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SZENT ISTVÁN UNIVERSITY

Animal Husbandry Science PhD School

UNRAVELLING THE ROLE OF CALCIUM SENSING RECEPTOR (CASR) IN NEUROLOGICAL PHENOTYPES

Thesis for Doctoral degree (PhD)

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ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
1,25(OH)2D	1,25-dihydroxyvitamin D
AA	ascorbic acid
Aβ	β-amyloid1,25-dihydroxyvitamin D
AC	adenylate cyclase
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
ADH	autosomal dominant hypocalcaemia
AICD	AβPP intracellular domain
AKT	serine/threonine protein kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	alkaline phosphatase
AP2σ	adaptor-related protein complex 2 sigma
APH1A	Aph-1 homolog A, gamma-secretase subunit
APOE	apolipoprotein E
AβPP	amyloid precursor protein
BACE1	beta-secretase 1
BCA	bicinchoninic acid
β2AR	β2-adrenergic receptor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CA1	cornu Ammonis 1
CA3	cornu Ammonis 3
cAMP	cyclic adenosine 3′,5′-monophosphate
CaSR	calcium sensing receptor
CHO	Chinese hamster ovary
CKD	chronic kidney disease
CM	conditioned medium
CNS	central nervous system
CSF	cerebrospinal fluid
CTFα	C-terminal fragment α
CTFβ	C-terminal fragment β
DAG	diacylglycerol
DAPI	4', 6-diamidino-2-phenylindole
DG	dentate gyrus
DMEM	Dulbecco's minimum Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOR	δ-opioid receptor
DTT	dithiothreitol
EOAD	early onset Alzheimer's disease
ERAD	Endoplasmic reticulum-associated degradation

ECD	extracellular domain
ECS	extracellular solution
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGL	external granule-cell layer
EGTA	ethylene glycol tetra acetic acid
ER	endoplasmic reticulum
ERK	extracellular signal–regulated kinase
ESC	embryonic stem cell
EPSC	excitatory post-synaptic current
fAD	familial Alzheimer's disease
FBS	foetal bovine serum
FGF	fibroblast growth factor
FHH	familial Hypercalcaemia Hypocalciuria
GABA	γ aminobutyric acid
GABAbR	γ aminobutyric acid b receptor
GCI	global cerebral ischemia
GCP	cerebellar granule-cell precursor
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GSK3B	glycogen synthase kinase 3 beta
HEK	human embryonic kidney
HCN	human cortical neuron
ICD	intracellular domain
ICC	immunocytochemistry
IgG	immunoglobulin G
IGL	internal granule cell layer
IP	intraperitoneal
IP3	inositol trisphosphate
iPSC	induced pluripotent stem cell
KLF4	Kruppel like factor 4
LIF	leukaemia inhibitory factor
LOAD	late onset Alzheimer's disease
MAPK	mitogen-activated protein kinase
MAPT	microtubule associated protein TAU
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
MEF	mouse embryonic fibroblast
mEPSC	miniature excitatory post-synaptic current
mGluR	metabotropic glutamate receptor
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
MT5-MMP	membrane-type 5- matrix metalloproteinases
MWM	Morris water maze
NAHA	normal adult human astrocytes
NCCs	nonselective cation channels

NEAA	nonessential amino acids
NES	nestin
NFT	neurofibrillary tangle
NDM	neural differentiation medium
NMM	neural maintenance medium
NPC	neural progenitor cell
NCS	neural stem cell
NS	nervous system
NSAID	nonsteroidal anti-inflammatory drug
NSHPT	neonatal severe hyperparathyroidism
OCT4	octamer-binding transcription factor 4 (POU5F1)
OPC	oligodendrocytes progenitor cell
P_o	opening state probability
PAX	paired box
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	phospholipase C
PNS	peripheral nervous system
PSC	pluripotent stem cell
PSEN	presenilin
PTH	parathyroid hormone
RIPA buffer	radioactive immunoprecipitation assay buffer
RNA	ribonucleic acid
ROS	reactive oxygen species
sAβPPα	soluble amyloid precursor protein-α
sAβPPβ	soluble amyloid precursor protein-β
SDS	sodium dodecyl sulphate
SOX2	sex determining region Y-box 2
TAL	thick ascending limb
TBI	traumatic brain injury
TGN	trans-Golgi network
TMD	transmembrane domain
TBS	tris-buffered saline
TUBB3	tubulin beta 3 class III
VFT	Venus Flytrap

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1 INTRODUCTION

1.1 Importance of the field

Animal and human *in vitro* cellular systems are providing novel tools for a better understanding of physiological and pathological processes, and development of new medical and veterinary interventions and drugs. Mouse and human pluripotent cell lines (PSCs) are well established and provide efficient tools while the development of non-rodent, companion and large animal pluripotent cell lines is still in progress. Therefore, the existing mouse and human cellular systems are widely applied for better understanding the pathology and progression of neurological diseases in mammalians or even to find potential drug targets.

G protein-coupled receptors (GPCRs) have crucial roles in almost every physiological process from cardiac function, immune responses and neurotransmission to sensory functions comprising sight, taste and smell. Aberrant GPCR activity or expression contributes to some of the most prevalent diseases (O'Hayre *et al.*, 2013) and GPCRs are the targets for >35% of all modern drugs (Sriram and Insel, 2018). Their importance is reflected by the 2012 Nobel Prize in Chemistry awarded for "Studies of G-protein-coupled receptors". The calcium-sensing receptor (CaSR) is a G protein-coupled receptor (GPCR) in the plasma membrane which enables the cell to sense extracellular calcium ion levels and modulate secondary messengers accordingly. It has emerged as a promising new target for the treatment of major non-communicable diseases either via traditional pharmacotherapeutic approaches aimed at receptor activation or inhibition, or via alternative pharmacotherapeutic approaches that modulate receptor expression and trafficking. The CaSR plays a pivotal role in the control of systemic calcium metabolism and has been successfully targeted in the treatment of various disorders of calcium metabolism using allosteric modulators (Leach *et al.*, 2014). The recognition that abnormal CaSR function, or expression contributes to, and promotes, the pathogenesis of major diseases including Alzheimer's disease (AD), cardiovascular disease (CVD), diabetes mellitus (DM), cancer, and sarcopenia; diseases that account for >25% of the global disease burden, led the researchers to consider the receptor a potential target for such disorders. However, the precise contribution of CaSR dysfunction to the pathophysiology of these diseases remains to be elucidated. Compounds targeting the CaSR may have therapeutic potential for these diseases which represent a global health burden and have veterinary importance as well.

The relevance of CaSR for physiology of the nervous system (NS) has been substantiated by a large body of evidence in the last 20 years. Consistent with the biased-signalling which is typical of G-protein coupled receptors, to whom CaSR belongs, the receptor can activate different

intracellular pathways in the NS depending on the developmental stage, cell type and pathological conditions (Ruat and Traiffort, 2013). Thanks to several studies which have employed genetic silencing and pharmacological approaches, it has been demonstrated that CaSR plays important roles in the development of cells of NS (Chattopadhyay *et al.*, 2008; Liu *et al.*, 2013; Vizard *et al.*, 2015; Tharmalingam *et al.*, 2016), in the regulation of ion channels currents and excitability (Vassilev *et al.*, 1997; Smith *et al.*, 2004; Phillips *et al.*, 2008) and in neurodegenerative processes (Xue *et al.*, 2017). In this regard, evidences supported that CaSR activation might contribute to exacerbate the amyloid Aβ accumulation and TAU phosphorylation which represent the key players in Alzheimer's disease (AD) (Dal Prà *et al.*, 2014). As consequence, the inhibition of CaSR signalling has been proposed as a relevant therapeutic strategy and results from *in vitro* experiments have recently showed the beneficial effects of the negative allosteric modulator NPS 2143 in the AD phenotype (Chiarini *et al.*, 2017a). Moreover, a CaSR overexpression in parallel with amyloid plaque deposition, which is a main AD hallmark, has been observed in a transgenic AD mouse model (Gardenal *et al.*, 2017), further substantiating the potential involvement of CaSR in the disease mechanisms.

According to these promising studies and considering that, despite tremendous efforts, research on AD still suffers with the lack of a comprehensive understanding and efficacious treatments, the investigation around the CaSR role in AD appears to be highly relevant, in order to identify new therapeutic targets and potential treatments.

1.2 Modelling neurological diseases with Pluripotent Stem Cells

One of the main problems when studying disorders of the nervous system is represented by the difficulty of adequately modelling the physiological mechanisms of the brain. Beside the limited accessibility to the brain tissue for research purposes, the attempt to model the neurodegenerative processes in transgenic animals or *in vitro* models, has sometimes proved to be poorly relevant.

Remarkably, development of iPSC technology revolutionized the way of studying neurological disorders, for example Alzheimer's disease (AD). Through the differentiation of adult somatic cells into neurons, this cutting-edge methodology allows to recreate a human-derived-neuronal system which promises to overcome the limitations of post-mortem analyses and transgenic models. Indeed, observations obtained from post-mortem samples give a knowledge only regarding the late stages of the disease, whereas the subtle cellular changes which cause AD, slowly progress over the years and are thought to start decades in advance before the appearance of the clinical symptoms. Moreover, while revealing important cellular processes underlying the disease mechanisms, the usage of animal models and transgenic immortalized cell lines often led

to not fully recapitulate the human disease and to overestimate the drug efficacy, respectively, which contributed to the failure of the clinical trials so far.

In contrast, iPSCs are obtained from adult differentiated cells, through a process called reprogramming. Thereafter, iPSCs are induced in neural precursors that are further terminally differentiated into neuronal cultures. Thus, by maintaining the individual genetic background, iPSC-derived neurons provide a more reliable platform for disease modelling and drug screening. Accordingly, researchers worldwide have generated iPSCs from sporadic and familial AD patients and from control individuals to study the disease. Moreover, the neuronal differentiated cultures are highly versatile systems, allowing to culture the cells in 2D or 3D format or even in co-culture with other cell types.

Interestingly, recent reports demonstrated that human derived neurons very well mimic the pathological hallmarks of the disease, including increased amyloid levels, TAU hyperphosphorylation, oxidative stress and cell death. Therefore, such promising evidences strongly encourage employment of iPSC-based system for researching Alzheimer's disease (Freude *et al.*, 2014; Mahairaki *et al.*, 2014; Penney *et al.*, 2019).

1.3 Objectives

The aim of the current study was to investigate the relevance of the CaSR in the pathomechanisms of neurological disorders, such as Alzheimer's disease (AD), by using stem cell derived neurons as a relevant *in vitro* system.

- The first aim was to explore the CaSR expression in the iPSC-derived neurons, for which any evidence exists, and its potential activated signalling in the neuronal cultures.
- The second aim was to characterize the iPSC derived neuronal cultures, especially focusing on the amyloid precursor protein (AβPP) processing and amyloid secretion, in order to substantiate the relevance of the iPSC derived neurons for disease modelling and drug testing.
- The final goal was to investigate the effects of CaSR inhibition with calcilytic on the AD pathomechanism recapitulated in iPSC derived neurons.

In order to reach our goals, the specific objectives of the research were:

- Differentiate the mouse ESCs and human iPSCs-derived neural precursor cells (NPCs) into neurons.
- Characterize the iPSC-derived neurons by detection of neuronal marker expression and assessment of functional properties.
- Detect the CaSR expression in mouse and human brain and kidney tissues for comparative analyses.
- Detect the CaSR expression in control and AD neuronal lines.
- Pharmacological modulation of the amyloid β secretion and AβPP processing by γ-secretase inhibitor in control and AD neurons.
- Investigate the effect of calcilytic on amyloid β secretion and sAβPPα release in control and AD cultures.
- Analyse the plasma membrane proteins expression in control and AD derived neurons exposed to calcilytic.

2 OVERVIEW OF THE LITERATURE

2.1 Discovery of the CaSR in parathyroid glands

The calcium sensing receptor (CaSR) is a member of class C G-protein coupled receptors (GPCRs) which exerts a main role in maintaining systemic Ca^{2+} homeostasis (Brown *et al.*, 1993). Despite the presence of a plasma membrane protein on parathyroid cells capable of stimulating intracellular Ca^{2+} mobilization in response to elevation of extracellular Ca^{2+} or divalent cations had been proposed already in late 1980's (Nemeth and Scarpa, 1987; Brown *et al.*, 1987), it was only in 1993 that Brown and co-workers molecularly identified the CaSR in parathyroid glands. Indeed, authors isolated a 5.3 kb complementary DNA (cDNA) clone, named BoPCaR1 (bovine parathyroid calcium sensing receptor) from a bovine parathyroid cDNA library (Brown *et al.*, 1993). When injected in *Xenopus laevis* oocytes, BoPCaR1 stimulated Cl^- inward currents in response to Ca^{2+}, Mg^{2+}, gadolinium (Gd^{3+}) and neomycin. Such currents were obtained through a phospholipase C (PLC)-mediated Ca^{2+} intracellular mobilization which in turn stimulated Ca^{2+}-activated Cl^- channels. These currents were similar to those mediated by the native CaSR of bovine parathyroid cells, which led Brown's group to confirm the molecular identity of the CaSR (Brown *et al.*, 1993). After that, the human CaSR was cloned from parathyroid gland of a patient affected with primary hyperparathyroidism (Garrett *et al.*, 1995), and the CaSR expression was demonstrated in several tissues other than parathyroids, including kidney (Riccardi *et al.*, 1995; Aida *et al.*, 1995), bone (Yamaguchi *et al.*, 2009), intestine (Chattopadhyay *et al.*, 1998a) and brain (Ruat *et al.*, 1995). Being expressed in several tissues, intensive research has been dedicated to unveiling the multiple functions of the receptor during the last three decades.

2.1.1 The role of CaSR in Ca^{2+} homeostasis and PTH secretion

The primary role of CaSR is to maintain systemic Ca^{2+} homeostasis by regulation of parathyroid hormone (PTH) secretion from parathyroid glands. The production and release of PTH is controlled by a feedback mechanism regulated by the extracellular Ca^{2+} ion concentration and mediated by the CaSR which serves as a "calciostat" (Conigrave, 2016). The set-point for this calciostat occurs at a plasma ionized Ca^{2+} concentration of around 1.1-1.2 mM. PTH secretion rates rise 2 to 4-fold as Ca^{2+}_o drops toward 1.0 mM and are effectively suppressed by >50% as Ca^{2+}_o rises toward 1.4 mM (Conigrave, 2016). Indeed, in case of hypocalcemia, the receptor is inactive while PTH secretion continues and restores Ca^{2+} concentration within the physiological range by promoting Ca^{2+} reabsorption from kidney and bone and Ca^{2+} absorption in the intestine (Conigrave, 2016). As soon as extracellular Ca^{2+} increases, CaSR is activated and it suppresses

PTH secretion through different mechanisms. On one hand, CaSR initiates the Gq/11- PLC signalling inducing the consequent intracellular Ca^{2+} mobilization, which inhibits vesicle fusion and exocytosis of PTH (Nemeth and Scarpa, 1987; Conigrave and Ward, 2013). On the other hand, the receptor operates through Gi/o-mediated inhibition of adenylate cyclase which suppresses cAMP intracellular synthesis and the consequent release of parathyroid hormone (Brown *et al.*, 1978). The existence of such Ca^{2+} sensing mechanism at parathyroid level was substantiated by *in vivo* observations, according to which surgical removal of parathyroid glands causes severe hypocalcemia in humans (Vasher *et al.*, 2010). Moreover, subjects receiving infusions of calcium or citrate, a Ca^{2+} chelator, to induce hypercalcemia or hypocalcemia respectively, displayed rapid negative and positive changes in the serum PTH concentration accordingly (Conlin *et al.*, 1989). In addition, *in vitro* evidence demonstrated that mammalian parathyroid cells in primary culture supported a robust endogenous secretion of PTH that was promptly blocked by elevation of extracellular Ca^{2+} (Conigrave, 2016).

2.1.2 Mineral disorders linked to CaSR mutations

The contribution of CaSR to the Ca^{2+} systemic homeostasis is clear from the mineral disorders which are associated to genetic mutations of the receptor. Inactivating mutations of the CaSR gene are responsible for familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT), two conditions in which CaSR is hypofunctional (Hendy *et al.*, 2009).

FHH is caused by heterozygous loss-of-function mutations in the CaSR gene or to mutations in other genes encoding for proteins involved in the CaSR-mediated signalling (Hannan *et al.*, 2016). The disease is characterized by an asymptomatic lifelong mild-to-moderate hypercalcemia associated with normal or mildly raised serum PTH levels and low urinary calcium excretion (Hannan *et al.*, 2016). According to the chromosome location, three genetic types of FHH have been identified. FHH type 1 (FHH1) accounts for ~65% of cases and is linked to mutations in the CASR gene. More than 130 different mutations of the CASR gene have been linked to FHH1 so far. Loss of CaSR function causes a reduction in the sensitivity of parathyroid and renal cells to calcium levels so hypercalcemia is perceived as normal. The remaining ~35% of FHH cases present mutations in two other genes both located on chromosome 19p13.3. The GNA11 gene encodes for the G-protein α-11 (Gα11) subunit and leads to FHH type 2 (Gorvin *et al.*, 2016) while the AP2S1 gene encodes the adaptor-related protein complex 2 sigma (AP2σ) subunit and causes FHH type 3 (Nesbit *et al.*, 2013). Studies demonstrated that Gα11 is a key mediator of downstream

CaSR signal transduction while AP2σ is involved in CaSR signalling and trafficking (Hannan *et al.*, 2016).

Differently from FHH, NSHPT is linked to homozygous or compound heterozygous deactivating mutations of the CaSR gene. NSHPT is characterized by a severe neonatal hypercalcaemia and elevations of serum PTH concentrations due to near-total failure of extracellular Ca^{2+}-mediated feedback control of PTH secretion. In addition, patients might develop skeletal demineralization along with pathological fractures. NSHPT can be fatal at birth and it has been proved that surgical removal of the parathyroid glands corrects the set point for calcium and restores normal or mild hypocalcaemia (Conigrave, 2016; Hannan *et al.*, 2016). Mouse models of FHH and NSHPT very well mimic the disease- related phenotypes observed in patients. *In vivo* studies revealed that heterozygous CaSR knockout (–/+) mice showed inappropriately high PTH levels and relative hypocalciuria resulting in elevated blood ionized calcium levels thus exhibiting a similar phenotype to FHH. In contrast, CaSR knockout (–/–) animals displayed markedly elevated serum calcium and parathyroid hormone levels, bone abnormalities, retarded growth and premature death like humans with neonatal severe hyperparathyroidism (Ho *et al.*, 1995).

Conversely, activating gain-of-function mutations of the CaSR cause autosomal dominant hypocalcaemia (ADH). This is a chronic benign condition characterized by mild-to-moderate hypocalcaemia and normal-to-low serum PTH levels. The activating mutations cause that CaSR is hyper functional, displaying enhanced sensitivity to extracellular Ca^{2+}. Around 70% of all ADH cases are linked to mutations in the CASR gene and are referred as ADH type 1. Patients affected with ADH1 may develop hypocalcemic symptoms such as paraesthesia, carpopedal spasms and seizures (Hannan *et al.*, 2016). More severe gain-of-function CaSR mutations are associated with Bartter's syndrome type 5 which is characterized by hypocalcemic metabolic alkalosis, renal salt wasting, elevated renin and aldosterone levels (Letz *et al.*, 2014). A second group of ADH, classified as ADH type 2, is associated with gain-of-function mutations in $G\alpha_{11}$ subunit. ADH2 patients typically present hypocalcemic symptoms such as paraesthesia, muscle cramps, carpopedal spasm and seizures (Piret *et al.*, 2016; Roszko *et al.*, 2016).

2.2 CaSR: from gene to protein structure

Fluorescence in situ hybridization studies revealed that human CASR gene maps on chromosome 3q13.3–21, while the receptor gene localizes to chromosome 11 in rats and to chromosome 16 in mice (Janicic *et al.*, 1995). Human CASR gene consists of eight exons, two non-coding exons (1A and 1B) and six coding exons (2-7). The gene has two promoters, P1 and P2, that produce alternative transcripts including either exon 1A or exon 1B 5'-untranslated region sequences that

splice to exon 2, which contains the ATG translation start site (Hendy and Canaff, 2016). Both promoters present vitamin D response elements (VREs) that confer responsiveness to the hormonally active secosteroid 1,25-dihydroxyvitamin D (1,25(OH)2D), but promoter P2 appears to be 2.5-fold more active than promoter P1 (Canaff and Hendy, 2002) (**Figure 1A** and **1B**).

The human CaSR protein is composed of 1078 amino acids (aa) and it presents the classic GPCR structure with a large extracellular N-terminal domain (ECD), a seven transmembrane domain (TMD) and an internal C-terminal domain (ICD) (Brown *et al.*, 1993) (**Figure 1C**).

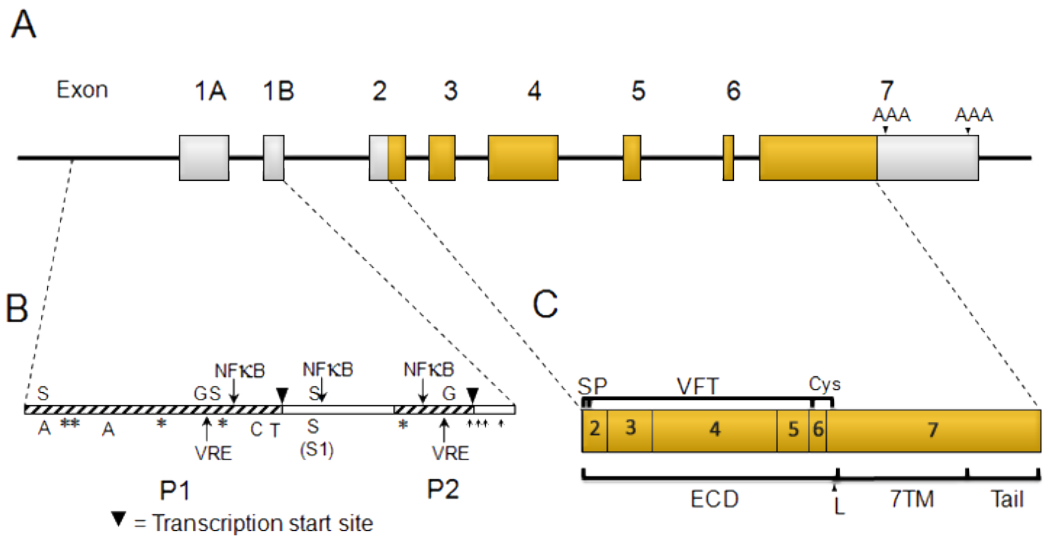

Figure 1. Schematic presentation of the CaSR (**A**) Exon-intron structure of CaSR gene. Exons that comprise the CaSR protein are shaded yellow; untranslated exons are left unshaded. The location of polyadenylation sites (AAA) within the untranslated region of exon 7 are arrowed. (**B**) promoters location and position of various transcription factor binding sites and regulatory elements on the CaSR gene: C, CCAAT box; T, TATA box; A, Ap-1 sites; *, serum response elements; VRE, vitamin D response elements; G, glial cells missing-2 response element; ↑, Sp1 sites; NFκB, NFκB response elements; S, STAT sites (S1 regulates IL-6 response). (**C**) CaSR protein structure composed of the extracellular domain (ECD) consisting of a signal peptide (SP), Venus Fly Trap (VFT) domain and cysteine-rich domain (Cys) connected by a small linker region (L) to the seven transmembrane domain (7TM) and intracellular tail. (*Adapted from http://atlasgeneticsoncology.org)*

The crystal structure of human CaSR-ECD has been recently reported (Zhang *et al.*, 2016) (Geng *et al.*, 2016). It consists of 612 amino acids and presents a bilobed Venus flytrap (VFT)-like structure through which the CaSR binds its physiological ligand, the calcium ion (Ca^{2+}) and various other agonists (Zhang *et al.*, 2015). The VFT presents two globular lobes, containing β-sheets, which oscillate between ‘open’ and ‘closed’ conformational states until the binding of

endogenous agonist in the cleft between the two lobes induces stabilization of the 'closed' conformation (Hu and Spiegel, 2007). The VFT domain is linked to the TMD through a cysteine-rich region which is important for signal transmission. Deletion of such cysteine -rich domain abolishes CaSR activation while preserving a certain level of receptor's expression (Hu *et al.*, 2000). The TMD is composed of seven α-helices (TM1–TM7) joined together by extracellular and intracellular loops, which contain additive binding sites for Ca^{2+} and allosteric modulators. The presence of allosteric sites in the TMD demonstrates that it plays a role in receptor activation (Hu and Spiegel, 2007). The ICD is composed of a 216 amino acids-tail and is needed to interact with signaling proteins (Huang and Miller, 2007), to mediate cell-surface expression and intracellular pathways (Chang *et al.*, 2001). Truncations and point mutations at the C-terminus exhibit a decrease in the affinity for extracellular Ca^{2+} and a reduction in cooperativity (Gama and Breitwieser, 1998). The C-terminal domain is also responsible for CaSR negative regulation. Indeed, phosphorylation at Thr^{888} mediated by protein kinase C (PKC) has been demonstrated to inhibit CaSR's activity in CaSR-HEK-293 cells (Jiang *et al.*, 2002) (**Figure 2**).

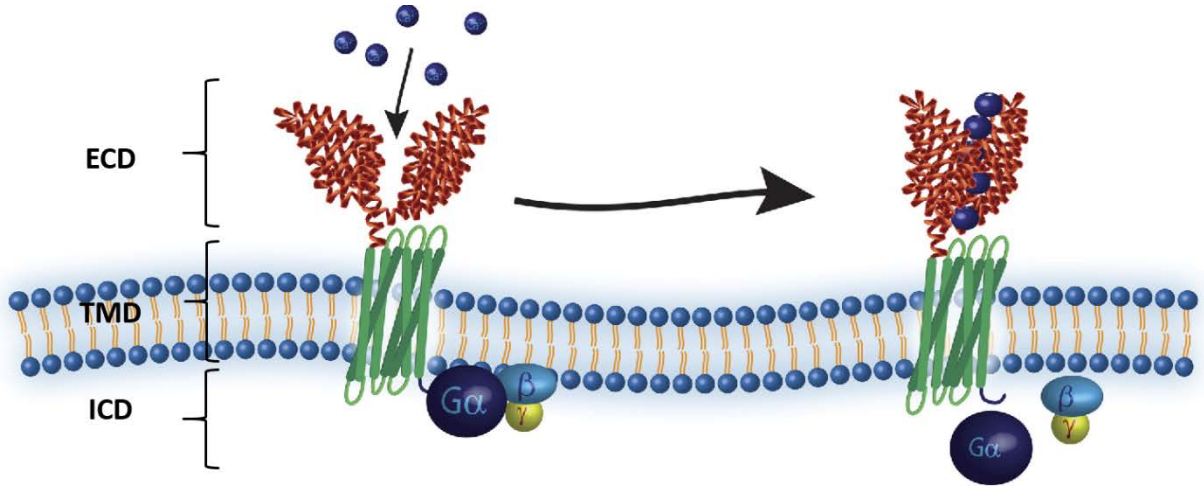

Figure 2. A schematic depicting of the receptor's protein structure. In the inactive state, shown to the left, the "Venus flytrap" is interacting with calcium ions, which leads to the activation of the receptor. The activation causes conformational changes, resulting in downstream signal transduction. ECD, Extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; [adapted from (Alfadda *et al.*, 2014)].

2.3 The Biased signalling of CaSR

The CaSR mediates a variety of distinct signalling pathways according to the tissue where it localizes and to the ligand and/or allosteric modulator which binds to the receptor. Such capability is known as "biased-signalling" and it is characteristic of GPCRs to which the CaSR belongs (Leach *et al.*, 2015). Indeed, different ligands stabilize preferred receptor's conformations which

in turn drive specific intracellular pathways. The multiplicity of the endogenous ligands and the variety of the receptor's mediated signalling explain the diversity of the functions that CaSR plays in different cell types and conditions (Alfadda *et al.*, 2014). As a GPCR, the CaSR couples to heterotrimeric G–proteins (**Figure 3**): $G_{q/11}$ which stimulates phospholipase C (PLC) to form diacyl glycerol (DAG) and inositol 1,4,5 tris- phosphate (IP_3), with consequent intracellular Ca^{2+} mobilizations and mitogen-activated protein kinase (MAPK) cascade (Kifor *et al.*, 1997; Kifor *et al.*, 2001); $G_{i/o}$ which inhibits cyclic adenosine monophosphate (cAMP) production (Kifor *et al.*, 2001; Thomsen *et al.*, 2012); $G_{12/13}$ which stimulates Rho kinase signalling (Davies *et al.*, 2006). In addition, CaSR can operate through G-protein independent mechanisms. For example, the receptor mediates recruitment of β-arrestins which stimulate the MAPK pathway (Thomsen *et al.*, 2012) and regulate CaSR desensitization (Pi *et al.*, 2005).

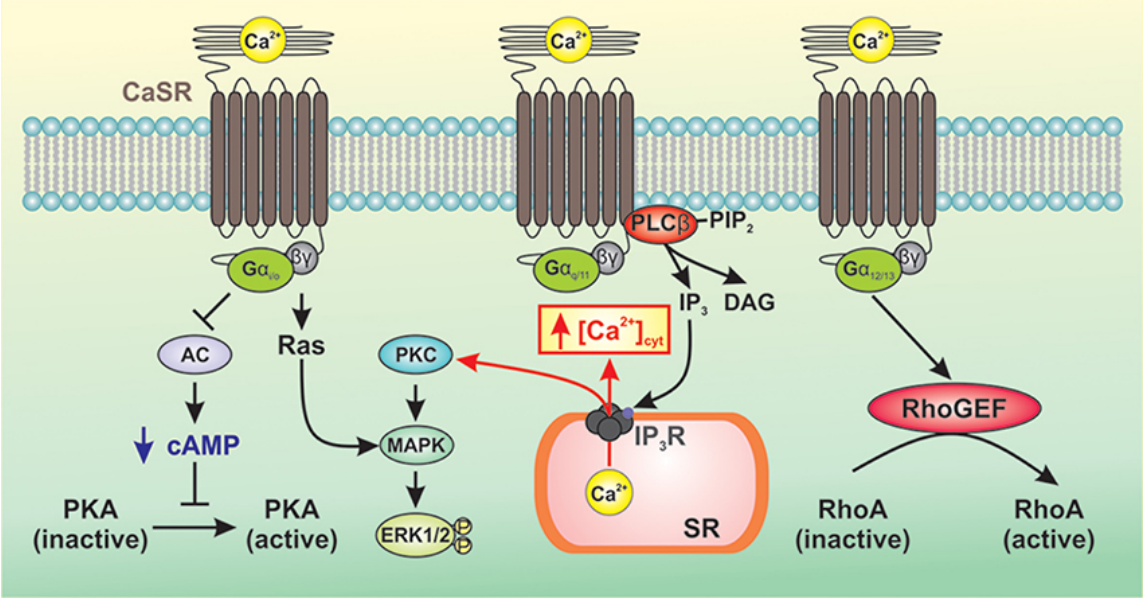

Figure 3. The CaSR-mediated signalling pathways. CaSR signals to downstream pathways via three main groups of heterotrimeric G-proteins, $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$. CaSR-mediated activation of $G_{q/11}$ leads to activation of PLCβ resulting in production of IP3 which mobilizes cytosolic Ca2+ from intracellular Ca2+ stores and phosphorylation of PKC, thereby activating MAPK and subsequent phosphorylation and activation of ERK1/2. CaSR-mediated activation of Gi/o inhibits AC, which reduces levels of cAMP and PKA activity. The β/γ subunits of $G_{i/o}$ activate Ras leading to MAPK activation and ERK1/2 phosphorylation. Activation of $G_{12/13}$ causes RhoGEF to translocate to the plasma membrane where it activates GEF [*Adapted from* (Smith *et al.*, 2016)].

2.4 The CaSR pharmacology: orthosteric agonists and allosteric modulators

In addition to its primary ligand, the ion Ca^{2+}, CaSR responds to a plethora of different agonists. Such molecules include many divalent and trivalent cations, as Mg^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}, Al^{3+}, Gd^{3+} (Gerbino and Colella, 2018), polyamines as spermine and spermidine, and positively charged peptides as the amyloid-β (Aβ). Interestingly, the receptor is also activated by some

aminoglycoside antibiotics, such as neomycin, G418, gentamycin and tobramycin (Hofer and Brown, 2003). All these receptor's activators are classified as orthosteric agonists because they are capable of binding and activating the CaSR on their own (Saidak *et al.*, 2009). Conversely, the allosteric modulators are compounds which modify the CaSR-mediated signalling only in the presence of an orthosteric ligand. Positive allosteric modulators potentiate the CaSR-activated response with the consequence to left-shift the calcium dose-response curve. Such positive modulators include some L-Amino acids, like L-phenylalanine and L-tryptophan, which, in response to extracellular Ca^{2+}, were found to markedly increase the intracellular Ca^{2+} mobilization and suppress the PTH secretion in human parathyroid cells (Conigrave *et al*., 2007). Moreover, positive synthetic modulators, named calcimimetics, have been developed to treat disorders of Ca^{2+} metabolism as hyperparathyroidism. A first-generation of calcimimetics is represented by the phenylalkylamines NPS R-567 and NPS R-568, which have been shown to decrease PTH secretion and plasma Ca^{2+} *in vivo* (Fox *et al.*, 1999). The second generation of CaSR positive modulators includes cinacalcet which is the only calcimimetic approved for clinical treatment of secondary hyperparathyroidism (SHPT). Cinacalcet effectively reduces PTH and improves biochemical control of mineral and bone disorders in patients with chronic kidney disease (CKD) (Nemeth and Goodman, 2016; Pereira *et al.*, 2018). Along with calcimimetics, negative allosteric modulators of the CaSR, known as calcilytics, have been developed. Such molecules, which include for example NPS 2143, NPS 89636 and Calhex, shift the concentration-response curve of Ca^{2+} to the right (Saidak *et al.*, 2009). NPS 2143 was the first calcilytic compound to be published (Nemeth *et al.*, 2001). Based on an amino alcohol structure, NPS 2143 binds an allosteric site in the TMD of the CaSR with high selectivity. Consistent with CaSR inhibition, NPS 2143 caused a concentration-dependent inhibition of the cytoplasmic Ca^{2+} response to extracellular Ca^{2+} in HEK 293 cells stably transfected with the human CaSR, while it efficiently increased PTH secretion from bovine parathyroid cells and infused rats (Nemeth *et al.*, 2001). Calcilytics were initially tested as therapeutics for osteoporosis. However, these compounds elicited a PTH over-secretion which stimulated in parallel both osteogenesis and osteolysis, which stopped any further clinical testing of calcilytics for osteoporosis (Nemeth and Shoback, 2013; Nemeth and Goodman, 2016). Interestingly, in the last decade, calcilytics have been indicated as potential therapeutics to mitigate autosomal dominant hypocalcaemia (Park *et al.*, 2013) and the airways hyper responsiveness and inflammation proper of asthma (Yarova *et al.*, 2015). Moreover, as it will extensively described in the next sections, several studies demonstrated the effect of calcilytic NPS 2143 to block the CaSR/Aβ signalling, supporting to repurpose this compound for Alzheimer's disease (Chiarini *et al.*, 2016; Chiarini *et al.*, 2017a). Such evidences prompted us to use this molecule for our study. Orthosteric ligands and allosteric modulators present different binding sites on the CaSR protein.

Whereas the endogenous ligands of the CaSR bind to the amino-terminal domain, most if not all of the synthetic modulators published so far bind to TMD (Jensen *et al.*, 2007; Zhang *et al.*, 2015). Finally, studies suggest that CaSR activity can be negatively modulated by low extracellular pH (Quinn *et al.*, 1998).

2.5 Calcium sensing receptor: tissue expression

The expression of CaSR has been extensively described in several tissues deeply involved in the maintenance of Ca^{2+} homeostasis, other than the parathyroid glands, like kidneys and bones. The CaSR' s presence in rat kidneys was demonstrated few years after receptor cloning from bovine parathyroid glands (Riccardi *et al.*, 1995). Later, by using different approaches including *in situ* hybridization, immunohistochemistry and proximity ligation assays, the receptor's distribution was reported in several renal segments including the thick ascending limb (TAL), the distal tubule and the collecting duct, with TAL having the highest levels (Graca *et al.*, 2016). Here, the CaSR regulates Ca^{2+} reabsorption, independently of the actions of PTH, through interaction with Claudin-14 tight junction protein. In addition, the renal- CaSR regulates Mg^{2+} and inorganic phosphate (Pi) transport by integrating multiple inputs from divalent cation concentration, osmolarity, and urine acidification (Riccardi and Valenti, 2016).

In vitro and *in vivo* studies provided considerable insight into the role of CaSR in skeletal homeostasis (Goltzman and Hendy, 2015). The CaSR mRNA has been found mainly in bone marrow cells, osteoblasts and osteocytes (Chang *et al.*, 1999). In osteoblast-cell lines, activation with high extracellular Ca^{2+} produced an increase of intracellular IP_3 and Ca^{2+} as consequence of PLC pathway mediated by the CaSR (Chang *et al.*, 1999). Moreover, evidences reported that high levels of extracellular Ca^{2+} or polycationic CaSR agonists (such as neomycin or gadolinium) stimulated osteoblast proliferation, differentiation and matrix-mineralization capacity via activation of mitogen-activated protein kinases (Goltzman and Hendy, 2015). Additionally, increased calcium gradient in the site of bone resorption favoured osteoblast precursors chemotaxis while inhibiting osteoclasts, which substantiated a role for CaSR in bone modelling and remodelling (Caudarella *et al.*, 2011).

2.6 The functions of CaSR in the nervous system

Interestingly, a growing body of evidences reported the expression of the CaSR in organs and cells not directly associated with the control of calcium homeostasis. Indeed, several studies revealed that CaSR can exert different functions according to cell type, developmental stage and physiological condition. Such capability of CaSR is valid also for the nervous system (NS), and

the receptor has been demonstrated to play crucial roles for both the developing and the adult NS, as it was recently reviewed by our group and it will be described in the next paragraphs. The first report of CaSR expression in the brain was provided in 1995, when Ruat and colleagues cloned the receptor from a rat striatal cDNA library and showed its presence in several brain areas such as the hypothalamus, striatum, hippocampus, cortex, cerebellum, and brainstem (Ruat *et al.*, 1995). Afterwards, *in vitro* and *in vivo* studies revealed that the CaSR plays a crucial function in several mechanisms, including differentiation and migration of neuronal and glial cells, neuronal excitability and pathological processes.

2.6.1 CaSR role in neurodevelopment

In vertebrates, the development of nervous system depends on highly sophisticated mechanisms, including a finely regulated calcium signaling. Studies in *Xenopus laevis* embryos have demonstrated that, during the specification of neural *versus* epidermal ectoderm, spontaneous elevations of intracellular Ca^{2+} occur in the dorsal ectoderm cells, which give rise to the neural progenitors, and do not occur in ventral ectoderm cells, which originate epidermis (Leclerc *et al.*, 2006; Moreau *et al.*, 2008). Moreover, calcium signalling also contributes to the proliferation and differentiation of neurons and to the neuro-glia switch (Leclerc *et al.*, 2012). Based on the relevance of Ca^{2+} signals for developing NS, the function of CaSR in neurodevelopment has been object of intense investigation. *In vitro* and *in vivo* studies demonstrated that the receptor plays crucial roles for differentiation of neurons and glial cells (**Table 1**).

In neurons, CaSR is involved in the growth of axons and dendrites. Studies on sympathetic neurons isolated from the mice superior cervical ganglion (SCG), revealed that CaSR mRNA expression reaches a peak at embryonic day18 (E18) which is the time when the axons grow to innervate their targets (Glebova and Ginty, 2004). Importantly, authors demonstrated that activating the CaSR with high extracellular Ca^{2+} or with the calcimimetic NPS R-467 significantly increased axonal growth (Vizard *et al.*, 2008). In contrast, treatment with the calcilytic NPS-89636, receptor deletion or expression of a dominant negative CaSR (DNCaSR), completely abolished the effect of high Ca^{2+} on neurite growth. Moreover, hippocampal neurons from DNCaSR mouse at post-natal day 4 (P4) developed significantly shorter dendrites than neurons from wild type animals (Vizard *et al.*, 2008). Later, the effect of CaSR on neurite growth of SCG-neurons was found to depend on the ERK1/2 activated signalling (Vizard *et al.*, 2015). Several studies showed a crucial role of CaSR also in neuronal migration and chemotaxis. A first proof of receptor's involvement in such mechanisms was demonstrated in two gonadotropin-releasing hormone (GnRH) neuronal cell lines, GN11 and GT1-7. Wild type cells stimulated with high extracellular Ca^{2+} presented an increased chemotaxis, while such effect was largely attenuated in cells expressing a DNCaSR

(Chattopadhyay *et al.*, 2007). In line, GT1-7 cells treated with high Ca^{2+} and spermine were found to release the monocyte chemoattractant protein-1 (MCP-1), which is known to support migration of rat neural stem cells (Widera *et al.*, 2004), whereas DNCaSR cells significantly reduced the MCP-1 production induced by Ca^{2+} (Chattopadhyay *et al.*, 2007). Additionally, *in vivo* evidence suggested that CaSR is required not only for the migration but also for the survival of the GnRH neuronal population. Indeed, mice knock-out for CaSR and PTH ($CaSR^{-/-}/PTH^{-/-}$) showed a 27% reduction of GnRH neurons in the preoptic area (POA) of the anterior hypothalamus compared to the wild type animals (Chattopadhyay *et al.*, 2007). Another example of the CaSR relevance for neuronal chemotaxis and migration was reported on cerebellar granule-cell precursor (GCPs) neurons (Tharmalingam *et al.*, 2016). During the cerebellum development, GCPs migrate within the external granule-cell layer (EGL) until they reach their final position in the internal granule cell layer (IGL) (Tharmalingam *et al.*, 2016). Interestingly, *in vivo* studies demonstrated that, in rat cerebellum, CaSR expression was up- regulated at P7 to P18, a period which coincides with peak GCP migration. Moreover, authors monitored the GCPs migration from the EGL into the IGL by BrdU-cells labelling and found that post-natal rats injected into the cerebrospinal fluid with calcimimetic presented an increased number of BrdU-positive GCPs into the IGL, which supported an increased migration from EGL into IGL. Conversely, animals injected with calcilytic increased the number of BrdU-positive GCPs in the EGL. *In vitro* experiments confirmed the positive and negative effects of calcimimetic and calcilytic respectively on GCP migration, and further demonstrated the implication of MAPK signalling in this process (Tharmalingam *et al.*, 2016).

Along with the described role of CaSR in neuronal development, several evidences demonstrated that the receptor is crucial for oligodendrocyte's differentiation. For example, CaSR-null mice presented reduced levels of Myelin basic protein (MBP) in the cerebellum, compared to wild type animals (Chattopadhyay *et al.*, 2008). Interestingly, comparison of CaSR levels among adult and fully differentiated cells of nervous system, revealed that oligodendrocytes had a higher CaSR expression level compared to neurons and astrocytes. In line with this trend, the expression of CaSR mRNA was found to be higher in rodent neural stem cells (NSC) undergoing differentiation towards the oligodendrocyte lineage than in cells differentiating in neurons or astrocytes (Chattopadhyay *et al.*, 2008). Similar to the role exerted in neuronal cells, CaSR is needed for the correct development of oligodendrocytes. Indeed, stimulation with high Ca^{2+} and spermine was found to positively induce the proliferation and the maturation of oligodendrocytes progenitor cells (OPCs), an effect that was significantly blunted by the expression of a dominant negative CaSR (Chattopadhyay *et al.*, 1998b; Chattopadhyay *et al.*, 2008). In addition, GalC-positive

oligodendrocytes isolated from 20 days postnatal rat brain and stimulated with high Ca^{2+} and with the positive allosteric modulator NPS R-568, presented an intracellular Ca^{2+} mobilization and inositol phosphate accumulation, demonstrating that CaSR activation couples to the phospholipase C pathway in these cells (Ferry *et al.*, 2000).

While large evidences support the relevance of CaSR in neurons and oligodendrocytes development, the role of the receptor in astrocytes differentiation is uncertain. This is mainly due to the lack of studies and to the usage of poorly relevant *in vitro* models. Two reports, based on experiments employing glial- derived tumours cell lines, suggested that stimulation of CaSR with agonists and calcimimetic induces the activation of nonselective cation channels (NCCs) (Chattopadhyay *et al.*, 1999a) and the opening of an outward K^+ channel (Chattopadhyay *et al.*, 1999b), but further studies are needed to clarify the role of CaSR for the differentiation of astroglial cells.

Table 1. Proposed functions of the CaSR in the cells of developing nervous system.

Cell types	Model	Role of CaSR	References
Neurons	Neurons differentiated from NSCs of newborn CaSR-/- mice	Serves for neurite growth.	(Liu *et al.*, 2013)
	Superior cervical ganglion (SCG) sympathetic neurons	Promotes axonal and dendritic growth and extension through ERK1/2 activation	(Vizard *et al.*, 2008) (Vizard *et al.*, 2015)
	GnRH neuronal cell lines GN11 and GT1-7	Induces neuronal migration and chemotaxis by the secretion of monocyte chemoattractant protein-1, MCP-1; supports the neuronal survival of GnRH neuronal population.	(Chattopadhyay *et al.*, 2007)
	Cerebellar granule-cell precursor (GCP) neurons	Stimulates GCPs migration through the activation of MAPK signaling.	(Tharmalingam *et al.*, 2016)
Oligodendrocytes	Oligodendrocytes differentiated from NSCs of newborn CaSR-/- mice	Serves for oligodendrocytes development.	(Liu *et al.*, 2013)
	Oligodendrocytes differentiated from rat NSCs	Favors oligodendrocyte commitment and lineage progression; stimulates oligodendrocyte proliferation; induces the opening of a Ca-activated K+ Channel.	(Chattopadhyay *et al.*, 1998) (Chattopadhyay *et al.*, 2008)
	20 days post-natal oligodendrocytes from rat brain	Mediates an intracellular calcium mobilization and inositol phosphate accumulation—PLC mediated.	(Ferry *et al.*, 2000)
Astrocytes	U373 and U87 astrocytoma cell lines	Increases cell proliferation; activates a nonselective cation channel (NCC). Stimulates the opening of an outward K+ channel.	(Chattopadhyay *et al.*, 1999a) (Chattopadhyay *et al.*, 1999b)

2.6.2 The role of CaSR in neuronal excitability

ADH patients present neonatal or childhood seizures occasionally (Hendy *et al.*, 2000). Considering that ADH is due to CaSR activating mutations, a correlation between the receptor mutation and the neurological phenotype appears plausible. In line, rare missense mutations were

identified in the CaSR gene of patients with idiopathic epilepsy (Kapoor *et al.*, 2008). In particular, the R898Q variant was found to increase plasma membrane targeting of the mutated receptor as expected from a gain-of-function mutation (Stepanchick *et al.*, 2010). Such observations in humans, strongly support a role for the CaSR in neuronal excitability and neurotransmission and studies conducted in this regard demonstrated an involvement of CaSR in regulation of several ion channels.

An increased opening state probability (P_o) of NCCs was observed in rat hippocampal neurons from WT animals stimulated with high extracellular Ca^{2+}, neomycin or spermine. Conversely, such increase did not occur in cells isolated from CaSR-/- mice (Ye *et al.*, 1996a; Ye *et al.*, 1997b). As a confirm of the CaSR-mediated effect on NCCs opening state, HEK-293 cells stably transfected with the CaSR similarly displayed an augmented P_o of NCCs in response to Ca^{2+} stimulation, which was not observed in non-transfected cells (Ye *et al.*, 1996b).

By modifying the P_o of neuronal ion channels, CaSR could actively influence the cell excitability and neurotransmission. However, controversy results have been reported so far. Smith and coworkers performed patch-clamp recordings from isolated mice cortical terminals, according to which a reduction in extracellular Ca^{2+} at synaptic cleft activated nonselective cation channels while an increase in extracellular Ca^{2+} reduced the channels currents, through the activation of CaSR (Smith *et al.*, 2004). In agreement with it, electrophysiological analyses on neurons from CaSR-/- mice displayed augmented excitatory post-synaptic currents (EPSC) compared to neurons from WT animals (Phillips *et al.*, 2008). Based on these evidences, CaSR activation would depress neurotransmission. Conversely, a decrease of CaSR activity would promote synaptic transmission. In contrast with this hypothesis, Vyleta and colleagues presented results supporting the idea that CaSR stimulation would promote the release of the excitatory neurotransmitter glutamate. Vyleta group found that CaSR stimulation with calindol and cinacalcet increased spontaneous vesicle fusion and miniature EPSCs (mEPSC) frequency, with consequent glutamate release, in cultured mouse neocortical neurons. Consistent with such results, the frequency of spontaneous synaptic transmission and glutamate secretion was decreased in neurons isolated from CaSR-/- mice (Vyleta and Smith, 2011).

In opposition to the described works, a recent study reported that potent allosteric modulators of CaSR had no effect on mEPSC frequency in adult mouse CA1 hippocampal pyramidal cells. Moreover, the authors of this study failed to detect any CaSR expression in adult hippocampal neurons (Babiec and O'Dell, 2018). The large discrepancy among the presented studies makes clear that further investigation is needed to clarify the role of CaSR in neuronal excitability and neurotransmission.

2.6.3 Heterodimerization of CaSR with other GPCRs in the brain

Studies showed that CaSR can form heterodimers with metabotropic glutamate receptors (mGluRs) and γ-amino isobutyric acid B receptors (GABABRs). These receptors, like CaSR, belong to the family C of G protein coupled receptors, and share significant homology with the calcium-sensing receptor (Brown *et al.*, 1993). Furthermore, mGluRs and GABABRs represent critical receptors for neuronal activity. In consideration of the CaSR role in excitability previously described, it is noteworthy that the heterodimerization of the CaSR and GABABRs or mGluRs has been observed in the brain, which may reflect a function of such heterodimers in neurotransmission. In 2001, Gama and collaborators reported the co-immunoprecipitation of the CaSR and mGluR1α from bovine brains, whereas the immunohistochemical co-localization of the receptors was showed in rat brain samples (Gama *et al.*, 2001). Such ability to heterodimerize was also confirmed *in vitro*. The receptors were found to form heterodimers in HEK-293 cells transiently transfected with both GPCRs, and the CaSR became sensitive to glutamate-mediated internalization when present in CaSR/mGluR1α heterodimers (Gama *et al.*, 2001). With similar experimental approaches, heterodimerization between the CaSR and GABABRs were demonstrated. Co-immunoprecipitation studies revealed the presence of heterodimers between the CaSR and GABA type B receptor in HEK-293 cells expressing both receptors and in mouse brain lysates (Chang *et al.*, 2007). Interestingly, authors also demonstrated that GABABR1 exerts a negative regulatory effect on CaSR levels, showing that the expression of CaSR was increased in lysates from GABABR1 knock-out mouse brains and in cultured hippocampal neurons with their GABABR1 genes deleted *in vitro* (Chang *et al.*, 2007). By highlighting the importance of family C GPCRs dimerization, these studies added a further level of complexity in elucidating the role of the CaSR in the brain and raised questions regarding i) how these interactions between GPCRs might affect ligand binding and sensitivity; ii) what might be the biological meaning of such dimerization in the nervous system; iii) how dimerization affects the pharmacology of the resulting receptor; and iv) how dimerization modulates the signalling networks and neurotransmission. To date, these questions are yet to be answered.

2.6.4 The role of CaSR in Ischemia and Hypoxia

Together with the cited roles in the nervous system physiology, CaSR has been demonstrated to have crucial functions in pathological conditions of NS like ischemia and hypoxia (**Figure 4**). Various studies reported that CaSR expression become up-regulated in the brains of animals subjected to ischemic and hypoxic stimuli (Noh *et al.*, 2015; Bai *et al.*, 2015). Moreover, the receptor increased expression followed to such noxious treatments likely mediates the astro-glial reaction observed in the injured brain zones (Pak *et al.*, 2016). In agreement, Kim and colleagues

reported an increased CaSR expression while a concomitant downregulation of GABABR1 levels in the hippocampal regions CA1 and CA3 of mice subjected to bilateral carotid artery occlusion to induce global cerebral ischemia (GCI) (Kim *et al.*, 2011). The changes in receptors expression was accompanied by an increased neuronal death. Interestingly, hypothermia efficiently prevented both receptors expression changes and cell death (Kim *et al.*, 2011). According to a previous work, which showed that GABABR1 receptors negatively regulated CaSR expression levels in mouse neurons (Chang *et al.*, 2007), Kim et al speculated that CaSR overexpression could have been caused by the reduction of GABABR1 following to GCI. Studies conducted on hippCaSR –/–mice (with CaSR KO targeted to hippocampus and activated 3 weeks after birth) were performed to test this hypothesis. HippCaSR –/– animals, undergoing transient global cerebral ischemia, showed to have a higher survival of neurons in the CA1, CA3 and DG regions of hippocampus compared to the neurons from wild-type mice (Kim *et al.*, 2014). Such evidences were further substantiated by pharmacological approaches. Intra-cerebroventricular (ICV) injections of calcilytics effectively protected the hippocampal neurons of wild type animals from ischemic injury. Moreover intraperitoneal (IP) injections of calcilytics preserved neurological abilities in ischemic mice, tested with the Morris Water Maze (MWM) behavioural test, by partly restoring GABABR1 expression (Kim *et al.*, 2014). Accordingly, co-injection of calcilytics with the GABABR agonist baclofen had a stronger effect in preserving neurons and cell viability from ischemic injury when compared to treatment with calcilytic alone, which demonstrated synergism of these compounds to enhance neuroprotection (Kim *et al.*, 2014).

A similar function of CaSR has been reported in hypoxia by several studies. Hypoxic treatment significantly decreased cell viability of rat hippocampal neurons, while concurrently increasing the expression levels of caspase–3, Bax, cytochrome C, which promoted cell death. Moreover, phosphorylation of ERK1/2 resulted to be increased following to hypoxic insult. Such effects were further increased by simultaneous treatment with the CaSR agonist gadolinium chloride (GdCl3) (Wang *et al.*, 2015). Interestingly, authors found that calcilytic NPS 2390 significantly reversed all the hypoxia-mediated effects on neuronal viability and ERK1/2 phosphorylation (Wang *et al.*, 2015). In agreement, another group reported calcilytic NPS 2390 to be beneficial in attenuating neuronal apoptosis induced by traumatic brain injury (TBI). Indeed, NPS 2390 efficiently counteracted the increase of caspase–3 levels, Bax and cytochrome C induced in the TBI-injured rat brains (Xue *et al.*, 2017).

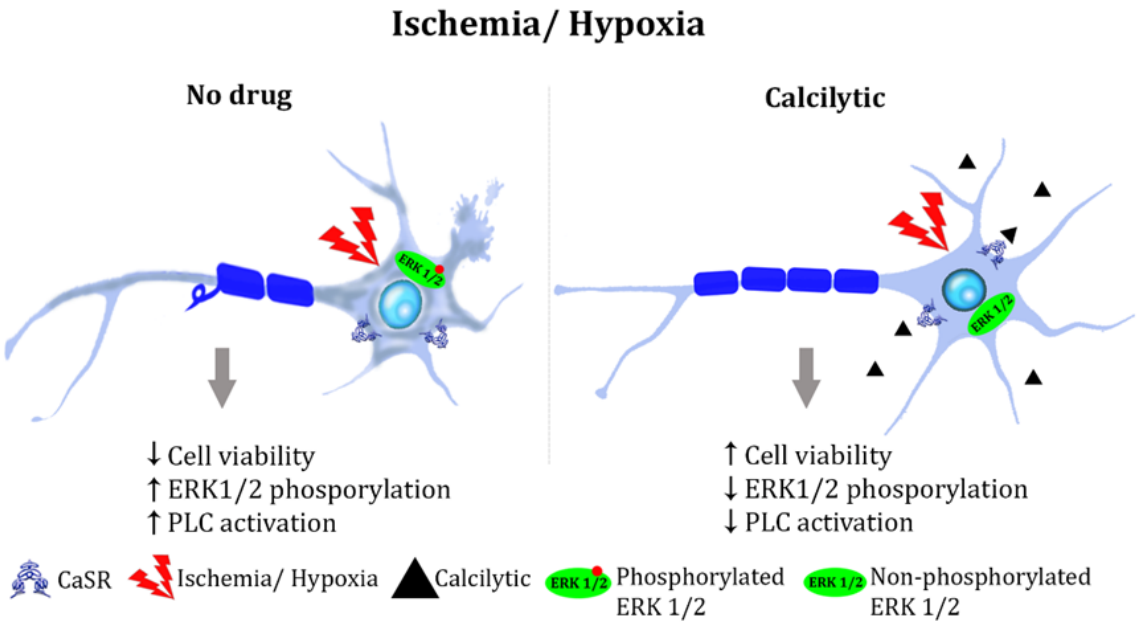

Figure 4. Hypothesized action of a calcilytic in ischemia and hypoxia. Neurons subjected to ischemic or hypoxic stress present reduced cell viability (due to the upregulation of cell death-related proteins such as caspase-3, Bax, and cytochrome C), increased ERK1/2 phosphorylation and phospholipase C (PLC)-activated-Ca^{2+} intracellular mobilization. Such events are likely mediated by the CaSR and concur with cell death (here represented by axonal demyelination and changes in cell morphology). Calcilytic treatment efficiently attenuates mitogen-activated protein kinase (MAPK)- and PLC-activated pathways and restores cell viability. [*Adapted from* (Giudice *et al.*, 2019)].

Interestingly, a study by Bai and colleagues showed that hippocampal neurons stimulated with hypoxia presented an activation of CaSR which coupled to intracellular Ca^{2+} mobilization and to increased levels of Beta-secretase 1 (BACE1), Aβ40 and Aβ42 amyloid species (Bai *et al.*, 2015). BACE1 is the enzyme which initiates the production of Aβ, the toxic peptide which accumulate in the brains of patients with Alzheimer's disease (Murphy and LeVine, 2010). Remarkably, Calhex 231, a blocker of CaSR, partly prevented the hypoxia- induced overexpression of BACE1 and amyloid peptides (Bai *et al.*, 2015), suggesting a contribution of CaSR to favour AD. An overview on AD and on the hypothesized involvement of CaSR in AD will be extensively discussed in the next paragraphs.

2.7 *In vitro* neurodegenerative diseases modelling

Neurodegenerative diseases represent a major threat to human health. These age-dependent disorders are becoming increasingly prevalent, in part because the elderly population has increased in recent years. Examples of neurodegenerative diseases are Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal dementia. Such disorders are diverse in their pathophysiology, with some causing memory and cognitive

impairments and others affecting a person's ability to move or speak, but usually these all have an insidious onset and chronic progression (Gitler *et al.*, 2017).

One way to learn about how a disease works is to develop a model system that recapitulates the hallmark characteristics of the disease. Powerful experimental model organisms such as the mouse, fruit fly, nematode worm, and even baker's yeast have been used for many years to study neurodegenerative diseases and have provided key insights into disease mechanisms (Krobitsch and Lindquist, 2000; Couthouis *et al.*, 2011; LaFerla and Green, 2012; Gitler *et al.*, 2017). Animal models offer the possibility to study both physiological and behavioral mechanisms. However, they do not always provide translatable results in pre-clinical drug screening for humans due to inter-species differences (McGonigle, 2014). An alternative is represented using human-based *in vitro* models. Indeed, *in vitro* systems allow to study critical cellular and molecular processes, which would be difficult to be analyzed in a whole animal. Therefore, cellular models provide important insights about the pathogenesis of neurodegenerative disorders and represent an interesting approach for the screening of potential pharmacological agents (Schlachetzki *et al.*, 2013).

2.8 Role of iPSCs in neurological disease modelling

The recently acquired ability to reprogram induced-pluripotent stem cells (iPSCs) from adult cells has made it possible to obtain patient- and disease-specific cell lines in a tissue culture dish, generating human models of human diseases (Han *et al.*, 2011). IPSCs technology was established for the first time in 2006 when Yamanaka and colleagues successfully converted mouse fibroblasts into pluripotent stem cells, a process called reprogramming, by introduction of four transcription factors, OCT4, SOX2, Kruppel like factor 4 (KLF4) and cellular-myelocytomatosis oncogen (c-MYC), in the differentiated cells (Takahashi and Yamanaka, 2006). As consequence, the newly generated stem cells, designated induced pluripotent stem cells, exhibited the morphology and growth properties of embryonic stem (ES)- cells and expressed ES cell marker genes. In the last few years, several protocols for iPSC generation have been developed (e.g. use of Sendai virus, RNA-based methods and episomes) using vectors or reagents that do not integrate or leave any residual sequences into iPSCs genome, and therefore create footprint-free iPSCs (Malik and Rao, 2013; Centeno *et al.*, 2018).

Similar to embryonic stem cells, iPSCs exhibit self-renewal and can originate cells from the three germ layers: endoderm, mesoderm, and ectoderm, which makes iPSCs an unlimited cell source, obviating the ethical problems associated with the use of human embryonic cells. Moreover, iPSCs

retain the genetic background of the donor, allowing for the creation of disease- or patient-specific cell lines with which to test potential therapeutics with minimal risk to the patient, enabling advances in drug screening in human *in vitro* models and personalized medicine (Ko and Gelb, 2014). These capabilities render iPSCs a highly versatile cellular model enabling a wide range of research studies and experimental designs like identifying transcriptomic and metabolomic markers of drug efficacy, screening in drug discover and advancing regenerative medicine (Engle *et al.*, 2018; Kuang *et al.*, 2019) (**Figure 5**).

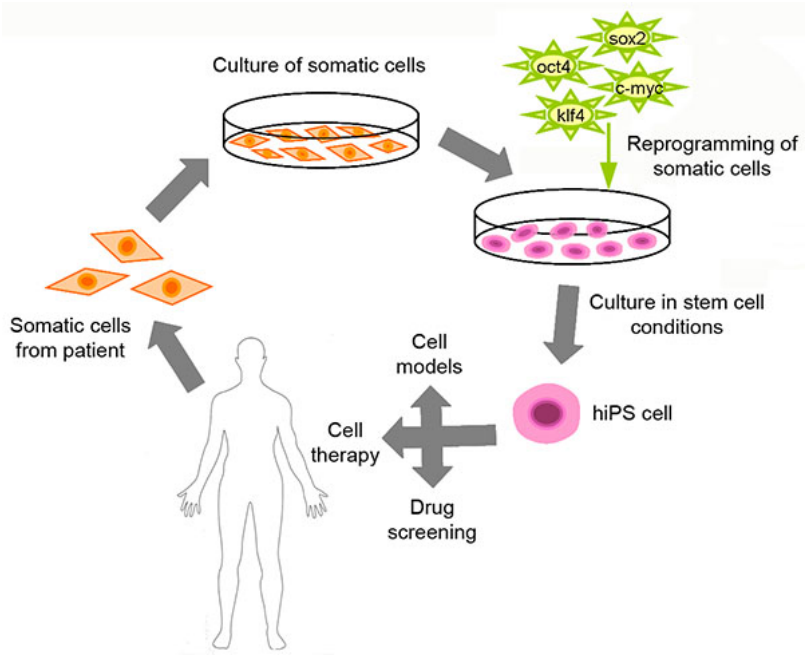

Figure 5. **Schematic representation of iPSC-technology and its applications.** Generation of iPSCs by reprogramming of adult somatic cells with OCT4, SOX2, KLF4 and c-MYC transcription factors. The newly generated iPSCs can be used as cell systems for disease modelling, cell therapy and drug screening.

In addition, one of the more relevant potentialities of iPSCs is represented by disease modelling. Recent evidences demonstrated that the number of disorders modelled via iPSCs is constantly increasing and various tissue-specific iPSC derivatives have been successfully generated for the studying of hematopoietic (Elbadry *et al.*, 2019) hepatic (Wang *et al.*, 2019) cardiovascular (Granéli *et al.*, 2019) and neurological (Li *et al.*, 2018) diseases. Moreover, the development of more sophisticated, 3D, multicellular systems, called organoids enabled to better represent the complexity of various tissues providing more relevant experimental models. Extremely important for studying neurological disorders, iPSC technology allowed the generation of all types of neural cells, including neural stem cells, neurons, astrocytes, oligodendrocytes and microglia. Moreover, by using specific differentiation protocols it is possible to generate disease-relevant neuronal

subtypes and promising reports already demonstrated that human derived cells faithfully modelled the disease related phenotypes. For example, iPSC-derived motor neurons obtained from familial Amyotrophic lateral sclerosis (ALS) patients showed mitochondrial dysfunctions (Kiskinis *et al.*, 2014) and neurite degeneration (Chen *et al.*, 2014) which are typical of ALS. In addition, evidences demonstrated that Parkinson's disease (PD) patients iPSC-derived dopaminergic (DA) neurons presented elevated *α*-synuclein levels and decrease of dopamine level and neuronal activity (Li *et al.*, 2018) characteristic of PD. Similarly, Alzheimer's disease has been successfully modelled with iPSCs as it will be extensively described in a next paragraph of this thesis. Overall, although the generation of iPSCs opened a new exciting era for the stem cells field, such technology presents some limitations. Indeed, there are some concerns regarding the lack of standardized protocols, the consequences of reprogramming protocols, and the possibility of epigenetic memory interference leading to great variability between clones and lineages and consequent doubts about reliability (Liang and Zhang, 2013; Ortmann and Vallier, 2017). However, there are strategies that may help to overcome these issues that include: obtaining cells from sources that contain less accumulated genetic mutations (i.e. younger tissues instead of aged ones); using safer reprogramming protocols (i.e. those that do not integrate into the iPSC genome or retain transgene sequences), detecting and monitoring variations in iPSC lineages, executing extensive characterization of cell lines, and standardizing protocols between laboratories (Centeno *et al.*, 2018).

2.9 An overview on Alzheimer's disease

Currently, around fifty million people worldwide have dementia, with nearly ten million new cases every year (source WHO). Alzheimer's disease is the most common form of dementia accounting for 60-70% of total cases. Recently, the Alzheimer's Association reported that between 2000 and 2017, deaths from heart disease have decreased 9% while deaths from Alzheimer's have increased 145%. Moreover, it has been estimated that dementia will cost the United States around 290 billion dollars in 2019 and these costs could rise as high as 1.1 trillion dollars by 2050 (source alz.org). Such impressive data clearly demonstrate that AD represents an urgent public issue with significant social and economic impact.

Although tremendous efforts were aimed at the development of novel therapeutic interventions for AD, any curative drug reached the market so far. One of the reasons for the lack of cure is a not fully comprehensive understanding of the disease mechanisms, due also to the uncertain etiology which characterizes majority of AD cases. Indeed, most cases (> 95%) are sporadic (sAD) with any identified cause and with an onset of the disease occurring during the seventh- eighth decade.

In contrast, a small proportion of patients (< 1%) have an early onset presenting the first clinical signs before age 65. Such cases are familial and due to genetic inherited mutations in genes encoding for the amyloid precursor protein (AβPP), presenilin 1 (PSEN1) or presenilin 2 (PSEN2) (Masters *et al.*, 2015). Patients suffering with AD develop progressive cognitive decline and abnormalities of memory, language and behaviour. The gradual cognitive loss is emblematic of the toxic changes which take place in the brain and slowly progress, leading to massive neuronal death over the years (Larson *et al.*, 1992). As consequence, a severe degeneration of multiple brain regions occurs in the brain of the patients, represented by substantial shrinkage of hippocampus and cerebral cortex and ventricles enlargement (**Figure 6A**). There are two main biochemical hallmarks which are found in the brains of people with Alzheimer's disease: the amyloid plaques (Hardy and Selkoe, 2002) and the neurofibrillary tangles, NFTs (Spillantini and Goedert, 2013) (**Figure 6B**).

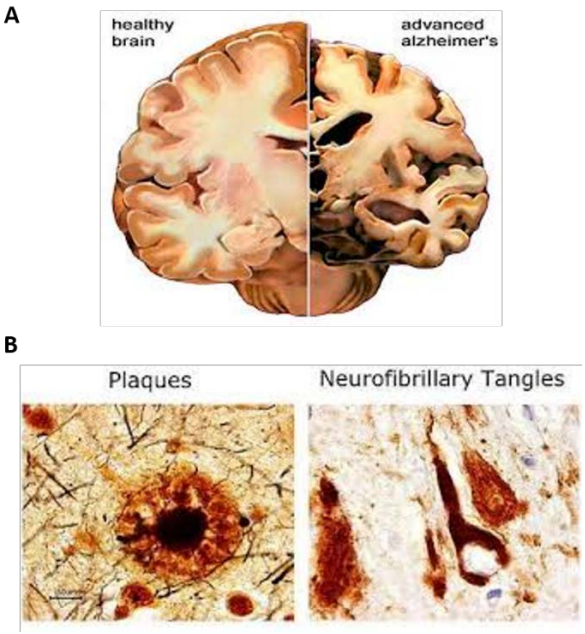

Figure 6. AD hallmarks. (**A**) Physiological differences between a healthy and AD brain section. The brain of AD patients is characterized by a severe atrophy of hippocampal regions and cerebral cortex together with evident enlargement of ventricles (*source: www.alz.org*). (**B**) Amyloid plaques and neurofibrillary tangles in Alzheimer's disease brain tissue.

Amyloid plaques were first isolated and partially characterized by Glenner and colleagues in 1984 (Glenner and Wong, 1984). These are extracellular aggregations of Aβ peptides, which are obtained from the cleavage of the amyloid precursor protein (AβPP). Longer forms of Aβ, particularly Aβ42, are especially toxic as more prone to aggregate in oligomers and plaques sequentially, which deposit in the brain of the patients (Murphy and LeVine, 2010) (**Figure 7**). Thus, studies pointed to the longer forms of Aβ (Aβ42, Aβ43…) as key players in the initiation of toxic Aβ species aggregation, which ultimately lead to neurodegeneration in AD (Haass and

Selkoe, 2007; Benilova *et al.*, 2012). The Aβ plaques constitute the assumption of the theory which has dominated Alzheimer's research for more than 25 years, the so called "amyloid hypothesis" (Hardy and Higgins, 1992). According to it, Aβ clumps represent the triggers of AD neurodegenerative processes, which lead to the loss of memory and cognitive ability.

The NFTs are instead intracellular aggregations of the hyperphosphorylated microtubule-associated protein TAU. Being encoded by MAPT gene, TAU mainly locates at the axons, promoting microtubule assembly and stability of neurons, supporting cell differentiation and polarization. During AD, TAU gets hyperphosphorylated, which causes microtubule destabilization and breakdown and the formation of intracellular tangles (Brunden *et al.*, 2009; Goedert, 2018). Recent evidences show that hyperphosphorylated TAU is also actively secreted in the extracellular space causing trans-cellular propagation and spread the TAU pathology (Yamada, 2017) (**Figure 7**).

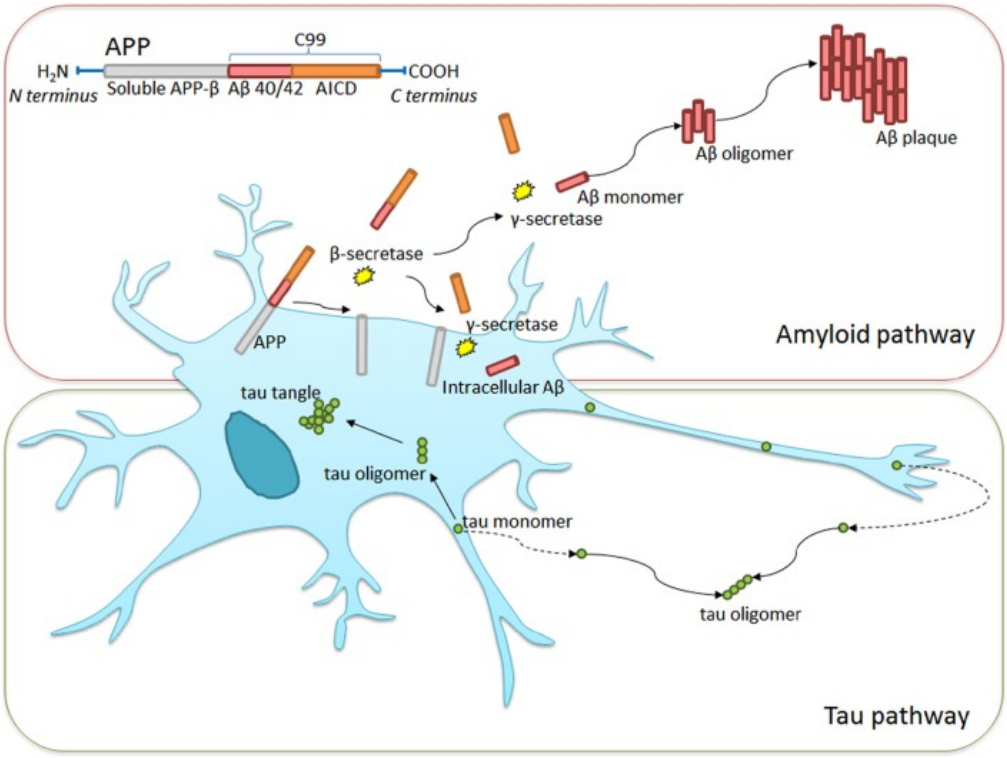

Figure 7. Alzheimer's disease hallmarks formation. AβPP protein undergoes amyloidogenic processing through sequential cleavage by β-secretase and γ-secretase, which ends with formation of Aβ peptides. The most amyloidogenic monomeric peptides (e.g. Aβ42) clump together, forming oligomers and plaques eventually. Similarly, hyperphosphorylated TAU monomers aggregate to form complex oligomers and eventual neurofibrillary tangles **[***Adapted from* (Lim *et al.*, 2016)].

Since the postulation of the "amyloid cascade hypothesis" (Hardy and Higgins, 1992), AD research has mainly focused on amyloid as the trigger of neurotoxic events, including TAU phosphorylation and NFTs. However, nowadays researchers have broadened the point of view on

AD and defined it as a multifactorial and complex disorder with several target proteins and cellular processes, contributing to its etiology (Gong *et al.*, 2018). Indeed, some hypotheses focusing to different targets, other than amyloid and TAU, have been proposed to explain Alzheimer's disease. These include for example the cholinergic hypothesis (Terry and Buccafusco, 2003), the mitochondrial hypothesis (Swerdlow *et al.*, 2014), the neuroinflammation hypothesis (Morales *et al.*, 2014), the calcium hypothesis (Berridge, 2010) and the brain-gut-microbiota axis hypothesis (Kowalski and Mulak, 2019). A common limit of these hypotheses is that they overemphasize the specific mechanism/pathway proposed and undervalue other mechanisms. Such a narrow focus appears to attribute to the failure of AD drug development during the last decades (Gong *et al.*, 2018). Moreover, another problematic aspect is the heterogeneity of AD patients. Indeed, a group of researchers has recently developed a new way to classify AD patients and identified subgroups of patients with substantial biological differences (Mukherjee *et al.*, 2018). Following to this study, researchers concluded that Alzheimer's disease should be considered as a mixture of distinctly different conditions instead of one single disease. Such novel way of thinking about AD might represent an important step toward developing more personalized treatments and better targeted clinical trials.

2.9.1 Amyloidogenic and non- amyloidogenic processing of AβPP

AβPP is a type-I trans-membrane protein ubiquitously expressed in human tissues with high expression in the central nervous system (CNS). Both AβPP751 and AβPP770 isoforms are mainly expressed in non-neuronal cells, while AβPP695 isoform is predominantly expressed in neurons. The three isoforms share a common structure with a large extracellular domain, a short transmembrane domain and an intracellular domain (Wang *et al.*, 2017). While trafficking through the secretory pathway, nascent AβPP is post-translationally modified by N- and O-linked glycosylation, ectodomain and cytoplasmic phosphorylation, and tyrosine sulphation (Haass *et al.*, 2012). Once AβPP reaches the cell surface, it is rapidly internalized and subsequently trafficked through endocytic and recycling organelles to the trans-Golgi network (TGN) or the cell surface (Haass *et al.*, 2012). AβPP cleavage can end up with amyloid peptide production, depending on the proteolytic processing that it undergoes. Indeed, AβPP can be processed according to different cleavages: -the amyloidogenic pathway, from which Aβ is generated; -the non-amyloidogenic pathway, which is the most abundant cleavage of AβPP that prevents Aβ generation (**Figure 8**).Such processing pathways depend on the activity of different proteolytic enzymes, α-, β-, and γ-secretase, which sequentially cleave AβPP (Haass *et al.*, 2012).

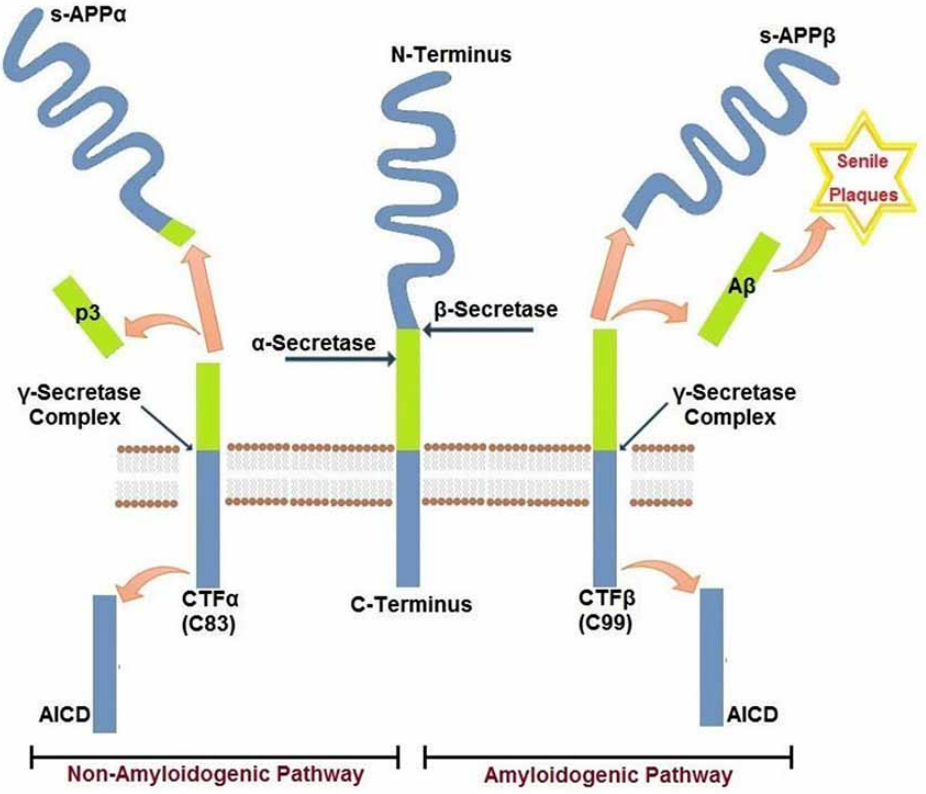

Figure 8. Amyloidogenic and non-amyloidogenic pathways of AβPP. Amyloid precursor protein AβPP is a single pass transmembrane glycoprotein. AβPP may be cleaved by β–γ secretases (amyloidogenic) releasing amyloid Aβ peptide(s) or by α–γ secretases (non-amyloidogenic). The two pathways are mutually exclusive and are detailed in the text**.** [*Adapted from* (Canobbio *et al.*, 2015)].

According to the non-amyloidogenic pathway, AβPP is cleaved by α-secretase to generate a N-terminal secreted AβPPα (sAβPPα) and a carboxy-terminal fragment (CTF) of 83 amino acids (C83). C83 is further cleaved by γ-secretase to release a 3 kDa product (P3) and AβPP intracellular domain (AICD) (**Figure 8**). Interestingly, sAβPPα, is thought to have neurotrophic and neuroprotective properties and its levels are strongly reduced in AD patients with one of two copies of APOE4 (Tackenberg and Nitsch, 2019). Therefore, pharmacological up-regulation of α-secretase and targeting its signalling mechanism has been proposed as an alternative therapeutic approach for AD (Bandyopadhyay *et al.*, 2007). Instead, in the amyloidogenic pathway, AβPP is primarily processed by β-secretase which generates secreted AβPPβ (sAβPPβ) and C99 (Wang *et al.*, 2017). Thereafter, γ-secretase further processes C99 to produce Aβ peptides of different lengths (**Figure 8**). The γ-secretase is a complex enzyme, whose catalytic core includes the PSENs. Interestingly, AD causative PSEN mutations consistently decrease γ-secretase processivity (number of cuts per substrate molecule), thereby shifting Aβ profiles toward longer and more amyloidogenic peptides (Chávez-Gutiérrez *et al.*, 2012; Szaruga *et al.*, 2017).

More recently, Willem and colleagues described a third physiological AβPP processing pathway, the so called η-Secretase processing (Willem *et al.*, 2015). According to it, the membrane-bound matrix metalloproteinases MT5-MMP cleaves AβPP695 at amino acids 504–505 to generate a higher molecular mass carboxy-terminal fragment of AβPP, termed CTF-η (C191). CTF-η is enriched in an AD mouse model and human AD brains and it can be further processed by ADAM10 and BACE1 generating Aη-α and Aη-β, respectively (Willem *et al.*, 2015). Interestingly, Baranger et al. reported that MT5-MMP is a pro-amyloidogenic secretase, promoting amyloid pathology and cognitive decline in AD model mice (Baranger *et al.*, 2016).

2.10 The difficulty of modelling AD with conventional systems

Over the last three decades AD has been the focus of intense investigations which led researchers to unravel the fundamental processes that characterize the disease. However, the lack of a cure indicates that we have still an incomplete understanding of the disease mechanisms, especially regarding the onset of AD.

The main difficulties in studying AD are represented by the limited access to human brain and the usage of post-mortem samples from the patients. Such analyses give limited or any information regarding the early stage of the disease, which is the most important to understand from a therapeutic point of view.

Since the late 90's, AD research mainly relied on usage of transgenic animal models. Numerous rodent models have been developed which express human AβPP protein with one or more fAD mutations (Cavanaugh *et al.*, 2014). The PDAPP mouse was first described in 1995 (Games *et al.*, 1995) and is considered one of the earliest mouse models of Alzheimer's disease. Carrying the so called "London" mutation in AβPP (V717I), from a young age this model exhibits cognitive deficits in a variety of tasks, including spatial working memory at four months as assessed by the Morris water maze (Hartman *et al.*, 2005). Such cognitive impairments present prior to plaque deposition, which occurs at approximately six months of age. Moreover, in other AβPP transgenic mouse models, there is evidence of inflammation in the brain, and the animals display cognitive and behavioural deficits compared to wild-type animals (Games *et al.*, 1995; Sturchler-Pierrat *et al.*, 1997; Verret *et al.*, 2007). In addition, doubly transgenic mice expressing both mutated AβPP and PSEN1 were found to develop Aβ plaques at a much earlier age than mice expressing mutated AβPP alone, with more severe Aβ plaque formation, neuroinflammation and cognitive decline (Casas *et al.*, 2004; Oakley *et al.*, 2006; Sasaguri *et al.*, 2017). However, crucial aspects of the disease, like NFT formation and severe neuronal loss, have never been convincingly demonstrated in AD transgenic models (Zhu and Zhang, 2017) thus failing to fully reproduce the entire biology

of the human disease (Mullane and Williams, 2019). Only the development of triple transgenic mice which, in addition to mutations in AβPP and PSENs, also included human mutated form of TAU protein reproduced the NFT formation (Oddo *et al.*, 2003). However, TAU protein mutations are associated with frontotemporal dementia, FTD, but not with AD, which raised some concerns about the relevance of these models for AD. More importantly, the relevance of these genetic models to the study of sporadic AD (sAD) patients, who do not carry mutations in AβPP or PSENs, is still a matter of debate (Zhu and Zhang, 2017).

Additionally, evidences demonstrated that usage of transgenic models overexpressing the fAD clinical mutations often led to an overestimation of the efficacy of candidate drugs. This was the case for a group of nonsteroidal anti-inflammatory drugs (NSAIDs), including flurbiprofen and indomethacin, which were highly efficacious in lowering the production of Aβ42 in transgenic immortalized cell lines (Weggen *et al.*, 2001), while failed to exert any positive effect in human derived neurons (Mertens *et al.*, 2013) and in clinical trials (de Jong *et al.*, 2008). Such limitation of transgenic models might explain why drugs that successfully treated transgenic mice did not work in people (King, 2018).

In light of these evidences, human iPSC-neuronal cells derived from patients promise to provide more relevant platforms for modelling AD and test novel therapeutics.

2.11 Modelling AD with iPSCs

Remarkably, development of induced pluripotent stem cell-(iPSC) technology represented a ground-breaking discovery which provided the opportunity to partially overcome the limitations of transgenic models and to alternatively approach AD studying.

Increasingly, laboratories are creating their own banks of iPSCs derived from diverse donors. Following controlled induction process, iPSCs can be turned into specific cell types, including cells from neuronal lineage. Such potentialities make iPSCs highly relevant for studying neurological disorders, as for example Alzheimer's disease. Several groups worldwide, including ours, have used iPSC technology to generate human iPSC-derived neurons from individuals with familial and sporadic AD. Overall, neuronal cells generated from patients displayed augmented amyloid levels and increased Aβ42/Aβ40 ratios compared to neurons obtained from healthy individuals (Yagi *et al.*, 2011; Muratore *et al.*, 2014; Mahairaki *et al.*, 2014; Ochalek *et al.*, 2017). In addition, iPSCs allowed to recapitulate other important mechanisms of the disease. An *in vitro* study employing iPSC-three-dimensional (3D) neurons revealed that 3D cultures from AD subjects presented altered levels of proteins involved in axon growth, mitochondrial function, and

oxidative stress, which were comparable to the dysfunctions found in post-mortem AD brain tissues (Chen *et al.*, 2018). Moreover, in a recent work, Arber and colleagues analysed matched CSF, post-mortem brain tissue, and iPSCs from the same individual with the AβPP V717I mutation (Arber *et al.*, 2019). Authors reported that the Aβ38/Aβ40 ratio measured in cerebrospinal fluid (CSF) from the patient was similar to the ratio measured in the cell culture medium of iPSC-neurons derived from the same subject (Arber *et al.*, 2019). Such result provides the first correlation between CSF profiles from patients and their own Aβ secretome in the iPSC-neuronal cultures, thus substantiating patient-differentiated neurons as a valid tool to mimic and study the mechanisms of the pathology (Kondo *et al.*, 2017). In addition to amyloid defects, evidences from patients-derived neurons showed that iPSC system allows also the modelling of TAU hyperphosphorylation and increased GSK3B activity (Israel *et al.*, 2012; Choi *et al.*, 2014) (Ochalek *et al.*, 2017; Chang *et al.*, 2018). Based on these evidences, iPSC-derived neuronal cultures enable to recapitulate the early AD pathogenic processes without any transgenic intervention or exogenous toxic treatment, which instead are required when using other conventional models as transgenic models or immortalized cell lines. Thus, iPSCs-based research promises to greatly contribute to advances in the understanding of neurodegenerative processes and identifying novel therapeutic targets.

2.12 The role of CaSR in Alzheimer's disease

As briefly anticipated in the previous paragraph, studies demonstrated that CaSR is involved in the processes underlying AD. A first evidence of such involvement dates back to late 1990's, when Ye and co-workers observed that Aβ peptides (Aβs) activated NCCs in cultured hippocampal neurons of wild type mice (Ye *et al.*, 1997a). Authors reported that NCCs activation was likely mediated by CaSR via elevation of intracellular Ca^{2+}, as it was not observed in neurons from mice with constitutive receptor deletion (CaSR-/-) (Ye *et al.*, 1997a). More recently, a discrete expression of CaSR protein has been reported in normal adult human astrocytes (NAHAs) (Armato *et al.*, 2013) and, through in situ proximity ligation assay, the binding between the receptor and Aβ has been showed to occur at the plasma membrane of astrocytic cells (Dal Prà *et al.*, 2014). Another study, using a luciferase-reporter gene assay, demonstrated that exogenous Aβ1-42 as well as APOE activated the CaSR signalling in Cos 1 cells transfected with the receptor (Conley *et al.*, 2009). Additionally, using a cohort of AD patients and control subjects, authors identified a genetic association for the CaSR with AD status only in subjects without an APOE4 allele (Conley *et al.*, 2009), which is known to be a susceptibility gene for late-onset Alzheimer's disease (De Luca *et al.*, 2019). Further, immunohistochemical analyses on a triple transgenic mouse AD model

(3xTg-AD) revealed an augmented expression of CaSR in hippocampal CA1 area and in dentate of AD mice compared to the receptor levels in WT animals. Such increment which was already significant at 9 months of age, continued to increase at 12 and 18 months in parallel with the accumulation of β-amyloid plaques, suggesting an interplay between the CaSR expression and amyloid accumulation (Gardenal *et al.*, 2017). However, any *in vivo* study tested the calcilytic effect on AD phenotype so far. Recently, new insights on the CaSR's contribution to AD have been obtained thanks to works which investigated the effects of calcimimetic and calcilytic drugs on amyloid production and secretion. ELISA and Western blot analyses of amyloid levels produced by NAHAs and human cortical postnatal neurons (HCN-1A) revealed that cells, exposed to exogenous fibrillary Aβ (fAβ25–35), presented an increased Aβ42 intracellular accumulation and secretion. This increment was further augmented by treatment with the calcimimetic NPS R-568 (Armato *et al.*, 2013). Noteworthy, incubation with calcilytic NPS 2143 significantly suppressed the fAβ25–35-mediated surges of endogenous Aβ42 secretion by astrocytes and neurons. Such results suggested that activation of CaSR could contribute to worsen AD by inducing amyloid accumulation, thus supporting the receptor's inhibition as a potential therapeutic approach for AD (Armato *et al.*, 2013). This idea was substantiated by further evidences which revealed new details regarding the calcilytic's mechanism of action in AD. Interestingly, NAHAs exposed to exogenous fAβ25–35 presented a dramatic reduction of secreted sAβPPα, in addition to the cited Aβ42 over secretion. The concomitant reduction of sAβPPα and increase of Aβ supported the prevailing of the amyloidogenic pathway of AβPP over the non-amyloidogenic pathway in fAβ-treated NAHAs (Chiarini *et al.*, 2017a). Together with suppressing Aβ release, NPS 2143 significantly increased the extracellular shedding of sAβPPα by driving the translocation of both AβPP and α-secretase ADAM10 to the plasma membrane. Therefore, calcilytic efficiently restored non-amyloidogenic processing of AβPP (Chiarini *et al.*, 2017a). Interestingly, an involvement of CaSR in the mechanism underlying phosphorylation of TAU protein has been recently proposed (Chiarini *et al.*, 2017). NAHAs exposed to fAβ25−35 exhibited an increased activity of glycogen synthase kinase 3 (GSK)-3β responsible to mediate TAU phosphorylation. As consequence, an increase of p-TAU oligomers, released extracellularly within exosomes, was observed in fAβ-treated astrocytes. Remarkably, incubation with NPS 2143 fully restored GSK-3β activity and totally blocked any increase in p-Tau levels (Chiarini *et al.*, 2017). Taken together, these evidences strongly support the hypothesis according to which the CaSR expressed in neural cells is activated by Aβ peptides overproduced during AD. Such CaSR/Aβ signalling would worsen AD phenotype by feeding a vicious cycle. Accordingly, treatment with calcilytic might block the CaSR/Aβ signalling and represent a potential therapeutic for AD, which constitutes the rationale behind this project thesis (**Figure 9**).

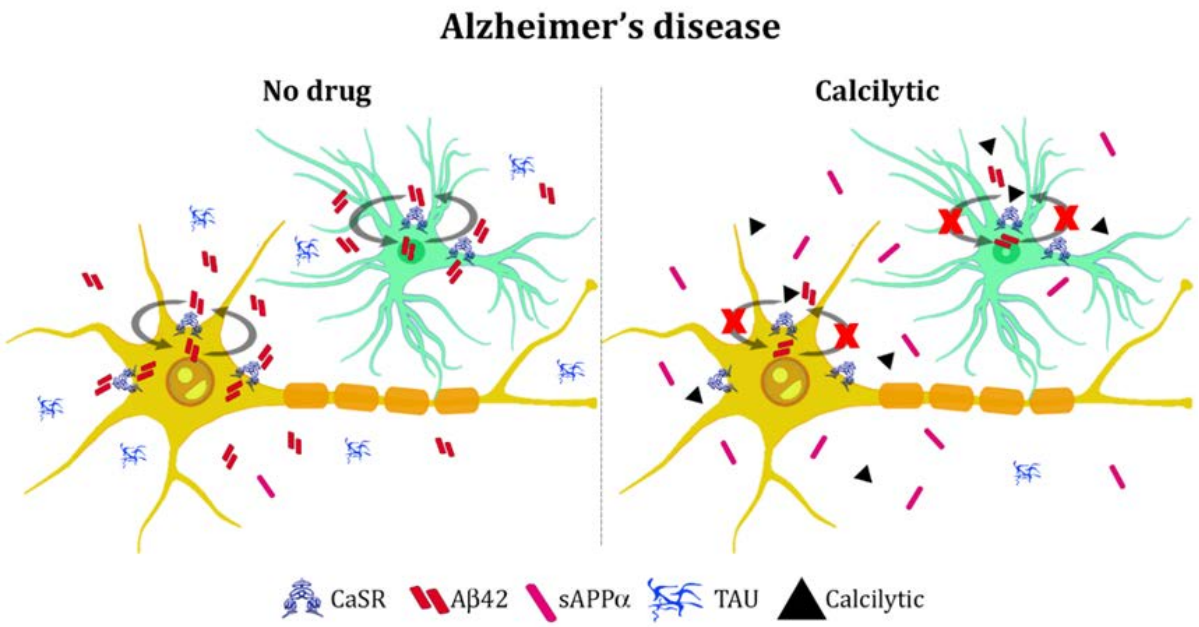

Figure 9. Model of the calcilytic effect in Alzheimer's disease. Extracellular Aβ42, is over-secreted during Alzheimer's disease (AD) and binds the CaSR at the plasma membrane of neurons (yellow cell) and astrocytes (green cell). The Aβ42/CaSR-activated signalling mediates a further release of de novo produced Aβ42 and hyperphosphorylated TAU, so feeding a vicious cycle. Moreover, the secretion of sAβPPα is dramatically reduced. Treatment with a calcilytic blocks this signalling by reducing Aβ42 and TAU secretion while increasing the release of sAβPPα [*Adapted from* (Giudice *et al.*, 2019)].

10.14751/SZIE.2020.001

3 MATERIALS AND METHODS

The chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and the cell culture reagents and culture plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA), unless specified otherwise.

3.1 Mouse embryonic stem cell culture

The mouse embryonic stem cell (mESC) line HM1 (129/Ola mouse strain origin) was used in the experiments. The pluripotent cells were maintained on early-passage mitotically inactivated (Mitomycin C treated) mouse embryonic fibroblast feeders (MEF) and cultured in mESCs medium: Dulbecco's modified Eagle Medium Nutrient Mixture F-12, containing 10% (vol/vol) FBS, 2 mM sodium pyruvate, 2 mM glutamax, 1X nonessential amino acids (NEAA), 50 μM β-mercaptoethanol (β-ME), Pen/Strep 50 U/mL, and mouse leukaemia inhibitor factor (LIF) 1000 U/mL. The cells were passaged before reaching 70% confluency (approximately every 2 Days).

3.2 Induction of neuronal differentiation of mouse ESCs

For neuronal differentiation, mESCs were starved for 12 h the night before passaging in mouse neuronal differentiation medium, short name: B27 media; composition: DMEM/F12, N2, B27, 1X non-essential amino acids (NEAA), 50 μM β-mercaptoethanol (β-ME), Pen/Strep 50U/mL, and 10 ng/ml bFGF. On the next morning cell cultures were trypsinized, and pre-plated onto cell culture dishes (10 cm dish) for 30 min (through microscope control). In this step MEF cells soon attach to the surface of the dish while the ESCs are still floating in the media, making easier the collection of mESCs. Thereafter, ESCs are carefully centrifuged, re-suspended in B27 medium and plated onto matrigel-coated dishes at 12.000 cells/cm^2 density (both for Western Blot, WB and immunocytochemistry, ICC). The medium was renewed every day during the 7-days differentiation.

3.3 Terminal differentiation of human iPSC-derived NPCs into neurons

Induced pluripotent stem cells, iPSCs, were previously reprogrammed from a familial AD (fAD) patient with a genetic mutation in PSEN1 gene which leads to an amino-acid change (p.Val89Leu), as published in details earlier (Nemes *et al.*, 2016). Afterwards, neuronal progenitors, NPCs, were obtained and neuronal differentiation was characterized and published recently (Ochalek *et al.*, 2017). In the present study, we used two control NPCs, named Ctrl-1 and Ctrl-2, derived from two healthy individuals, and the PSEN1 mutant iPSC derived NPCs (named fAD-1), which we used in our previous study (Ochalek *et al.*, 2017).

Control and fAD NPCs were plated on poly- L-ornithine/laminin (POL/L) -coated dishes, and maintained 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 mML-glutamine, 50 U/ml penicillin/ streptomycin) supplemented with 10 ng/ml EGF and 10 ng/ml bFGF. To differentiate neuronal progenitors into neurons, NPCs were cultured for 6 weeks in NMM supplemented with 0.2 mM ascorbic acid and 25 μM β-mercaptoethanol, with medium changed every 3-4 days. Cells were plated on coverslips at a seeding density of 40.000 cells/cm^2 for ICC and calcium imaging and on 6-well dishes at density of 100.000 cells/cm^2 for ELISA, Western blot and Biotinylation experiments. To confirm differentiation of NPCs into neurons, neuronal expression markers and functional properties were assessed by ICC and calcium imaging respectively.

3.4 Calcium imaging

Functional properties of the neurons were assessed after 4 weeks of differentiation. Ctrl-1 and fAD-1 cells were incubated with 1 μM fura-2-AM for 30 minutes at 37°C. Coverslips were then placed in a perfusion chamber mounted upon an Olympus IX71 inverted microscope equipped with a Cairn monochromator-based epifluorescence system (Cairn Instruments, Faversham, UK). Solutions and agonists were locally applied to the neurons using a rapid solution changer (RSC160, Intracel RSC160, Intracel, Royston, UK) as follows: 60 mM KCl solution to determine expression of functional voltage- gated Ca2+ channels; 100 μM GABA in physiological extracellular solution, (ECS), or in reduced Cl- solution (isosmotic replacement of NaCl with Na-isethionate in extracellular solution, resulting in 7.5 mM Cl- concentration) to test whether GABA induced an excitatory or inhibitory response, representative of immature or mature neuron respectively; 100 μM AMPA and 100 μM Kainic acid to assess expression of ionotropic glutamate receptors. Fura-2 was alternately excited with light of 340 and 380 nm and images were captured at 510 nm using an Orca CCD camera (Hamamatsu Photonics, Welwyn Garden City, UK). Following background subtraction of the emission intensities evoked by each excitation wavelength, emission ratios (340/380) were calculated offline. These experiments were performed in the laboratory of School of Biosciences, Cardiff University, with the supervision of Professors Daniela Riccardi and Paul J. Kemp.

3.5 Immunocytochemistry

Neuronal differentiation and CaSR expression were evaluated in mouse ESC-derived neurons and human iPSC-derived neurons through immunocytochemical analyses. Both mouse and human cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). Cells were washed twice with phosphate buffered saline (PBS) and permeabilized with 0.2% Triton X-

100 in PBS for 15 minutes. Then, cells were blocked with 3% bovine serum albumin (BSA) in the presence of 0.2% Triton X-100 in PBS for 60 minutes at RT. The respective primary antibodies were applied overnight at 4°C (Supplementary **Table 1**). To detect the signal, cells were incubated for 1 hour at RT with the appropriate secondary antibodies (Supplementary **Table 1**). Cell nuclei were visualized using Vectashield Mounting Medium with DAPI (1.5 µg/ml; Vector Laboratories). Samples were analysed under fluorescent microscope equipped with 3D imaging module (Axio Imager system with ApoTome; Carl Zeiss MicroImaging GmbH) controlled by AxioVision 4.8.1 software (Carl Zeiss). Alternatively, images were acquired with a Zeiss LSM 880 confocal laser scanning microscope using the ZEN Imaging Software (Carl Zeiss AG, Germany).

3.6 Lysis of mouse ESC-neurons and human iPSC-neurons

Mouse ESC-derived neurons and human iPSC-derived neuronal cultures were collected at the end of the differentiation process (mouse: TD7; human: TD42). Cells were lysed with RIPA Lysis and Extraction Buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail and Pierce™ Universal Nuclease for Cell Lysis. Lysed samples were sonicated and centrifugated at 13,000 rpm for 20 min to pellet unbroken nuclei and cell membranes. Cell lysates (supernatants) were transferred in new tubes and stored at -80°C. Protein extracts were quantified using a Pierce BCA Protein Assay Kit following the manufacturer's protocol.

3.7 Lysis of brain and kidney from human and mouse tissues

Mouse brain and kidney tissue samples were kindly provided by Prof. Daniela Riccardi (University of Cardiff, UK). Human brain cortex sample was provided by the Human Brain Tissue Bank, HBTB (Semmelweis University, Budapest, Hungary). Mouse and human tissue samples were homogenized in RIPA Lysis buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail and Pierce™ Universal Nuclease for Cell Lysis. Cell lysis was carried out using a tissue homogenizer. Following lysis, homogenates were centrifuged at 13,000 rpm for 20 min to remove insoluble debris. Tissue lysates (supernatants) were transferred into new tubes and stored at -80°C until use. Protein extracts were quantified using the Pierce BCA Protein Assay Kit. Human kidney lysate was purchased from Abcam.

3.8 Transfection of SH-SY5Y neuroblastoma with pcDNA-5-FRT-HA-tag-hCaSR

SH-SY5Y neuroblastoma cell line was grown with culture medium (1:1 vol/vol mixture of Minimum Essential Medium Eagle and Ham's F-12 Nutrient Mixture, complemented with 10% fetal bovine serum (FBS), 1× NEAA, 2 mM L-glutamine, 50 U/ml penicillin/ streptomycin), at 37°C and 5% CO_2. Cells were transfected with pcDNA-5-FRT-HA-tag-hCaSR (provided by Prof.

Hans Bräuner-Osborne, University of Copenhagen, Denmark) using Lipofectamine® 2000 reagent. Transient transfections were performed according to manufacturers' instructions using approximately 70-80% confluent cells. Briefly, DNA-Lipofectamine® 2000 complexes were prepared in Opti-MEM® Reduced Serum Medium and incubated at room temperature for 5 minutes. The DNA-lipid complexes were then added to the cells which were incubated at 37°C and 5% CO_2. After 48 h of transfection, cells were processed for cell lysis and protein quantification similarly to the iPSC-derived neuronal cultures (see paragraph 3.6).

3.9 Immunoblotting

Cell lysates (5-50 μg) from human iPSC-derived neuronal cultures, SH-SY5Y-HA-tag-hCaSR and from brain and kidney tissues were separated on 12% or 7.5% precast gels and transferred to Immun-Blot® PVDF Membrane (Bio-Rad). The membranes were blocked by Tris-buffered saline with Tween 20, TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% BSA or non-fat milk and then incubated with the respective primary antibody solution overnight at 4°C (Supplementary **Table 2**). After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT (Supplementary **Table 2**). Signals were detected with SuperSignal™ West Dura Extended Duration Substrate by KODAK Gel Logic 1500 Imaging System and Kodak MI SE imaging software. Densitometry measurement of protein bands intensity was carried out using Image Studio™ Lite software (LI-COR).

To investigate the extracellular release of sAβPPα, equal volume of conditioned medium samples was separated on 12% precast gels. Protein transfer to PVDF Membrane, filter blocking and antibodies incubation were performed similarly to the cell lysate samples. Densitometry measurement of sAβPPα bands were normalized to the total protein content of the cell lysates.

As it will be discussed in the Results section, the full-length AβPP along with the AβPP-C-terminal fragment were detected in cell lysates through immunoblotting with anti-AβPP CTF antibody (Supplementary **Table 2**), while the secreted sAβPPα were immunodetected with the anti- anti-β-Amyloid, 1-16 Antibody (6E10) (Supplementary **Table 2 and Figure 10**).

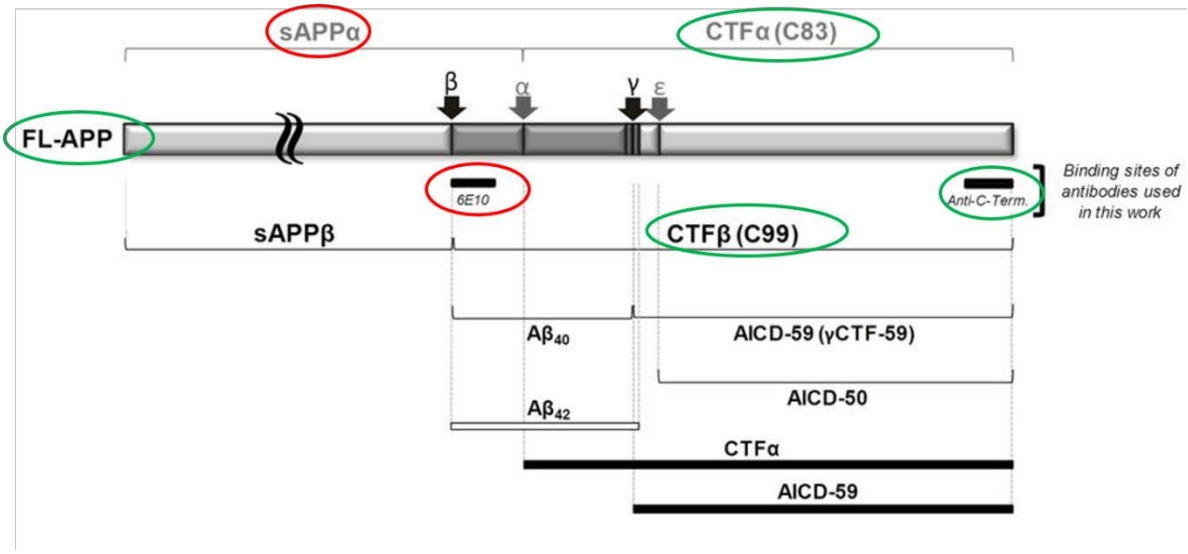

Figure 10. Schematic representation of AβPP processing products, including the binding sites of the antibodies utilized in this work. The binding sites on AβPP and its post processing products, of 6E10 (red) and anti AβPP-C-terminal (green) antibodies are shown. [*Adapted from* (Branca *et al*., 2015)].

3.10 Treatment with γ-secretase inhibitor DAPT and calcilytic NPS 2143

Neuronal cultures differentiated for 6 weeks were treated with γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-Lalanyl]- S-phenylglycine t-butyl ester;) or with calcilytic NPS 2143 hydrochloride (2-chloro-6-[(2R)-3-1,1-dimethyl-2-(2-naphtyl)ethylamino-2-hydroxypropoxy]-benzonitrile hydrochloride; Tocris Bioscience). Both DAPT and NPS 2143 were dissolved in DMSO and next diluted in NMM medium at a final concentration of 1 μM. In the experiments with the γ-secretase inhibitor, cells were treated with DAPT or vehicle for 48 h, followed by media collection and harvesting for protein. For the experiments with NPS 2143, cells were added with fresh media containing NPS 2143 or vehicle. After 24 h, the conditioned media were temporarily collected in sterile tubes and neuronal cultures were exposed for 30 minutes to either NPS 2143 or vehicle dissolved in fresh medium. Then, the previously cell-conditioned media, supplemented with a second pulse of calcilytic or vehicle, were added again to the plates. At the end of treatments (48 h), collection of conditioned media and cell lysates or biotinylation and isolation of cell surface proteins were performed.

3.11 ELISA of Aβ40 and Aβ42 secreted in conditioned media

After 48 h treatment with DAPT or NPS 2143, conditioned media were collected. Extracellular Aβ40 and Aβ42 levels were measured using Human β-Amyloid (1-40) ELISA Kit and Human β-Amyloid (1-42) ELISA kit, highly sensitive (FUJIFILM Wako Pure Chemical Corporation of Japan), according to the manufacturer's instructions. The signal was detected with Varioskan Flash

Multimode Reader (Thermo Fisher Scientific). The secreted Aβ levels were normalized to total protein content of cell lysate.

3.12 Biotinylation and isolation of plasmalemmal proteins

Following to 48 h treatment with calcilytic, biotinylation and isolation of proteins at cell surface, were performed using the Pierce TM Cell Surface Protein Isolation Kit. Briefly, after collection of cell culture media, cells were washed twice with ice-cold PBS and incubated with Biotin solution for 30 minutes at 4°C with gentle agitation. Biotinylation reaction was stopped by adding Quenching Solution. Cells were harvested by gentle scraping and pelleted by centrifugation at 500×g for 5 minutes at 4°C. After washing with TBS, cell pellets were lysed using the provided Lysis Buffer (Pierce) containing a protease inhibitor cocktail for 30 minutes on ice with intermittent vortexing. Lysates were centrifuged at 10,000×g for 2 minutes at 4°C and clarified supernatants were incubated for 1 h at RT to allow the biotinylated proteins to bind to the NeutrAvidin Gel. The unbound proteins, representing the intracellular fractions named "Flow-troughs" (FT), were collected by centrifugation of the column at 1,000×g for 2 minutes. Finally, the biotinylated surface proteins were incubated with SDS-PAGE Sample Buffer for 1 h at RT and were collected by column centrifugation at 1,000×g for 2 minutes as "Eluate" fractions (E). FT and E samples were loaded on precast gels for WB analyses.

3.13 Statistical Analysis

Data were analysed using GraphPad Prism 5 software. Analysis of data was presented in the form of mean±S.E.M. (standard error of the mean). Statistical significance was tested by either Student's t-test (two-tailed) or one-way ANOVA with Tukey's post-test. In all cases, significance was noted at $*p<0.05$.

4 RESULTS

4.1 Characterization of mouse-ESCs derived neurons

As a first experiment, we investigated the expression of CaSR in mouse stem cell derived neuronal cultures. Therefore, pluripotent mouse ESC cell line HM1 were successfully differentiated into neurons using B27 supplement induction method (Bian *et al.*, 2016) within 7 days. Immunocytochemistry (ICC) staining revealed the expression of pluripotency markers OCT4 (POU domain, class 5, transcription factor 1, Pou5f1), SRY (sex determining region Y)-box 2 (SOX2) and Nanog homeobox (NANOG) in the cells before neuronal induction, proving the pluripotent state of the PSCs before the differentiation. Cells also express the Stage Specific Embryonic Antigen-1 (SSEA-1) which is specific for the mouse PSCs (**Figure 11**)

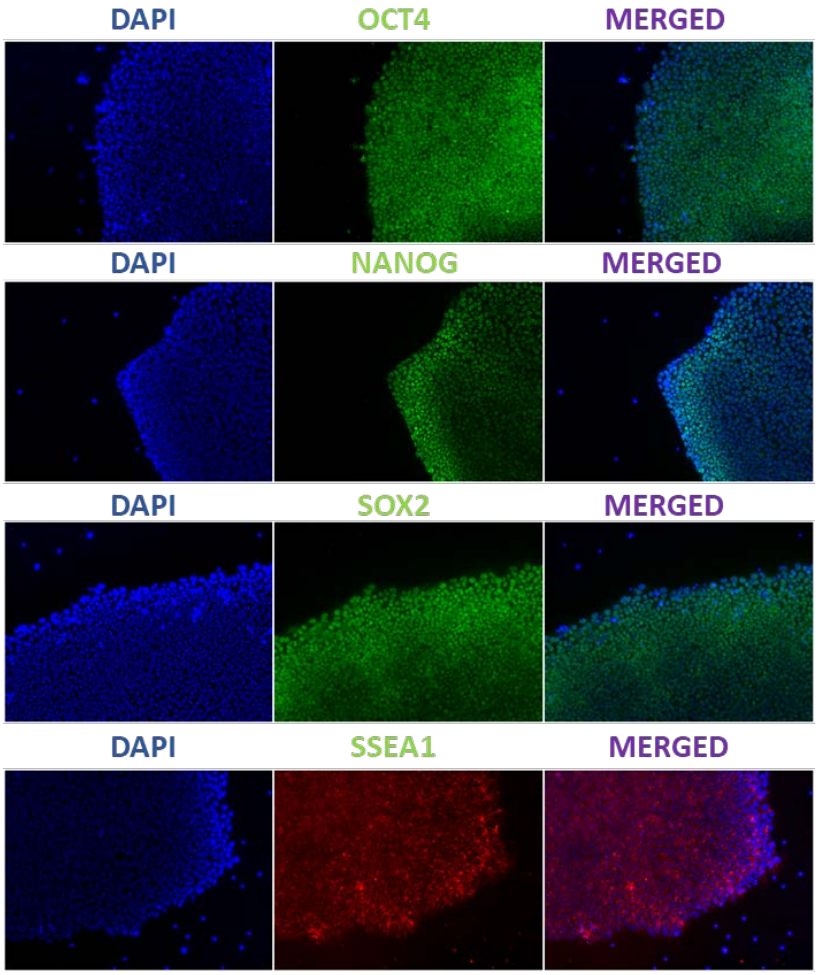

Figure 11. Immunostaining of pluripotent stem cells (PSCs). Representative immunostaining of mESC line expressing pluripotency markers OCT4, NANOG, SOX2 and SSEA-1, specific for mouse stem cells.

Neuronal precursor cells (NPCS) were positive for intermediate filament Nestin (NES) and the relevant early marker of neuroectodermal lineage commitment, the paired box protein Pax-6 (PAX6) and SRY (sex determining region Y)-box 1 (SOX1) transcription factor expression. OCT4

expression was still detectable in differentiating cells at Day2, but disappeared until Day6 **(Figure 12**), as differentiation proceeded. While tubulin, beta 3 class III (TUBB3) was observed at day 6 of the differentiation in line with microtubule-associated protein 2 (MAP2), representing the differentiated neurons (**Figure 12**). We have to note that, although the differentiation of mouse PSCs were very fast compared to human cells (see details in later chapters), however, the cell population was mixed, early NPCs and maturing neurons were detectable at the same time with similar density in 6 days cultures (**Figure 12**).

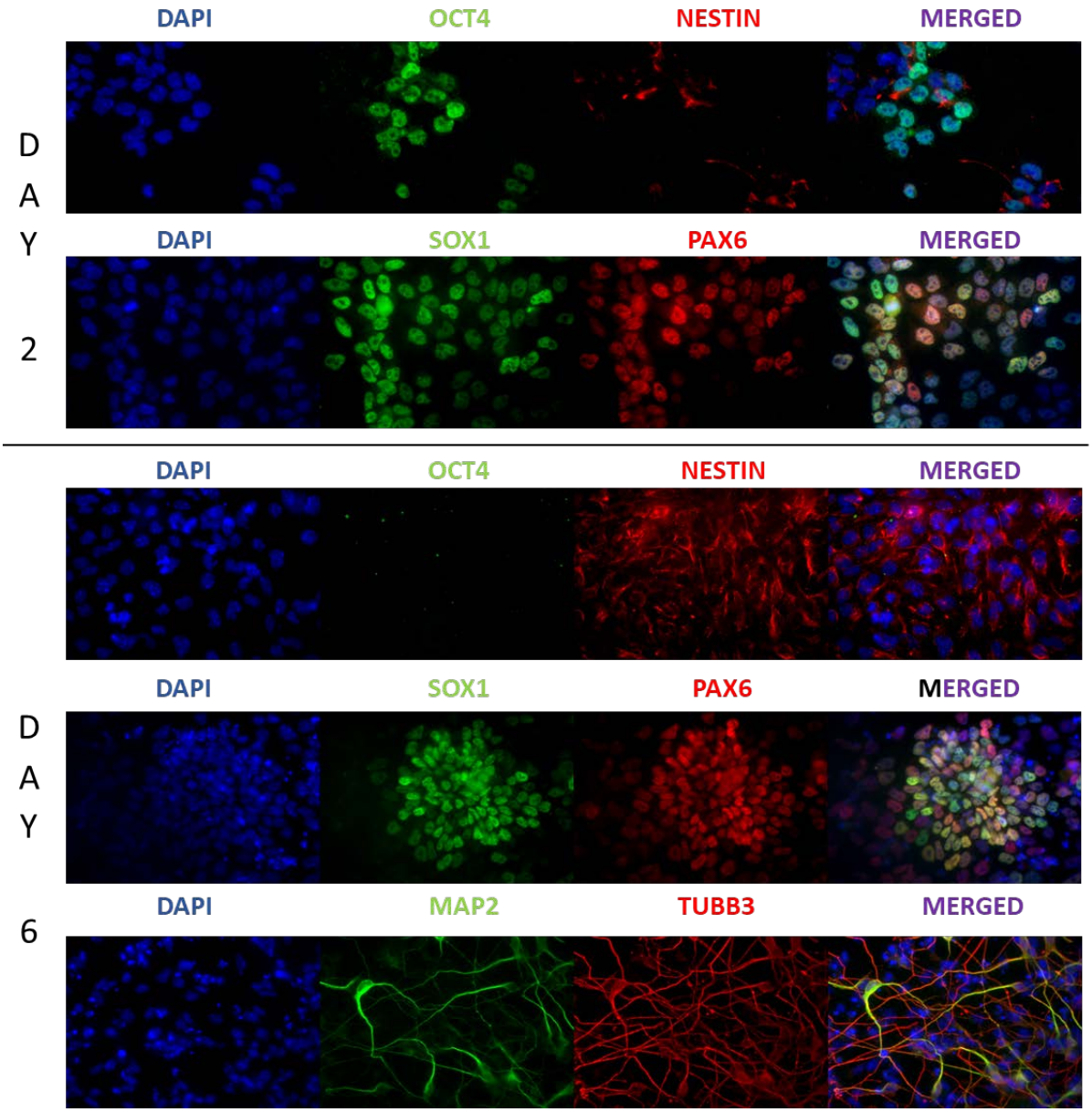

Figure 12. Neuronal differentiation of mESCs (**A**) Representative immunocytochemical analyses showing the differentiation of mESCs, expressing the pluripotency markers OCT4 (green) early neuronal markers PAX6 (red), SOX1 (green) and NESTIN (red) at Day 2 of neuronal differentiation. Until day 6, OCT4 expression disappeared from the cells, keeping PAX6, SOX1 and NESTIN active, while neuronal markers TUBB3 (red) and MAP2 (green) appeared in the neurites. Nuclei were counterstained with DAPI (in blue).

The results demonstrated a successful neuronal differentiation of the mouse PSC which was the first step towards to identify the expression of the receptor in *in vitro* mouse tissues.

4.2 Validation of anti-CaSR antibody specificity on SH-SY5Y-HA-Tag-hCaSR neuroblastoma

In order to validate the specificity of anti-CaSR antibody, first we tested the antibody on SH-SY5Y neuroblastoma cells transiently transfected with HA-Tag-hCaSR. Evidences from literature showed that CaSR is low or not expressed in neuroblastic tumors due to epigenetic silencing (Casalà *et al.*, 2013) which makes neuroblastoma cell lines suitable for studying the exogenous overexpression of the receptor, without "background" endogenous expression. SH-SY5Y-HA-tagged-hCaSR were immunoblotted with an anti-HA tag antibody or with the anti-CaSR antibody (ADD clone). Both antibodies recognized similar bands at ~130-140 kDa and ~260-280 kDa which represent putative monomers and dimers of the receptor (**Figure 13**).

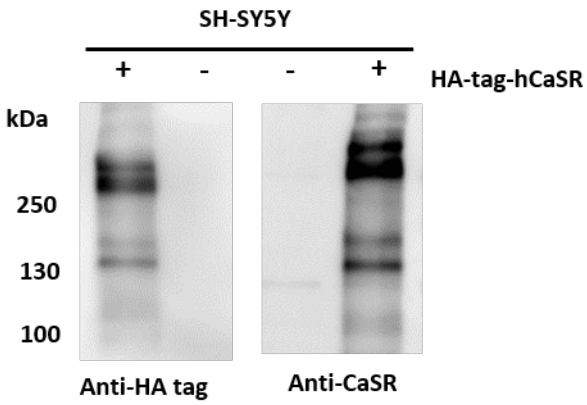

Figure 13. Validation of anti-CaSR antibody in human neuroblastoma SH-SY5Y transiently transfected with the HA-tagged human CaSR. SH-SY5Y-HA-tagged-hCaSR lysates were immunoblotted with the anti-HA tag antibody or with the anti-CaSR antibody (ADD clone). Both antibodies recognized similar bands which validated specificity of CaSR protein.

4.3 Expression of CaSR in mouse-ESCs derived neurons and in mouse brain and kidney tissues

After validation of specificity of anti-CaSR antibody, the receptor expression was investigated in HM1 neuronal cultures by ICC. The staining revealed the presence of CaSR in mouse derived neurons (**Figure 14A**) a result with is in accordance with evidence from literature (Chang *et al.*, 2007). Moreover, Western blot analyses confirmed the expression of CaSR in mouse brain and kidney tissue lysates with kidney lysate having a higher receptor expression than the brain lysate,

as expected. The blot shows several bands between ~100 and ~130 kDa, which represent the putative non-glycosylated and glycosylated CaSR monomers in both tissue samples. Additional bands, representative of CaSR dimers, are observed at higher molecular weight (~250-260 kDa) in brain and kidney. Overall, in both tissue samples, we found a predominance of monomers over dimers (**Figure 14B**). The observation of CaSR expression in mouse ESC-derived neurons and in brain and kidney tissue represents an important evidence which constitutes the premise for the next step of our research aimed at investigating the receptor's role in neurological disease.

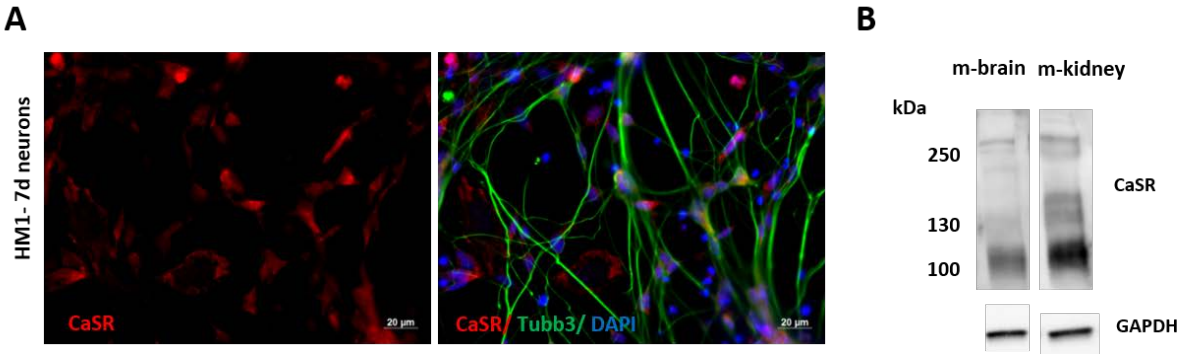

Figure 14. CaSR expression in mESCs-derived neurons and in mouse brain and kidney tissues. (**A**) Representative immunocytochemical analyses showing the expression of CaSR (red) in TUBB3-positive neurons derived from mESCs. Nuclei were counterstained with DAPI (in blue). Scale bar 20 µM. (**B**) Representative Western blot of mouse brain and kidney tissues revealing expression of CaSR at monomeric (~100-130 kDa) and dimeric form (~260 kDa).

4.4 Characterization of iPSC- derived neurons

Human iPSC lines, reprogrammed from a fAD patient and from non-AD control individuals, were previously characterized by our group and successfully converted in neural progenitor cells (Nemes *et al.*, 2016; Ochalek *et al.*, 2017). Here, we let control and fAD NPCs to terminally differentiate towards neuronal cells for 6 weeks (**Figure 15A**). By immunostainings we confirmed the differentiation of NPCs, stained with the specific markers PAX6 and NESTIN, into neurons expressing MAP2 and TAU (**Figure 15B**).

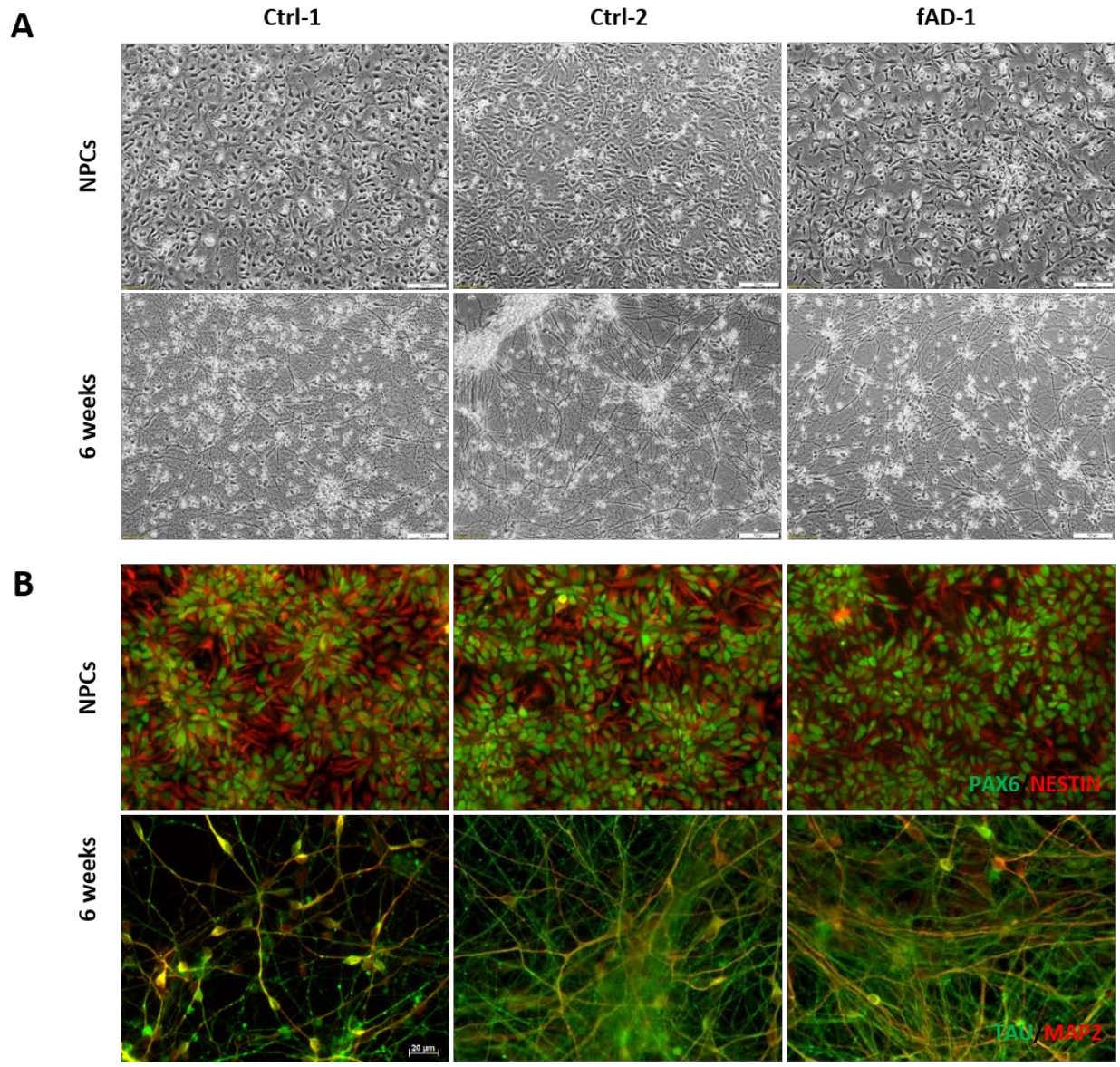

Figure 15. Terminal differentiation of Ctrl and fAD iPSC-derived neural progenitors into neurons. (**A**) Bright field images presenting the morphology of iPSC-derived NPCs and neurons differentiated for 6 weeks from the three cell lines. Scale bar 100 µM. (**B**) Representative immunocytochemical analyses showing the differentiation of NPCs, expressing the specific markers PAX6 in nuclei (green) and NESTIN cytoplasmic intermediate filaments (red), in neurons, stained with MAP2 (red) and TAU (Green). Scale bar 20 µM.

Moreover, Ca^{2+} imaging analyses revealed that 4 weeks-differentiated control and fAD cells positively respond to stimulation with high KCl, GABA, AMPA and Kainic acid, demonstrating that neurons express functional voltage gated ion channels, GABA receptors and ionotropic glutamate receptors (representative traces from control and fAD cells are shown in **Figure 16**). In accordance with our previous findings (Ochalek *et al.*, 2017) no evident differences between neuronal cultures, differentiated from control and fAD iPSC were observed, which prompted us to use these cell lines for further experiments.

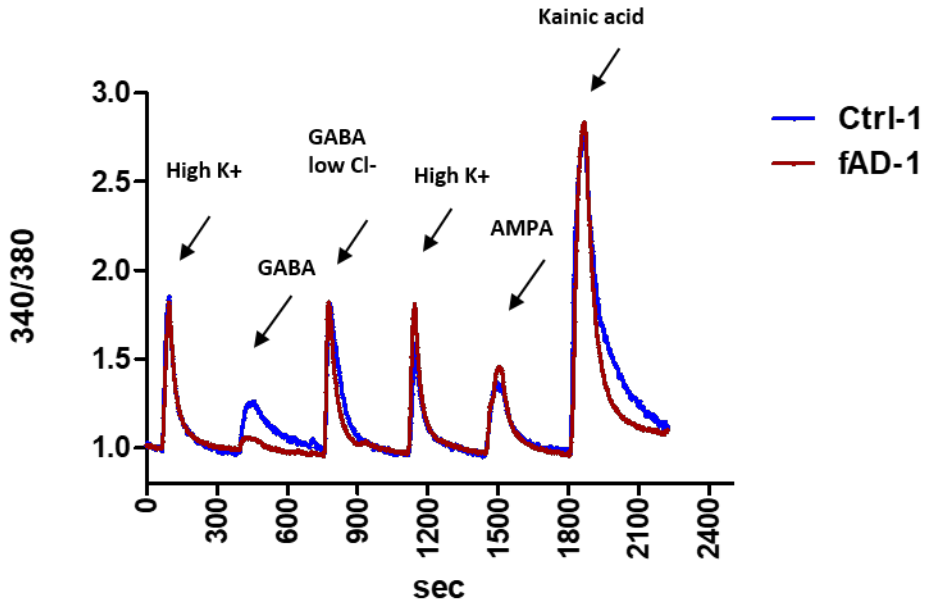

Figure 16. Calcium intracellular influx recorded from fura-2 loaded 4-weeks old neurons in response to 60 mM KCl, 100 µM GABA in physiological extracellular chloride (GABA), 100 µM GABA in 7.5 mM chloride (GABA in low Cl-), 100 µM AMPA and 100 µM Kainic acid, demonstrating functional expression of voltage gated ion channels, GABA receptors and ionotropic glutamate receptors.

4.5 Modulation of AβPP processing and amyloid secretion by ɣ-secretase inhibitor

In order to use patient derived neurons as a platform for testing potential anti-amyloid drugs, we first assessed the effects of the potent ɣ-secretase inhibitor DAPT on AβPP physiological processing and Aβ secretion. In line with our previous report in which multiple control, sporadic and familial AD cell lines were analysed (Ochalek *et al.*, 2017), ELISA of conditioned media confirmed that PSEN1 mutant neurons presented a higher Aβ42/Aβ40 ratio compared to control cells in this study (**Figure 17B**, Vehicle condition). Data from each Aβ species showed that this ratio change was primarily due to a ~2-fold increase in production of Aβ42 in fAD neurons respect to control cell lines (**Figure 17A**, Vehicle condition). Importantly, 48 h treatment with DAPT (1 µM) drastically reduced the secretion of Aβ40 and Aβ42 from both control and fAD neurons (**Figure 17A**, DAPT condition). Nevertheless, the ratio between Aβ42 and Aβ40 in fAD cells treated with DAPT remained higher than the ratio observed in control cells (**Figure 17B**, DAPT condition). Consistent with inhibition of ɣ-secretase activity, western blot analyses of lysates from healthy and PSEN1 mutant neurons demonstrated that DAPT treatment led to a strong accumulation of AβPP- C terminal fragment (AβPP- CTF), which constitutes the substrate of ɣ-secretase (Morohashi *et al.*, 2006) (**Figure 17C**). These observations demonstrated that AβPP- and amyloid- processing of control and patient-derived neurons efficiently respond to pharmacological

modulation with ɣ-secretase inhibitor, thus allowing to use this cellular model to explore the potential of calcilytic in these processes.

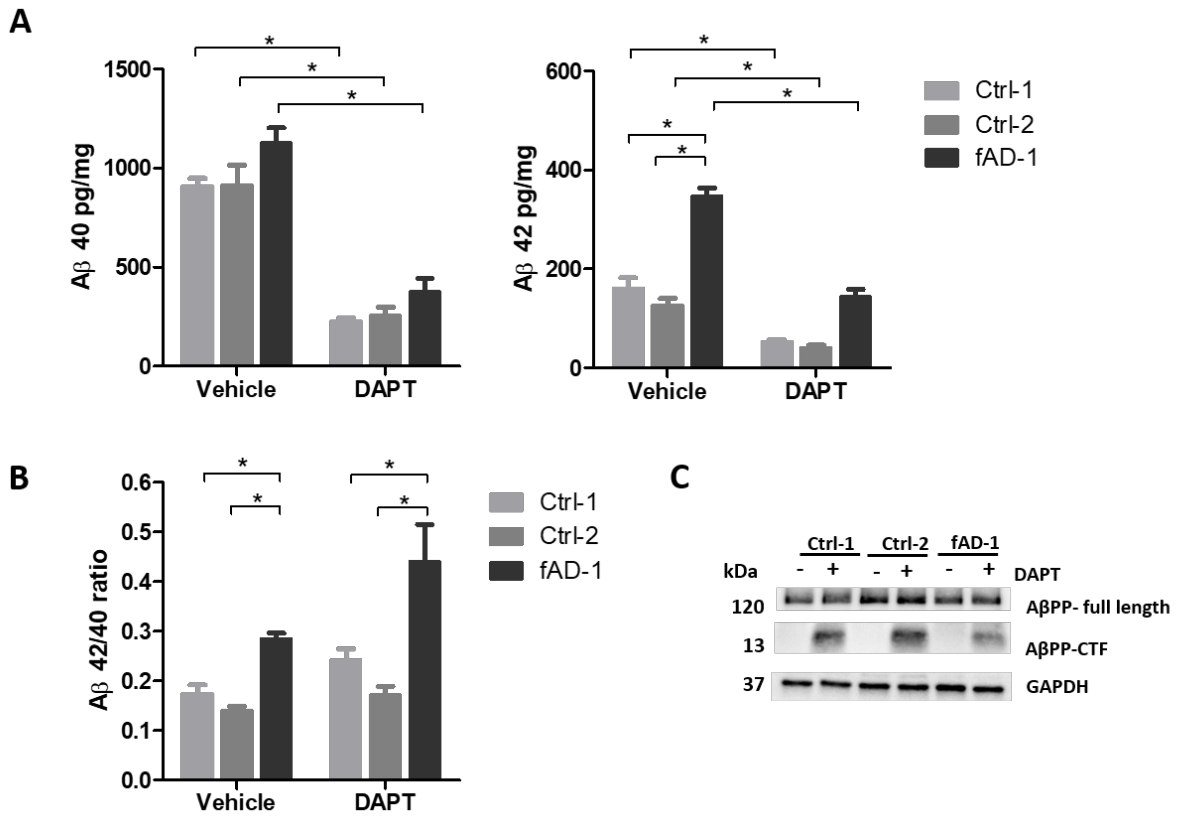

Figure 17. Pharmacological modulation of amyloid secretion and AβPP processing by DAPT treatment. (**A**) ELISA analyses of Aβ40 and Aβ42 endogenously secreted into conditioned media from controls and fAD cell lines ± treatment with 1μM DAPT for 48 h. Data normalized to total mg of protein. (**B**) Ratio between Aβ42 and Aβ40 secreted from controls and fAD cell lines ± treatment with 1μM DAPT for 48 h. (**C**) Representative western blot analysis of total lysate showing the accumulation of AβPP C-terminal fragment upon treatment with 1μM DAPT for 48 h; In A and B, two-tailed t-tests were performed when comparing different conditions (vehicle and DAPT) within the same cell line, while one-way ANOVA performed with Tukey's multiple comparisons test. * $p<0.05$; Error bars= SEM;

4.6 CaSR expression in human derived neurons

After validation of the specificity of the anti-CaSR antibody, the next step was to investigate the CaSR expression in human derived samples. Although evidences exist showing that CaSR is expressed in several regions of human brain (Kapoor *et al.*, 2008) and in human astrocytes (Chattopadhyay *et al.*, 2000; Dal Pra *et al.*, 2005) no studies have investigated the receptor presence in human iPSC derived neuronal cultures. By ICC, I showed that CaSR is expressed in 6-weeks old MAP2 positive neurons differentiated from control and fAD human iPSCs (**Figure 18**).

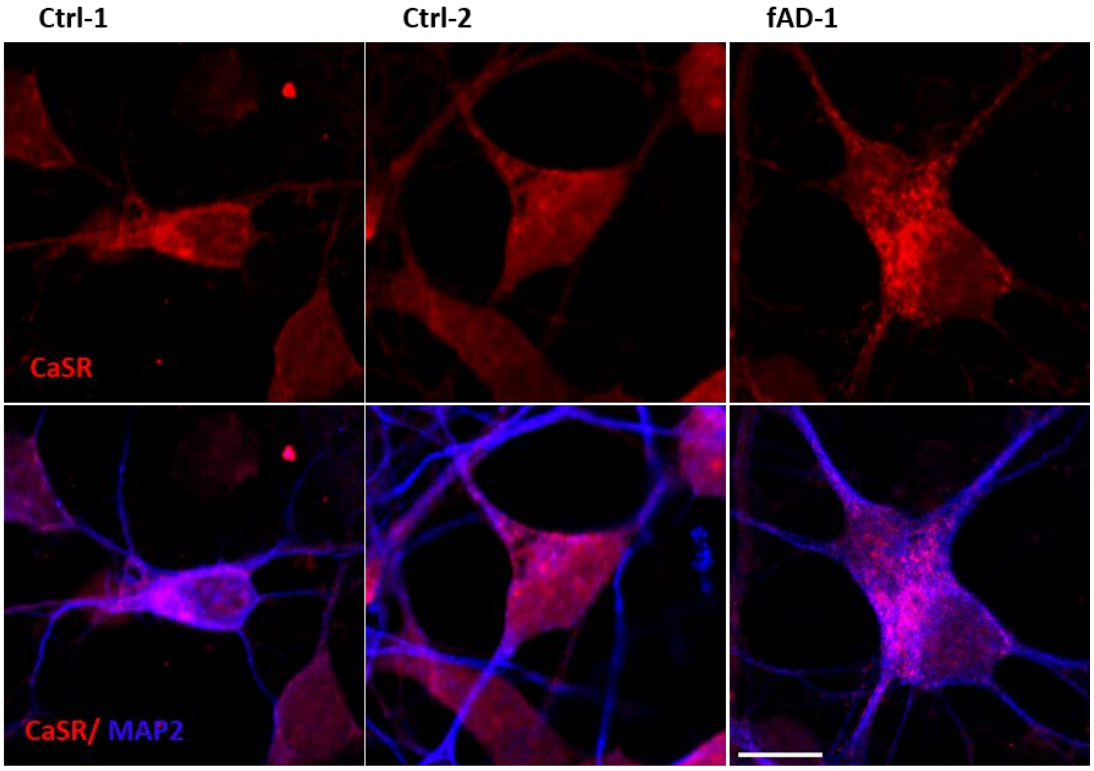

Figure 18. Expression of CaSR protein in iPSC differentiated neural cultures. Immunocytochemical analysis showing the CaSR expression in neurons (upper panel, in red), co-stained with MAP2 (lower panel, in blue) differentiated from iPSCs for 42 days (TD42). Scale bar 10 µM.

Furthermore, WB analyses confirmed that control and fAD cells present CaSR-specific protein, representing both the monomeric form at ~120-130 kDa and the dimeric form at ~ 250-260 kDa (**Figure 19A**). No evident differences were observed between healthy and PSEN1 mutant cells regarding the expression of the receptor. Moreover, I analysed the CaSR presence in lysate of adult human brain tissue. Although at lower level of expression, CaSR is present in adult human brain, mostly in the monomeric form (~130 kDa). Indeed, dimer at ~260 kDa are weakly visible only at higher exposure time acquisition, as indicated by the black arrow in **Figure 19B**.

Moreover, as further positive control, I also assessed CaSR expression in human kidney lysate, where similar molecular weight bands representative of monomers and dimers are observed. Indeed, together with parathyroid glands, kidney is the organ of human body with the higher expression of the receptor. Western blot of commercially available human kidney lysate revealed similar pattern of CaSR expression with abundance of monomers and dimers (**Figure 19C**). In conclusion, we detected the expression of CaSR on iPSC derived human neurons at the first time both in control and familial AD cells.

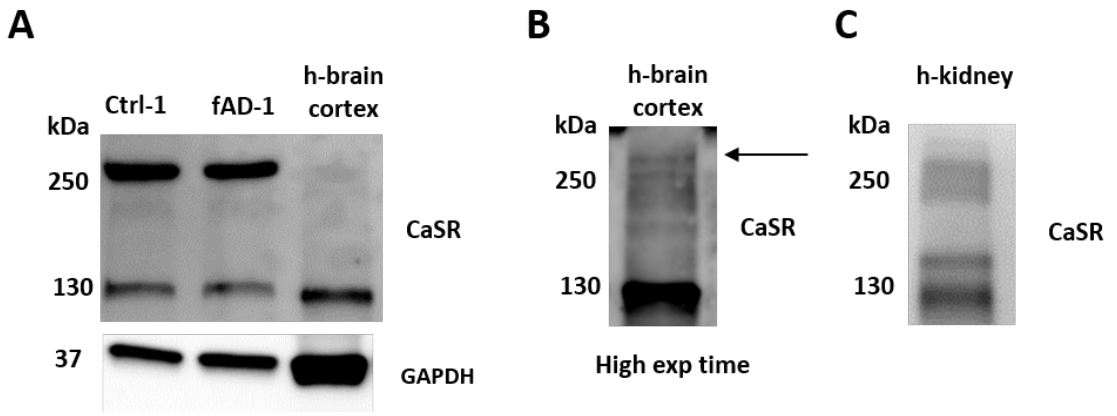

Figure 19. Expression of CaSR in differentiated neural cultures detected with Western-blot. (**A**) Representative western blot of control and fAD lysates demonstrating the receptor positive bands at ~130kDa and ~260 kDa which should represent the monomeric and dimeric forms of CaSR. Human adult brain cortex lysate has been loaded as positive control of CaSR expression in human brain. (**B**) At high exposure time acquisition, dimers of CaSR are visible in human brain cortex sample. (**C**) CaSR expression in human kidney lysate.

4.7 ERK1/2 phosphorylation in iPSC-neurons treated with calcilytic NPS 2143

Based on several studies, the CaSR activation promotes ERK1/2 phosphorylation signalling (Casalà *et al.*, 2013; Vizard *et al.*, 2015). To investigate whether a similar intracellular pathway is endogenously activated in human iPSC-neurons, the ERK1/2 phosphorylation levels were evaluated in cells exposed to calcilytic for 48 h. Immunoblotting results showed that both Ctrl-1 and fAD-1 cells presented discrete amounts of ERK1/2 phosphorylation, which remained unchanged after treatment with NPS 2143 (**Figure 20**).

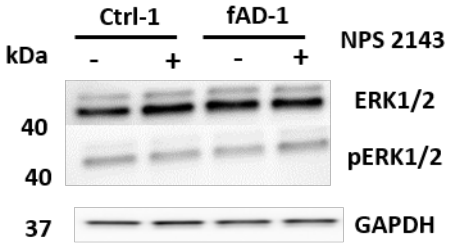

Figure 20. ERK1/2 phosphorylation in iPSC-neurons treated with calcilytic NPS 2143. Representative western blot of control and fAD lysates showing total expression of ERK1/2 and the phosphorylated form, which were not modified by NPS 2143 treatment.

4.8 Modulation of amyloid secretion in fAD neurons treated with calcilytic NPS 2143

Evidences reported that calcilytic inhibited Aβ accumulation and secretion from human astrocytes treated with exogenous amyloid (Armato *et al.*, 2013; Chiarini *et al.*, 2017a). To evaluate the effect of calcilytic in iPSC derived neurons, I treated 6 weeks old control and fAD cells with NPS 2143 for 48 hours. ELISA analyses of conditioned media revealed that treatment with calcilytic had no significant effect on Aβs secretion in control cell lines. Conversely, NPS 2143 reduced by ~25% the Aβ40 and Aβ42 in the conditioned media of fAD cells compared to levels present in the vehicle treatment **(Figure 21A)**. Moreover, as calcilytic caused a similar reduction of both amyloid species in fAD neurons, the resulting ratio between the Aβ42 and Aβ40 in PSEN1 mutant cells treated with NPS 2143 was not significantly changed compared to the treatment with vehicle, remaining higher than the ratio displayed by the control cell lines **(Figure 21B)**. The overall decrease of amyloid species in fAD cells would suggest a decrease of the AβPP- amyloidogenic pathway induced by calcilytic in these cells.

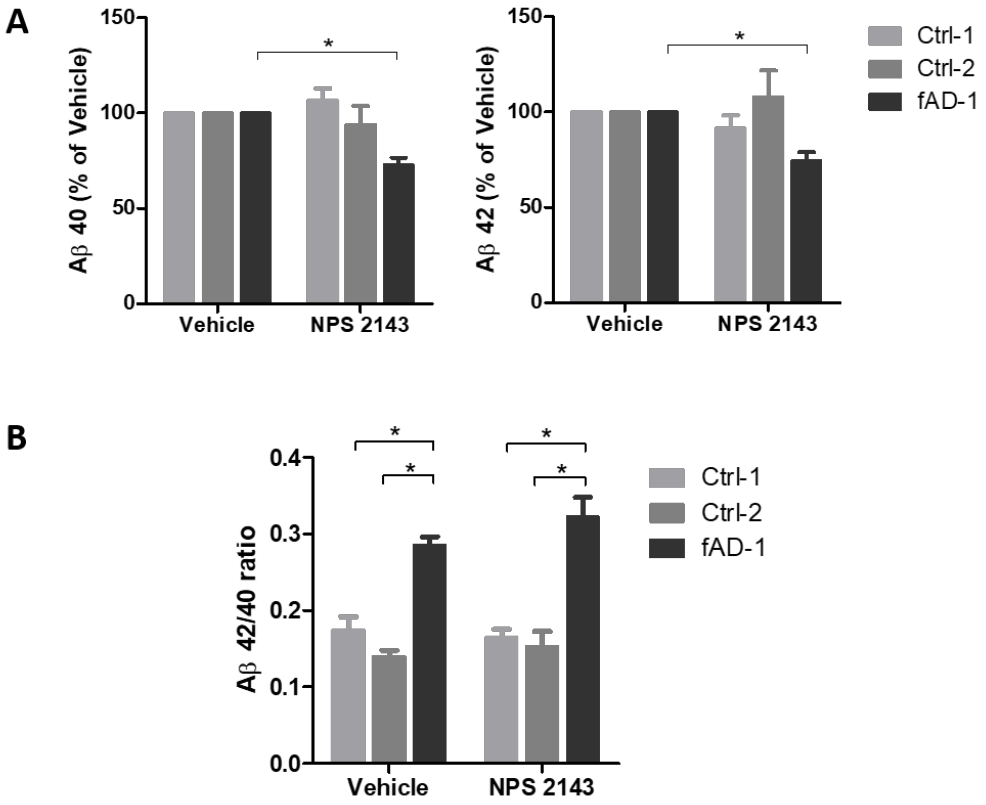

Figure 21. Modulation of amyloid secretion by NPS 2143 treatment. (**A**) ELISA analyses of Aβ40 and Aβ42 endogenously secreted into conditioned media ± treatment with 1 µM NPS 2143 for 48 h. Two-tailed t-tests were performed. * $p<0.05$; Error bars= SEM. (**B**) Ratio between Aβ42 and Aβ40 endogenously secreted from controls and fAD cell lines upon the treatment with 1 µM NPS 2143 for 48 h. One-way ANOVA performed with Tukey's multiple comparisons test, * $p<0.05$; Error bars= SEM.

Considering the effect of calcilytic in reducing amyloid levels secretion in fAD cells, I investigated whether NPS 2143 acted similarly to the γ-secretase inhibitor DAPT. Therefore, I assessed AβPP full-length and AβPP-C-terminal fragments level in cell lysates after treatment with calcilytic drug. As shown in **Figure 22A and B**, AβPP full-length and AβPP-C-terminal fragments were not modified by incubation with NPS 2143, demonstrating that calcilytic does not act in a γ-secretase inhibitor fashion.

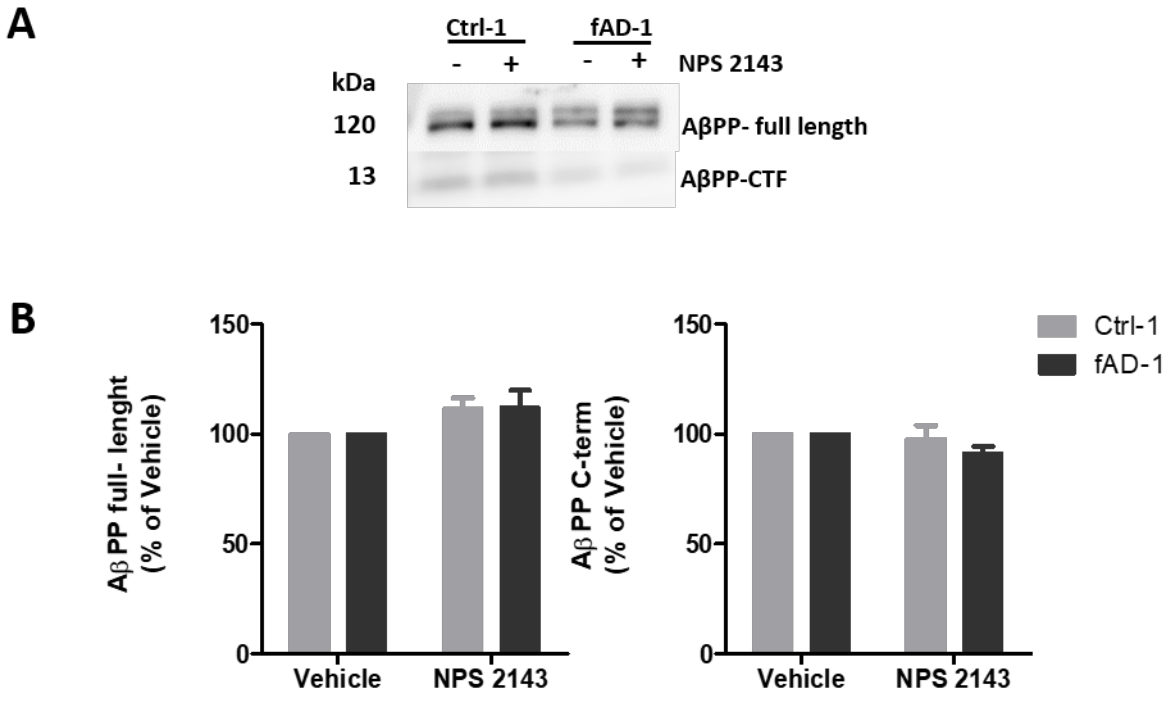

Figure 22. NPS 2143 does not act as γ-secretase inhibitor. (**A**) Representative western blot of Ctrl-1 and fAD-1 lysates showing AβPP full-length and AβPP-C-terminal fragment upon treatment with 1 μM NPS 2143 for 48 h. (**B**) No significant changes were observed of the target proteins were observed, as demonstrated by the densitometric analyses.

4.9 Modulation of sAβPPα release in fAD neurons treated with calcilytic NPS 2143

A recent study demonstrated that NPS 2143 increased the sAβPPα release from human astrocytes treated with exogenous Aβ (Chiarini *et al.*, 2017a). Therefore, I investigated the sAβPPα released extracellularly by iPSC-neurons after 48 h incubation with calcilytic. Interestingly, western blot analyses of conditioned media demonstrated that fAD neurons secreted significantly lower amount of sAβPPα compared to the control cell lines (**Figure 23A**). Interestingly, calcilytic strongly increased the release of sAβPPα from fAD cells whereas no evident effect was observed in control cells (**Figure 23B**). All together such observations would indicate that NPS 2143 favoured AβPP non-amyloidogenic α-pathway while it decreased the amyloidogenic β-processing only in cells with PSEN1 mutation.

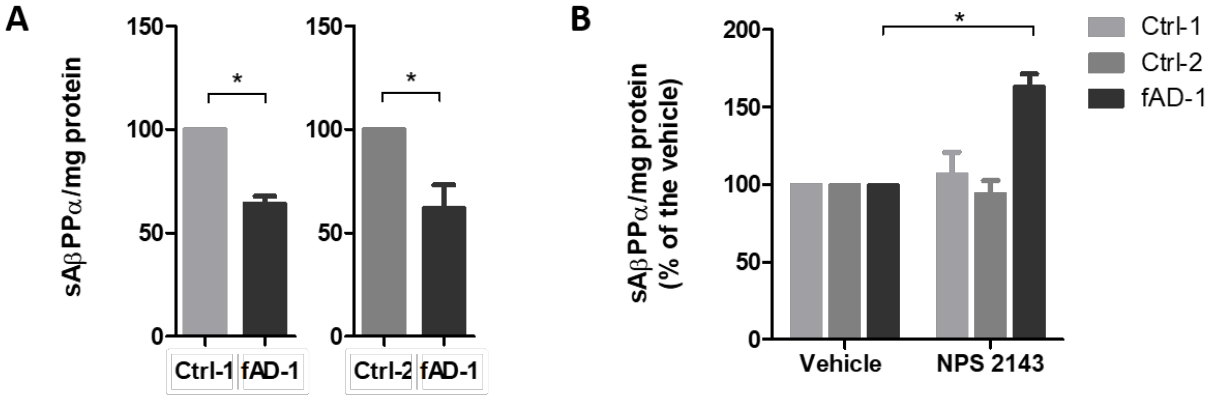

Figure 23. Modulation of sAβPPα by NPS 2143 treatment. (**A**) sAβPPα secreted into conditioned media detected by immuno-blot with 6E10 antibody. Densitometric values normalized to total amount of protein; Two-tailed t-tests were performed. ∗ P < 0.05; Error bars= SEM. (**B**) sAβPPα secreted into conditioned media ± treatment with 1μM NPS 2143 over 48h detected by immuno-blot with 6E10 antibody. Densitometric values normalized to total amount of protein; Two-tailed t-tests, * p<0.05; Error bars= SEM.

4.10 Modulation of CaSR and PSEN1 expression at the plasma membrane of fAD neurons treated with calcilytic NPS 2143

To explore whether the effects of calcilytic on AβPP processing observed in PSEN1 mutant cells were due to changes in AβPP - or secretase- expression at plasma membrane, 6 weeks old cultures were treated with NPS 2143 and biotinylation of surface's proteins was performed. I validated efficient isolation of biotinylated proteins at cell surface by using Integrin α7 as a positive control for plasma membrane expression. WB analyses of flow-through (FT) and eluted (E) samples confirmed that Integrin α7 was exclusively in the eluted fractions (**Figure 24A**- lanes 2, 4, 6, 8). Immunoblot bands showed that AβPP and CaSR resulted to be expressed at both intracellular and plasma membrane level (**Figure 24A**). Moreover, we noted that in control and fAD cells, AβPP expression at cell surface was not significantly changed following the treatment with calcilytic (**Figure 24A**, lane 4 vs lane 2; lane 8 vs lane 6 and **Figure 24B**). Interestingly, fAD cells treated with NPS 2143 presented significantly reduced levels of CaSR in the eluted fraction compared to vehicle treatment (**Figure 24A**, lane 8 vs lane 6 and **Figure 24C**). In contrast, cell surface expression of CaSR was not affected by calcilytic in control line (**Figure 24A**, lane 4 vs lane 2 and **Figure 24C**). In addition, we observed that PSEN1 was mainly expressed intracellularly but it also presented discrete levels at plasma membrane (**Figure 24A**). Similar to what we observed for CaSR, cell surface expression of PSEN1 in fAD cultures incubated with calcilytic was decreased compared to treatment with vehicle (**Figure 24A**, lane 8 vs lane 6 and **Figure 24D**).

However, NPS 2143 had no evident effect on PSEN1 plasma membrane presence in control cells (**Figure 24A**, lane 4 vs lane 2 and **Figure 24D**).

Although the reduction of CaSR and PSEN1 at the cell surface of fAD cells treated with calcilytic, we did not detect evident changes in the levels of these proteins in the corresponding FT fractions (**Figure 24E** and **Figure 24F).**

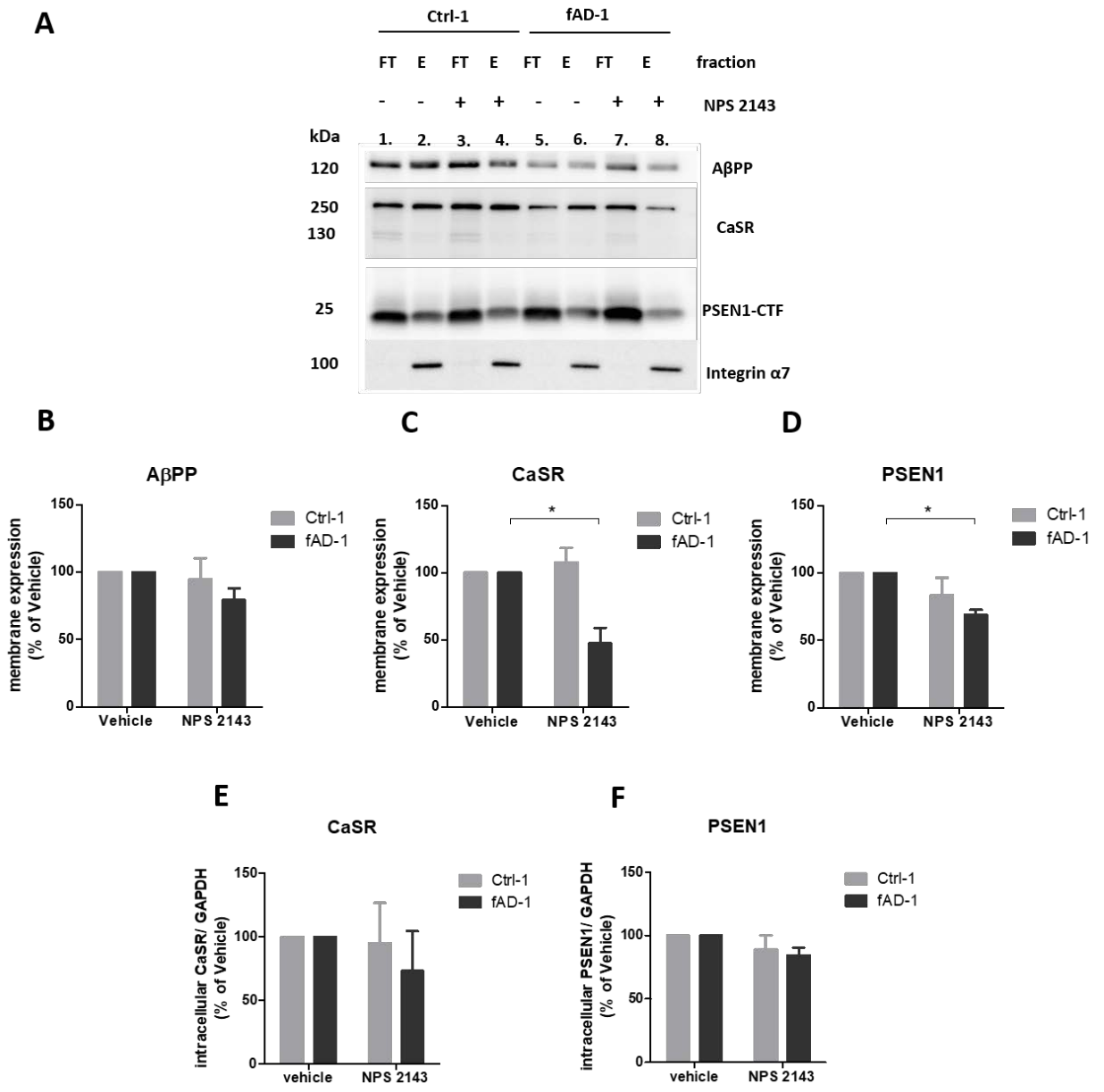

Figure 24. Modulation of AβPP, CaSR and PSEN1 at the plasma membrane by NPS 2143 treatment. (**A**) Representative western blot bands representing AβPP, CaSR and PSEN1 present in the intracellular (flow through fractions, FT) and in the plasma membrane (Eluate, E) fractions of biotinylated samples ± treatment with 1 µM NPS 2143 for 48 h. The Integrin α7 is a plasma membrane marker and it has been used as a control of the Biotinylation's efficiency. (**B**) The E fractions bands of AβPP, (**C**) CaSR and (**D**) PSEN1 proteins, normalized to Integrin α7 (only present in the E fractions), presented as a percent of the vehicle. Two-tailed t-tests, * $p<0.05$; Error bars= SEM. (**E**) and (**F**) The FT fractions relative bands of CaSR and PSEN1 were normalized to GAPDH and presented as a percent of the Vehicle.

Finally, levels of α-secretase ADAM10 and β-secretase BACE1 were assessed. Active form of ADAM10 at ~70 kDa was mainly present in the eluted fractions in control cells (**Figure 25**, lanes 1-4), while its expression in the FT and E fractions was similar in fAD neurons (**Figure 25**, lanes 5-8). Instead BACE1 resulted to be expressed almost exclusively in the flow-through fractions of healthy and PSEN1 mutant cells (**Figure 25**). However, as evident from the blot analyses, cellular localization of ADAM10 and BACE1 was not affected by calcilytic treatment in both cell lines. Based on the results of biotinylation studies, NPS 2143 modulated the presence of CaSR and PSEN1 at the plasma membrane of fAD neuronal cultures only.

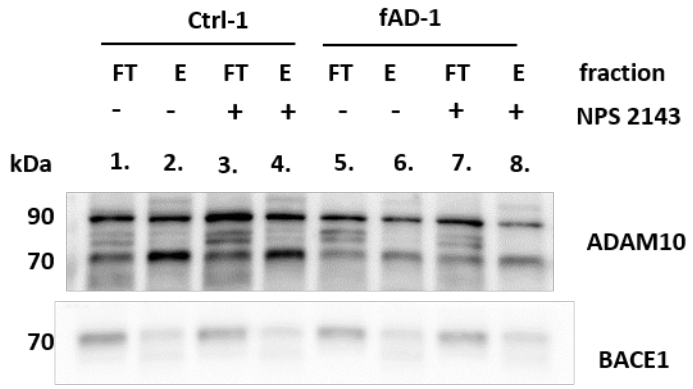

Figure 25. ADAM10 and BACE1 expression in biotinylated samples treated with NPS 2143. Representative western blot bands representing ADAM10 and BACE1 present in the "flow through" (FT) and in the "Eluate" (E) fractions of biotinylated samples ± treatment with 1 µM NPS 2143 for 48 h.

5 NEW SCIENTIFIC RESULTS

In this research, the role of CaSR in Alzheimer's disease was investigated. After successful differentiation of mouse and human stem cells into neurons, the expression of the CaSR was detected in both species. Next, the AD phenotype in diseased iPSC-derived neurons were characterized and the effects of CaSR's negative modulation with calcilytic on AD cellular mechanisms were investigated. The following new scientific achievements were obtained:

1. For the first time, I detected the protein expression of CaSR in both control and fAD iPSC-derived neurons.
2. I found that calcilytic treatment did not modify the ERK1/2 phosphorylation in iPSC-derived neurons.
3. As a novel finding, I showed that PSEN1 mutant iPSC-derived neuronal cultures secrete lower amounts of sAβPPα compared to the control cell lines, which represent an important key feature of AD that is recapitulated in our *in vitro* system.
4. By evaluating the amyloid Aβ levels of conditioned media, I demonstrated the effect of calcilytic NPS 2143 in decreasing Aβ secretion in fAD neuronal cells only.
5. I reported that treatment with calcilytic NPS 2143 significantly increases the release of sAβPPα from PSEN1 mutant neuronal cells.
6. Finally, I found that calcilytic NPS 2143 changes the cellular localization of the CaSR and PSEN1 in fAD-neurons, by reducing their presence at the plasma membrane.

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6 DISCUSSION

In the present thesis, we studied the role of the CaSR in the CNS using PSC lines of mouse and human species and differentiate them towards the neuroectodermal lineage to generate neurons. In this *in vitro* cellular system, we investigated the role of CaSR in neurological diseases, with special focus on Alzheimer's disease, the most frequent kind of dementia form worldwide. Here, we used the potential of PSCs which makes it possible to generate all kind of tissues and cell types from a single cell source efficiently and unrestrictedly. PSCs represent a highly versatile system allowing a multiplicity of applications. In the field of nervous system research, animal and human PSCs are widely used for investigating neurogenesis and neurodevelopmental disorders (del Pino *et al.*, 2018). Moreover, in the last decades, iPSCs have been intensively applied for studying neurodegenerative diseases, as AD. Encouraging data, obtained from different laboratories worldwide, seem to converge to the conclusion that patient-derived neuronal cultures constitute more relevant platforms for disease modelling and drug testing (Mertens *et al.*, 2018), obviating some limitations of conventional systems.

To this aim, we first provided evidence of the expression of CaSR in neurons derived from mouse ESCs. Indeed, the presence of the receptor in rodent neural cells from developing and adult nervous system has been extensively described (Chang *et al.*, 2007; Chattopadhyay *et al.*, 2007), as recently reviewed by our group. However, any study on the receptor expression in HM1-derived neuronal cultures has been published so far. Protein analyses revealed the CaSR presence in 7-days mouse differentiated neurons, which represented the premise for the next investigation step based on human iPSC-derived neurons. The switch from the animal- to the human- system was due to the need to model *in vitro* the neurodegenerative mechanisms of Alzheimer's disease. Our group has previously demonstrated the relevance of human iPSC-derived neurons as a suitable system for studying the cellular mechanisms responsible for AD (Ochalek *et al.*, 2017). Moreover, here, we further extended the investigation of AD processes that are recapitulated in human derived neurons. Experiments were conducted on two iPSC- cell lines, derived from two healthy individuals and one fAD cell line, obtained from a patient with PSEN1 mutation and early onset AD, whose a wide characterization was published in our previous work (Ochalek *et al.*, 2017). In this study, we confirmed the efficient differentiation of iPSC-derived neuronal progenitors into neurons. Immunocytochemistry analyses demonstrated expression of two main neuronal markers, MAP2 and TAU, from the three cell lines at 6 weeks of differentiation. Further, we performed calcium imaging on 4 weeks-old cells, which showed that both healthy and PSEN1 mutant neurons express functional ion channels and neurotransmitter receptors, with no evident difference between the cell lines.

In agreement with our previous study (Ochalek *et al.*, 2017), here we confirmed that iPSC-neurons derived from a fAD patient presented higher secretion of Aβ42 and higher Aβ42/Aβ40 ratio compared to healthy neurons. Such findings are characteristic of AD phenotype and are in line with results from other groups, which also reported increased amyloid levels and amyloid ratios in iPSC-neurons derived from AD patients (Yagi *et al.*, 2011; Muratore *et al.*, 2014; Choi *et al.*, 2014). It has to be highlighted that, in contrast to transgenic animal or cellular models which, by overexpressing fAD-mutations very often led to overestimate the efficacy of potential drugs, human iPSC derived neurons secrete physiologically relevant levels of amyloid, representing a more reliable system for drug discovery which could improve the therapy translation from preclinical studies to patients.

In order to further substantiate the validity of iPSC-neurons as a robust platform for drug screening, we assessed the effect of the potent γ-secretase inhibitor DAPT on AβPP processing and amyloid production. Indeed, inhibition of γ-secretase activity lead to blocking the cleavage of C-terminal fragment, preventing amyloid production (Haass *et al.*, 2012). Through ELISA assays, we demonstrated that iPSC neurons from healthy and fAD donors drastically reduced endogenous secretion of Aβ40 and Aβ42 in response to DAPT treatment, an effect observed also from other groups (Koch *et al.*, 2012; Muratore *et al.*, 2014). Additionally, DAPT induced an intracellular accumulation of AβPP -C-terminal fragment, as expected (Koch *et al.*, 2012). Despite the great potential, γ-secretase inhibitors turned out to be unsuccessful from a therapeutic point of view (Wolfe, 2014), producing serious side effects *in vivo* (Searfoss *et al.*, 2003). This is mainly due to the fact that γ-secretase is responsible for cleaving several substrates other than AβPP, as Notch, AβPP -like proteins and N- and E-cadherins (Haapasalo and Kovacs, 2011), therefore its inhibition seriously affects other important signalling. In addition, γ-secretase blockage produces accumulation of AβPP-C-terminal fragments, which represent another problematic aspect as these AβPP intermediates also become toxic when accumulated (Mitani *et al.*, 2012). However, γ-secretase inhibitors are still largely used as useful tools for biological research. In this case, the effects mediated by DAPT allowed to confirm that iPSC-neurons present a functional γ-secretase activity and AβPP processing, which can be pharmacologically modulated, prompting to test the potential of calcilytic on these cells.

As a next step, we demonstrated the protein expression of CaSR in human iPSC derived neurons for the first time. ICC analyses showed that the receptor is expressed in both control and familial AD neurons at 6 weeks of differentiation. We demonstrated that CaSR is expressed in form of monomers and dimers in both Ctrl and fAD cells, with no evident differences between the cell lines. In contrast, a recent study which analysed the receptor expression in a triple transgenic AD mouse model reported an augmented CaSR expression in the hippocampus of AD animals

compared to that of control rodents (Gardenal *et al.*, 2017). However, due to the limited number of studies, any conclusion can be done on it. When comparing CaSR expression of human neurons to that of adult human brain, we found that the receptor is lower expressed in brain lysate. Moreover, in the adult tissue, CaSR is almost completely present as monomers. In this regard few studies have showed the CaSR protein expression in adult brain. In their work, Kapoor et colleagues presented Western blot analysis of tissue lysates from different regions of human brain, including temporal lobe, frontal lobe, parietal lobe, hippocampus and cerebellum (Kapoor *et al.*, 2008). In all different regions, the receptor protein presence was represented by a 120 kDa molecular weight band, which is consistent with the analyses presented in this study. Similarly, a discrete expression of the 120 kDa CaSR protein was demonstrated in adult human astrocytes (Armato *et al.*, 2013). Although, as mentioned, any evidence from literature exists regarding the receptor expression in human derived neurons, the specificity of the CaSR dimers observed in the lysates of iPSC-neurons has been strongly substantiated in this study, by usage of several positive controls which showed consistent results.

The validation of CaSR expression in iPSC-derived neuronal cells led to the next step, aimed to evaluate the effects of the calcilytic NPS 2143 on AD phenotype. ELISA analyses of amyloid species released in the culture media, demonstrated that calcilytic significantly reduced the levels of Aβ40 and Aβ42 secreted from PSEN1 mutant neurons. In contrast, amyloid levels were not modified in control cell lines. Further, to rule out the possibility that calcilytic could act in a "γ-secretase inhibitor fashion", as DAPT, the AβPP-C-Terminal levels were evaluated after treatment with NPS 2143. Results showed that neither AβPP full-length neither AβPP-C-Terminal were modified by calcilytic, suggesting different mechanism of action of calcilytic compared to DAPT.

The observed reduction of amyloid species mediated by calcilytic only in fAD cells raises the question of how/why NPS 2143 produces such effect selectively on AD cells. In our fAD model, the increased Aβ42 secretion compared to the healthy counterpart could explain the selective effect of NPS 2143 obtained in these cells. Indeed, according to the hypothesized role of Aβ as an activator of the CaSR (Dal Prà *et al.*, 2014) and due to the higher presence of Aβ42 in the medium of fAD cells, the receptor of AD cultures might result to be more activated compared to the receptor of healthy cells, thus justifying the evident effect of NPS 2143 observed only in fAD neurons. In agreement, when non-tumorigenic cortical adult human astrocytes, NAHAs, were treated with NPS 2143 alone, it did not modify the basal levels of the intracellular and the secreted amyloid Aβ42 (Armato *et al.*, 2013). Only when cells were exposed to synthetic Aβ, which caused a de novo production and release of amyloid, thus mimicking AD condition, addition of calcilytic was beneficial in counteracting the Aβ-mediated noxious effects (Armato *et al.*, 2013). In the

indicated study, cells were treated with 20 μM synthetic fAβ25-35, an Aβ42 proxy, to induce an AD phenotype. Indeed, following to this treatment, NAHAs displayed an augmented accumulation and secretion of Aβ42 while in parallel, cells presented higher intracellular amounts of Aβ40 and unchanged extracellular Aβ40 levels (Armato *et al.*, 2013). Authors found that co-treatment of fAβ25-35 with NPS 2143 fully abolished the Aβ42 intracellular accrual accumulation and release. Instead, co-treatment of fAβ25-35 and calcilytic only partially decreased the Aβ-induced intracellular increase of Aβ40 while it promoted its secretion at the same time (Armato *et al.*, 2013). The results from Armato and co-workers suggested that calcilytic differently modulates the secretion of Aβ40 and Aβ42 from fAβ25-35 exposed astrocytes. In contrast with this report, we found that NPS 2143 induced a similar reduction of both Aβ40 and Aβ42 species in the cellular medium of our fAD cellular system. Moreover, considering that NPS 2143 modified the amyloid levels endogenously secreted from iPSC neurons without any exogenous amyloid treatment, the effects exerted by calcilytic might be physiologically relevant.

Further, as a new result, we reported that PSEN1 mutant iPSC derived neurons endogenously displayed lower levels of sAβPPα in the conditioned media compared to control cells. This observation represents a remarkable feature of AD phenotype which is recapitulated in our *in vitro* system. Soluble AβPPα is obtained from the α secretase-mediated cleavage of amyloid precursor protein, which initiates the non-amyloidogenic pathway of AβPP (Lammich *et al.*, 1999). Interestingly, evidences reported the loss of sAβPPα during AD (Colciaghi *et al.*, 2002) and its beneficial role as neurotrophic factor, thus modulation of α-cleavage might be an alternative therapeutic target approach (Habib *et al.*, 2017). Similar to what observed for amyloid modulation, NPS 2143 was effective in modifying sAβPPα secretion only in fAD cells. Indeed, treatment with calcilytic significantly increased the release of soluble AβPPα from PSEN1 mutant cells while it did not change the sAβPPα levels secreted from healthy neurons. Overall the amyloid reduction accompanied with the sAβPPα increase observed in PSEN1 mutant neurons treated with NPS 2143, would suggest a role of calcilytic in decreasing the β-amyloidogenic processing of AβPP and promoting the α-non-amyloidogenic pathway, substantiating the hypothesis corroborated by Chiarini and colleagues (Chiarini *et al.*, 2017a). Indeed, in addition to the previously mentioned effect of calcilytic in modulating amyloid accumulation and secretion in NAHAs (Armato *et al.*, 2013), it has been recently reported a role of NPS 2143 also in modifying the sAβPPα of NAHAs (Chiarini *et al.*, 2017a). According to this study, exposure to 20 μM fAβ25-35 induced a reduced secretion while a concomitant intracellular accumulation of endogenous sAβPPα in human adult astrocytes. Interestingly, adding NPS 2143 to fAβ25-35 restored the physiological secretion of sAβPPα from the cells whereas it almost completely abolished its intracellular accumulation. The overall effects of NPS 2143 in reducing Aβ42 levels, and partially

Aβ40, and increasing the sAβPPα shedding led authors to hypothesize that calcilytic might favour the non-amyloidogenic AβPP processing versus the amyloidogenic cleavage (Chiarini *et al.*, 2017a). Chiarini and co-workers further demonstrated that this "switch" from amyloidogenic to non-amyloidogenic AβPP processing exerted by NPS 2143 was due to the capacity of calcilytic to drive the translocation of AβPP and ADAM10 from intracellular compartment to the plasma membrane. Indeed, authors found that NPS 2143 increased the expression of AβPP and ADAM10 at cell surface of fAβ25-35 treated astrocytes (Chiarini *et al.*, 2017a). In contrast with these evidences, NPS 2143 had no effect in modifying the cellular location of AβPP and ADAM10 in iPSC derived neurons, in this study. However, we demonstrated that calcilytic significantly reduced the expression of CaSR and PSEN1 at cell surface of fAD neurons, an effect which was not observed in healthy cells. Moreover, the intracellular levels of these proteins were not significantly modified. Interestingly, a capacity of calcilytic to mediate CaSR expression reduction has been reported also by other groups. Huang and Breitweiser reported that allosteric modulators can regulate turnover of the CaSR, modifying the receptor expression levels (Huang and Breitwieser, 2007). Indeed, authors found that in HEK293 cells transfected with a wild-type FLAG-CaSR, incubation with NPS 2143 for 12 h led to a reduction of CaSR protein expression compared to DMSO-treated cells. Such decrease was partially rescued when the proteasomal inhibitor MG132 was added to NPS 2143 treatment, suggesting that calcilytic destabilizes CaSR and increases ER-associated degradation (ERAD). Further, treatment with calcilytic modified CaSR function by reducing the CaSR expression at cell surface and the receptor-mediated ERK1/2 phosphorylation (Huang and Breitwieser, 2007). Moreover, Armato and colleagues observed a transient decrease of the total CaSR protein at 24 h in astrocytes treated with NPS 2143 alone, which was rescued at 48 and 72 h. Instead, the receptor levels fell rapidly and were kept steadily reduced between 24 and 72 h in cells co-treated with NPS 2143 and fAβ25-35 (Armato *et al.*, 2013). Such effect of NPS 2143 on CaSR expression of human astrocytes, which became strongly evident when calcilytic was added to exogenous Aβ, is in line with the reduction of CaSR observed at the cell surface of fAD-neurons, supporting the idea that calcilytic might have anti-amyloidogenic capacity only in the presence of an AD phenotype.

Moreover, the reduction of CaSR at the plasma membrane associated with the decrease of PSEN1 at cell surface is intriguing and it might indicate the probability of a physical interaction between the two proteins. Interestingly, several evidences demonstrated the capability of some GPCRs to physically associate with γ-secretase, modifying its expression and activity. For instance, co-immunoprecipitation assays revealed a physical association of β2-adrenergic receptor (β2AR) with PSEN1 in transfected HEK293 cells (Ni *et al.*, 2006). This study reported that agonist of β2AR induced co-internalization of the receptor and PSEN1, which enhanced γ-secretase activity and Aβ

production. Conversely, antagonization of β2AR inhibited Aβ production and reduced cerebral plaques in a transgenic Alzheimer's mouse model (Ni *et al.*, 2006). Similarly, coimmunoprecipitation assays demonstrated interaction of the δ-opioid receptor (DOR) with PSEN1 and BACE1 which formed a secretases/receptor complex (Ni *et al.*, 2006; Teng *et al.*, 2010). The activation of DOR mediated co-endocytic sorting of the GPCR/secretase complex together with an increased activity of secretases and Aβ production. Interestingly, knockdown or antagonization of DOR reduced secretase activities and ameliorated Aβ pathology and Aβ-dependent behavioural deficits, without affecting the processing of Notch, N-cadherin or APLP, in AD model mice (Teng *et al.*, 2010).

Although an interaction-based mechanism between CaSR and PSEN1 has not been demonstrated so far, it would represent an attractive possibility to explain the calcilytic-mediated concomitant reduction of these proteins at cell surface in fAD neurons. Based on this study and on evidences from literature, we can only speculate that the beneficial effect of calcilytic in ameliorating the AD phenotype of PSEN1 mutant cells is correlated to the CaSR putative heterocomplex with secretases or/and to its internalization and potential degradation mediated by NPS 2143. However, further studies are needed to investigate such complex processes.

Altogether, here we provided evidences that calcilytic positively counteract amyloid secretion and sAβPPα loss in a relevant model of familial AD. Based on our data and other reports, the therapeutic effect of NPS 2143 observed in fAD neurons might be due to the fact that these cells secrete higher levels of Aβ42 compared to healthy neurons. Thus, such an over-production of Aβ42 would represent the premise for CaSR to be activated and for calcilytic to exert its therapeutic effect. Considering that several studies demonstrated that iPSC-neurons generated from sporadic AD patients also present increased levels of Aβ42 secretion (Ochalek *et al.*, 2017; Lin *et al.*, 2018), a major perspective would be to explore whether NPS 2143 produces similar effects in sporadic patient derived neurons. Indeed, sporadic patients represent majority of AD cases, counting about 95% of total patients, whereas only up to 5% are familial cases. Therefore, testing the calcilytic in sporadic iPSC-neurons would be highly relevant from a therapeutic perspective. Moreover, considering that NPS 2143 was recently found to efficiently reduce the phosphorylated-TAU release and the GSK3β activation from Aβ-exposed astrocytes (Chiarini *et al.*, 2017), and that both these processes can be recapitulated in AD iPSC-neurons (Israel *et al.*, 2012; Ochalek *et al.*, 2017), further studies would be needed to investigate the potential of calcilytic in such AD-related processes in patient-derived neurons. In case of positive outcome, calcilytic treatment would represent an attractive therapeutic strategy in the medication of Alzheimer's disease.

7 SUMMARY (EN)

The goal of our research was to investigate the expression and the potential role of CaSR in neuronal cells through an *in vitro* cellular model, using pluripotent stem cells. Moreover, we aimed to explore the role of the receptor in neurological diseases, through the most common form of dementia, the Alzheimer's disease, using our unique cellular system. The failure of the clinical trials so far made clear the limitations of the conventional AD modelling systems, leading the researchers to re-think about the way of studying it. In this perspective, the advent of iPSC technology allowed to alternatively approach the study of neurological disorders, including AD. Indeed, several groups worldwide demonstrated that the key processes of the disease can be recapitulated in AD patients-derived neuronal cultures, which makes human derived neurons a powerful tool not only to better model the disease, but also to identify new therapeutic targets. Interestingly, recent evidences indicated the CaSR as a potential player in AD. The receptor's main role is to maintain systemic Ca^{2+} homeostasis by regulating the PTH secretion from parathyroid glands, while in the adult nervous system it is involved in neurodegeneration. Indeed, some studies reported that CaSR promotes amyloid and TAU pathological accumulation when activated by Aβ extracellularly. In accordance, inhibition of the CaSR/ Aβ signalling with calcilytic was found to counteract AD pathology. In this study, we provided evidence that iPSC-neurons present a functional processing of AβPP, which can be pharmacologically modulated by GSI, supporting that iPSC derived neurons constitute a suitable platform for drug testing. Here, we confirmed that PSEN1 mutant neurons secrete higher levels of Aβ42 and have increased Aβ42/Aβ40 ratios compared to control neurons, which is in line with the results presented in our previous work and from other groups. In addition, this study demonstrated that AD-neurons secrete lower amounts of sAβPPα compared to the healthy counterpart. Then, the CaSR expression was demonstrated in human iPSC-neurons for the first time, which entitled us to further test the effect of calcilytic in our *in vitro* system. Interestingly, we found that treatment with calcilytic NPS 2143 significantly decreased Aβ40 and Aβ42 secretion while it increased the sAβPPα release from AD cells only. These results suggested that calcilytic promoted the non-amyloidogenic pathway while at the same time, it blocked the amyloidogenic processing of AβPP, substantiating previous observations of the literature. Finally, we demonstrated a reduction of CaSR and PSEN1 levels at the plasma membrane of AD cells while this effect was not observed in control cells. Altogether, the results of this study strongly substantiate the potential of iPSC-derived neurons to model AD *in vitro* and to test novel drugs. Moreover, the beneficial effect of calcilytic observed in our fAD cellular system could indicate the calcilytic as a promising AD candidate therapeutic.

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8 SUMMARY (HU)

Kutatásunk során legfőbb célunk a CasR fehérje expressziójának és lehetséges funkciójának idegsejtekben történő vizsgálata volt, amelyeket egy pluripotens őssejteken alapuló in vitro sejtes rendszerben állítottuk elő. Célul tűztük ki továbbá a receptor idegrendszeri betegségekben betöltött szerepének a vizsgálatát, melyet a leggyakoribb demencia, az Alzheimer kór (AD) példáján keresztül elemeztünk, saját fejlesztésű egyedi sejtes rendszerünk segítségével. Az eddigi klinikai vizsgálatok kudarcai világossá tették a tradicionális AD modell rendszerek hiányosságait, ami arra ösztönözte a kutatókat, hogy újra gondolják a betegség tanulmányozását. Ennek megfelelően, az iPSC technológia megjelenése egy ilyen alternatív megközelítést hozott az idegrendszeri betegségek, köztük az AD vizsgálatára. Ilyen módon, világszerte több kutatócsoport is demonstrálta, hogy a betegség főbb folyamatai megmutatkoznak az AD betegekből származó újraprogramozással létrehozott őssejtekből (iPSC-k) differenciáltatott neurális kultúrákban, így megfelelően modellezhető a betegség, továbbá új terápiás célpontok is azonosíthatók. A legújabb kutatások arra utalnak, hogy a CasR fehérjének is szerepe lehet a betegségben. A receptor fő feladata a szervezet Ca2+ homeosztázisának fenntartása a mellékpajzsmirigy PTH szekréciójának szabályozásával, míg a felnőtt idegrendszerben a neurodegenerációban van szerepe. Ezt alátámasztva több tanulmány is beszámolt róla, hogy az extracellulárisan adott Aβ által aktivált CaSR elősegíti a kóros amiloid és TAU felhalmozódását. Ezenfelül a CasR/Aβ szignalizáció kalcilitikus vegyülettel történő gátlása ellensúlyozza az AD kórképét. Támogatva az elméletet, miszerint az iPSC-ből származó idegsejt kultúra megfelelő modell gyógyszertesztelésre, ebben a tanulmányban igazoltuk, hogy az iPSC-idegsejtekben jelen van az AβPP funkcionális változata, ami farmakológiailag módosítható GSI-vel. Megerősítve a korábbi eredményeket, kontroll neuronokhoz képest a PSEN1 mutáns idegsejtek Aβ42 szekréciója, így Aβ42/Aβ40 aránya is magasabbnak bizonyult. Ezen kívül megmutattuk, hogy az egészséges kontrollhoz képest az AD neuronok alacsonyabb sAβPPα expressziót mutatnak. Valamint először mutattuk meg a CaSR expressziót humán iPSC-idegsejtekben és azt találtuk, hogy az NPS 2143 kalcilitikus vegyülettel történő kezelés csökkentette az Aβ40 és Aβ42 szekrécióját, míg növelte az sAβPPα felszabadulását, kizárólag AD sejtekből. Ezek az eredmények arra engedtek következtetni, hogy a vegyület előmozdította a nem-amiloidogén útvonalat, míg ezzel egyidejűleg blokkolta az AβPP amiloidogén processzálását. Végül, azt is megmutattuk, hogy a kontroll sejtekkel szemben, az AD sejtek plazma membránjában a CaSR és PSEN1 szintje lecsökken. Eredményeink bizonyították az iPSC-ből származó idegsejtek alkalmasságát in vitro AD modellezésre és gyógyszertesztelésre. Továbbá, az fAD sejtes rendszerünkben megfigyelt kalcilitikus vegyület kedvező hatása az ilyen típusú vegyületek AD terápiában való hasznosságát is jelezhetik.

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9 APPENDICES

M1: BIBLIOGRAPHY

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M2: PUBLICATIONS

International paper publications:

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- **Lo Giudice M**, Mihalik B, Kobolák J, Dinnyés A. *Studies on the role of the CaSR in human iPSC-derived neuronal cells.* 3rd International Symposium on The Calcium-Sensing Receptor (CaSR) May 2017, Florence, Italy.
- **Lo Giudice M**, Mihalik B, Riccardi D, Kemp PJ, Kobolák J, Dinnyés A. *Expression of the Calcium sensing receptor (CaSR) in neuronal cultures generated from healthy and familial Alzheimer's disease iPSC.* 11th FENS Forum of Neuroscience, July 2018, Berlin, Germany.
- **Lo Giudice M**, Ochalek A, Mihalik B, Téglási A, Bock I, Kobolák, Dinnyés A. *Characterization of iPSC-derived neuronal cells from Alzheimer's disease patients reveal functional APP processing and increased amyloid levels.* 14th International Conference on Alzheimer's and Parkinson's Diseases, March 2019, Lisbon, Portugal.

- **Lo Giudice M**, Mihalik B, Kobolák J, Dinnyés A. *Studies on the role of the CaSR in human iPSC-derived neuronal cells.* FENS Regional Meeting September 2017, Pecz, Hungary.

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- **Lo Giudice M**. *CaSR and Alzheimer's disease*. Lecture on 1st ETN School of CaSR Biomedicine. Oxford, UK. 12-14 December 2016.
- **Lo Giudice M**. *CaSR and Alzheimer's disease*. Lecture at 1st Mid-term annual Meeting CaSR Biomedicine. Manchester, UK. 14-15 September 2017.
- **Lo Giudice M**. *The CaSR in iPSC derived neuronal cells*. Lecture at 4th ETN School of CaSR Biomedicine Training Network, Gödöllő, Hungary. May 2018.
- **Lo Giudice M**, Kobolák J, Dinnyés A. *Modelling Alzheimer's disease with patient derived induced pluripotent stem cell (iPSCs) to reveal the role of Calcium Sensing Receptor (CaSR)*. Lecture at ECRIN Laboratory at the Department of Dermatology and Allergology, Faculty of Medicine, University of Szeged, Hungary. February 2019.

M3: SUPPLEMENTARY TABLES

Supplementary Table 1. Antibodies used for immunocytochemistry (ICC)

	Antibody	Dilution	Company
Primary Antibodies	Mouse anti-NESTIN	1: 1000	Merck Millipore
	Rabbit anti-PAX6	1: 500	Covance
	Mouse anti-MAP2	1: 1000	Merck Millipore
	Rabbit anti-TAU	1: 1000	Dako
	Rabbit anti-MAP2	1: 1000	Abcam
	Mouse anti-CaSR	1: 100	Thermo Fisher Scientific
	Goat anti-NANOG	1: 50	Santa Cruz Biotechnology
	Rabbit anti-Oct-3/4	1: 50	Santa Cruz Biotechnology
	Rabbit anti-Sox-2	1: 50	Santa Cruz Biotechnology
Secondary Antibodies	Alexa Fluor 488 donkey anti-goat IgG	1: 2500	Thermo Fisher Scientific
	Alexa Fluor 488 donkey anti-rabbit IgG	1: 2500	Thermo Fisher Scientific
	Alexa Fluor 594 donkey anti-rabbit IgG	1: 2500	Thermo Fisher Scientific
	Alexa Fluor 594 donkey anti-mouse IgG	1: 2500	Thermo Fisher Scientific

Supplementary Table 2. Antibodies used for Western blotting (WB)

	Antibody	Dilution	Company
Primary Antibodies	Rabbit anti-APP-CTF	1: 1000	Thermo Fisher Scientific
	Mouse anti-CaSR	1: 500	Thermo Fisher Scientific
	Rabbit anti-PSEN1	1: 1000	Cell Signaling
	Mouse anti- anti-β-Amyloid, 1-16 Antibody	1: 1000	Biolegend
	Goat anti-Integrin α7	1: 1000	Santa Cruz Biotechnology
	Rabbit anti-GAPDH	1: 10000	Sigma- Aldrich
	Rabbit anti-ADAM10	1: 1000	Cell Signaling
	Rabbit anti-BACE	1: 1000	Cell Signaling
	Mouse anti-HA 11 epitope Tag	1: 1000	Covance
	Rabbit anti-p44/42 MAPK (ERK1/2)	1: 1000	Cell Signaling
	Rabbit anti-phospho p44/42MAPK (ERK1/2)	1: 1000	Cell signaling
Secondary Antibodies	Goat anti-Rabbit IgG, Cross-Adsorbed, HRP	1: 2000	Thermo Fisher Scientific
	Rabbit anti-Goat IgG, Cross-Adsorbed, HRP	1: 2000	Thermo Fisher Scientific
	Goat anti-Mouse IgG, Cross-Adsorbed, HRP	1: 2000	Thermo Fisher Scientific

M4: MEDIA COMPOSITIONS

Media compositions for human iPSC-derived cells

1. Thawing Medium (TM)

Reagent	Concentration	For 10 ml
Complete NMM media	100 %	10 ml
Rock inhibitor (10 mM)	end concentration 10 µM	

2. Freezing Medium (FM)

Reagent	Concentration	For 10 ml
FBS	90 %	9 ml
DMSO	10 %	1 ml
Rock inhibitor (10 mM)	end concentration 10 µM	

3. Neural Maintenance Medium (NMM)

Reagent	Concentration	For 500 mL
DMEM/F12	50 %	235 mL
Neurobasal medium	50 %	235 mL
Nonessential amino acids	1 %	5 ml
B-27 (50x)	2 %	10 ml
N-2 (100x)	1 %	5 ml
L-Glutamine (200 mM)	1 %	5 ml
Pen/Strep	1 %	5 ml
EGF 100 µg/ml	end concentration 10 ng/mL	
bFGF 100 µg/ml	end concentration 10 ng/mL	
β-mercaptoethanol	end concentration 100 µM	

4. Neuronal Differentiation Medium (NDM)

Reagent	Concentration	For 500 ml
DMEM/F12	50 %	235 ml
Neurobasal-A medium	50 %	235 ml
Nonessential amino acids	1 %	5 ml
B-27 (50x)	2 %	10 ml
N-2 (100x)	1 %	5 ml
L-Glutamine (200 mM)	1 %	5 ml
Pen/Strep	1 %	5 ml
Ascorbic Acid	end concentration 0.2 mM	
β-mercaptoethanol	end concentration 25 µM	

Media compositions for mouse ESCs

1. **Thawing Medium (TM)**

Reagent	Concentration	For 10 ml
Mouse ESC media	100 %	10 ml
Rock inhibitor (10 mM)	end concentration 10 µM	

2. **Mouse Embryonic stem cell medium (mESC) medium**

Reagent	End concentration
DMEM/F12(d-(+)-glucose)	50 %
FBS	10 %
Nonessential amino acids	1X
Sodium - Pyruvate	2 mM
Glutamax	2 mM
Pen/Strep	50 U/ml
β-mercaptoethanol	50 µM
LIF	1000 U/ml

3. **Mouse Neuronal Differentiation Medium (NDM)**

Reagent	End concentration
DMEM/F12(d-(+)-glucose)	50 %
Neurobasal medium	50 %
Nonessential amino acids	1 %
B-27 (50x)	1 %
N-2 (100x)	0.5 %
Glutamax	2 mM
Pen/Strep	50 U/ml
bFGF 100 µg/ml	10 ng/mL
β-mercaptoethanol	50 µM

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