Hungarian University of Agriculture and Life Sciences

Doctoral School of Animal Biotechnology and Animal Science

IN VITRO PRODUCED NEURONAL NETWORK STUDIES ON THE ROLE OF P2X7 RECEPTOR AND ITS RELEVANCE TO ALZHEIMER'S DISEASE

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Linda Francistiová

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Name: Doctoral School of Animal Biotechnology and Animal Science

Discipline: Animal Science

Leader of the school: Professor Dr. Miklós Mézes, D.V.M., Member of the HAS

Head of Department,

Hungarian University of Agriculture and Life Sciences, Institute of Physiology and Animal Nutrition, Department of Nutritional Safety

Supervisor: Professor Dr. András Dinnyés, D.V.M, D.Sc.

Hungarian University of Agriculture and Life Sciences, Institute of Physiology and Animal Nutrition, Department of Physiology and Animal Health

Co-supervisor: Dr. Julianna Kobolák, PhD,

Senior Research Fellow, Hungarian University of Agriculture and Life Sciences, Institute of Aquaculture and Environmental Safety

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Approval of the PhD School leader

……………………..…………… ………………………………..…

Approval of the Supervisor Approval of the Co-supervisor

1. INTRODUCTION AND GOALS

Neuroinflammation is one of the major underlying pathologies of many neurodegenerative diseases, including Alzheimer's disease (AD). It results from various effects such as tissue damage, pathogen infiltration, autoimmune conditions, deposition of pathologic proteins such as amyloid-β, stress, and neuronal hyperexcitability during seizures. Subsequently, neuroinflammation leads to the biosynthesis and release of various signaling molecules, including cytokines, chemokines, and reactive oxygen species (ROS). Neuroinflammation is an essential protective process in the central nervous system (CNS), as it contributes to the elimination of the negative factor that primarily caused it and to the restoration of the normal tissue homeostasis. However, in the case of prolonged and large magnitude inflammation, like the one that takes place during AD, it can lead to further cellular damage amplifying the existing inflammation and causing irreversible tissue damage. During neurodegeneration, when neuronal cells are damaged or die, ATP is released into the extracellular environment. This ATP serves as a damage-associated molecular pattern (DAMP) which is detected by microglia cells via their purinergic receptors. From amongst both ionotropic (P2X) and metabotropic (P2Y) ATP-gated purinergic receptors, the P2X7R has attracted most attention due to its unique characteristics such as its low affinity to ATP and the suggested involvement in a broad range of neurodegenerative diseases such as AD, epilepsy, schizophrenia, Huntington's disease and many others. Moreover, another property of the P2X7R is to form a macropore following exposure to high concentrations of ATP. The formation of such a macropore in the cell membrane induces cell death and can be experimentally demonstrated by the uptake of large molecules e.g. Yo-Pro-1 by the cells.

Since P2X7R is mainly expressed on immune cells, it is involved mostly in the context of immune system-related effects. In the CNS parenchyma, the immune cells are represented by microglia – the brain tissue macrophages. These cells are involved in many physiological processes such as developmental synaptic pruning and immune surveillance during adulthood. The expression and activity of the P2X7R on microglia have been widely examined and validated. A role of the P2X7R was also suggested in the pathogenesis of Alzheimer’s disease, as a general driver of

neuroinflammation, amyloid peptide-dependent neuroinflammation or via modulation of chemokine signaling.

While the expression of the P2X7R on neurons has been suggested, an irrefutable conclusion is not yet available. One of the reasons behind the difficulties in detecting the P2X7R on neurons is the limitations in the detection methods, such as the lack of specificity of the available anti-P2X7R antibodies. Another way to examine the presence of the receptor on cells is by performing pharmacological studies. One of the most reliable and specific P2X7R antagonists with high affinity is JNJ 47965567, which has been used successfully in assessing the functionality of the P2X7R.

Human induced pluripotent stem cells (hiPSCs) are generated from somatic cells via genetic reprogramming. They resemble embryonic-like stem cells and can be differentiated into all three germ layers (endoderm, ectoderm, and mesoderm) and could give rise to any type of human cells under proper signals and culture conditions. Over the past decade, hiPSC technology became broadly used and presents an invaluable tool in connecting the biological data obtained from rodent models to human disease. hiPSCs are particularly useful in modelling otherwise hardly accessible tissues, like the human brain. Protocols for the generation of neurons and astroglia from hiPSC are readily available and utilized routinely in several laboratories worldwide including ours. However, microglia generation from hiPSCs has been problematic for a long time due to microglia's unique yolk sac origin. Currently, there are several well-established protocols available for the generation of microglia-like cells.

In the present study, we investigated the expression and function of the P2X7R in hiPSC-derived cortical type neuronal cells obtained from a healthy donor and from a patient with familial Alzheimer's disease, as well as in healthy donor-obtained hiPSC-derived microglia-like cells. Using immunocytochemistry, immunoblot detection, and RT-qPCR analysis, we showed the expression and localization of P2X7R on neuronal and microglial cells. Furthermore, by employing pharmacological assays using the highly specific P2X7R antagonist JNJ 47965567, we investigated the activation of P2X7R by administering ATP and BzATP on both cell types. Thus, the results of our study show for the first time P2X7R expression and function on different hiPSC-derived cell types of the CNS.

Objectives of this study

The overall aim of this study was to find answers to the following scientific questions:

- Is the P2X7R present and functional on human induced pluripotent stem cell-derived neuronal cells?
- Does the expression of P2X7R change during the differentiation of the hiPSC-derived neuronal progenitor cells towards mature neuronal cells?
- Is the expression pattern different in hiPSC-derived neurons differentiated from hiPSCs obtained from Alzheimer's disease patients compared to those obtained from healthy donors?
- Does the cultivation of the cells in the presence of P2X7R antagonists improve the quality of the AD cell lines and their viability upon application of large concentrations of ATP?
- What impact does the addition of microglia cells have on the overall quality of the control versus AD patient-derived neuronal cell cultures? Does the presence of microglia change the response of the cell culture on the application of P2X7R agonists?

Specific objectives of the research:

- Establishment and long term maintenance of hiPSC-derived neuronal cell cultures and their characterisation by detection of the expression of neural markers
- Verification of the presence of Alzheimer's disease-related pathology in the patient-derived *in vitro* cell cultures
- Detection of the P2X7R on the hiPSC-derived neurons
- Analysis of the subcellular localisation and the functions of the P2X7R
- Evaluation of the effects of P2X7R-modifying compounds on the overall quality of cell cultures and comparing the results between the control and Alzheimer's disease cells
- Investigation of the impact of microglia cells on the cell culture response on the P2X7R modulation

2. MATERIALS AND METHODS

2.1 hiPSC lines

Mononuclear blood cells donated by an Alzheimer's disease patient (from the Institute of Genomic Medicine and Rare Disorders, Semmelweis University, Budapest, Hungary) or healthy, non-demented donor (assessed by a clinical evaluation) were isolated from the peripheral whole blood samples and subject to reprogramming to generate hiPSC lines, according to national laws and ethical permissions. Both cell lines were established and characterised previously (Nemes et al., 2016; Ochalek et al., 2017), which is not part of the current thesis. In the current study, both cell lines were maintained under identical conditions. The hiPSCs were cultivated on Matrigel (BD Matrigel; Stem Cell Technologies) in mTESR1 (Stem Cell Technologies) culture media, with daily media change and cell passage every 5-7 days using Gentle Cell Dissociation Reagent (Stem Cell Technologies), according to the manufacturer's instructions.

2.2 Neural induction of hiPSCs

Generation of the neuronal progenitor cells (NPCs) was performed via dual inhibition of the SMAD signalling pathway using the LDN193189 and SB431542 compounds (Chambers et al., 2009) as we described previously (Ochalek et al., 2017). In detail: neural induction took place after the cell culture of the hiPSCs reached the desired confluence. hiPSC culture was maintained on Matrigel-coated plates in the neural induction medium (NIM) (1:1 vol/vol mixture of DMEM/ and neurobasal medium, 1x N2 supplement, 1x B27 supplement, 1x non-essential amino acids [NEAA], 2 mM L-glutamine, 50 U/ml penicillin/ streptomycin, 100 µM mercaptoethanol, 5 µg/ml insulin), which was supplemented with 5 ng/ml basic fibroblast growth factor (bFGF), 0.2 µM LDN193189 (Selleckchem, Houston, TX, USA), and 10 µM SB431542). Medium change was performed every day until day 10, when the neural rosettes were picked manually and replated on poly-L-ornithine/laminin (POL/L) (Sigma-Aldrich)-coated plates, and expanded in neural maintenance medium (NMM) (1:1 vol/vol mixture of DMEM/F12 and neurobasal medium, 1× N-2 supplement, 1× B-27 supplement, 1× NEAA, 2 mM-glutamine, 50 U/ml penicillin/streptomycin), and supplemented with 10 ng/ml epidermal growth factor and 10 ng/ml bFGF.

2.3 Neural differentiation of NPCs

Neuronal progenitor cells (NPCs) were plated on culture dishes coated with poly-L-ornithine/laminin (Sigma-Aldrich) and were cultured for proliferation and propagation in neuronal maintenance medium (NMM) (1:1 vol/vol DMEM:F12 and neurobasal medium, 1x N-2 supplement, 1x B-27 supplement, 1x non-essential amino acids, 1 mM L-glutamine, 50 U/ml penicillin/streptomycin) supplemented with 10 ng/ml of EGF and 10 ng/ml bFGF. To induce differentiation into mature neurons, NPCs were plated at a density of 40.000 cells/cm^2 for immunocytochemistry and 100.000 cells/cm^2 for Western blot and RT-qPCR experiments and were cultured in NMM without the addition of bFGF and EGF. The medium was changed every 3-4 days during the terminal differentiation that took place for 66 days. Samples were collected weekly.

2.4 Differentiation of hiPSCs towards microglia-like cells

hiPSCs were plated on 96-well low attachment plates and grown in mTeSR1 (STEMCELL Technologies) supplemented with 50 ng/ml BMP-4 (Thermo Fisher), 50 ng/ml VEGF-165 (Merck), 20 ng/ml SCF (R&D) and 1x Revitacel (ThermoFisher) to promote differentiation towards mesodermal hematopoietic precursors until the formation of 3D embryoid bodies. The medium was changed for mTeSR1 with 50 ng/ml BMP-4 (Thermo Fisher), 50 ng/ml VEGF-165 (Merck), 20 ng/ml SCF (R&D on day 2 by 75% change, on day 3 by 50% change. After the embryoid bodies have formed the medium was changed for X-VIVO-15 medium (Lonza) supplemented with 1x GlutaMax, 100 U/ml penicillin/ streptomycin, 50 µM 2-Mercaptoethanol, 100 ng/ml M-CSF, 25 ng/ml IL-3 (all supplements are from Thermo Fisher) to induce the production of microglial progenitor by the embryoid bodies termed as “the Factories”. The Factories were placed into new 6-well plates and divided into 10-12 Factories per one 6-well plate well and maintained in medium promoting the production of the microglia-like progenitor cells: X-VIVO-15 medium (Lonza) supplemented with 1x GlutaMax, 100 U/ml penicillin/ streptomycin, 50 µM 2-Mercaptoethanol, 100 ng/ml M-CSF, 25 ng/ml IL-3 (all supplements are from Thermo Fisher). The medium was not changed for one week to allow undisturbed cell differentiation. After one week, the medium was changed. Emerging microglial progenitors produced by the Factories could be observed at this stage. The cells were returned to the culture of Factories, where they provided support for further potency

of the Factories. After week 4, the Factories were producing a sufficient amount of well-differentiated microglia-like cells. These cells were collected and used for further experiments. Factories can be maintained and active for prolonged periods, with 50% medium change once a week for X-VIVO-15 medium (Lonza) supplemented with 1x GlutaMax, 100 U/ml penicillin/ streptomycin, 50 µM 2-Mercaptoethanol, 100 ng/ml M-CSF, 25 ng/ml IL-3.

2.5 Immunocytochemistry

Cells were cultured on glass coverslips, and at the required time, they were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT), washed three times with PBS, and permeabilised with 0,2% Triton X-100 in PBS. Next, cells were blocked with a blocking buffer (5% bovine serum albumin in 0,1% Tween-PBS) for 1 hour at RT. Primary antibodies were applied overnight at 4°C. Subsequently, appropriate secondary antibodies were applied to the cells for 1 hour at RT. Stained cells were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen/ThermoFisher Scientific, Waltham, MA, USA) and analysed under a fluorescence microscope (Axio Imager system with ApoTome; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) controlled by AxioVision 4.8.1 software (Carl Zeiss MicroImaging GmbH).

2.6 Lysis of the cells

Cells were collected from the culture and lysed with the RIPA Lysis, and Extraction Buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail and Pierce™ Universal Nuclease for Cell Lysis (Thermo Fisher Scientific). Individual cell lysates were collected from one well of a six-well plate, lysed on ice for 30 minutes, sonicated, and centrifuged at 13000 rpm for 20 minutes to pellet the cellular debris, and the protein-containing supernatant was collected and stored at -80°C. The concentration of proteins in the lysate supernatants was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions using the Varioskan Flash Multimode Reader (Thermo Fisher Scientific).

2.7 Immunoblotting (Western blot)

Cell lysates (2-30 µg) were separated by electrophoresis on 10% SDS-polyacrylamide precast gels (Bio-Rad) and transferred to Immuno-Blot® PVDF

Membranes (Bio-Rad). The membranes were blocked with TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% BSA or 5% non-fat milk and then incubated with the respective antibody solution overnight at 4°C. After the incubation with primary antibodies, membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. Signals were obtained after treatment with SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific) and detected using the KODAK Gel Logic 1500 Imaging System and Kodak MI SE imaging software. Densitometry measurement of the bands' intensities was carried out via the Image Studio™ Lite software (LI-COR).

2.8 Biotinylation assay for detection of membrane-bound proteins

To determine the membraneous localisation of the P2X7R protein, a biotinylation assay was performed. Cells were washed with ice-cold PBS and incubated with Biotin (Thermo Fisher Scientific) solution for 30 minutes at 4°C. After 30 minutes, a Quenching Solution (Thermo Fisher Scientific) was added to stop the reaction. Cells were collected, and pellets were lysed as previously described (see 3.6 pharagraph). Lysate supernatants were incubated with the NeutrAvidin Gel (Thermo Fisher Scientific) for 1 hour at RT to allow the biotinylated proteins to bind to NeutrAvidin. The unbound intracellular proteins were collected by centrifugation (300g for 2 min at 4°C) of the separation column. The biotinylated membrane-bound proteins were incubated with SDS-PAGE Sample Buffer (Bio-Rad) for 1 hour at RT and were eluted from the column. IC flow-through and MB eluted samples were loaded on precast 10% SDS-polyacrylamide gels (Bio-Rad) and analysed by western blot.

2.9 Functional assays and compound applications

Control hiPSC derived NPCs, terminally differentiated neuronal cells and microglia cells were cultured on 96-well plates at 35.000 cells/cm^2 density and treated with different concentrations of ATP (A7699, Sigma-Aldrich) and BzATP (B6396, Sigma-Aldrich) and incubated at standard cultivation conditions for 24 hours. Alternatively, the cells were pre-treated with P2X7R antagonist JNJ 47965567 (5299, Tocris). The working concentration of JNJ 47965567 was consistently 1 µM. The viability of the cultures was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen) according to the manufacturer’s protocol. The fluorescent signal was

measured using the Varioskan Flash Multimode Reader (Thermo Fisher Scientific). The cell survival was represented as a percentage of untreated cells (100% viability), while cells killed by exposure to water (positive control) represented 0% viability and analyzed with Prism 9 software (GraphPad, Software, La Jolla, CA, USA).

2.10 Reverse-transcription quantitative PCR (RT-qPCR)

Cells pellets were collected and snap-frozen until lysis. Total RNA was isolated from the lysates collected at different time points using the RNeasy Plus Mini Kit (Qiagen) in accordance with the manufacturer's instructions. One µg of RNA was transcribed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). The PCR conditions were: 94°C, 3 min, initial denaturation; followed by 40 cycles of 95°C, 5 seconds, denaturation; 60°C 15 seconds, annealing and 72°C 30 seconds, elongation.

The amplification was carried out in a total volume of 15 µL using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). RT-qPCR was run on the Rotor-Gene Q 5plex Platform (QIAGEN) using oligonucleotide primers. The data was analyzed using the REST software (2009 V2.0.13).

2.11 LPS challenge

The cell cultures of microglia-like cells maturated for two weeks were incubated in the presence of 1 µg/mL LPS (Invitrogen) for 24 hours under standard cultivating conditions. After 24 hours, the cells were washed 1x with PBS and lysates were prepared for Western blot analysis. The Western blots were performed and analyzed as described above (section 3.7).

2.12 Statistical analysis

For statistical analysis, GraphPad Prism 9 software was used. Data are presented as means± standard deviation (SD). Student's T-test or Tukey's multiple comparisons test were used (as stated in the figures' description) to determine the statistical differences between the samples. Significance was accepted at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3. RESULTS

3.1 Western blot detection of P2X7 receptor in neuronal cells

Western blot analysis was performed to investigate the expression of the P2X7R in the cell cultures during neuronal differentiation. Two anti-P2X7R antibodies were used for this purpose. Alomone labs (APR-004) antibody that recognises the intracellular part of the P2X7R at the C-terminal tail and the Santa Cruz (sc-514962) antibody that recognizes the extracellular part of the receptor. The results of Western blot analysis using both antibodies suggest and decreasing signal intensity along the neuronal cultures' differentiation times, which is represented in a graph of values obtained after densitometric analysis of the blots and normalised to GAPDH. Finally, a regression analysis of the obtained data shows a significant moderate to a strong negative correlation between the differentiation timeline and the signal intensity, confirming the observation of decreasing P2X7R expression.

3.2 Investigation of the subcellular localisation of P2X7 receptor in neuronal and microglial cellular models

Proteins of the P2X receptor family are all membrane-bound ion channels, and thus the localization is crucial for their proper ion channel function. To investigate whether the detected P2X7R signal originates from its expected subcellular localization – the cell membrane, high-resolution confocal microscopy was performed on NPCs, TD35 and TD63 neuronal cells from both control and fAD cells, as well as on hiPSC-derived microglia-like cells. Our results suggest that the signal from the NPCs and neurons is of intracellular origin, while the signal detected from microglia-like cells clearly outlined the whole surface of the cell, indicating membranous expression of the receptor.

The intracellular localization of the P2X7R has been reported by others (Gu et al., 2000; Sarti et al., 2021). Therefore, to further investigate the localization of the P2X7R on our cells, cell surface protein biotinylation and isolation assay was performed. The results confirmed the observations from the immunocytochemistry experiments and showed the presence of P2X7R in the fraction containing the intracellular proteins (IC) in the case of neuronal cells examined at stage TD35 (both

control and fAD cell lines). In contrast, the receptor was present in the membrane-bound proteins' fraction (M) in microglia-like cells and in NPCs as well. Altogether, our findings indicate that the P2X7R is localized on the cell membrane of microglia and NPCs but not on the neuronal cells, which raises the question about its functionality and purpose in the neuronal cells.

3.3 Examination of P2X7 receptor's functionality using pharmacological assays

Control and fAD neuronal cells (at NPC and TD30 stage) and the microglia-like cells (at 14 DIV) were treated with different concentrations of P2X7R agonists ATP and BzATP or pre-treated with the highly specific P2X7R antagonist JNJ 47965567 (Bhattacharya et al., 2013). The results show that the ATP application had a negative effect on the overall viability of the NPCs, while in the case of the cells treated with a lower concentration of ATP (4 mM), this negative effect was slightly reduced upon the application of the P2X7R antagonist JNJ 47965567. No difference was observed between the control and fAD NPC cultures. The viability of the cells treated with lower concentrations of BzATP (3 mM) was not different from the untreated control, but the JNJ 47965567 pre-treated cells showed decreased viability. The viability of the cells treated with higher concentrations of BzATP (5 mM) was significantly decreased, and pre-treatment with JNJ 47965567 had no effect on the measured viability. Neuronal cultures maturated for 30 days did not show a decrease in viability upon treatment with ATP, while treatment with BzATP significantly decreased their viability. However, the pre-treatment of the cell cultures with JNJ 47965567 had no effect on the cells' viability. In microglia-like cells, different concentrations of both ATP and BzATP treatment of the cells caused a significant decrease in cell viability. In contrast to neuronal cells, pre-treatment of microglia-like cells with JNJ 47965567 significantly increased the cells' viability, suggesting a P2X7R-driven mechanism of toxicity.

4. NEW SCIENTIFIC RESULTS

In this research, the expression, subcellular localisation, and the role of the purinergic receptor P2X7R was investigated. hiPSC-derived neuronal cell cultures were established successfully, and hiPSC-derived microglia-like cells were generated. These two cell types were effectively co-cultivated, and we confirmed that only in the presence of neuronal cells are microglia cells capable of fully developing their resting morphology, ramify, and display their characteristic *in vivo*-like phenotype. This allowed us to use microglia cells as a control for all P2X7R-related experiments, results of which provided the following new scientific findings:

1. I have shown that hiPSC-derived microglia-like cells can be co-cultured with both control and fAD neuronal cell cultures.
2. For the first time, I have detected the P2X7R in both control and fAD hiPSC-derived cortical neurons, showing a gradually decreasing signal with the differentiation of the neuronal cells.
3. For the first time, I have detected and reported the presence of P2X7R in human hiPSC-derived microglia-like cells.
4. I have presented data suggesting an unexpected intracellular localisation of the P2X7R in neuronal cells.
5. Finally, I have provided evidence that the P2X7R is, in contrast with microglia cells, not involved directly in the ATP-mediated toxicity in neurons.

5. DISCUSSION AND FUTURE PERSPECTIVES

In the present study, we used human induced pluripotent stem cell-derived models to investigate the expression, localisation and role of the purinergic receptor P2X7. hiPSC-derived cellular models used in this study were neuronal progenitor cells differentiated towards mature neuronal cells with inclusion of astrocytes, and microglia-like cell cultures. We confirmed that hiPSC-derived neurons and astrocytes provide a well-established and suitable *in vitro* modelling system for studying different CNS-related mechanisms. Moreover, by including hiPSC-derived microglia-like cells in this system and our experiments, we have further improved this CNS *in vitro* model and obtained an important control cell for the P2X7R-related experiments. We presented novel data by investigating the ATP-gated purinergic P2X7R in a human neurodegenerative disease model.

The value of this research rests upon the fact that the majority of the currently available data on the P2X7R originates from research done using rodent models or transgenic cell lines. While the resulting information are extremely valuable and paved the way for further investigation in the field, our work on hiPSC-derived cells presents a valuable contribution, extending the work on animal models to the human cellular system and towards clinical translation.

There is an intense discussion regarding the presence and potential function of the P2X7R on neuronal cells (Illes et al. 2017; Miras-Portugal et al. 2017). While P2X7R is a well-described immune receptor, it is questionable whether its expression and, even more importantly, functionality on neuronal cells can be confirmed. Here we showed that the P2X7R could be detected in the human hiPSC-derived NPCs and neuronal cultures when detecting the P2X7R *via* Western blot analysis.

Next, we continued in exploring the reasons for the apparent differences in the P2X7R signal's localisation in neuronal and microglial cells. While on the microglia-like cells the localisation seems to be membranous, outlining the overall shape of the cell body, in the case of neuronal cells we have seen areas of more intensive signal, typically in the middle of the cell body. The intracellular localization of the P2X7R has been reported by others (Gu et al., 2000; Sarti et al., 2021). Therefore, to further investigate the localization of the P2X7R on our cells, cell surface protein

biotinylation and isolation assay was performed. The results confirmed the observations from the immunocytochemistry experiments and showed the presence of P2X7R in the fraction containing the intracellular proteins (IC) in the case of neuronal cells examined at stage TD35. In contrast, the receptor was present in the membrane-bound proteins' fraction (M) in microglia-like cells and NPCs as well. Altogether, our findings indicate that the P2X7R is localized on the cell membrane of microglia and NPCs but not on the neuronal cells, which raises the question about its functionality and purpose in the neuronal cells.

After performing the biotinylation assay aimed for identification of the membranous localisation of proteins only in the case of NPCs, we were able to localize the receptor on the membrane and to observe some functionally relevant responses on P2X7R activation. We confirmed the validity of the measurements by using microglia cells. The results confirm the well-known notion of microglia being the primary source of P2X7R in the CNS. Moreover, the gradual decrease of the P2X7R signal we show in both Western blot detection and ICC visualization is indeed in line with the proposed assumption that P2X7R has a role in neurogenesis which is executed by NPCs (Tang and Illes, 2017). Thus the presence of the P2X7R on the NPCs is in agreement with previously reported research showing the presence and functional importance of P2X7R in NPCs during neurogenesis (Leeson et al., 2018). It would therefore make sense that with the progression of the NPC differentiation towards mature neurons, the P2X7R disappears from the cells' surface and the overall expression decreases.

In the pharmacological assays presented in this study, P2X7R-dependent effects of ATP and BzATP on the microglia-like cells' viability were observed. These observations are in agreement with the knowledge that the P2X7R is primarily related to immunological events and thus is mainly present on macrophages and microglia (Janks et al. 2018). The observed inability of the P2X7R antagonist JNJ to prevent BzATP-induced cell death of neuronal cells might suggest P2X7R independent activity of BzATP, for example by activation of other P2X receptors. In the case of neuronal progenitor cells, it is known that the expression of the P2X7R on NPCs can have multiple roles, from phagocytosis in the absence of ATP to modulation of the cells' proliferation rates, depending on the ATP concentration (Leeson et al., 2018; Lovelace et al., 2015). The fact that some effect was observed in NPCs treated with ATP and BzATP when pre-treatment with JNJ 47965567, is in line with our protein

localization study that showed the presence of P2X7R in both intracellular and membrane-bound forms. We assume that a delicate balance of the ATP/BzATP concentration is needed to consistently regulate these processes in NPCs, while the concentrations used in this study might not satisfy this requirement. Overall, the efficiency of the highly specific P2X7R antagonist JNJ 47965567 implies the involvement of the P2X7R in microglia and a lesser amount in NPCs, but not in neuronal cells. It is important to note, however, that it could be possible that there is a brain region-dependent variation in P2X7R expression. While the expression dynamics in our cortical-type neuronal cultures suggest a gradual decrease in P2X7R along with the neuronal differentiation, in other brain area-specific neurons, this might differ. Research shows substantial regional and developmental heterogeneity of protein expression in the brain in health and disease (Collado-Torres et al., 2019; Dauth et al., 2017; Herrero-Navarro et al., 2021), and therefore P2X7R expression in brain area-specific hiPSC-derived neuronal cultures could be another important direction for future investigation.

In conclusion, in this study, we examined the expression and functionality of the P2X7R in human hiPSC-derived cortical neuronal cultures and microglia-like cells. We demonstrated that P2X7R is expressed in an active form on microglia-like cells and neuronal progenitor cells. While it is possible to detect the receptor in the intracellular compartment of neurons, no P2X7R-specific functional response was detected on neurons. Despite being known primarily as a plasma membrane channel, P2X7R has been previously identified in the intracellular compartment. It has been reported to, for example, span the nuclear membrane (Atkinson et al., 2002), participate in phagosomes stabilization by influencing the actin assembly on the surface of the phagosome (Kuehnel et al., 2009) and mitochondria (Sarti et al., 2021). Nevertheless, the intracellular roles of P2X7R are not very well explored yet.

Thus, we propose that the main cells in the CNS P2X7R signaling pathway are microglia cells. Nevertheless, it would be important to identify the role of the P2X7R in the intracellular compartments of neuronal cells in further studies.

6. PUBLICATIONS

International paper publications:

- **Francistiová, L.**, Bianchi, C., Di Lauro, C., Sebastian-Serrano, A., de Diego-Garcia, L., Kobolák, J., Dinnyés, A., and Diaz-Hernandez, M. *The role of P2X7 receptor in Alzheimer's disease*. Front. Mol. Neurosci. 2020 Jun 03, 13, 94. doi:10.3389/FNMOL.2020.00094. Impact factor: 5.639
- **Francistiová, L.**, Klepe, A., Curley, G., Gulya, K., Dinnyés, A., and Filkor, K. *Cellular and Molecular Effects of SARS-CoV-2 Linking Lung Infection to the Brain.* Front. Immunol. 2021 Aug 13, 12, 3248. doi:10.3389/fimmu.2021.730088. Impact factor: 7.561
- **Francistiová, L.**, Vörös, K., Csáky, Z., Dinnyés, A., Kobolák, J. *Detection and functional evaluation of the P2X7 receptor in hiPSC derived neurons and microglia-like cells*. Front. Mol. Neurosci. 2022 Jan 12, 14, 351. doi:10.3389/fnmol.2021.793769. Impact factor: 5.639

International abstract and poster presentations:

- **Francistiová L**, Téglási A, Turi Z, Kobolák J, Dinnyés A. Modeling Alzheimer's disease progression with disease-specific human induced pluripotent stem cells. The 3rd Cellfit annual meeting, October 2019, Athens, Greece.
- **Francistiová L**, Téglási A, Turi Z, Kobolák J, Dinnyés A. Modeling Alzheimer's disease progression with disease-specific human induced pluripotent stem cells. 3. Sejt-, Fejlődés-, és Őssejt- Biológusok Éves találkozója, October 2019, Gödöllő, Hungary.
- **Francistiová L**, Téglási A, Kern Z, Kobolák J, Dinnyés A. Human induced pluripotent stem cell-based approach for the study of Alzheimer's disease pathology and neuroinflammation. IBRO Workshop, January 2020, Szeged, Hungary.
- **Francistiová L**, Téglási A, Kern Z, Kobolák J, Dinnyés A. The expression of P2X7 receptor in human induced pluripotent stem cell-derived cellular model of Alzheimer's disease. Alzheimer's Association International Conference, July 2020, online conference.
- **Francistiová L**, Téglási A, Kern Z, Kobolák J, Dinnyés A. The role of P2X7 receptor in Alzheimer's disease pathology in human induced pluripotent stem cell-derived neurons and microglia cells. 12 FENS Forum of Neuroscience, July 2020, Virtual forum.