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Investigation of molecules with potential implications in neurodegenerative pathology

SUMMARY OF DOCTORAL THESIS

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I. GENERAL PART

Current state of knowledge

Alzheimer's disease is a neurodegenerative disorder, mainly characterised by the progressive development of cognitive/mnesic disorders and a decline in the ability to carry out daily activities [1]. Alzheimer's disease has a significant impact worldwide, with the prevalence of Alzheimer's disease increasing with age, with the majority of cases being diagnosed in people over 65 [2],[3]. The burden of Alzheimer's disease is significant and complex. It affects not only the health and well-being of those diagnosed, but also places a substantial economic burden on healthcare systems, families and society [3]. Looking at the big picture surrounding this pathology, research efforts have focused on understanding the pathogenic mechanisms in order to develop effective treatments but also on identifying ways of early (pre-clinical) diagnosis with the aim of preventing or delaying the onset of specific symptoms. Early detection and intervention are essential to improve the prognosis and quality of life of people with Alzheimer's disease.

Over time, the pathogenic mechanisms of Alzheimer's disease have been extensively researched and multiple hypotheses have been formulated, the best known being the amyloid and tau hypothesis [4],[5]. Recently, the neuroinflammation hypothesis has been brought to attention as a potential mechanism involved in the initiation and progression of Alzheimer's disease [4].

The neuroinflammation hypothesis in Alzheimer's disease [6] includes several key aspects listed below:

- Microglia activate in the presence of beta-amyloid and generate the release of pro-inflammatory molecules (cytokines, chemokines) and thus maintain oxidative stress [7], [8].
- Chronic release of pro-inflammatory molecules in the brain causes damage to neurons and secondary synaptic dysfunction, which are essential for maintaining normal cognitive function [9], [10].
- The blood-brain barrier in Alzheimer's disease is affected. This allows immune cells and inflammatory molecules easier access to the brain, potentiating the local effects mentioned above [11].

Neuroinflammation research suggests a new possible important player in processes related to beta-amyloid uptake and accumulation, initiation and maintenance of neuroinflammation and neurovascular dysfunction, this being the scavenger receptor CD36 [12].

CD36, is a transmembrane protein that plays a key role in the uptake of long-chain fatty acids and oxidized low-density lipoproteins (oxLDL) into cells, as well as in the binding of hydrophobic amyloid fibrils found in the brain affected by Alzheimer's disease [11][13]. CD36 has also been observed to influence the inflammatory profile of human and mouse microglia, suggesting a possible interaction with the neuroinflammation cascade. CD36 has been correlated with cognitive impairment in aged mice, where it has been shown that peripheral inflammation can induce age-related neuroinflammation and increase CD36 levels in the mouse hippocampus [14]. CD36 has also been found to promote amyloid deposition in blood vessels, leading to cerebral vascular damage, neurovascular dysfunction and cognitive deficits [11].

Studies of the pathogenesis of Alzheimer's disease have repeatedly shown that activated microglia play a major role in the initiation of the pro-inflammatory pathway and the importance of the CD36 receptor in microglial activation. However, the role of astrocyte in initiating a pro-inflammatory response in the context of beta-amyloid accumulation via CD36 has not been explored. Although astrocytes do not express CD36 under basal conditions, it has been observed that they can produce cytokines in response to fatty acid treatments. Several previous studies have pointed to the role of the CD36 receptor in the autophagy and phagocytosis processes of microglia and astrocytes, suggesting its possible involvement in beta-amyloid clearance in Alzheimer's disease. [15].

It is also known that long-chain fatty acids (saturated and unsaturated) interact with numerous transcription factors, including peroxisome proliferator-activated receptors (PPARs) and NF-kB, triggering inflammatory reactions [16] [17] [18].

However, it has been observed that at increased concentrations saturated fatty acids, particularly palmitic acid, induce the production of pro-inflammatory cytokines in cultured astrocytic cells in a dose-dependent manner [19] [20] [21]. It is also shown in some studies that palmitic acid plays a role in disease progression by promoting inflammation and secondary neurodegeneration [22]. Regarding the effects of oleic acid, studies have not recorded a dose-dependent pro-inflammatory effect [23].

The second part of the experiment looked at cognitive effects in relation to CD36 receptor modulation. There are studies that have shown that CD36 activation had positive effects on cognition, in tests performed on murine models of AD [24] [25]. Conversely, CD36 deficiency has been observed to reduce macrophage-induced inflammation [26]. Therefore, CD36 has been investigated as a possible therapeutic target in Alzheimer's disease [27]. Although it has been targeted in several studies, no definite conclusion has been reported regarding the role that CD36 plays in cognitive modulation in mice. According to published data from a single experiment, CD36 KO mice were observed to exhibit anxiety-like behavior [28]. The literature review on cognitive behaviour in CD36KO mice identified few studies and most of them focused on short-term memory testing.

Understanding the complex connection between fatty acids, neuroinflammation, neurodegeneration and the role of CD36 proteins is essential for the development of novel therapeutic strategies to treat neurodegenerative disorders. By revealing the mechanisms underlying neuroinflammation processes regulated by CD36, future prospects target novel therapeutic molecules with the aim of reducing neuroinflammation levels and maintaining neuronal integrity. This PhD work aims to make a significant contribution to this emerging area of neurodegeneration studies by examining in detail the role of certain fatty acids in astrocyte-derived inflammatory processes and by analysing the neurocognitive consequences in CD36-deficient (CD36 knockout) mice, as detailed in the original contributions section.

The research activity was carried out at the National Institute for Research and Development in Pathology and Biomedical Sciences "Victor Babes" Bucharest, Laboratory of Biochemistry-Proteomics. The research activity and publication of the results obtained was also partly supported by the project Net4SCIENCE: Network of applied doctoral and postdoctoral research in the fields of intelligent specialization Health and Bioeconomy Funding contract POCU/993/6/13/154722, Programme 1: Improvement of the National Research and Development System, Subprogramme 1.2: Institutional Excellence - Funding of Excellence Projects in RDI, Contract No 7PFE/16.10.2018, and Core Programme PN 1N/2019_19.29.01.02, PN 19.29.01.04 (Core Programme), COP A 1.2.3., Grant ID: P_40_197/2016, Programme 1, Improvement of the National Research and Development System, Subprogramme 1.2, Institutional Excellence, Funding of Excellence Projects in RDI, Contract No 31PFE/30.12.2021

II. PERSONAL CONTRIBUTIONS

Working assumptions

The following hypotheses were the basis of the PhD thesis:

1) The member receptor CD36 participates in the induction of neuroinflammation through astrocyte-dependent mechanisms.

2) Fatty acids play an important role in modulating neuroinflammation in Alzheimer's disease, with a particular focus on the role of CD36 in mediating these effects.

3) The CD36 receptor is involved in the modulation of cognitive-behavioural processes in Alzheimer's disease.

General objectives of the experiments

The main objective of my PhD thesis was to investigate the involvement of the scavenger receptor CD36 in neurodegeneration-associated neuroinflammatory processes and its role in modulating neurocognitive processes.

The milestones were as follows:

1) Assessment of fatty acid (oleic acid and palmitic acid) uptake in human astrocytes (NHA).

2) Evaluation of the anti-inflammatory effect of fatty acids on cell cultures (human astrocytes).

3) Comparative evaluation of APP brain distribution in two different murine models of neuroinflammation (NRF2 ko and CD36 ko).

4) Choice of two *in vivo* models to study neuroinflammatory effects on cognition and behaviour.

5) Evaluation of the neuroinflammatory effect on learning ability using two transgenic mouse models (CD36 ko and NRF2 ko).

6) Evaluation of the neuroinflammatory effect on the modulation of anxiety behaviour using two transgenic mouse models (CD36 ko and NRF2 ko).

STUDY 1 - Evaluation of the effect of CD36 on the inflammatory response of astrocytes

Materials and method

The first experiment aimed to assess the accumulation of fatty acids (oleic acid and palmitic acid) in human astrocyte cultures (NHA). Therefore, glial cell cultures (normal human astrocytes, Lonza) grown in ABM Basal Medium (CC-3187) supplemented with AGM SingleQuotsTM Supplements (CC-4123) and maintained in a cell culture incubator (5% CO2, 37 °C) were used. These were seeded in 6-well plates at 5000 cells/cm2 for 1 week, with periodic medium changes.

The experimental methodology involved treatment of astrocyte cultures with unsaturated fatty acids, with oleic acid (OA) and saturated fatty acids such as palmitic acid (PA) being chosen for this purpose. Following a review of the literature and experimental samples, it was finally decided to treat the cell cultures with OA at a concentration of 40 µM and PA at a concentration of 20 µM. We also wanted to evaluate the effect of SSO in the studied cultures, thus opting for a pre-treatment with 20 µM SSO for 10 minutes, and then continuing with the fatty acid treatment protocol mentioned above. SSO is known as an inhibitor of fatty acid uptake with a role in reducing inflammation.

Control samples of the experiment were incubated with vehicle (medium supplemented with 0.07% ethanol).

The maintenance of the cell cultures involved their passage, every 4 days, according to the protocols mentioned in extenso in the PhD thesis.

In order to quantify the inflammatory effect of fatty acids on astrocyte cultures we used the following materials and methods.

Luminex

From cell cultures of that were treated with oleic acid, palmitic acid and SSO, 100 µL supernatant was collected at time 0 (before addition of fatty acids) and supernatant was collected at 1 hour, 2 hours, 4 hours, 6 hours, 24 hours and 7 days.

Inflammatory markers (MIP-1a, IL-8, IL-10, IL-1b, IL4, IL-6, TNFa) were detected and quantified from the harvested supernatant using the Multiplex Magnetic Luminex Assay

Human Premixed Multi-Analyte kit (R&D Systems, Minneapolis, MN, USA and Luminex®200TM platform (Luminex Corp, Austin, TX 78727, USA) for experimental data acquisition. We used xPONENT 4.2 software for data analysis; calibration curves were generated with a 5-parameter logistic fit.

BioStation video microscopy

For real-time monitoring of fatty acid-treated astrocytic cultures, we used the BioStation IM system (Nikon, Japan) and NikonNIS Elements software for real-time image acquisition.

For this purpose, we counted and divided 10,000 cells into 4 flasks with 4 chambers (80416, Ibidi) and incubated overnight. These were pre-treated with two different concentrations of SSO (20 and 10 µM) for a duration of 10 min. After treatment, the SSO medium was replaced with vehicle (medium) or OA 40 µM. Nikon Biostation set for acquisition every 15 minutes was used to obtain the required images, and the nude area was evaluated with NiS Elements BR software.

ELISA

Another experimental step was to measure IL-6 using the commercial Legend Max quantitative tests (BioLegend, San Diego, CA, USA). The experiment consisted of treating NHA cultures with 0.2 µM beta-amyloid 1-42 and pre-incubated for 48 h at 37 °C for fibril formation. Cell supernatant was collected at 4 and 24 h. Quantitative IL-6 was determined from the harvested supernatant in order to follow the ability of astrocytes to produce a pro-inflammatory response. We added cell culture supernatant (50 µL) collected from samples treated at different times to the plates and incubated at room temperature for 2 h with shaking at 200 rpm. We then followed the ELISA protocol according to the manufacturer's specifications.

OILRED colouring

To highlight lipid droplets from cultured and fatty acid-treated astrocytes we used the Oil Red staining technique. As explained in extenso in the thesis this technique involves staining lipid-rich structures with Oil Red dye resulting in a red staining.

Statistical analysis

Statistical analysis of the data was performed using GraphPad v7 (one-way ANOVA, Dunnett's multiple comparisons) with the data being compared to the control. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

1. Astrocytes have the capacity to assimilate saturated as well as unsaturated fatty acids, Pre-treatment with SSO does not influence this effect.

Knowing that SSO is a blocker of fatty acid uptake, we wanted to test whether SSO would affect human astrocytes in astrocytic cell culture treated with SSO and fatty acids (oleic and palmitic). The extracted observation is that while SSO affects cell proliferation, the presence of saturated as well as unsaturated fatty acids prevents this effect.

2. In the presence of oleic acid, short-term SSO treatment does not affect long-term astrocyte viability.

The observation extracted from the experiment is that the mobility or viability of astrocytes did not vary significantly between the conditions tested (NHA cultures for 10 min-with SSO in two different concentrations (10 and 20 μM) and subsequent substitution of the medium with OA induction in 40 μM concentration, for all conditions tested). The results obtained support that a lower concentration of SSO leads to a higher surface coverage. This effect can be justified by a slightly higher number of splits.

The experimental results show a statistically significant progression of cell proliferation for the situations tested at SSO concentration of 5 μM ($p<0.1$) and 10 μM ($p<0.001$), respectively.

3. Astrocytes can produce pro-inflammatory cytokines in cell culture, and their synthesis is reduced by SSO treatment

Consistent with our premise, astrocytic cultures treated with beta-amyloid 1-42 induced IL-6 formation at 24 h, a statistically significantly higher value than the control ($p < 0.01$). This effect was diminished by SSO treatment.

The next experimental step was to investigate the production of other pro-inflammatory cytokines (IL-1b, IL-4, IL-6, IL-8, IL-10, TNFa, MIP1a) in the harvested supernatant, even more motivated by the observed effect of SSO in decreasing cytokine

production under all conditions tested. Despite the use of an extensive panel of inflammatory markers, IL-6 and IL-8 were among the only cytokines detected in NHA cell culture supernatant. Importantly, the secretion of these cytokines followed a different pattern of secretion relative to time.

STUDY 2- Assessment of the impact of CD36 on cognitive function

Materials and method

For behavioural and cognitive testing purposes we used two transgenic strains of CD36-/- (B6.129S1-Cd36tm1Mfe/J) and NRF2-/- mice at different ages. C57BL/6 mice of *Nrf2-/-* and *Nrf2+/+* genotypes were created from animals from the laboratory of Prof. A. Cuadrado, Universidad Autonoma de Madrid and CD36 -/- mice were purchased from Jackson Laboratoires US.

Adult mice were tested using the open field test and novel object recognition test. As for the elderly mouse groups, they were tested using the 8-arm radial maze test to analyse anxiety behaviour and memory dysfunction. Mice were housed differentially by gender and transgenic model in single cages under a normal 12 h light/dark cycle, constant temperature and humidity, with *ad libitum* access *to* food and water.

The studies were carried out in accordance with the guidelines of the European Directive 2010/63/EU and approved by the National Authority for Veterinary Research in Romania, authorisation no. 588/13.01.2022 and no. 385/09.02.2018 respectively.

Open Field Test Protocol

The experimental protocol consisted of acclimatizing all animals for 30 minutes in the test room in their cages to minimize stress. Each animal was placed in the middle of the arena and allowed to explore for 5 minutes. Using Smart 3.0 video tracking software we recorded the trajectory in the central area versus the periphery of the test paddock.

Novel Object Recognition (NOR) test protocol

The protocol for testing the new objects took place over 3 consecutive days:

- On the first day, each animal was placed in the centre of the empty arena and allowed to explore for 5 minutes.

- On the second day, mice were placed for 5 minutes in the arena and offered to explore 2 similar objects placed in diametrically opposite corners.

- On the 3rd day, one of the objects was exchanged for a new one (different shape and colour) and each mouse was allowed to explore both the old and new objects for 5 minutes.

Note that this experiment did not involve starvation of the animals. Animal behaviour was tested using SMART 3.0 video tracking software.

Radial maze test protocol

The radial maze experiment involved three stages:

- the accommodation phase - each animal was placed for 5 minutes on the central platform of the maze, with all arms closed; this phase ran for 5 successive days.
- Two-stage training phase - for the first 7 days, each mouse was placed in the central platform with free access to only 3 baited arms for 5 minutes. Over the next 14 days, mice had full access to all 8 arms, of which the same 3 were baited with food; this phase ran for 21 days.
- the final testing phase - involved placing each mouse on the central platform with all arms open; this phase was carried out over three consecutive days.

  Animal behaviour was recorded using MazeSoft8 to assess memory errors and SMART 3.0 video tracking software to analyse trails, speed and distance.

The experiment involved food deprivation for 12 hours prior to testing to increase motivation and performance.

Statistical analysis.

The database was obtained using both automatic generation of results in SMART and using the Excel database. Statistical analysis was performed using the following software Prism7 (GraphPad Software 9.1.0) with OneWay ANOVA and Student's t-test functions. Data are expressed as mean ± SD. Groups were considered significantly different when $P < 0.05$ ($*P < 0.05$, $**P < 0.01$).

Results

1) Open field test results

The results obtained in terms of total distance, distance in the centre and speed of movement of the NRF2-/- group were statistically significantly higher compared to the control group, and these results were interpreted as an anxious, hyperactive behaviour of NRF2-/- mice. In the CD36-/- group we obtained a slightly higher distance and time spent in the centre compared to the control group but without reaching statistical significance. The speed of movement and total distance were similar when testing both control and CD36-/- groups.

2) New Object Recognition (NOR) test results

The novel object recognition test shows opposite behaviour of the two animal models. While NRF2-/- animals are more interested in exploiting the novel object, CD36-/- animals avoid it, and this avoidance behaviour is even more pronounced than in the control group.

3) 8-arm radial maze test results

We tested and recorded the distance and maximum speed travelled in the initial radial maze at the beginning of testing (days 2-4) and in the mid-term (days 5-14). Both CD36-/- and NRF2-/- group subjects made errors, with all arms visited before the end of the testing period, however the NRF2 -/- group had fewer long-term memory errors.

Analysis of the experimental data showed some cognitive protection (with respect to age-related memory impairment) in CD36-/- mice. NRF2 blockade did not show any changes in cognitive abilities compared to the control group.

Finally, we wanted to investigate whether aging is an important variable in the evolution of behaviour and memory capacity for the chosen knockout groups. To this end, we examined distance and speed in aged knockout mice compared to the control group. We also assessed both working memory errors for recent memory assessment and baseline memory errors for long-term memory.

We observed an age-dependent decrease in NRF2-/- exploratory behaviour. Distance travelled was shorter than in WT, while maximum speed was comparable to control, but without memory difficulties. Regarding the performance of the CD36-/- group, no

significant differences were observed compared to the control. However, it is possible that CD36 deficiency in older animals provides some protection against memory impairment.

Similar results of both groups were also achieved in terms of distance covered and maximum speed. Regarding the production of reference memory errors, CD36 -/- performed slightly better, but without the differences being statistically significant. No statistically significant changes were observed in the NRF2 group related to distance, maximum speed or learning errors.

Conclusions and future perspectives

1) Astrocytes are able to take up both saturated (palmitic acid) and unsaturated (oleic acid) fatty acids. Fatty acid uptake is not affected by SSO pre-treatment, which specifically inhibits CD36's ability to bind these ligands. This result indicates that astrocytes also rely on other membrane transporters for GA translocation activity.

2) Astrocytes are capable of producing pro-inflammatory cytokines in cell culture, and their synthesis is reduced by SSO treatment

3) In small amounts, oleic acid has a stimulatory effect and causes early release of IL-8.

4) APP had a predominantly vascular brain expression in the CD36ko mouse model.

5) Removal of protection against oxidative stress by NRF2 deficiency influences the anxiety-like behaviour observed over time in young animals.

6) Enhanced protection against neuroinflammation by blocking the CD36 receptor potentially has a favourable effect, with some cognitive protection (in terms of age-related memory impairment) observed in CD36-/ mice.

7) NRF2 deficiency had no impact on cognitive abilities.

Based on these conclusions, I propose the following research directions:

- further study of the role of astrocytes in neuroinflammation by generating a CD36+ astrocytic cell line (e.g., by transfection or gene editing)

- Assessing the involvement of neurovascular dysfunction in amyloidogenesis by studying CD36+ astrocytes and the relationship with other blood-brain barrier participants on brain organoids.

- Further evaluation of cerebral amyloid angiopathy (CAA) in CD36-/- mice and its implication in the prevention and prognosis of cerebral haemorrhage secondary to CAA.

- continue the study on cognitive abilities of old CD36-/- mice by increasing the tested group and including a group of females (given the known impact of hormonal differences).

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Co-authored article

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