

Plasticity of Calcium Signalling Cascades in Human Embryonic Stem Cell-Derived Neural Precursors

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Abbreviations

ATP - adenosine-5'-triphosphate; **α,β -MeATP** - α,β -Methyleneadenosine 5'-triphosphate lithium salt; **BzATP** - 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt; **[Ca²⁺]_i** - intracellular Ca²⁺ concentration; **CM** - culture medium; **CNS** - central nervous system; **CPA** - cyclopiazonic acid; **EBs** - embryoid bodies; **ESCs** - embryonic stem cells; **GABA** - γ -aminobutyric acid; **GVIA** - ω -conotoxin GVIA; **hESC NPs** - human embryonic stem cell-derived neural precursors; **hrEGF** - human recombinant epidermal growth factor; **hrFGF** - human recombinant fibroblast growth factor; **HVA** - high voltage activated Ca²⁺ channels; **InsP₃R** - inositol-1,4,5-trisphosphate receptor; **LVA** - low voltage activated Ca²⁺ channels; **MEFs** - mouse embryonic fibroblasts; **MVIIC** - ω -conotoxin MVIIC; **NMDA** - N-Methyl-D-aspartic acid; **NPM** - neural precursor medium; **NPs** - neural precursors; **PBS** - phosphate-buffered saline;

PPADS - pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate; **P6-P10** - passage 6-10; **RU** - ratio units; **ROCC** - receptor-operated Ca^{2+} channels; **RyR** - ryanodine receptor; **SERCA** - sarcoendoplasmic reticulum Ca^{2+} -ATPase; **VOCC** - voltage-operated Ca^{2+} channels.

Abstract

Human embryonic stem cell-derived neural precursors (hESC NPs) are considered to be a promising tool for cell-based therapy in central nervous system (CNS) injuries and neurodegenerative diseases. The Ca^{2+} ion is an important intracellular messenger essential for the regulation of various cellular functions. We investigated the role and physiology of Ca^{2+} signalling to characterize the functional properties of CCTL14 hESC NPs during long term maintenance in culture (*in vitro*). We analyzed changes in cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) evoked by high K^+ , ATP, glutamate, GABA and caffeine in correlation with the expression of various neuronal markers in different passages (P6 through P10) during the course of hESC differentiation. We found that only differentiated NPs from P7 exhibited significant and specific $[\text{Ca}^{2+}]_i$ responses to various stimuli. About 31% of neuronal-like P7 NPs exhibited spontaneous $[\text{Ca}^{2+}]_i$ oscillations. Pharmacological and immunocytochemical assays revealed that P7 NPs express L- and P/Q-type Ca^{2+} channels, P2X_2 , P2X_3 , P2X_7 and P2Y purinoreceptors, glutamate receptors and ryanodine (RyR1 and RyR3) receptors. The ATP- and glutamate-induced $[\text{Ca}^{2+}]_i$ responses were concentration-dependent. Higher glutamate concentrations (over 100 μM) caused cell death. Responses to ATP were observed in the presence or in the absence of extracellular Ca^{2+} . These results emphasize the

notion that with time in culture, these cells attain a transient period of operative Ca^{2+} signalling that is predictive of their ability to act as stem elements.

Introduction

Human embryonic stem cells are pluripotent cells derived from the inner cell mass of a preimplantation embryo [1]. *In vitro*, these cells are able to maintain a normal euploid karyotype, differentiate into derivatives of all three germ layers and proliferate extensively [2,3]. These properties make them a unique candidate for cell transplantation, research into growth factors and early human development as well as for drug discovery. Substantial progress has been made recently in the differentiation of hESCs into a neuronal phenotype [3-5] this being a promising strategy for cell-based therapy of CNS injuries and neurodegenerative diseases. However, to date, several important questions remain unanswered: i) when and at what stage of differentiation should cells be transplanted; ii) what are the functional properties (ion channels, receptors and second messengers) of these cells and how are they regulated; and iii) how compatible are these properties with the physiological or pathological environment at the site of transplantation and treatment? Hitherto, with a few exceptions, the quality of stem cells is generally evaluated by determining the expression of various genes and key proteins during the process of differentiation; these however, although being present in the cell, may be physiologically inactive. Therefore, the aim of this study was, for the first time, to determine and characterize Ca^{2+} signals activated by physiological stimulation of neural precursors derived from hESCs.

Ca^{2+} is a ubiquitous second messenger involved in the regulation of almost all known cellular processes and, above all, in defining the life and death of every cell [6-10]. Signals mediated by Ca^{2+} are fundamental for fertilization, cell differentiation, proliferation [11], intercellular signalling, transcription factor activation and various death programs including necrosis and apoptosis [12]. Ca^{2+} can enter the cytoplasm from two sources: either by an influx via plasmalemmal voltage-operated and receptor-operated Ca^{2+} channels (VOCC and ROCC respectively) or by release from intracellular stores, such as the endoplasmic reticulum, through endomembrane Ca^{2+} channels classified as inositol-1,4,5-trisphosphate receptors (InsP_3Rs) and ryanodine receptors (RyRs). The variety of functions executed by Ca^{2+} depends on the speed, amplitude and spatio-temporal pattern of Ca^{2+} signals as well as by interactions between Ca^{2+} and other signalling pathways [9]. For example, changes in $[\text{Ca}^{2+}]_i$ following the activation of purinoceptors (P2X_3 , P2X_4 , P2Y_1 , P2Y_2) promote cell proliferation in murine embryonic stem cells (ESCs) [13]. On the other hand, glial excitability depends on Ca^{2+} waves that often occur as a result of ATP-mediated signalling through P2Y receptors [14]. The entry of Ca^{2+} through VOCC and the release of Ca^{2+} from internal stores modulate neuronal excitability [15]. A transient increase in $[\text{Ca}^{2+}]_i$ regulates cellular secretion and cellular motility during neuronal development [16]. Both GABA and glutamate have been shown to influence neural precursor cell proliferation during development [17,18]. Hence, a detailed characterization of the Ca^{2+} signalling cascades that are activated by various stimuli is useful in determining the functional state of the cell and may even predict its fate.

In previous work we have described a novel protocol for the efficient generation

of neural precursors from hESCs [19]. Our results showed that i) hESC NPs are able to differentiate into a neuronal phenotype and to develop into functionally active neurons; ii) P8 hESC NPs are the appropriate candidate for transplantation compared to undifferentiated hESCs and to P1, P5 and P10; iii) long term maintenance *in vitro* decreases tumorigenicity, although simultaneously attenuates proliferative activity and differentiation potential. Our study further revealed that the profile of NPs changes with the length of maintenance in culture.

To date, the role of Ca^{2+} , its homeostasis and signalling potential in ESCs differentiated to a neuronal phenotype have not been studied. To our knowledge there are only a few reports available on mouse ESCs [20,21] and on human ESCs differentiated towards neurones [22-25]. Therefore, we undertook the present study to determine whether the functional properties of NPs change during long term maintenance in culture. We also asked at what stage of differentiation are these cells in ideal physiological condition? We identified and analyzed molecular cascades of $[\text{Ca}^{2+}]_i$, homeostasis and Ca^{2+} signalling in correlation with human embryonic stem cell differentiation into a neuronal phenotype.

Materials and Methods

Drugs and solutions

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA): accutase, laminin, cadmium chloride, nickel chloride, nifedipine hydrochloride, ATP, α,β -Methyleneadenosine 5'-triphosphate lithium salt (α,β -MeATP), suramin sodium salt,

pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS), 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt (BzATP), L-Glutamic acid potassium salt monohydrate, N-Methyl-D-aspartic acid (NMDA), kainic acid, GABA, adenosine, β -mercaptoethanol, bovine serum albumin, Triton-X 100; from GIBCO (Rockville, MD, USA): DMEM/F12, L-glutamine, penicillin/streptomycin, fetal bovine serum, collagenase type IV and B27; from Invitrogen (Carlsbad, CA, USA): human recombinant fibroblast growth factor (hrFGF), Fura-2 AM 1mM solution in anhydrous DMSO cell permeant and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI); from R&D Systems (Minneapolis, MN, USA): human recombinant epidermal growth factor (hrEGF); from Molecular Probes (Eugene, OR, USA): goat anti-mouse IgG conjugated with Alexa-Fluor 594, goat anti-rabbit IgG conjugated with Alexa-Fluor 488 and Pluronic F-127; from Tocris Bioscience (Bristol, UK): NF279; from Polysciences, Inc. (Warrington, PA, USA): Aqua Poly/Mount mounting medium; from Alomone Labs Ltd. (Jerusalem, Israel): caffeine, ryanodine, CPA, ω -conotoxin MVIIC (MVIIC), ω -conotoxin GVIA (GVIA).

Concentrated stock solutions of nifedipine, glutamate and ryanodine were prepared in DMSO, while the remaining stock solutions of agonists/antagonists were dissolved in dH₂O. For each experiment, caffeine was freshly dissolved in Normal Locke's buffer (NL) at 37°C and vortexed until the caffeine crystals dissolved. All concentrated stock solutions were stored at -20 °C. Test solutions were prepared daily using aliquots from frozen stocks to obtain the working concentrations. All buffers and solutions in this study were made using ion-free dH₂O from Merck - Germany.

Human embryonic stem cell and human embryonic stem cell-derived neural precursor culture

Human ESCs line CCTL14 (complete information on the derivation and characterization of CCTL14 line of hESCs is available at <http://www.isscr.org/science/sclines.htm>) were cultured on a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts (MEFs) in gelatin-coated tissue culture dishes. The culture medium (CM) was Dulbecco's Modified Eagle Medium (D-MEM/F-12 without L-glutamine) supplemented with 15% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, penicillin and streptomycin at 50 U/ml, 0.1 mM β -mercaptoethanol, and 4 ng/ml human recombinant fibroblast growth factor (hrFGF). Colonies of hESCs were passaged every 4-7 days using either mechanical scraping with a glass pipette to provide low-density cultures of undifferentiated cells or enzymatic dissociation with collagenase type IV to provide high-density cultures of undifferentiated cells [19].

Neural precursors were derived from the CCTL14 line of human ESCs as described previously [19]. Briefly, to induce neural differentiation, clumps of undifferentiated hESCs were plated in agarose-coated tissue culture dishes with CM without hrFGF for 4 days, then with CM supplemented with Noggin for the next 4 days. At this time, 70% - 90% of the colonies formed embryoid bodies (EBs). Aggregates whose diameter exceeded 0.5 mm were dissected into smaller clumps with 20 G surgical blades, then replaced in serum-free medium or neural precursor medium (NPM) and cultured for 6 days. At this stage, cells in the EBs were defined as passage

1. NPM consisted of DMEM/F12 medium (1:1), B27 supplement (1:50), 2 mM L-glutamine and penicillin and streptomycin at 50 U/ml, supplemented with 20 ng/ml hrEGF and 20 ng/ml hrFGF. For the long-term propagation of hESC NPs, the EBs were dissociated by accutase and the cells were plated onto laminin-coated culture dishes. NPs were cultured in NPM and passaged by accutase each 5 - 7 days.

Antibodies and immunocytochemistry

Cells plated onto laminin-coated coverslips were washed in phosphate-buffered saline (0.1M PBS, pH 7.2) and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were washed twice in PBS prior to immunostaining. Permeabilization and blocking were carried out in a blocking buffer consisting of 0.4% Triton-X 100, 10% bovine serum albumin in 0.1M PBS for 45 min at room temperature (RT). Primary antibodies (See Table 1) were diluted in buffer consisting of 0.1% Triton-X 100, 2% bovine serum albumin in PBS overnight at 4°C. After 2 washes with PBS, appropriate secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200), or Alexa Fluor 594-conjugated goat anti-mouse IgG (1:200), were applied for 30 min at RT. To visualize the cell nuclei, the coverslips were incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes at RT. Finally, the coverslips with cells were mounted using Aqua Poly/Mount mounting medium and examined using a ZEISS LSM 510 DUO confocal microscope (Carl Zeiss, Germany).

Dye loading and measurements of intracellular $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ measurements on single cells were performed as previously reported [26-31]. Briefly, cultures of hESC NPs, plated on 24 mm glass-bottom dishes (WillCo Dishes-BV, Amsterdam, Netherlands) coated with laminin, were incubated with 2.5 μ M Fura-2 AM with 0.02% Pluronic F-127 in culture medium at 37°C and 5% CO₂ for 40 min. Loaded cells were then washed and the culture medium replaced with Normal Locke's buffer containing (in mM): NaCl, 140; KCl, 5; MgCl₂, 1.2; CaCl₂, 2.2; glucose, 10; HEPES-Tris, 10; pH 7.25) and kept at 37°C throughout the time course of the experiment. The osmolarity of the buffer was 298-300 mosmol/l⁻¹. Fluorescence measurements of $[Ca^{2+}]_i$ were performed with a fast fluorescence microspectrofluorimetry system based on an inverted microscope (Axiovert, Zeiss-Germany) equipped for epifluorescence (Plan-Neofluar 100x /1.30 oil immersion objective). To achieve fast switching between different excitation wavelengths, a rotating filter wheel was mounted in the excitation light path. The cells were alternately illuminated (200 Hz) at 340 \pm 10 and 380 \pm 10 nm. In order to minimize the background noise of the Fura-2 signal, successive values were averaged to a final time resolution of 320 ms. A measuring amplifier was synchronized to the filter wheel to measure the fluorescence intensities resulting from different wavelengths. The FFP software controlled the acquisition of the intensity data and provided functions for adjusting the signal values and the display and storage of the measured data. A CCD camera was used to visualize the cells. The $[Ca^{2+}]_i$ measurement values are expressed as the ratio units (RU) between the fluorescence obtained with two excitation wavelengths, 340 nm (A) and 380 nm (B). Fura-2 calibration was performed in these cells in vitro following the

procedure described by Lambert et al [32] and Jamen et al [33], which yielded $R_{min} = 0.08$, $R_{max} = 2.02$, $\beta = 1.757$ and $K_d = 224$ at 37°C .

Drug application

As described previously [28,34,35], the control and test solutions were applied using a temperature controlled multichannel polypropylene capillary perfusion system (Warner Instruments, Inc., USA). A single outlet capillary tubing (100 μm inner diameter) with a flow rate of 250 $\mu\text{l}/\text{min}$ was positioned close to the tested cell (< 0.5 mm). The selected cell was subjected to a constant flow of control buffer or test solutions. Each capillary was fed by a reservoir 45 cm above the bath and connected to a temperature control device (Harvard-France). The temperature of all solutions was maintained at 37°C . In this approach, switching the flow from one capillary to the next resulted in complete solution exchange within 1-3 seconds. After each application of the tested drug, the cells were washed with control buffer. This method allowed for the fast and reliable exchange of the solution surrounding the selected cell under observation without exposing the neighbouring cells.

Data analysis and statistical methods

Origin 8.5.1 was employed for plotting and statistical procedures. The results are expressed as mean \pm SEM. The number of the sample size (n) given is the number of cells tested according to the same protocol (control, test drug, recovery) for each group. The figures (traces) show on-line single cell measurements of the $[\text{Ca}^{2+}]_i$ levels before

and after the application of test substances, while bar diagrams and numerical data are given as mean \pm S.E.M. and present the peak amplitude of the $[Ca^{2+}]_i$ increase as a ratio between the fluorescence values of 340/380 nm excitation wavelengths. The results were analysed using one-way ANOVA. Differences were considered statistically significant if $p \leq 0.05$.

Results

$[Ca^{2+}]_i$ responses in undifferentiated hESC

Undifferentiated hESCs (n = 34) from two independent cultures were subjected to the application of K^+ (50 mM), ATP (100 μ M), glutamate (50 μ M), GABA (10 μ M) and CPA (10 μ M). These cells were only partially responsive to ATP. To identify the specific involvement of the type(s) of active purinergic receptors in undifferentiated hESC, we tested the effect of ADP (100 μ M) and adenosine (2 μ M). ADP had no effect, whereas adenosine caused rise in $[Ca^{2+}]_i$ in only 18% (n = 11). P2 receptor antagonist suramin (300 μ M) inhibited ATP-induced $[Ca^{2+}]_i$ by $33.3 \pm 23\%$ (n = 4), PPADS (10 μ M) had no effect (n = 3).

$[Ca^{2+}]_i$ responses in hESC NPs change during long term propagation in culture

The functional properties of hESC NPs were studied during long term propagation *in vitro* starting from P6 NPs to P10 NPs. All data within each passage were

collected and analyzed from a minimum of 3 independent experiments. Cells were chosen randomly and subjected to the same experimental protocol. The cells from each passage were subjected to various stimuli such as K^+ (50 mM), ATP (100 μ M), glutamate (50 μ M), GABA (10 μ M) and caffeine (20 mM; Table 2). The results indicate that P6 NPs were sensitive to the application of glutamate and K^+ ($27 \pm 1.3\%$ and $11 \pm 1\%$, respectively). Only a few cells were sensitive to other stimuli such as GABA or ATP ($7 \pm 1\%$ each) and to caffeine ($4 \pm 2\%$). The percentages of P7 cells responding to K^+ , ATP, glutamate, GABA and caffeine were $50 \pm 3.9\%$, $46 \pm 2.2\%$, $57 \pm 0.3\%$, $5 \pm 2.3\%$ and $18 \pm 2.1\%$, respectively. Similarly, the percentages of P8 NPs responding to K^+ , ATP, glutamate, GABA and caffeine were $32 \pm 4\%$, $24 \pm 3.5\%$, $9 \pm 1.3\%$, $16 \pm 1.1\%$ and $16 \pm 1\%$, respectively. The cells from P9 were responsive to ATP ($9 \pm 1.9\%$) and non-responsive to GABA. Less than 5% of cells were sensitive to other stimuli. P10 NPs were not sensitive to glutamate or GABA, while $5 \pm 2.3\%$ of cells were sensitive to ATP and 2.3% (1 out of 44 cells tested) to K^+ or caffeine. Immunocytochemical staining for Ca^{2+} channels and ryanodine receptors revealed the expression of L-type and P/Q-type VOCC, ryanodine receptors RyR1 and RyR3 and the weak expression of RyR2 in all passages (Table 3). Since the number of cells responding to physiological stimuli was significantly higher in passage P7 NPs ($59 \pm 2.5\%$) than in other passages (P6 NPs: $41 \pm 1.6\%$; P8 NPs: $49 \pm 1.1\%$; P9 NPs: $15 \pm 1.7\%$; P10 NPs: $7 \pm 0.2\%$), these cells were chosen for further experimentation in this study.

Characterization of $[Ca^{2+}]_i$ transients mediated by Ca^{2+} channels in P7 hESC NPs

We monitored Ca^{2+} entry through VOCC as $[\text{Ca}^{2+}]_i$ transients evoked by depolarization with 50 mM K^+ . The application of high K^+ solution evoked a rapid increase in $[\text{Ca}^{2+}]_i$ in 50% ($n = 145$) of the tested cells. Pre-incubation with Cd^{2+} (100 μM), a non-specific blocker of high-voltage activated (HVA) Ca^{2+} channels (L- N- P- Q- and R-types), together with Ni^{2+} (50 μM), a more specific blocker of low-voltage activated (LVA) Ca^{2+} channels (T-type), for 5 min significantly reduced the $[\text{Ca}^{2+}]_i$ responses induced by K^+ in all cells tested by $85.3 \pm 7.5\%$ ($n = 7$) indicating the involvement of voltage-activated Ca^{2+} channels in depolarization-induced Ca^{2+} entry (Fig. 1 A, B). In order to further characterize the involvement of specific sub-types of HVA Ca^{2+} channels, we used specific Ca^{2+} channel blockers such as nifedipine (for L-type), ω -conotoxin MVIIC (for P/Q type) and ω -conotoxin GVIA (for N-type). Application of 10 μM nifedipine reduced $[\text{Ca}^{2+}]_i$ responses in 67% of tested P7 NPs ($n = 12$). In 2 out of 8 cells nifedipine totally abolished the $[\text{Ca}^{2+}]_i$ responses (Fig. 1 C, D). In the other 6 cells, it significantly reduced the amplitude of the responses by $54 \pm 19.5\%$ ($p = 0.03$; $n = 6$), suggesting the contribution of L-type Ca^{2+} channels in P7 NPs. The application of 300 nM ω -conotoxin MVIIC (Fig. 1 E, F), which is known to block the P/Q-type of VOCC [36], reduced the $[\text{Ca}^{2+}]_i$ responses by $92 \pm 32\%$ ($p = 0.004$; $n = 5$) in all cells. The application of MVIIC at a higher concentration (1 μM), which was reported to also block N-type Ca^{2+} channels, completely inhibited the K^+ -induced responses in the tested cells ($n = 3$). Further, we used another specific N-type VOCC blocker, ω -conotoxin GVIA, at two different concentrations (500 nM and 800 nM), which only partially and reversibly reduced the K^+ -induced responses by $20 \pm 14.6\%$ ($p = 0.47$; $n = 11$) and $48 \pm 31.8\%$ ($p = 0.27$; $n = 9$), respectively. To confirm the above $[\text{Ca}^{2+}]_i$

measurement results, we performed a series of immunocytochemical analyses. No positive immunostaining for the $\alpha 1B$ subunit of the N-type of VOCC was observed, but positive immunostaining for the $\alpha 1C$ subunit of the L-type of VOCC (Fig. 1 G) and the $\alpha 1A$ subunit of the P/Q-type of VOCC (Fig. 1 H) was observed, suggesting the presence of L- and P/Q-type Ca^{2+} channels in P7 NPs.

[Ca²⁺]_i signalling through purinergic receptors in P7 hESC NPs

The application of ATP (100 μ M) induced a rapid $[Ca^{2+}]_i$ increase in 33 out of 71 (47%) NPs tested with the mean amplitude of 0.8 ± 0.12 (ratio units; RU) (Fig. 2 A). The rise in $[Ca^{2+}]_i$ in response to ATP was significantly inhibited (by $65 \pm 25\%$) by the broad-spectrum P2 receptor antagonist suramin (300 μ M) ($p = 0.01$; $n = 8$) (Fig. 2 B, C). Another P2 receptor antagonist, PPADS, had a similar effect at a concentration of 10 μ M, decreasing the $[Ca^{2+}]_i$ response to ATP by $73 \pm 20\%$ ($p = 0.02$; $n = 8$), (Fig. 2 D, E). These results suggest that functional P2 purinoceptors are present in P7 NPs.

To further identify which P2 receptors (ionotropic P2X or metabotropic P2Y) operate in hESC NPs, we studied the effect of the P2X agonist α, β -me ATP. In 64% of the cells, the application of α, β -meATP (100 μ M) transiently increased $[Ca^{2+}]_i$ by a mean amplitude of 0.86 ± 0.14 , $n = 16$ (Fig. 2 A). The application of 20 μ M BzATP, another P2X receptor agonist, evoked a $[Ca^{2+}]_i$ increase in 18% of the tested cells with the mean amplitude of 0.28 ± 0.07 , ($n = 22$; Fig. 2 A). The P2X receptors antagonist, NF279, was ineffective at concentrations from 20 nM to 1 μ M at which it is specific agonist for P2X₁ receptors (Fig. 2 F), whereas at a higher concentration (100 μ M), it inhibited the ATP-

induced $[Ca^{2+}]_i$ increase by $69 \pm 20.6\%$, $p = 0.04$; $n = 3$ (Fig. 2 G). Immunocytochemical staining with antibodies directed against P2X₂, P2X₃, P2X₄, P2X₆ and P2X₇ receptors revealed expression of P2X₂ (Fig. 2 H), P2X₃ (Fig. 2 I) and P2X₇ R (Fig. 2 J). Finally, to investigate P2Y-mediated Ca^{2+} signalling, the effects of ATP were examined in Ca^{2+} -free medium. In the absence of extracellular Ca^{2+} , the application of ATP induced an increase in $[Ca^{2+}]_i$ in 86% of the tested cells with a mean amplitude of 0.34 ± 0.15 , $n = 7$ (Fig. 2 A). The application of 100 μ M ADP, a P2Y₁ and P2Y₁₂ agonist, elevated $[Ca^{2+}]_i$ by 0.38 ± 0.08 ($n = 5$) in 56% of the tested cells (Fig. 2 A). These data suggest the involvement of both the P2X (P2X₂, P2X₃, P2X₇) and P2Y purinoceptors in Ca^{2+} signalling in P7 NPs.

Ca²⁺ release from intracellular Ca²⁺ stores and spontaneous [Ca²⁺]_i transients in P7 hESC NPs

To check for functional intracellular Ca^{2+} stores in P7 NPs, we used caffeine (20 mM), which in millimolar concentrations acts as an inhibitor of intracellular receptors for IP₃ while being a potent activator of RyR. We also applied ryanodine at 1 μ M, the concentration which opens the ryanodine receptor. A brief application (10 s) of either caffeine or ryanodine induced a $[Ca^{2+}]_i$ rise in 18% ($n = 44$) and 22% ($n = 9$) of P7 hESC NPs, respectively. Immunostainings for RyR1 (Fig. 3 A) and RyR3 (Fig. 3 B) were positive in all tested cells. The application of 10 μ M CPA, a potent, selective and reversible inhibitor of the sarco-endoplasmic reticulum Ca^{2+} -ATP_{ase} pump (SERCA),

caused an intracellular $[Ca^{2+}]_i$ increase in 12 out of 13 tested cells (92%) with a mean amplitude of 0.38 ± 0.05 .

Spontaneous $[Ca^{2+}]_i$ transients were observed in 51 out of 164 of tested cells (31%) in the absence of any stimuli (Fig. 3 C). The amplitude of the spontaneous $[Ca^{2+}]_i$ transients was 0.74 ± 0.05 ($n = 91$), and they appeared with a mean frequency of 1 per 3.2 min. These transients were partially inhibited by the application of VOCC blockers (100 μ M Cd^{2+} , 50 μ M Ni^{2+} , 10 μ M nicardipine) or by the removal of extracellular Ca^{2+} (Fig. 3 D).

$[Ca^{2+}]_i$ responses to glutamate in P7 hESC NPs

We tested the $[Ca^{2+}]_i$ responses of P7 NPs to various concentrations of glutamate (1 - 1 mM; $n = 118$). Out of 118 cells 80 cells, the glutamate caused an elevation of $[Ca^{2+}]_i$ in 80 cells (68%). Glutamate at 1 mM caused cell death which was determined by the loss of Ca^{2+} signals (Supplemental Fig.1). Therefore the concentration-dependent elevation of $[Ca^{2+}]_i$ was determined for the doses ranging between 1 and 100 μ M. The amplitude of the glutamate-induced $[Ca^{2+}]_i$ responses at various concentrations ranged, respectively: at 1 μ M = 0.27 ± 0.04 , $n = 6$; at 10 μ M = 0.29 ± 0.04 , $n = 6$; at 50 μ M = 0.56 ± 0.15 , $n = 29$; and at 100 μ M = 0.95 ± 0.06 , $n = 31$ (Fig. 4 A, B). When the cells were exposed briefly, 10 s, to 1 mM glutamate, there was a robust increase in $[Ca^{2+}]_i$, the Ca^{2+} level was high, sustained without any decay and did not return to resting level even after wash of glutamate for a longer duration (supplemental Fig. 1.) This phenomenon was observed in all 8 cells tested.

Glutamatergic signals in the nervous system are mediated by ionotropic (NMDA, AMPA, kainate) and metabotropic (mGlu) receptors. Two out of 9 tested cells were sensitive to the application of NMDA (100 μ M), while only 1 out of 6 cells was sensitive to kainic acid (100 μ M). In order to determine the contribution of metabotropic glutamate receptors, we applied glutamate in the absence of extracellular Ca^{2+} ; this caused a $[Ca^{2+}]_i$ increase in only 1 out of 9 cells suggesting that the sensitivity of P7 NPs to glutamate is mediated mainly by ionotropic glutamate receptors.

Expression of neural markers in neural precursors derived from hESC

The expression of various neural markers was analyzed in P6 - P10 NPs. Table 1 shows the list of primary and secondary antibodies used. All immunocytochemical results were obtained from two independent stainings with each antibody. The results from P6 - P10 are summarized in Table 3. The expression of various neural markers in P7 NPs is shown in Fig. 5. The neuronal markers NeuN and β III tubulin were expressed in all passages, with the highest expression in P6 - P8. Immunostaining for neuronal marker MAP-2 was negative in all passages. Staining for nestin, a marker of neural progenitor cells, revealed the presence of numerous nestin-positive cells in all passages of hESC. Glial cells markers such as GFAP (Fig. 5 Db, Gb) and astrocytic marker S100 (Fig. 5 Ab, Bb, Cb, Hb) were also expressed throughout all passages. However another astrocytic marker glutamine synthetase (Fig. 5 Gb), oligodendrocyte marker OLIG (Fig. 5 Fb) were detected only in P7 NPs. P7 NPs also expressed microglial marker Iba1 (Fig. 5 Eb). In contrast, P10 NPs, but not other passages showed positive

immunostaining with anti-NG2 chondroitin sulphate proteoglycan. Immunostaining with anti-glutamate and anti-GABA was positive in P6 NPs, intensely positive in P7 NPs (Fig. 5 Ha) and absent in passages 8 - 10 (Table 3). The low expression of GABA was observed only in P6 and P8 NPs.

Discussion

Remodelling of the $[Ca^{2+}]_i$ signalling toolkit in hESC NPs

Here we demonstrated the functional remodelling of hESC NPs during propagation *in vitro*. While undifferentiated hESC were partially responsive only to ATP, pre-differentiated cells expressed more sophisticated Ca^{2+} signalling mechanisms, characteristic of a neural phenotype. P7 and P8 hESC NPs express functional Ca^{2+} channels, ATP receptors, glutamate receptors, ryanodine receptors and also demonstrate spontaneous Ca^{2+} oscillations. We found that the highest activity of Ca^{2+} signalling systems was observed at P7 and P8. This reflected data obtained *in vivo*, which showed that P8 NPs yielded the best results in terms of functional improvement after transplantation [19]. Thus we may suggest that the behaviour and fate of P7 and P8 hESC NPs after transplantation *in vivo* correlate with their elevated functional state as revealed by Ca^{2+} signalling. This rapid and transient remodelling of the Ca^{2+} the signalling toolkit represents, in our view, the most important finding of the present study. This shows that physiologically hESCs cells may acquire a neuron-specific pattern of Ca^{2+} signalling but only for a short period limited to only 2 passages (Fig. 6). We have

also determined the expression of a number of neuroal markers in undifferentiated and various passages of differentiated hESCs (Fig. 7) which the differential expression of various markers in hESC-NPs. We may further contemplate that the evaluation of Ca^{2+} signals in stem cells can be used to predict the fate of the cells during differentiation and can serve as an important criterion for the assessing the quality of stem cells before their use in cell replacement therapy.

NPs derived from the CCTL14 line of hESCs at various passages show differences in their expression of genes and cell markers, as well as differences in the behaviour of the cells after transplantation into the brain following middle cerebral artery occlusion in rats [19]. Therefore, our main attention was focused on studying the functional properties of NPs at different passages during maintenance *in vitro* that would be appropriate for transplantation.

Our results obtained from $[\text{Ca}^{2+}]_i$ measurements in single cells indicated that P6 NPs were sensitive mostly to glutamate (27%), while only a small number of P6 NPs responded by an increase in $[\text{Ca}^{2+}]_i$ to other agonists, such as 50 mM K^+ (11%), ATP (7%), GABA (7%) or caffeine (4%), suggesting an insignificant role for VOCCs and the absence of ATP-, GABA- and ryanodine receptor-generated Ca^{2+} signals in P6 NPs. Likewise only a small population of P9 NPs was sensitive to K^+ (4%), ATP (9%) or caffeine (4%). The number of cells sensitive to glutamate decreased significantly to 9% in P9 NPs when compared to P6 NPs (27%), and GABA was without any effect on $[\text{Ca}^{2+}]_i$. Unlike all other passages, P10 NPs were not sensitive to any of the applied stimuli (ATP, 5%; K^+ , 2%; caffeine, 2%; GABA, 0%). These results suggest that P10 NPs are generally devoid of Ca^{2+} signalling machinery. The highest number of cells

responsive to at least one of the applied stimuli was found in NPs from passages 7 and 8 (59% and 49%, respectively). P7 NPs contained a significantly higher number of cells sensitive to glutamate (57% in P7 NPs vs. 9% in P8 NPs), K^+ (50% in P7 NPs vs. 32% in P8 NPs) and ATP (46% in P7 NPs vs. 24% in P8 NPs). Almost equal populations of P7 NPs and P8 NPs were sensitive to caffeine (18% and 16%, respectively). The sensitivity to GABA increased to 16% in P8 NPs in comparison with P6 NPs (7%); however, it should be noted that P9 NPs and P10 NPs were totally insensitive to GABA. These findings are in agreement with immunocytochemical results showing the expression of glutamate only in passages 6 and 7, faint expression of GABA in passages 8 and 6, but not in other passages (Table 3). The reasons for such peculiar signalling patterns remain unclear. Interestingly, the number of cells sensitive to various stimuli was consistently the highest in P7 NPs when compared to the other passages (Table 2, Figure 6). Therefore, P7 NPs were chosen for the further detailed characterization of Ca^{2+} signalling mechanisms.

Expression of VOCC in P7 hESC NPs

Ca^{2+} entering the cytosol via VOCC regulates enzyme activity, gene expression and other biochemical processes in cells. In neurons, VOCC also initiate synaptic transmission [37]. While L- and T-type currents are found in a wide range of cells, N-, P, Q-, and R-type Ca^{2+} currents are most prominent in neurones. Depending on their localization in the cell, VOCC carry out different functions. For example, L-type VOCC

located in the cell bodies and proximal dendrites induce gene activation, while N- and P/Q-type VOCC trigger the release of neurotransmitters at synaptic endings [9].

Previous studies have demonstrated the strong enhancement of neurogenesis by Ca^{2+} influx through L-type VOCC [38,39]. In neuronal cells derived from ESCs, Yu and co-workers [40] showed that cooperation between L-type VOCC and RyR2 is crucial for the activity-dependent neurogenesis induced by GABA signalling. In neural progenitors derived from hESCs after 30 days in culture, Malmersjo and colleagues observed an increase in $[\text{Ca}^{2+}]_i$ evoked by 50 mM K^+ , but they did not specify which type of VOCC was activated [22]. Using RT-PCR studies, others have shown the up-regulation of VOCC expression in differentiated hESCs [23]. L-type channels are also expressed in neural precursors derived from human [25] and mouse ESCs [24].

Here we identified the functional expression of L- and P/Q-type VOCC in P7 NPs. Our findings are in accordance with the reports mentioned above; however, to the best of our knowledge, our study is the first to document the functional expression of P/Q Ca^{2+} channels in neural precursors derived from hESCs. Another interesting observation in this study was the lack of an effect of GVIA on K^+ -induced $[\text{Ca}^{2+}]_i$ responses and the absence of immunocytochemical staining for the $\alpha 1\text{B}$ subunit of the N-type of Ca^{2+} channels.

Our data show that P/Q-type channels were functional in 50% of the cells tested (responsive to 50 mM K^+) from P7 NPs. The application of 300 nM up to 2 μM MVIIC was reported to block several P/Q- and N-type Ca^{2+} channel-regulated physiological functions [36,41,42]. In our case, high K^+ induced a $[\text{Ca}^{2+}]_i$ increase that was blocked by 1 μM MVIIC in all tested cells, although the application of GVIA (500 and 800 nM), a

selective N-type VOCC blocker, was not effective in blocking the K^+ -induced $[Ca^{2+}]_i$. This could be explained by the presence of P/Q channels, which are sensitive to MVIIC but not to GVIA [30,36,43]. Indeed, the application of MVIIC at a 300 nM concentration, effective in blocking the P/Q-type of VOCC, reduced the Ca^{2+} increase in all tested cells. In addition, immunocytochemical staining for the $\alpha 1B$ subunit of the N-type of VOCC was negative, while immunostaining for the $\alpha 1A$ subunit of the P/Q-type of VOCC was positive. Therefore, we conclude that P7 NPs express functional P/Q-, but not N-type, Ca^{2+} channels. An interesting finding was that there was no correlations between the immunocytochemical expression of VOCC and their functional activity. L- and P/Q-types of VOCC could be identified immunocytochemically in all passages without any significant differences, while functionally only P7 and P8 showed the expression of VOCC, while in other passages (P6, P9 and P10) VOCC were inactive.

It was shown previously that mouse embryonic stem cell-derived neurones in the early stages of differentiation possess a complex pattern of VOCC, with a shift in channel contribution from N- and L-types in apolar cells to P/Q- and R-type channels in bi- and multipolar cells [21]. In our study the $[Ca^{2+}]_i$ increase in response to depolarization by K^+ was partially reduced by nifedipine, suggesting a possible role for L-type VOCC.

Purinergic receptor activation in P7 hESC NPs

Purinergic receptors are widely distributed in the body and participate in the regulation of virtually all physiological processes. ATP acts as a fast excitatory

neurotransmitter and has a potent long term role in cell proliferation, growth and development and also in disease and cytotoxicity [44,45]. As well as nervous system development, including progenitor cell proliferation, cell migration, neuronal and glial interactions and differentiation and synaptic network formation are also controlled by purines [13,46].

In our study we used a series of agonists and antagonists to determine which subtypes of purinergic receptors are functional in P7 NPs. Suramin is generally selective as an antagonist for P2 receptors versus other types of receptors, but it does not discriminate between P2X and P2Y receptors [47]. In the present study, suramin reversibly inhibited the ATP-induced $[Ca^{2+}]_i$ increase by 65% in all tested cells, suggesting that P2 receptors are functional in P7 NPs. To discriminate between different types of P2X receptors, the selective P2X_{1,3,5,7} agonist α,β -me ATP was used; it displayed a similar high potency as ATP, which is typical for P2X₁ and P2X₃ receptors. The P2X_{1,2,3,5} selective antagonist PPADS effectively blocked the ATP-induced $[Ca^{2+}]_i$ increase in 75% of the tested cells. Another antagonist NF279, was not effective in low concentrations (less than 100 μ M), suggesting the absence of functional P2X₁ and P2X₂ receptors. BzATP was effective in 18% of the cells tested. These data suggest the presence of functionally active P2X₃ and P2X₇ receptors, which were also confirmed by immunocytochemistry. While we were unable to characterize the function of P2X₂ receptors pharmacologically, immunocytochemical staining showed the presence of P2X₂ receptors in P7 NPs.

To the best of our knowledge there is only one study by Young and co-workers [23] that has demonstrated the presence of purinergic receptors in hESC-derived neural

progenitors. Though the authors did not perform functional studies on P2 receptors, they showed by RT PCR that in the hESC line WA09, the P2X₄ subunits are up-regulated in hNPs but down-regulated in differentiated hNPs, while P2X₇ was not expressed at any stage. In contrast, in our study CCTL14 hESC NPs expressed P2X₇, but not P2X₄ receptors. This might be explained by the difference in the cell lines used and by differences between the differentiation protocols themselves, since it is now well established that the effect of various neurotrophic factors such as GDNF, BDNF, NGF, NT-3, etc., varies in dependence with a targeted cell type [48]. Others have shown that GABAergic neurones derived from mouse ESCs elevate [Ca²⁺]_i predominantly via the activation of P2X₂, P2X₄ and P2Y₁ receptors [49]. In our study we observed a [Ca²⁺]_i increase in response to ATP also in the absence of extracellular Ca²⁺, suggesting the involvement of metabotropic P2Y receptors in the functioning of P7 NPs. We hypothesize that the functional properties of neural precursors are highly dependent on the origin of the cells and the differentiation conditions they are exposed to.

Ca²⁺ stores in P7 hESC NPs

In the nervous system Ca²⁺ release from internal stores plays an important role in regulating synaptic plasticity, neurite outgrowth, neurodegeneration and secretion [50-52] and is also essential for triggering Ca²⁺ waves and oscillations in astrocytes [53]. It is regulated by two types of receptors, inositol-trisphosphate and ryanodine receptors, that are localized on the endoplasmic reticulum and in mitochondria [54,55]. Millimolar concentrations (5-20 mM) of caffeine modulate intracellular Ca²⁺ signalling through the

activation of RyR and at the same time the inhibition of InsP₃R [56]. Therefore, depending on the relative densities of RyR and InsP₃R in a particular cell, caffeine can either stimulate or block Ca²⁺ release from intracellular stores [57]. The release of Ca²⁺ from intracellular stores is well documented in embryonic stem cell-derived cardiomyocytes [58,59]. In human embryonic stem-cell derived dopamine neurones, it was shown that dihydroxyphenylglycine-induced [Ca²⁺]_i increase was observed in the absence of extracellular Ca²⁺ suggesting the involvement of intracellular stores [22]. Other reports have shown that the RyR2 receptor, acting through GABA_A receptors and L-type Ca²⁺ channels, induces neurogenesis in embryonic stem cells [40]. In our experiments, the application of caffeine caused an increase in [Ca²⁺]_i, suggesting the activation of RyR; these findings were further confirmed by positive immunostaining for the RyR1 and RyR3 receptor. The application of CPA in P7 NPs resulted in a slow, long-lasting [Ca²⁺]_i increase. These data suggest that P7 NPs possess mature and functional ER Ca²⁺ stores.

Spontaneous [Ca²⁺]_i oscillations in P7 hESC NPs

Another interesting phenomenon that we observed is that hESC-NPs exhibited spontaneous oscillations in [Ca²⁺]_i. Spontaneous [Ca²⁺]_i activity is an essential feature of developing neurones [15,35,60]. The elevation of [Ca²⁺]_i in developing neurones in the form of Ca²⁺ spikes or Ca²⁺ waves regulates neuronal differentiation, axonal outgrowth, the development of potassium currents, the expression of GABA, etc. We observed spontaneous [Ca²⁺]_i transients in 31% of tested cells from P7 NPs with a mean

frequency of 3.2 min. These spontaneous transients were not abolished by either the removal of extracellular Ca^{2+} , or by the presence of VOCC blockers (N-, L-, P/Q-type). Of interest, Spitzer and co-workers identified a few types of spontaneous $[\text{Ca}^{2+}]_i$ transients in developing neurones, including Ca^{2+} spikes and Ca^{2+} waves [60,61]. Ca^{2+} spikes functionally have been found to regulate the development of potassium currents and the expression of GABA [62,63], while Ca^{2+} waves regulate neurite outgrowth [64]. We would classify the spontaneous Ca^{2+} elevations, based on their characteristics, observed in P7 NPs as Ca^{2+} waves. These data clearly indicate that P7 hESC NPs function in a similar manner as early neuronal cells. Similarly, it was reported that post mitotic neurons from hESCs exhibit spontaneous $[\text{Ca}^{2+}]_i$ transients, similar to $[\text{Ca}^{2+}]_i$ waves, and are mediated by $\text{Gd}^{3+}/\text{La}^{3+}$. Blocking these transients led to a significant reduction in progenitor cell proliferation [25].

Glutamate and GABA receptors in P7 hESC NPs

Since glutamate and GABA are important neurotransmitters and play a role in neuronal development [65], we next tested whether P7 NPs are sensitive to these two substances. Only 5% of tested cells ($n = 44$) were sensitive to the application of GABA. According to some reports [66-68], the $[\text{Ca}^{2+}]_i$ increase induced by GABA causes neuronal depolarization mainly in cells undergoing neuronal differentiation and only in a fraction of precursor and progenitor cells; also, the number of responding cells decreases with time. In addition, there are some concerns about using $[\text{Ca}^{2+}]_i$ to monitor the depolarizing action of GABA. Glutamate regulates proliferation and neuronal

differentiation and also acts as a positive regulator in neurogenesis [69]. Glutamate acts via ionotropic NMDA, AMPA and kainate receptors as well as metabotropic mGlu receptors. Due to such a diversity of pathways, glutamate plays various roles in neurogenesis starting from the early stages of development [69,70]. In our study we observed a $[Ca^{2+}]_i$ increase even in response to low concentrations of glutamate in a majority of cells tested (68%). Similar findings have been reported previously in a few studies, for example in neural precursor cells derived from human embryonic stem cells differentiated into dopaminergic neurones [22]. Young and co-workers described the presence of AMPA- and kainate- and the absence of NMDA-mediated Ca^{2+} responses in hESC-derived neural progenitors [23]. In our study, only a few P7 NPs were sensitive to NMDA and kainic acid, 22% and 17% respectively. We did not make further attempts to test the responses to AMPA suggesting that glutamate responses were most likely not mediated by NMDA or kainate glutamate receptors. Other authors have reported that the application of 500 μ M glutamate caused a rise in $[Ca^{2+}]_i$ in 34 out of 68 tested human neural precursor cells, while only 3 out of 68 cells were depolarized by 50 mM K^+ [71]. In contrast, our results show that neural precursor cells are sensitive to the application of even low (1 μ M) concentrations of glutamate, and 50 % of cells were depolarized by 50 mM K^+ . Glutamate, when applied at 1 mM caused Ca^{2+} overload led to cell death (supplemental Fig 1). In the absence of extracellular Ca^{2+} , the application of glutamate had no effect, suggesting the absence of functional mGlu receptors. Additionally, we found positive immunoreactivity for the NR2D subunit of NMDA receptors in P7 NPs (supplemental Fig.2). This subunit has been shown to play an important role in synaptic transmission in the early stages of brain development.

Furthermore, immunocytochemical results revealed that P7 NPs consist of heterogeneous cell populations including neural progenitor cells (nestin-positive), which have the capacity for self-renewal, and more mature cells showing a neuronal or glial phenotype. This heterogeneity is also reflected in the variety of Ca^{2+} channels and receptors present in P7 NPs. Some overlap in expression of neuronal marker β III tubulin and glial marker GFAP was observed in all immunostaining throughout all passages. Of interest, it was reported previously [72-74] that both neurons and glial cells at certain time points can co-express β III tubulin and GFAP. Interestingly, there was no significant difference between the immunocytochemical expression of VOCC and ryanodine receptors among P6 - 10 NPs, although the number of cells sensitive to the agonists of these receptors varied from P6 to P10, with significantly higher activation in P7 and P8 NPs.

Conclusions

Remarkable progress has been made recently in differentiating ESCs and other pluripotent stem cells into a neuronal phenotype [2-5,75,76]. To date, various growth factors and morphogenes, as well as cell markers, necessary for neuronal differentiation and development have been identified [77]. Nevertheless, many questions remain unanswered, in particular concerning the physiological development and functional activity of transplantable neural precursors derived from ESCs. Here, we correlate the histochemical and morphological features of hESC NPs with their physiological properties. Our findings clearly indicate that the pre-differentiation of ESCs leads to an

activation of Ca^{2+} signalling cascades and enhances the functional activity of the cells. We also showed that the Ca^{2+} signalling mechanisms and the physiological properties of hESC-derived neural precursors change during maintenance *in vitro* (Fig. 6). The mechanisms and factors that underlie these processes need to be established. Studying the functional properties of stem cells *in vitro* may help to predict their behaviour and the fate of their physiopathological status *in vivo* and may serve as criteria to evaluate the quality of such cells. The preliminary results of this work have appeared as abstracts [78, 79]. We conclude that the criteria to establish the histochemical characteristics and to identify the markers of differentiating cells do not reflect their functional state in terms their signalling mechanisms. The evaluation of homeostatic signalling mechanisms could be considered as key element in determining the ‘quality of stem cells’. Therefore, understanding the physiology of stem cells may allow us to better control their regenerative potential, which in turn may help to improve strategies for their use in transplantation and the treatment of neurodegenerative diseases.

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Author Disclosure Statement

The authors declare they have no conflicts of interest.

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Table Legends

Table 1. Antibodies used for immunocytochemistry

Table 2. The number of cells responding to various physiological stimuli in different passages of human embryonic stem cell-derived neural precursors (P6-P10 NPs)

The number of cells from different passages of hESC NPs responding to various physiological stimuli (e.g., 50 mM K⁺; 100 μM ATP, 100 μM Glutamate; 10 μM GABA; 20 mM caffeine) by a rise in [Ca²⁺]_i. Cells responding to one or more of the applied stimuli were considered as physiologically active.

Table 3. Expression of various neural markers and Ca²⁺ sensitive channels in P6 - P10 hESC NPs

Figure Legends

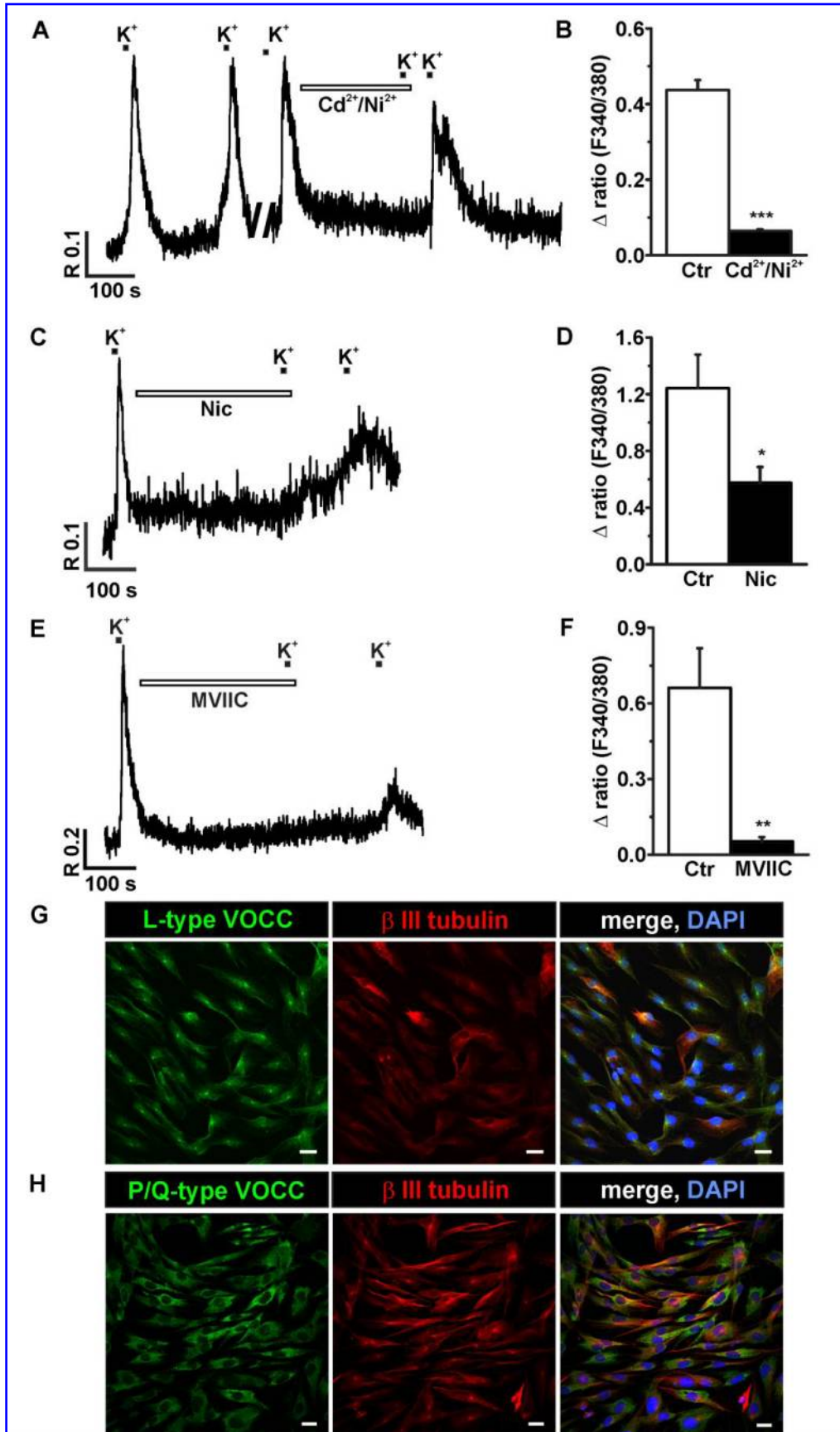


Fig 1. Voltage-operated Ca²⁺ channels in P7 hESC NPs

Example of traces **(A, C, E)** from individual cells for each experimental design show the block of K⁺ responses by specific Ca²⁺ channel antagonists. The cells were first exposed to control K⁺ depolarization and the [Ca²⁺]_i responses were monitored. Note that the amplitude of the control responses to high K⁺ is identical without any run down. Subsequently, the same cell was pre-incubated for 5 min with Ca²⁺ channel blockers (Cd²⁺/Ni²⁺; Nic: nifedipine and MVIIC) as indicated on the trace and then again challenged with high K⁺. **(B)** Pre-incubation of cells with 100 μM Cd²⁺ together with 50 μM Ni²⁺ significantly reduced [Ca²⁺]_i responses in all tested cells (n = 7). The trace **(C)** and bar diagram **(D)** show the reduction of the high K⁺-induced [Ca²⁺]_i responses by the L-type Ca²⁺ channel blocker 10 μM nifedipine (n = 6). The trace **(E)** shows the K⁺-induced [Ca²⁺]_i responses in the presence of and after pre-incubation with the P/Q-type Ca²⁺ channel blocker, ω-conotoxin MVIIC (300 nM). **(F)** Bar diagram shows the nifedipine inhibition of the high-K⁺ responses (n = 6). **G and H** are confocal images of P7 hESC NPs, co-stained for βIII tubulin **(G, H middle panel)** and for L-type Ca²⁺ CP α1C (H-280) **(G, left panel)** or P/Q-type Ca²⁺ CP α1A (H-90) **(H, left panel)**. Merged images are presented in the right panels **(G, H)**. Scale bars = 20 μm. * p = 0.05; ** p = 0.005; *** p = 0.0005.

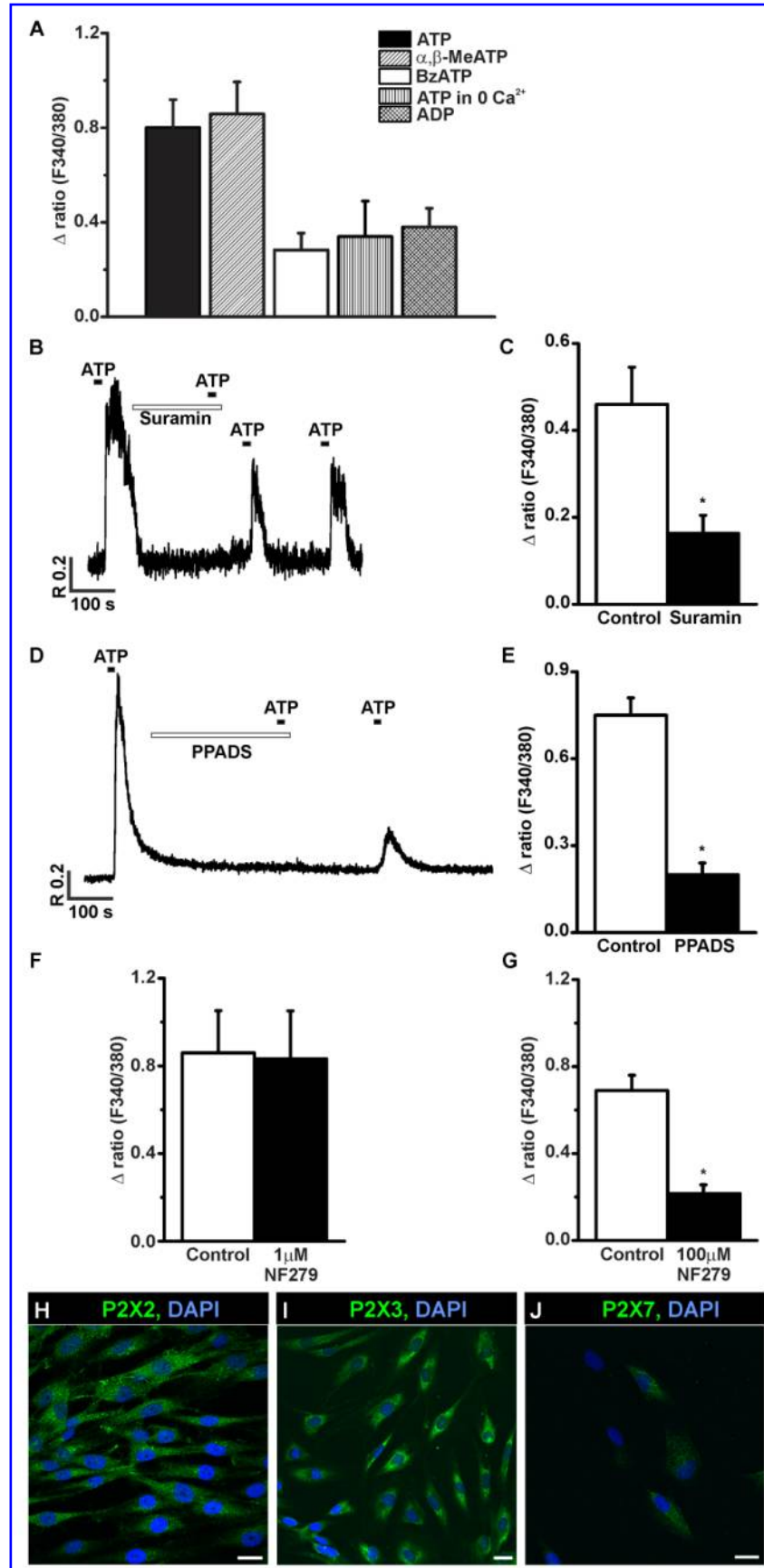


Fig. 2. Purinergic responses in P7 hESC NPs

P7 NPs express functional P2X and P2Y purinergic receptors. **(A)** Bar diagram showing the mean amplitude of the $[Ca^{2+}]_i$ increase in response to various purinergic receptor agonists in P7 hESC NPs. The cells were exposed to 100 μ M ATP, 100 μ M α,β -meATP, 20 μ M BzATP and 100 μ M ADP. The application of ATP also resulted in a rise in $[Ca^{2+}]_i$ in the absence of external Ca^{2+} . **(B and D)** Representative traces show the ATP-induced $[Ca^{2+}]_i$ responses obtained in the presence of and after the wash-out of the purinergic receptor antagonists suramin and PPADS. Incubation with 300 μ M suramin or 10 μ M PPADS after control ATP application significantly inhibited the ATP responses. **(C, E)** Bar diagrams showing the amplitude of the $[Ca^{2+}]_i$ response to ATP before (control) and after incubation with the antagonists **(C)** 300 μ M suramin ($p = 0.01$, $n = 8$) and **(E)** 10 μ M PPADS ($p = 0.02$, $n = 8$). **(F, G)** Bar diagrams showing the effect of NF279 in P7 NPs. At 1 μ M concentration NF279 had no effect **(F)**, while at 100 μ M concentration **(G)** it significantly inhibited ATP-induced $[Ca^{2+}]_i$ responses ($p = 0.04$; $n = 3$). Confocal images **(H, I, J)** showing the staining for P2X₂ **(H)** P2X₃ **(I)** and P2X₇ **(J)** receptors. Cell nuclei are visualized with DAPI staining. Scale bars = 20 μ m.

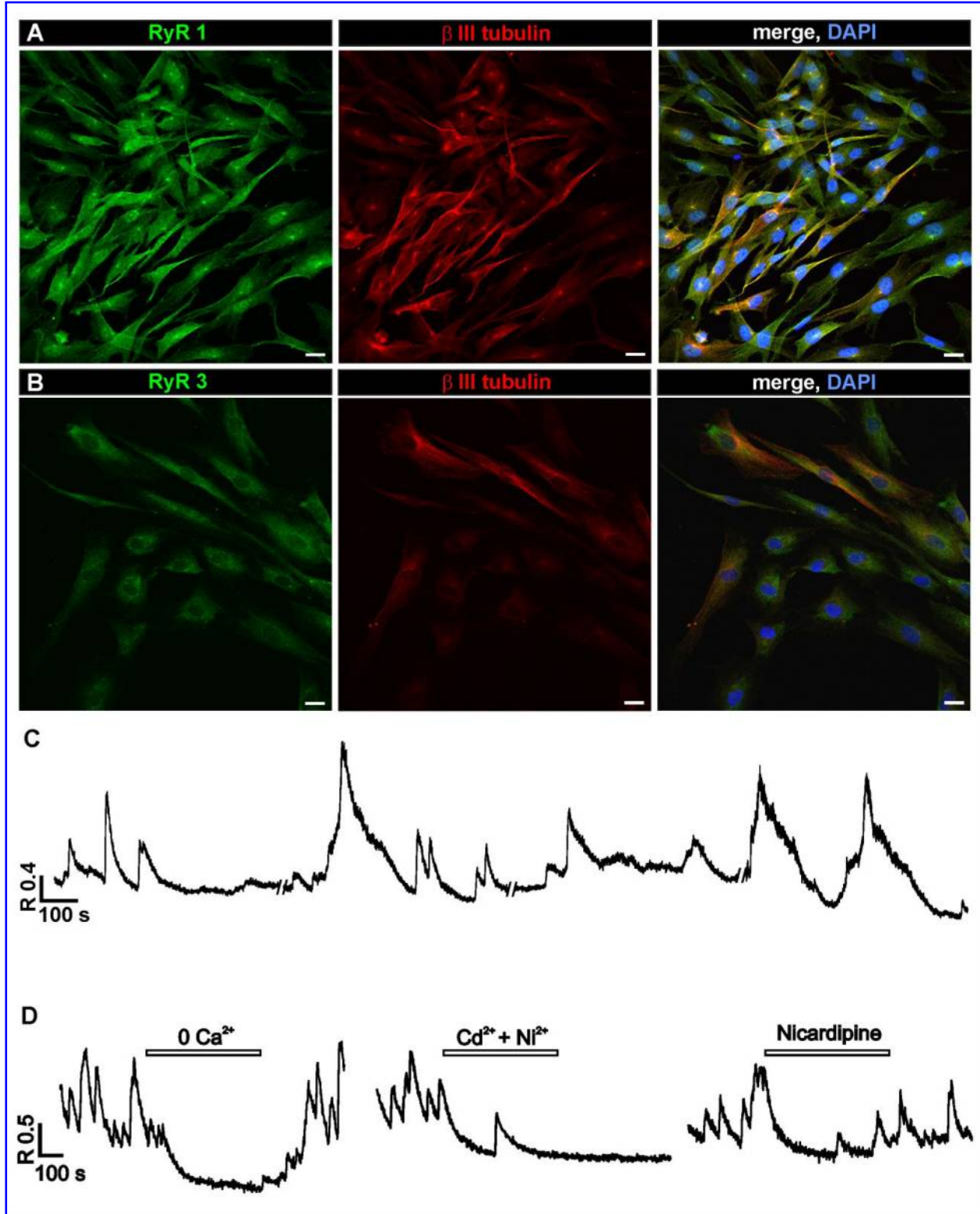


Fig. 3. Ca^{2+} release from intracellular Ca^{2+} stores and spontaneous $[\text{Ca}^{2+}]_i$ transients in P7 hESC NPs

(A, B) Confocal images of P7 hESC NPs. **(A)** RyR 1 and **(B)** RyR 3 receptors are present in all cells and are co-localized with immunostaining for β III tubulin. Scale bars = 20 μm . **(C)** A representative trace showing spontaneous $[\text{Ca}^{2+}]_i$ oscillations observed in the same cell. These types of oscillations were observed in about 31% of tested P7 hESCs. **(D)** Traces showing the inhibition of spontaneous $[\text{Ca}^{2+}]_i$ activity after removal of extracellular Ca^{2+} , application of VOCC blockers Cd^{2+} (100 μM) and Ni^{2+} (50 μM) and L-type VOCC blocker nicardipine (10 μM).

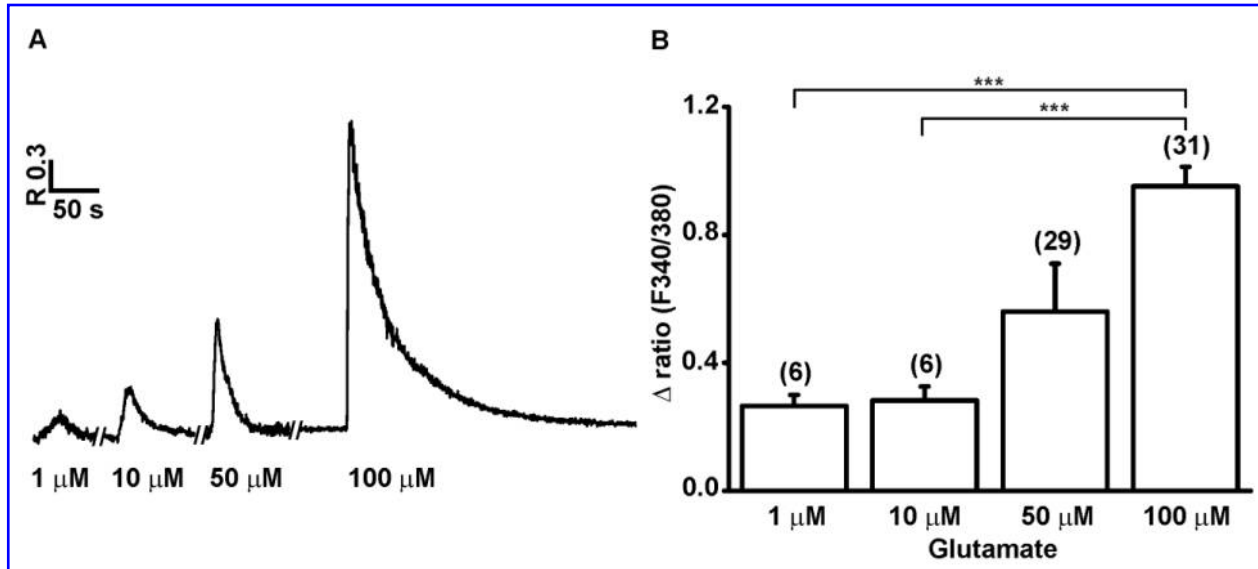


Fig. 4. Effect of glutamate in P7 hESC NPs

A representative trace (**A**) showing the $[Ca^{2+}]_i$ responses after the application of different concentrations of glutamate (1-100 μ M). A bar diagram (**B**) showing the amplitude (mean \pm SEM) of the $[Ca^{2+}]_i$ responses to the application of increased concentrations of glutamate. The number of cells tested is given in the parentheses. *** $p = 0.0005$

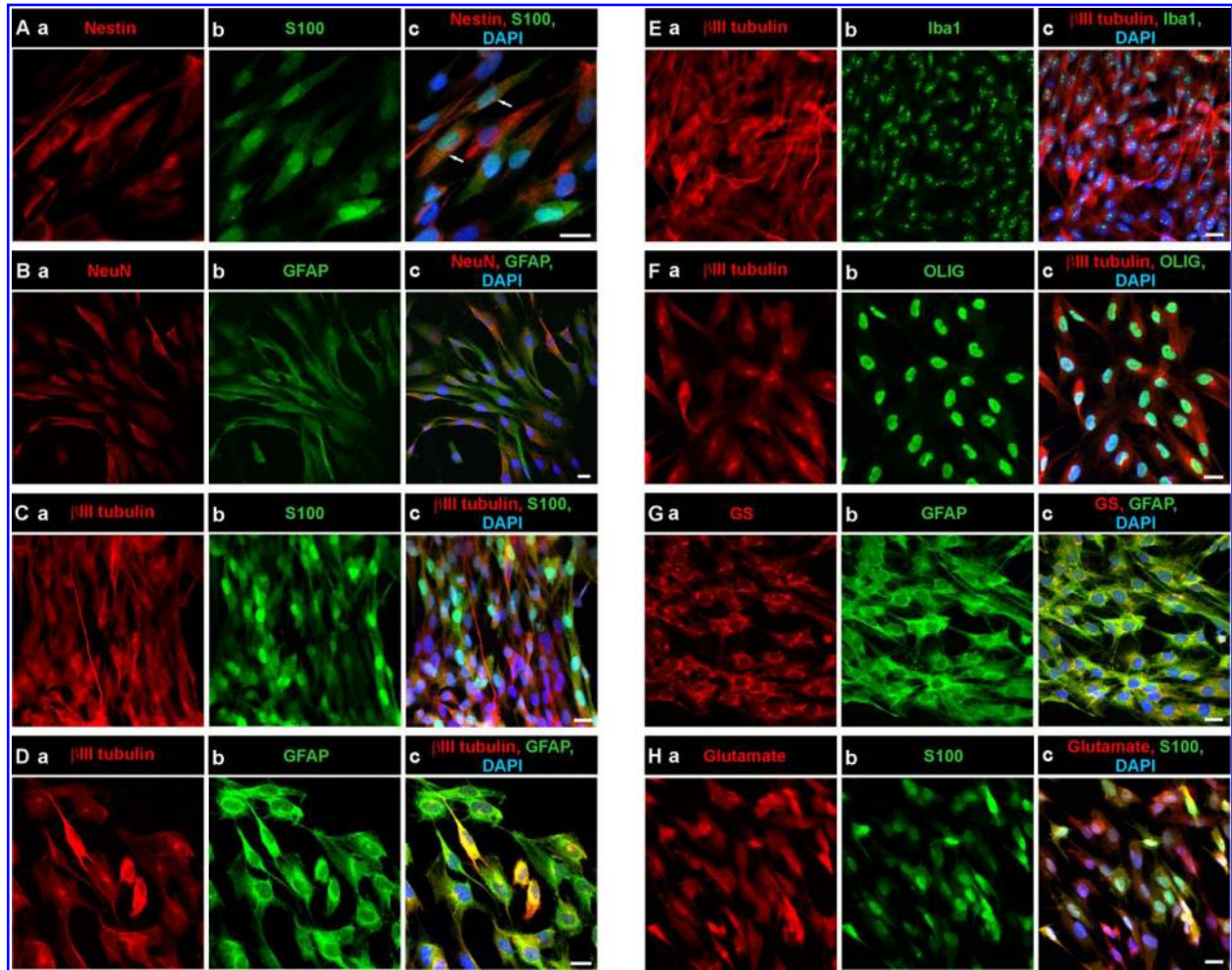


Fig. 5. Confocal microscopy analysis of the expression of neural cell markers in P7 NPs

P7 NPs express a number of neuron- and glia-specific markers. **(A)** The proliferative marker nestin **(Aa)** is expressed in a majority of P7 NPs and in some cells (indicated by arrows) is co-localized **(Ac)** with the glial marker S100 **(Ab, Ac)**. **(B)** P7 NPs express neuronal markers: NeuN, a marker of postmitotic neurons **(Ba)** and β III tubulin **(Ca, Da, Ea, Fa)**. The glial markers S100 **(Ab, Cb, Hb)**, GFAP **(Bb, Db, Gb)**, and glutamine

synthetase (**Ga**), the oligodendrocyte marker OLIG (**Fb**) and the microglial marker Iba1 (**Eb**) are also expressed in P7 NPs. The majority of cells from P7 NPs show positive staining for glutamate (**Ha**). Nuclei were labelled with DAPI. Scale bars = 20 μ m.

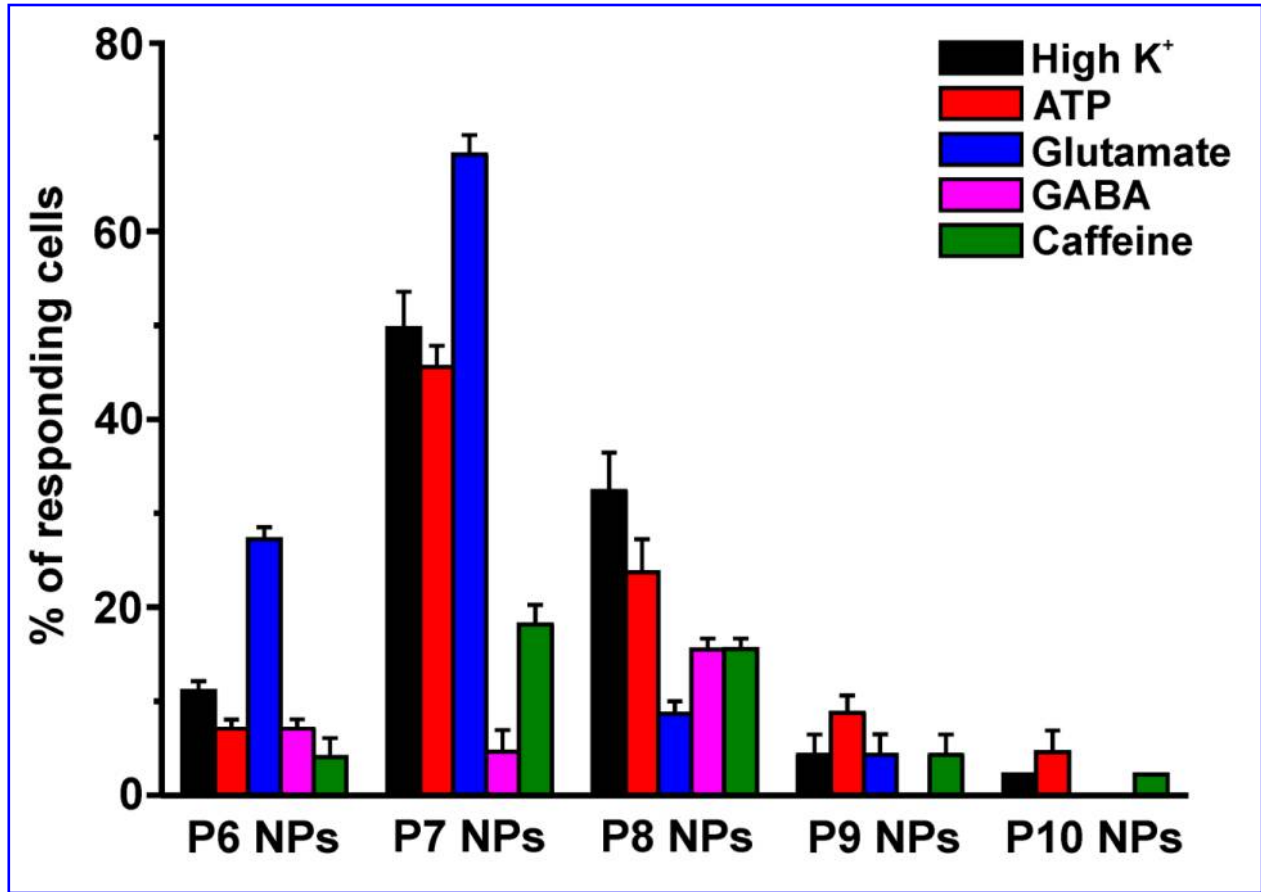


Fig. 6. The number of cells responding to various physiological stimuli in different passages of human embryonic stem cell-derived neural precursors (P6-P10 NPs)

The number of cells from different passages of hESC NPs responding to various physiological stimuli (e.g., 50 mM K⁺; 100 μM ATP, 50 μM Glutamate; 10 μM GABA; 20 mM caffeine) by a rise in [Ca²⁺]_i. Cells responding to one or more of the applied stimuli were considered as physiologically active.

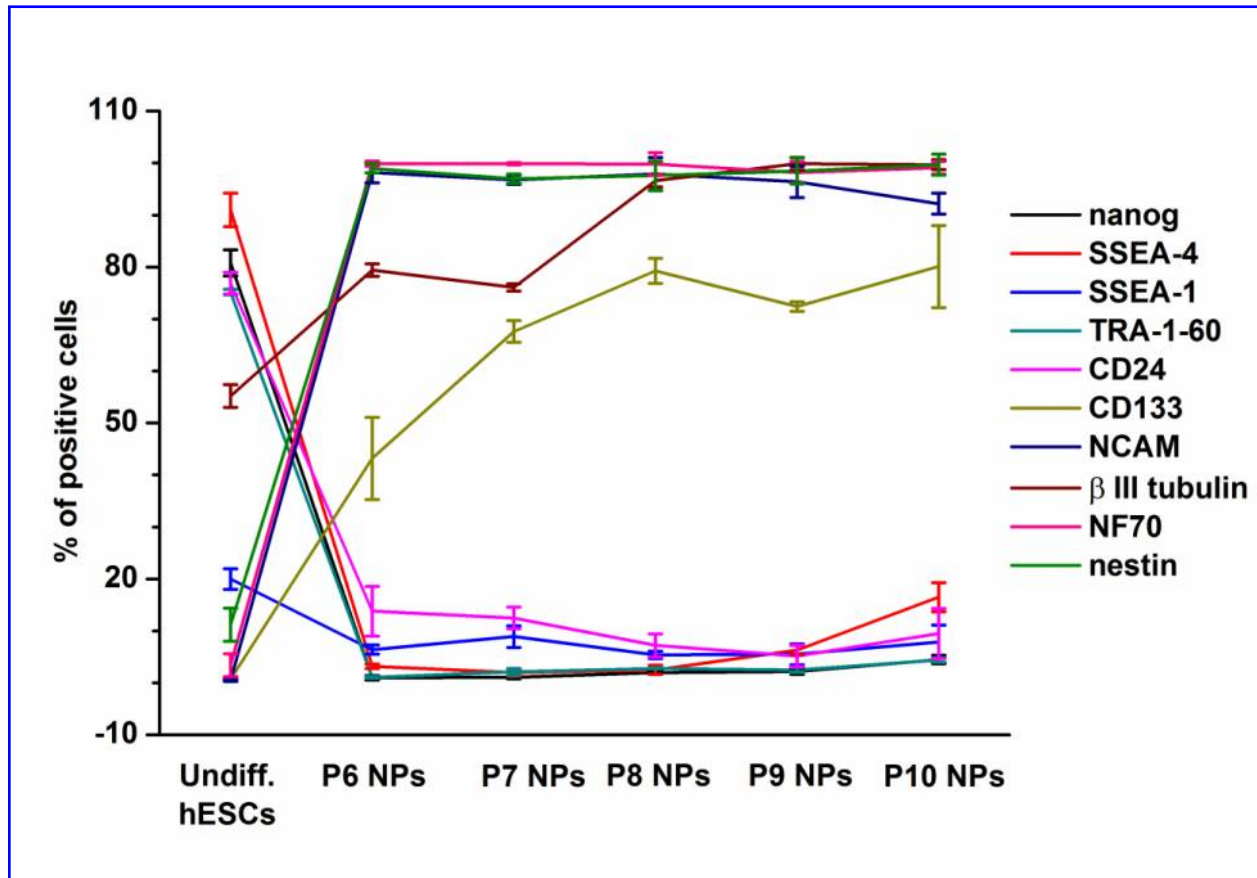


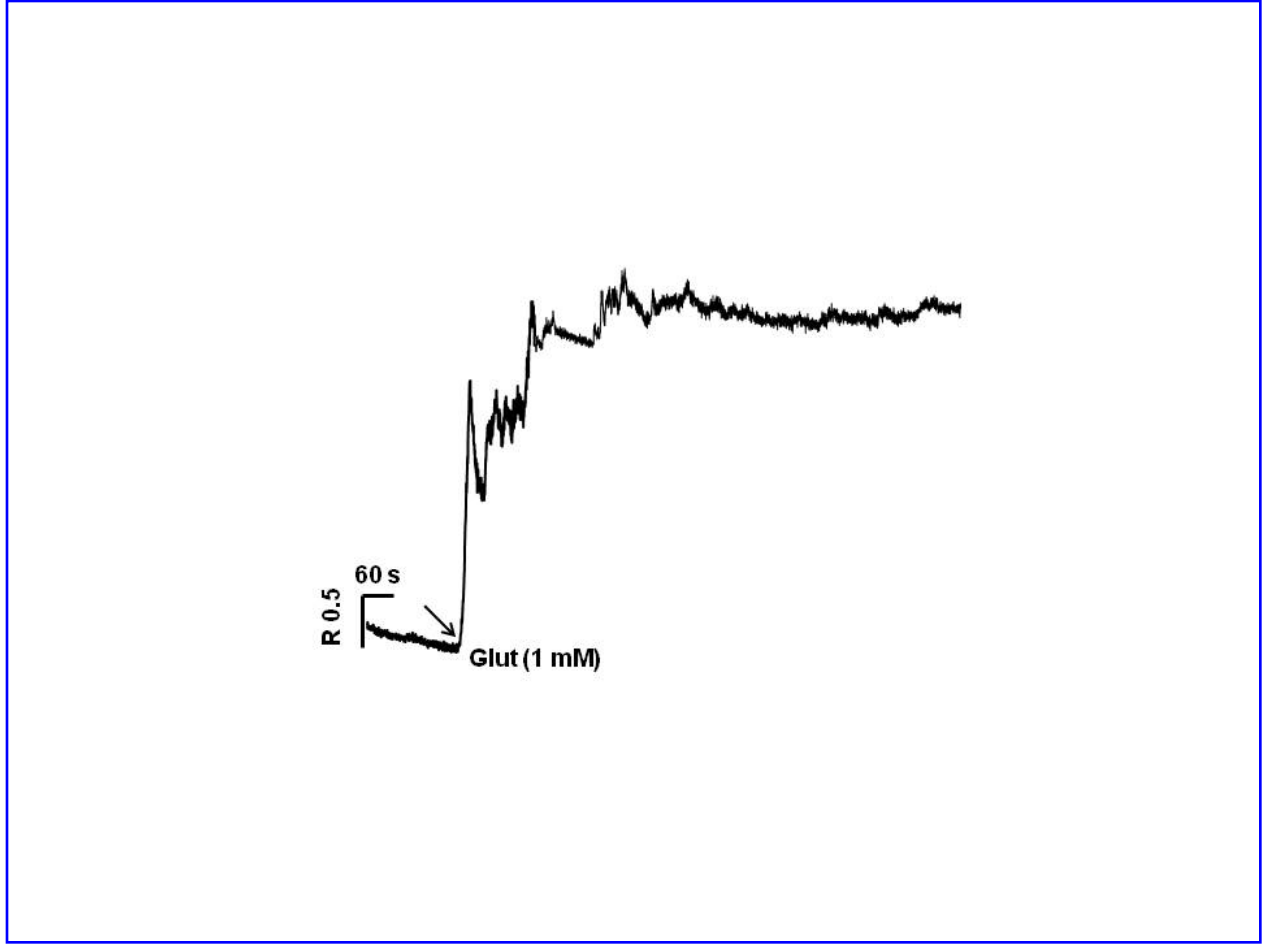
Fig. 7. Fluorescence-activated cell sorting profiles of pluripotent and neural markers in undifferentiated hESCs and hESC-derived NPs (P6 - P10) during long-term propagation in vitro

Pre-differentiation of hESC led to the down regulation of pluripotent markers (nanog, SSEA-4, SSEA-1, TRA-1-60, CD24) and up-regulation of neural markers (CD133, NCAM, β III tubulin, NF70 and nestin).

Legend to Supplemental Fig. 1. Effect of glutamate at 1 mM in P7 NPs

A representative trace showing $[Ca^{2+}]_i$ response induced by 1 mM glutamate applied for 10 s. The $[Ca^{2+}]_i$ levels in the cytosol were very high and did not return to resting level even after several minutes of washing out glutamate suggesting the loss of Ca^{2+} signal/homeostasis. A similar phenomenon was observed in all cells tested in different cultures of P7 NPCs.

Legend to Supplemental Fig. 2. Confocal image showing the expression of NR2D subunit of NMDA receptor in P7 NPs. Nuclei are visualized with DAPI. Scale bar = 20 μ m.



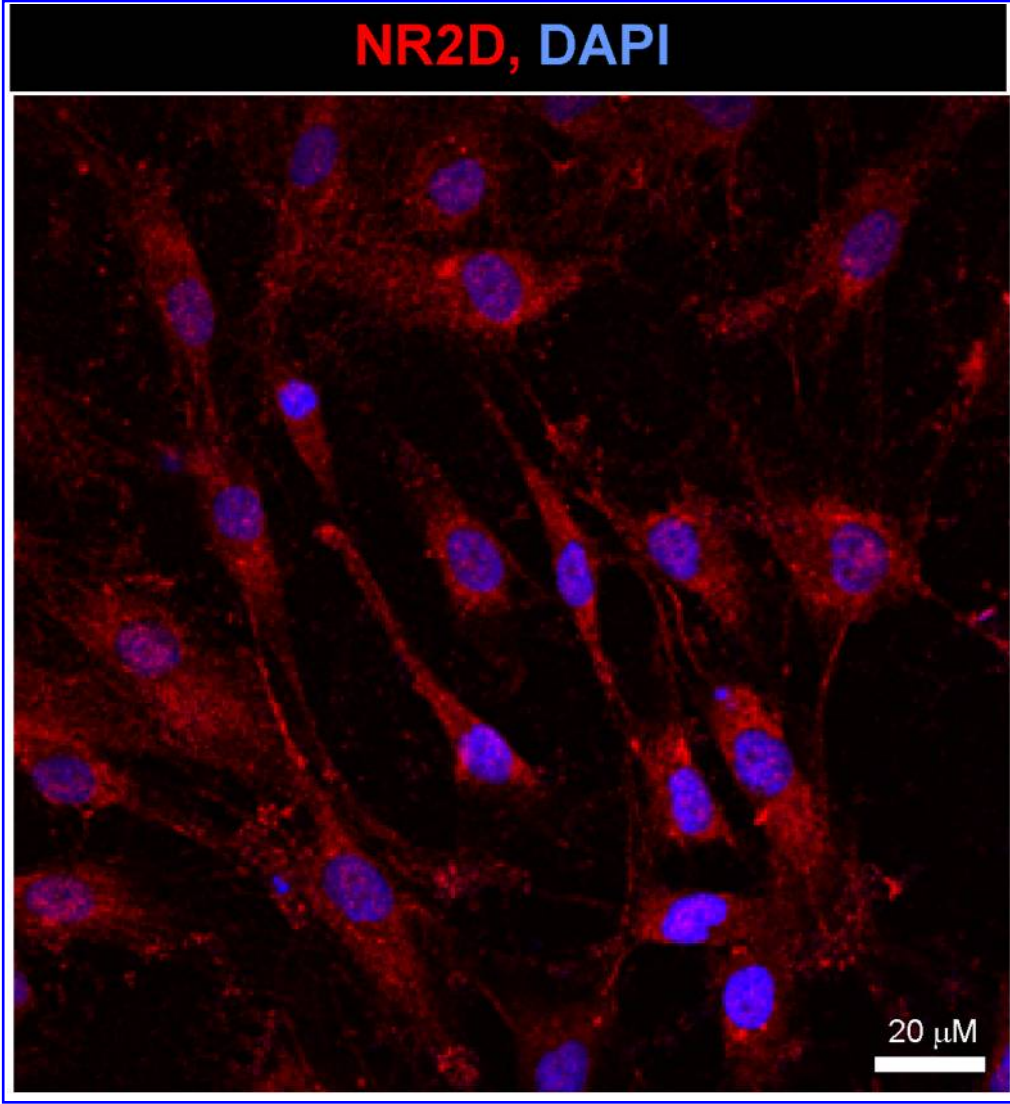


Table 1 Antibodies used for immunocytochemistry

| Antibody | Dilution | Host /Antibody type | Company | Secondary antibody used |
|--|----------|---------------------|--------------------------|--------------------------------------|
| Anti-NeuN | 1:100 | Mouse monoclonal | Merck Millipore | Alexa Fluor 594 goat anti-mouse IgG |
| Anti- β -tubulin isotype III | 1:1000 | Mouse monoclonal | Sigma-Aldrich | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-MAP-2 | 1:1000 | Mouse monoclonal | Merck Millipore Chemicon | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-Nestin | 1:2000 | Mouse monoclonal | Merck Millipore | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-Myelin/Oligodendrocyte specific protein | 1:250 | Mouse monoclonal | Merck Millipore Chemicon | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-S100 | 1:400 | Rabbit polyclonal | DAKO Denmark | Alexa Fluor 488 goat anti-rabbit IgG |
| Anti-Glial Fibrillary Acidic Protein (GFAP) | 1:80 | Rabbit | Sigma-Aldrich | Alexa Fluor 488 goat anti-rabbit IgG |
| Anti-glutamine synthetase | 1:1000 | Mouse monoclonal | Merck Millipore | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-Glutamate | 1:1000 | Mouse monoclonal | Sigma-Aldrich | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-GABA | 1:500 | Mouse monoclonal | Sigma-Aldrich | Alexa Fluor 594 goat anti-mouse IgG |
| Anti-Iba1 | 1:1000 | Rabbit polyclonal | Wako Chemicals GmbH | Alexa Fluor 488 goat anti-rabbit IgG |
| Anti-NG2 | 1:400 | Rabbit polyclonal | Merck Millipore | Alexa Fluor 488 goat anti-rabbit IgG |
| Anti-L-type Ca^{2+} CP α 1C | 1:100 | Rabbit polyclonal | Santa Cruz Biotechnology | Alexa Fluor 488 goat anti-rabbit IgG |
| Anti-N type Ca^{2+} CP α 1B | 1:100 | Rabbit polyclonal | Santa Cruz Biotechnology | Alexa Fluor 488 goat anti-rabbit IgG |

| | | | | |
|--|-------|----------------------|-------------------------------|---|
| Anti-P/Q-type Ca ²⁺ CP α1A | 1:100 | Rabbit polyclonal | Santa Cruz Biotechnology | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-P2X2 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-P2X3 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-P2X4 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-P2X6 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-P2X7 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-Ryanodine Receptor 3 | 1:100 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-Ryanodine Receptor 2 | 1:200 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| Anti-Ryanodine Receptor 1 | 1:200 | Rabbit polyclonal | Alomone Labs | Alexa Fluor 488 goat anti- rabbit IgG |
| NMDAε4 | 1:100 | Rabbit polyclonal | Santa Cruz Biotechnologies | Alexa Fluor 594 goat anti- rabbit IgG |

Table 2. Number of cells responding to various physiological stimuli in different passages of human embryonic stem cell-derived neural precursors (P6 - P10 NPs).

| | P6 NPs | P7 NPs | P8 NPs | P9 NPs | P10 NPs |
|--|------------------|------------------|------------------|------------------|-----------------|
| Number of cells tested | n = 44 | n = 44 | n = 45 | n = 45 | n = 44 |
| Cells sensitive to one or more of applied stimuli | 41 ± 1.6% | 59 ± 2.5% | 49 ± 1.1% | 15 ± 1.7% | 7 ± 0.2% |
| K⁺ 50 mM | 11 ± 1% | 50 ± 3.9% | 32 ± 4% | 4 ± 2.2% | 2.3% |
| ATP 100 μM | 7 ± 1% | 46 ± 2.2% | 24 ± 3.5% | 9 ± 1.9% | 5 ± 2.3% |
| Glutamate 50 μM | 27 ± 1.3% | 57 ± 0.3% | 9 ± 1.3% | 4 ± 2.2% | 0 |
| GABA 10 μM | 7 ± 1% | 5 ± 2.3% | 16 ± 1.1% | 0 | 0 |
| Caffeine 20 mM | 4 ± 2% | 18 ± 2.1% | 16 ± 1.1% | 4 ± 2.1% | 2.3% |

Table 3. Expression of various neural markers and Ca²⁺-sensitive channels in P6-P10 of hESC NPs

| | P6 NPs | P7 NPs | P8 NPs | P9 NPs | P10 NPs |
|---|--------|--------|--------|--------|---------|
| Neural markers | | | | | |
| Nestin | ++ | ++ | ++ | ++ | ++ |
| NeuN | ++ | ++ | ++ | + | + |
| β III tubulin | +++ | +++ | +++ | ++ | ++ |
| MAP2 | - | - | - | - | - |
| Glutamate | + | +++ | - | - | - |
| GABA | + | - | + | - | - |
| GFAP | ++ | +++ | ++ | + | - |
| S100 | + | ++ | ++ | ++ | ++ |
| OLIG | - | ++ | - | - | - |
| GS | - | ++ | - | - | - |
| NG2 | - | - | - | - | + |
| Ca²⁺-sensitive channels | | | | | |
| L-type VOCC | ++ | +++ | ++ | ++ | ++ |
| N-type VOCC | - | - | - | - | - |
| P/Q-type VOCC | ++ | +++ | ++ | ++ | ++ |
| RyR 1 | +++ | +++ | +++ | +++ | +++ |
| RyR 2 | + | + | + | + | + |
| RyR 3 | ++ | +++ | ++ | ++ | ++ |

“-“ - Negative immunostaining

“+” - Faint positive immunostaining / few cells express the marker

“++” - Positive immunostaining / many cells express the marker

“+++” - Very positive / majority of cells express the marker