



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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The PhD program

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Discipline: Animal Husbandry Science

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1. INTRODUCTION AND GOALS

Disease modelling is extremely important to understand the pathological processes which cause a given disease, to find potential targets and to develop new medical interventions. One of the main problems when studying disorders of the nervous system is the difficulty of adequately modelling the physiological mechanisms of the brain. Indeed, beside the limited accessibility to the human brain for research purposes, the usage of animal models and transgenic immortalized cell lines have major limitations, failing to fully recapitulate the human neurological diseases and often leading to overestimate the drug efficacy, respectively.

Development of iPSC technology revolutionized the way of studying neurological disorders, including Alzheimer's disease (AD). Through the differentiation of adult somatic cells into neurons, this cutting-edge methodology allows to obtain human-derived neuronal cells which maintain the individual genetic background. Interestingly, several reports demonstrated that human derived neurons very well mimic the pathological hallmarks of AD, including increased amyloid levels, TAU hyperphosphorylation, oxidative stress and cell death, thus supporting that iPSC-derived neurons provide a more relevant platform for disease modelling, drug screening and identification of new targets.

Recently, the calcium sensing receptor (CaSR) has been indicated as a potential key player in AD processes. The CaSR is a G-protein coupled receptor (GPCR) mainly expressed in parathyroid glands where it exerts its main role in controlling Ca^{2+} homeostasis and parathyroid hormone (PTH) secretion. In addition, the CaSR plays several functions in the nervous system in both physiological and pathological conditions. Concerning AD, the receptor's activation was found to promote amyloid $\text{A}\beta_{42}$ -extracellular secretion from exogenous amyloid-exposed adult human astrocytes (NAHAs). The extracellular accumulation of amyloid $\text{A}\beta_{42}$ constitutes the premise for amyloid plaques formation, which is a main hallmark of AD. Interestingly, inhibition of CaSR with calcilytic NPS 2143, a negative allosteric modulator of CaSR, efficiently counteracted the release of amyloid, by forcing the non-amyloidogenic processing of amyloid precursor protein ($\text{A}\beta\text{PP}$) over the amyloidogenic processing. Such evidences encouraged to repurpose calcilytic as a potential treatment for AD.

Based on these evidences, the aims of the current study were to validate our cellular in vitro system as a platform for drug screening and to study the effects of calcilytic NPS 2143 on AD phenotype.

Objectives of this study

The main aims of the study were:

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

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 A β PP processing by γ -secretase inhibitor in control and AD neurons.
- Investigate the effect of calcilytic on amyloid β secretion and sA β PP α release in control and AD cultures.
- Analyse the plasma membrane proteins expression in control and AD derived neurons exposed to calcilytic.

2. 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.

2.1 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 containing mouse leukaemia inhibitor factor (LIF) 1000 U/mL. The cells were passaged before reaching 70% confluency (approximately every 2 Days).

2.2 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 (B27 medium). 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 attached to the surface of the dish while the ESCs were still floating in the media, making easier the collection of mESCs. Thereafter, ESCs were carefully centrifuged, re-suspended in B27 medium and plated onto matrigel-coated dishes for Western Blot (WB) and immunocytochemistry (ICC). The medium was renewed every day during the 7-days differentiation.

2.3 Terminal differentiation of human iPSC-derived NPCs into neurons

In the present study, we used iPSCs derived-neural precursor cells (NPCs) from two healthy individuals, named Ctrl-1 and Ctrl-2, and from a fAD patient with a mutation in PSEN1 gene, named fAD-1. Control and fAD NPCs were plated on poly- L-ornithine/laminin (POL/L) -coated dishes, and maintained in neural maintenance medium (NMM) 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 μ M β -mercaptoethanol, with medium changed every 3-4 days. Cells were assessed through ICC, Calcium imaging, ELISA, WB and Biotinylation experiments.

2.4 Calcium imaging

Functional properties of the 4-weeks differentiated Ctrl-1 and fAD-1 neurons were evaluated by calcium imaging. Cells grown on coverslips were first incubated with 1 μ M fura-2-AM for 30 minutes at 37°C, and 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 -60 mM KCl; 100 μ M GABA in physiological extracellular solution, (ECS); 100 μ M GABA in reduced Cl⁻; 100 μ M AMPA; 100 μ M Kainic acid- were locally applied to the neurons using a rapid solution changer (RSC160, Intracel RSC160, Intracel, Royston, UK). 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.

2.5 Immunocytochemistry

Neuronal differentiation and CaSR expression were evaluated in mouse ESC-derived neurons and human iPSC-derived neurons by ICC. Both mouse and human cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT), then permeabilized and blocked for 60 min at RT. The cells were incubated with specific primary antibodies overnight at 4°C. To visualize the signal, isotype specific secondary antibodies conjugated with appropriate fluorophores were applied, while nuclei were counterstained with DAPI. 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).

2.6 Lysis of differentiated neuronal cells and tissues from brain and kidney

Mouse ESC-derived neurons and human iPSC-derived neuronal cultures were collected at the end of the differentiation process (mouse: TD7; human: TD42). Mouse and human tissue brain and kidney samples were homogenized using a tissue homogenizer. Neuronal cells and tissue homogenates were lysed with RIPA Lysis buffer supplemented with Protease and Phosphatase Inhibitor cocktail. After centrifugation, lysates (supernatants) were transferred in new tubes for protein quantification and WB analyses.

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

SH-SY5Y neuroblastoma cells were grown up to 70-80% confluence. Thereafter, cells were transiently transfected with pcDNA-5-FRT-HA-tag-hCaSR (provided by Prof. Hans Bräuner-Osborne, University of Copenhagen, Denmark) using Lipofectamine® 2000 reagent. After 48 h of transfection, cells were processed for cell lysis and protein quantification similarly to the differentiated neuronal cells (see paragraph 2.6).

2.8 Immunoblotting

Lysates (5-50 μ g) 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 with TBST containing 5% BSA or non-fat milk and then incubated with the respective primary antibody solution overnight at 4°C. After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. 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).

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

Neuronal cultures differentiated for 6 weeks were treated with γ -secretase inhibitor DAPT or with calcilytic NPS 2143 hydrochloride. Both DAPT and NPS 2143 were dissolved in DMSO and next diluted in NMM medium at a final concentration of 1 μ M. 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.

2.10 ELISA of A β 40 and A β 42 secreted in conditioned media

After 48 h treatment with DAPT or NPS 2143, conditioned media were collected. Extracellular A β 40 and A β 42 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 A β levels were normalized to total protein content of cell lysate.

2.11 Biotinylation and isolation of plasmalemmal proteins

Following to 48 h treatment with calcilytic, cell culture media were collected. Cells were washed twice with ice-cold PBS and incubated with Biotin solution for 30 minutes at 4°C. After adding Quenching Solution, Biotinylation reaction was stopped. Cells were collected and pellets were

lysed for 30 minutes on ice with intermittent vortexing. Then, lysates were centrifuged and 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. Finally, the biotinylated surface proteins were incubated with SDS-PAGE Sample Buffer for 1 h at RT and were collected by column centrifugation as “Eluate” fractions (E). FT and E samples were loaded on precast gels for WB analyses.

2.12 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 * $p < 0.05$.

3. RESULTS

3.1 Characterization of mouse-ESCs derived neurons

Before neuronal induction, mouse ESCs expressed major pluripotency markers (OCT4, SOX2, NANOG and SSEA-1) as demonstrated by ICC analyses. At Day2 of differentiation, cells underwent the neuroectodermal lineage commitment and were positively stained for NESTIN, PAX6 and SOX1 proving the differentiation of ESCs in NPCs, although OCT4 expression was still detectable. Finally, differentiated neurons expressing TUBB3 and MAP2 were observed in the cultures at Day6 of differentiation. However, at the same time with similar density, NPCs were still largely present in the cultures.

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

The specificity of anti-CaSR antibody was first validated in SH-SY5Y neuroblastoma cells transiently transfected with HA-Tag-hCaSR. WB analyses revealed expression of the CaSR in form of monomers (~130-140 kDa) and dimers (~260-280 kDa) in the transfected cells. After that, the receptor protein expression was demonstrated in HM1-differentiated MAP2-positive neurons by ICC. Moreover, WB 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 showed 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, were observed at higher molecular weight (~250-260 kDa) in brain and kidney samples. Next step was to investigate the receptor's role in neurological phenotype.

3.3 Characterization of iPSC- derived neurons

Control and fAD NPCs were successfully differentiated towards neuronal cells after 6 weeks. By immunostainings we confirmed the differentiation of NPCs, stained with the specific markers PAX6 and NESTIN, into neurons expressing MAP2 and TAU. Moreover, Ca 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 expressed functional voltage gated ion channels, GABA receptors and ionotropic glutamate receptors already at this stage of differentiation. No evident differences between Ctrl and fAD neuronal cultures were observed, which prompted us to use these cell lines for further experiments.

3.4 Modulation of A β PP processing and amyloid secretion by γ -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 γ -secretase inhibitor DAPT on A β PP physiological processing and A β secretion. In line with our previous report, ELISA of conditioned media confirmed that PSEN1 mutant neurons presented a higher A β 42/A β 40 ratio compared to control cells. Data from each A β species showed that this ratio change was primarily due to a ~2-fold increase in production of A β 42 in fAD neurons respect to control cell lines. Importantly, 48 h treatment with DAPT (1 μ M) drastically reduced the secretion of A β 40 and A β 42 from both control and fAD neurons. Nevertheless, the ratio between A β 42 and A β 40 in fAD cells treated with DAPT remained higher than the ratio observed in control cells. Consistent with inhibition of γ -secretase activity, western blot analyses of lysates from healthy and PSEN1 mutant neurons demonstrated that DAPT treatment led to a strong accumulation of A β PP- C terminal fragment (A β PP- CTF), which constitutes the substrate of γ -secretase. These observations demonstrated that A β PP- and amyloid- processing of control and patient-derived neurons efficiently respond to pharmacological modulation with γ -secretase inhibitor, thus allowing to use this cellular model to explore the potential of calcilytic in these processes.

3.5 CaSR expression in human derived neurons

Although evidences exist showing that CaSR is expressed in several regions of human brain and in human astrocytes, no studies have investigated the receptor presence in human iPSC derived neuronal cultures. By ICC, the CaSR expression was demonstrated in 6-weeks old MAP2 positive neurons differentiated from control and fAD human iPSCs. 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. No evident differences were observed between healthy and PSEN1 mutant cells regarding the expression of the receptor. Similar expression of CaSR was demonstrated in lysates of adult human brain and kidney tissues.

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

Based on several studies, the CaSR activation promotes ERK1/2 phosphorylation signaling. 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.

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

Evidences reported that calcilytic inhibited A β accumulation and secretion from human astrocytes treated with exogenous amyloid. To evaluate the effect of calcilytic in iPSC derived neurons, 6 weeks old control and fAD cells were treated with NPS 2143 for 48 hours. ELISA analyses of conditioned media revealed that treatment with calcilytic had no significant effect on A β s secretion in control cell lines. Conversely, NPS 2143 reduced by ~25% the A β 40 and A β 42 in the conditioned media of fAD cells compared to levels present in the vehicle treatment. Moreover, as calcilytic caused a similar reduction of both amyloid species in fAD neurons, the resulting ratio between the A β 42 and A β 40 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.

3.8 Modulation of sA β PP α release from fAD neurons treated with calcilytic NPS 2143

According to recent evidences, NPS 2143 promoted the sA β PP α release from human astrocytes treated with exogenous A β . Therefore, we investigated the sA β PP α secreted by iPSC-neurons after 48 h incubation with calcilytic. Interestingly, western blot analyses of conditioned media demonstrated that fAD neurons released significantly lower amount of sA β PP α compared to the control cell lines. In addition, we found that calcilytic strongly increased the release of sA β PP α from fAD cells whereas no evident effect was observed in control cells. Such observations would indicate that NPS 2143 favored A β PP non-amyloidogenic α -pathway while it decreased the amyloidogenic β -processing only in cells with PSEN1 mutation.

3.9 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 A β PP processing observed in PSEN1 mutant cells were due to changes in A β PP - or secretase- expression at plasma membrane, 6 weeks old cultures were treated with NPS 2143 and biotinylation of surface's proteins was performed. Efficient isolation of biotinylated proteins at cell surface was validated by using Integrin α 7 as a positive control for plasma membrane expression. WB analyses showed that A β PP expression at cell surface remained unchanged in control and fAD cells following treatment with calcilytic. Interestingly, fAD cells treated with NPS 2143 presented significantly reduced levels of CaSR and PSEN1 in the eluted fraction compared to vehicle treatment. In contrast, cell surface expression of CaSR and PSEN1 was not affected by calcilytic in the control cell line. 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. Finally, cellular localization of ADAM10 and BACE1 was not affected by calcilytic treatment in both cell lines.

4. 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 sA β PP α 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 A β levels of conditioned media, I demonstrated the effect of calcilytic NPS 2143 in decreasing A β secretion in fAD neuronal cells only.
5. I reported that treatment with calcilytic NPS 2143 significantly increases the release of sA β PP α 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.

5. DISCUSSION AND FUTURE PERSPECTIVES

In the present thesis, we studied the role of the CaSR in the nervous system using pluripotent stem cell (PSC) lines of mouse and human species to generate neurons. By using this cellular system, we investigated the role of CaSR in neurological diseases, with special focus on Alzheimer's disease, the most common form of dementia worldwide. In the last years, iPSCs technology has been intensively applied for studying neurodegenerative diseases, as AD. Encouraging data from different laboratories worldwide, seem to converge to the conclusion that iPSC-derived neuronal cultures obtained from patients constitute more relevant platforms for disease modelling and drug testing, obviating some limitations of conventional systems, and allowing for identification of potential targets. Recently, several studies indicated that CaSR might be involved in AD mechanisms and that its inhibition with calcilytic NPS 2143 might help to reduce amyloid secretion and to increase sA β PP α release.

To this aim, we first provided evidence of the expression of CaSR in neurons differentiated from mouse ESCs, 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. 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). By using ICC and Ca imaging approaches, we first confirmed the efficient differentiation of iPSC-derived neuronal progenitors into neurons expressing the two main neuronal markers MAP2 and TAU together with functional ion channels and neurotransmitter receptors. Moreover, in agreement with our previous study (Ochalek *et al.*, 2017), here we confirmed that fAD-1 iPSC-neurons presented higher secretion of A β 42 and higher A β 42/A β 40 ratio compared to healthy neurons, which are characteristic of AD phenotype.

Then, in order to 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 A β PP processing and amyloid production. Through ELISA and WB analyses, we demonstrated that DAPT treatment drastically reduced endogenous secretion of A β 40 and A β 42, while it concomitantly induced an intracellular accumulation of A β PP -C-terminal fragment in control and fAD neurons. The effects mediated by DAPT allowed to confirm that iPSC-neurons present a functional γ -secretase activity and A β PP processing, which can be pharmacologically modulated.

As a next step, we demonstrated the protein expression of CaSR in control and fAD iPSC derived neurons for the first time, prompting to test the potential of calcilytic on these cells. Through

ELISA analyses of amyloid species released in the culture media, we demonstrated that calcilytic NPS 2143 significantly reduced the levels of A β 40 and A β 42 secreted from PSEN1 mutant neurons, while any effect was observed in control cell lines. Moreover, as a new result, we reported that PSEN1 mutant iPSC derived neurons endogenously displayed lower levels of sA β PP α in the conditioned media, compared to control cells. This observation represents a further remarkable feature of AD phenotype which is recapitulated in our *in vitro* system. Interestingly, we found that NPS 2143 significantly increased the release of soluble A β PP α from PSEN1 mutant cells while it did not change the sA β PP α levels secreted from healthy neurons. Finally, we showed 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.

Altogether, we provided evidences that calcilytic positively counteracted amyloid secretion and sA β PP α 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 A β 42 compared to healthy neurons. Thus, such aberrant over-production of A β 42 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 A β 42 secretion, a major perspective would be to explore whether NPS 2143 produces similar effects in sporadic 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 point of view. Moreover, considering that NPS 2143 was recently found to efficiently reduce the phosphorylated-TAU release and the GSK3 β activation from A β -exposed astrocytes, and that both these processes can be recapitulated in AD iPSC-neurons, further studies would be needed to investigate the potential of calcilytic in such AD-related mechanisms in patient-derived neurons. In case of positive outcome, calcilytic treatment would represent an attractive therapeutic strategy against Alzheimer's disease.

6. PUBLICATIONS

International paper publications:

- Ochalek A, Mihalik B, Avci HX, Chandrasekaran A, Téglási A, Bock I, **Giudice ML**, Táncos Z, Molnár K, László L, Nielsen JE, Holst B, Freude K, Hyttel P, Kobolák J, Dinnyés A. *Neurons derived from sporadic Alzheimer's disease iPSCs reveal elevated TAU hyperphosphorylation, increased amyloid levels, and GSK3B activation*. *Alzheimers Res Ther*. 2017 Dec 1;9(1):90. doi: 10.1186/s13195-017-0317-z. Impact Factor: 5.015
- Crestini A, Vona R, **Lo Giudice M**, Sbriccoli M, Piscopo P, Borrelli A, Rivabene R, Ricceri L, Mancini A, Confaloni A. *Differentiation-Dependent Effects of a New Recombinant Manganese Superoxide Dismutase on Human SK-N-BE Neuron-Like Cells*. *Neurochem Res*. 2018 Nov 23. doi: 10.1007/s11064-018-2686-5. Impact Factor: 2.782
- Kobolák J, Molnár K, Varga E, Bock I, Jezsó B, Téglási A, Zhou S, **Giudice M L**, Hoogeveen-Westerveld M, Phanthong P, Pijnappel WWM P, Varga N, Kitiyanant N, Freude K, Nakanishi H, Laszlo L, Hyttel P, Dinnyes A. *Modelling the neuropathology of lysosomal storage disorders through disease-specific human induced pluripotent stem cells*. *Exp Cell Res*. 2019 Jul 15;380(2):216-233. doi: 10.1016/j.yexcr.2019.04.021. Impact Factor: 3.329
- **Giudice ML**, Mihalik B, Dinnyés A, Kobolák J. *The Nervous System Relevance of the Calcium Sensing Receptor in Health and Disease*. *Molecules*. 2019 Jul 12;24(14). pii: E2546. doi: 10.3390/molecules24142546. Impact Factor: 3.06
- **Giudice ML**, Mihalik B, Dinnyés A, Kobolák J. *Calcilytic NPS 2143 reduces amyloid secretion and increases sA β PP α release from PSEN1 mutant iPSC derived neurons* *Journal of Alzheimer's Disease*. vol. Pre-press, no. Pre-press, pp. 1-15, 2019 doi: 10.3233/JAD-190602

International abstract and poster presentations:

- **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.

Oral presentations:

- **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.

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