hNCAM and NSE, and in addition, the neuronal precursor cell marker, Nestin (Fig. 2A; Suppl. Fig. 2). No glial markers GFAP or S100b were detected. The images obtained by anti-synapsin antibody staining clearly visualized abundant branching dendrites that were not visible in phase contrast (Suppl. Fig. 2).

Functional Characterization of NGN2-Induced Human Neurons

Comparison of the brightfield (Fig. 3A) and green fluorescence images (Fig. 3B) revealed that the cells had drastically different levels of GCaMP6s fluorescence, probably due to differences in cell morphology and different levels of transgene expression. Therefore, the cells were loaded with the ratiometric (dual-wavelength) Ca$^{2+}$ indicator Fura-2.
(Fig. 3C), which permitted measurement of [Ca$^{2+}$]$_i$ in all the cells without interfering with the GCaMP6s signals in those cells that expressed a sufficiently high level of this Ca$^{2+}$ sensor (see Methods for details).

Addition of Glu (100 µM, 10 µM glycine, 0 Mg$^{2+}$) led to an increase in [Ca$^{2+}$]$_i$ (Fig. 4A). In these conditions Glu is capable of activating both the ionotropic glutamate receptors and the metabotropic ones. The presence of ionotropic glutamate receptors was revealed by a decrease in the [Ca$^{2+}$]$_i$ response to Glu in the presence of the receptor inhibitors: CNQX, an inhibitor of AMPA- and kainate receptors, and/or MK-801, an inhibitor of NMDA receptors [21, 22]. The profiles of the [Ca$^{2+}$]$_i$ responses to Glu differed between cells (Fig. 4A, B, E, F), therefore, instead of the amplitudes of the [Ca$^{2+}$]$_i$ increases, the areas under the curves of [Ca$^{2+}$]$_i$ changes were used as a quantitative measure of the [Ca$^{2+}$]$_i$ changes.

If [Ca$^{2+}$]$_i$ was measured with Fura-2, the increase in [Ca$^{2+}$]$_i$ in the presence of CNQX was not significantly different compared to the effect of Glu alone (difference 0.9 ± 4.9%, Mean ± SD; n = 33, p = 0.20, paired t-test). Measurements of the GCaMP6s signals (Fig. 4B), showed that CNQX significantly decreased Glu-induced [Ca$^{2+}$]$_i$ rise by 8.7 ± 7.0% (n = 27, p = 0.0015, paired t-test).
These data indicate the presence of AMPA and/or kainate receptors in the studied culture. The differences in Glu-induced $[\text{Ca}^{2+}]_i$ elevations as well as the differences in the relative decreases of $[\text{Ca}^{2+}]_i$ in the presence of iGluRs inhibitors (Fig. 4) are probably due to an almost twofold difference in the dissociation constants of complexes of these probes with $\text{Ca}^{2+}$ (Fura-2, $K_d = 224$ nM [23]; GCaMP6s $\sim 150$ nM. [24]. Therefore, GCaMP6s is more sensitive than Fura-2 to small changes in $[\text{Ca}^{2+}]_i$ near the basal level.

Next, we tested for the presence of NMDA-type ionotropic glutamate receptors and metabotropic glutamate receptors associated with $\text{Ca}^{2+}$ release from intracellular compartments (mGluRs type 1) in the cells. Adding Glu to cells in a nominally calcium-free buffer, in which the $\text{Ca}^{2+}$ concentration was reduced by replacing $\text{Ca}^{2+}$ with EGTA (100 μM), abolished the effect of Glu on $[\text{Ca}^{2+}]_i$ (Fig. 4E, F). Therefore, GCaMP6s is more sensitive than Fura-2 to small changes in $[\text{Ca}^{2+}]_i$ near the basal level.

Subsequent exchange of $\text{Ca}^{2+}$-free buffer with a $\text{Ca}^{2+}$-containing one and the addition of Glu revealed a distinct increase in $[\text{Ca}^{2+}]_i$, which could be prevented by the NMDA receptor inhibitor MK-801 (9 μM). Inhibition was significant using both the synthetic $\text{Ca}^{2+}$ indicator Fura-2 (43.4 ± 6.5%, $n = 21$, $p = 0.0012$, paired t-test) (Fig. 4E, G) and the “endogenous” $\text{Ca}^{2+}$ sensor GCaMP6s (49.1 ± 4.6%, $n = 21$, $p < 0.0001$, paired t-test) (Fig. 4F, H). The almost two-fold decrease of the signals from both $\text{Ca}^{2+}$ probes is consistent with the fact that NMDA receptors provide the main $\text{Ca}^{2+}$ and $\text{Na}^+$ influx during Glu action in cultured CNS neurons [25–28].

Simultaneous $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ Measurements

To further verify that the cells were expressing functionally active NMDA receptors, we performed simultaneous measurements of $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$, by recording fluorescence signals from GCaMP6s and the synthetic $\text{Na}^+$ indicator SBFI. The addition of Glu led to a rapid increase in both $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ (Fig. 5A, C), followed by a decrease in $[\text{Ca}^{2+}]_i$ to a level determined by balancing the influx of $\text{Ca}^{2+}$ and its removal back to the buffer, as well as through its uptake by the endoplasmic reticulum [14, 29, 30] and mitochondria [25, 27, 31] (Fig. 5A). In contrast to the changes in $[\text{Ca}^{2+}]_i$, the $\text{Na}^+$ concentration continued to rise, albeit at a slower rate, until the Glu was washed out (Fig. 5C). Removal of Glu rapidly restored $[\text{Ca}^{2+}]_i$ to the basal level, whereas $[\text{Na}^+]_i$ decreased noticeably more slowly (Fig. 5A, C). Similar differences in the dynamics of the post-glutamate changes of $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ have been observed in primary neuronal cultures from rat brain [32].

The addition of Glu in the presence of the cocktail of ionotropic Glu receptor inhibitors MK-801 and CNQX (both 9 μM) caused a decrease of both $[\text{Ca}^{2+}]_i$ by 74.2 ± 3.7% (Fig. 5A, B) and of $[\text{Na}^+]_i$ by 33.9 ± 0.7% (two independent experiments, $n = 47$, $p < 0.0001$, paired t-test) (Fig. 5C, D). A similar difference in the effect of ionotropic glutamate receptor inhibitors on $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ rises has also been observed for cultured rat hippocampal neurons upon NMDA receptor activation [28].

Neurons express potential-dependent $\text{Ca}^{2+}$ channels of various types [33, 34], the presence of which can be confirmed by reducing the transmembrane gradient of the potassium ion concentration and thereby depolarizing the plasma...
The addition of KCl (50 mM) caused a transient rise in \([\text{Ca}^{2+}]_i\) in all cells stained with Fura-2 (Fig. 6A; two independent experiments, \(n = 90\)). Similar changes in \([\text{Ca}^{2+}]_i\) were observed in those cells that expressed GCaMP6s at a level sufficient to measure its fluorescence signal (Fig. 6B; two independent experiments, \(n = 83\)).

Fig. 4 Changes in \([\text{Ca}^{2+}]_i\) induced by glutamate (Glu) alone and in the presence of inhibitors of ionotropic Glu receptors in neural culture. Only the graphs for those cells that responded to Glu by an increase in \([\text{Ca}^{2+}]_i\) are depicted. Glu was added at a concentration of 100 μM in the presence of 10 μM glycine (in Mg²⁺-free buffer). Changes in \([\text{Ca}^{2+}]_i\) are presented as the ratios of the Fura-2 signals upon fluorescence excitation at 340 and 380 nm (A, E, C, G) and as the GCaMP6s fluorescence signals (B, F, D, H). The Fura-2 fluorescence ratio (F340/F380) was considered to be 0 in resting cells and 1 at a saturating Ca²⁺ concentration in the presence of the Ca²⁺ ionophore Ionomycin (Iono, 2 μM, 5 mM CaCl₂ in the buffer). GCaMP6s fluorescence signals (f) are normalized relative to the basal level in resting cells (F/F₀). To quantitatively compare the Ca²⁺ responses of the cells to Glu alone and in the presence of inhibitors, the areas under the curves of the \([\text{Ca}^{2+}]_i\) changes (AUC, rel.un.) were calculated for CNQX (C, D) and MK-801 (G, H). GCaMP6s fluorescence was excited at 442 nm. The fluorescence signals of Fura-2 and GCaMP6s were recorded at 544 nm (see methods for details). To calibrate the maximum signals of Fura-2 and GCaMP6s, the Ca²⁺ ionophore ionomycin (2 μM in the presence of 5 mM CaCl₂) was added at the end of experiments. Measurements were made in cultures at 23 DD.
Neurons of different types can contain purinergic P2-type receptors [14]. In the culture we examined, the P2Y- and P2X-receptor agonist, ATP (100 μM), caused [Ca<sup>2+</sup>]i rises in only ~20% of cells (17 of 90) (Fig. 6B). ATP, in contrast to glutamate, induced this [Ca<sup>2+</sup>]i rise as rapidly and transiently as the [Ca<sup>2+</sup>]i rise induced by plasmalemma depolarization with KCl (Fig. 6).

Discussion

Improving and simplifying the methods of iPSC differentiation into target cells, including neurons, is an important theoretical and practical problem. There are many methods of neuron generation and they all lead to slightly different results [1]. Moreover, as it turned out, using the same method of differentiation into neurons with NGN2 overexpression meant that slightly different cultures were obtained from different protocols. The type and morphology of neurons depends on the specific cultivation conditions, the pluripotent stem cell line used, the presence of growth factors in the culture medium, and the type of substrate [6, 35–37]. Thus, the neuronal cultures obtained from rat iPSCs on different substrates (poly-D-lysine, Geltrex, gelatin and poly-laminin) were different in morphology and maturity [35]. NGN2-induced neurons that were cocultured on a Matrigel-coated substrate with mouse glial cells from day 2 of differentiation showed a fairly homogeneous culture, with features of excitatory neurons that expressed telencephal markers representative of 2/3 of the cortical layers. However, the resulting neurons can only partially be called glutamatergic [6]. When differentiated by the same method but on a
substrate of poly-L-ornithine and murine laminin followed by co-culture with murine astroglial cells, heterogeneous cultures with a predominance of cortical sensory neurons were obtained [36].

In our work, we obtained NGN2-induced neurons on a substrate sequentially coated with poly-D-lysine and Matrigel, without co-culturing with astroglial cells. Although, morphologically, the culture seemed fairly heterogeneous, it nevertheless, not only stained for neuronal marker proteins (Tuj1, Nestin, Synapsin, Synaptophysin, NeuN, NSE) but also expressed major neuronal marker genes such as TUBB3, TH, MAP2, NES, MAP and BRN2 (Fig. 2B). Moreover, the expression of BRN2 in the neurons we obtained indicates that they belong to the excitatory-type neurons of the brain, which is consistent with the previously obtained results [6, 38]. As in the cultures obtained by Thoma and colleagues, high expression of neural progenitor genes such as PAX6 and SOX1 was detected in our neural cultures [38]. Forced expression of NGN2 in pluripotent cells leads to activation of the PAX6 and SOX1 genes, despite the fact that in vivo their expression precedes that of NGN2 and not vice versa [39, 40]. Moreover, PAX6 has been shown to be involved in NGN2 regulation, and NGN2 expression is repressed in Pax6(-/-) murine neuroblastoma cells [39, 41]. It is likely that PAX6 and SOX1, which are activated by NGN2, then trigger the expression of various transcription factors, including NGN2 itself, and activate the molecular cascades required for neural differentiation. Indeed, transcriptome analysis showed that during the first 4 days of differentiation, NGN-induced cells managed to undergo some stages of neural precursors in an "accelerated format," and the identified differentiation pathways were similar to those in vivo. Moreover, significant similarity has been found between the transcriptomes of differentiating neurons at the 4th DD with the developing human brain [42]. Thoma et al., suggest that neurons obtained by NGN2 hyperexpression are mainly glutamatergic because they are positive for the vesicular glutamate transporter (vGLUT1) as well as for NMDA receptor 1 [38]. Zhang et al. came to the same conclusion; their neurons expressed AMPA receptor subunit genes for glutamate, although not NMDA receptor genes [6]. Our functional tests using calcium imaging also showed responses to glutamate. Despite the fact that the study of cell morphology and methods of immunocytochemistry can initially identify the type of neurons, the maturity of neurons in vitro is poorly determined by the expression of specific markers. More appropriate evidence of the maturity and functionality of the obtained neurons is the presence of receptors in these cells, the stimulation of which leads to a rapid change in the intracellular concentration of ions that serve as secondary messengers, as well as of those ions that ensure the propagation of an electrical impulse along dendrites and axons. These are, primarily, calcium, sodium and potassium ions [26].

In the central nervous system (CNS), the main excitatory agonist of neurons is glutamate (Glu), which is able to activate ionotropic receptors (iGluRs), as well as metabotropic receptors (mGluRs). The iGluRs provide an influx of Ca\(^{2+}\) and Na\(^{+}\) into the cytosol from the environment through channels that are internal parts of the receptor structure [26, 43, 44], whereas mGluRs trigger enzymatic processes. Activation of the so-called type 1 mGluRs (mGluR1 and mGluR5) leads to mobilization of Ca\(^{2+}\) into the cytosol from intracellular depots (from the endoplasmic reticulum, ER) [45, 46]. We tested the ability of the NGN2-induced neurons to respond to Glu. To the best of our knowledge, this was the first time that Glu has been shown to cause rises in both [Ca\(^{2+}\)]\(_i\) and [Na\(^{+}\)]\(_i\) in NGN2-induced neurons (Fig. 5).
dynamics of these processes differ markedly at the time of Glu action and, especially, after its removal. The recovery of low [Na\(^+\)] during the post-glutamate period is much slower than that of [Ca\(^{2+}\)] (Fig. 5). A similar characteristic of the changes in both the [Ca\(^{2+}\)] and [Na\(^+\)] themselves, and in the differences between them has been described for primary cultures from rat cerebral cortex [32]. Differences in the dynamics of [Ca\(^{2+}\)] and [Na\(^+\)], changes after Glu washing are due to the much greater consumption of ATP by Na/K-ATPase compared to the Ca-ATPases of the plasma membrane and endoplasmic reticulum, taking into account the dramatic decrease in cytosolic ATP [32, 47].

It should be noted that the amplitudes of the [Ca\(^{2+}\)], rises induced by Glu do not exceed half of the maximum increase observed under the action of ionomycin (Fig. 4). Given that Fura-2 is a high-affinity Ca\(^{2+}\) probe, having a Kd = 224 nM [23], the amplitudes of the [Ca\(^{2+}\)] rises induced by Glu can be estimated as not exceeding 300 nM. In primary neuronal cultures Glu in the same concentration (100 µM in the presence of 10 µM glycine) induces [Ca\(^{2+}\)] rises by units of micromoles/liter, and during prolonged exposure, by tens of micromoles/liter [27, 48, 49]. It is probable that, in the neural cultures we obtained, the content of NMDA and AMPA/kainate receptors was lower than in primary neuronal cultures from the brain. The absence of a Glu-induced [Ca\(^{2+}\)] rise in calcium-free buffer, and the suppression of [Ca\(^{2+}\)] rise by ~74% upon addition of a mixture of MK-801 and CNQX indicates a dominant role of ionotropic glutamate receptors in Ca\(^{2+}\) signaling (Fig. 5A, C). Ca\(^{2+}\) entry from the buffer is provided by NMDA-type receptors and, to a lesser extent, by AMPA/kainate-type receptors.

Some evidence suggests that AMPA and kainate receptors are present and active not only in neurons but also in neuroprogenitor cells, even more so than are NMDA receptors. AMPA/kainate receptors are highly expressed in proliferative zones during embryogenesis; in addition, kainate causes currents in cultured hippocampal precursors. Meanwhile, proliferating NSC cultures are insensitive to NMDA [50]. All these facts indicate that there are no functional NMDA receptors on hippocampal NSCs [50]. However, all cells responding to an NMDA stimulus have been shown to stain for the neuronal marker Tuj1 [51]. Thus, the prevalence of NMDA-type receptors in our neural culture evidently suggests a lack of neural precursors.

The mGluR1 and/or mGluR5 metabotropic glutamate receptors, functionally associated with Ca\(^{2+}\) mobilization, into the cytosol from calcium stores, does not seem to occur in the NGN2-induced neurons we obtained. At least we were unable to detect their functioning using single-cell fluorescence microscopy. This observation can also be seen as an argument in favor of the mature status of the neurons in the culture we obtained, since it is known that mGluR1/5 activation controls the proliferation, survival and differentiation of cultured neural progenitor cells isolated from adult mouse SVZ [52]. The mGluRs non-coupled to Ca\(^{2+}\) mobilization from intracellular stores, such as mGluR2 and mGluR3 stimulate proliferation of neural progenitors [53], accelerate progenitor differentiation into the astroglial lineage [54], and collectively, prevent neuroprogenitor cell apoptosis. Nevertheless, more detailed analysis of specific receptor expression by transcriptome is required for more specific assertions in the future.

The method we use allows us to quickly obtain a model of functionally active human neurons in vitro, which can be used for various purposes. Nevertheless, this model, like all other in vitro models of human neurons derived from pluripotent stem cells, has some significant limitations.

First, it should be understood that it is still an artificial cell system, which not only does not have some of the properties of the human brain, but can also carry its own characteristics not related to the model object at all. Even more so, given that, it is a 2D model that does not reveal all of the brain’s intercellular interactions, nor does it have vascularization [55]. However, these are still human neurons that have genes, proteins, and receptors specifically human, not rat or mouse. Because researchers do not have the opportunity to study the native human brain from the inside out, a comprehensive approach is needed to study various processes in brain cells, including pathophysiological processes, including both animal models, which map processes in a complete natural environment, and a model of human neurons, but in an artificial in vitro system. The second limitation of such in vitro models is the fact that the composition of cultures, the ratio of neuron types, unfortunately, can depend on the type and lineage of pluripotent stem cells used. Different PSC lines have different mutations, which inevitably arise in the process of cultivation, and in addition, lines can have different epigenetic landscape. As a result, PSC lines can have a tendency to differentiate into different cell types depending on the cell culture condition, genetic differences of donors, reprogramming method, and even stochastic epigenetic differences between clones [56, 57]. To more or less avoid this drawback, continuous monitoring of cell cultures, increasing the number of controls and donors, as well as analysis of the obtained neural cultures at the transcriptome level, including, for example, using the single-cell RNA-seq method, which is now becoming more popular and available to a growing number of laboratories. All these points must be taken into account when planning and conducting such studies.
Conclusion

In this manuscript, we have reported on the development of a method to obtain human neurons with the GCaMP6s calcium indicator, based on a human iPSC line with the TetON–NGN2 transgene complex. The protocol we developed allowed us quickly, conveniently and efficiently to obtain significant amounts of human neurons suitable for the study of various neurochemicals and their effects on specific neurophysiological activity that could be easily registered using fluorescence microscopy.

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Author Contributions DEB designed and supervised the study; GAA, SAM, SRR, BZV, ZD, DDA and SKM. developed the methodology and performed experiments; DEB, GAA, BZV and SAM analyzed and interpreted the data; DEB, GAA, SRR, BZV and SAM wrote the manuscript. All authors have read and approved the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest The authors have no conflicts of interest.

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