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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LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APOE	apolipoprotein E
APP	amyloid precursor protein
APP-CTF	C-terminal fragment of the amyloid precursor protein
ASD	autism spectrum disorder
ATP	adenosine triphosphate
BACE1	beta-secretase 1
BAMs	border-associated macrophage cells
BBB	blood-brain barrier
BBG	brilliant blue G
bFGF	basic fibroblast growth factor
BMP-4	bone morphogenetic protein 4
BORGs	3D brain organoid
BzATP	3'-O-(4-benzoyl)benzoyl adenosine triphosphate
C1q	complement component 1q
C3	complement component C3
CA3	Cornu ammonis 3
CD	cluster of differentiation
CHAT	choline acetyltransferase
CNS	central nervous system
CR3	complement receptor 3
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C motif chemokine receptor 1
DAMP	damage-associated molecular patterns
DAMs	disease-associated microglia cells
DAP12	DNAX activation protein of 12 kDa
EB	embryoid body
EC50	half maximal effective concentration
EGF	epidermal growth factor

fAD	familial Alzheimer's disease
FTD	frontotemporal dementia
GABA	gamma-aminobutyric acid
GAD1	glutamate decarboxylase 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GRIN	glutamate receptor ionotropic 1
GSK3-β	glycogen synthase kinase 3 beta
HEK	human embryonic kidney
hiPSC	human induced pluripotent stem cells
HSCs	hematopoietic stem cells
IBA1	ionized calcium binding adaptor molecule 1
IL	interleukin
IRF	interferon regulatory factor
KCl	potassium chloride
KLF4	Kruppel-like factor 4
LPS	lipopolysaccharide
LYVE1	lymphatic vessel endothelial hyaluronic acid receptor 1
MAP2	microtubule associated protein 2
MAPT	microtubule associated protein tau
M-CSF	macrophage colony-stimulating factor
MHCII	major histocompatibility complex
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa B
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NMM	neural maintenance medium
NO	nitric oxide
NOS	nitric oxide synthase
NPC	neural progenitor cells
Oct4	octamer-binding transcription factor 4

PARP	poly-ADP ribose polymerase
PAX6	paired box protein pax-6
PBS	phosphate-buffered saline
PD	Parkinson's disease
PFA	paraformaldehyde
PSEN1/2	presenilin 1/2
PU.1	purine rich box-1 (is encoded by the SPI1 gene)
ROS	reactive oxygen species
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RUNX1	runt-related transcription factor 1
sAD	sporadic Alzheimer's disease
SALL1	Spalt-like transcription factor 1
SALL3	Spalt-like transcription factor 3
SCF	stem cell factor
SCI	spinal cord injury
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SMAD	small mothers against decapentaplegic
SNP	single-nucleotide polymorphism
SOX2	SRY-box transcription factor 2
SOX9	SRY-box transcription factor 9
TAU	microtubule associated protein
TD	terminal differentiation
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase
TMEM119	transmembrane protein 119
TNF	tumor necrosis factor
TREM2	triggering receptor expressed on myeloid cells 2
TUBB3	tubulin teta 3 class III
VEGF	vascular endothelial growth factor
V-GLUT	vesicular glutamate transporter

1 INTRODUCTION

Evidence shows that multiple neurodegenerative and neurological diseases have common features, and the ATP-gated purinergic receptor P2X7R seems to be one of the connecting links. This receptor is activated by markedly elevated levels of extracellular ATP, which is usually a sign of an ongoing pathological process, and its activation often leads to necrosis or apoptosis of the cell. Therefore, P2X7R could potentially be targeted by drugs. While primarily described to be present and functional on microglia cells contributing to inflammatory responses, some reports suggest a neuronal expression of the receptor as well. However, the presence and the role of this receptor in neuronal cells remains a matter of debates, as well as its downstream signalling partners. Moreover, the presence and the characteristics of the P2X7R have not yet been investigated on human iPSC-derived cells (hiPSC). Here, we present experimental results showing P2X7R to be expressed on hiPSC-derived microglia cells, hiPSC-derived neuronal progenitors and in hiPSC-derived maturated neuronal cells. By applying cell surface protein detection assays, we show that P2X7R, despite being detected in neuronal cells, is not localized on the cell membrane and thus may not be available for directly mediating neurotoxicity. On hiPSC-derived microglia cells, a clear membranous expression was detected. Additionally, we have not observed differences in P2X7R functions between control and familial Alzheimer's disease (AD) patient-derived neuronal cells. Functional assays employing a P2X7R antagonist JNJ 47965567 confirm these findings by showing P2X7R-dependent modulation of microglia viability upon treatment with P2X7R agonists ATP and BzATP, while the same effect was absent from neuronal cells. Since the majority of P2X7R research was done on rodent models, our work on hiPSC-derived cells presents a valuable contribution to the field, extending the work on animal models to the human cellular system and towards clinical translation.

However, in the case of prolonged and extended 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 (Villegas-Llerena et al., 2016). 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 (Huang et al., 2019), the P2X7R has attracted most attention due to its unique characteristics such as its low affinity to ATP (Surprenant et al., 1996) and the suggested involvement in a broad range of neurodegenerative diseases such as AD (Francistiová et al., 2020; Martin et al., 2019), epilepsy (Conte et al., 2020; Engel et al., 2012; Morgan et al., 2020), schizophrenia (Calovi et

al., 2020), Huntington's disease (Ollà et al., 2020) and many others. Moreover, another property of the P2X7R is its ability to induce macropore formation upon exposure to high concentrations of ATP (Di Virgilio et al., 2018). 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 (Faria et al., 2005; Virginio, Mackenzie, et al., 1999).

Since P2X7R is mainly expressed on immune cells, it is involved mostly in the context of immune system-related effects. In the central nervous system (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 (Parkhurst et al. 2013; Paolicelli et al. 2011; Schafer et al. 2012b) and immune surveillance during adulthood (Ransohoff and Khoury, 2016; Sierra et al., 2014). The expression and activity of the P2X7R on microglia have been widely examined and validated (Amadio et al., 2017; Janks et al., 2018; Monif et al., 2009). The P2X7R was also suggested in the pathogenesis of Alzheimer's disease, as a general driver of neuroinflammation (McLarnon et al., 2006; Sáez-Orellana et al., 2016), amyloid peptide-dependent neuroinflammation (Martínez-Frailes et al., 2019) or via modulation of chemokine signaling (Martin et al., 2018).

While the expression of the P2X7R on neurons has also been suggested (Miras-Portugal et al. 2017; Illes et al. 2017; Deuchars et al. 2001a), 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 sensitive and specific anti-P2X7R antibodies (Kaczmarek-Hajek et al., 2018; Nicke et al., 2009). 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 rodent P2X7R. The high selectivity of this antagonist was probed against a panel of 50 other receptors and thus can be considered reliable for *in vitro* pharmacological assays (Bhattacharya et al., 2013).

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, and immunoblot detection, we demonstrate the expression and localization of P2X7R in 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.

1.1 Objectives of the PhD

In this study, we aimed to investigate the expression and the localisation of P2X7R on human neuronal cells derived from human induced pluripotent stem cells and its function in the development of neuronal networks. To do this, we developed a cellular system from hiPSCs obtained from healthy donors and Alzheimer's disease patients, respectively. Cells were cultured, and P2X7R expression monitored from the stage of neuronal progenitors during the development towards mature neuronal cells using RT-qPCR, Western blot and immunocytochemistry in the first stage. Next, the biochemical properties of the P2X7R were investigated via proteomic assays and glycosylation status of the P2X7R was determined.

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 LITERATURE OVERVIEW

2.1 P2X7 receptor

The P2X7 receptor (P2X7R) belongs to the group of P2X receptors – all of which are membrane-bound, ligand-gated (ATP-gated) ion channels (Rassendren et al., 1997; Surprenant et al., 1996). The result of their activation is an opening of an ion channel that allows the passage of small cations into (Na^+, Ca^{2+}) and out from the cell (K^+). Despite being functionally and structurally similar, the P2X7R differs from other P2X receptors by that the affinity of ATP to P2X7R is over 100-fold lower than to other P2X receptor subtypes (Sperlágh and Illes, 2014). While all P2X receptors are trimeric and structurally similar containing intracellular N- and C-termini, two transmembrane helices (TM1 and TM2) that form the channel, and an extracellular loop containing the ATP binding site (Karasawa and Kawate, 2016). P2X7R is most structurally distinct from the other receptors in this family (**Figure 1.**). It contains a unique, 200 amino acid long cytoplasmatic domain that can interact with different proteins other than P2X receptors which is also critical for the function of the receptor – e.g. for the fact that P2X7R does not undergo desensitization and thus can initiate apoptosis (McCarthy et al. 2019). This intriguing structural feature contains two additional elements compared to other P2X receptors: c-cys anchor, which is a cysteine-rich region at the end of the second transmembrane domain (TM2) that contains at least five palmitoyl groups and anchors the pore-lining helix of the receptor to the membrane; and the cytoplasmic ballast that is the 120 amino acid long C-terminal "tail" which is characteristic for the P2X7R and enables the receptor to undergo pore dilation and initiate cytolytic signalling (Costa-Junior et al., 2011). Interestingly, the cytoplasmic ballast have been shown to contain a dinuclear zinc ion complex and a high-affinity guanosine nucleotide-binding site – the functions of which are not yet known, but based on experiments with receptor constructs missing the entire cytoplasmic ballast, they do not modulate the basic ion channel properties of the P2X7R, and thus could present a novel framework for future P2X7R investigations (McCarthy et al. 2019). Nevertheless, the P2X7R remains a scientifically engaging subject with plenty of yet unanswered features and roles in physiology and disease.

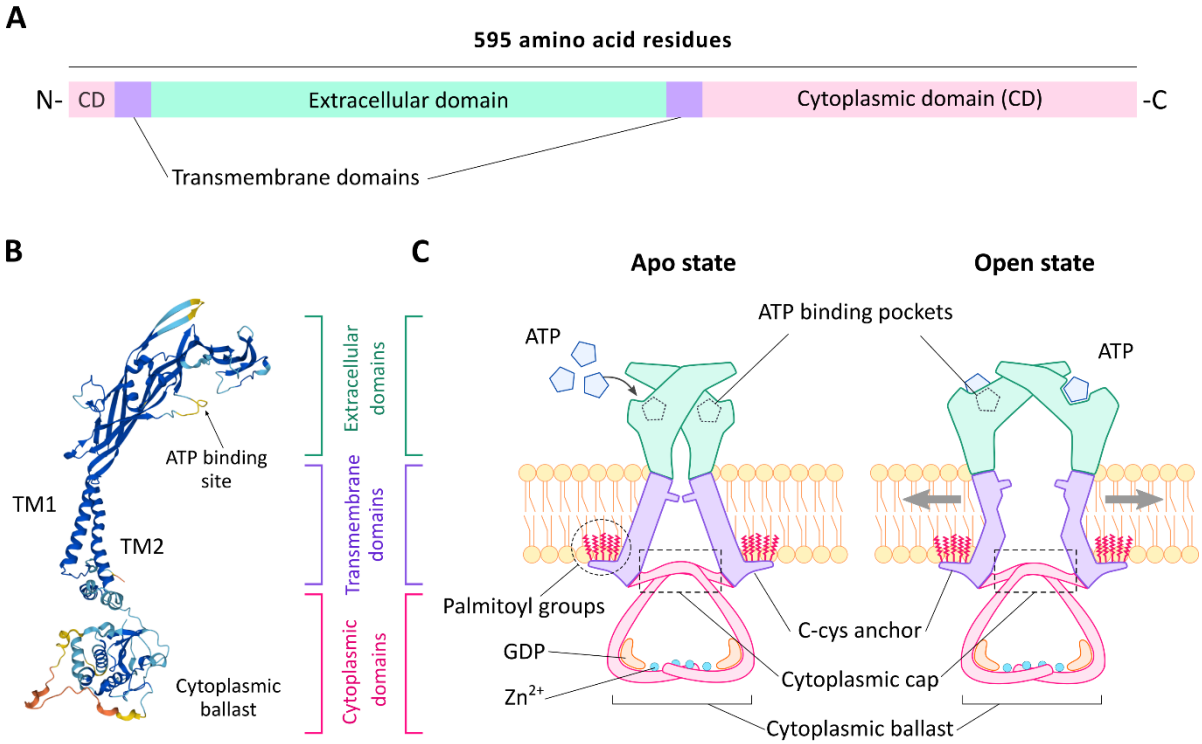

Figure 1. Overview of the P2X7R's structure. (**A**) The structure of the P2X7R peptide showing the cytoplasmic, transmembrane, and extracellular domains. (**B**) A ribbon model of the P2X7 monomer (AlphaFold entry AF-Q99572-F1) with the main regions pointed out. (**C**) Schematic model summarizing the gating mechanism of P2X7R. TM, transmembrane domain; ATP, adenosine triphosphate; GDP, guanosine diphosphate. [The Figure (**C**) was prepared based on (McCarthy et al. 2019)].

Moreover, P2X7R is the only P2X receptor that is directly involved in multiple inflammatory and cytotoxic pathways such as caspase-1 activation and IL-1β maturation, activation of epithelial membrane proteins, shedding of membrane proteins, recruitment of protein kinases, and modulation of lipid metabolism (Costa-Junior et al., 2011). Therefore, the P2X7R had been described to play a role in multiple different diseases such as cardiovascular (Shokoples et al. 2021), autoimmune diseases (Cao et al., 2019), oncological conditions (Pegoraro et al. 2021), and neuropsychiatric diseases including epilepsy (Jimenez-Mateos et al., 2015), schizophrenia (Koványi et al., 2016), depression (Deussing and Arzt, 2018), Huntington's disease (Ollà et al., 2020) and Alzheimer's disease (Illes et al., 2019).

2.2 Human P2X7 receptor

Human P2X7R protein is a 595 amino acid (AA) protein, encoded by the P2RX7 gene, located on chromosome 12 (12q24.31 locus) spanning 53,733 bases (NCBI, 2017). Alternative splicing takes

place during the gene's transcription that may give rise to at least ten different splice variants of P2X7R, described to date (Adinolfi et al., 2010; Cheewatrakoolpong et al., 2005; Feng et al., 2006; Rassendren et al., 1997; Skarratt et al., 2005; Sluyter and Stokes, 2011).

Genetically, the human P2RX7 gene is known to be highly polymorphic, although only a small number of the described nonsynonymous SNPs are altering the functionality of the P2X7R (Fuller et al., 2009; Stokes et al., 2010; Wiley et al., 2003). Site-directed mutagenesis studies showed that introduction of SNP mutations to human P2X7R previously identified in individuals with neuropsychiatric disorders could profoundly alter the P2X7R's key functions which are critical for the physiological role of the receptor (Roger, Mei, et al., 2010), supporting the role of P2X7R in the pathogenesis of these diseases.

Structurally, the P2X7R differs from other members of the P2X receptor family, mainly by its long C-terminal sequence and low agonist sensitivity, while, it is very similar to the P2X4R, showing a 47% identity and 64,8% similarity between the sequences of these two receptors (Alves et al., 2014). Therefore, P2X4R served as a template for initial structural studies of the P2X receptor family (Hattori and Gouaux, 2012; Kawate et al., 2009) and thus elucidating structural properties of P2X7R as well.

The functional properties of the P2X7R were mostly studied on rodent models. However, substantial differences between the rodent (rP2X7R) and human P2X7R (hP2X7R) are known to exist. Such as the hP2X7R having lower sensitivity to agonists and the time of the inward currents flow after a short-term application of agonists, where the receptor deactivation and closing were faster in the case of hP2X7R (Rassendren et al., 1997). Interestingly, agonist sensitivity does not seem to be dependent only on the extracellular agonist-binding part of the receptor. When the human receptor was experimentally modified by changing the intracellular C-terminal domain to the sequence of the rat receptor's C-terminal domain, the receptor became as sensitive as the wild-type rat receptor. However, the large difference in Yo-Pro dye uptake was not altered by this exchange (Rassendren et al., 1997). These data together with results from site-directed mutagenesis of the C-terminal sequence which resulted in the reduction of ATP-induced current responses in extracellular calcium-free solution, strongly suggest that C-terminal fragment is probably important also for calcium sensitivity regulated via calmodulin-dependent signalling (Roger, Mei, et al., 2010). Another difference in rP2X7R and hP2X7R is the electrophysiological profile where the activation of human P2X7R with BzATP or ATP is followed with a faster deactivation. At the same time, the EC50 values for the agonists are about twenty times higher than those for the rodent receptor (Donnelly-Roberts et al.,

2009; Rassendren et al., 1997). Differences in intracellular regulation were identified as well: it has been shown that the human P2X7R has relatively small calcium facilitation while rodent P2X7R displays Ca^{2+}-calmodulin-dependent inward current (Roger et al. 2010).

Downstream activation pathways of the P2X7R are still to be elucidated. However, a number of events have been previously described, and these include effects such as alterations in cell morphology (Cohn and Parks, 1967), interleukin-1β release (Ferrari, Chiozzi, et al., 1997), NFκB activation (Ferrari, Wesselborg, et al., 1997), increase in ROS/NOS formation (Hewinson and MacKenzie, 2007), phospholipase D activation (Humphreys and Dubyak, 1996), inflammasome NLRP3 activation (Franceschini et al., 2015), are might induce glutamatergic signalling in neurons (Sperlágh et al., 2002) or could induce apoptosis (Virginio, MacKenzie, et al., 1999). These events are described in detail later in this introduction.

2.3 Microglia

Microglia is an innate immune cell of the brain parenchyma (Tremblay et al., 2012; Ransohoff and El Khoury, 2015). Microglia cells are active cells that continually survey their local microenvironment and can rapidly change their phenotype in response to any disturbance in the CNS, including dying cells, infiltration of foreign entities or upon detection of pathologically changed proteins. Such flexibility allows these cells to protect the brain and contribute to the maintenance of its homeostasis. Moreover, microglia cells have a crucial role in various aspects of brain development, modulation of neuronal synapses, as well as in ageing and modifying the potential outcomes of neurodegenerative diseases (Bennett and Bennett, 2020; Saijo and Glass, 2011).

2.3.1 Origin and role of microglia in the developing brain

The presence of functional microglia is crucial during mammalian neuro-development since it finetunes the synaptic connections in neuronal networks, as well as during physiological conditions when microglia maintain the precisely defined extracellular microenvironment of the brain. Microglia differs from all other CNS cells in many ways. The first important distinction is its origin.

Microglia arises from myeloid lineage and is of a mesodermal origin (Geissmann et al., 2010). An important publication revealing the developmental fate of microglial cells was published in 2010 by Ginhoux and colleagues, where lineage tracing studies were performed, and it has been proven that

microglia are ontogenically distinct not only from other CNS cells but from other mononuclear phagocytes as well. They showed that microglia arises from primitive myeloid progenitor before E8.0 (embryonal day 8) in mice (**Figure 2.**), indicating that this takes place long before spleen and bone marrow hematopoiesis and even before fetal liver hematopoiesis, which starts at E11.5 in mice.

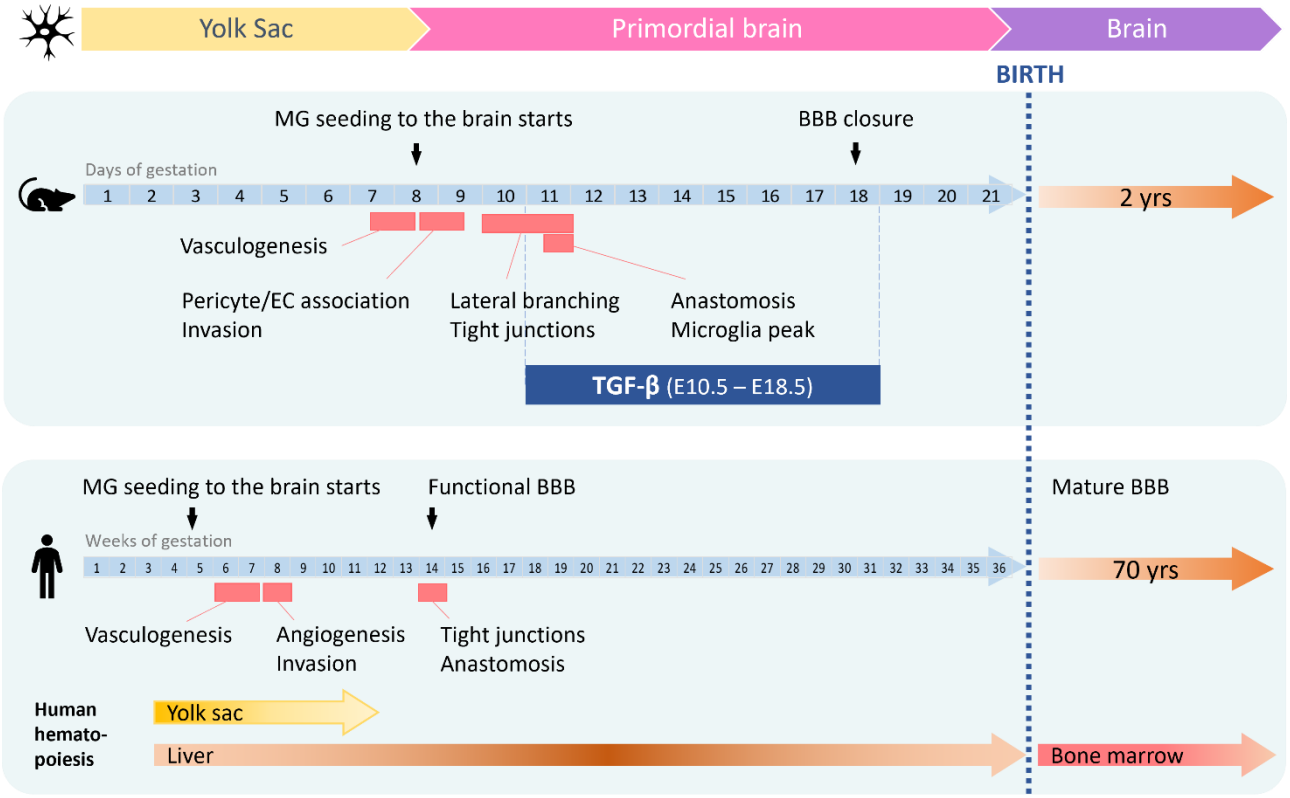

Figure 2. An overview of mouse and human microglia development. The transition of microglial cells from the yolk sac to the primordial brain coincides with the vasculogenesis, pericytes and endothelial cells (EC) association and related signalling – mainly high levels of TGF-β produced by the EC. Microglial infiltration peaks along with anastomosis and is finalised before the closure of the BBB. Microglial infiltration to the human brain could thus follow a similar pattern. [Figure prepared based on: (Matcovitch-Natan et al., 2016; Utz et al., 2020)]

To prove the yolk sac origin of microglia, an *in vivo* fate mapping of yolk sac-derived cells using tamoxifen-inducible Cre recombinase in ROSA26 mice was applied. The experiments showed that microglia progenitors indeed originate from the yolk sac and subsequently migrate to the developing neuroectoderm in a vasculature-dependent manner since the migration did not occur in transgenic embryos without a heartbeat (Koushik et al., 2001). This transfer seems to be mediated through metalloproteinases' (MMP) MMP and MMP9 activities and microglia spreading towards the brain rudiment were diminished after treatment with Batimastat (*CAS Number: 130370-60-4*), a broad MMP inhibitor (Kierdorf et al., 2013).

Ginhoux's work also confirmed the previously anticipated probability that transcription factor PU.1 (PU.1, also known as SPI1) and interferon regulatory factor 8 (IRF8) transcriptional factors might be crucial for microglial development. First, authors identified two types of microglial precursor cells termed uncommitted A1 precursor and mature "pre-microglial" A2 cells, which are distinct in a characteristic expression of CD45 (a pan-leucocyte surface antigen with tyrosine phosphatase activity and a regulator of signal transduction during hematopoiesis), c-kit (CD117; a hematopoietic stem cells surface receptor) and CX3CR1 (a chemokine fractalkine receptor) as follows: A1 ($CD45^+$, c-kit^{low} and $CX3CR1^-$), A2 ($CD45^+$, c-kit^- and $CX3CR1^+$). Then, to determine which transcription factors are required for microglial development, mRNA levels of multiple transcription factors were measured on sorted EMPs, A1 and A2 precursor cells. The studied factors were MYB, RUNX1, SPI1, IRF8, IRF4, ID2, BATF3, and KLF4. All of the factors were only scarcely expressed on $CD45^-$/c-kit^+ EMPs, while elevated levels of IRF8, BATF3, and KLF4 were measured in A1 and A2 cells, and embryonal microglia. The PU.1 was continuously expressed in all studied developmental stages. In further investigations of the transcription factors roles, by using respective mutant mouse models, it was revealed that PU.1- mice were lacking any microglia, both A1 and A2. In *Irf8* mutants, a significant reduction of parenchymal microglia took place. However, A1 cells were preserved. This finding signifies a maturation defect caused by lack of IRF8 and, therefore the importance of this transcriptional factor during microglial development and maturation. As concluded, this investigation demonstrated that microglia develop from IRF8 and PU.1 dependent, MYB, ID2, BATF3, and KLF4 independent EMPs of the yolk sac (Kierdorf et al., 2013).

Apart from microglia, another population of macrophages resides in the brain and is known as the border-associated macrophages (BAMs). These macrophages occupy the non-parenchymal borders of the CNS, such as the choroid plexus, meninges, and perivascular spaces (Bedi et al., 2013; Utz et al., 2020). While, as mentioned, dysregulation of microglia has been linked to neurodegenerative diseases while modifying its molecular signature and morphological appearance (known as disease-associated microglia (DAMs) (Keren-Shaul et al., 2017)). Due to the high similarities of DAMs and BAMs, together with the neurodegeneration-driven change of marker expression, it has been difficult to assign specific contributions to microglia and BAMs to development and pathology. A recent study using multiparametric single-cell analysis revealed the possibility of distinguishing BAMs from microglia by differential expression of certain markers. While the adult BAMs express Siglec1 (CD169), the mannose receptor CD206, lymphatic vessel endothelial hyaluronic acid receptor 1 (Lyve1), CD38, MHCII, CD11c, and the scavenger receptor CD163 (Mrdjen et al., 2018), microglia

are negative for these markers, and in contrast with BAMs express: Tmem119, P2ry12, Sall3, and Sall1 (Butovsky et al., 2014; Buttgereit et al., 2016). These characteristic markers are detectable reportedly from E12.5. Moreover, different responses to interruption of TGF-β signalling in microglia versus in BAMs further support the existence of two autonomous developmental pathways. While TGF-β signalling is essential for developing microglia and the absence of it leads to loss of microglial identity, BAMs can develop in an unbiased manner in the absence of TGF-β (Utz et al., 2020). The understanding of the differences between the border-associated macrophages and true microglia is important in designing approches for microglia generation from iPS cells as described later in section 2.4.5.

When the contribution of hematopoiesis to the adult microglial pool within the CNS was investigated, it turns out that less than 5% was originated from the circulating macrophage population in a parabiotic mice model, in contrast with experiments using sub-lethally irradiated mice models where 95% of microglia were of host origin. These results imply that circulating cells do not contribute to the adult microglial pool, the recruitment of the bone marrow-derived cells were caused by irradiation-induced brain injuries rather than natural reasons (Ginhoux et al., 2010).

While during embryonal development, a part of the microglia population migrating from the yolk sac resides in the brain parenchyma, the other part – the juxtavascular microglia – remains in close proximity to the vasculature and via extended cellular processes ensures the contact with the basal lamina of the blood vessels (Lassmann et al., 1991). During fetal development, the majority of microglial cells are localized near the vascular system, but in further stages, it organises close to the developing telencephalon where the first neurons begin to appear, and the synaptogenesis and subsequently the formation of the thalamocortical projection pathways begin. At this point, microglia perform their first functional task, the elimination of redundant synaptic connections. The synapse elimination occurs because, during embryonal development, far more synaptic connections are being established than are maintained in the adult brain. This elimination is an activity-dependent developmental program and is executed by microglial engulfment of synapses (Paolicelli, Bolasco, Pagani, Maggi, Scianni, et al., 2011). Clearly, for this engulfment to happen a mechanism that helps microglia distinguish synapses that are supposed to be eliminated is needed. A vital signalling molecule that facilitates communication between neurons and microglia is the chemokine fractalkine (CX3CL1, a ligand for the CX3CR1), protein as a possible regulator of synapse removal. It can be expressed as a membrane-bound protein as well as in a soluble form, however, it has been a question whether this molecule is of a beneficial or detrimental effect in the neuron-glia signalling (Lauro et al. 2015). During development, it plays an essential role in the functional maturation of synapses,

controls microglial entry, and functions in thalamocortical networks (Hoshiko et al., 2012). However, for a long time, an outstanding question was whether it plays a role in synaptic pruning as well (Hoshiko et al., 2012; Maggi et al., 2011; Zhan et al., 2014). Nevertheless, new research by Lowery et al. shows that loss of fractalkine signalling does not alter microglia-synapse dynamics. Using germline knockouts of CX3CR1 and examining the microglia-neuron interplays in the visual system, the group demonstrates that no defects in microglial density, morphology, or dynamics occur in CX3CR1-null mice. Moreover, the results imply that while fractalkine signalling is needed for microglial homeostatic behaviour, it is not required for postnatal or adolescent experience-dependent plasticity in the visual system. Based on these findings, the group concludes that fractalkine is not a universal regulator of developmental plasticity, but it has roles in specific areas and ages (Lowery et al., 2017).

To this day, the prevailing opinion is that synapse labelling for removal and the pruning mechanism itself involves complement cascade components C1q and C3 (Stevens et al., 2007), which means that aberrant synapses are tagged by complement, recognized, and phagocyted by microglia. The study of Schafer et al. confirms that microglia phagocyte the neural synapses and confirms that adult C3 knock-out mice had a 1.3-fold increase in VGlut2-containing synapses compared to wild-type littermates. Similarly, the number of VGlut2-positive puncta showed a 1.8-fold increase in CR3 (complement receptor 3) knock-outs as well. In both knock-outs resulted in defects in synaptic circuitry. Therefore, genetic (knock-out) and pharmacological perturbations of C3 complement signalling in microglia abolish synaptic pruning and thus leads to sustained deficits in brain wiring (Schafer et al., 2012b).

2.3.2 Microglia's role in adulthood

When embryonal development is completed, microglial cells are localised in the brain parenchyma where they acquire their typical branched morphology (**Figure 3.**), which is adjusted for the execution of microglia's main and long-term function, the immune surveillance and execute an action if appropriate signals are detected (Kostoví and Judaš, 2002). After migration to the area of the embryonal brain, microglia distribute equally in the brain parenchyma, while different numbers of microglia were described in distinct brain areas (Böttcher et al., 2018; Tan et al., 2020). Once the microglial cells are settled, under physiological conditions, they remain in their permanent locations and start monitoring the extracellular space of the brain by continual extending of their processes. Nimmerjahn et al. proved this phenomenon by using long-term two-photon *in vivo* imaging (Nimmerjahn et al., 2005). This research showed that microglial cells are diffusely and evenly

distributed in the neural tissue, approximately 50-60 µm distance between each other and a cell density of 6500 cells/mm^3. The position of the soma is mostly stable, and only the processes are motile. This motility emerged to be remarkably vivid, and cellular projections are engaged in cycles of continual prolongation, retraction, and *de novo* formation at the speed of 1.47 ± 0.10 µm/min. In this fashion, microglial cells successfully scan the whole extracellular space of the brain parenchyma every few hours (Nimmerjahn et al., 2005). The purpose of this intensive movement is to scan for possible foreign particles such as pathologic proteins, cellular debris, microbes, or other molecules that belong to the group of damage-associated molecular patterns (DAMP) or pathogen-associated molecular patterns (PAMP) molecules (Kierdorf and Prinz, 2013). One type of activation is mediated via soluble factors produced by other cells or by a lack of neuronal ligands, which under physiological conditions serve as inhibitors of activation, which leads to a sterile inflammation (Wilcock, 2014). These particles and signalling molecules are recognised by microglia via receptors that are present on the ends of the cellular processes. After such an interaction, the microglial cell becomes activated. Depending on the nature of the activating stimulus, different types of microglial activation with different phenotypic attributes have been described (**Figure 3.**). To depict these phenotypic similarities and differences analogously to activational types of peripheral macrophages, they were named M1 type and M2 type (M2a, M2b, and M2c, respectively) of activation. These two polarised types represent the two ends of an activational continuum (Mantovani et al., 2004). According to this terminology, the M1 macrophages and microglia are characterised by expressing pro-inflammatory cytokines such as nitric oxide (NO), tumour necrosis factor (TNF), and IL-1β and are prone to cause inflammatory tissue damage.

Moreover, studies on peripheral macrophages demonstrate a shift in M1 cellular metabolism from oxidative phosphorylation to aerobic glycolysis. Under such conditions, cells adopt glycolysis over catabolic mitochondrial pathways in order to generate and conserve metabolic resources and still produce a sufficient amount of ATP (Vander Heiden et al., 2009).

On the contrary, M2 types express growth factors (e.g. insulin-like growth factor, IGF-1) and anti-inflammatory cytokines (e.g. IL-10) and are aimed towards initiation of phagocytosis and tissue repair (Orihuela et al., 2016). A firm disagreement with the M1/M2 nomenclature can be found in the field. The central objection is referring to the fact that the terminology is based on peripheral macrophages which are of multiple types and functions (unlike microglia) and does not take into account the yolk sac origin of microglia. Furthermore, it is mainly established on monitoring one or a few gene products only, which might not be enough. Therefore some authors suggest reassessing the M1/M2 terminology (Martinez and Gordon, 2014; Ransohoff, 2016a).

Another type of activated microglia is the so-called “primed” microglia. Since even the smallest disturbance in the microenvironment can cause microglial activation, the same applies to the presence of signalling molecules originating from the periphery that are able to cross the blood-brain barrier and interact with microglial receptors in the brain. This interaction can happen during prolonged systemic inflammation and causes priming of microglia. It means that microglia become pre-activated and its reaction for the second stimulus is substantially stronger than that of a naïve, resting microglia (Perry and Holmes, 2014).

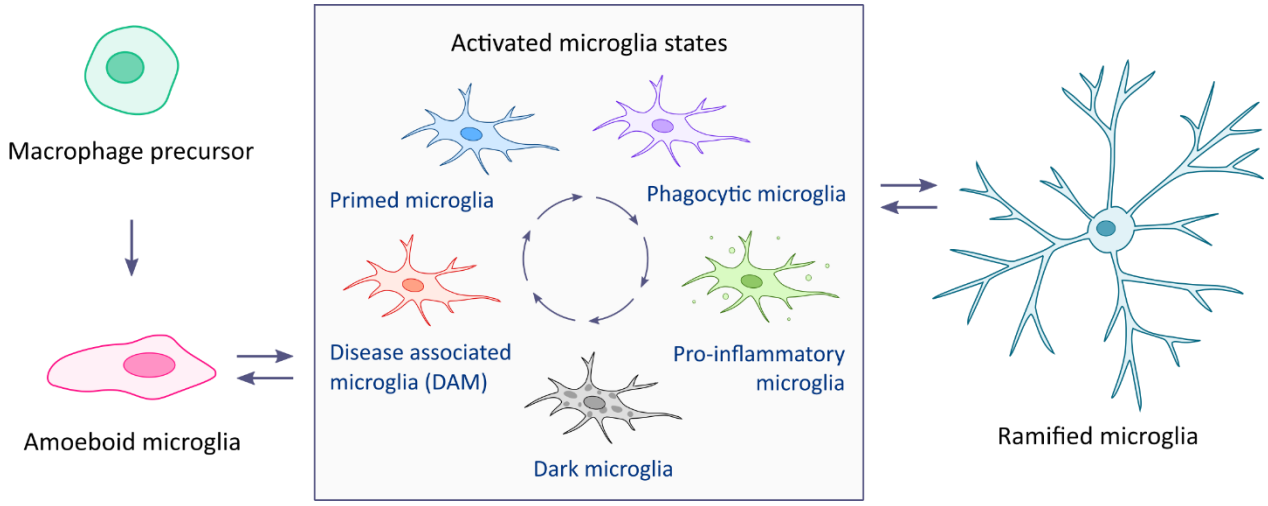

Figure 3. Outline of microglial morphological states. Globular macrophage precursors from the yolk sac transform into amoeboid cells in order to migrate towards the primordial brain. In the brain, microglial cells exist primarily in the resting state and show ramified morphology with motile projections. After activation, microglia change their morphology and overall phenotype depending on the activating stimulus. Microglial activation states can be characterized as a spectrum within which the phenotype changes occur interchangeably.

Morphology and phenotype of primed microglia are hard to spot and are still not fully described. Nevertheless, this concept might explain the role of systemic inflammation on the progression of neurodegenerative disorders and proposes a possible modulation of neuroinflammation by treating peripheral inflammation (Lunnon et al., 2011). Regardless of the nomenclature, microglial cells are sensitive immune cells capable of the potent inflammatory response and thus have the potential to damage the brain tissue under pathological conditions.

2.3.3 Microglia play an important role in inflammation-accompanied neurodegeneration

After we listed the essential functions of microglia in the healthy brain, it is clear that the consequences of any malfunction might be significant. In hereditary microglia dysfunction, many of

its developmentally important tasks may be impaired. These alterations are often linked to conditions such as autism spectrum disorder (ASD) (Voineagu et al., 2011), schizophrenia (van Berckel et al., 2008), and possibly many other neuropsychiatric disorders. However, we intend to focus on neurodegenerative diseases, such as sporadic Alzheimer's disease (sAD) and other dementias, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). It is well known that microglial activation is one of the principal features of Alzheimer's disease (AD). While moderate inflammation is a fundamental and natural reaction directed to the protection of the CNS, prolonged or uncontrolled neuroinflammation is an immensely detrimental process and has a direct effect on neurodegenerative diseases. In AD patients with high levels of pro-inflammatory cytokines and complement in the cerebrospinal fluid, a conversion from mild cognitive impairment to fully developed dementia is faster (Wyss-Coray et al., 2002). The aged brain microenvironment is typical for high levels of pro-inflammatory molecules and by low levels of microglial inhibitors, such as neuronal CD200, immunoglobulin expressed on neurons which upon interaction with microglial CD200R receptor hinders the microglial activation (Cox et al., 2012) and fractalkine, a chemokine expressed by neurons, recognized by microglial CX3CR1 that has inhibitory effects on microglial activation as well (Cardona et al., 2006; Lana et al., 2017). This elevation in levels of pro-inflammatory molecules causes a gradual accumulation of pathology-associated factors. Another factor that most probably contributes to the pathology of neurodegenerative diseases is the extraordinary longevity of microglia (Füger et al., 2017) which consecutively leads to a rich abundance of senescent and less reactive microglia cells in aged brains (Galatro et al., 2017; Streit et al., 2004).

To design the most authentic neurodegeneration model, the pivotal cellular player must not be left out. For neuronal cells there are several functional and robust protocols available (Chambers et al., 2009; McKinney, 2017; Paşca et al., 2014; Shi et al., 2012) as well as protocols recapitulating hematopoietic development towards monocytes (Grigoriadis et al., 2010; Karlsson et al., 2008; van Wilgenburg et al., 2013). However, to generate microglial cells which match the *in vivo* identity, much fewer publications are available (Abud et al., 2017a; Brownjohn et al., 2018; Douvaras et al., 2017; Haenseler et al., 2017; Muffat et al., 2016; Pandya et al., 2017). Moreover, it is necessary to develop and maintain microglia from healthy donors as well as from patients with brain dysfunctions in order to be able to facilitate the investigation of these diseases *in vitro*.

2.3.4 Microglia in Alzheimer's disease

Despite the long-standing notion of M1/M2 activation states of microglia cells, currently available findings imply that this model might be somewhat oversimplified considering the complexity of

microglial effector roles in both health and disease. Although microglia is activated during AD, and it does show different activation states during AD progression in terms of pro-inflammatory versus protective/pro-phagocytic (Nizami et al., 2019), research focused specifically on microglia phenotypes in neurodegenerative environments suggests the existence of specialized microglial cell type (Keren-Shaul et al., 2017; Krasemann et al., 2017). Thus, during neurodegeneration, microglia acquire a unique (non-M1/M2) activation pattern that includes signalling pathways driven by the Triggering receptor expressed on myeloid cells 2 (TREM2)-Apolipoprotein E (APOE) interactions. In many recently published studies, this interaction shows great importance in the offset and the development of Alzheimer's disease. Particularly in the context of mediating and controlling the properties of amyloid-β plaques. In recent years, a growing number of studies point towards the importance of a macrophage-specific receptor TREM2 on microglia. TREM2 is a cell surface receptor, in the CNS expressed only on microglia, which binds polyanionic ligands such as phospholipids and transmits intracellular signals through the associated adaptor protein DAP12 (DNAX activation protein of 12kDa) (Bouchon et al. 2000). The physiological role of TREM2 is to detect lipidic entities, such as cellular debris and bacterial LPS, and initiate appropriate cellular response needed for clearance – activation and phagocytosis (Poliani et al., 2015). Moreover, in the inflammatory environment of neurodegenerative diseases such as AD, TREM2 has been shown to participate in the response to the presence of amyloid-β in a way, that enhances the capacity of microglia to phagocyte amyloid and in later stages to cluster around amyloid plaques and form a physical barrier around them and enclose neuritic plaques in a TREM2-dependent manner (Condello et al., 2015; Wang et al., 2016). This encapsulation of plaques is believed to provide some level of protection of neurons from toxic amyloid-β species by keeping the plaques in a compact, non-diffusing structures what provides a mechanism of dampening their toxic effects on neurons (Meilandt et al., 2020; Wang et al., 2016; Yuan et al., 2016). In TREM2 knock-out mice, this effect was lost, and neuritic damage was greater – proving the critical role of TREM2 in amyloid recognition and processing by microglia cells (Wang et al. 2016). In line with the knock-out animal findings, studies investigating the impact of a TREM2 variant R47H (loss-of-function variant recognised as one of the strongest single-allele genetic risk factors in AD (Guerreiro et al., 2013)) have demonstrated that R47H allele reduces the expression of TREM2 and compromises the myeloid cell response to amyloid-like pathology and thus enhances neuritic dystrophy (Cheng-Hathaway et al., 2018). Research exploring these events in human samples has shown similar results, proving that TREM2-R47H microglia has a markedly reduced capacity to encapsulate amyloid plaques, leading to the formation of less compact plaques with amyloid fibrils extending towards the surface of adjacent neurites and damaging them (Yuan et al., 2016). Taken together, these studies suggest that

not only pro-inflammatory or phagocytic responses of microglia play decisive roles in AD but also the seemingly non-reactive and purely mechanistic function of plaque encapsulation is of critical importance. A possible reason for the tremendous impact of TREM2 on plaque processing could be the alleged interaction of TREM2 with APOE. At the same time, APOE binds to amyloid-β and thus enhances its uptake by microglia (Yeh et al., 2016) while this role is disrupted in TREM2 absence or loss of function. Furthermore, the role of TREM2 in microglia metabolism has been suggested (Ulland et al., 2017). These findings were later confirmed and expanded in studies using human AD patient-derived iPSC-microglia (Piers et al., 2020). Naturally, a single molecule with such a great impact on amyloid toxicity has captured the interest of clinical research scientists as a potential therapeutic target or a diagnostic biomarker (Zhong and Chen, 2019).

2.3.5 Microglial P2X7 receptor

The P2X7R is known to be present on macrophages and microglia cells, where it contributes to several disease-related processes such as oxidative stress, pro-inflammatory events including inflammasome activation, migration towards damaged cells, and phagocytosis (**Figure 4.**). According to several studies, one of the consequences of the activation of P2X7R *via* ATP on immune cells including microglia, is the efflux of K^+ from the cell to the extracellular environment which then initiates the oligomerisation of the NLRP3 inflammasome complex and subsequent IL-1β processing and release (Perregaux et al. 2000; Laliberte et al. 1999). The role of P2X7R-induced K^+ efflux and subsequent NLRP3 activation was confirmed by experiments where microglia cells were pre-treated with potassium chloride (KCl), while the presence of potassium prevented the K+ efflux and thus inhibited the NLRP3 activation (Gustin et al., 2015). While any damaging insult to the surrounding cells can be the source of the ATP, it had been also shown that also neuronal cells in the proximity of amyloid-β peptide can be the source of the ATP (Gonçalves et al., 2019). Moreover, additional research showed that amyloid-β can directly cause NF-kB activation and NLRP3 expression in a P2X7R-dependent manner (Chiozzi et al., 2019). Therefore, the P2X7R's involvement in AD pathobiology gained relevance in recent years.

ATP presents a universal extracellular signalling molecule and when it is present in high concentrations, it serves as a DAMP and a so-called "find me and eat me" signal (Di Virgilio et al., 2017). While the main purinergic receptors that mediate the microglial migration towards the source of ATP gradient are mainly metabotropic receptors such as P2Y12R, P2Y1, or P2Y6, evidence shows, that ionotropic receptors including P2X7R are involved in migration of the cells as well (Martínez-Frailes et al., 2019).

Additionally, a number of reports point towards the possibility that P2X7R is also involved in the regulation of the equilibrium in the levels of reactive oxygen species (ROS) in the microglia cells. Experimental evidence showed that stimulation of rat microglia cells with ATP or BzATP induced O^{2-} release in an NADPH oxidase activation-dependent manner, while the inhibition of phosphatidylinositol 3 kinase attenuated BzATP-induced H_2O_2 release (Parvathenani et al., 2003). Furthermore, reports say that fibrillary $A\beta_{1-42}$ causes an ATP release from microglial cells and activation of the P2X7R in an autocrine manner (Liu et al., 2020). In line with these findings, experiments with P2X7R-deficient microglial cells confirmed that Aβ-induced mitochondrial toxicity requires P2X7R (Chiozzi et al., 2019) and that *in vivo* administration of a selective P2X7R antagonist A438073 led to the suppression of ROS production induced by P2X7R (Munoz et al., 2017).

Along with ROS responses, inflammasome activation, and migration, P2X7Rs also play a role in microglial phagocytosis as confirmed by several studies that used both P2X7R genetic depletion and pharmacological inhibition using P2X7R inhibitor BBG (Janks et al. 2018; Martínez-Frailes et al. 2019). The results of these studies showed evidence that the silencing or inhibition of P2X7R significantly increased the phagocytosis as opposed to microglia cells activated by the application of P2X7R agonist BzATP (Janks et al. 2018).

These experiments and their results place the P2X7R firmly at the forefront of microglial responses to pathological events in the brain and strongly support the importance of the P2X7R-focused research in the scope of neuropsychiatric and neurodegenerative diseases.

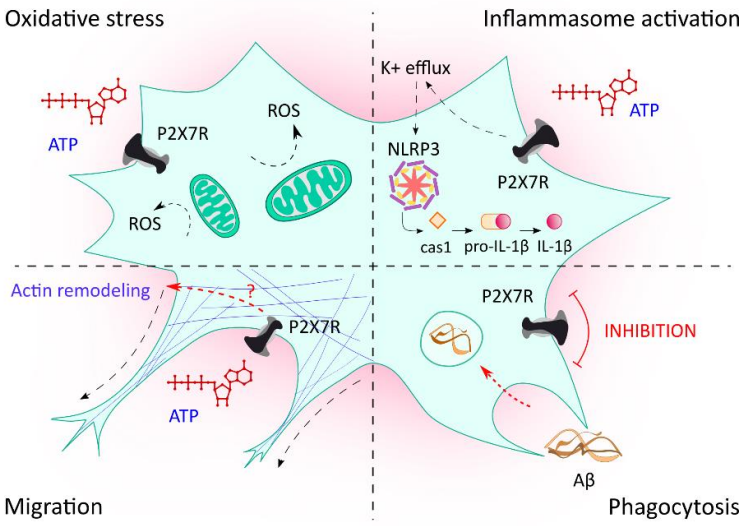

Figure 4. Proposed roles of P2X7R in microglia cells. In microglia, the P2X7R plays a role in oxidative stress modulation, immune responses via the activation of inflammasome complex and subsequent interleukin release, migration of the cells towards the source of ATP gradient, and in the regulation of phagocytosis of foreign or pathological entities.

2.3.6 Neuronal P2X7 receptor

As discussed above, P2X7R's expression on macrophages and microglia is comprehensively published. However, in the case of neuronal expression, the conclusion is not unequivocal since the initial investigations of the P2X7R's presence on the neurons were mostly based on immunofluorescent detection using antibodies, it was necessary to question and verify the specificity of the used antibodies. Especially after the finding that in P2X7R knock-out mice, the anti-P2X7R antibodies still provided a detectable signal (Miras-Portugal et al., 2017). Later, based on these findings, it has been found that these mouse models (Sim et al., 2004; Solle et al., 2001) retain a functional splice variant of the receptor (P2X7R(k)), that is able to escape the knocking out and at the same time preserve functionality (Nicke et al., 2009). Altered functional consequences of P2X7R deficiency were reported in the respective knock-outs which were not in agreement with each other, suggesting that a possible explanation could be the presence of P2X7R isoforms that evaded the gene

knock-out (Illes et al., 2017). However, reports of unspecific staining keep showing up (e.g. Nadal-Nicolás et al., 2016).

Due to the high importance of ATP signalling the organisms have to be able to sense them as early in their development as possible. Therefore purinergic receptors are said to be amongst the first ones expressed on the cells of early embryos and have been even reported to be an important regulator of stemness in stem cells (Burnstock and Ulrich, 2011). The presence of P2X7R has been described in mice neural progenitor cells (NPC), in which P2X7R activation-induced necrosis accompanied by impaired mitochondrial dysfunction (Delarasse et al., 2011). It has also been implied that during early development, P2X7R activation inhibits NPC proliferation and induces differentiation while favouring differentiation into glial cells, suggesting its importance for neural differentiation (Yuahasi et al., 2012). In contrast, another study showed that P2X7R activation in NPCs induces neuronal differentiation (Tsao *et al.*, 2013). The exact effect of P2X7R on NPCs differentiation remains to be clarified; however, these reports clearly indicate a role of P2X7R in the signalling about the switch from proliferative NPCs to differentiating NPCs.

Interestingly, since the NPCs appear in the developing embryonal brain earlier than the microglial cells colonise it and serve as brain-specific phagocytes, P2X7R expressing NPCs are capable of executing the phagocytic function and thus regulate the environment of the neurogenic niche (Gu et al., 2015). Research has shown that the binding of P2X7R agonists inhibits the P2X7R mediated phagosome formation and thus the phagocytosis (Gu et al., 2010). In adult mouse NPCs, P2X7R stimulation with high amounts of BzATP induced pore formation, caspase-3 activation, and cell death via apoptosis (Leeson et al., 2018; Messemer et al., 2013). Taken together, the P2X7R's role on NPCs appear to execute different functions depending on the extracellular ATP concentrations, wherein the absence of ATP, P2X7R expressing NPCs are phagocytic and contribute to the maintenance of neurogenic zones' environment by removal of apoptotic bodies; low levels of ATP will cause a decrease in NPC proliferation; high levels of ATP lead to the formation of the transmembrane macropore and subsequent cell death (Leeson et al., 2019).

In the mature central nervous system ATP plays a fundamental role as a signalling molecule that can mediate the information of cellular damage to microglial cells, and so it makes more sense to anticipate the presence of the P2X7R on microglial cells in agreement with their involvement in brain repair and maintenance of the extracellular space. However, a growing body of research supports the presence of this receptor on neurons as well. Some of these works imply the role of P2X7R in excitatory presynaptic terminals in rat spinal cord and brainstem (Deuchars et al., 2001b), presynaptic

terminals of CA3 mossy fibres where the activation of the P2X7R causes depression of synaptic transmission (Armstrong et al., 2002) or can lead to neuronal cell death (Ohishi et al., 2016).

ATP can have a strong detrimental effect on neurons if it is present in high concentrations. This effect is referred to as ATP-induced excitotoxicity, and it is accompanied by an elevated release of glutamate and subsequent influx of calcium, resulting from the stimulation of Ca^{2+}-dependent enzymes, mitochondrial dysfunction, increase in the levels of reactive oxygen species (ROS) and loss of mitochondrial membrane potential, which could lead to neuronal injury or even neuronal death (Prentice *et al.*, 2015). In line with the hypothesised presence of the P2X7R on neuronal cells, the study of Arbeola et al. shows that after brain ischemia, the receptor seems to be involved in neuronal death and tissue damage via mediating the ATP excitotoxicity. In contrast, blockade of the receptors attenuated this effect (Arbeloa et al., 2012).

A role of P2X7R in synaptic transmission has been described as well, and some studies indicate that it might be functionally targeted at the excitatory presynaptic terminals of the central nervous system as well as neuromuscular junctions in the peripheral nervous system (Deuchars *et al.*, 2001). The research of Deuchars and colleagues supports the theory of P2X7R's involvement in excitatory toxicity by showing that at the neuromuscular junctions, activation of P2X7R induces the release of neurotransmitters (Deuchars *et al.*, 2001). Another study demonstrates the presence of the P2X7R at the axonal growth cones of cultured hippocampal neurons and its regulation of axonal growth, while it is inhibited by ATP and rescued by P2X7R inhibition (Díaz-Hernandez et al., 2008).

Spinal cord injury (SCI) research revealed the presence of P2X7R on spinal cord neurons as well as motor neurons. It showed another implication for the P2X7R in neuronal death, via ATP release and Ca^{2+} signalling in SCI. Importantly, this study showed a positive effect on the neuronal damage after P2X7R inhibition, suggesting this receptor as a potential therapeutic target for acute SCI (Wang et al. 2004). Nadal-Nicolas and colleagues reported a similar finding. They reported the P2X7R to be an important component in the neuronal death model of optic nerve axotomy, where it plays a role as 'mediator' of the neuronal loss in the contralateral retinas. There the P2X7R controls the phagocytosis by microglial cells, an effect that was not present in P2X7R-deficient mice and at the same time this effect was suppressed by the administration of a selective P2X7R antagonist A438079 (Nadal-Nicolás et al., 2016).

Interestingly, not only cell membrane's increased permeability seems to be the consequence of P2X7R activation and the cause of further cellular damage, but also the pronounced mitochondrial dysfunction and PARP (poly-ADP ribose polymerase) activation. In a model of ATP-induced

neuronal death, mitochondrial permeability transition pore inhibitor completely rescued the cells from ATP-induced death. The same study showed that the magnitude of the ATP-induced death is dependent on the expression levels of P2X7R and that the presence of astrocytes further exacerbated the neuronal injury (Ohishi et al., 2016). Likewise, in dorsal horn neurons, ROS production was elevated after ATP treatment and could be eliminated with a P2X7R antagonist. This effect has been proven in both, *in vitro* culture and *in vivo*. However, this study reports, that the selective P2X7R antagonist A438079 attenuated the ROS increase only partially, suggesting an involvement of other purinergic receptors in ATP-induced ROS production (Munoz et al., 2017). Moreover, inhibition of the formation of P2X7R/Pannexin-1 macropore was reported to suppress depolarisation and lessen neuroinflammation by inhibiting the expression of pro-inflammatory markers in the cortex (Chen et al., 2017).

All these findings together endorse the importance of P2X7R in the process of neurodegeneration and strongly suggest the presence of this receptor on neuronal cells. It remains to be uncovered what is the primary purpose of the presence of P2X7R on neuronal cells and what are the exact consequences of the activation of neuronal P2X7R.

2.4 Modelling Alzheimer's disease

Alzheimer's disease (AD) is a progressive, incurable neurodegenerative disease, characterized by an impairment of learning and memory, progressing towards complex defects of attention, executive functions, language, visuospatial compartment (Ballard et al., 2011). Impaired mobility, hallucinations and seizures may emerge, and death is on average 8.5 years after the emergence of first symptoms (Lane et al., 2018). The estimated number of people suffering from AD is 44 million worldwide (Lane et al., 2018), which presents a substantial economic impact as well as non-economic costs of unpaid assistance by caregivers and, ultimately, the lost lives of the affected patients.

The two main pathological hallmarks of AD are amyloid plaques and Tau protein aggregates - neurofibrillary tangles. In the brain tissue, Aβ first accumulates in highly metabolically active areas, such as association cortices and then spreads from the neocortex to the allocortex to the brainstem, eventually reaching the cerebellum. Tau pathology first appears in the transentorhinal cortex of the hippocampus and then spreads to limbic areas and to the neocortex (van der Kant et al., 2020). Another major hallmark of AD and many other neurodegenerative diseases is chronic neuroinflammation. Neuroinflammation is mediated by glial cells of the CNS – astroglia and

microglia cells (Madore et al., 2020). Microglia, in particular, are the cells responsible for maintaining brain homeostasis. During neurodegeneration, the presence of pathological proteins and degenerating neurons activate microglia, leading to initiation or exacerbation of neuroinflammation (Hemonnot et al., 2019; Ransohoff, 2016b; Villegas-Llerena et al., 2016). Amyloid-beta pathology and deposition, tau pathology and neuroinflammation are therefore the main targets in the efforts towards the development of therapeutics to treat AD.

2.4.1 The current trends in the development of in vitro brain models

The current state of advancements in *in vitro* models of brain tissue for developmental, physiological, and disease studies is mostly based on primary cells from rodents, immortalised cell lines, or very rarely primary human cells. These models suffer from serious limitations. For example, in the case of primary cells from rodents (or other mammals), the species barrier is often considered a drawback. While it is possible to create transgenic animals that bear some of the disease-specific mutations, their proteome, immunological properties, and metabolic activity is still different from humans, as well as the complexity of the disease phenotype (Smith and Dragunow, 2014). Although primary human cell cultures might be close to a perfect *in vitro* model, human cells are challenging to acquire. If they are obtained *post mortem*, usually they represent the phenotype of the final stage of the disease (Pacitti et al., 2019). In 2006 a new biological approach was developed, the so-called induced pluripotent stem cell (iPSC) technology (Takahashi et al., 2007). By applying the technology of genetic reprogramming, we are now able to generate different types of cells using de-differentiation of differentiated mature cells (e.g. fibroblasts or blood cells) into a pluripotent stage and then induce a new differentiation towards the desired cellular fate. The iPSC technology in the research of neurodegeneration is progressing fast (Wu et al., 2019) as we are able to generate such cell lines from patients' cell sources. The generation of cells derived from patient-specific iPSCs provides valuable insight into specific molecular phenotypes of neurodegenerative diseases (McKinney, 2017) because their main advantage is that the cells possess the complete genetic background of the patient, including genetic mutations. For disease modelling, iPSCs can be derived from patients bearing proven disease-causing mutations (e.g. MAPT mutation in Frontotemporal dementia -FTD) or from patients with complex disease phenotypes with an unknown genetic background (e.g. sporadic Alzheimer's disease, Schizophrenia). Moreover, healthy individuals' derived iPSCs can be genetically modified to introduce disease-specific genetic patterns or vice-versa the mutant cell line – in case of a well-defined mutation – can be corrected *in vitro*. In the research field of neurodegenerative and neuropsychiatric disorders, the most prevalent approach is to compare the

biochemical, morphological and functional properties of patient-derived cells to healthy donor-derived cells.

Many neurodegenerative disease models are available to date. For example, Alzheimer's disease patient-derived iPSCs (sporadic and familial) are being used by many research groups, including ours (Israel et al., 2012a; Ochalek et al., 2017; Sullivan and Young-Pearse, 2017). Parkinson's disease modelling is based on various alpha-synuclein mutations (Byers et al., 2011; Singh Dolt et al., 2017; Soldner et al., 2011) and samples from patients with idiopathic Parkinson's disease (Sánchez-Danés et al., 2012). Even though these models are noticeably superior to primary animal cell culture and immortalised cell lines, they still lack the inclusion of microglia cells in their *in vitro* cellular systems. The iPSC-based microglia differentiation is currently at its early stage with few publications and many protocol versions. The first attempts to produce microglia from iPSCs were based on the differentiation of hematopoietic stem cells (HSCs). However, this approach generates blood-borne macrophages and some tissue-resident macrophage populations, but the microglial origin is restricted to EMPs in the yolk sac, which creates a complication for generating this cell type.

Next, we will discuss the limitations of neurodegenerative disease models, the importance and possible implications of microglial cells in these models, and the probable causes of the hardships in iPSC-derived microglia development. Finally, we will describe and compare the presently available protocols that use different approaches for iPSC-derived microglia differentiation.

2.4.2 Animal models of Alzheimer's disease and their limitations

Modelling diseases on animal models is a crucial step in every scientific endeavour that aims to elucidate the origin, development, and progress of any given disease. It is desired to have an animal that provides the best option based on criteria such as organ structure similarity to humans, ease of adaptability to experimental conditions, the generalizability of the results, transferability of the information, genetic uniformity and ethical implications, and the cost and availability (Davidson et al., 1987). In the case of neurodegenerative diseases, it would be valuable to utilise higher-order mammals or non-human apes. While spontaneous cerebral amyloidosis and neurofibrillary tangles have been described in non-human primates when used in neurodegeneration studies, they do not fully develop the typical neuropathological phenotypes of neurodegeneration nor clinical symptoms and manifestations (Stancu et al., 2014). Though, they show many aspects that are similar to a healthy human brain – such as neuroanatomy, physiology, cognition, emotion, social behaviour, ageing, and others. These represent an advantage over rodents and are of a higher translational value. It is not surprising that primates provide the most relevant models for diseases like Huntington's (Yang et al.,

2008), Parkinson's disease (Zhang et al., 2000), focal cerebral ischemia (Fukuda and del Zoppo, 2003), Alzheimer's disease (Gralle et al., 2014; Huang et al., 2010), or as models for studies of consequences of AD vaccination (Gandy and Walker, 2004) (see reviews from (Van Dam and De Deyn, 2017; Heuer et al., 2012; Hutchison and Everling, 2012; Toledano et al., 2014). However, there are many limitations to their use, e.g. complexity and high costs of their breeding, long generation intervals, and the existence of severe ethical barriers. These are the result of the fact that non-human primates present a highly intelligent species, especially the Great Apes are often identified to have a "personhood" due to their ability of self-recognition, rudimentary language abilities, deep emotional attachments, and other human-like social tendencies (Prescott, 2010)(Phillips et al., 2014).

Mouse models present one of the best *in vivo* systems available. However, in the case of uniquely human diseases, such as neurodegenerative or psychiatric disorders, the large species barrier might be insurmountable due to different physiology as well as the distinctive properties of the molecular targets (Saito et al., 2016). This affects the translatability of the pre-clinical animal experiments to human clinical tests (Doody et al., 2013; Frölich et al., 2011; Gold et al., 2010; Salloway et al., 2009). AD-like neurodegeneration does not occur in mice and rats, and thus, for research purposes, it must be induced via the introduction of some genetic anomaly. After generating a transgenic rodent model, it is not uncommon that it still does not show the same neuronal loss as it occurs in the degenerating human brain (Ribeiro et al., 2013). The majority of the transgenic rodent models are designed to overexpress some of the pathological proteins typical for the given disease. E.g. Alzheimer's disease models overexpress the mutant amyloid precursor protein (APP), presenilin (PSEN1 or 2), Microtubule-associated protein tau (TAU/MAPT), or various combinations of these (Götz et al. 2018). Mouse models of AD underwent substantial development over the last few decades. Even though there is no doubt about the considerable contribution of the animal models towards the elucidation of neurodegenerative diseases pathogenesis, regarding the pharmacological research, they have not been proven effective in drug development, since no effective drug for dementias such as Alzheimer's disease has been developed based on animal pre-clinical tests (Cummings, 2018).

However, even if the human versions of the proteins are introduced to these models, in reality, they still fail to induce a human-like form of dementia in mice (Bertram and Tanzi, 2008). An example that underscores the importance of this issue is the failed clinical trial of the active immunotherapeutic strategy using the AN-1792 antibody targeted against amyloid-β (ÉLAN, Ireland) (Gilman et al., 2005; Thatte, 2001; Vellas et al., 2009). In this case, clinical trials were terminated after several patients developed aseptic meningoencephalitis as a result of the antibody administration. This side effect was not predicted by the pre-clinical trials on the animal models (Lee et al. 2005).

2.4.3 In vitro cellular models of neurodegeneration

The majority of the presently available *in vitro* neurodegenerative disease models are primary cell cultures from animals, immortalised cell lines, or cells acquired from *post mortem* human brains – none of these recapitulates the pathological processes of neurodegeneration satisfactorily. Despite the species aptness and the appropriate genetic background, cells from deceased patients are not only tough to get, but there are many doubts about their biological fitness after, often, long periods between cell isolation and examination. Moreover, they present mainly the final stage of the neuropathological process (Wu et al., 2019).

In the case of the immortalised cell lines (e.g. murine BV2 cell line (Blasi et al., 1990) and human HMC3 (Janabi et al., 1995), these are tumorous cells that do not stop dividing or artificially manipulated cells that proliferate indefinitely and can be cultured over many generations (Obinata, 2007). Their major advantage is the well-established culture system, easy genetic modification and well-characterised phenomenon. However, as a consequence of immortalisation, the genomes of these cells are often impaired too much to be eligible for modelling normal, *in vivo* conditions (Carter et al. 2015; Timmerman et al. 2018).

2.4.4 Alzheimer's disease modelling using induced pluripotent stem cells

The currently available knowledge regarding Alzheimer's disease is mostly based on results acquired from animal studies, resulting in a significant amount of knowledge. However, in order to verify these data and gain new, a need for a human-derived system arose. Since human brain tissue is extremely hard to get, especially if there is a need for early-onset, not post-mortem tissue. Thus, the Nobel prize awarded induced pluripotent stem cell (iPSC) technology that allows the genetic reprogramming of mature somatic cells into pluripotent stem cells (**Figure 5.**) (Takahashi et al., 2007) became widely utilised. This revolutionary discovery enables the generation of a theoretically unlimited number of PSCs from somatic cells. Reprogramming is made possible with the forced expression of certain reprogramming factors such as OCT4, SOX2, KLF4, and c-MYC (known as OKSM factors). Since the first iPSC generation research article was published, many modifications of the original procedure enabling increased reprogramming efficiency were described, such as expression of RNA-binding protein LIN28, p53shRNA and l-MYC in combination with the OCT4, SOX2 and KLF4 (Okita et al., 2011). It has been revealed that the hypoxic environment during reprogramming increases reprogramming efficiency as well (Yoshida et al., 2009). Other factors were shown to be beneficial for the iPSC yield maximisation, such as the addition of ascorbic acid in combination with GSK3-β inhibition, inhibition of histone-deacetylases with sodium butyrate or valproic acid, activation of Wnt

signalling, to name a few (Bar-Nur et al., 2014; Mali et al., 2010; Marson et al., 2008). An important development was the application of non-integrative reprogramming methods, where the reprogramming factors are not integrating into the genome. Therefore, the original genome of the donor was not disturbed. Such approaches include plasmid- (Okita et al., 2008), mRNA- (Warren et al., 2010), episome- (Jia et al., 2010), adenovirus- (Stadtfeld et al., 2008), and Sendai virus-based (Ye et al., 2013) reprogramming methods. Among these, the Sendai virus-based reprogramming is considered an efficient method in the delivery of the reprogramming factors to the cells while it is easy to eliminate due to its non-replicative nature, it dilutes out from the cells during passaging (Ye et al., 2013).

The obtained iPSCs are defined by the ability of self-renewal and remaining in an undifferentiated state while in optimal culture conditions and proliferate unlimitedly until differentiation towards one of the three germ layers is not triggered, e.g. by adding inducing factors into their cultivation environment. It means PSCs have the potency to differentiate into any specialised cell type, including cortical neurons. In order to obtain them, a neurodevelopmental-like spatial and temporal expression of patterning factors must be modelled *in vitro*. The central signalling pathways of neuroectodermal differentiation include the transforming growth factor β (TGF-β) and bone morphogenic protein (BMP) (Muñoz-Sanjuán and Brivanlou, 2002). In both pathways, the essential components are SMAD transcription factors (SMAD1/5/8 and SMAD2/3, respectively). Inhibition of both classes of SMAD transcription factors leads to neurulation and, finally, the formation of the neural tube *in vivo*, and thus this approach is being used *in vitro* as well to induce neural fate in iPSCs (Chambers et al., 2009).

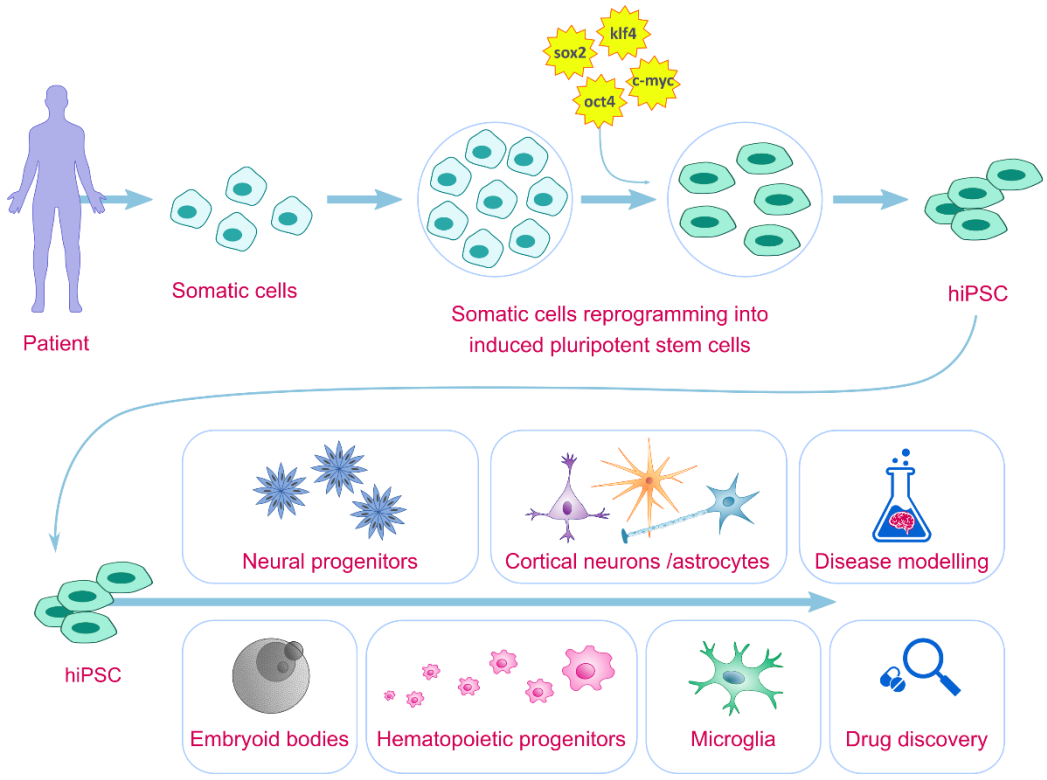

Figure 5. Overview of the hiPSC technology and its utilization in disease modelling. hiPSCs are generated via reprogramming, using OCT4, KLF4, SOX2, and c-MYC transcription factors. The generated hiPSCs can be further differentiated and used for various purposes, including disease modelling and drug discovery.

Considering that these cells originate from an adult individual, Alzheimer's disease patients could become donors, and thus the obtained cells will carry the donor's unique and disease-relevant genetic background, including both genetic and epigenetic factors. Hence, this technique has the potential to reform the whole field of Alzheimer's disease research, and since its inception, it has been widely used. (McKinney, 2017).

It might seem counter-intuitive to use stem cells to model 'old age' diseases; however, even in the early stages after differentiation, these cells exhibit AD-related phenotypes such as increased amyloid-β (Aβ) secretion and TAU protein accumulation. Many neurodegenerative disease models are available to date. For example, Alzheimer's disease patient-derived hiPSCs (sporadic and familial) are being used by many research groups, including ours (Arber et al., 2019; Chang et al., 2019; Lo Giudice et al., 2019; Israel et al., 2012b; Kondo et al., 2017; Ochalek et al., 2017; Sullivan and Young-Pearse, 2017). Parkinson's disease modelling is based on various alpha-synuclein mutations (Byers *et al.*, 2011; Soldner *et al.*, 2011; Singh Dolt *et al.*, 2017) and samples from patients with idiopathic Parkinson's disease (Sánchez-Danés et al., 2012).

To focus on AD research that used hiPSC-derived cells, it is worth mentioning that many important findings stemmed from this approach. For instance, describing for the first time an autophagic dysfunction due to lysosomal depletion and suggesting that modifying the lysosomal biogenesis could present a novel therapeutic intervention (Lee et al., 2014), showing that hiPSC derived cells respond very differently on drug treatment than APP-overexpressing cell lines and thus suggests that it can be a better option for preclinical screening of compounds (Liu et al., 2014), proving that β-secretase has a higher affinity for neuregulin than for APP, which means that it might be possible to inhibit Aβ production via BACE1 processing but without affecting BACE1 interactions with its other substrates (Ben Halima et al., 2016). Importantly, hiPSC has proven to be a suitable for compound screening as demonstrated in this principal study where the correlation between CSF profiles from patients and their own Aβ secretome in the differentiated neuronal cultures is provided, showing the relevance and 'reliability' of iPSC derived systems in AD modelling (Kondo et al., 2017).

Previous results from our laboratory demonstrated that γ-secretase inhibitor DAPT treatment on healthy and PSEN1 mutant hiPSC-derived neurons resulted in reduced endogenous amyloid levels and intracellular accumulation of APP-C-terminal fragment (Lo Giudice et al., 2019), in agreement with previous results (Ochalek et al., 2017). In hiPSC-derived neurons obtained from sporadic AD (sAD) patients, constitutional metabolic changes in ROS production without mitochondrial fission and fusion proteins damage have been described, suggesting that increased ROS production might have a more important role in amyloid and tau pathology than anticipated (Birnbaum et al., 2018). More supporting evidence of the previous findings of TAU protein species propagation patterns where tau oligomers, but not monomers, induce accumulation of pathological, hyperphosphorylated tau in human neurons (Usenovic et al., 2015). Our studies led to conclude that there is no evident difference except the secreted Aβ1-40 levels in phenotype between familial AD (fAD) and sporadic AD (sAD) patients' hiPSC derived neuronal cultures, while hyperphosphorylation of tau protein and an increased level of active glycogen synthase kinase 3 beta (GSK3B), a physiological kinase of TAU, was detected in both patient groups with similar incidence (Ochalek et al., 2017).

Generation of 3D cell cultures has supported the logical assumptions that a three-dimensional culture will more reliably imitate the brain tissue, cell interactions, the microenvironment, and at the same time, all the processes acquire proper dimensions as well – for example, gradients of signalling molecules (Zhang et al., 2014). The power of 3D hiPSC-derived neuronal cell cultures has been demonstrated in an outstanding paper (Choi et al., 2014) that describes human neurons derived from AD patients, cultured in a 3D format to produce extracellular deposits of Aβ (including plaques), as well as high levels of detergent-resistant aggregates of phosphorylated tau. In this culture system, the

attenuation of Aβ generation led to a decrease of Aβ pathology and tau pathology as well (Choi et al., 2014). It is important to note that until this study was published, no other *in vitro* AD model succeeded in linking these two pathological events together. Thus, future steps in the field of iPSC-based cellular models will likely continue to improve differentiation protocols as well as the protocols for obtaining the neuronal cells. Better standardisation of protocols and conditions will contribute to reducing the experimental variability (Sullivan et al., 2018). Particularly for neurodegeneration research, where 3D cultures present a clear goal in order to promote the formation of specific neuronal cell types and their interactions and the development of AD pathologies that are not found in 2D cell cultures (Mungenast *et al.*, 2016).

2.4.5 iPSC-derived microglia in AD modelling

Eight landmark papers describing the differentiation of microglial cells from hiPSCs (outlined and compared in **Supplementary table 1.A and 1.B**) will be discussed in the next section while the aim is to compare these protocols based on media composition, culture parameters, length of the differentiation, the yield of the protocol, and finally, upon the characterisation of the differentiated cells.

The first of these is the composition of the media system. The majority of the described protocols used chemically defined, serum-free media and did not use a feeder-cell layer during cultivation (**Supplementary table 1.A and 1.B**). More importantly, all research groups use sequential media changes to trigger the different stages of the differentiation, using different cocktails of growth factors, cytokines, vitamins, or other components (see details in **Supplementary table 1.A and 1.B**). While Haenseler and Abud used monolayer-based hiPSC induction, all the other groups used Embryoid Body (EB) based differentiation for starting. The differentiation times vary from 4 up to 8 weeks, which affects the maturity of the outcome.

As the first line of characterisation of the cells, all authors completed screening of Butovsky's panel of human primary microglia signature genes (a comprehensive overview of these markers is available in **Table 1.**) and the expression of characteristic proteins. The expression of these markers was assessed by either RNA-sequencing (Abud, Douvaras, Haenseler, Muffat) or by RT-qPCR (Abud, Haenseler, Pandya).

Table 1. Butovsky's panel of human primary microglia signature genes

Human primary microglia signature genes				
Marker	**Protein**	**Expression**	**Role**	**Ref**
C1QA	Complement C1q subcomponent subunit A	"Intracellular until secreted"	Recognition subunit of the classical complement pathway	(Ruiz et al., 1995)
GAS6	Growth arrest-specific 6	"Intracellular until secreted"	Ligand for the Axl (TAM receptor)	(Lemke, 2013)
PROS1	Protein S	"Intracellular until secreted"	Ligand of the TAM receptor family, anticoagulant	
MERTK	MER proto-oncogene, tyrosine kinase	Transmembrane protein receptor	Member of the TAM receptor family;	
P2RY12	Purinergic receptor P2Y12	Transmembrane protein	G-protein coupled purinergic receptor	(Sasaki et al., 2003)
GPR34	Probable (orphan) G-protein coupled receptor 34	Integral membrane protein	Intracellular signal mediation via activation of a G-protein	(Engemaier et al., 2006)

To verify the phagocytic activity of microglia all of the reviewed studies implemented some type of phagocytosis assays (e.g. using E.coli, human synaptosome, pH-sensitive fluorescent zymosan particles, labelled microspheres), which were often complemented with the determination of cytokine profile (Abud, Douvaras, Haenseler, Muffat et al.) Abud and Douvaras determined calcium production as well.

To reveal the *in vivo* functionality of the differentiated cells, hiPSC-derived microglia were transplanted into mice brains. Abud and co-workers additionally tested the ability of microglia to co-exist with hiPSC-derived 3D brain organoids ("*BORGs*") consisting of neurons, astrocytes and oligodendrocytes. Results reveal that after three days, the microglial cells showed rapid chemotaxis towards the CNS-like environment of the organoid and embedded themselves into the tissue of the *BORGs*. Afterwards, they were not detectable in the medium anymore. Moreover, the embedded cells extended ramified processes that are a typical feature of microglia *in vivo*. To further test the microglial cells, Abud et al. made a lesion to the organoids with a needle. After wounding the organoid, microglia clustered near the injury site as an *in vivo*-like response to a neuronal injury, while they endorsed an amoeboid morphology. All of these features are consistent with the behaviour of microglial cells *in vivo* and thus support the quality of microglial cells generated by the group (Abud et al., 2017b). The cell yield varies from 0,5 – 4-times of the initial hiPSC number (Muffat)

up to 30 – 40-times of the starting hiPSC cell number (Abud). Pandya et al., on the other hand, embraced another strategy as they transplanted the murine iPSC-derived microglial cells into the brains of glioma-positive mice. In this fashion, the authors demonstrated the ability of microglia to migrate towards and infiltrate gliomas (Pandya et al., 2017).

3 MATERIALS AND METHODS

3.1 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.

3.2 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.

3.3 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 (**Figure 6.**). Samples were collected weekly.

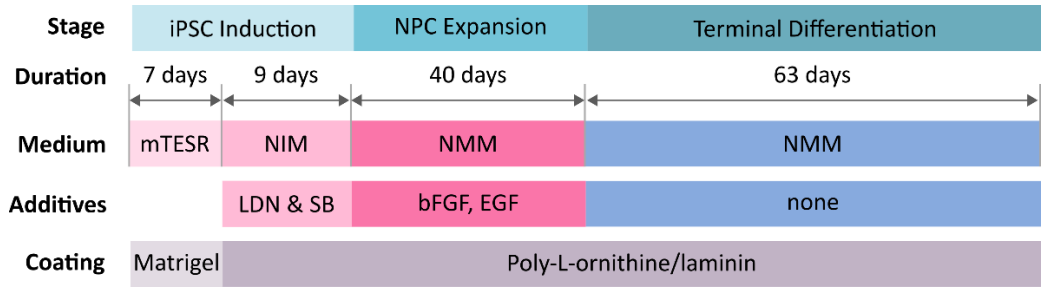

Figure 6. Outline of the generation of the neuronal cells from hiPSC showing the timeline, used media, additives, and plate coating at each stage. NIM, neural induction medium; NMM, neural maintenance medium; LDN, LDN193189; SB, SB431542; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

3.4 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. However, these cells are not yet of sufficient quality and maturation level. Thus they 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 (**Figure 7.**). 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 .

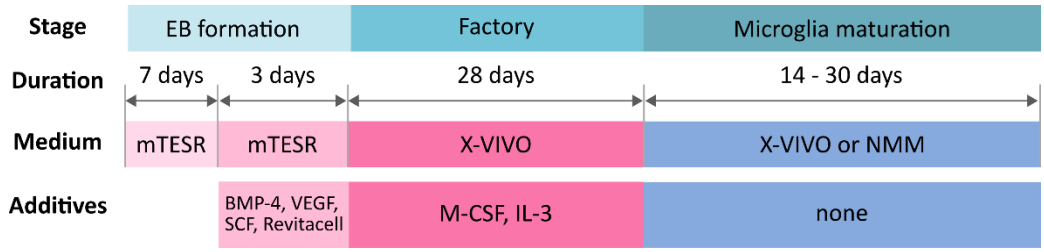

Figure 7. Outline of the generation of the microglia-like cells from hiPSC showing the timeline, used media, additives, and plate coating at each stage. BMP-4, bone morphogenetic protein 4; VEGF, vascular endothelial growth factor; SCF, stem cell factor; M-CSF, macrophage colony-stimulating factor; IL-3, interleukin 3.

3.5 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 (**Supplementary Table 2. and 3.**). 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).

3.6 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).

3.7 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 (**Supplementary Table 2. and 3.**). 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).

3.8 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 (named “IC” as Intra-Cellular) were collected by centrifugation (300g for 2 min at 4°C) of the separation column. The biotinylated membrane-bound proteins (named “MB” as membrane-bound) 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.

3.9 De-glycosylation assay

The cells were lysed as described (**3.6**) and processed in the same way as for Western blot (as described in **3.7**). Before loading the samples on the separation gels, 0.5 µl of de-glycosylating enzyme PNGase F (P0704S, New England Biolabs) per 30 µl of a sample. The samples were incubated with the enzyme at 37°C for 30 minutes and then loaded on the gel and further processed in the same way as during a Western blot procedure.

3.10 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) (**Figure 8.**). 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).

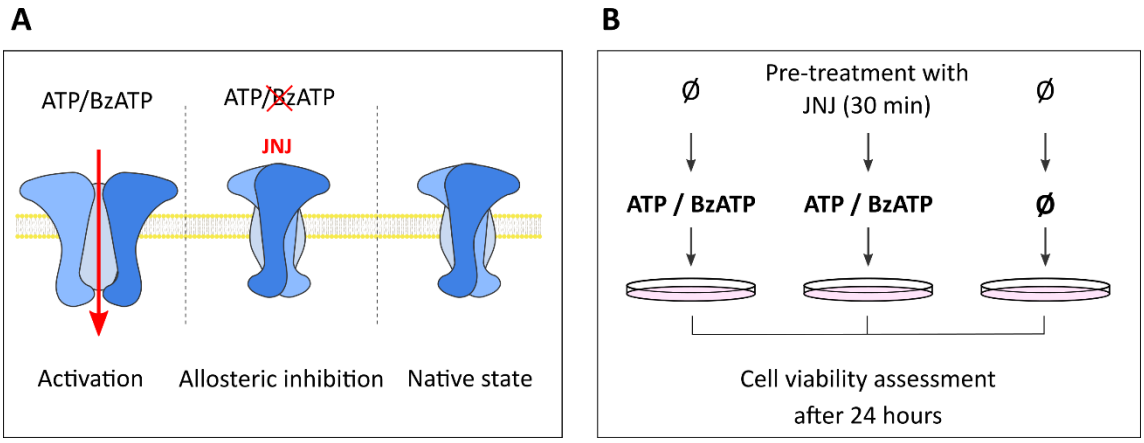

Figure 8. Outline of the experimental design. (**A**) Proposed effects of the ATP/BzATP and the antagonist JNJ on P2X7R. (**B**) Experimental design - cells were treated for 24 hours with ATP or BzATP in different concentrations to promote P2X7R activation; in the samples treated with the P2X7R antagonist JNJ 47965567, the pre-treatment was performed for 30 minutes before the application of ATP or BzATP to induce blockage of the receptor. Cell cultures were monitored as representative phase-contrast photographs show. Cell viability was measured using PrestoBlue cell viability assay after the treatment.

3.11 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 detailed in **Supplementary Table 4.** Human GAPDH was used as a reference gene. The data was analyzed using the REST software (2009 V2.0.13).

3.12 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).

3.13 Fractal analysis

Microglia ramification was analyzed using the fractal analysis as previously described (Morrison et al., 2017), which among others, quantifies cell complexity (fractal dimension, D_B), cell size (density) and rotational variance (lacunarity, λ). Fractal analysis is typically applied to single cells. Therefore, we selected 8 representative microglia cells from both early and late microglia-neuron co-cultures. Individual microglia images were made binary in the FIJI software (Schindelin et al., 2012), and any additional structures surrounding the cell of interest were manually excluded from the image. Binary images were then converted to outlines using FIJI, and FracLac plugin was applied (FracLac plugin for FIJI). FracLac applies a box plot protocol that quantifies the number of pixel detail with increasing scale. For density estimation, FracLac generates a measure of the convex hull, which circumscribes each microglia cell outline with a polygon and a circle that bounds the convex hull. Density (cell size) is the ratio between the number of pixels encompassed by the cell outline to the area (in pixels) of the convex hull. Lacunarity measures heterogeneity to complement fractal dimensions in describing complexity. It uses box mass instead of box count – as it is well described in the FIJI reference guide, section for FracLac https://imagej.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm .

3.14 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 *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4 RESULTS

4.1 Characterisation of hiPSC-derived neuronal cells

Familial Alzheimer's disease patient, bearing a PSEN1 mutation and a healthy control individual-derived hiPSC lines were established and characterised previously in our laboratory (Chandrasekaran et al., 2017; Nemes et al., 2016; Ochalek et al., 2017). These cell lines were used to investigate the *in vitro* pathology of AD as previously reported by us (Lo Giudice et al., 2019) therefore not part of the current thesis.

At the beginning of this work, neuronal precursor cells (NPCs) were generated from hiPSCs, from which a cell bank was generated and was further used in all experiments. NPCs identity was confirmed by immunocytochemical detection of neuroepithelial markers Paired box protein pax-6 (PAX6) and NESTIN and neural stem cell marker SRY-box transcription factor 9 (SOX9) (**Figure 9.**). In the presented work, we used these cells directly for experiments and further differentiation to mature neuronal cells for 63 days (9 weeks).

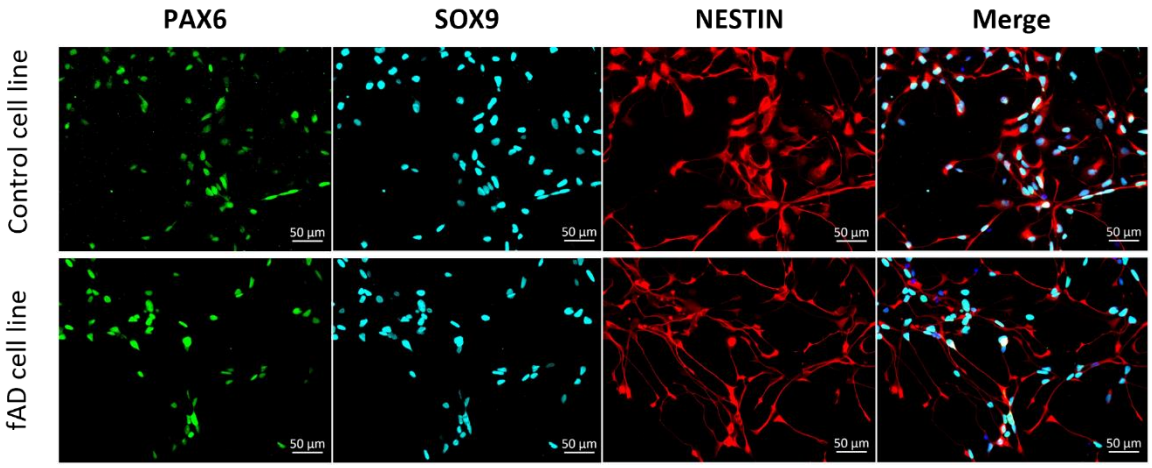

Figure 9. Immunostaining of neuronal progenitor cells (NPC). Representative immunostaining of NPCs expressing stem cell markers PAX6, SOX9 and NESTIN. The scale bar is 50 µm.

The cultured cells show the increasing complexity of the networks formed by the growing and maturing cellular processes. While both used cell lines (control and fAD) were of good quality, more disorderly patterns were observed in the cultures of cells generated from the fAD cell line with ageing (**Figure 10.**).

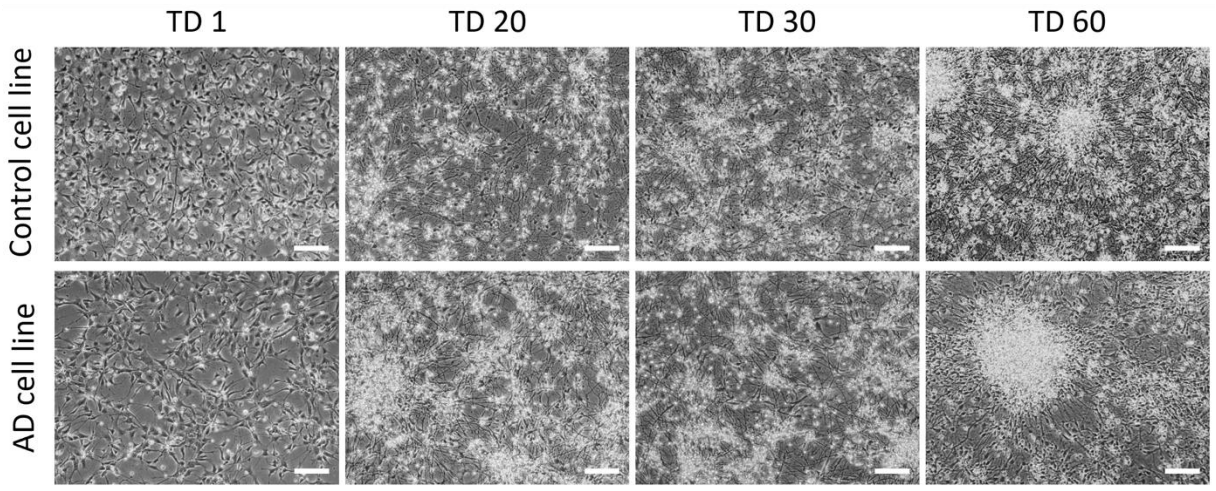

Figure 10. Terminal differentiation of control and fAD hiPSC-derived neuronal progenitors into neurons for 9 weeks. The representative brightfield images show the cell cultures' morphologies and increasing complexity (more connections) along the time of differentiation. The scale bar is 100 µm.

To confirm the identity of the differentiated cells, Western blot detection of main neuronal markers (TUBB3, Tubulin Beta 3 Class III and MAP2, Microtubule associated protein 2) and glial marker (GFAP, Glial fibrillary acidic protein) was performed (**Figure 11.**). Expression of these markers proves that the main cellular component of our cellular systems is neuronal cells with the presence of GFAP-positive glial cells. While no major difference between the two cell lines was visible in the detected amounts of TUBB3, there was a clear distinction in the levels of MAP2 and GFAP. The ratio of these two markers was skewed towards the GFAP in the case of the fAD cell line.

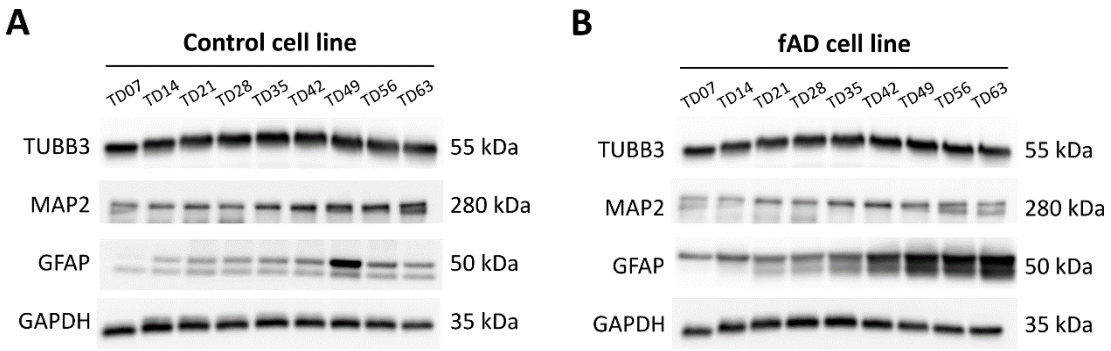

Figure 11. Western blot detection of main neuronal and astrocytic markers in (**A**) control and (**B**) fAD cell lines. Representative Western blot results show the expression of TUBB3, MAP2, and GFAP in the neuronal cultures. 10 µg of protein per lane was used, and GAPDH was used as a loading control.

Additionally, RT-qPCR was performed (**Figure 12.**) weekly during the differentiation period and the samples were tested for stem cell markers (NESTIN, PAX6 and KI-67), pan-neuronal (TUBB3 and

MAP2) and neuronal subtype-specific markers (GRIN1, glutamate receptor ionotropic 1; CHAT, choline acetyltransferase and GAD1, glutamate decarboxylase 1 [marker of GABAergic neurons]) in both cell lines. The examination showed a gradual decrease in expression of the stem cell markers in accordance with the expected disappearance of the immature, differentiating cells from the cell culture. At the same time, the pan-neuronal markers (TUBB3 and MAP2) increased during the early weeks of differentiation and remained highly expressed during the differentiation and maturation, confirming the stability of the neuronal cultures. Additionally to the differences in GFAP levels between the two cell lines detected *via* Western blot analysis (**Figure 11.**), the RT-qPCR results also showed this distinction in GFAP expression, with much higher levels detected in the fAD cell line, especially in the later stages of the cells' maturation.

The levels of the neuronal subtype-specific markers were present in later stages of the differentiation, thus reflecting the progressive maturation of the cellular system. Interestingly, while the markers of glutamatergic neurons variate in relatively similar levels in both cell lines, it seems that there is a marked difference in the amount of the cholinergic marker CHAT, levels of which are 4-5-fold larger in the fAD cell line.

Neuronal subtypes were further examined by immunocytochemistry (**Figure 13.**) which revealed the presence of markers of dopaminergic neurons (TH, tyrosine hydroxylase), cholinergic neurons (CHAT, choline acetyltransferase), GABAeric neurons (GABA, gamma-aminobutyric acid) and glutamatergic neurons (V-GLUT, vesicular glutamate transporter). In the here presented experiments, it was not possible to quantify and compare the precise numbers of cells expressing the individual neuronal subtype markers, but upon empirical observations, it seems that the most abundant subtypes were the cholinergic and GABAergic neurons, while the least abundant were the TH-positive dopaminergic neurons.

In conclusion, both control and fAD cell lines were differentiated into neuronal cells that are possible to cultivate for long periods. Moreover, in the mature cell cultures, specialised neuronal subtypes appear in both cell lines.

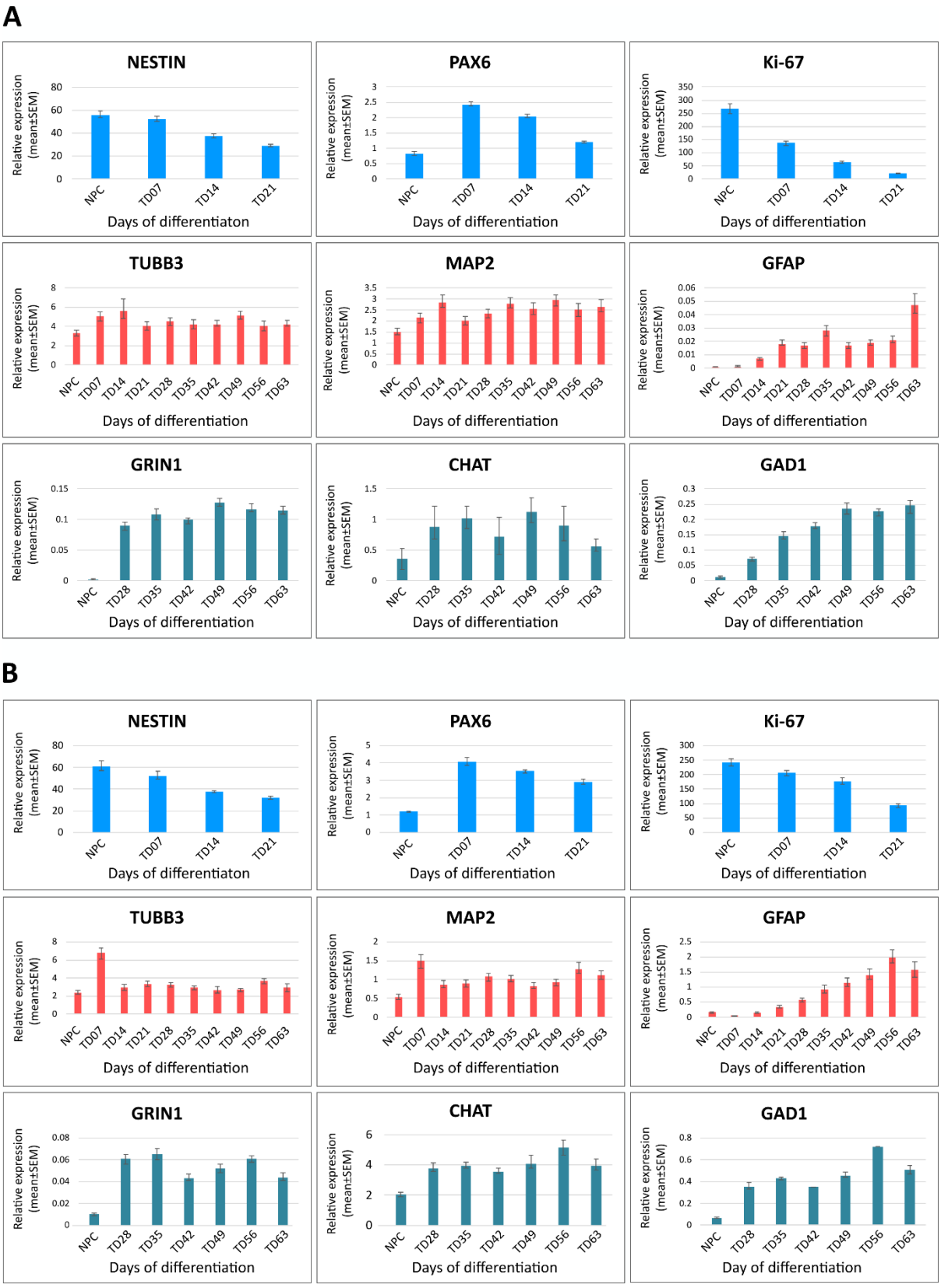

Figure 12. RT-qPCR measurements of stem cell, pan-neuronal and neuronal subtype-specific markers in a control cell line from NPC stage until terminal differentiation day 63 (TD63) at weekly intervals in control (**A**) and fAD (**B**) cell lines. Bars are representing the relative expression data of 3 independent experiments (mean ± SEM). The figure shows a gradual decrease of the stem cell markers (NESTIN, PAX6 and KI-67) and an

increase of astrocytic (GFAP) and neuronal subtype-specific markers (GRIN1, CHAT and GAD1) along with the cell differentiation and maturation timeline. The pan-neuronal markers (TUBB3 and MAP2) increase during the early weeks of differentiation and remain highly expressed during the differentiation and maturation. Data were normalized with the positive control GAPDH and calculated as a relative amount of mRNA compared to the human cortical RNA reference sample. Note that the y-axes ranges are not the same in all graphs.

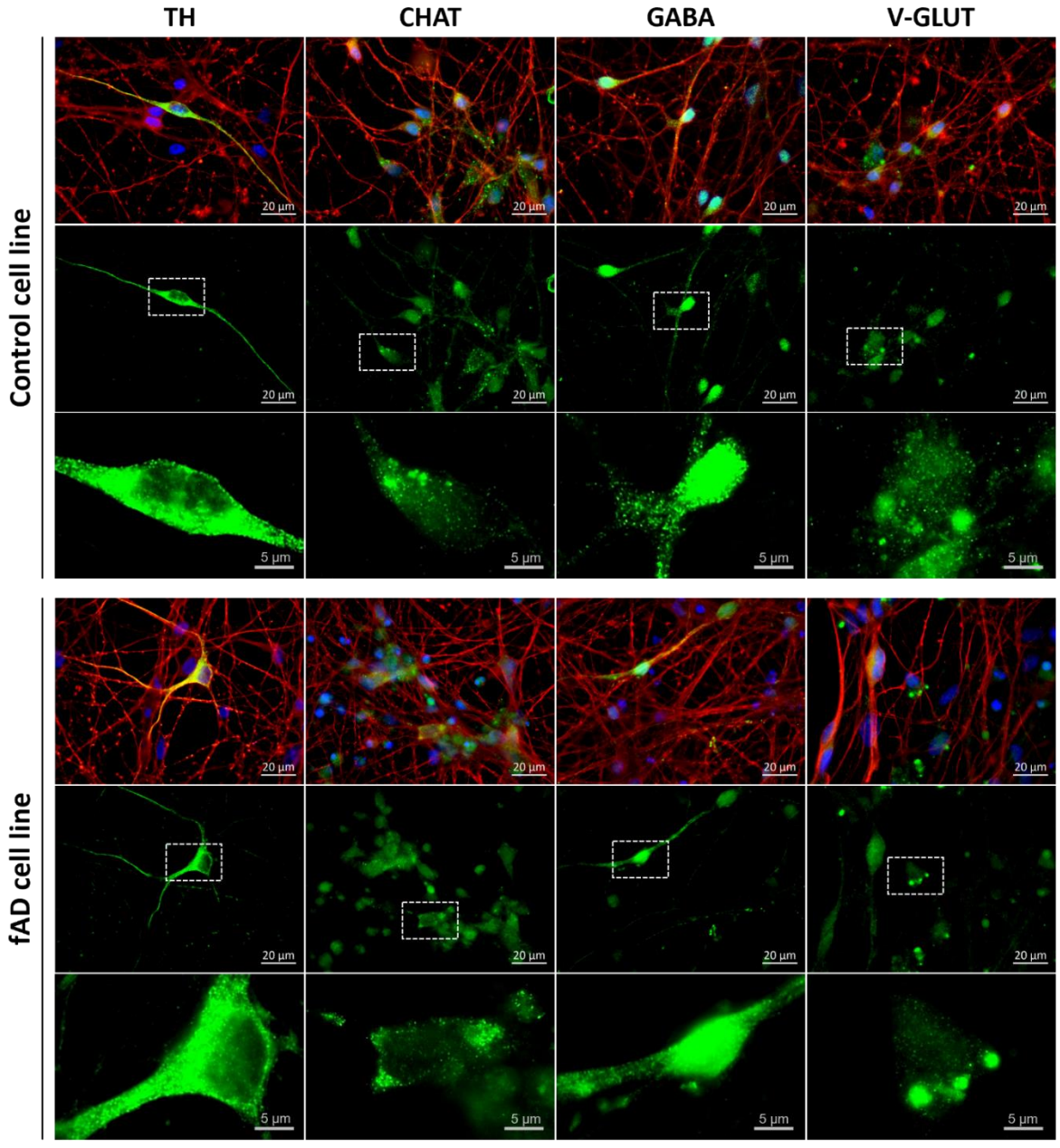

Figure 13. Differentiation of neurons for 63 days is accompanied by the emersion of various neuronal subtypes characterized by the expression of markers such as tyrosine hydroxylase (TH) in dopaminergic neurons, choline acetyltransferase (CHAT) in cholinergic neurons, gamma-aminobutyric acid (GABA) in GABAergic neurons and vesicular glutamate transporter (V-GLUT) in glutamatergic neurons. Scale bar is 20 µm and 5 µm in the magnified images.

After the basic characterisation of the cell lines and their capacity to differentiate into mature neurons, Alzheimer's disease phenotype was tested. In line with the previous reports from our laboratory (Lo Giudice et al., 2019; Ochalek et al., 2017), which showed Alzheimer's disease-like effects on APP processing. Here we show a representative demonstration of the AD pathology as the presence of a 25 kDa C-terminal fragment of the amyloid precursor protein (APP-CTF) in the fAD cell line (**Figure 14.A**) but not in the control cell line (**Figure 14.B**). 25 kDa sized APP fragment is the typical fragment produced in patients with AD linked to PSEN1 mutations (García-Ayllón et al., 2017).

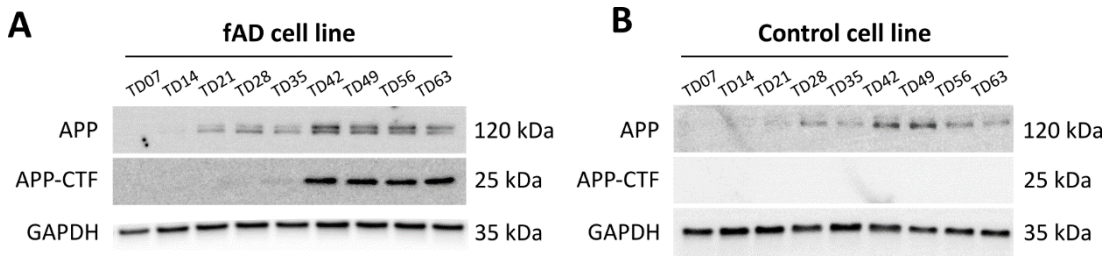

Figure 14. Detection of APP and APP-CTF expression during neuronal differentiation. Representative Western blots are confirming the presence of Alzheimer's disease-related proteins in our culture system. The presence of the C-terminal fragment of APP (APP-CTF) in the fAD cell cultures but not in the control cell line cultures signifies the ongoing Alzheimer's disease phenotype. 5 µg of protein per lane was used. As a loading control, GAPDH was used.

4.2 Characterisation of hiPSC-derived microglia-like cells

In the next part of this work, iPS-cells obtained from the same healthy control individual as used for generating the neuronal cells described above (**Chapter 4.1**) were used to produce microglia-like cells. Following the protocol established by Haenseler et al. (2017), mature microglia-like cells were generated as described above (**Chapter 3.4**). The generated cells were co-cultured with neuronal cells, and they showed an excellent capacity to incorporate themselves into the neuronal network. Not only do they populate the neuronal culture evenly and without damaging it (**Figure 15.A**), the cells establish themselves within the network by growing ramifications and intertwine them between neuronal projections (**Figure 15.B**). This phenomenon resembles the microglial behaviour *in vivo* and thus supports the good quality of our system.

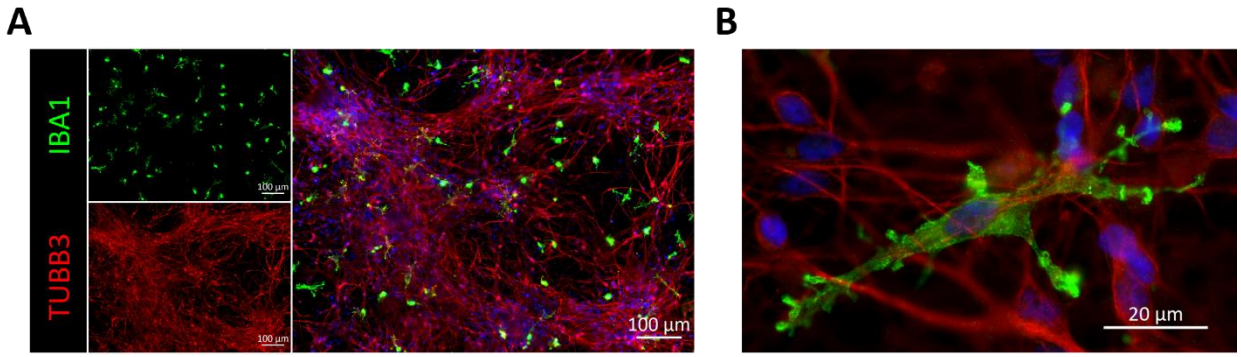

Figure 15. iPSC-derived microglia-like cells in co-culture with neurons. (**A**) Microglia-like cells evenly populate the neuronal culture and interweave its processes between the neuronal network (**B**). The scale bar is 100 µm and 20 µm, respectively.

To verify and quantify the presence of the main microglia-specific markers and thus examining the similarity of our cells with microglia, RT-qPCR detection was performed. Detection of microglia-specific markers after two weeks showed that the cells in monoculture were expressing microglia-specific markers (**Figure 16.A**) such as CD11b, IBA1 and TMEM119. However, relatively high levels of CD45 suggest a similarity to macrophages rather than microglia, which are usually defined as $CD45^{low}$ or $CD45^{int}$ (Honarpisheh et al., 2020; Rangaraju et al., 2018). Also, the typical microglial markers CX3CR1 and P2RY12 show low expression levels at this stage. CX3CR1 encodes the receptor for fractalkine, which serves as a main microglia-neuron signalling molecule and is typically expressed in later stages of microgliogenesis (Kierdorf et al., 2013). When the microglia-like cells that had maturated for two weeks were treated with 1 µg/ml LPS for 24h, they became activated and showed significant upregulation of NLRP3 inflammasome (**Figure 16.B, C**), which proves the capacity of the cells to become activated and be immunologically engaged.

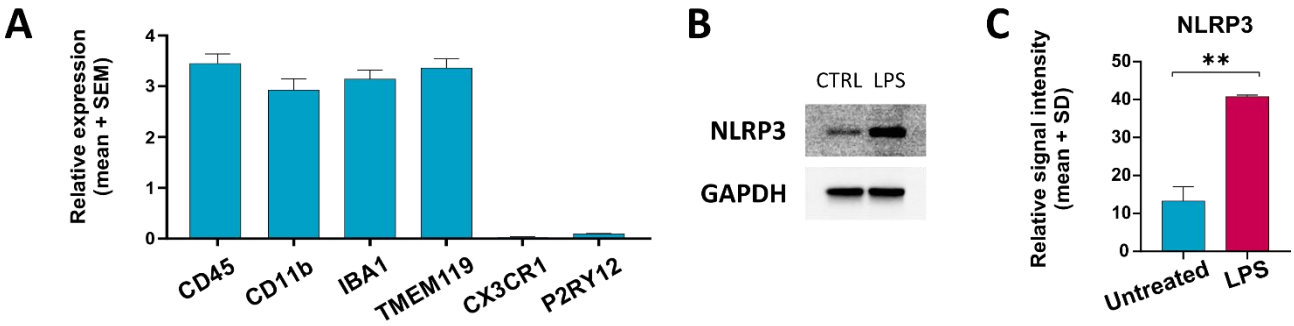

Figure 16. hiPSC-derived microglia-like cells characterisation. (**A**) Detection of the expression of microglia-specific markers via RT-qPCR. Expression values were normalised to GAPDH and calculated as a relative expression value related to the total human cortical mRNA reference sample. (**B**) Verification of microglia-like cells' capacity to become activated upon the treatment with 1 µg/ml of LPS. Activation was estimated through the detection of NLRP3 protein's upregulation via western blot and evaluated after (**C**) densitometric

analysis. 10 µg of protein were loaded on the gel, and GAPDH was used as a loading control. Error bars represent the mean + SEM or SD of three measurements. p** < 0.01

When microglia cells were co-cultured with neurons, an outstanding integration of the microglia-like cells into the neuronal network and subsequent transformation of the microglia cells' morphology from round to amoeboid to branched was observed. To compare and quantify the two distinct morphologies, fractal analysis of the cells was conducted (**Figure 17 .A, B, C**), which showed a significant difference in the morphologies of the two microglia populations: amoeboid (early co-cultures – less than 14 days) and ramified (long-term co-cultures – over 30 days) (**Figure 17.B**). Based on the fractal analysis, the analyzed cells were divided into two groups that correlate with the length that microglia cells spent in co-culture with neurons (**Figure 17.C**). These results demonstrate that our hiPSC-derived microglia-like cells are well-tolerated in co-culture with hiPSC-derived neurons. Moreover, the ramification and active interweaving of the microglial processes into the network of neuronal cells suggest that microglia acquire a morphology that resembles the resting microglial phenotype.

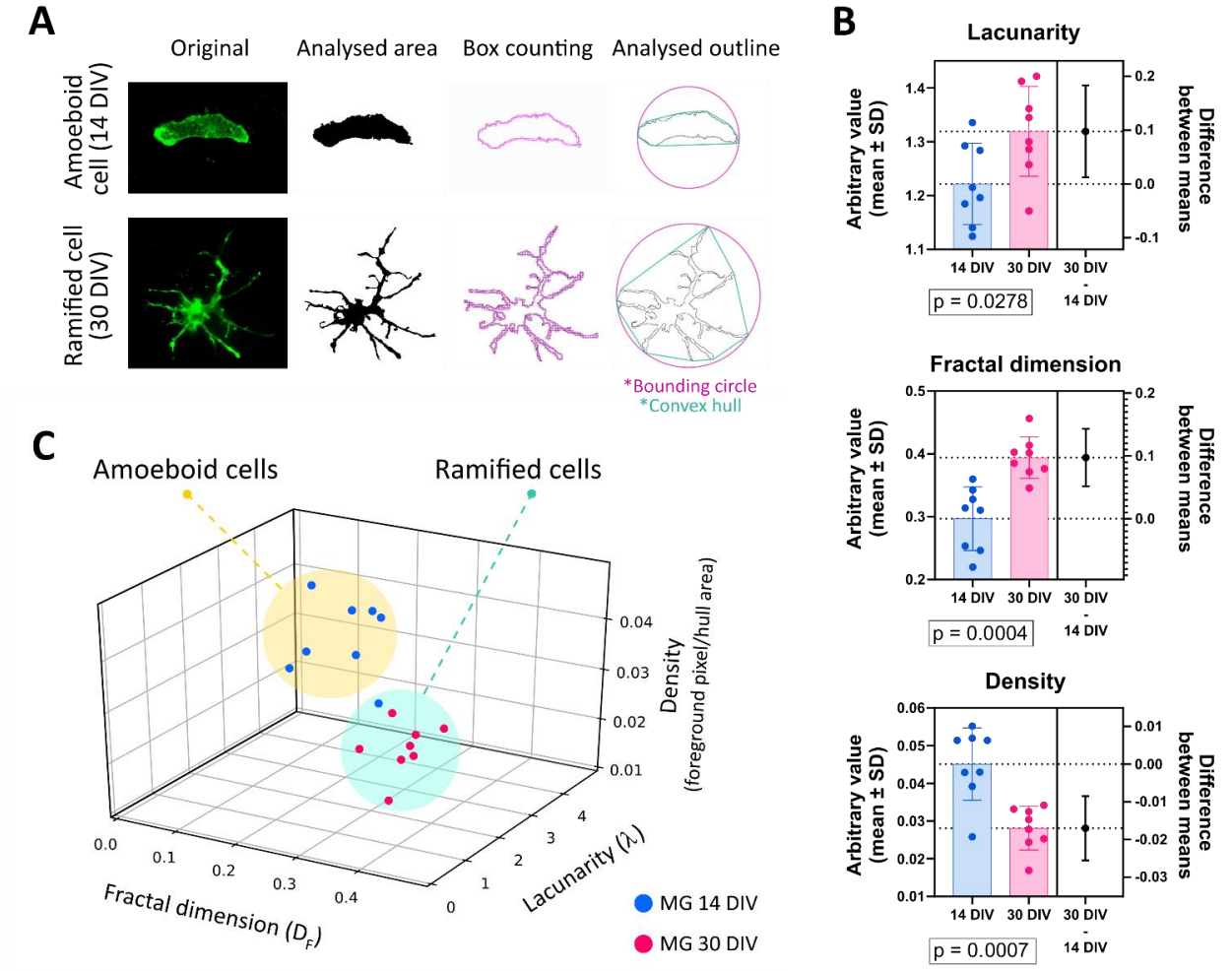

Figure 17. Fractal analysis of the shapes of microglial cells. (**A**) Example of the image analysis using FracLac box-counting method for microglia ramification assessment. Original immunocytochemistry pictures were subjected to uniform FIJI protocols, binary picture, and cell outline preparation. Box counting (pink squares along the cell outline), convex hull (turquoise polygon), and bounding circle (pink) were used by FracLac for subsequent calculations. (**B**) Comparison of the individual variables between cells from early and late co-cultures. n=16. Error bars represent the mean ± SD. Statistical analysis was performed with an unpaired Student's t-test. p values are indicated below the respective graphs. (**C**) Plotting the values on a 3D plot based on the relationship between the three variables (fractal dimension, lacunarity, density) distributes the individual data points (representing individual microglia cells) into two populations marked as amoeboid cells and ramified cells. The two groups' distribution correlates with the time-lengths of microglia-neuron co-cultivations.

4.3 Examination of the P2X7 receptor expression in the neuronal and microglial cellular models

After the characterisation of the used cellular cultures, the next step was to investigate the expression of the P2X7R both in hiPSC-derived neuronal (control and fAD) cell cultures, as well as in hiPSC-derived microglia-like cells. In the beginning, verification of the most refered antibody (anti-P2X7R, APR-004, Alomone labs) was performed.

4.3.1 Antibody validation

Verification of the anti-P2X7R antibody was performed by pre-incubation of the antibody with the control peptide (synthetic peptide against which the antibody was raised consisting of 21 amino acid residues CKIRKEFPKTQGQYSGFKYPY) and subsequent treatment of the Western blot membranes in parallel with the control peptide-free antibodies. NPCs from the control cell line were used as well as human embryonic kidney (HEK) cells overexpressing P2X7R (HEK P2X7^{+}). This test approved the specificity of the antibodies for their epitope, and thus, they were considered appropriate for further experiments (**Figure 18.A**). Additionally, this control peptide was also loaded on a gel as a sample to show that the antibody can recognise it in this way as well (**Figure 18.B**). As the last step of antibody validation, the fact that the native P2X7R is glycosylated was utilised. A protein extract from hiPSC-derived microglia-like cells was used and treated with PNGase F. The reduction in the size of the detected band confirms that removal of glycans occurred and that the detected protein is a P2X7R protein (**Figure 18.C**).

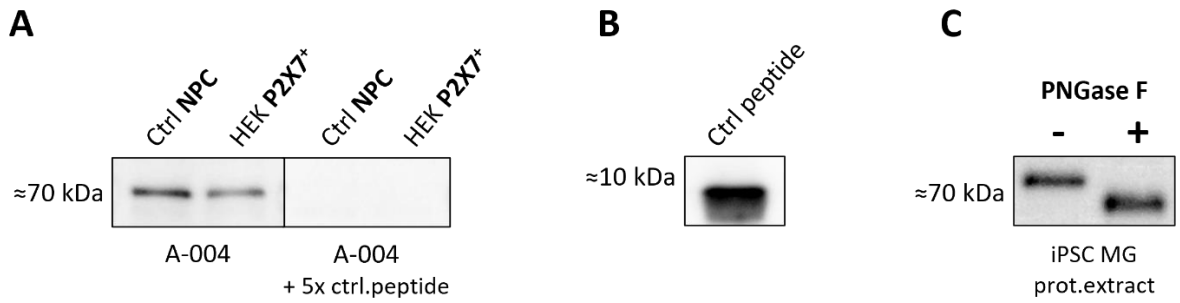

Figure 18. APR-004 antibody validation. (**A**) Pre-incubation of the antibody with the control peptide prevents the antibody from binding to its epitope in the used samples. (**B**) Control peptide was loaded on a gel, and antibody recognized it as its epitope during western blot analysis. (**C**) De-glycosylating enzyme caused the P2X7R band's reduction in size after the glycans were removed.

4.3.2 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 (amino acids 576 – 595; epitope: KIRKEFPKTQGQYSGFKYPY) (**Figure 19.A-1**) and the Santa Cruz (sc-514962) antibody that recognizes the extracellular part of the receptor (amino acids 81-105; epitope: KKLVHSVFDTADYTFPLQGNSFFVM) (**Figure 19.B-1**). These two antibodies were used because, despite the Alomone labs (APR-004) is an antibody broadly used in the field,

including for P2X7R detection in human samples, it was originally raised against rat P2X7R peptide. Therefore, analysis using the Santa Cruz (sc-514962) antibody was added, as this antibody is raised against an epitope of human origin.

The results of Western blot analysis using both antibodies suggest and decreasing signal intensity along the neuronal cultures' differentiation times (**Figure 19.A-2, B-2**), which is represented in a graph of values obtained after densitometric analysis of the blots and normalised to GAPDH signal (**Figure 19.A-3, B-3**). 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 (**Figure 19.A-4, B-4**), confirming the observation of decreasing P2X7R expression. The results of the regression analysis are reviewed and explained in **Table.2**.

Table 2. Results and data comparison of the regression analysis of the Western blot measurements.

	Alomone lab (APR-004)		**Santa Cruz (sc-514962)**	
	Control line	**fAD line**	**Control line**	**fAD line**
Coefficient of determination (R^2)	0.6301	0.4044	0.6398	0.8016
Correlation coefficient (r)	-0.79	-0.64	-0.8	-0.9
p-value (p)	0.01	0.066	0.009	0.001

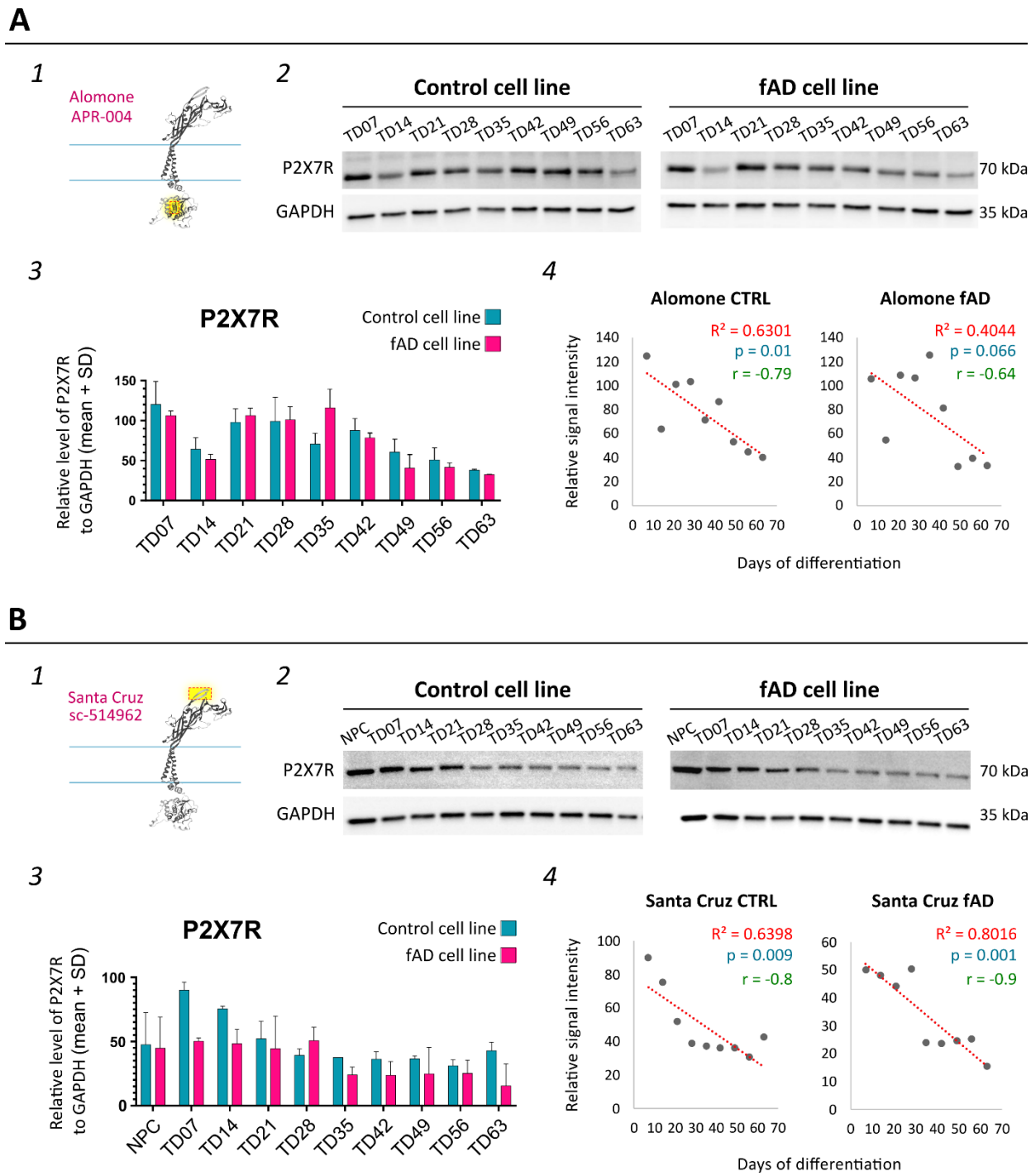

Figure 19. Western blot detection of the expression of P2X7R in control and fAD neuronal cells using the Alomone APR-004 (**A**) and the Santa Cruz sc-514962 antibody (**B**). (***1***) Highlighted part of the protein (yellow box) represents the epitope recognized by the antibody. (***2***) Representative results of Western blot analysis of the P2X7R in control and fAD cell neuronal cells differentiated for 63 days and a sample collected weekly in 3 independend experiments. GAPDH was detected as a loading control. (***3***) The Western blot was analysed densitometrically and plotted. (***4***) Regression analysis showing the correlation between the decrease of the P2X7R's signal intensity along the time of differentiation of neurons. *(*Note: the NPC stage was not included in the B-4 regressesion analysis, in order to the analysis be comparable with A-4 dataset which did not contain the NPC stage either.)*

4.3.3 RT-qPCR detection of P2RX7 gene expression

Expression of the P2RX7 gene was measured using the RT-qPCR (**Figure 20.**). In contrast with the results of Western blot analysis, these results show a gradual increase of the P2RX7 mRNA signal along the differentiation timeline in both control and fAD cell lines with a more pronounced increase in the fAD cell line. Nevertheless, the relative expression ranges are very low (control cell line neurons: 0.006 – 0.075; fAD cell line neurons: 0.029 – 0.024; microglia: 0.587).

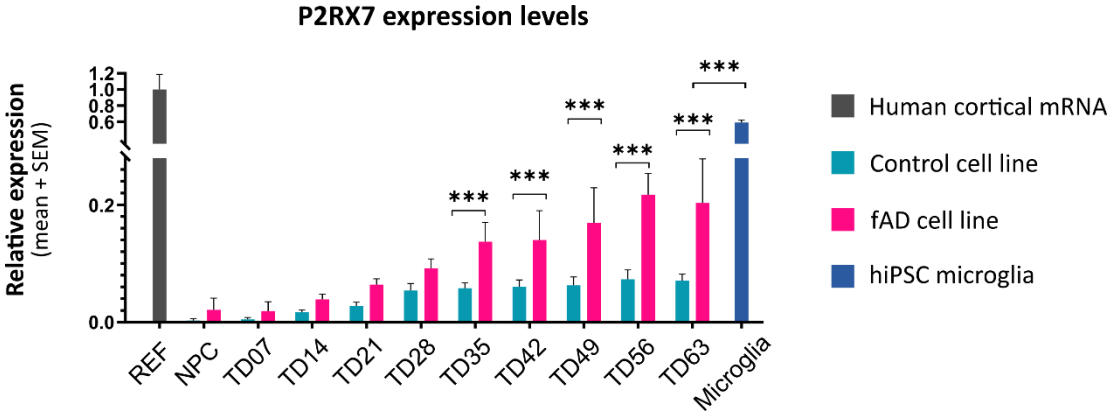

Figure 20. P2RX7 gene expression was measured by RT-qPCR in control and fAD neurons, and hiPSC-derived microglia-like cells. Measurements were performed using the hiPSC-derived microglia cells and on neuronal cultures from the NPC stage until TD63 stage at weekly intervals. Data were normalized with the positive control GAPDH and calculated as a relative amount of mRNA compared to the human cortical mRNA reference sample. Bars are representing the relative expression data (mean + SEM). ***p<0.001

4.3.4 Immunocytochemical detection of P2X7 receptor

Immunocytochemical investigation revealed the presence of P2X7R-positive signal in neurons of both control and fAD cell lines in the neuronal progenitor stage and throughout all the differentiation stages, up to 9 weeks (TD63). However, we have noticed that while in the NPC and early terminal differentiation stage of neurons (TD7), the expression seems to be membranous, in the later differentiation stages, the signal weakened and disappeared from the cell membrane of neurons (positive for TUBB3 in colocalization). Instead, it became strongly centralized in the intracellular compartments of the cells (**Figure 21.**). No differences were observed in the P2X7R localisation detected on control and fAD cells in the immunocytochemical experiments. The shift from membranous to intracellular staining was similar in both cell lines.

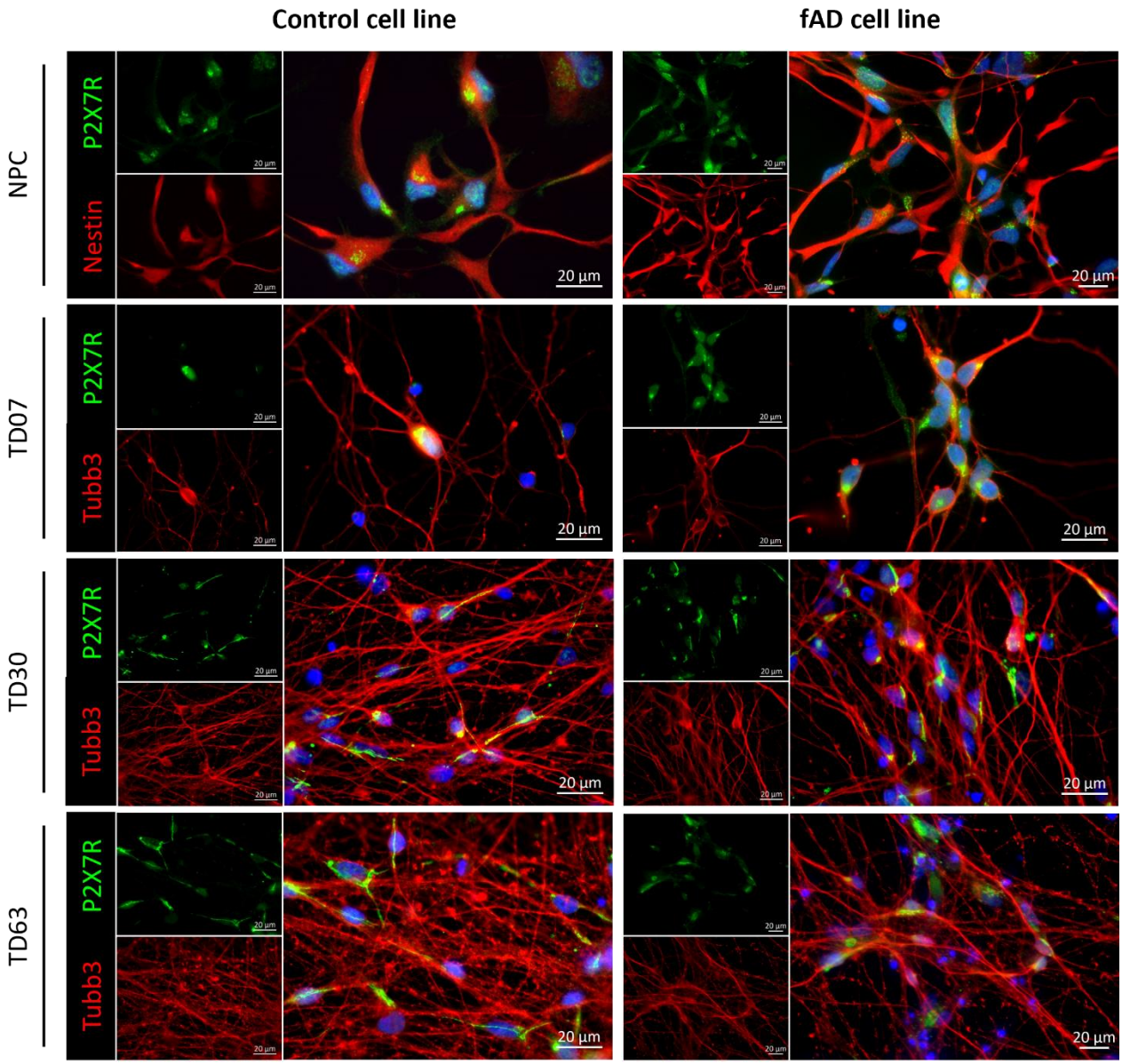

Figure 21. Wide-field fluorescence microscopy detection of the P2X7R in hiPSC-derived NPC and differentiated neuronal cell cultures. Immunocytochemical detection of P2X7R in different differentiation stages of control and fAD hiPSC-derived neurons show the presence of the P2X7R (green) in early stages of neuronal differentiation (NPC and TD07) and weaker signal in later differentiation stages (TD35, TD63). The scale bar is 20 µm.

In the case of microglia-like cells, a clear P2X7R signal was detected on both monoculture and co-cultivated microglia-like cells. Moreover, the P2X7R's localization on microglia-like cells was uniformly membranous in all examined conditions (**Figure 22.A, B**).

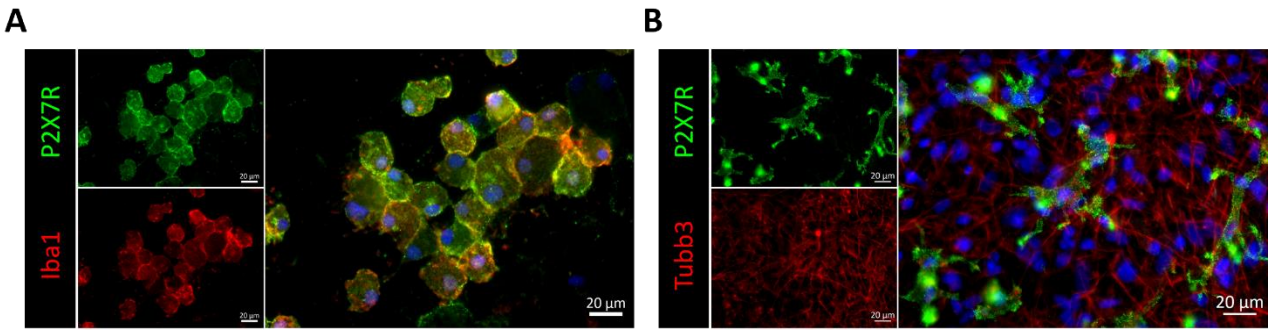

Figure 22. Detection of the P2X7R in hiPSC-derived microglia-like cells in monoculture (**A**) and co-culture with differentiated control neuronal cells (**B**). Both conditions show membranous localisation of the P2X7R signal. The scale bar is 20 µm.

4.4 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 (**Figure 23.A**). 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 (**Figure 23.B**).

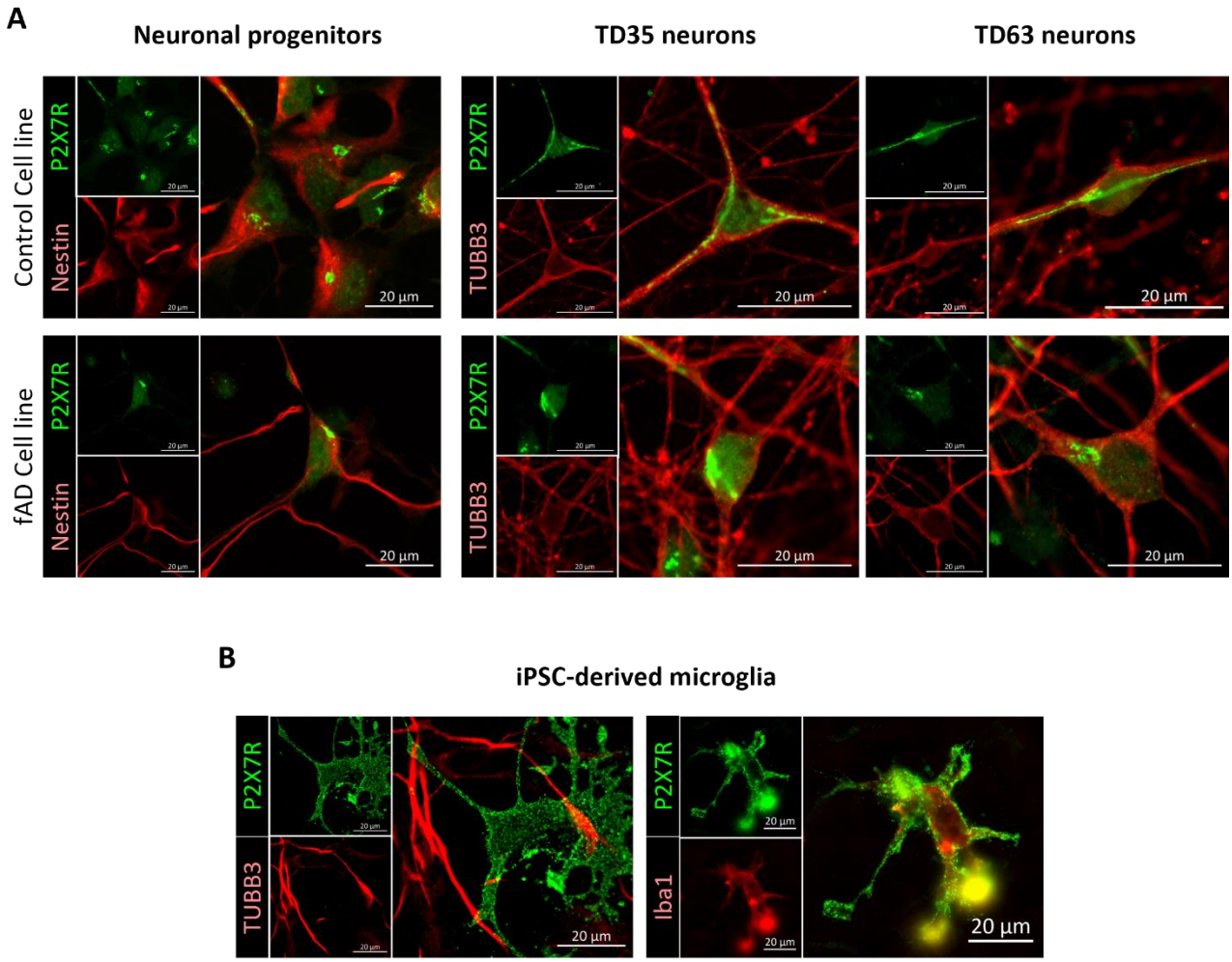

Figure 23. Representative confocal images are suggesting intracellular localisation of the P2X7R's signal (in green) in (**A**) Ctrl and fAD neuronal cells (labeled with TUBB3 (in red)) and (**B**) membranous localisation in NPCs (labeled with nestin (in red)) and microglia-like cells (labeled with Iba1 (in red)). Each staining is a representative picture of at least six independent experiments. Microglia-like cells were co-stained with IBA1 (red). Scale bar: 20 µm

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 (**Figure 24.A**). 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) (**Figure 24.C**). In contrast, the receptor was present in the membrane-bound proteins' fraction (M) in microglia-like cells (**Figure 24.D**) and in NPCs as well (**Figure 24.B**). 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.

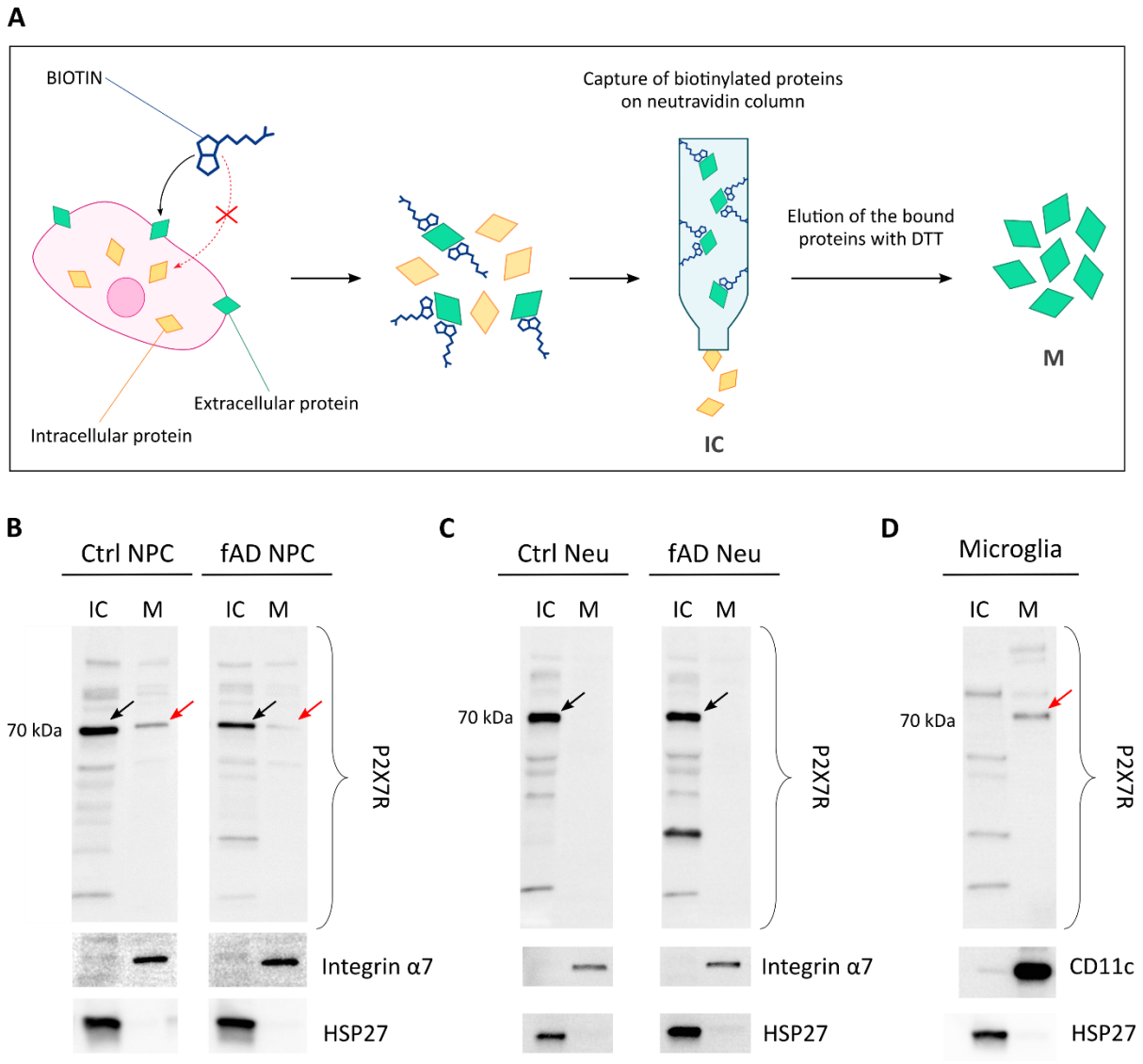

Figure 24. Cell surface protein biotinylation and isolation. The outline (**A**) shows the general procedure of the method. The representative western blot results of biotinylation analysis (**B**, **C**, **D**). Arrows indicate the bands representing the canonical ≈70 kDa sized P2X7R localised in the IC (intra-cellular) fraction in the case of Ctrl and fAD neurons' (TD53) samples (**C**) and the M (membrane) fraction of microglia samples (**D**) and both fractions in NPC samples (**B**). Red arrows indicate the presence of the P2X7R band in the M fractions. The Integrin α7 (**B**, **C**) and CD11c (**D**) are plasma membrane proteins present only in the M factions. HSP27 is a nuclear protein present only in the IC fractions (**B**, **C**, **D**). Integrin α7 and HSP27 were used as controls of the efficiency of biotinylation and the separation of biotinylated proteins. Western blot measurements were performed as biological triplicates. Ctrl, control; Neu, neuron; NPC, neuronal progenitor cell; IC, intra-cellular; M, membrane.

Because the results of biotinylation experiments suggested an intracellular localisation of the P2X7R in neurons and NPCs, a co-localisation experiment was conducted (**Figure 25.A, B**). The co-localisation experiment was focused on the endoplasmic reticulum (ER) and mitochondria (MT). Therefore, the cells were stained using the calnexin antibody (ER) or mitotracker (MT) and co-stained in both cases with anti-P2X7R antibody (**Figure 25.A**). The co-localisation coefficient was determined using the FIGI software add-in Coloc2, and the results were plotted on a comparative graph (**Figure 25.B**). The resulting values of the colocalisation coefficient do not confirm the co-localisation of P2X7R signal with ER nor MT since the coefficient values between 0.3 – 0.7 are considered to be a medium probability of co-localisation.

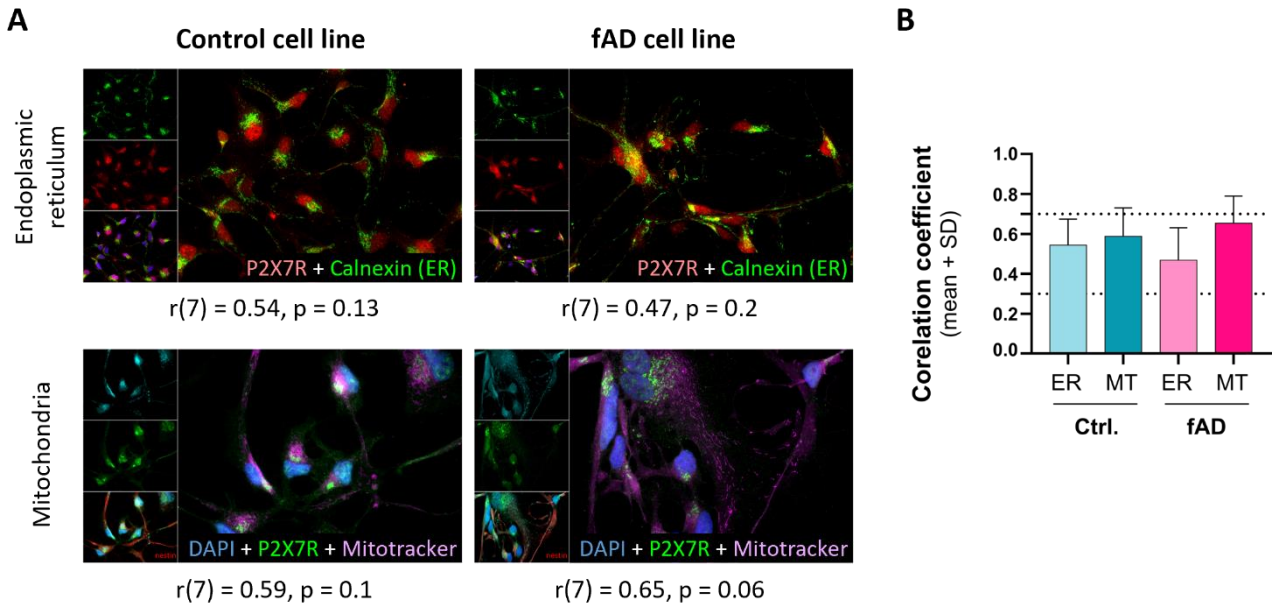

Figure 25. Detection of subcellular localisation of P2X7R in NPCs. (**A**) cells were co-stained for P2X7R (red in ER and green in mitochondria group) and either with calnexin (endoplasmic reticulum, green) or mitotracker (mitochondria, magenta) in both control and fAD cell line neuronal progenitors, and after co-localisation analysis, correlation analysis was performed (**B**).

4.5 Examination of P2X7 receptor's functionality using pharmacological assays

To examine whether the observations from the localization studies are reflected in the functional assays, we performed pharmacological assays. First, a preliminary screening of the best ATP and BzATP concentrations was performed. The P2X7R channel opening had been reported to be activated at micromolar ranges (BzATP EC50 = 0.7 µM; ATP EC50 = 100 µM). Therefore, a series of micromolar concentrations (ATP = 0.5 – 1000 µM; BzATP = 0.5 – 500 µM) was applied on NPCs to

examine if any effect on the viability of the cells would be detectable, but the results show no such effects (**Figure 26.A, B**). In hiPSC-derived microglia, higher concentrations were used (ATP = 0.5 – 5 mM; BzATP = 0.25 – 5 mM), and the results showed and gradual decrease of cell viability as a response to the growing concentration of the compounds (**Figure 26.C**). Since for the purpose of this experiment, inducing a strong response was desired, the highest and predicted medium concentrations were selected for further use (ATP = 5 and 4 mM; BzATP = 5 and 3 mM).

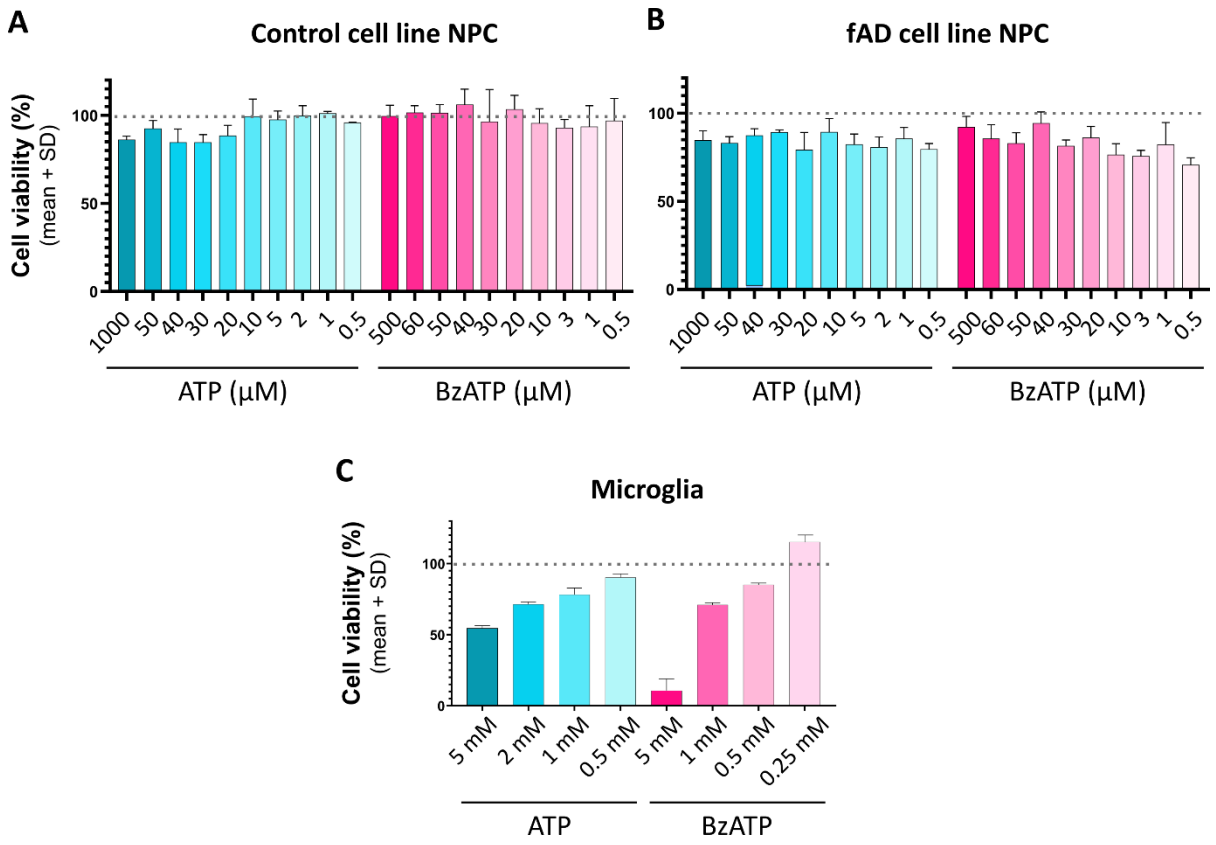

Figure 26. Results of preliminary investigation of the proper P2X7R agonists' concentration. Micromolar concentrations of ATP and BzATP were tested on both control and fAD NPCs with no effect on cell viability (**A, B**) and millimolar concentrations on microglia cells with a decrease in cell viability upon treatment with higher concentrations of the compounds (**C**). The viability of the untreated cells represents 100% viability, and the viability of positive controls (cells treated with water to induce total cell death) represents 0% viability. Error bars represent the mean+SD values.

After selecting the most appropriate concentrations of the studied compounds, functional analyses were executed. 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). Our

results show that the ATP application had a negative effect on the overall viability of the NPCs (**Figure 27.**), 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 BzATP application had various effects on the NPCs. 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. This effect was similar in both control and fAD cell lines. Neuronal cultures maturated for 30 days did not show a decrease in viability upon treatment with ATP (**Figure 28.**), 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. Viability levels were similar in control and fAD cell lines. In microglia-like cells, different concentrations of both ATP and BzATP treatment of the cells caused a significant decrease in cell viability (**Figure 29.**). 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.

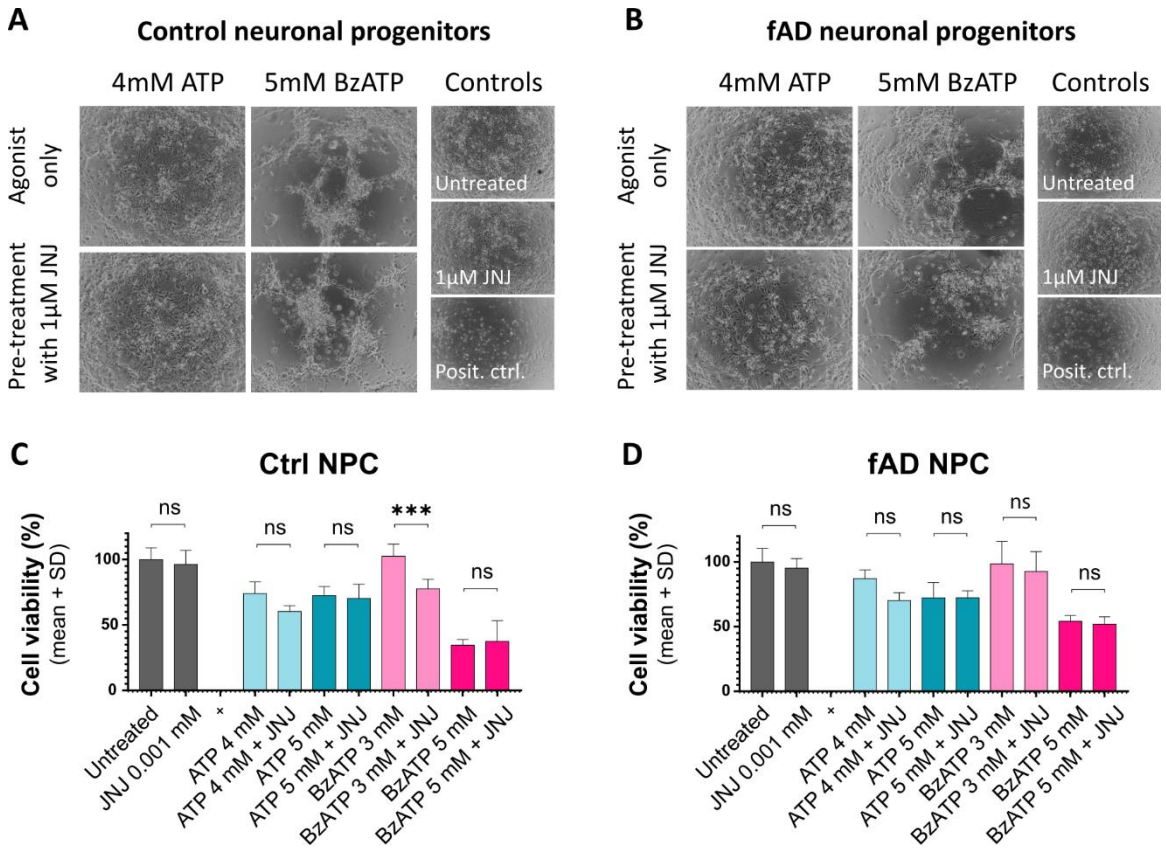

Figure 27. Functional assay results of both control and fAD NPCs. Results are obtained after the application of P2X7R agonists ATP and BzATP alone or upon pre-incubation with the highly specific P2X7R antagonist JNJ 47965567. The working concentration of JNJ 47965567 was 1 µM in all conditions. (**A**) Brightfield images show the condition of the cell cultures after the treatment and the respective controls. (**B**) The viability of the untreated cells represents 100% viability, and the viability of positive controls (cells treated with water to induce total cell death) represent 0% viability. All the measured values were normalised to the two controls. Error bars represent the mean+SD values. Statistical analysis was performed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test; p-value *<0.05; ***< 0.001

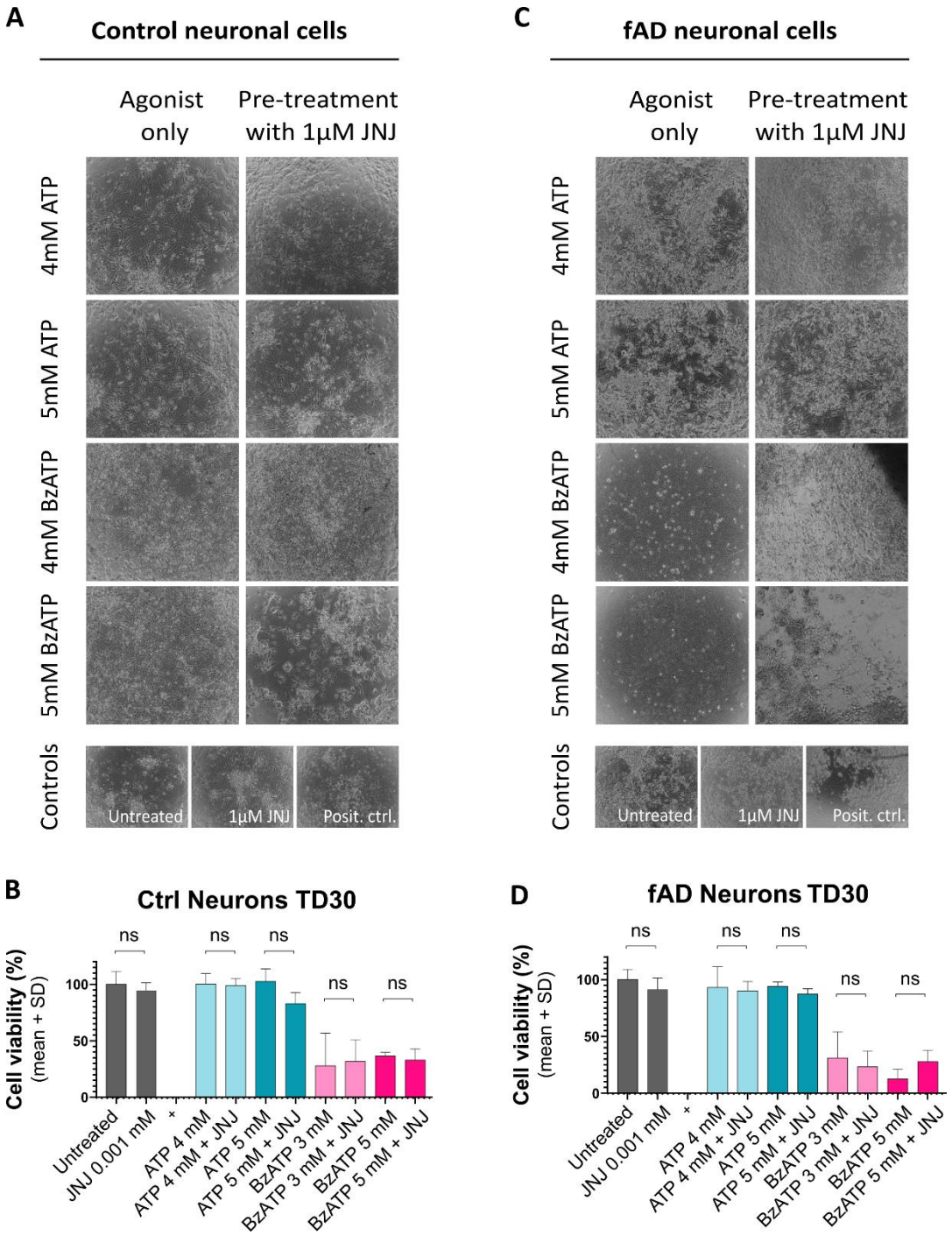

Figure 28. Functional assay results of control (**A, B**) and fAD (**C, D**) neuronal cells differentiated for 30 days. Results are obtained after the application of P2X7R agonists ATP and BzATP alone or upon pre-incubation with the highly specific P2X7R antagonist JNJ 47965567. The working concentration of JNJ 47965567 was 1 µM in all conditions. (**A, C**) Brightfield images show the condition of the cell cultures after the treatment and the respective controls. (**B, D**) The viability of the untreated cells represents 100% viability, and the viability of positive controls (cells treated with water to induce total cell death) represent 0% viability. All the measured values were normalised to the two controls. Experiments were performed in biological triplicates. Error bars represent the mean+SD values. Statistical analysis was performed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test; p-value *<0.05; ***< 0.001

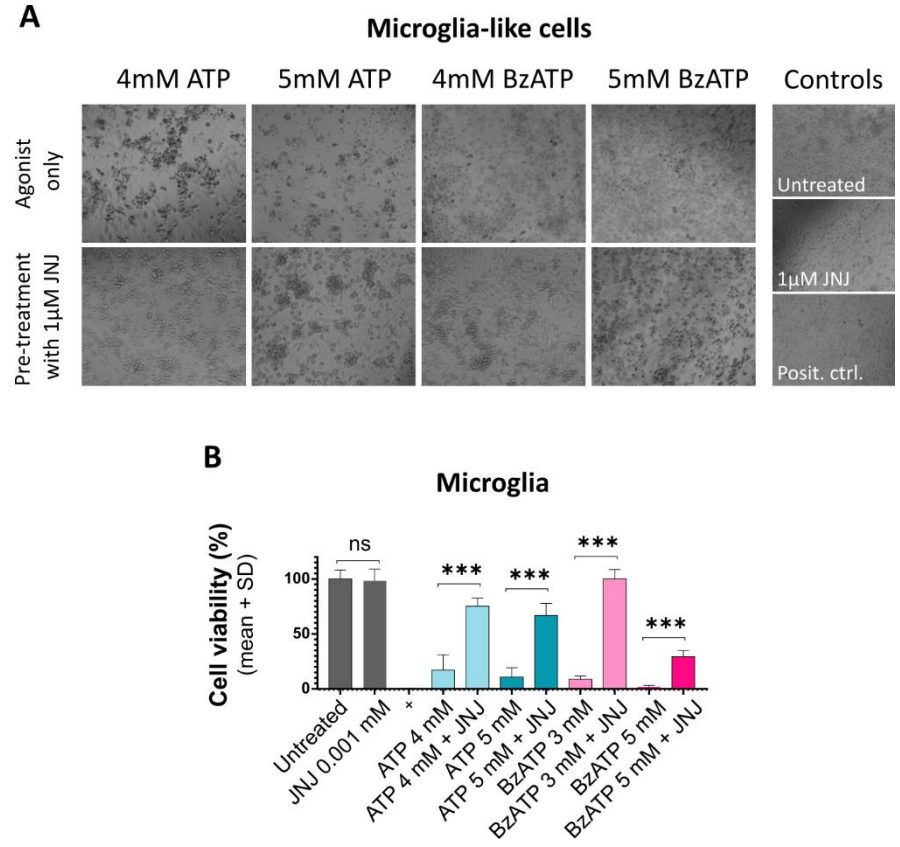

Figure 29. Functional assay results of hiPSC-derived microglia-like cells. Results are obtained after the application of P2X7R agonists ATP and BzATP alone or upon pre-incubation with the highly specific P2X7R antagonist JNJ 47965567. The working concentration of JNJ 47965567 was 1 µM in all conditions. (**A**) Brightfield images show the condition of the cell cultures after the treatment and the respective controls. (**B**) The viability of the untreated cells represents 100% viability, and the viability of positive controls (cells treated with water to induce total cell death) represent 0% viability. All the measured values were normalised to the two controls. Experiments were performed in biological triplicates. Error bars represent the mean+SD values. Statistical analysis was performed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test; p-value *<0.05; ***< 0.001

Overall, these results show that in microglia-like cell cultures, ATP and BzATP induced cellular death, which can be effectively prevented by pre-treatment of the cells with P2X7R antagonist JNJ 47965567. This protection against toxicity was not observed in the case of neuronal cells or NPCs.

5 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.

6 DISCUSSION

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.

Therefore, in this work, we first showed the basic characteristics of the used neuronal cells, such as morphology of the cell cultures, expression of main neuronal markers on both protein- and mRNA-level. We have shown that the neuronal cell cultures consist mainly of neuronal TUBB3- and MAP2-positive cells, contain increasing levels of GFAP-positive cells (astrocytes) and various neuronal subtypes were also detectable. In this regard, we found that the predominant neuronal subtypes in both control and fAD cell cultures were cholinergic neurons. Moreover, in the case of fAD cell line, we have detected 4-5-times higher levels of CHAT (a marker for cholinergic neurons) in the fAD cell line than in the control. This is a very interesting observation since the cholinergic system is known to be negatively affected in Alzheimer's disease and the number of cholinergic cells to be decreased to such extent, that cholinergic loss had been termed as the most prominent component of Alzheimer's disease neuropathology (Hampel et al., 2018). Thus, the question arises why is cholinergic loss not reflected in our fAD model system. We hypothesise, that one of the reasons could be the fact that the loss of cholinergic neurons in Alzheimer's disease is driven mainly by the presence of accumulated abnormally phosphorylated TAU in the form of neurofibrillary tangles or pre-tangles in the cholinergic neurons (Mesulam et al., 2004). While in the previously published work from our lab, we

have shown the presence of pathologically phosphorylated TAU protein (Ochalek et al., 2017), we did not observe any neurofibrillary lesions.

Next, we have shown that the main AD-pathology-related trait – APP-CTF expression was also detected in our fAD, but not control cells. Additionally, the increased astroglia/neurons ratio in the fAD cultures (**Figure 11.A, B**) is likely another consequence of the ongoing AD-related pathological processes. 25 kDa sized APP fragment is the typical fragment produced in patients with AD linked to PSEN1 mutations (García-Ayllón et al., 2017). Interestingly, the appearance of the 25 kDa APP-CTF in the fAD cells at TD35 correlates with the rise of the GFAP levels fortifying the possible association between the onset of AD pathology and the increase of the number of astroglia cells. The AD-phenotype of the neuronal cells used in this study was extensively studied and assessed in our previous published work. Previously, we reported the establishment of a hiPSC-based cellular platform able to model *in vitro* the major pathological events of both the familial and the sporadic form of Alzheimer's disease in neuronal cell cultures. We reported an increased $A\beta_{42}/A\beta_{40}$ ratio, TAU hyperphosphorylation and oxidative stress sensitivity (Ochalek et al., 2017), which agreed with others' earlier results (Penney et al. 2020). Moreover, we reported the modulation of AβPP processing and amyloid secretion upon γ-secretase inhibitor, DAPT and the calcilytic NPS 2143 (acting through CaSR) treatment (Lo Giudice et al., 2019). To increase the similarity of our *in vitro* system to the *in vivo* conditions as much as it is possible, in this study, we included hiPSC-derived microglia-like cells in the experiments. Moreover, the here studied purinergic P2X7R, and its functions are best described on immune cells (De Torre-Minguela et al. 2016; Wewers and Sarkar 2009; Janks et al. 2018). Therefore, the addition of human hiPSC-derived microglia cells serves as an excellent control for our experimental conditions.

In the present experiments, we showed that our hiPSC-derived microglia-like cells closely resembled the characteristics of *in vivo* microglia cells. The microglia-like cells expressed several microglia-specific markers, including IBA1, CD11b and TMEM119. The absence of CX3CR1 expression, high levels of CD45 but the presence of other microglial markers might suggest an early developmental stage of the analyzed microglia cells sample. Low expression of the P2RY12 is most probably due to the absence of neuronal cells in the culture, namely the lack of TGF-β signaling (Arnold et al., 2019; Butovsky et al., 2014). For true microglia, however, the expression of CX3CR1 and P2YR12 genes are desired. However, the here presented detection of these markers was performed in cells that were kept in monoculture for easier processing. Thus, microglia did not interact with neuronal cells as it is

in vivo, which probably resulted in decreased expression of CX3CR1, the fractalkine receptor, which is one of the main mediators of neuron-microglia interaction (Pawelec et al., 2020; Sheridan and Murphy, 2013). It has been proven that the lack of CX3CR1/CX3CL1 signaling leads to loss of the suppression of microglial activation (Bolos et al., 2017), and thus possibly influences the expression of the P2Y12R as well. The P2Y12R is most highly expressed in mature microglia, and its expression levels depend on microglial activation. In "M2" activated microglia, it is upregulated and participates in the chemotaxis of microglial cells towards the source of the injury (Haynes et al., 2006). In contrast, during pro-inflammatory activation of microglia, the expression of P2Y12R is decreased (Moore et al., 2015). Therefore, low levels of CX3CR1 and P2Y12R in our microglia-like cells are likely due to the combination of the early developmental stage of the cells, lack of neuronal signaling, and the activated/primed state of the cells. Nevertheless, the microglia-like cells respond to activation cues such as LPS treatment which we demonstrated by the detection of NLRP3 levels (**Figure 16.B, C**). It is well known that co-culture of microglia with neurons and glia cells improves the identity of the microglia cells to better resemble *in vivo* cells (Grubman et al., 2020). And indeed, our microglia-like cells closely mimic microglial morphology and interactions with neuronal networks when placed in co-culture. In this study, we present the gradual morphological changes from amoeboid to ramified occur upon placing microglia-like cells in co-culture with neuronal cells. These events are identical to those observed in re-population studies *in vivo* (Svoboda et al., 2019).

Still, microglia-like cells used in this study had proven themselves appropriate and useful in the detection of P2X7R and related and functional studies. Using immunocytochemistry, cell surface protein isolation assay and functional assays, we demonstrate that in the human hiPSC-derived microglia-like cells, the P2X7R is abundantly present, properly localized, and functional.

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, we utilised two anti-P2X7R antibodies APR-004 (Alomone labs) and sc-514962 (Santa Cruz Biotechnology). The APR-004 antibody recognises the intracellular C-terminal fragment of the receptor and was primarily raised against rat P2X7R protein. Nevertheless, the immunizing peptide shares 16/20 (80%) amino acid compatibility with the human form of the receptor. This antibody is routinely used in P2X7R research, has been thoroughly validated (Nicke, 2008), and has been utilised in other human-based research as well (Martínez-Frailes et al., 2019; Ollà et al., 2020). Despite that,

questions could be raised regarding the specificity of this antibody human-derived samples and that is why we used more than one antibody to support our analyses. Thus the finding that both of the tested antibodies provide similar results and in both cases a decrease in detected signal during the differentiation times of the cells was confirmed is very important for the credibility of the conclusion of both western blot and ICC analyses.

We have investigated the P2X7R's expression levels using RT-qPCR as well (**Figure 20.**). Surprisingly, the results of this analysis were not consistent with our observation using other detection methods. The observed discrepancy between the protein detection-based approaches and RT-qPCR measurements might suggest that the regulation of the expression of the P2RX7 gene relies on the regulation of translation rather than transcription. Alternatively, the cells may possess an intracellular reserve pool of non-functional P2X7R, and thus sustained levels of mRNA could be needed. But it is also important to notice, that the ranges of the relative expression are very low (suggesting very low levels of respective mRNA) and thus the measurement could be below the optimal detection sensitivity levels.

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. As we reported in section **4.4**, 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.

Interestingly, there is a number of articles reporting the presence of P2X7R in astrocyte cells (Gao et al., 2017; Kamatsuka et al., 2014; Khan et al., 2019). Some of these reports suggest that the astrocytic P2X7R indirectly regulates neuronal activity (Khan et al., 2019). A good avenue for future experiments could be the examination of P2X7R in hiPSC-derived pure astrocytes and its interposed impact on neuronal cells. Ideally, a model system consisting of neurons, astrocytes, oligodendrocytes and microglia could provide valuable data about the effects of intercellular communication on P2X7R signaling in the human brain in health and disease.

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). However, the different protective capacity of the P2X7R antagonist JNJ in the case of 3 mM and 5 mM BzATP might indicate either that the JNJ concentration was not sufficient to protect the cells from such high BzATP concentrations or that 5 mM BzATP is so far exceeding the physiological range, that the toxic effect is not only the result of P2X7R activation but also other mechanisms (other purinergic receptors' activation). 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 (**Figure 24.**). 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 (**Figure 30.**). 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.

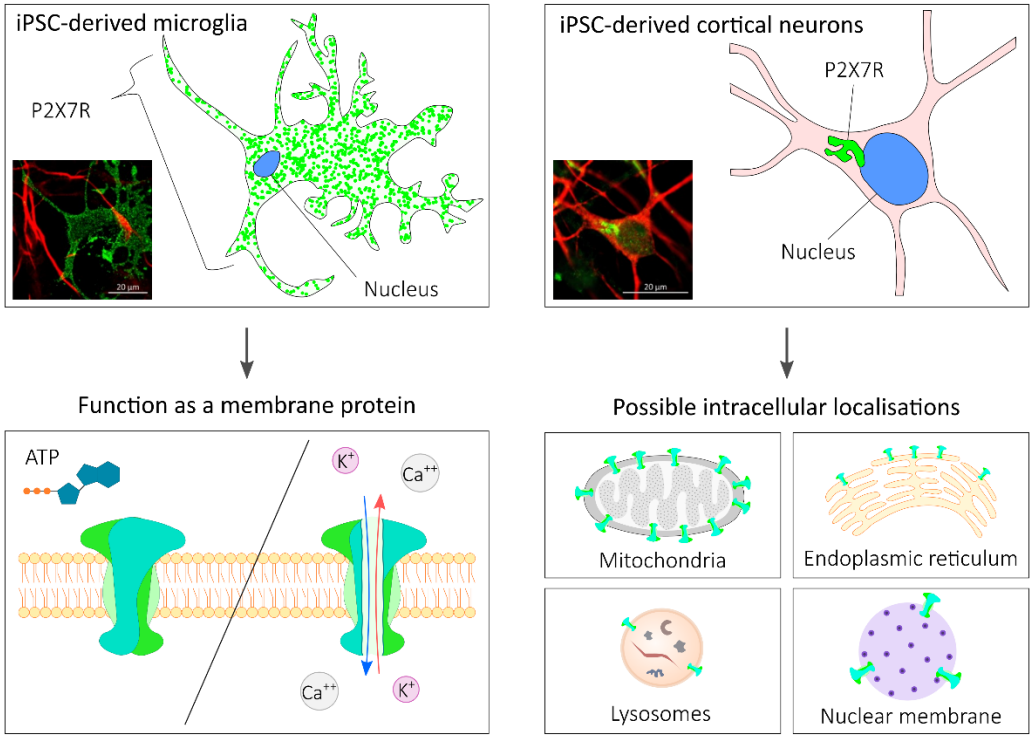

Figure 30. Outline of the described membrane localisation of P2X7R in microglia cells and of possible intracellular localisations of the P2X7R in neuronal cells.

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.

7 SUMMARY (EN)

The goal of the presented study was to investigate the expression and function of the P2X7R in hiPSC-derived cortical type neuronal cells and hiPSC-derived microglia-like cells. P2X7R is a transmembrane protein that functions as an ATP-gated ion channel, with relatively low sensitivity to ATP, which allows the activation of this receptor during the presence of very high concentrations of ATP – typically present during pathological events, such as cell death. Downstream activation pathways of the P2X7R are still to be elucidated. However, a number of events have been previously described, such as alterations in cell morphology, interleukin-1β release, NFκB activation, increase in ROS/NOS formation, phospholipase D activation, inflammasome NLRP3 activation, and possibly glutamatergic signalling in neurons. Interestingly, this receptor does not undergo desensitization; therefore a prolonged activation can lead to apoptosis of the cell. While the P2X7R is well described to be present and functional primarily on macrophages - including microglia cells of the CNS - contributing to their inflammatory responses, some reports suggest a neuronal expression of the receptor as well. Moreover, evidence suggests the involvement of the P2X7R in neurodegenerative diseases, including Alzheimer's disease. Therefore we have used neuronal cells derived from healthy as well as familial Alzheimer's disease hiPSC lines to examine the differences in the expression and function of this receptor in health and Alzheimer's disease. We presented experimental results showing P2X7R to be expressed on human hiPSC-derived microglia like cells, hiPSC-derived neuronal progenitors and hiPSC-derived matured neuronal cells. By applying cell surface protein detection assays, we show that P2X7R is not localized on the cell membrane, despite being detected in neuronal cells and thus may not be available for directly mediating neurotoxicity. On hiPSC-derived microglia-like cells, a clear membranous expression was detected. Additionally, we have not observed differences in P2X7R functions between control and familial Alzheimer's disease patient-derived neuronal cells. Functional assays employing a P2X7R antagonist JNJ 47965567 confirm these findings by showing P2X7R-dependent modulation of microglia-like cells viability upon treatment with P2X7R agonists ATP and BzATP, while the same effect was absent from neuronal cells. Since the majority of P2X7R research was done on rodent models, our work on human hiPSC-derived cells presents a valuable contribution to the field, extending the work on animal models to the human cellular system and towards clinical translation. The results of our study show for the first time P2X7R expression and function on different hiPSC derived cell types of the CNS.

8 SUMMARY (HU)

A bemutatott tanulmány célja a P2X7R expressziójának és működésének vizsgálata volt hiPSC eredetű kérgi típusú neuronsejtekben és hiPSC eredetű mikroglia-szerű sejtekben. A P2X7R egy transzmembrán fehérje, amely ATP-függő ioncsatornaként működik, viszonylag alacsony érzékenységgel az ATP-vel szemben, ami lehetővé teszi a receptor aktiválását magas ATP koncentráció jelenléte esetén is – mely jellemzően kóros körülmények közt, például sejthalál esetén fordul elő. A P2X7R downstream aktiválási útvonalai még nincsenek teljes mértékben feltárva, korábban azonban számos lehetséges módozatot leírtak. Aktiválást eredményezhetnek többek közt a sejtmorfológiában bekövetkezett változások, interleukin-1β felszabadulása, NFκB aktiváció, emelkedett ROS/NOS képződés, foszfolipáz D aktiváció, gyulladásos NLRP3 aktiváció és feltételezések szerint a glutamáterg jelátvitel neuronokban. Érdekes módon ez a receptor nem esik át deszenzitivizáción, ezért az elhúzódó aktiváció a sejt apoptózisához vezethet. A P2X7R a szakirodalom szerint elsősorban a makrofágokon van jelen és aktív, beleértve a központi idegrendszer mikroglia sejtjeit is, hozzájárulva azok gyulladásos válaszához. Egyes eredmények azonban a receptor neuronális expressziójára is utalnak. További bizonyítékok utalnak vannak arra, hogy a P2X7R szerepet játszik a neurodegeneratív betegségekben, beleértve az Alzheimer-kórt is. Ezek alapján egészséges és örökletes Alzheimer-kóros hiPSC sejtvonalakból származó neuronális sejteket használtunk, hogy megvizsgáljuk a receptor expressziójában és működésében mutatkozó különbségeket. Kísérleti eredményeink kimutatták, hogy a P2X7R expresszálódik hiPSC-eredetű mikroglia-szerű sejteken, hiPSC-eredetű neuronális progenitor sejteken és hiPSC-eredetű érett idegsejteken. Sejtfelszíni fehérje detektálási módszerek segítségével bemutattuk, hogy a P2X7R ezen sejttípusokban bár jelen van, azonban nem a sejtmembránon lokalizálódik, így elképzelhető, hogy közvetlenül nem vesz részt a neurotoxicitás közvetítésében. A hiPSC-eredetű mikroglia-szerű sejteken egyértelmű membrán-lokalizált expressziót észleltünk. Az egészséges és az örökletes Alzheimer-kóros betegektől származó idegsejtek között nem figyeltünk meg különbségeket a P2X7R működésében. A JNJ 47965567 jelű P2X7R antagonistát alkalmazó funkcionális vizsgálatok megerősítik ezeket az eredményeket. Ezek kimutatták a mikroglia-szerű sejtek életképességének P2X7R-függő modulációját P2X7R agonistákkal; ATP-vel és BzATP-vel történő kezelés után, miközben ugyanez a hatás nem volt észlelhető az idegsejtekben. Mivel a P2X7R kutatások többsége rágcsálómodelleken történt, a hiPSC-eredetű sejtekkel kapcsolatos munkánk értékes hozzájárulást jelent a területhez, kiterjesztve az állatmodelleken végzett munkát az emberi sejtrendszerre és az események lehetséges klinikai felhasználásához vezethet. Kutatásunk eredményei első alkalommal

mutatták ki a P2X7R expresszióját és működését a központi idegrendszer különböző hiPSC-eredetű sejtjeiben.

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10 PUBLICATIONS LIST

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.

11 APPENDICES

Appendix A: Supplementary table 1.A Comparison of media composition used in the 8 landmark iPSC-microglia generation protocols (Part 1/2)

→ Chronological direction of differentiation		Muffat et al. 2016	Abud et al. 2017	Douvaras et al. 2017	Haenseler et al. 2017
iPSC stage	Basis of the medium	DMEM/F12	TeSR-E8	mTeSR1 (custom mTeSR medium)	mTeSR1
	Added factors	bFGF		BMP4	
Embryoid body stage	Basis of the medium	modified Neurobasal medium	no embryoid body stage	no embryoid body stage	mTeSR1
	Added factors	iron-loaded transfferin; pyruvate; lactic acid; glucose; galactose; glutamine; biotin and lipid loaded albumin (Albumax); glutamate was omitted			BMP4; VEGF; SCF
Haematopoietic progenitor stage	Basis of the medium	modified Neurobasal medium	Iscove's modified Dulbecco's medium	StemPro-34 SFM	X-VIVO 15 (Lonza)
	Added factors	iron-loaded transferrin; pyruvate; lactic acid; glucose; galactose; glutamine; biotin and lipid loaded albumin (Albumax); glutamate was omitted	insulin; holo-transferrin; sodium selenite; ethanolamine; L-ascorbic acid 2-phosphate magnesium; monothyoglycerol; PVA; chemically-defined protein concentrate	bFGF; SCF; VEGF	M-CSF; IL-3
Macrophage/ microglia precursor stage	Basis of the medium	modified Neurobasal medium	E8	StemPro-34	X-VIVO 15 (Lonza)
	Added factors	IL-34; CSF1	Y-27632 ROCK Inhibitor; FGF2; BMP4; Activin-A; LiCl; VEGF; TPO; SCF; IL-6; IL-3	SCF; IL-3; TPO; M-CSF; Flt3 ligand	M-CSF
Precursor → microglia transition stage	Basis of the medium	modified Neurobasal medium	phenol-free DMEM/F12 (1:1)	StemPro-34	DMEM:F12
	Added factors	IL-34; CSF1	insulin; holo-transferin; sodium slenite; B27; monothioglycerol; Glutamax; additional insulin	M-CSF; Flt3 ligand; GM-CSF	IL-34; GM-CSF
Microglia	Basis of the medium	modified Neurobasal medium	complete phenol-free DMEM/F12 (1:1)	SF-Microglial Medium	aDMEM:F12
	Added factors	IL-34; CSF1	M-CSF; IL-34; TGFβ-1; CD200; CX3CL1	GM-CSF; IL-34	IL-34; GM-CSF

Supplementary table 1.B Comparison of media composition used in the 8 landmark iPSC-microglia generation protocols (Part 2/2)

→ Chronological direction of differentiation		Pandya et al. 2017	Brownjohn et al. 2018	McQuade et al. 2018	Konttinen et al. 2019
iPSC stage	Basis of the medium	mTeSR → OP9 differentiation medium (ODM)	mTeSR-E8	TeSR-E8	Essential 8 Medium
	Added factors	FBS; MTG			BMP4, Activin A, CHIR 99021, Y-27632
Embryoid body stage	Basis of the medium	Myeloid progenitor expansion medium	Embryoid body medium	no embryoid body stage	DMEM/F12
	Added factors	GM-CSF	ROCK inhibitor, BMP-4, SCF, VEGF		FGF2, VEGF, SB431542, insulin
Haematopoietic progenitor stage	Basis of the medium	Astrocyte differentiation medium	X-Vivo TM15	"Medium A"	EMP
	Added factors	FBS; GM-CSF; M-CSF; IL-3	M-CSF, IL-3	authors do not provide details	FGF2, VEGF, IL-3, SCF, thyroid peroxidase, insulin
Macrophage/ microglia precursor stage	Basis of the medium	Media A	RPMI1640 complete microglia medium	"Medium B"	Iscove's Modified Dulbecco's medium
	Added factors	STEMdiff APEL + hVEGF; hBMP4; hCSF; hActivin	10% FBS, IL-34, GM-CSF	authors do not provide details	inactivated FBS
Precursor → microglia transition stage	Basis of the medium	Media B	RPMI1640 complete microglia medium	DMEM/F12	DMEM/F12
	Added factors	Media A + hSCF; hFlt3L; hIL-3; hIL-4; hG-CSF; hBMP4	10% FBS, IL-34, GM-CSF	IL-34; TGFβ1; M-CSF	N2, B27, insulin, MCSF, IL-34
Microglia	Basis of the medium	Media C	RPMI1640 complete microglia medium	DMEM/F12	Microglia maturation medium
	Added factors	IMDM + FBS; hIL-3; hGM-CSF; hM-CSF	10% FBS, IL-34, GM-CSF	IL-34; TGFβ1; M-CSF; CD200; CX3CL1	MCSF, IL-34

Supplementary Table 2. Primary antibodies used in this work for immunocytochemistry (ICC) and Western blot (WB) analyses

Target of the antibody	Host	Catalogue number and vendor	Application	Working dilution
APP	Mouse	803001 (Biolegend)	WB	1:1000
APP-CTF (CT695)	Rabbit	512700 (Thermofisher Scientific)	WB	1:1000
Calnexin	Mouse	MA3-027 (Thermo Fisher Scientific)	ICC	1:500
CD11c	Rabbit	ab52632 (Abcam)	WB	1:2000
CHAT	Goat	AB144P (Millipore)	ICC	1:500
GABA	Rabbit	ab43865 (Abcam)	ICC	1:1000
GAPDH	Rabbit	G9545 (Sigma-Aldrich/Merck)	ICC, WB	1:5000
GFAP	Mouse	MA5-12023 (Thermo Fisher Scientific)	ICC, WB	1:500
HSP27	Goat	sc-1049 (Santa Cruz Biotechnology)	WB	1:500
Iba1	Goat	ab5076 (Abcam)	ICC	1:500
Integrin α-7	Goat	sc-27706 (Santa Cruz Biotechnology)	WB	1:1000
MAP2	Chicken	AB5392 (Abcam)	ICC	1:2500
MAP2	Mouse	MAB3418 (Merck)	WB	1:1000
NESTIN	Mouse	MAB5326 (Sigma-Aldrich/Merck)	ICC	1:1000
NLRP3 (D4D8T)	Rabbit	15101S (Cell Signalling)	WB	1:500
P2X7R	Rabbit	APR-004 (Alomone labs)	ICC, WB	1:1000
P2X7R	Mouse	sc-514962 (Santa Cruz Biotechnology)	WB	1:500
PAX6	Rabbit	901301 (Biolegend)	ICC	1:250
SOX9	Goat	sc-17319 (Santa Cruz Biotechnology)	ICC	1:50
TUBB3	Mouse	ab78078 (Abcam)	ICC	1:1000
TUBB3	Rabbit	802001 (Biolegend)	ICC, WB	1:1000
Tyrosine hydroxylase	Rabbit	AB152 (Millipore)	ICC	1:500
V-GLUT	Rabbit	AB79774 (Abcam)	ICC	1:500

Supplementary Table 3. Secondary antibodies used in this work for immunocytochemistry (ICC) and Western blot (WB) analyses

	Antibody	Catalogue number	Working dilution
ICC	AF® 488 donkey anti-rabbit	A21206	1:2000
	AF® 488 donkey anti-mouse	A21202	1:2000
	AF® 488 donkey anti-goat	A11055	1:2000
	AF® 594 donkey anti-rabbit	A21207	1:2000
	AF® 594 donkey anti-mouse	A21203	1:2000
	AF® 647 donkey anti-rabbit	A31573	1:2000
	AF® 647 donkey anti-mouse	A31571	1:2000
	AF® 647 goat anti-chicken	A21449	1:2000
	AF® 647 donkey anti-goat	A214470	1:2000
WB	Horse anti-mouse IgG, HRP-linked	7076 (Cell Signalling)	1:2000
	Goat anti-rabbit IgG, HRP-linked	7074 (Cell Signalling)	1:2000
	Mouse anti-goat IgG, HRP-linked	31400 (Thermo Fisher Scientific)	1:2000

* AF® = Alexa Fluor®; all Alexa Fluor® antibodies were purchased from Thermo Fisher Scientific

Supplementary Table 4. Primers used in this work for RT-qPCR analyses

Gene	Forward sequence	Reverse sequence
CD11b	GACTCTTCACAGCCTTGTTTCC	GTCCTCACCATCATTTCTCACA
CD45	GACACGGCTGACTTCCAGATA	AGCCAAATGCCAAGAGTTTAAG
CHAT	GCCTGCTGCAATCAGTTCTT	GTCCTCGTTGGAAGCCATT
CX3CR1	CCTCTTCTGGACACCCTACAAC	ATGAGAGGATTCAGGCAACAAT
GAD1	GCACAGGTCATCCTCGATTT	TTGATGTCAGCCATTCTCCA
GFAP	TGAAAGAGATCCGCACGCAG	CGTCTGTCAGGTCTGCAAAC
GRIN1	GCAACACCAACATCTGGAAG	ATCCGCATACTTGGAAGACA
IBA1	GAGACGTTCAGCTACCCTGACT	CTTCAATCCCATCATCCCTTT
KI-67	ACGGATTATACCTGGCCTTC	AGGAAGCTGGATACGGATGT
Map2	TTGTCTCTAACCGAGGAAGCA	TCGTTGTGTCGTGTTCTCAA
NESTIN	ACTGAAGTCTGCGGGACAAG	CAGTGGTGCTTGAGTTTCTG
P2RX7	CTTCCGAGAAACAGGCGATA	CCAACGGTCTAGGTTGCAGT
P2RY12	CTCTGTCCCAGGACAATAGGAA	ACTTAGCGCTTTGCTTTAACGA
PAX6	GCCAGCAACACACCTAGTCA	TGTGAGGGCTGTGTCTGTTC
TMEM119	GTCTCTCTTGTTAGCCCAGGAA	GAGTGTCAGGAAGCAGTCAGG
TUBB3	AACGAGGCCTCTTCTCACAA	GGCCTGAAGAGATGTCCAAA

Appendix B:

Compositions of media used in this work:

1. **Thawing medium**

Reagent	Final concentration
Complete NMM media	100%
ROCK inhibitor (10 mM)	10 µM

2. **Freezing medium**

Reagent	Final concentration
NMM	90%
DMSO	10%

3. **NPC maintenance medium**

Reagent	Final concentration
DMEM/F12	50%
Neurobasal medium	50%
Non-essential amino acids	1%
B-27 supplement (50X)	2%
N-2 supplement (100X)	1%
L-Glutamine (200 mM)	1%
Pen/Strep (10 000 U/ml)	1%
EGF (100 µg/ml)	10 ng/ml
bFGF (100 µg/ml)	10 ng/ml

4. Neuronal maintenance medium (NMM)

Reagent	Final concentration
DMEM/F12	50%
Neurobasal medium	50%
Non-essential amino acids	1%
B-27 supplement (50X)	2%
N-2 supplement (100X)	1%
L-Glutamine (200 mM)	1%
Pen/Strep (10 000 U/ml)	1%

5. Embryoid body formation-supporting medium (EB medium)

Reagent	Final concentration
mTeSR™-1	100%
BMP-4 (100 µg/ml)	50 ng/ml
VEGF-165 (100 µg/ml)	50 ng/ml
SCF (100 µg/ml)	20 ng/ml
Revitacell (100X)	1x

6. Microglia factory medium

Reagent	Final concentration
XVIVO-15	100%
GlutaMax (100X)	1x
Pen/Strep (10000 U/ml)	100 U/ml
2-Mercaptoethanol (50 mM)	50 µM
M-CSF (100 µg/ml)	100 ng/ml
IL-3 (100 µg/ml)	25 ng/ml

7. Microglia maturation medium

Reagent	Final concentration
XVIVO-15	100%
GlutaMax (100X)	1x
Pen/Strep (10000 U/ml)	100 U/ml
M-CSF	100 ng/ml

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