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Expression and Functional Properties of NMDA and $GABA_A$ Receptors during Differentiation of Human Induced Pluripotent Stem Cells into Ventral Mesencephalic Neurons

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Abstract—Ionotropic glutamate and GABA receptors regulate the differentiation and determine the functional properties of mature neurons. Both insufficient and excessive activity of these neurotransmission systems are associated with various nervous system diseases. Our knowledge regarding the expression profiles of these receptors and the mechanisms of their regulation during the differentiation of specialized human neuron subtypes is limited. Here the expression profiles of the NMDA and $GABA_A$ receptor subunits were explored during *in vitro* differentiation of human induced pluripotent stem cells (iPSCs) into ventral mesencephalic neurons. The correlation between the neuronal maturation and the expression dynamics of these genes was investigated, and the functional activity of these receptors was assessed by calcium imaging. The role of NMDA and $GABA_A$ receptors in neurite outgrowth and the development of spontaneous activity was analyzed using the viral transduction of neural progenitors with the reporter genes *TagGFP* and *TagRFP*. The data indicate that agonists of the investigated receptors can be employed for optimization of existing protocols for neural differentiation of iPSCs, in particular for acceleration of neuronal maturation.

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Keywords: NMDA receptors, $GABA_A$ receptors, induced pluripotent stem cells, mesencephalic neurons, differentiation

The derivation of human induced pluripotent stem cells (iPSCs) [1] and their directed differentiation into specific neuron subtypes provide unique opportunities for studying the pathogenesis of neurodegenerative diseases *in vitro* [2]. At the same time, the conditions and time periods in which neurons differentiate during ontogenesis cannot be completely reproduced *in vitro*, leading to phenotypic deviations of cultured neurons [3] and limiting their utility for modeling specific physiological and pathological processes.

The functioning of neurons as active elements of the nervous system depends on the expression of neurotransmitter receptors, among which important roles belong to $GABA_A$ receptors ($GABA_A$-R) and glutamate NMDA receptors (NMDA-R). Impaired functions of these receptors are associated with various mental and neurological diseases [4-7]. NMDA-R and $GABA_A$-R play important roles in nervous system development by regulating cell proliferation, migration, and differentiation [8, 9].

A number of studies have shown that functional NMDA-R and $GABA_A$-R are expressed in neurons derived from embryonic stem cells (ESCs) and human iPSCs [10-12]. At the same time, the functions of these receptors in the differentiation of iPSCs into specialized neuron subtypes, as well as the similarity of the expression profiles of these genes *in vitro* and *in vivo*, remain poorly investigated.

The purpose of this work was to analyze the expression profile of NMDA-R and $GABA_A$-R subunits during the differentiation of human iPSCs into ventral mesen-

Abbreviations: BDNF, brain derived nerve growth factor; (c)NPs, (committed) neural progenitors; eNPs, early neural progenitors; ESCs, embryonic stem cells; GABA, α-aminobutyric acid; $GABA_A$-R, $GABA_A$ receptors; GDNF, glial cell derived nerve growth factor; iPSCs, induced pluripotent stem cells; NMDA, N-methyl-D-aspartic acid; NMDA-R, NMDA receptors; RT-PCR, reverse transcription polymerase chain reaction; TD, terminal differentiation; VM neurons, ventral mesencephalic neurons.

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cephalic neurons and to study the functional activity of these receptors.

MATERIALS AND METHODS

iPSC cultures and neuronal differentiation. IPSHD1.1S and IPSPDL2.15L iPSC lines were obtained by reprogramming human skin fibroblasts using lentiviral vectors carrying the *OCT4*, *SOX2*, *Nanog*, and *Klf4* genes [13]. Differentiation into ventral mesencephalic (VM) neurons was performed in DMEM-F12 medium containing 2 mM L-glutamine (PanEco, Russia), and 1× insulin-transferrin-selenite supplement (PanEco) according to a previously published protocol [14] consisting of three stages: (i) neural induction using double inhibition of the SMAD pathway using 10 μM SB431542 (Stemgent, USA) and 80 ng/ml recombinant Noggin (Peprotech, USA); (ii) regional specification using 100 ng/ml Sonic hedgehog (Shh) and 20 ng/ml FGF8 (fibroblast growth factor 8; Peprotech); (iii) terminal differentiation (TD), which was performed either in a monoculture in medium supplemented with 20 ng/ml BDNF (brain derived nerve growth factor) and 20 ng/ml GDNF (glial cell derived nerve growth factor) (Peprotech) or in co-culture with mouse astrocytes derived from the brain of 19-20 day embryos, in standard DMEM-F12 medium without additives (except 2 mM L-glutamine).

Calcium imaging. Intracellular Ca^{2+} measurements were performed with Fluo-4 AM dye (ThermoFischer, USA) using an LSM 510 confocal microscope (Zeiss, Germany) [15]. L-Glutamate (Sigma-Aldrich, USA) was applied simultaneously with 15 μM glycine (NMDA-R co-agonist).

iPSC transduction. The cells were transduced with Lv-Tag-GFP2 and Lv-Tag-RFP (Evrogen, Russia) following manufacturer's protocol.

Real-time RT-PCR. RNA samples were extracted with a RIBO-sol-A kit (Interlabservice, Russia) following the manufacturer's instructions. Real-time PCR was performed using a MiniOpticon amplifier (Bio-Rad, USA) with a qPCRmix-HS SYBR kit (Evrogen) and primer pairs specific to the indicated genes (DNA-Synthesis, Russia). Primer sequences are given in the table. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method [16].

Immunofluorescent staining. The staining was performed as previously described [15] using rabbit polyclonal antibodies to Glun2A (Sigma-Aldrich) at 1 : 300 dilution and mouse monoclonal antibodies to Nestin (Millipore, USA) at 1 : 750 dilution. The detection was performed using goat polyclonal antibodies to mouse and rabbit immunoglobulins conjugated with Alexa 546 and Alexa 488 dyes, respectively, at 1 : 1000 dilution (ThermoFischer).

TUNEL staining. Apoptotic cells were stained according to the method described by Zhao and Darzynkiewicz [17] using Br-dUTP (DNA-Synthesis) and terminal nucleotidyltransferase (NEB, UK). BrdU was detected using mouse monoclonal antibodies (1 : 200; Santa Cruz, USA). To confirm the specificity of the

Real-time RT-PCR primers

Gene	Forward primer, 5′→3′	Reverse primer, 5′→3′
gapdh	AGCCACATCGCTCAGACACC	GTACTCAGCGGCCAGCATCG
grin1	AGGATGGGGACCGGAAGTTCG	AGGGCTCCTGGTGGATCGTCA
grin2a	GAAGTAATGGCACCGTCTCACC	GATTCTGGACAGGCACGGAGTT
grin2b	TCATCCCTGAGCCCAAAAGCAG	CCTCCAGGGTCACAATGCTCAG
grin2c	CATCAGTGTGATGGTGGCTCGCA	CCCGGACTTCTTGCCTCTGGTGA
grin2d	CGCTCGTGCTCACGCCCAA	GTAGGACAGGAAGGCCCGGTGG
grin3a	AAGGGGAGGGGATGTGGTAAGCG	GGCCACAACCTTGCTTTGCCTT
grin3b	CCCGGGGCGCTTCTTGGCA	AAGTCCAGCTGGCCGTCCCG
gabra1	ATGCCCATGCTTGCCCACTA	TGTTGAGCCAGAAGGAGACTTGTG
gabra2	AAAGAGGATGGGCTTGGGATGGG	GGCTTCTTGTTGGGTTCTGGCGT
gabra3	GCTGCGACCTGGGCTTGGAGAT	TGGAAGAAGGTGTCCGGTGTCCA
gabrb1	ATTCGCTTGCGGCCGGACT	GCAGCTGTGGTTGTGATTCGGA
gabrb2	ACGATGCTTCAGCTGCAAGGGTG	GGGCCCCCTCCCAAAGAAGATGT
gabrg1	GAGCTAGTGCAGCACACGTA	ACCCAGGTTTTGTTCACCGT
gabrg2	GAGCTAGTGCAGCACACGTA	CACGTGGATAGCCATAACTGGA
slc12a2	TTCCAGAAGTTTAGGGCCCGA	CGTGGCCCAAAGTTTTCTGCAA
slc12a5	CCTCAGTCACAGGGATCATGGC	CACAATGCCATCCCTCGAGATG

reaction, staining of $TUNEL^+$ cells was correlated with their nuclear morphology.

Fluorescent microscope Imager Z1 (Zeiss) and ImageJ 1.49p software (NCBI, USA) were used for visualization and image analysis, respectively.

Statistical data processing was performed using SigmaPlot 11 software (Systat Software Inc, USA).

MK-801, muscimol, and monosodium glutamate (Sigma-Aldrich) were employed in calcium imaging, cell survival, and neurite outgrowth studies.

RESULTS

Expression of NMDA-R and $GABA_A$-R subunits and NKCC1 and KCC2 chloride transporters during differentiation of human iPSCs into VM neurons. To study the subunit composition of NMDA-R and $GABA_A$-R in human VM neurons, directed iPSC differentiation was carried out as previously described [14], and cell populations were obtained with a significant proportion (>35%) of dopaminergic neurons immunopositive for tyrosine hydroxylase, along with a small proportion of $GABA^+$ neurons (5-10%).

Using real-time RT-PCR, the transcription levels of the NMDA-R and $GABA_A$-R subunits as well as the *slc12a2* and *slc12a5* genes were measured in these cultures at three consecutive stages of differentiation: early multipotent neural progenitors (eNPs), committed VM neural precursors (cNPs), and neurons at the terminal differentiation stage (TD). The last stage of differentiation was carried out under two different conditions: in medium supplemented with recombinant neurotrophic factors BDNF and GDNF (TD BDNF) or in the co-culture of the studied cells with mouse astrocytes in the standard medium (TD astr.). The gene expression at the TD stage was analyzed on the day 15. These two differentiation conditions are widely employed in modern protocols for obtaining specific neuron subtypes from iPSCs [12, 18]. The fold changes in the expression levels were calculated relative to the expression level in eNPs, taken as one unit.

According to the ΔC_T values, the ratio of transcription levels of the NMDA-R subunit genes was as follows (from highest to lowest): *grin1* >> *grin2a* = *grin2b* > *grin2c* > *grin3a* > *grin2d* > *grin3b*.

The expression changes were similar in both investigated iPSC lines, thus here we present the data only for the IPSHD1.1S line (Fig. 1a). The transcription level of these genes was significantly higher at the TD stage with recombinant BDNF/GDNF compared to mouse astrocyte co-cultures. *Grin2b* transcription level remained stable at all examined differentiation stages and was not dependent on the TD conditions.

Out of the seven studied $GABA_A$-R subunits, the most pronounced changes in transcription were observed for the *gabra2*, *gabrb1*, *gabrg1*, and *gabrg2* genes (Fig. 1b). Under TD with the recombinant neurotrophic factors, a higher transcription level of *gabrb1* and *gabrg2* was found, while under TD in co-culture with mouse astrocytes, a higher transcription level of the *gabra2*, *gabrb2*, and *gabrg1* was observed.

The differentiation of the investigated cells was accompanied by a significant increase in *slc12a5* transcription level, which was most pronounced under TD in mouse astrocyte co-cultures. The expression level of *slc12a2* remained stable through the course of differentiation (Fig. 1c).

The Glun2A subunit of NMDA-R is expressed in human neural progenitors and immature neurons. In the mouse, the Glun2A subunit of the NMDA-R encoded by the *grin2a* gene was shown to be specifically expressed in mature neurons [19]. Using immunofluorescent staining, the Glun2A expression was analyzed at consecutive stages of neuronal differentiation of human iPSCs. Glun2A staining was absent in human undifferentiated iPSCs (data not shown). In eNP cultures, only single Glun2A-expressing cells were found, while all cNPs were Glun2A-positive. Glun2A immunoreactivity was also observed in all cells at the TD stage, with the strongest staining found in neurites (Fig. 2a).

It was found that in the rosette-forming human NPs, which represent a specialized type of multipotent NPs [20], both in adherent culture and in suspension neurospheres the Glun2A immunoreactivity was localized in the apical pole of the cells (Fig. 2b).

To compare the Glun2A expression profiles during the neural differentiation in human and murine cells, the immunofluorescent staining of primary mouse NP cultures was performed using antibodies to Glun2A and the NP marker Nestin (Fig. 2c). In these cultures, Glun2A immunoreactivity was observed in neurites, but no co-localization with Nestin was found.

To compare the Glun2A expression in human NPs *in vitro* and mouse *in vivo*, the immunofluorescent staining of mouse embryonic brain sections was performed. Immunoreactivity of variable intensity was present in the cortical plate, polymorphic, and molecular layers of the hippocampus – the areas where neuronal maturation takes place (Fig. 2d). At the same time, Glun2A staining was absent in the neocortical subventricular zone, the hippocampal neuroepithelium, and the dentate gyrus, which represent germinal zones containing the proliferating NP populations (Fig. 2d, inset).

Expression of NMDA-R and $GABA_A$-R subunits in human VM neurons *in vitro* is associated with formation of functional receptors in these cells. NMDA-R are cation channels permeable to Ca^{2+} [21]. $GABA_A$-R are anion channels, whose activation in immature neurons causes cell membrane depolarization and opening of voltage-gated calcium channels [22]. Thus, NMDA-R and $GABA_A$-R activity is associated with changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

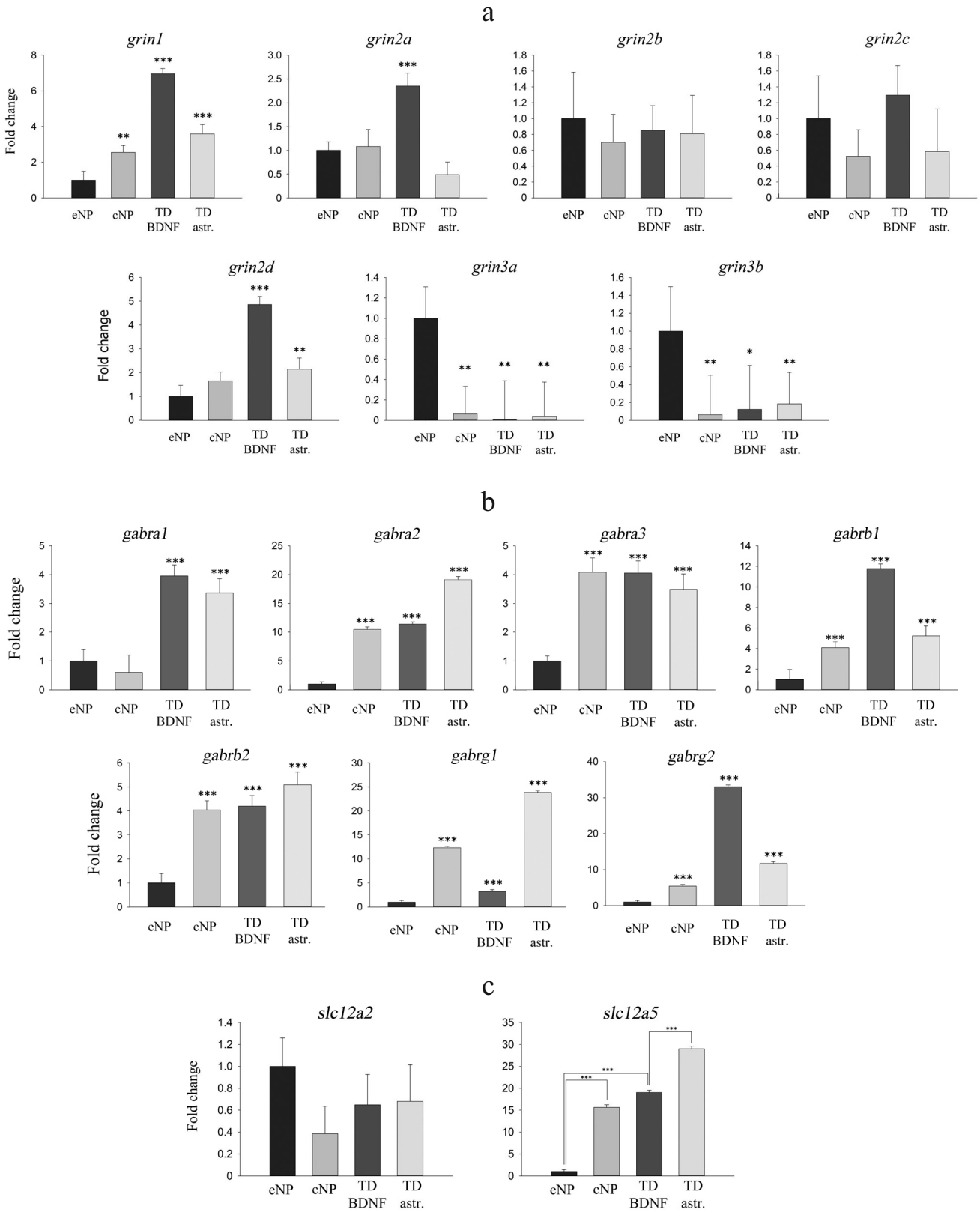

Fig. 1. Gene transcription dynamics during neuronal differentiation of iPSCs (IPSHD1.1S line). eNPs, early multipotent precursors; cNPs, committed VM precursors; TD BDNF, terminal differentiation in medium supplemented with recombinant neurotrophic factor; TD astr., terminal differentiation in mouse astrocyte co-culture. a) NMDA-R subunits; b) $GABA_A$-R subunits; c) chloride transporters *slc12a2* (NKCC1) and *slc12a5* (KCC2); $n = 4$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's *t*-test.

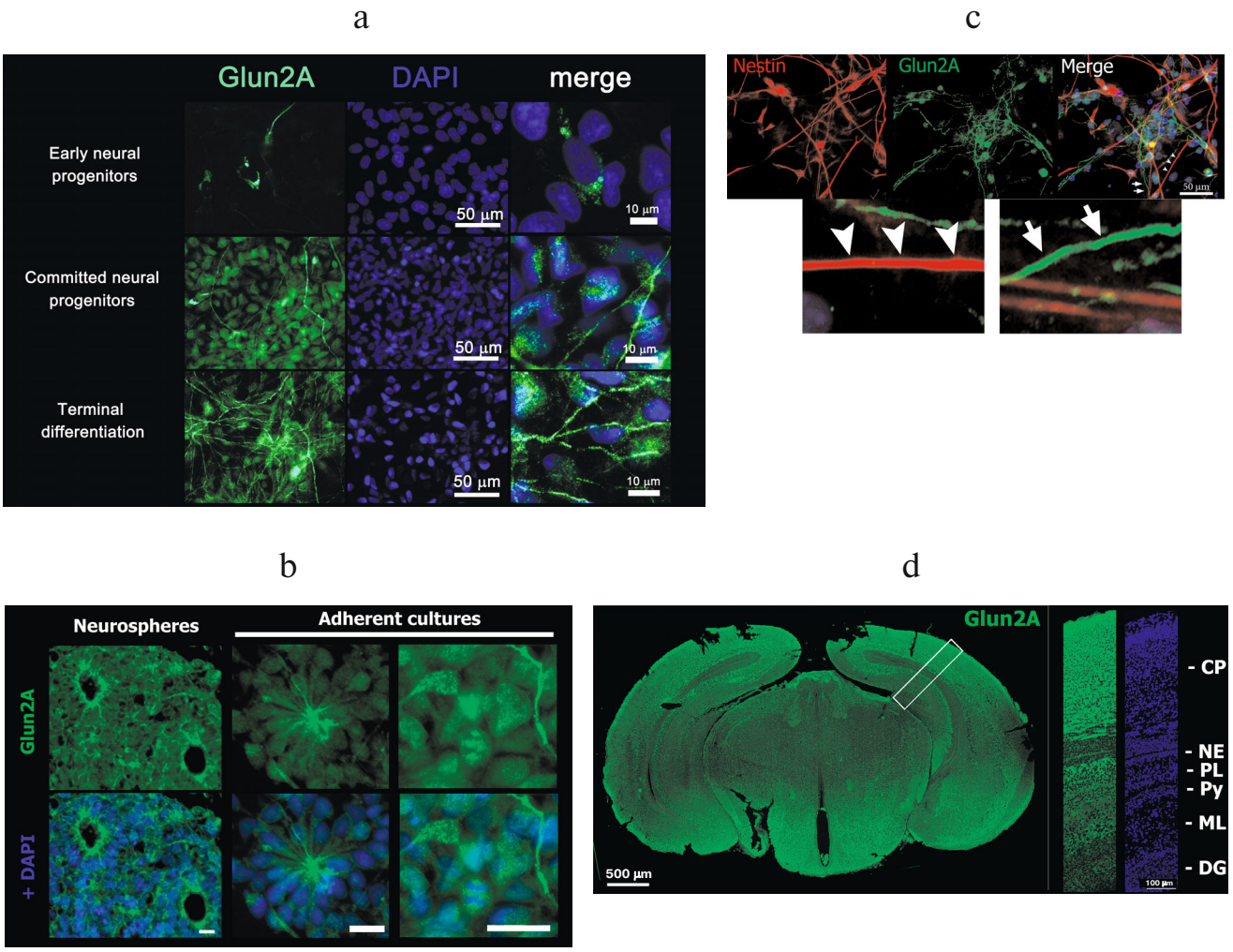

Fig. 2. a) Immunofluorescent staining of Glun2A during neuronal differentiation of iPSCs. Left column, Glun2A; middle column, nuclear staining (DAPI); right column, magnified sections of the combined images. b) Localization of Glun2A in the apical region of rosette-forming NPs. Both in suspension neurospheres (left) and in adherent culture (central column), Glun2A staining intensity prevails in the apical pole of the cells. In other human NP subtypes (right), Glun2A does not exhibit the asymmetric distribution. Scale bar, 20 μm. c) Double immunofluorescent staining of mouse primary NP cultures using Glun2A (green) and Nestin (red) antibodies. $Glun2A^+$ staining (long arrows) shows no co-localization with Nestin-positive processes (short arrows). The bottom row shows enlarged image sections. d) Immunofluorescent staining of Glun2A (green) in the brain of a 19-day mouse embryo. The framed area of the image is enlarged on the right. CP, cortical plate; NE, hippocampal neuroepithelial layer; PL, polymorphic layer of the hippocampus; Py, layer of the hippocampal pyramidal neurons; ML, molecular layer of the hippocampus; DG, dentate gyrus of the hippocampus. Nuclei were counterstained with DAPI (blue).

To establish a correlation between the expression of the genes encoding the NMDA-R and $GABA_A$-R subunits and the formation of functional receptors in the investigated cells, $[Ca^{2+}]_i$ was measured using the Ca^{2+} indicator Fluo-4 in the cells at the TD stage during the application of glutamate receptor and $GABA_A$-R agonists.

Previously, we have demonstrated that from days 8 to 30 of TD, the activation of $GABA_A$-R elicits excitatory effect in the iPSC-derived neurons, causing an increase of $[Ca^{2+}]_i$ [15]. In the present study, a similar excitatory effect of GABA and the selective $GABA_A$-R agonist muscimol was observed on day 15 of the TD in neurons co-cultured with mouse astrocytes. In the same cells, the application of L-glutamate (100 μM) in a medium containing 0.7 mM Mg^{2+} caused no changes in $[Ca^{2+}]_i$ (Fig. 3a). In the absence of Mg^{2+}, the application of 10 μM L-glutamate caused significant elevation of $[Ca^{2+}]_i$ in a large number of cells (Fig. 3b). Since it is known that Mg^{2+} induces voltage-dependent block of NMDA-R, without affecting the functioning of other types of ionotropic glutamate receptors [21], this result indicates the presence of functional NMDA-R in the studied cells, but not the other types (AMPA or kainate) of Ca^{2+}-permeable ionotropic glutamate receptors.

According to the literature, NMDA-R represents the most abundant type of glutamate receptors in developing

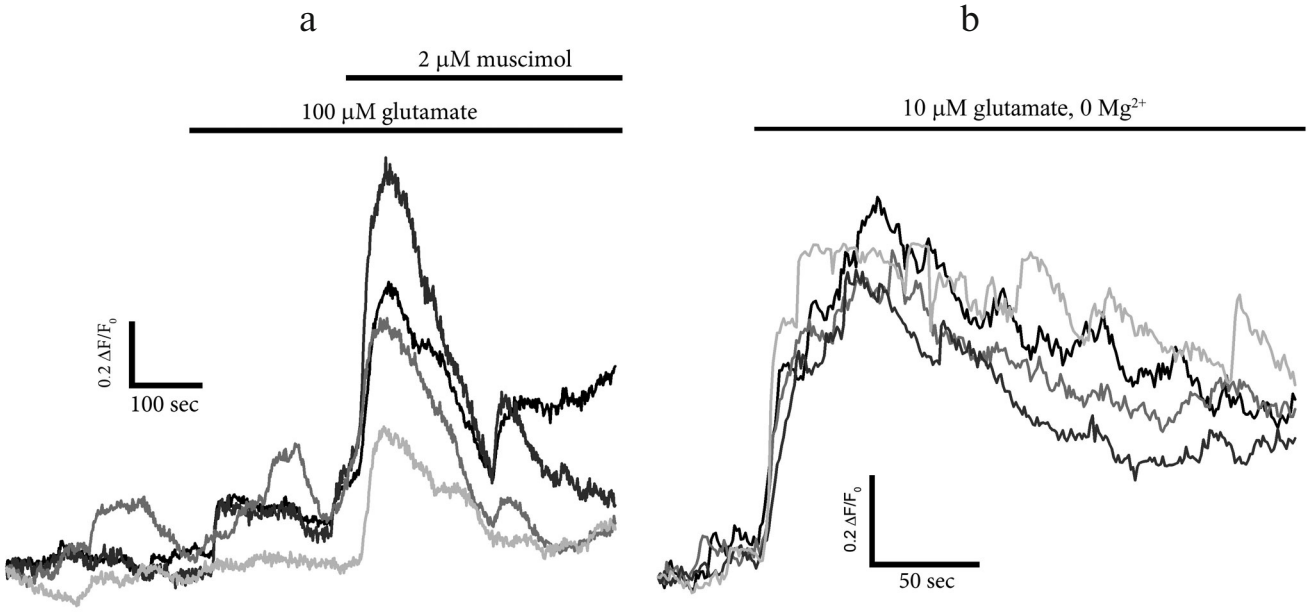

Fig. 3. The $[Ca^{2+}]_i$ dynamics at day 15 of TD in the investigated cells co-cultured with mouse astrocytes: a) application of glutamate and muscimol in standard medium (Hanks' solution). Graphs of Fluo-4 fluorescence intensity versus time in four representative cells; b) application of glutamate in Mg^{2+}-free medium (Hanks' solution prepared without Mg^{2+}). Representative fluorescence intensity changes of Ca^{2+} indicator Fluo-4 versus time in four cells.

neurons [23], while the AMPA-R are predominant in mature neurons [24]. The results of this study indicate that a similar predominance of NMDA-R is also observed during the differentiation of human VM neurons.

Glutamate exerts moderate toxicity in VM neurons derived from human iPSCs. Previously, Gupta et al. [25] demonstrated the excitotoxic effect of glutamate in human ESC-derived neurons of unidentified subtype. Glutamate susceptibility was absent in 2-week-old neurons and progressively increased during differentiation, reaching a maximum level in 8-week-old cultures [25]. Data on glutamate toxicity in human iPSC-derived VM neurons is not available in the current literature.

Here, the TUNEL method was employed to identify the apoptotic cells in cultures of human VM neurons. In our experiments, 500 μM glutamate was introduced into the culture medium on day 14-15 of TD. It was found that in these cultures there was initially a relatively high basal level of apoptotic cell death: the proportion of $TUNEL^+$ nuclei was 20.1 ± 6.3% (Fig. 4). In the presence of 500 μM glutamate, the proportion of $TUNEL^+$ nuclei increased to 36.9 ± 5.4%. This result indicates the existence in the studied cultures of a neuron subpopulation that is vulnerable to the toxic effects of glutamate.

Participation of NMDA-R in development of spontaneous activity in VM neurons. Spontaneous activity is critical for the maturation of various neuron subtypes [26]. The generation of the spontaneous activity was considered in a number of studies as a hallmark for assessing the functional maturity of neurons derived from human iPSCs [12, 27, 28]. Since NMDA-R are known to be involved in the regulation of the maturation of many neuron subtypes [29, 30], the participation of this receptor in the development of spontaneous cell excitability was analyzed by measuring the maximum amplitude of Ca^{2+} spikes.

Since the basal DMEM-F12 medium employed for cell culture in this study contains 50 μM L-glutamate, MK-801 (a selective NMDA-R antagonist) at a concen-

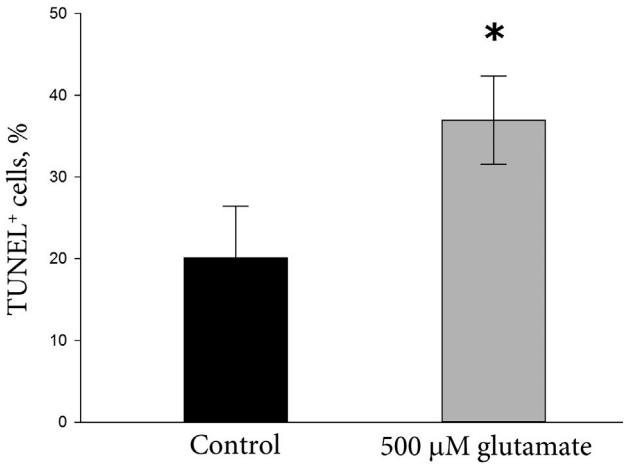

Fig. 4. Assessment of glutamate toxicity in human VM neuron cultures using the TUNEL method. The total number of cells was determined by the number of nuclei stained with DAPI; * $p < 0.001$; $n = 3$.

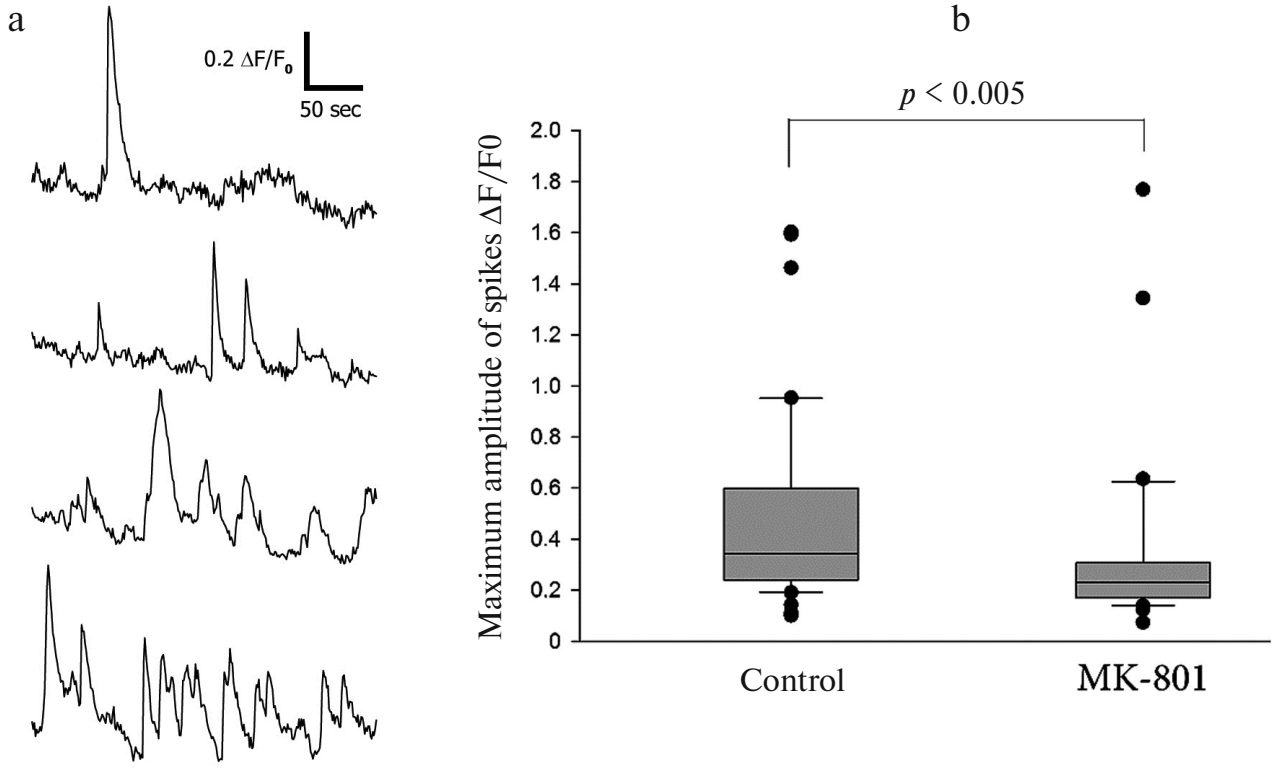

Fig. 5. Spontaneous changes of $[Ca^{2+}]_i$ in cultures of human VM neurons at day 15 of the TD stage. a) Representative graphs show Fluo-4 fluorescence intensity in individual cells as a function of time. Spontaneous activity was observed in the form of single Ca^{2+} spikes, bursts, or pacemaker-like activity. b) Decrease in the maximum amplitude of spontaneous Ca^{2+} spikes in neurons after 10 days of differentiation in MK-801 supplemented medium. Median values were calculated for 70 or more neurons for each replicate, $n = 4$. The significance of differences was assessed using the Mann–Whitney U-test.

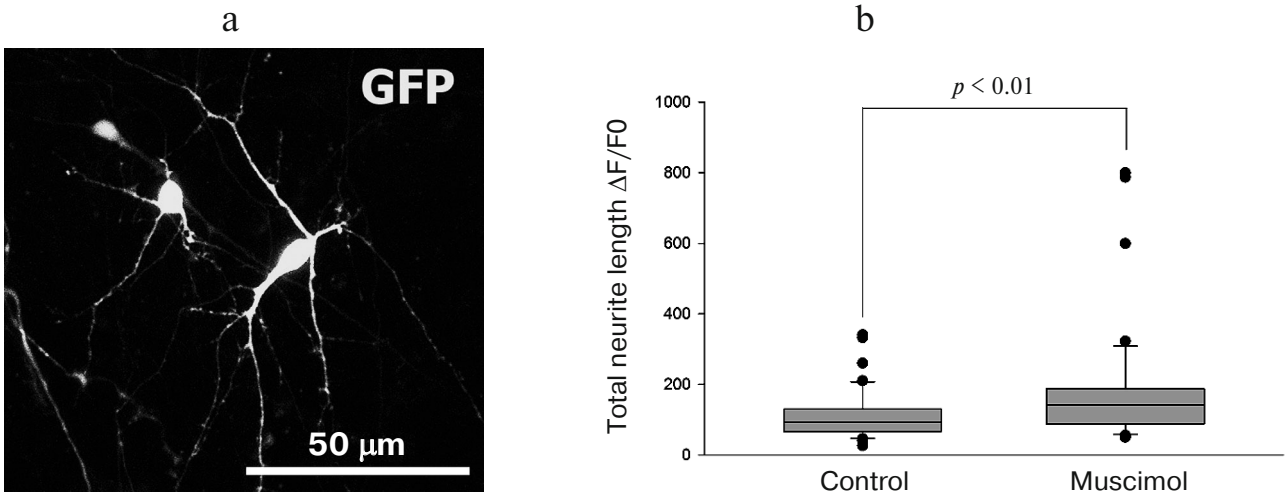

Fig. 6. Effect of muscimol on neurite length of individual cells. In total, >300 neurons were analyzed in three independent experiments. a) Representative image of neurons expressing Tag-GFP; b) analysis results of total neurite length per cell on day 4 of the TD.

tration of 10 μM was used to study the role of NMDA-R in the neuronal maturation. TD was performed in the presence of MK-801, and 24 h before the registration of the spontaneous activity, the inhibitor was washed out. The spontaneous activity properties were determined by measuring the dynamics of $[Ca^{2+}]_i$ changes.

The appearance of spontaneous activity in iPSC-derived neurons was observed on days 10-15 of the TD.

Representative records of the spontaneous activity in the individual cells are shown in Fig. 5a. It was found that the amplitude of spontaneous Ca^{2+} spikes was significantly lower in neurons differentiated in the medium supplemented with MK-801 compared to the control (Fig. 5b). These results indicate the participation of NMDA-R in the regulation of the functional maturation of human VM neurons.

Role of $GABA_A$-R in regulation of neurite outgrowth in human VM neurons. To study neurite outgrowth in single cells, we have transduced iPSC-derived NPs with lentiviral vectors carrying the *Tag-GFP2* and *Tag-RFP* genes. The immunofluorescent staining for cytoskeletal proteins βIII-tubulin and MAP-2 did not allow us to establish the identity of neurites to particular individual cells due to the growth of these cultures in a relatively high density and their predisposition to the formation of multilayered structures and dense neurite plexuses. Tag-GFP2- or Tag-RFP-labeled cells were mixed and cultured with non-transduced cells in 1 : 10 proportion, which provided the simplicity of identification and the tracing of individual labeled neurites of cells while maintaining optimal for growth cell culture density (Fig. 6a). The statistically significant differences of the average neurite length at the consecutive timepoints of differentiation (4, 7, and 11th days of TD) highlight the efficiency of this approach for studying factors affecting neuritogenesis (data not shown).

Differentiation in the presence of 2 μM muscimol, a selective $GABA_A$-R agonist, caused a significant increase of total neurite length per cell at the TD stage (Fig. 6b). This result is consistent with the previously described effects of $GABA_A$-R agonists for other types of neurons [31] and indicates an important regulatory role of $GABA_A$-R in the phenotype determination of the differentiating human VM neurons. Since the increase of neurite length is one of the distinguishing features that accompany neuronal maturation [22], $GABA_A$-R agonists can be considered as stimulators of human VM neuron maturation. This finding is in accordance with the data of Rushton et al. [32], who reported the ability of GABA to stimulate the maturation of human iPSC-derived neurons of an unidentified regional phenotype.

DISCUSSION

It is known that the differentiation and maturation of neurons involve a switch of NMDA-R and $GABA_A$-R subunit composition [9, 33]. Despite a significant number of studies on the directed differentiation of iPSCs into VM neurons, the neurotransmitter receptor functions in the resulting cells have not been investigated in detail [34, 35]. In this study, the subunit composition and functional activity of NMDA-R and $GABA_A$-R in human iPSC-derived VM neurons were analyzed for the first time. The *grin2a*, *gabra1*, and *slc12a5* genes represent potential mature neuron markers. It was shown that in the investigated cultures, the expression profiles of these genes can considerably vary depending on the conditions of the TD and are not predetermined by the regional phenotype of neurons. This observation suggests that the maturation of neurons is not a single process – the acquisition of individual mature phenotypic characteristics may involve independent regulation.

According to our data, Glun2A is expressed in all human cNPs and post-mitotic VM neurons. A similar expression profile is observed in human embryonic forebrain, where Glun2A is co-expressed with markers of various NP subtypes and young post-mitotic neurons [36]. In addition, Glun2A expression was demonstrated in immature neurons of an unidentified regional phenotype derived from human iPSCs [11]. Our data on Glun2A expression in murine cells *in vitro* and *in vivo* are consistent with literature data [33] and emphasize that Glun2A can be considered as a mature neuron marker in mouse, but not in human.

It is known that *in vivo* during the maturation of various neuron subtypes, including VM neurons, the transcription level of the *gabra2* gene encoding the predominant embryonic $GABA_A$-R α-subunit decreases. This phenomenon is accompanied by an increase of *gabra1* expression, the prevailing α-subunit in the adult central nervous system [37]. Here it was demonstrated that *in vitro* at the TD of human VM-neurons there is a significant increase of *gabra2* expression level, which exceeds the expression level of *gabra1*. Thus, regardless of the TD conditions, embryonic type $GABA_A$-R prevail in the investigated cells at this timepoint. The expression dynamics of the *grin1*, *gabra2*, and *gabrb1* genes described here are consistent with the results of the full-transcriptome analysis of human iPSC-derived VM neurons [38]. Similarly to our results, Momcilovic et al. have shown that *gabra2* transcription level exceeds *gabra1* level at the TD stage [38]. At the same time, no increase of *grin2a* and *grin2d* transcription was found by the transcriptome analysis at the investigated differentiation stages (eNP, cNP, and TD) [38]. These inconsistencies between our results might be due to the differences in the employed differentiation protocols.

It was found that during the differentiation, the expression of the *slc12a5* gene is increased, which was similarly observed by Momcilovic et al. [38]. This phenomenon is characteristic for the maturation of most types of neurons in the CNS [22] except for rat mesencephalic dopamine neurons [39]. It can be assumed that the expression profile of *slc12a5* differs in this neuron subtype between humans and rodents. It has been reported that in mature neurons, BDNF treatment suppress *slc12a5* expression [40], which might probably explain the differences in the transcription level of this gene depending on the TD conditions.

The measurements of the $[Ca^{2+}]_i$ dynamics have revealed that functional NMDA-R and $GABA_A$-R are already present at day 15 of TD in the investigated cells (Fig. 3). This result indicates the possibility of studying the effects of GABAergic and glutamatergic drugs in human VM neuron cultures at this timepoint of differentiation, omitting the extended differentiation protocols for obtaining fully mature neurons [27].

Activation of $GABA_A$-R in the investigated cells leads to an increase in $[Ca^{2+}]_i$, which is a distinctive feature of immature neurons [22]. This type of response is observed until day 30 of TD. Glutamate-induced Ca^{2+} current, sensitive to Mg^{2+}, indicates the presence of functional NMDA-R in the investigated cells (Fig. 3a). On days 10-15 of TD, the continuous application of glutamate in a non-flow-through cell chamber causes a transient increase of $[Ca^{2+}]_i$ in neurons. At the later timepoints of differentiation, an elevated $[Ca^{2+}]_i$ persists for an extended period of time. Thus, during the maturation of human VM neurons *in vitro*, NMDA-R become less prone to desensitization.

Excitotoxicity is considered as one of the primary causes of neuronal death in ischemic stroke and neurodegenerative diseases [4-6]. Modeling of this process *in vitro* is essential for studying the pathogenesis of these diseases and the search for new therapeutic agents. Excitotoxicity is mediated through the hyperactivation of glutamate receptors and an irreversible increase of $[Ca^{2+}]_i$ [41]. Glutamate toxicity can also be mediated through the inversion of the cystine–glutamate antiporter [42]. It was shown here that on days 14-15 of TD, the application of high glutamate concentrations induced cell death (Fig. 4), but the percentage of the vulnerable cells is much lower compared to primary rodent VM neuron cultures [43]. Most probably, these differences are explained by the heterogeneity of the investigated cultures and the asynchronous maturation of neurons.

In this study, it was demonstrated for the first time that the neurite outgrowth and the spontaneous activity of neurons, i.e., the properties that are tightly associated with neuronal maturation, are regulated in human VM neurons via NMDA-R and $GABA_A$-R (Figs. 5b and 6b). Thus, agonists of these receptors may be employed for optimization of the existing iPSC neural differentiation protocols, in particular, to accelerate the maturation of neurons.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Guidelines

Animal experiments were carried out in accordance with the applicable international, national, and/or institutional guidelines for the care and use of laboratory animals.

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