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Analysis of calcium homeostasis in fresh lymphocytes from patients with sporadic Alzheimer's disease or mild cognitive impairment☆

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ABSTRACT

Alzheimer's disease (AD) is the most widespread, age-related neurodegenerative disorder. Its two subtypes are sporadic AD (SAD) of unknown etiology and genetically encoded familial AD (FAD). The onset of AD is often preceded by mild cognitive impairment (MCI). Calcium dynamics were found to be dysregulated in FAD models, but little is known about the features of calcium dynamics in SAD. To explore calcium homeostasis during the early stages of SAD, we investigated store-operated calcium entry (SOCE) and inositol triphosphate receptor (IP_3R)-mediated calcium release into the cytoplasm in unmodified B lymphocytes from MCI and SAD patients and compared them with non-demented subjects (NDS). Calcium levels in the endoplasmic reticulum and both the rising and falling SOCE slopes were very similar in all three groups. However, we found that SAD and MCI cells were more prone to IP_3R activation than NDS cells, and increases in calcium levels in the cytoplasm were almost twice as frequent in SAD cells than in NDS cells. MCI cells and SAD cells exhibited an enhanced magnitude of calcium influx during SOCE. MCI cells but not SAD cells were characterized by higher basal cellular calcium levels than NDS cells. In summary, perturbed calcium homeostasis was observed in peripheral cells from MCI and SAD patients. Thus, lymphocytes obtained from MCI subjects may be promising in the early diagnosis of individuals who will eventually develop SAD. However, no conclusions are made regarding SAD due to the limited number patients. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. Introduction

Alzheimer's disease (AD) is the most common age-related, neurodegenerative disorder, characterized by progressive neuronal loss that leads to cognitive, memory, and behavioral impairments. AD has been diagnosed through patient history of cognitive decline and postmortem findings of extracellular plaques of amyloid β (Aβ) protein aggregates and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. At least two types of AD can be distinguished: sporadic Alzheimer's disease (SAD), which accounts for most cases, and familial Alzheimer's disease (FAD), which affects up to 5% of all AD patients and is caused by mutations in amyloid precursor protein (APP) or presenilin proteins 1 or 2 (PS1, PS2) [1]. These proteins participate in Aβ production, in which presenilins are part of a larger protein complex (i.e., γ-secretase) that is responsible for the final cleavage of APP to generate Aβ. Most mutations in these proteins favor the production of a toxic, amyloidogenic form of Aβ, Aβ42 [2].

The molecular mechanism of FAD pathology appears to be well understood and enabled the construction of many animal models. However, the etiology of SAD is still unknown. Until recently, the deposition of protein aggregates was widely accepted to be a proximal cause of pathogenesis in both FAD and SAD [3]. However, some differences between these two types of AD have lately been identified (reviewed in [4,5]). The appearance of plaques has been suggested to be a result of earlier pathological events and is not the only factor responsible for the pathology of the disease (reviewed in [6]). Currently, very few experimental studies have directly compared FAD and SAD or the etiology of SAD itself. SAD is particularly difficult to study because of a lack of appropriate animal models of the disease. The only material available for study is obtained from patients. Since SAD is believed to be a systemic disorder, changes might occur not only in neurons but also in peripheral cells, such as fibroblasts or blood cells [7–9].

Altered calcium homeostasis has recently emerged as one of the early events responsible for AD. Calcium can be linked both to FAD pathology, mostly by interactions between mutated presenilins and several molecules involved in ER calcium homeostasis or by loss of

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presenilin function as a leak channel (reviewed in [10–12]) as well as to SAD pathology, directly by altering Aβ generation and tau protein hyperphosphorylation or indirectly, mostly by calcium changes connected with aging (reviewed in [13,14]). The most convincing evidence showing the involvement of calcium aberration in AD is the fact, that one of the two drugs which are effective in AD treatment is memantine—a partial antagonist of N-methyl-D-aspartate receptor (NMDAR, nonselective cation channel).

The correlation between increased cytosolic calcium level, increased calcium fluxes and higher production of Aβ is well established [15,16]. It appears that almost all ER calcium related proteins are connected with Aβ production. The inhibition of SERCA pump leads to the inhibition of Aβ generation, whereas overexpression of SERCA increases Aβ accumulation [17]. Additionally inhibition of SOCE causes increased Aβ42 production [18]. Furthermore, knockdown of the IP_3R reduces [19], while expression of RyR increases [20] Aβ production. In vivo imaging of neurites and spines of APP overexpressing mice revealed, that spines which are located in proximity to Aβ plaques showed increased basal calcium levels. The consequences include abnormal spines morphology and disrupted calcium compartmentalization [21].

Many recent studies have shown a strong correlation between PS1 and PS2 mutations in FAD models and calcium dyshomeostasis. Such changes in calcium handling are mostly associated with calcium ion flux from the endoplasmic reticulum (ER) via two types of calcium channels: inositol triphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs).

IP_3 receptor activity was found to be significantly increased in *Xenopus laevis* oocytes that overexpress mutant PS1 and PS2 [22,23], mouse PS1 knock-in cortical neurons [24], and lymphoblasts derived from FAD patients [25]. A recent study showed that the expression of the FAD mutant PS constitutively activates cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and influences CREB-responsive gene expression in both cultured neuronal cells and mouse models of AD [26]. This was one of the first studies that showed a causal link between calcium dyshomeostasis and alterations in signaling pathways that possibly lead to neuronal dysfunction.

Increased RyR expression and exaggerated RyR calcium signaling was found in several AD mouse models that bore mutated PS1 [27–30]. An altered RyR expression pattern was found in human brain AD specimens and correlated with tau and amyloid pathology [31]. Presenilin-linked disruptions in RyR signaling and subsequent calcium-induced calcium release via NMDAR-mediated calcium influx was shown to alter synaptic function, providing additional evidence of a strong link between calcium handling and the functioning of neurons in FAD [32].

Store-operated calcium entry (SOCE) is a mechanism that enables the replenishment of depleted ER calcium stores. SOCE was shown to be downregulated in mouse PS1 knock-in fibroblasts [33], human neuroblastoma that overexpressed PS1 [34], cortical neurons from different FAD models [35], human microglia [36], and lymphoblasts obtained from FAD patients [37]. Mutations in presenilins, therefore, may disrupt various aspects of calcium homeostasis and contribute to neuronal failure.

Interestingly, some changes in calcium homeostasis were also observed in peripheral cells obtained from SAD patients [38–41]. A more comprehensive dataset that reveals aberrant calcium signaling in peripheral cells derived from SAD patients may help better understand the molecular basis of the disease and relationship between FAD and SAD.

Mild cognitive impairment (MCI) is defined as the presence of cognitive impairments that are more severe than those expected at a certain age but not significant enough to interfere with everyday activities [42]. Mild cognitive impairment does not fulfill the criteria of AD or any other dementia. It is often observed in individuals who develop AD later in life and therefore may be considered a risk factor for AD [42–44]. Little is known about calcium dysregulation in MCI, but RyR expression patterns were shown to be different between MCI and AD groups [45].

The present study investigated the changes in calcium handling observed in unmodified lymphocytes derived from SAD patients and MCI subjects. We focused on the two major cytoplasmic calcium entry mechanisms: SOCE and IP_3R-dependent flux. To our best knowledge, there was no study concerning IP_3R mediated signals in unmodified lymphocytes B from SAD patients, despite very promising results obtained for FAD lymphocytes bearing presenilin mutations. Also, no broad spectrum analysis including kinetics of the SOCE reaction was made in cells obtained from SAD patients. The use of fresh lymphocytes B is critical, because most of the experiments to date were carried on immortalized lymphocytes B from AD patients and it is known, that the process of immortalization interferes with cell metabolism and calcium handling [46,47]. Our results showed that cells derived from the MCI group were characterized by increased SOCE calcium influx and basal calcium levels. The cells derived from SAD patients exhibited a higher response to IP_3R stimulation. These data confirm some of the previous observations in FAD cells and provide new information about early calcium aberrations that likely lead to SAD.

2. Materials and methods

2.1. Subjects

Samples of peripheral venous blood were obtained from 16 SAD patients aged 57–86 years (mean, 75 ± 8 years), 28 MCI subjects aged 58–84 years (mean, 69 ± 9 years), and 22 non-demented subjects (NDS) aged 60–84 years (mean, 73 ± 7 years). All of the subjects were considered for the IP_3R calcium signaling measurements. Due to the failure of hardware during SOCE measurements the samples from three SAD patients and one MCI subject could not be collected.

The subjects' demographic and clinical characteristics are reported in Table 1. The SAD patients and MCI subjects were recruited from the Alzheimer's Disease Department at the Ministry of Internal Affairs Hospital, Warsaw, Poland. The non-demented subjects were recruited from the Cardiovascular Disease Clinic at Banacha's Hospital, Warsaw, Poland. The SAD and MCI subjects underwent detailed medical, neuropsychological, neurological, and psychiatric examinations. Close informants were interviewed, and laboratory testing and computed tomography or magnetic resonance imaging of the brain were performed. The clinical diagnosis of probable AD was made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association [48]. Mayo Clinic criteria were used to diagnose MCI [49]. Two MCI and two AD patients suffered from infections or immune diseases. The exclusion of the results obtained for these subjects doesn't change the statistical significance of the results. The NDS group consisted of subjects with no apparent neurological disease or psychiatric syndrome, no complaints of memory impairment, and a Mini-Mental State Examination score ≥ 27. The exclusion criteria for NDS included autoimmune diseases, neoplasms, and acute or chronic inflammation. Because NDS were recruited from the Cardiovascular Disease Clinic, most of them suffered from cardiovascular diseases (CVD). CVD is one of the many risk factors for AD [50] and aging is one of the risk factors for both AD and CVD [50]. Therefore, aged cardiovascular patients who showed no signs of dementia can be considered as AD "escapees". All of the participants were informed of the purpose of the study and gave their informed written consent. The project was approved by the Bioethical Committee for Studies on Human Subjects at Central Clinical Hospital MSWiA in Warsaw (approval no. 08/2011).

2.2. Preparation of cells

Blood samples were collected between 8:00 and 9:00 AM. Peripheral blood mononuclear cells were isolated from 6 ml of venous blood collected in lithium heparin-treated tubes (BD Vacutainer, BD Biosciences) by standard density gradient centrifugation (LSM 1077 Lymphocyte, PAA). Briefly, blood was mixed in a 1:1 ratio with phosphate-buffered

Table 1
Demographic and clinical information for Alzheimer's disease patients (SAD group), mild cognitive impairment subjects (MCI group), and non-demented subjects (NDS subjects).

SAD				MCI				NDS	
Age (years)/ sex	MMSE	CDR	GDS	Age (years)/ sex	MMSE	CDR	GDS	Age (years)/ sex	MMSE
57/M	19	1/2	5	54/F	30	0/0.5	3	57/M	29
62/F	24	1	3/4	56/F	25	0.5	2/3	60/M	30
69/M	20	1/2	4	57/M	29	0.5	3	62/M	30
70/F	26	0.5/1	4	58/F	30	0.5	3	64/F	30
74/F[a]	23	1/2	4	58/M	28	0.5/1	2	65/F	30
74/M	20	1/2	4	58/F	25	0	2/3	65/M	29
75/F	25	1	4	60/M	25	0.5	3	66/F	30
76/F	20	1	3/4	60/M	28	0	2/3	67/F	28
77/F[a]	17	1	3/4	62/M	28	0.5	3	67/F	29
78/F	22	1	3/4	63/M	25	0.5	3	70/M	30
79/M	17	2	4/5	64/F	28	0/0.5	3	72/F	30
80/F[a]	26	0.5	3	66/M	27	0/0.5	3	73/F	27
80/M	23	1	3/4	68/M	27	0.5	3	74/F	30
83/F	26	1	3	70/F	28	0.5	3	74/F	29
84/F	23	1	3/4	70/F	26	0.5	3	75/M	29
86/M	22	1/2	4/5	70/M	25	0.5	3	77/M	30
				70/F	30	0/0.5	3	78/M	30
				72/M	30	0/0.5	3	78/F	29
				72/M	25	0.5	3	80/F	29
				72/M	30	0.5	3	81/F	30
				73/M	27	0.5	3	81/F	27
				75/F[a]	29	0.5	2	84/M	29
				81/M	28	0	3		
				81/M	28	0	3		
				81/F	26	0.5	3		
				82/M	29	0.5	2		
				83/M	27	0.5	3		
				84/F	27	0.5	3		
Average		Mode		Average		Mode		Average	
75 ± 8	22 ± 3	1	3.5	69 ± 9	28 ± 2	0.5	3	73 ± 7	29 ± 1

F, female; M, male; MMSE, Mini-Mental State Examination; CDR—Clinical Dementia Rating; GDS—Global Deterioration Scale.
[a] Subjects absent from the SOCE analysis.

saline (PBS), overlaid on Ficoll, and centrifuged. The interphase that contained mononuclear cells was washed twice with PBS. Human B lymphocytes were isolated with the Dynabeads Untouched Human B Cells kit (Invitrogen, Life Technologies) according to the manufacturer's protocol. The cells were cultured overnight in RPMI 1640 medium with 1% HEPES and 1% penicillin–streptomycin supplemented with 10% fetal bovine serum and 2% L-glutamine (all from Sigma-Aldrich). The next day, the cells were attached to poly-L-lysine-coated Lab-Tek Chambered Coverglass eight-well slides (Nunc) for 2 h.

2.3. Calcium measurements

Fura-2 digital fluorescence microscopy was used to determine changes in intracellular Ca^{2+} levels [51]. Briefly, before the experiment, the culture medium was replaced for 30 min by 2 μM Fura-2 AM (Molecular Probes, Life Technologies) in a solution that contained 145 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES (pH 7.4), and 1 mM $MgCl_2$ (standard medium) supplemented with 2 mM $CaCl_2$ (high-calcium medium) at 37 °C. The cells were then washed by 15 min incubation with standard medium supplemented with 0.5 mM ethylene glycol tetraacetic acid—EGTA (low-calcium medium) for SOCE or 2 mM $CaCl_2$ for IP_3R calcium signaling measurements. The Lab-Teks were mounted on an Olympus Scan^R/Cell^R imaging system equipped with an IX81 inverted microscope, MT20 illuminator (Xenon lamp, high-speed filter wheel with 340 and 380 nm filters), and cage incubator (Life Imaging Services, Basel, Switzerland) set at 37 °C. Images were taken with a 40×/0.95 objective and Hamamatsu C9100-02 EM-CCD camera. Data acquisition and analysis were conducted using Olympus Cell^R software. SOCE measurements were initiated in the low-calcium medium. After 3 min, 1 μM thapsigargin (TG) was added (Sigma-Aldrich). After 5 min, calcium was added to yield a final concentration of 2 mM. Measurements of IP_3R calcium responses began in a calcium medium that was replaced 7.5 min later by the same one that contained 5 μg/ml Gt F(ab')2 Anti-Human IgM antibody (Invitrogen). The experiment was carried out in parallel for subjects from various groups and no differences in highest and lowest Fura-2 ratios were detected. In human lymphocytes calcium measurements were performed by others to analyze dialysis patients and with chronic renal failure [52].

2.4. Data processing

Fura-2 signals were analyzed using a custom computer program (details and software available upon request: j.dzbek@gmail.com). This automated approach allowed for the elimination of personal bias in the data evaluation and made the extraction of a broad range of parameters feasible from a large dataset of available signals, in which a total of 1500 single cell responses were recorded. Using this custom software, the following parameters were quantified:

- SOCE: Calcium signal baseline value (basal), area-under-the-curve (AUC) after the addition of TG to the low-calcium medium (AUC ER, representing the releasable calcium pool in the ER), AUC in the high-calcium medium (AUC SOCE, showing Ca^{2+} influx in SOCE), calcium peak height (peak ΔCa^{2+}), maximum rise and maximum fall rates of SOCE peak ($\uparrow Ca^{2+}$ slope and $\downarrow Ca^{2+}$ slope, respectively), and calcium level at the end of the measurement (final Ca^{2+}).
- IP_3R signals: Presence of spontaneous peaks before the addition of IgM antibody, presence of response of the cell to anti-IgM stimulation, presence of an additional calcium increase after anti-IgM stimulation.

The acquired calcium time-courses were initially down-sampled and de-noised using a Wiener filter. Several custom-developed heuristic algorithms were then applied. The first step involved finding the local maxima of the signal and its derivative and other locally estimated values. In the second step, the results from the first step were properly aggregated to acquire the value of the specific signal parameter. The aggregation step was controlled by parameters that were manually adjusted on a subset of representative signals. Their values were selected to ensure the best algorithm specificity and sensitivity. Finally, all of the calculated signal parameter values were graphically inspected to avoid obvious errors that may have arisen from insufficient signal quality or algorithm problems.

2.5. Statistical analysis

The statistical analysis was performed using PRISM software version 5.02 (GraphPad Software, San Diego, CA) and one-way analysis of variance (ANOVA) followed by Tukey's multiple test for comparing three groups. A normal distribution of the data from all of the experiments was indicated using the D'Agostino–Pearson normality test. Differences were considered significant at a level of $p<0.05$. Each symbol represents the mean value of four independent experiments. The number of cells measured in four independent experiments ranged from 10 to 223 each (average, 70 ± 46). Horizontal lines in the figures represent mean values within a group. The bars indicate the standard deviation ($^{*}p<0.05$, $^{**}p<0.01$).

3. Results

3.1. B lymphocytes exhibit heterogeneous IP_3R-mediated calcium response

B lymphocytes were purified from blood samples obtained from SAD patients, MCI subjects, and non-demented subjects (NDS; Table 1). For each type of cell, two calcium entry routes into the cytoplasm were

tested: calcium entry from intracellular stores via IP_3R and SOCE from the extracellular space. The collected data were analyzed, and the features and quantitative parameters of the responses were compared between each of the three groups (Table 2). When IP_3 receptors were activated with anti-IgM antibodies (anti-IgM) via the B cell receptor (BCR) pathway, two types of calcium responses in human B lymphocytes were observed. In 48% of responding cells, a single, transient calcium peak was recorded (Fig. 1, black line). In remaining responding cells, anti-IgM stimulation triggered a calcium peak that was followed by a sustained increase in calcium levels (Fig. 1, gray line). 54% of cells exhibited spontaneous calcium oscillations in a high-calcium medium (Fig. 1, dotted line). Spontaneous calcium oscillations were dependent on extracellular calcium, as in a calcium free medium none or very few oscillations were detected (data not shown).

3.1.1. Fraction of cells with spontaneous oscillations is decreased in SAD cells

We first compared the number of cells that exhibited spontaneous calcium oscillations. The relative number of oscillating cells was significantly lower in SAD cells and slightly lower in MCI cells than in NDS cells (Fig. 2A).

3.1.2. SAD lymphocytes are more sensitive to anti-IgM-induced activation

We next compared the responses of B lymphocytes to anti-IgM-induced activation (i.e., whether IP_3Rs are more prone to activation in SAD or MCI cells than in NDS cells). As shown in Fig. 2B, 17% of NDS cells responded to the stimulation, whereas the percentages were 23% and 27% in cells from MCI subjects and SAD patients, respectively. All of the response types were considered. The difference between SAD and NDS was statistically significant.

3.1.3. Fraction of cells that respond with cytoplasmic calcium influx after anti-IgM-induced activation is doubled in SAD cells

As shown in Fig. 1, the activation of IP_3R in some cells was followed by sustained calcium influx. The relative number of these cells in the SAD group was increased by approximately 16% compared with 8.5% in NDS cells and 11% in MCI cells (Fig. 2C). The difference between SAD and NDS was statistically significant. A summary of these data is shown in Table 2.

3.2. Quantitative parameters of SOCE in B lymphocytes

B lymphocytes were treated with thapsigargin (TG), the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor, in a low-calcium medium to empty ER calcium stores. After store depletion, the medium was enriched with calcium to induce SOCE. Several parameters of calcium dynamics were derived from the recorded signals. Basal calcium levels were estimated before the addition of TG. The AUC in the low-calcium medium in the presence of TG (AUC ER) reflected the amount of calcium released from the ER, whereas the AUC in the high-calcium medium reflected the extent

Table 2
Summary of parameters of calcium dynamics in lymphocytes from non-demented subjects (NDS), sporadic Alzheimer's disease patients (SAD), and subjects with mild cognitive impairment (MCI).

Parameter	Comparison between groups	Fold change	Figure number
Fraction of cells with spontaneously occurring oscillations (%)	NDS > SAD	1.4*	2A
Fraction of cells with activation of IP_3 receptors (%)	SAD > NDS	1.6*	2B
Fraction of cells with calcium influx from the medium after IP_3R activation (%)	SAD > NDS	1.9*	2C
Basal calcium level in cytoplasm (Fura-2 ratio)	MCI > NDS	1.1**	4
ER calcium efflux (AUC ER)	SAD = MCI = NDS	–	5
Calcium influx during store-operated calcium entry (AUC SOCE)	MCI > NDS	1.3*	6A
Calcium peak height (Fura-2 ratio)	MCI > NDS MCI > SAD	1.2* 1.3*	6B

Asterisks (*, **) indicate statistical significance.

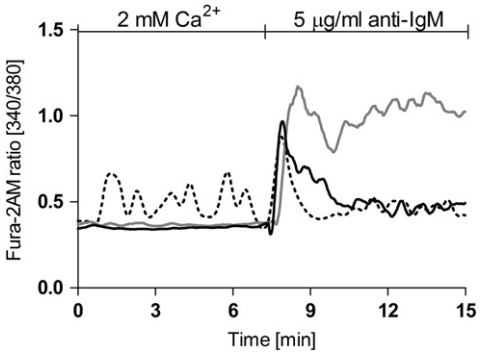

Fig. 1. Representative IP_3R-mediated calcium signaling induced by immunoglobulin M antibody (anti-IgM). Two types of responses were triggered by anti-IgM stimulation. The black line indicates a single calcium peak followed by a decrease in calcium concentration. In some cases, the IP_3R signals were followed by additional calcium influx, indicated by the gray line. IP_3R-mediated calcium signaling in B lymphocytes was elicited by binding the B cell receptor (BCR) with a high concentration (5 µg/ml) of anti-IgM in the high-calcium medium. The dotted line indicates a representative cell response with spontaneous calcium oscillations observed in the high-calcium medium before anti-IgM stimulation.

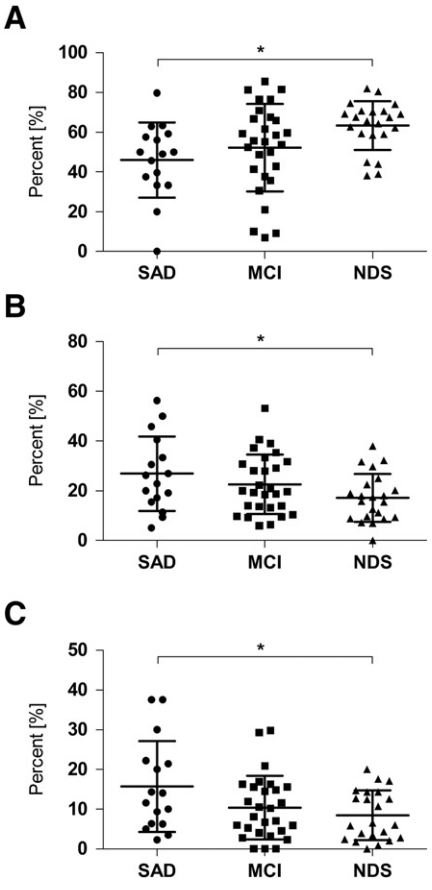

Fig. 2. Percentage of B lymphocytes cells with various increases in calcium. (A) Percentage of lymphocytes with spontaneous oscillations. Spontaneous oscillations were observed in the high-calcium medium for 2 min before the addition of anti-IgM (*$p<0.05$). (B) Percentage of the cells shown in Fig. 1 that responded to IgM antibody. Lymphocytes were incubated in a high-calcium medium. After 7.5 min, anti-IgM was added at a final concentration 5 µg/ml (*$p<0.05$). (C) Percentage of cells in which anti-IgM triggered influx of calcium from medium. In the majority of the cases, IP_3R stimulation was followed by calcium influx from the high-calcium medium (*$p<0.05$).

of calcium influx defined as SOCE (AUC SOCE). Other parameters calculated in the high-calcium medium are shown in Fig. 3, including peak amplitude (peak ΔCa^{2+}), the rising slope of the calcium peak ($\uparrow Ca^{2+}$ slope), and the falling slope of the peak ($\downarrow Ca^{2+}$ slope). All of these parameters were compared between the three groups (Figs. 4–6).

3.2.1. Basal calcium levels are increased in lymphocytes from MCI subjects

We compared basal calcium levels in resting cells. In MCI cells, the level was significantly higher than in NDS cells (Fig. 4). The exclusion of the outliers in the MCI group did not change the statistical significance of the results (Table 2).

3.2.2. Releasable calcium pool in the ER is unchanged between groups

To empty ER calcium stores, TG was added to the low-calcium medium. The amount of calcium stored in the ER was estimated by measuring the AUC (AUC ER). No differences were found in the releasable calcium pool from the ER in SAD, MCI, and NDS cells (Fig. 5, Table 2).

3.2.3. SOCE is enhanced in MCI cells

We next compared the dynamics parameters of SOCE calcium influx evoked by calcium enrichment of the medium. Two of the analyzed parameters differentiated the cells from MCI patients and NDS individuals, specifically the overall amount of calcium that entered the cell (AUC SOCE) and calcium peak amplitude (Fig. 6A, B). Additionally, the calcium peak amplitude was significantly lower in the SAD group than in the MCI group. The other three parameters did not significantly discriminate the cells from the analyzed groups (i.e., rise and fall rates of the SOCE peak [$\uparrow Ca^{2+}$ slope and $\downarrow Ca^{2+}$ slope, respectively]; Fig. 6C, D; calcium level at the end of the measurement [final Ca^{2+}]; data not shown).

4. Discussion

Alzheimer's disease is one of the most extensively studied neurodegenerative disorders. Most research has focused on animal models or post mortem human brain specimens. However, post mortem analysis provides information about the latest stages of the disease, and animal models enable the investigation of FAD but not necessarily SAD. The majority of animal models were generated based on the amyloid and tau hypothesis, which insufficiently explains the induction of sporadic AD pathology (reviewed in [53]). Most of the drugs developed based on the amyloid hypothesis (i.e., drugs that seek to reduce Aβ amyloid production, prevent Aβ aggregation, clear amyloid deposits, or target tau phosphorylation and assembly) have failed in late clinical trials (reviewed in [54]). Therefore, expanding our view of SAD and searching for other possible causes of the disease that are responsible for its onset are necessary. The establishment of early diagnosis and reliable biomarkers is critical, and the use of peripheral cells may be a solution, in addition to brain imaging and cerebrospinal fluid biomarker analyses.

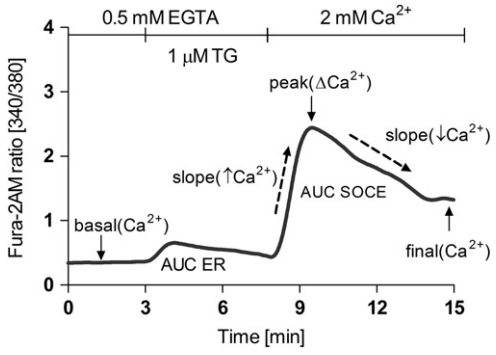

Fig. 3. Single-cell calcium traces during store-operated calcium entry. Cells were incubated in a low-calcium medium. SOCE was evoked by the addition of 1 μM thapsigargin (TG) followed by the addition of a high-calcium medium. Each of the analyzed parameters is indicated.

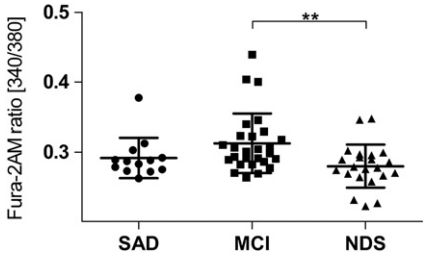

Fig. 4. Basal calcium level in human B lymphocytes. Lymphocytes were incubated in a low-calcium medium to avoid the occurrence of spontaneous peaks. Relative basal calcium levels were measured during the first 2 min of the experiment before the addition of thapsigargin (TG) (**$p<0.01$).

Alzheimer's disease is considered a brain disease, but the periphery also appears to be involved. Indeed, AD shows typical peripheral effects, with changes in fibroblasts and blood cells, including enhanced oxidative stress, impaired cellular calcium dynamics, and mitochondrial dysfunction [55,7,56–58]. All of these pathological processes observed in peripheral cells long precede plaque formation in neurons, making them perfect tools for early diagnosis [59,24]. A strong correlation exists between mitochondrial dysfunction, which leads to oxidative damage, and changes in cellular calcium homeostasis. Interference with metabolism increases cytosolic free calcium and alters intracellular calcium dynamics [60]. Thus, these processes might be considered to be linked in a chain of events.

In the search for possible biomarkers, a very helpful approach is to use peripheral cells obtained from MCI subjects. The rate of progression from MCI to AD is estimated to be almost seven-fold higher than the progression to AD in individuals without cognitive impairment [43]. Unfortunately, very few studies have compared changes in peripheral cells from AD and MCI subjects. Increased DNA damage, particularly attributable to oxidized purines and pyrimidines, was found in peripheral cells from AD and MCI subjects [61]. A reduction of the complex IV activity of platelets was reported in AD and MCI [62]. Enhanced apoptotic death, elevated SOD mRNA, and an elevated Bax/Bcl2 ratio were found in peripheral blood mononuclear cells from MCI and AD subjects [63]. Plasma antioxidant levels were also shown to be decreased in both AD and MCI [64]. Mitochondrial dysfunction in lymphocytes from AD and MCI patients without any Aβ or tau pathology points to the relevance of these changes in the cascade of events that leads to brain failure.

An exaggerated IP_3R response in AD samples was reported in the early 1990s, and diagnostic criteria for fibroblasts from both FAD and SAD patients were proposed [40]. More recently, a possible mechanism was discovered. Presenilins 1 and 2 were shown to interact with IP_3Rs, and mutations in PS enhanced the gating of IP_3R Ca^{2+} channels through a gain-of-function effect that was independent of γ-secretase activity. These data were obtained in FAD lymphoblasts and murine neurons

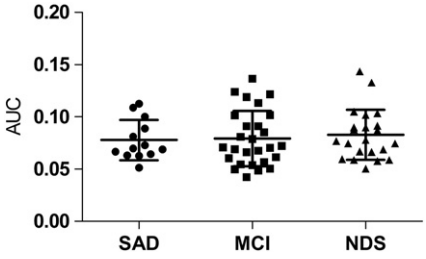

Fig. 5. Releasable calcium pool from the endoplasmic reticulum in AD, MCI, and NDS cells. The AUC was calculated for cells treated with 1 μM thapsigargin (TG) in a low-calcium medium for 5 min. No changes were observed between the three groups of cells.

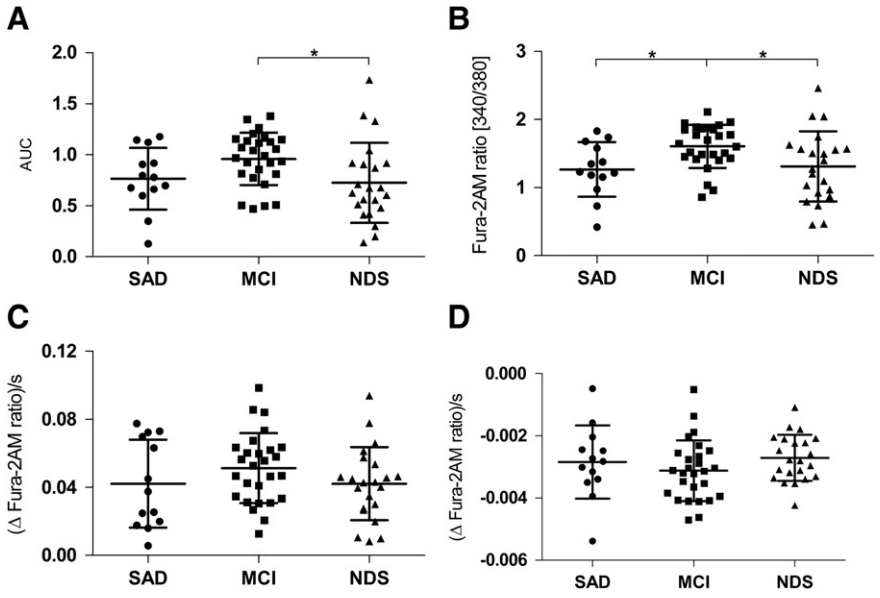

Fig. 6. SOCE increased in B lymphocytes obtained from MCI subjects. (A, B) AUC–SOCE and maximum calcium level during SOCE (peak ΔCa^{2+}) were calculated during 8 min of calcium influx in a high-calcium medium after blocking the SERCA pump with 1 μM thapsigargin (TG) (*$p<0.05$). (C, D) Rising and falling slopes of SOCE reaction, respectively. The slope was calculated as a change in calcium concentration over time.

[25]. However, these observations did not explain why similar changes were observed in SAD cells, in which presenilin was not mutated.

The present study found an exaggerated IP_3R response in unmodified lymphocytes from SAD patients. The percentage of cells in which IgM triggered a calcium response followed by additional calcium influx was also higher in SAD cells. Such a prolonged and enhanced calcium presence in cells may influence basic cell pathways and contribute to oxidative changes. For example, dysregulated phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II-α was found in the hippocampus of subjects with MCI and SAD [65]. An interesting observation was that the percentage of cells with spontaneous activation was smaller in SAD and MCI cells than in cells from non-demented subjects. There is an inverse correlation between the number of spontaneous peaks and the magnitude of the response to anti-IgM stimulation, but there is no inverse correlation between the occurrence of the oscillations and the presence of the anti-IgM response. This may indicate that the behavior of SAD and MCI cells changes because of an ongoing inflammatory process. This would support involvement of inflammation in SAD pathology (reviewed in [66]).

Notably, we observed a gradation of changes in the case of IP_3R mediated responses in all three groups, with the most pronounced changes seen in SAD and fewer in MCI. This may support the hypothesis that MCI is an intermediary step between non-dementia status and SAD. In the case of SOCE and basal calcium level, NDS and SAD group shared similar results, with outstanding results obtained for the MCI group. As discussed later, the SAD data may indicate that after the initial pathological increase in SOCE and basal calcium levels observed during the MCI stage, the compensating mechanism occurs during later stages of the disease. However, SAD cells were initially obtained from 16 patients, but 3 samples could not be analyzed because of hardware problems. This brought down the number of patients to 13, but two of them had infections, which could affect calcium homeostasis. Therefore, the final number of samples is low to make conclusive remarks about physiological significance of these observations. SOCE has been reported to be decreased in FAD cells, but some reports have found either no changes or an increase in SOCE (reviewed in [11]). The present study found no changes in the magnitude and kinetics of SOCE in SAD cells, but MCI cells were characterized by enhanced SOCE. We hypothesize that exaggerated SOCE occurs at the beginning of the disease and then is compensated (in the case of early symptomatic stages of SAD) or downregulated (in the case of advanced SAD) by some yet unknown mechanism. The releasable pool of calcium from the ER was unchanged in all three groups, consolidating the notion that the "store overload hypothesis" does not explain the increase in calcium responses from the ER observed in SAD but rather exaggerated IP_3R single-channel activity [25] or RyR overexpression [30]. We showed that basal calcium levels were increased in MCI cells. Such increases were not observed in SAD cells, consistent with the data reported earlier in fibroblasts from SAD patients and control subjects [67]. Enhanced calcium signaling may therefore be a triggering mechanism in SAD pathology that occurs at the very early stages of the disease. Enhanced SOCE and increased basal calcium levels in MCI subjects can significantly interfere with cellular metabolism and consequently impair many signaling pathways, activate calcium-dependent enzymes, and trigger pathological processes, such as oxidative stress and apoptosis.

Some reports show that pathological changes observed in AD are not restricted to neurons, but can be observed in fibroblasts and blood cells. Such peripheral cells have been therefore considered as cellular material for drug screening, and as a potential easily available diagnostic material. Some changes are observed in subjects with MCI that may be considered an AD risk factor. To follow the clinical outcome of the MCI subjects analyzed in these studies would be very interesting to see whether the patients with the highest calcium changes develop AD. If so, then lymphocytes obtained from MCI subjects may be a promising material for early diagnosis in groups at-risk for SAD.

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