Calcium dysregulation in mononuclear cells from patients with Amyotrophic lateral sclerosis

Dissertation

zur Erlangung des akademischen Grades

doctor medicinae (Dr. med.)

vorgelegt dem Rat der Medizinischen Fakultät
der Friedrich-Schiller-Universität Jena

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geboren am 15.08.1986 **in** Pingliang

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Tag der öffentlichen Verteidigung: 17.Oktober 2017

Content

List of abbreviations

Abbreviation	Description
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
CHOP	C/EBP homologous protein
CNS	Central neuro system
CPA	Cyclopiazonic acid
CSF	Cerebrospinal fluid
ER	Endoplasmic reticulum
ERMCC	ER-mitochondrial Ca^{2+}cycle
fALS	Familial ALS
FIG4	Polyphosphoinositide phosphatase
FUS/TLS	Fused-in sarcoma protein/translated in liposarcoma
HAND	HIV-associated neurocognitive disorders
IHC	Immunohistochemistry
IRE1	Inositol-requiring enzyme1
LMN	Lower motor neuron
LPS	Lipopolysaccharide
MNs	Motor neurons
NLRP3	Nucleotide-binding oligomerization domain receptors
PBMC	Peripheral blood mononuclear cells
PERK	Protein kinase RNA-like ER kinase
ROS	Reactive oxygen species
RyR	Ryanodine receptors
sALS	Sporadic ALS
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SETX	Senataxin
SOD1	Copper-zinc superoxide dismutase 1
TDP-43	DNA-binding protein-43
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor
UMN	Upper motor neuron

UPR	Unfolded protein response
VAPB	Vesicle-associated membrane protein-associated protein B
VCP	Valosin containing protein

Abstract

Amyotrophic lateral sclerosis (ALS) is a heterogeneous multisystemic disease involving the selective loss of upper motor neuron (UMN) and lower motor neuron (LMN). Unsuitable environment for MNs is largely correlated to ALS pathogenesis. It has been shown that improving MNs´ surrounding environment at the initial stage could exert a protection effect. Moreover, immune abnormalities have been found in the blood and CSF of ALS patients. Mononuclear cells from peripheral blood have been revealed as a vital source for establishing disease-specific biomarkers. In ALS it has been described as a condition in which the endoplasmic reticulum (ER)-mitochondria Ca^{2+} balance is deregulated and misfolded proteins are aggregated above the toxicity threshold. Similar calcium homeostasis dysfunction is also present in peripheral blood cells of ALS patients. In peripheral blood monoculear cells (PBMCs), a large variety of Ca^{2+} channels, receptors and Ca^{2+} binding protein are involved in the regulation of intracellular Ca^{2+} level. Furthermore, P2X receptors (P2XRs), expressed on PBMCs, are highly related to deregulate intercellular Ca^{2+} homeostasis.

In the present dissertation the aim was to find a link between P2XRs and Ca^{2+} dysregulation in PBMCs by performing the measurement of intracellular Ca^{2+} levels in vitro. Furthermore, the Ca^{2+}-binding protein (calnexin) expression level in monocytes from ALS patients was compared to controls.

Blood samples were collected from 82 ALS patients (male=48, female=32) and 40 age- and gender-matched controls (male=19, female=21) and used for PBMC isolation. The expression of P2X4R and P2X7R was analyzed by immunohistochemistry (IHC) and western blot. Fura-2, a ratiometric fluorescent dye specifically bounded to free Ca^{2+} was used to detect intercellular Ca^{2+}. Moreover, exogenous adenosine triphosphate (ATP), inflammatory stimulus lipopolysaccharide (LPS) and thapsigargin were used for Ca^{2+} elicitation. To analyze changes in ER Ca^{2+}-binding proteins of monocytes, differences in calnexin levels between patients and controls were measured by the integrated density.

Western blot analyses revealed no significant difference in protein level of P2X4 expression between patients and controls. However, the expression of P2X7R decreased in ALS patients compared with controls. In the monocytes from patients

with ALS, the increase of cytosolic Ca^{2+} induced by different concentrations of ATP was significantly lower than controls. Similarly, the Ca^{2+} level of controls was significantly higher in terms of elevation of LPS-evoked Ca^{2+}. By contrast, thapsigargin-evoked Ca^{2+} elevation during acute sarco/endoplasmic reticulum calcium ATPase (SERCA) SERCA inhibition were extremely high in ALS patients. More abundant calnexin was visualized in monocytes from patients, suggesting an ER-related protein aggregation with ER stressor exposures.

This work elucidates Ca^{2+} disturbance in monocytes from ALS patients. Calnexin is involved in the Ca^{2+}dysregulation in monocytes during immune activation and ER stress. At the initial stage, aggregation exerts protective effects, but may become a potential cytotoxic event resulting from impaired ER at the advanced stages of ALS. The results also offer an explanation for overloaded Ca^{2+} in monocytes which may be buffered by an increase of Ca^{2+}-binding protein.

Zusammenfassung

Die Amyotrophe Lateralsklerose (ALS) ist eine heterogene, multisystemische Erkrankung, die mit dem selektiven Untergang der oberen und unteren Motoneurone einhergeht. Ein schädigendes Milieu in der Umgebung der Motoneurone wird als bedeutender Faktor in der Pathogenese der ALS gesehen. Eine Verbesserung dieser Umgebungsbedingungen in der initialen Phase der Erkrankung erweist sich als protektiv. Außerdem sind immunologische Veränderungen im Blut und Liquor von ALS-Patienten nachweisbar. Die Isolation von mononukleären Zellen des peripheren Blutes (peripheral blood mononuclear cells, PBMCs) ist eine wichtige Methode zur Erforschung erkrankungsspezifischer Biomarker. In der Pathologie der ALS wird ein Zustand beschrieben, in dem das Ca^{2+}-Gleichgewicht zwischen Endoplasmatischem Reticulum (ER) und Mitochondrien dereguliert ist und fehlgefaltete Proteine oberhalb toxischer Konzentrationsschwellen aggregieren. Dies könnte ein Schlüsselprozess sein, der zum Zelluntergang von Motoneuronen führt. Ähnliche Dysregulationen in der Calcium-Homöostase zeigen sich auch in den peripheren Blutzellen der ALS-Patienten. In PBMCs ist eine Vielzahl unterschiedlicher Ca^{2+}-Kanäle, Rezeptoren und Ca^{2+}-bindender Proteine vorhanden, die an der Regulation intrazellulärer Ca^{2+}-Konzentrationen beteiligt sind. Vor allem die auf PBMCs exprimierten P2X-Rezeptoren (P2XRs) spielen eine große Rolle bei der Deregulation der intrazellulären Ca^{2+}-Homöostase.

Das Ziel der vorliegenden Arbeit bestand darin, eine Verbindung zwischen den P2XRs und der Calcium-Dysregulation in PBMCs durch die Messung intrazellulärer Ca^{2+}-Konzentrationen in vitro zu finden. Außerdem wurde das Expressionslevel des Ca^{2+} bindenden Proteins Calnexin in Monozyten von ALS-Patienten gemessen und mit denen gesunder Kontrollen verglichen.

Dazu wurden Blutproben von 82 ALS-Patienten (48 männliche, 32 weibliche) und 40 alters- und geschlechtskorrelierte Kontrollpersonen (19 männliche, 21 weibliche) gesammelt, um PBMCs zu isolieren. Die Expression von P2X4R und P2X7R wurde mithilfe immunhistochemischer Färbungen und dem Western Blot-Verfahren analysiert. Fura-2 als radiometrischer, fluoreszierender Farbstoff, der spezifisch freies Ca^{2+} bindet, wurde verwendet, um intrazelluläres Ca^{2+} zu detektieren. Dabei wurden exogenes Adenosintriphosphat (ATP), Lipopolysaccharid (LPS) als inflammatorischer Stimulus und Thapsigargin genutzt, um Ca^{2+}-Ströme zu induzieren.

Die Untersuchung von Veränderungen in der Konzentration Ca^{2+}-bindender Proteine des ERs in Monozyten erfolgte anhand immunhistochemischer Messung des Calnexin-Levels in Monozyten von ALS-Patienten und Kontrollen.
Die Auswertung der Western Blot-Ergebnisse ergab keine signifikanten Unterschiede im Proteinlevel der P2X4R-Expression zwischen ALS-Patienten und den Kontrollpersonen, wo hingegen die Expression von P2X7R bei ALS vermindert war. Die Monozyten der ALS-Patienten zeigten nach Induktion durch unterschiedlich hohe ATP-Konzentrationen einen signifikant geringeren Anstieg in der zytosolischen Calcium-Konzentration im Vergleich zu Kontrollen. Ebenso wurde durch LPS ein signifikant geringerer zytosolischer Calcium-Anstieg bei ALS-Patienten ausgelöst. Im Gegensatz dazu konnten massiv erhöhte Calcium-Elevationen nach Thapsigargin-Applikation bei den Monozyten der ALS-Patienten gemessen werden, welches als akuter Inhibitor der Sarco/Endoplasmatisches Reticulum-Calcium-ATPase (SERCA) wirkte. Darüber hinaus wurde Calnexin nach Inkubation mit ER-Stressoren in größerem Ausmaß bei ALS-Patienten nachgewiesen, was auf eine Proteinaggregation im ER hinweist.
Diese Dissertation belegt eine Störung des Calcium-Haushaltes in Monozyten von ALS-Patienten. Calnexin ist bei der Calcium-Dysregulation in Monozyten während der Immunaktivierung und des ER-Stresses involviert. Im initialen Stadium stellt die Proteinaggregationen zwar einen protektiven Effekt dar, kann jedoch durch Störung des ERs zu Zytotoxizität im fortgeschrittenem Stadium der ALS führen. Die Ergebnisse dieser Arbeit erklären außerdem auch die Calcium-Überladung in den Monozyten, was durch eine erhöhte Konzentration an Calcium bindenden Proteinen kompensiert sein könnte.

1 Introduction

1.1 The pathology of ALS

ALS is a rapidly progressive neurodegenerative disease. Primarily characterised by destruction of both upper and lower MNs in the motor cortex, brain stem and spinal cord, this kind of disease can lead to muscular atrophy, paralysis and respiratory failure (Wijesekera and Leigh, 2009). The prevalence is approximately 4-6 cases in 100,000, with the mean age of onset between 48 and 70 years. Approximately 90% of ALS cases are sporadic ALS (sALS), which is the most common form of the disease (Gros-Louis et al., 2006). However, about 10% have genetic disorders clarified as hereditary ALS. Besides, more than 60% of patients die within 3-5 years after primary onset due to respiratory issue, while only 5-10% of patients could survive longer than 7 years (Heiman-Patterson et al., 2015).

In the last decade, many studies have focused on identifying genetic mutations which would lead to ALS. Copper-zinc superoxide dismutase 1(SOD1) is the first gene which has been found with the possibility of resulting in classical adult-onset form of ALS (Chen et al., 2013). In ALS, there are around 20% fALS patients and 2-4% sALS patients are SOD1mutated ALS cases (Chen et al., 2013, Gros-Louis et al., 2006). Evidence of newly identified pathogenic mutations is useful for further research. For example, additional studies have expanded the genetic bank, including TAR (the transactive response), TDP-43 (DNA-binding protein-43), VCP (valosin containing protein), FUS/TLS (fused-in sarcoma protein/translated in liposarcoma (FUS/TLS), FIG4 (polyphosphoinositide phosphatase), SETX (the senataxin), UBQLN2 (ubiquitin-like protein ubiquilin2) and VAPB (vesicle-associated membrane protein-associated protein B) (Chen et al., 2013), which would further provide insights into the mechanisms of pathogenesis in ALS (Heiman-Patterson et al., 2015). Previous studies showing the genetic disorders are not only concerned with fALS but also related to sALS. Furthermore, some neuropathological processes and mechanisms of sALS overlap with fALS, such as abnormal RNA metabolism, protein degradation and several pathogenetic pathways (Ling et al., 2013). However, the aetiologies of minority sALS are complex, which means diverse (Al-Chalabi and Hardiman, 2013), known and unknown genetic variations, epigenetics programming, gender and age are potentially involved (Simpson and Al-Chalabi, 2006). It is highly probable that

these elements may combine and interact with each other further increased risk of ALS by different populations at different rates.

The diagnosis of ALS is based on clinical assessment of LMN and UMN degeneration, electrophysiological or neuropathologic examination, conventional imaging recourses, which updated by Andersen et al. on 2015 as revised El Escorial criteria (Ludolph et al., 2015). With the relatively fast emergence of the clinical symptoms, no effective treatment is available in case when therapeutic intervention occurs after illness onset. In absence of cure, current therapies are largely restricted to symptom control, with the primary purpose of prolonging survival and improving quality of life as much as possible. Until now the only approved drug certified in different clinical trials is Riluzole (Mitsumoto et al., 2014), which is a glutamate antagonist and prolongs lifespan for approximately 3 months. Several drugs such as minocycline and creatine have been employed to extend the lifespan of animal models but failed on clinic trials (Gordon, 2013). Therefore, understanding the pathogenesis processes, early detection, fast diagnosis and individualized treatments are essential for improving ALS patient survival probability and reducing socioeconomic impacts.

1.2 Role of Calcium disturbances and ER stress in ALS

The pathological process that contributes to MNs death in ALS is noticeable earlier than the appearance of the obvious symptoms. The major mechanisms of pathogenesis in ALS comprise the unsuitable surrounding environment for MNs from spinal cord and brain, which is selectively degenerated due to either functional loss or changes of molecular pathway. As for the cellular pathophysiologic events include mitochondrial damage, oxidative stress, excitotoxicity, misfolded protein accumulation, inflammatory, disturbed axonal transport and activation of apoptosis-related molecules are include (Grosskreutz et al., 2010). In fact, it has become clear that the abnormal Ca^{2+} oscillation in MNs of ALS is a prominent molecular signaling that implicates variously pathophysiologic function of neurodegeneration in ALS (Kawamata and Manfredi, 2010, Jaiswal, 2013).

Earlier investigations have noted that Ca^{2+} signals, mediated by ligand- and voltage-gated Ca^{2+} channels, Ca^{2+} transporters and also Ca^{2+}-related proteins, could reflect the process of responding to various stimulations. Enhanced cytosolic calcium shows the Ca^{2+} entry through plasma membrane which induces additional Ca^{2+} release from

intracellular ER Ca^{2+} stores via activating IP_3 receptor and ryanodine receptor (Clapham, 1995). In the meanwhile, the calcium pump takes Ca^{2+} back into the ER store via SERCA receptors. Moreover, the ER also interacts with adjacent organelles, particularly lysosome and mitochondria, maintaining a balance of internal Ca^{2+} homeostasis. According to studies in MNs of ALS, Ca^{2+} transforming back and forth between ER and mitochondria has been determined as ER-mitochondrial Ca^{2+} cycle (ERMCC) effects MNs degeneration (Grosskreutz et al., 2010). Following studies targeting ERMCC having demonstrated altering Ca^{2+}-Na^{+} channel showed significant positive effects on MNs after acute exposure SOD1 over-expressed MNs to toxic substances (Lautenschlager et al., 2013).

ER, as major calcium store not only modulates Ca^{2+} signaling, but also participates in protein metabolism, including protein synthesis, chaperone-assisted protein folding protein degradation and post-translation modification (Paschen, 2001). Based on previous studies, the disturbance of Ca^{2+} homeostasis and accumulation of misfolded protein would lead to functional abnormalities in ER, and this pathological process is termed ER stress (Schroder and Kaufman, 2005). Furthermore, a subsequent unfolded protein response (UPR) pathway triggered by ER stress is an adaptive response. In the process, misfolded protein can be accommodated to cope with ER stress in the prime stage. However, continuous activation of UPR pathway has enhanced the ER stress level, which is quite toxic to link it to apoptosis (Jaronen et al., 2014). Many studies demonstrated the existence of ER stress in postmortem tissue from ALS patients. In MNs of lumbar spinal cord from ALS patients, aggregated granular materials and abnormal distributed ribosomes can be observed in swollen and distended ER (Matus et al., 2013). In addition, several upregulated proteins have been involved in modulation of ER stress, so as to further describe the enhanced level of UPR. It has been suggested that there is a critical crosstalk between the accumulation of unfolded proteins and ER Ca^{2+} during the execution of UPR in ALS (Schroder and Kaufman, 2005). With the increase of Ca^{2+} concentration in ER, the Ca^{2+}dependent protein, such as calnexin and calreticulin, can reduce ER stress levels by altering their protein activity (Jaronen et al., 2014).

1.3 Peripheral blood mononuclear cells

1.3.1 Systemic alteration of PBMC in ALS

Recently, it has been recognized that innate immune response is involved in ALS. Innate immunity is a nonspecific response which can contribute to adaptive immune responses. Inflammatory environment of ALS can be modulated during immune response through the activation of non-neuronal cells, such as microglia, astrocytes, monocytes and T-lymphocytes.

PBMCs are important monitors in the circulation system. They are a mixed population with round nuclei, including monocytes and lymphocytes. According to various studies on PBMC, direct relationships have been found between activated mononuclear cells and neurological diseases, such as Alzheimer's disease, Parkinson's disease and HIV-associated neurocognitive disorders (HAND) (Zhou et al., 2012). Blood-derived immune cells can control the balance between immunoprotection and immunoinjury through a complex mechanism (Zhang et al., 2005, Banati et al., 1995). PBMCs also play a crucial role in pathogenesis of ALS, as identified in both animal models and human.

Mouse or rat models overexpressing genetic mutations are widely considered and used to study the pathology of ALS. Several studies have demonstrated a systemic immune activation via detecting the alternation of different population in PBMC of ALS. It has been examined that there is a reduced number of CD4+ T cells were examined in mSOD mice, including disease onset, plateau phase and end stage, thus showing that the state of activated PBMC is associated with disease progression (Beers et al., 2008). Further studies show increased CD8+ cytotoxic T lymphocyte appears at the end of the disease to perform immune surveillance in murine models of ALS (Lewis et al., 2012). Isolated PBMC from slow stage mice with special immunophenotypic provided a protective strategy which can prolong the survival of mSOD1 mice before disease onset (Zhao et al., 2012).

Following changes were observed in rodents model of ALS, investigaitons were also conducted on the potential roles of PBMC in ALS patients. It was observed that there was an increase in CD8+ T cell and decrease in CD4+ T cell in peripheral blood of ALS patients. The population of regulatory T cells was decreased as observed in rapidly progressing ALS patients, thereby exerting the functional role of T-cell

activation (Lewis et al., 2012, Rentzos et al., 2012). Down regulation of the anti-apoptotic molecule Bcl-2 on lymphocytes suggested its protective pathway is deregulated in ALS (Mantovani et al., 2009, Cova et al., 2006). As suggested in accumulating evidence, T lymphocytes may be harmful to motor neuron through secretion of inflammatory cytokines, such as IL-2, IL-4, IL-6, IL-10, IL-13, IL-21 and INF-γ (Rentzos et al., 2012, Beers et al., 2011). Within the CNS, surrounding microglia and monocyte/macrophages can be damaged through the toxicity of cytokine produced by lymphocytes as well.

An increasing number of evidences support that activated monocytes have been identified within spinal cord of patients with ALS. According to those studies, immune activators were significantly changed in circulating monocytes and macrophages from patients with ALS, which directly contributes to the pathogenesis of neurodegeneration (Zhang et al., 2005, Miller et al., 2014). For instance, an increasing level of monocyte-chemoatractant protein-1 (MCP-1) has been detected, which has exhibited disease severity in diagnosed ALS (Wilms et al., 2003). CCR2 is the dominant receptor of MCP-1 which plays an important role in the recruitment of monocytes/macrophages. Down-regulation of CCR2 on monocytes from ALS patients suggested a relationship between the activation of monocyte/macrophage and the deregulation of MCP-1 (Robelin and Gonzalez De Aguilar, 2014, Zhang et al., 2006). Inflammatory chemokines and cytokines released from activated monocyte may also induce injury of MNs (Sumegi et al., 2011).

Cross-talk between lymphocyte activation and Ca^{2+} events has been implicated in lymphocytes from ALS patients. Disturbed oxidative metabolism may affect cytosolic Ca^{2+} throught the alternation of mitochondrial Ca^{2+} response. However, the regulation of Ca^{2+} in monocytes from ALS patients is still obscure.

1.3.2 The importance of PBMC and biomarkers in ALS

Although extensive researches have been carried out, regarding definition of ALS clinical features, an instant investigation has been conducted on the genetic alternation genetic alternation, neuromuscular electrodiagnostic studies, neurophysiology and neuroimaging features of ALS (Wijesekera and Leigh, 2009). Efficient tests are still in urgent need for ALS diagnosis, prognosis and follow up. A rapid and conclusive early diagnosis is obviously conducive, due to that it can not only potentially reduces the cost and duration of clinical drug trails, but also provides

a biological measurement for tracking the course of therapeutic intervention during the prodromal phase. Disease biomarkers are potential tool for the diagnosis of ALS at the early stage. It is common that biomarker can be obtained from various tissues and body fluids, such as blood, cerebrospinal fluid (CSF) and urine.

Skeletal muscle defect is an important part of the disease process in ALS; skin would also represent a valuable source for the study. The tissues mentioned above are used for diagnosis, staging and evaluating treatment. Due to highly invasive biopsy procedure and accompanied pain, frequent collection of those tissues from patients and controls are not feasible. Accordingly, human biofluid, which is suitable for biomarker sources, is easily accessible. Previous published studies focused on CSF, serum, and the exploration of potential biomarkers of ALS. CSF is considered to reflect the pathogenic results in CNS (Ryberg et al., 2010). However, low protein concentration and invasion of lumbar puncture are disadvantages that have to be considered. In a recent study, gene expression profiling has demonstrated the feasibility of PBMCs as ideal protein source on neurodegenerative and neuroinflammatory studies (Achiron and Gurevich, 2006). It can be inferred from the obvious alteration of gene expression on PBMC-derived protein indicates that PBMCs become one of the key approaches to find the differentially expressed protein between disease and control groups. It can thus prove the great advantage that proteins are more stable and abundant than that obtained from CSF (Zhang et al., 2011, Kruger et al., 2013). Based on the proteomic analysis of PBMC from patients, further studies validated a series of candidate biomarkers for ALS, some of which were verified through diagnostic/prognostic tests and can be used to identify different disease states (See table 1) (Nardo et al., 2011, Mougeot et al., 2011, Robelin and Gonzalez De Aguilar, 2014).

Table1. Investigated Biomarkers in PBMC for ALS

Protein name	ALS-specific biomarkers	ALS-progression biomarkers	ALS-transnational biomarkers
CLIC1	+		+
CypA	+	+	+
CALR	+		+
TDP-43		+	

GSTO1	+		+
ERp57		+	
FUBP1			+
mGLUR2			+
PRDX2			+
IRAK4	+		
Actin NT			+
CCL2		+	
CCR2			+

1.4 Purinergic signaling in neurological disease

Purinergic signaling involves the activation of purinergic receptors and functional effects on neighbouring cells. According to the evidences from in vitro and in vivo experimental studies over the last decade, extracellular ATP can contribute to nucleotides related disturbance of purinergic signalling, which may promote neurodegenerative and neuroinflammatory disorders, including ALS, Alzheimer's disease, multiple sclerosis, and Parkinson's disease (Volonte et al., 2003, Puchalowicz et al., 2014).

As the main energy in cells, ATP remains a relatively constant at the range of 2-5 mM in the cytosol. However, under physiological stresses or pathological stimulation, enormous amount of ATP released from stressed, apoptotic or necrotic cells into extracellular, is endowed with dramatic cytotoxicity and has chemotactic and excitatory effects on cells. Extracellular ATP modulates molecular signals through binding to P2 receptors (P2R), the family of this membrane receptor including ionotropic P2XRs and G-protein-coupled P2YRs. In this sense, there is a difference between different cell types and functional response to nucleotides in terms of expression. P2XRs containing 7 subtypes (P2X1-7) with common similar transmembrane structure are widely expressed in a variety of cell types. As P2XRs are fast ion-selective channels. These receptors regulate the influx and efflux of key cations, such as K^+, Na^+ and especially Ca^{2+}. In CNS, ATP-evoked P2XRs activation in neurons can lead to an elevation of intracellular Ca^{2+} and cell depolarization, which can thus contribute to adjacent signal transduction (Figure 1). In addition, upregulated P2XRs are capable of moderating ATP-dependent cytokine from

immune cells. For example, activated P2XR on microglia promotes the secretion of interleukin-1β (IL-1β) and tumor necrosis factor (TNF- α), which implicates the process of neurodegeneration.

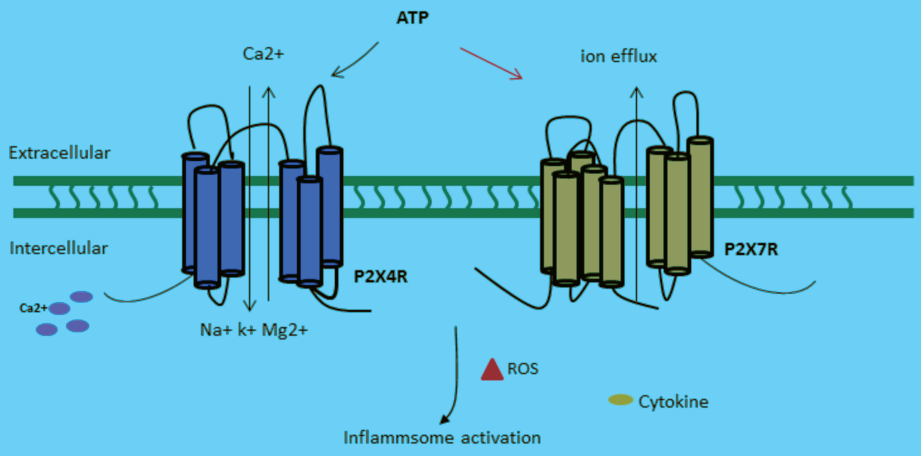

Figure1

Functional role of ATP and P2X4/7R

P2XRs are transmembrane domains protein. As ATP-gate ion channels, they allow different cations across the plasma membrane. One the extracellular loop, there are ten conserved cysteine residues which supplied several ATP- binding sit. P2XRs can be directly activated by ATP or external high-frequency stimulation. Upon pathway activations, P2XRs increase their permeability to cations with sustained current, thus promoting the production of proinflammatory cytokine and inflammsome activation.

Two subtypes of P2XRs, P2X4R and P2X7R, are proposed as dominant P2X subunits involved in neurodegenerative disease (Varma et al., 2009, Delarasse et al., 2011, Le Feuvre et al., 2002). Both P2X4R and P2X7R serve as ligand gated cation-selective channel response to external ATP. Interestingly, P2X4 and P2X7 are usually co-expressed in immune cells, implicating an interaction between two receptors (Apolloni et al., 2013).

In contrast to other P2XRs, P2X4R only has one potential subunit located at chromosome 12q24.31. C-terminal domain determined its complex and relative stable structure. Moreover, P2X4R serves as sensitive cation-selective channel response to the external environment with a high permeability. For instance, when the P2X4R is stimulated with ATP/ADP for milliseconds, activated ionophores can continually enhance the permeability of the membrane to cations. It can thus ensure the passing-through of more ion exchange and large molecules (Coddou et al., 2011). P2X7R has the same gene localization as P2X4R, which implies that the

similar structure with genetic heterogeneous contributes to coordinated functions (North, 2002). Indeed, P2X4 and P2X7 are usually co-expressed in immune cells such as microglia and recruited monocytes which has been demonstrated in the CNS of SOD1 mice (Apolloni et al., 2013). Both receptors have found to be up-regulated in microglia/macrophage from autopsy spinal cords (D'Ambrosi et al., 2009). In response to the inflammatory stimulation, two receptors may interact with each other in terms of performing surveillance. In the meanwhile, they might participate in apoptosis by alternation of Ca^{2+} homeostasis.

1.5 Neuorinflammation in ALS

Inflammation is a protective response against different infections, injuries or other pathological conditions, which in turn can lead to detrimental effects during long-term inflammatory process. In the CNS, systemic inflammatory activation is a prominent response observed in neurodegenerative processes (Pizza et al., 2011). Neuroinflammatory functions in ALS are common marks occurring in many cells, including microglia, astrocytes, T cells and monocytes. The activation of these non-neural cells is highly involved in the pathogenesis of ALS (Rizzo et al., 2014).

As resident macrophage, microglia presents the mainly cellular factors of immune response of surrounding MNs. An increasing level of cytokines and chemokines can induce proinflammatory profiles to promote different activations of microglia, aiming to prolong neuronal survival at the initial stage (Philips and Robberecht, 2011). However, continuous reaction of microglia may accelerate the toxicity of T cells and reduction of trophic factors (Rich, 1992). For instance, classically activated microglia display a proinflammatory phenotype which can produce IL-1β, IL-6, IL-12, IL-23, NO and TNF- α. It further indicates the essential role of neuronal damage in CNS already mentioned above. At the same time, alternatively activated microglias play a protective role in secreting anti-inflammatory mediators and interact with other immune cells like lymphocytes and monocytes, activing innate and adaptive immune response, reducing the release of cytokine and producing multiple neurotrophins (Hooten et al., 2015). Similarly, triggers of astrocytes reaction can also provoke the inflammatory response of MNs in surrounding microenvironment and produce multiple cytokines/chemokines (Glass et al., 2010). Therefore, the activation of non-neuronal cells exerts either neurotoxic or neuroprotective effects on MNs.

It is well characterized that LPS activates immune cells. It rapidly binds to LPS-binding protein and initiates signals via multiple intracellular signal transduction pathways (Hamilton and Adams, 1987). Based on these observations, LPS-related signaling in different immune cells can be involved in inflammatory response by a systemic activation of these cells. The production of LPS-induced mediator such as TNF-α and NO, can amplify the inflammatory response and results in immunotoxicity. As for LPS-related signaling, like an indirect mediator, it can exert a selective effect of inducing a protective function against toxicity. Thus, selective neuronal damage may also be the consequence of LPS-evoked progressive neuroinflammation and neurodegeneration (Qin et al., 2007). Based on recent investigations, antioxidants can reduce the damage of neuron induced by exogenous LPS in immune cells and promote the production of trophic elements able to contribute to neurodegeneration slowdown (Tyagi et al., 2008, Rees et al., 2011). Regardless of classic stimulus in CNS (microglial and astrocytes activation), the LPS induced monocyte inflammatory response may also be associated with MNs damage.

1.6 Aim of the study

ALS is the most common and aggressive form of adult motor neuron (MN) degeneration. Several reports indicate that Ca^{2+} perturbation in immune cells is linked to dysregulation of the environment surrounding MNs but the underlying mechanism of how this affects PBMC is currently unknown. Rapid and conclusive diagnosis of ALS could be potentially determined from analysis of molecular pathways in PBMC. In this thesis propose that calcium perturbation in PBMC is linked to ALS, and that changes in the expression of purinergic receptors and ER calcium storage capability are the underlying mechanisms.

The purpose of this dissertation was to test potential Ca^{2+} disorders in monocytes isolated from ALS patients. To do this, the expression of P2X4R and P2X7R was determined. ATP-induced Ca^{2+} influx via purinergic signaling regulation and LPS-induced Ca2+ influx were examined in monocytes isolated from both ALS patients and control patients. Additionally, thapsgargin-triggered depletion of ER Ca^{2+} and the expression of Ca^{2+} binding protein calnexin that partially reflects ER calcium storage capability were evaluated in ALS and control patients.

2 Publication

Neuroscience Letters 630 (2016) 77–83

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Down-regulation of purinergic P2X7 receptor expression and intracellular calcium dysregulation in peripheral blood mononuclear cells of patients with amyotrophic lateral sclerosis

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HIGHLIGHTS

- Expression of purineric receptors (P2X4 and P2X7) and intracellular Ca^{2+} changes were analyzed in PBMCs of ALS patients.
- We observed a downregulation of P2X7 receptors in PBMCs of ALS patients compared to controls.
- Intracellular Ca^{2+} dysregulation as a main characteristic of motor neurons in ALS is detectable in PBMCs.
- Stimulation of PBMCs with different concentrations of ATP revealed decreased intracellular Ca^{2+} levels in ALS patients.

ARTICLE INFO

Article history:
Received 20 April 2016
Received in revised form 11 July 2016
Accepted 20 July 2016
Available online 21 July 2016

Keywords:
Amyotrophic lateral sclerosis
Peripheral blood mononuclear cells
Purinergic receptors
Calcium homeostasis

ABSTRACT

Background: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder associated with intracellular Ca^{2+} dysregulation. The P2X receptor family is comprised of ligand-gated ion channels that respond to extracellular adenosine triphosphate (ATP) and increases permeability of calcium into the cell. The underlying mechanisms of purinergic signalling on peripheral blood mononuclear cells (PBMCs) in ALS remain unclear. Herein, we studied the expression of P2X4/P2X7 receptors and calcium homeostasis in blood cells of ALS patients.

Methods: We used PBMCs from 42 ALS patients and 19 controls. Purinergic receptors P2X4 (P2X4R) and P2X7 (P2X7R) were examined using western blot analysis. The effect of exogenous ATP on intracellular Ca^{2+} homeostasis in monocytes was measured using fluorimetry by Fura-2 on a single-cell level.

Results: Western blot analysis revealed stable P2X4R expression in patients and controls. P2X7R expression was significantly reduced ($p = 0.012$) in ALS patients. Repetitive long-term ATP stimulation caused a sustained decrease in Ca^{2+} levels in the ALS group as measured by the area under the curve, peak amplitude and peak height.

Conclusion: These results confirm our hypothesis that Ca^{2+} abnormalities in ALS are measurable in immune cells. These findings suggest that the reduction of P2X7 receptor expression on PBMCs leads to intracellular calcium dysregulation. Our study improves the understanding of ALS pathophysiology and proposes PBMCs as a non-invasive source to study ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, primarily characterised by degeneration of upper and lower motor neurons (MNs) resulting in generalised muscular weakness and paralysis. Disease onset usually appears between the ages of 40 and 70 years. The median survival rate is 3–5 years following symptoms onset, with respiratory failure being the most frequent cause of death [1].

The pathophysiological mechanisms that contribute to MN cell death include mitochondrial dysfunction, oxidative stress, excitotoxicity, immune factors and apoptosis. A critical feature of MN death is the dysregulation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ [2]. The endoplasmic reticulum (ER) interacts with adjacent structures and plays an important role in Ca^{2+} signalling [3].

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http://dx.doi.org/10.1016/j.neulet.2016.07.039
0304-3940/

Ca^{2+} circulates between the ER and mitochondria; described as the ER–mitochondria Ca^{2+} cycle (ERMCC) [4]. These mechanisms are essential for normal function of MNs; however, they might also cause the demise of MNs under pathological conditions [5,6]. The function and survival of MNs is also dependent on the environment comprised of non-neuronal cells including astrocytes, microglia, T lymphocytes and monocytes.

ALS is regarded as a multi-systemic disorder. Typical changes of the disease can be observed in peripheral blood mononuclear cells (PBMCs) [7]. Abnormally activated monocytes were found in blood specimens from patients with sporadic ALS, suggesting that these cells play a role in the pathogenesis of ALS and could serve as biomarkers [7,8]. Furthermore, increased activation of immune cells was detected in spinal cords and surrounding tissues from ALS patients [9]. Regarding PBMCs, disturbances in purinergic signalling may promote neurodegeneration [10] and the expression of P2X receptors (P2XR) are of special interest [11]. P2XRs are involved in neuronal processes and their activation by adenosine triphosphate (ATP) leads to elevation of intracellular Ca^{2+} and cellular depolarisation. Specifically, P2X4R and P2X7R are key receptors in neurodegenerative diseases [12,13].

Various studies report that immune cells, including PBMCs, affect the survival of MNs [14], however detailed mechanisms underlying the activation of the peripheral purinergic signalling in ALS remain unclear.

This study was performed to investigate the expression of purinergic P2X4 and P2X7 receptors and to determine putative Ca^{2+} dysregulation in PBMCs via activation of P2X4 R and P2X7 R by ATP.

2. Materials and methods

2.1. Subjects and blood samples

The study was performed at the Department of Neurology at the Jena University Hospital and including 42 ALS patients diagnosed according to the revised El Escorial criteria [15] and 19 healthy controls. Disease severity was assessed by the revised ALS Functional Rating Scale (ALSFRS-R). Disease progression was calculated as (48-ALSFRS-R)/disease duration. ALS patients received Riluzole but no psychotherapeutic drugs. 18 ml of blood was drawn into vacuum EDTA tubes. The study was approved by the local ethics committee (Ethics Committee of the Friedrich-Schiller-University Jena; Number: 3633-12/12).

2.2. Cell isolation and cell culture

The blood was diluted in phosphate-buffered saline (PBS) using the Leucosep tube (Greiner Bio-One, Germany). Afterwards, the blood was centrifuged at 1000 x *g* at room temperature for 10 min and the PBMC layer was collected. PBMCs were washed three times with PBS. Cells were cultured by plating the PBMCs on poly-D-lysine (Sigma-Aldrich, Germany) coated 12 mm dishes (Marienfeld, Germany) at a density of 100,000 cells/dish in RPMI-1640 medium (Gibco, UK) supplemented with 10% fetal bovine serum (Pan-Biotech, Germany) and penicillin (10 U/mL)/streptomycin (10 µg/mL, Gibco) in an atmosphere of 5% CO_2 at 37 °C. Monocytes were selected by their adherence to the coverslips followed by washing the wells thoroughly with media after 4 h and 48 h. Adherent cells were harvested from the plates and cell pellets were stored at −80 °C for subsequent protein extraction.

2.3. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde diluted in PBS (pH 7.4) for 30 min. Nonspecific binding sites were blocked by 10% nor-

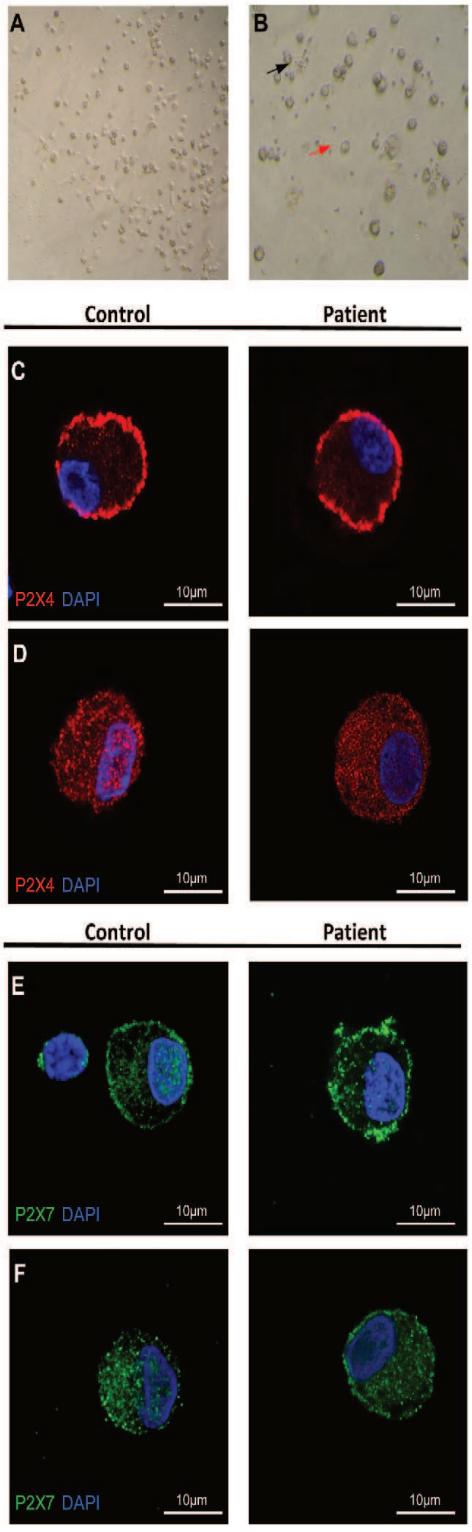

Fig. 1. Morphology and purinergic receptor expression of monocytes. The images (10 x (**A**) and 20 x magnification (**B**)) reveal the morphology of PBMCs from one ALS patient: monocytes (red arrow) and lymphocytes (black arrow). Monocytes from study subjects were examined by immunofluorescence staining against P2X4R (**C**/**D**) and P2X7R (**E**/**F**). (**C**) P2X4R immunostaining (red) on the cell surface of monocytes and (**D**) in the cytosol. P2X7R immunoreactivity (green) (**E**) on the cell surface and (**F**) internal of monocytes co-labelled with the nucleus marker DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

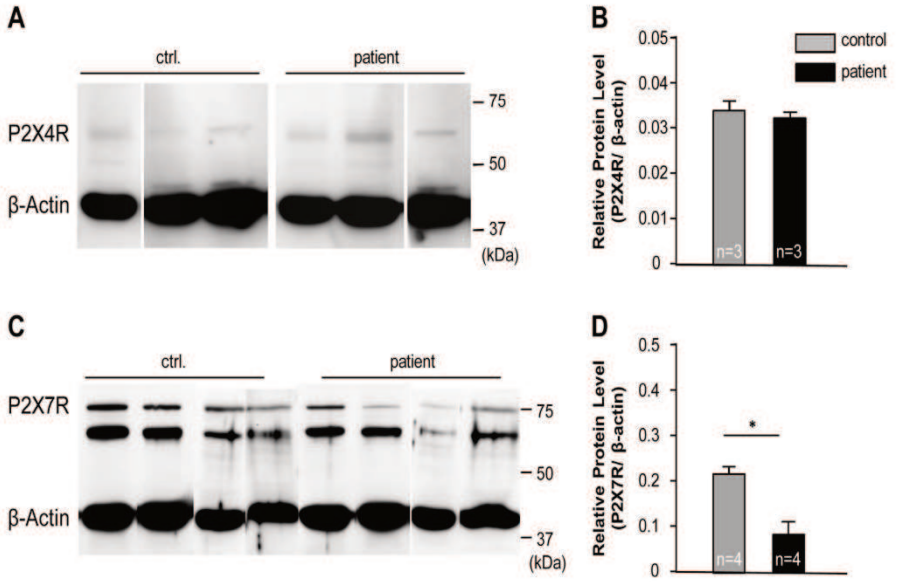

Fig. 2. Western blot analysis of P2X4R and P2X7R. (A) Western blot of PBMCs shows equal content of P2X4R (63 kDa) in ALS patients and controls. (**B**) Quantitation of P2X4R protein level by densitometry, adjusted to actin and compared between the groups ($p = 0.69$). (**C**) Western blot analysis of P2X7R (75 kDa, full-length receptor, 65 kDa non-glycolisated receptor) of ALS patients and controls. (**D**) The full-length receptor showed a significant reduction of P2X7R expression in ALS patients. ($p = 0.012$). Bars represent mean ± SD. Asterisks indicate significant differences.

mal donkey serum (NDS), 1% BSA, and 0.05% Triton X-100 for 2 h. To detect P2X4R and P2X7R, cells were incubated with primary antibodies goat anti-P2X4R polyclonal antibody 1:200 (APR024, Alomone Labs; Israel), rabbit anti-P2X4R polyclonal antibody 1:200 (APR002, Alomone Labs, Israel), rabbit anti-P2X7R polyclonal antibody 1:200 (sc-25698, Santa Cruz; USA) and goat anti-P2X7R polyclonal antibody 1:200 (sc-15200, Santa Cruz, USA), diluted 3% NDS, 1% BSA and 0.05% Triton X-100 overnight at 4 °C. Cells were washed and incubated with secondary antibodies Alexa 488 donkey anti-goat and Alexa 488 donkey anti-rabbit (Invitrogen, UK) and DAPI (Sigma-Aldrich). The specimens were examined using confocal microscopy (LSM 710, ZeissGmbH, Germany).

2.4. Western blots

For protein extraction cells were lysed using SDS-PAGE loading buffer. Proteins were dissolved in SDS dissociation buffer and boiled for 5 min. Proteins were separated by 5–20% gel electrophoresis and transferred to nitrocellulose filters. After blocking with 1% bovine serum albumin, the filters were incubated overnight with primary antibodies P2X4R 1:50 (APR002, Alomone Labs), P2X7R 1:50 (sc-15200, Santa Cruz), and β-actin rabbit polyclonal antibody 1:2000 (Abcam; Germany). Secondary antisera comprised horseradish peroxidase conjugated goat anti-rabbit 1:2000 IgG (sc-2030, Santa Cruz). Immunoreactivity was visualized by enhanced chemiluminescence ECL detection system (BioRad; Germany).

2.5. Determination of cytosolic Ca^{2+}

Monocytes were adhered to Poly-D-lysine 12 mm coverslips and loaded with 8 μM fluorescent Ca^{2+} dye fura-2-acetoxymethyl ester (fura-2-AM, Sigma-Aldrich) and incubated at 37 °C, 5% CO_2 for 25 min. Adherent monocytes were used to mimic activated monocytes. After de-esterification at room temperature, coverslips were fixed and perfused with extracellular solution containing (mM): NaCl 129.1, KCl 5.9, glucose 11.5, $MgCl_2$ 1.2, $CaCl_2$ 3.2 and adjusted to pH 7.3 with NaOH. The fluorescence was measured at excitation wavelengths of 350 and 380 nm and ratiometric fluorescent images were monitored by Till Vision Imaging System (TillPhotonics; Germany). Calcium concentration was calculated from the fluorescence ratio as follows: $[Ca^{2+}] = KD \times \beta \times (R - Rmin)/(Rmax - R)$ [16]. The coefficient was obtained by extracellular and intercellular Ca^{2+} correction, KD = 245, β = 3.6, Rmin and Rmax are ratios at zero and saturating Ca^{2+}, respectively (Rmax = 0.8, Rmin = 0.08). Cytosolic Ca^{2+} changes were recalibrated after adjustment to the baseline. ATP (Sigma-Aldrich) was applied using a customized solution application system described previously [2]. Extracellular solution was applied for 10 s after onset of whole-cell recording, once sufficient equilibration of Fura-2 has been reached. At least 10 cells of 3 coverslips from each study subject were measured.

2.6. Statistical analysis

For Ca^{2+} imaging, 9 ± 2 replicate data points from at least three independent experiments were included. Values are presented as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics 20. Distribution was analysed by Shapiro–Wilk and Kolmogorov-Smirnov test. Mann-Whitney *U* test was used for data from non-Gaussian distribution and Student's *t*-test was used for normally distributed data. $P < 0.05$ was considered significant.

3. Results

3.1. Patients

The mean age of the ALS patients (27 male and 15 female subjects) was 61.8 ± 11.7 years. The mean ALSFRS-R score was 36.7 ± 8.1 from a maximum score of 48 in healthy individuals. The mean disease duration was 21.8 ± 13.0 months and the mean progression rate was 0.75 ± 0.64. 26 patients had spinal onset, whereas 16 had bulbar onset. The control group consisted of 10 females and 9 males with a mean age of 58.6 ± 12.0 years.

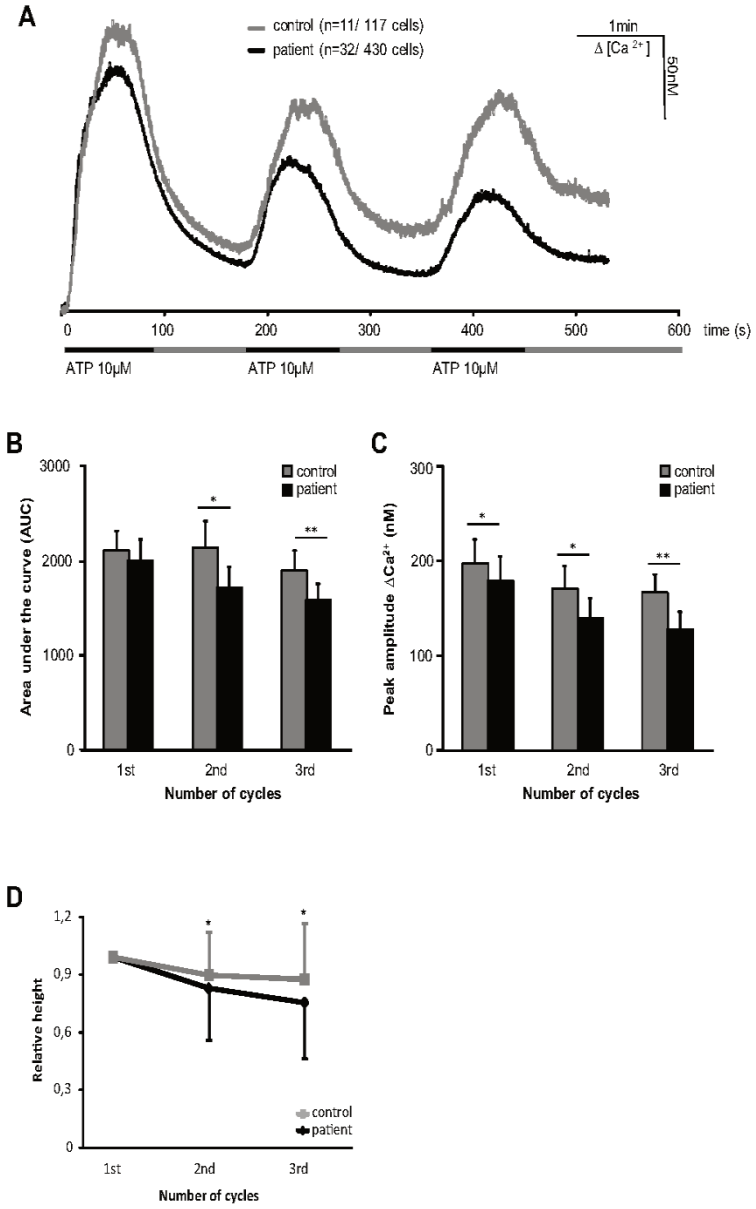

Fig. 3. Low concentration of ATP decreased calcium influx in monocytes from ALS patients. (**A**) 10 μM ATP was applied for 1 min with an interval of 2 min on monocytes from ALS patients and controls. On each ATP application, intracellular Ca^{2+} level increased. (**B**/**C**) The cytosolic calcium load, calculated as the AUC referring to the peak amplitude was decreased in ALS patients. (**D**) The relative peak height decreased more in patients than in controls.

3.2. Downregulation of P2X7R expression in monocytes

Typical PBMC morphologies were observed using light microscopy (Fig. 1A). The lymphocytes were washed away by medium change. P2XR subtype location was assessed by immunofluorescence staining. Both, P2X4R and P2X7R, were located intracellular (Fig. 1D,F) and in the cell membrane (Fig. 1C,E). To verify the protein level, we performed western blot analysis. Contradictory to P2X4R which showed no differences between the groups (Fig. 2A/B), P2X7R expression was reduced ($p = 0.012$) in ALS patients (Fig. 2C/D).

3.3. Reduced intracellular Ca^{2+} levels in monocytes from ALS patients

The effects of ATP on the intracellular Ca^{2+} levels were determined using 430 cells from 32 independent preparations of ALS patient and 117 cells from 11 preparations of controls.

The addition of 10 μM ATP produced a marked Ca^{2+} influx in both groups. Intracellular Ca^{2+} rapidly peaked and then decayed to a sustained elevated phase. Both groups failed to return the Ca^{2+} level to the original baseline (Fig. 3A). The area under the curve (AUC) (Fig. 3B) as well as the peak amplitude (Fig. 3C) revealed a decreased reactivity to the stimulus during the agonist application in both groups. ALS patients showed a significant decrease in the Ca^{2+} level

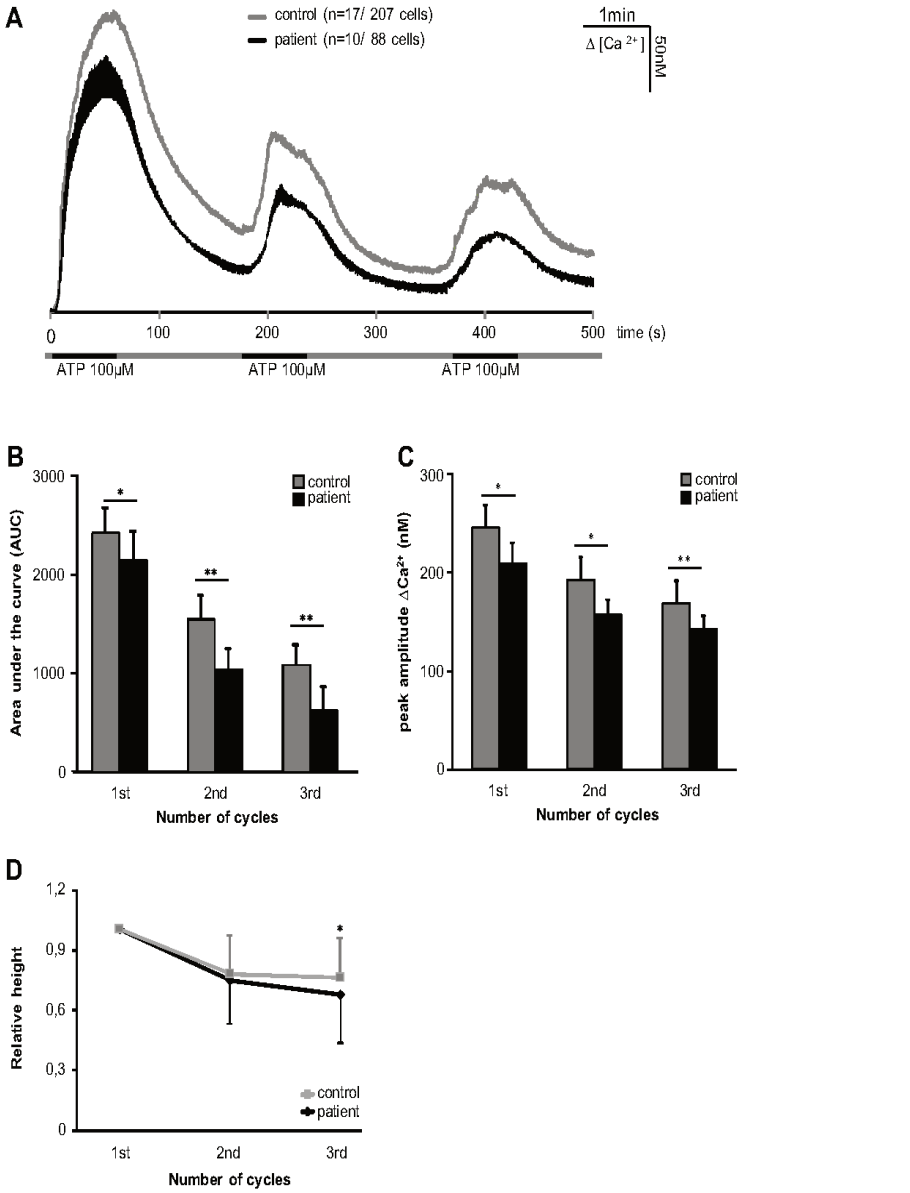

Fig. 4. High concentration of ATP disturbs calcium influx in monocytes from ALS patients. (**A**) Repetitive stimulation with 100nµM ATP reduced intracellular Ca^{2+} peaks in monocytes of ALS patients. The cytosolic calcium load was decreased in ALS patients. (**B**/**C**) The intracellular Ca^{2+} was higher in controls. (*$p < 0.05$, **$p < 0.001$) (**D**) The decay increased within the first cycles of ATP application, but decreases after one interval.

at the second cycle ($P = 0.03$) and third cycle ($P < 0.001$) (Fig. 3B). The relative peak height is shown in Fig. 3D, where a striking decrease was observed in the PBMCs of ALS patients.

We applied 100 µM ATP under the same experimental conditions. The intracellular Ca^{2+} level and the relative peak height are shown in Fig. 4A and D. The application of a high concentration of ATP caused a response in 87% of monocytes −2.3% more monocytes compared to the application of 10 µM ATP (data not show). There was no variation in the number of responding cells between patients and controls. Non-responding cells were not included into the analysis. There were significant differences in the AUC and peak amplitude of ALS patients and controls in the three cycles (Fig. 4B and C).

4. Discussion

ALS is a multisystemic disease in which pathological processes extend beyond MNs. Specifically, PBMCs display traits of the disease including changes in immunophenotype [17], increased oxidative stress [18] and glutamatergic dysfunction [19]. PBMCs could serve as a promising biomarker based on the low invasiveness and high availability of material.

The vulnerability of MNs and intracellular Ca^{2+} disturbances are known features in ALS and have been studied in ALS animal models. MNs are characterised by a low Ca^{2+} buffering capacity due to low endogenous expression of Ca^{2+} buffering proteins [20]. Inflammation and oxidative stress also play crucial roles in ALS

pathogenesis. Degeneration of motor neurons produce signals that release free radicals and pro-inflammatory cytokines. Extracellular ATP was also described to induce pro-inflammation. Major players responding to extracellular ATP are the purinergic receptors P2X4 and P2X7. The extent of P2XR subtype expression and response to extracellular ATP on monocytes of ALS patients remains unclear.

In our study, the expression of P2X7R in monocytes was reduced in ALS patients. Studies have shown that P2X7R in microglia is mainly involved in pro-inflammatory responses including NADPH oxidase activity, TNFα and COX-2 [21–23]. Furthermore, the knock-out of P2X7R aggravates ALS pathogenesis suggesting a dual role of this purinergic receptor [24]. The lack of P2X7R increased astrocytosis and motor neuron death in the spinal cord of SOD1-G93A mice combined with an elevation of microglia cytotoxic markers such as NOX2 and iNOS [25,26]. The specific involvement of P2X7R in ALS may contribute to other pathways. A reduction of P2X7R could lead to an involvement of additional P2 receptor subtypes. Specifically, the P2X4R subunit is co-expressed with P2X7R in ALS microglia and was upregulated in SOD1-G93A/P2X7 knockout mice. Although the P2X7R protein was reduced in monocytes of ALS patients in the present study, an increase of the P2X4R was not observed. Apolloni et al. analysed the effects of the P2X7 receptor in the SOD1-G93 microglia. Stimulation of P2X7 receptor by ATP leads to oxidative stress in SOD1-G93 microglia. Specific blocking of P2X7 suppresses early and late inflammation [27]. The role of P2X7 in ALS remains unclear and needs further investigation.

Studies also confirmed the expression of P2XRsubtypes in human PBMCs [28,29]. Consistent with these findings, we revealed co-localisation of P2X4R and P2X7R expression at the extra and intracellular level of PBMCs from ALS patients and controls.

Our study showed that adherent monocytes responded to ATP application, which increased intracellular Ca^{2+} in both groups. After initial ATP stimulation, [Ca^{2+}] rapidly peaked and then decayed to a sustained elevated level. Our data indicates that the PBMCs of ALS patients have a lower Ca^{2+} buffering ability and capacity for Ca^{2+} mobilisation. A previous study [30] showed that low concentrations of ATP act primarily via P2X4R, suggesting the disturbances in PBMCs are associated with purinergic signalling. ATP is considered as the energy supply of cells; however, organelle damage can also lead to ATP release as a danger signal. In this case, ATP concentrations are high in the cytoplasm but relatively low at the extracellular level; consequently, newly synthesised ATP is transported out of the plasma membrane. In our study, 100 μM of ATP could have been an ideal concentration to evoke P2X4R reactivity and also the subthreshold for the activation of human P2X7R. P2X7R has a lower ATP affinity compared to other subtypes expressed on immune cells. As a low-sensitivity component, the activation of P2X7R requires higher concentrations ATP. Several studies [31,32] indicate that high levels of extracellular ATP activate monocytes and increase cell toxicity. Our study detected down-regulation of P2X7R expression on monocytes combined with the reduction of intracellular calcium stores. However, it is unclear to which extent the changes of P2X7R expression influence calcium homeostasis in monocytes. The release of pro-inflammatory cytokines such as mature IL-1β in ALS [33,34] by activation of P2X7 receptor in monocytes is an important issue for further analysis.

5. Conclusion

The study confirms that Ca^{2+} abnormalities in ALS do not only exist in motor neurons but also in immune cells. Purinergic signalling events regulate the activation and functional responses of PBMCs. Our study improves the understanding of ALS pathophysiology on a systemic level and suggests that PBMCs could serve as non-invasive surrogate markers in ALS. To confirm the direct link between reduced P2X7R expression and Ca^{2+} disturbances in PBMCs of ALS patients functional studies are required.

Competing interests

The authors declare that there is no financial or non-financial competing interests.

Author's contributions

JL, TP and JG were responsible for the experiments measurement and statistical analysis. JL, TP, JG and OW were responsible for the design and planning of the study. BS and TP analysed the clinical data. JL, TP, BS and SK drafted the manuscript. JG, TP, TR, AG, NG, AM and VT were responsible for enrolment of subjects into the study and sample collection.

Acknowledgements

We thank Svetlana Tausch for technical assistance and Mandy Arnold for blood collection. We express thanks to Nasim Kroegel for language editing. This study was undertaken in cooperation with the German MND-NET. The project is supported by the German Bundesministerium für Bildung und Forschung (BMBF) grant SOPHIA to JG under the aegis of the EU Joint Programme – Neurodegenerative Disease Research (JPND – www.jpnd.eu) and a BMBF grant PYRAMID to JG in the framework of the ERANET E-RARE program (www.e-rare.eu). This study was supported by a Deutsche Gesellschaft für Muskelkranke (DGM) grant and ALS grant to TP.

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3 Further study

3.1 Materials and methods

Subject consent and blood sample

The study population was comprised of 40 patients with ALS and 21 gender- and age-matched healthy controls. All subjects were recruited at the University Hospital Jena. Revised criteria from the El Escorial World Federation of Neurology were used for the diagnosis of ALS. The degree of severity was measured using an outcome measure called the "ALS Functional Rating Scale Revised (ALSFRS-R).” The disease progression rate was calculated as follows: (48 -ALSFRS-R score)/disease duration. All subjects with a history of acute heart failure, pre-eclampsia, stroke, diabetic peripheral neuropathy, active infection, and those receiving anti-inflammatory drugs, antioxidants or other treatments were excluded. 12 ml of peripheral blood was collected from each patient and healthy control into vacuum EDTA tubes (Sarstedt, Germany). The study was approved by the local ethics committee.

Preparation of PBMC and culture

Whole blood samples from patients and controls were diluted 1:2 with balanced salt solution (PBS) in a Leucosep tube (Greiner Bio-One, Germany). Leucosep tube contains biologically inert barrier and special separation medium. PBMCs were isolated by gradient centrifugation at 1000xg at room temperature for 10 min and the PBMC layer was harvested from the plasma-separation medium interface. Mononuclear cells were washed three times with pre-warmed PBS and isolation medium to remove erythrocytes. Cells were cultured by plating the PBMCs on poly-D-lysine (Sigma, Germany)-coated 12-mm dishes (Marienfeld GmbH & Co. KG, Germany) in RPMI-1640 medium (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (Pan-Biotech, Germany) and penicillin (10 U/mL)/streptomycin (10 µg/mL, Gibco, UK) in an atmosphere of 5% CO_2 humidified incubator at 37 °C. After 1 day, monocytes were separated by the adherence to the coverslips and thorough washing of the wells with culture media. The culture was maintained at a density of 100,000 cells/well.

Induction of ER Stress

Cells were treated with 5µM and 10µM reversible SERCA inhibitor cyclopiazonic acid (CPA, Sigma, Germany) and 500nM of the irreversible SERCA inhibitor thapsigargin (Appli Chem, Germany) for 6 h.

Immunocytochemistry and detection of ER stress

Monocyte cultures were fixed in 4% paraformaldehyde (pH=7.4). After 3 washes with PBS, dishes were incubated with 0.3% Triton X-100 and 2% normal donkey serum for 2 h at room temperature to block nonspecific binding. Cultures were incubated overnight with mouse anti-calnexin monoclonal antibody 1:1000 (ab31290, Abcam). The cells were washed and incubated with the secondary antibody Alexa 488 goat anti-mouse. DAPI (Sigma, Germany) was applied for 5 min for the detection of the nucleus. The specimens were examined and recorded using the laser confocal scanning microscope (LSM710, Carl Zeiss Germany). This was performed for 9 cells per sample from at least 3 independent cultures.

Measurements of intracellular Ca^{2+}

For cytosolic calcium measurements, monocytes were loaded with the membrane permeable ester form of the high-affinity ratiometric calcium dye fura-2AM (2uM) (Sigma, Germany) in the incubator at 37°C for 25 min. Incubation for additional 5 min at room temperature in the dark allowed the de-esterification. Following de-esterification and washing, cells were placed onto a recording chamber and continuously perfused with extracellular solution containing (mM): NaCl 129.1, KCl 5.9, glucose 11.5, MgCl2 1.2, and CaCl2 3.2 and then adjusted to pH 7.3 with NaOH. The fluorescence was captured by a 40×/0.8 W water immersion objective at excitation wavelengths of 350 and 380 nm and ratiometric fluorescent images were monitored by Till Vision Imaging System (TillPhotonics, Gräfelfing, Germany) with a CCD camera cooled to -50°C (iXONEM+, ANDOR™, Belfast, UK). The exposure time was set to 5 ms, the binning was 2 × 2, and the frequency for imaging was set to 5 Hz. The concentration of free intracellular Ca^{2+} is proportional to the ratio of fluorescence at 340/380 as described by Grynkiewicz: Ca^{2+} concentration =KD × β × (R − R_{min}) / (R_{max} − R) where K_d = 245 nM, R = 340/380 ratio, and R_{max} and R_{min} are the 340/380 ratios under Ca^{2+} saturating and Ca^{2+} free conditions, respectively. The intracellular Ca^{2+} concentration was recalibrated after adjustment to the background. 40ng of LPS (Sigma, Germany) or 500nM thapsigargin was applied as described previously (Grosskreutz et al., 2007). The extracellular solution was applied 30

seconds after sufficient equilibration of Fura-2. In each experiment, 1-3 cells in the field of view were evaluated and each subject included 9±2 replicate cells from at least three independent experiments.

Statistical analysis

Staining fluorescence intensity was quantified from each slice with same interval (Z-stacks). Quantitative analysis of staining was performed using the software Fiji with the Z project plug-in. Data are represented as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics 20 and Sigma plot 13. Distribution of groups was analyzed by Shapiro–Wilk normality and Kolmogorov-Smirnov test. For multiple comparisons, one way ANOVA with Bonferroni correction was applied. Mann–Whitney-U test was used for data from non-Gaussian distribution and student's t-test was used for normally distributed data. For all analysis, a *p*-value <0.05 was considered as significant.

3.2 Results

Characteristics of the study population

In total, 61 subjects were recruited for this study including 40 patients (male=22, female=18) and 21 controls (male=8, female=13). The mean age of patients and controls was 59.1 (SD=11.4) years and 53.0 (SD=19.8) years, respectively. The mean ALSFRS-R score was 34.8 (SD=7.4). 27 patients had spinal onset ALS and 13 had bulbar-onset ALS. The mean progression rate was 0.65 (SD=0.5).

Monocytes of ALS patients lose the function of Ca^{2+} mobilization

To investigate Ca^{2+} modulation under immune activation, we used LPS, a typical monocyte activator. LPS induced a cytosolic Ca^{2+} increase in monocytes from both patients and controls (patient subjects: n=8 patients /65 cells; control subjects: n = 11 controls/85 cells). No differences were observed in the response rate between patients and controls with LPS treatment. As shown in Figure 1, intracellular Ca^{2+} increased slowly, plateaued for a short time, and then decreased gradually. Treatment for 3 min was sufficient for the activator to exert its maximal effects as indicated by the maximum peak of Ca^{2+} occurred around 1.5 min after the initiation of stimulation (Fig. 1A). The time required to reach the maximum peak height was similar for the two groups (data not shown). To estimate the calcium load, the area under the curve (AUC) was calculated (Fig. 1B). There was a marginally significant difference in Ca^{2+} dynamics between patients and controls (p<0.001; Fig. 1C).

Next, the changes in ER luminal Ca^{2+} stores were investigated. Following the exposure of monocytes to 3μM thapsigargin as performed previously, Ca^{2+} was depleted from the ER by the inhibition of the SERCAR. A steady increase in Ca^{2+} levels was detected within 3 min after the initiation of stimulation. However, Ca^{2+} levels decreased for several seconds and then remained at a high level showing complete emptying of the ER Ca^{2+} stores in both groups (Fig. 1D). The basal Ca^{2+} did not recover after 3 min of washing with extracellular solution. We observed significant differences in the levels of cytosolic Ca^{2+} between patients and controls as shown in Fig. 1 E by means of the AUC (p<0.001). The time required for Ca^{2+} to reach the highest value (peak amplitude/time) was significantly faster in the monocytes from ALS patients (p<0.001) (Fig. 1F).

ER stress and calcium homeostasis induced changes in calnexin expression

In the measurements, all monocytes expressed calnexin. Figure 2 shows that the expression of calnexin was higher in the ALS patients than in the control group. Following treatment with thapsigargin, the density of cytosolic calnexin was obviously enhanced in both patients and controls (Fig 2. A, B, G, H).

The difference in the calnexin levels of monocytes from patient and control was quantified (Fig. 3). Untreated cells of patients showed higher calnexin expression than untreated control cells (p<0.01). After treatment with the reversible SERCA inhibitor cyclopiazonic acid (CPA; 5μM or 10μM) or the irreversible SERCA inhibitor thapsigargin (500nM), we found that the level of calnexin density in patients was always greater than in controls. All monocytes responded equally to the two agonists. Monocytes from the patient group that were treated with 10μM CPA or 0.5μM thapsigargin showed a significantly elevated amount of calnexin compared with the levels in monocytes from untreated subjects (p<0.01 and p<0.001, respectively). In the control group, there was no significant difference between the inhibitors (P>0.05, ANOVA). The treatment with thapsigargin resulted in higher calnexin expression than the levels in untreated control cells (p=0.035).

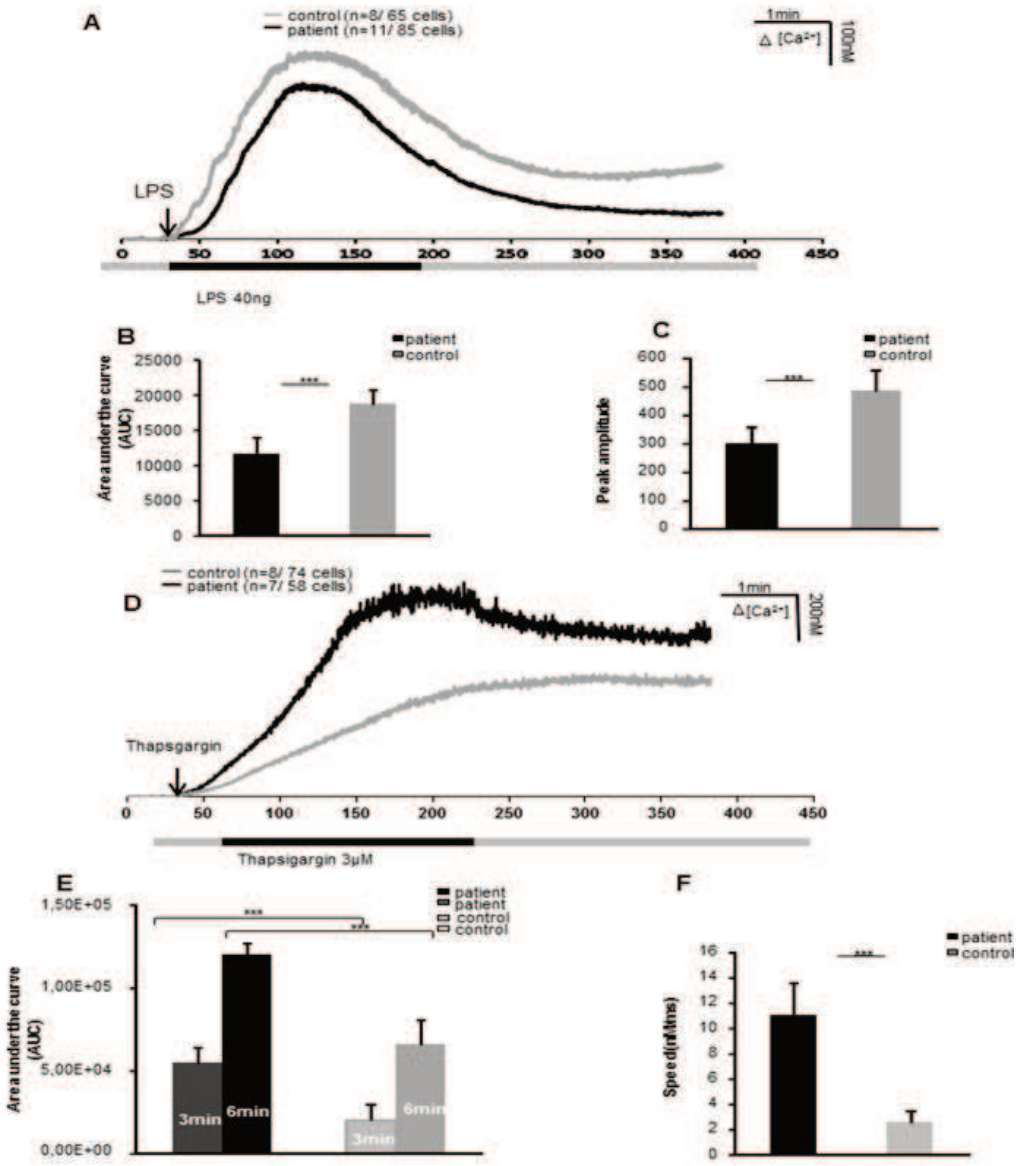

Figure1.

The elevated Ca $^{2+}$ concentration in monocytes

LPS 40ng was applied for 3min after 30s observation. The intercellular Ca^{2+} continuously increased in the first 1 min and then start to decrease before the application device was removed (A). The traces indicate the calcium levels did not return to baseline levels after stimulation in subjects from either group. Cytosolic Ca^{2+} concentration as indicated by AUC and peak amplitude was significantly higher in the controls (B.C). A persistent Ca2+ elevation was elicited by the application of 3μM thapsgargin using the same protocol as above (D). There was no recovery of Ca^{2+} after the application device was removed in subjects from both groups. The time (Δt) required to reach maximum Ca^{2+} level (peak amplitude) upon thapsgarain exposure indicated the fast Ca^{2+} release process. ER Ca2+ loading (E) and Ca^{2+} transient were higher in ALS patients (F) (*p < 0.05, **p < 0.01).

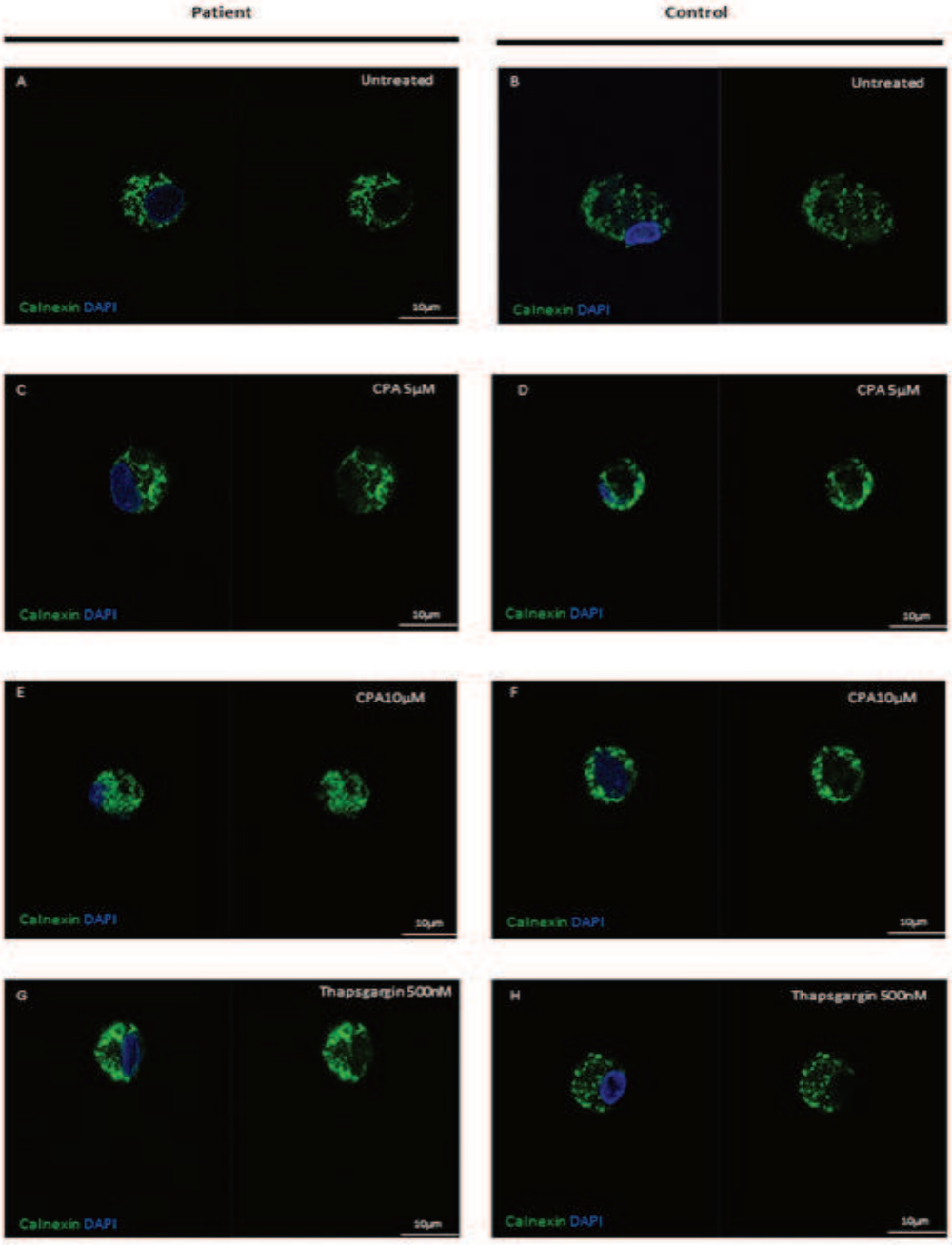

Figure2.

Expression of calnexin on monocytes

Representative immunofluorescence images of indicated ER stained with calnexin (green) and the nucleus was visualised with DAPI (blue). Cells from ALS patients and controls were grouped by untreated (A), pretreated with CPA5Mm (B), CPA10μM (C) and thapsgargin (D) 500nM for 6 hours, respectively.

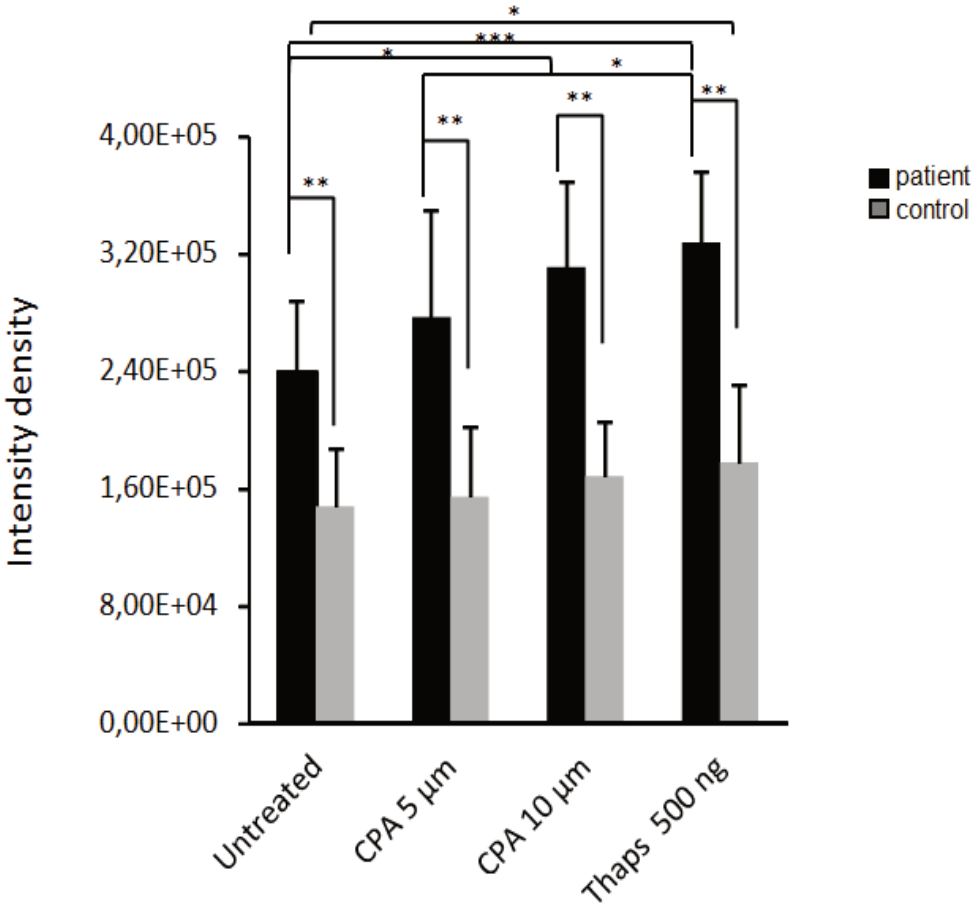

Figure3.

Comparison of calnexin expression

The expression level of calnexin on monocytes was measured by fluorescence density under basal condition and after treated with compounds. (*p < 0.05, **p < 0.01, ***p<0.001).

4 Discussion

4.1 P2X4R and P2X7R in PBMC of ALS

P2X4R and P2X7R are key receptors that are highly expressed in immune cells and play a major role in cellular nucleotide metabolism, inflammasome modulation, immune activation, and Ca^{2+} signaling mediation. Previous studies reported the expression of P2XR in monocytes and lymphocytes (Gu et al., 2000). Further evidence showed that P2X4R is most highly expressed in PBMCs, followed by P2X7R (Wang et al., 2004).

Due to the limitations of microglia extraction from the human body, peripheral blood mononuclear cells are considered as an ideal material alternative to microglia to study ALS in vitro. Activated monocytes are microglial precursors that normally impart protective immunity during the initial stages of CNS injury. P2X4R and P2X7R receptors can be activated by changes of stimuli or transmitters such as ATP that induce a rapid increase of Ca^{2+} in the cytosol and promote interaction between cells and chemokines to recruit immune cells at the site of injury. In this study, the distribution and expression of P2X4R and P2X7R in monocytes was ascertained by immunohistochemistry (IHC) and western blot. Both the extracellular and intracellular components of the receptors were detectable in the monocytes from ALS patients and controls. This study did not find any significant difference in P2X4R expression between patients and controls by IHC or at the protein level by western blot. This result is inconsistent with the finding that P2X4R is upregulated in mutant SOD1 microglia (Parisi et al., 2013), the different findings may be due to the different species and cell types used. It is very interesting that the expression of P2X4R on the cell surface is associated with the lysosome (Beggs et al., 2012). In addition, the CC chemokine receptor (CCR2) is the regulator of P2X4R, is mainly expressed on the surface of cells, and can control P2X4R levels via endocytosis and the degradation of the lysosome. Therefore, it is reasonable to infer that the levels of P2X4R protein might be maintained via the modulation of CCR2. The functional interaction between CCR2 and P2X4 in ALS requires further investigation (Zhang et al., 2006, Toyomitsu et al., 2012). P2X7R has been studied across various neurodegenerative diseases and is considered to be a target for treatment (Takenouchi et al., 2010). P2X7R may contribute to the expression and function of other P2 subtypes types, particularly

P2X4R. Studies have shown that the activation of the microglia in the ALS SOD1 model is mediated by P2X7 (Aga et al., 2002). A defective or absent P2X7 receptor is associated with microglia damage and motor neuron death in SOD1 mice and an increase of microglia inflammatory markers including NO, iNOS, and TNFα (Kim et al., 2013, Valdmanis et al., 2008, Zhao et al., 2012). It was reported that the disease severity worsened at the end stage for P2X7R knockout SOD1 mice (Apolloni et al., 2014). The results of this study showed that the P2X7R protein is a 75kDa protein, which is the full-length size of the receptor. The band seen at 65kDa is from non-glycosylated P2X7R. Although a decrease of P2X7R expression in ALS patients was observed in this study, the P2X4R protein level did not change. Inhibition of P2X7R activation or a decrease inextracellular ATP can reduce neurotoxicity in SOD1 mice, suggesting that the antagonist of P2X7R might reduce neuroinflammation in ALS (Gandelman et al., 2010).

Activation of the P2XR by ATP results in intracellular calcium release. To analyse the Ca^{2+} alterations in monocytes, the first experimental study was focused on the P2X4R receptor for two reasons: first, this subtypes clearly showed Ca^{2+} transients in the monocytes of patients with ALS; second, P2X4-like immunoreactivity was associated with degenerating MNs in mSOD1G93A mice (Casanovas et al., 2008). As a powerful and selective agonist, ATP binds to cysteine-rich loops and activates P2X4R over a wide range of concentrations (8–100 μM) (North, 2002). Activated P2X4R can trigger Ca^{2+} release from intracellular Ca^{2+} stores and activates plasma lemma Ca^{2+} influx (Yamamoto et al., 2000, Huang et al., 2014). This supports the model that P2X4R is highly Ca^{2+}-permeable and directly activated by ligand binding linked to channel opening and fast ATP signalling (Coddou et al., 2011). P2X4 ion channels become increasingly permeable to Ca^{2+} when the application of ATP is continued for 50 s (Li and Fountain, 2012). To examine the Ca^{2+} transients in P2X4R activation, 10μM ATP were applied 1 minute for every 2 minutes in 3 cycles. In the early phase, cytosolic Ca^{2+} increased slowly. After 1-3 seconds, it rapidly reached maximal peak and then exhibited a progressive decline but failed to reach the previous baseline. Because no difference was observed between patients and controls following the initial stimulation, one can conclude that ATP-induced Ca^{2+} mobilisation reflects a compensatory reaction. Therefore, P2X4R in ALS may be unlikely to maintain a functional Ca^{2+} dynamic balance. To investigate the

mechanism further, a higher dose of ATP (100 μM) was applied to stimulate intracellular Ca^{2+} transients. Indeed, under regular physiological conditions, the oscillation of extracellular ATP was very limited. A high dose of ATP is most likely to be produced in pathological processes. Compared to P2X4R, P2X7R is thought to be specific with a relatively lower affinity for ATP. The activation of P2X7R requires high concentrations of ATP (≥100μM) under pathological conditions. High concentrations of ATP are typically used experimentally for P2X7R activation and the production of cytokines. Dose-dependent effects of ATP by intracellular Ca^{2+} were observed. The ALS and control samples did not differ significantly during the first cycle when ATP was applied at 10 μM. However, differences after applying ATP at 100 μM were observed. In patients, the baseline of Ca^{2+} mobilisation increased steadily and the decay of Ca^{2+} became progressively faster during the stimulation cycles. In striking contrast to the patients with ALS, the Ca^{2+} concentration of controls decreased rapidly after the peak and the baseline remained relatively high. Cytosolic Ca^{2+} in ALS patients was lower than that in the controls, implying that the capability of P2X4R/7R-related Ca^{2+} modulation in monocytes is significantly decreased in the ALS patients. The obtained results are consistent with the findings that P2X4R and P2X7R contribute to the regulation of receptor activity and Ca^{2+} homeostasis in immune cells (Smart et al., 2003, Buell et al., 1996).

Many studies have focused on ATP signaling in immune response and the roles of possible regulators that are involved in the etiopathology of neurodegenerative process (Le Feuvre et al., 2002, Volonte et al., 2003). Extracellular ATP is released from damaged cells due to conditions such as hypoxia, organelle stress, and inflammasome activation. Accumulation of ATP is quite toxic, and ATP levels can regulate monocytic response by recruiting monocytes to the injured sites. This may suggest that systemic reduction in ATP level could be another target for treatment of ALS by decreasing the production of ATP or by accelerating the degradation of ATP. Since Ca^{2+} has repeatedly been described as a key player of ATP production, the data reported suggest a role for both P2X4 and P2X7 receptors to potentially mediate extracellular ATP-induced cell damage and cell death. The results of this study imply that P2X4- and P2X7-modulated Ca^{2+} perturbation participates in the damage of monocytes in ALS patients.

4.2 Role of Ca^{2+} in monocytes activation in patient with ALS

The activation and recruitment of monocytes are crucial for the eradication of infections and inflamed tissue as part of the innate immune defense system. Although inflammatory responses and immune-mediated mechanisms have been held responsible for progression of ALS, the correlation of these processes with Ca^{2+} homeostasis is not exactly clear (Lewis et al., 2012, Zhang et al., 2009).

An experimental study reported that exceptionally high amount of LPS and TNFα are present in the circulating blood of ALS patients (Cereda et al., 2008). MNs in CNS are susceptible to inflammatory injury after systemic exposure to LPS or TNF-α. The influence of LPS is to act as potentially endotoxin binding toll-like receptors (TLR) as part of the immune response or induce inflammation by cell activation, but also extends to causing an abnormal change in the levels of cytosolic Ca^{2+}. Indeed, the elevation of Ca^{2+} indirectly results from LPS. LPS can trigger activation of monocytes, macrophages, and B lymphocytes and promote the release of pro-inflammatory factors. The activated cells lead to systemic immune response and primary Ca^{2+} release. LPS is also a potent activator of phospholipase C (PLC)-ɣ enzyme, which can subsequently accelerate the production of IP_3. The produced IP_3 binds to IP_3 receptors and then induces a second phase of Ca^{2+} release from the intracellular stores (McLeish et al., 1989). Additionally, Ca^{2+} could enter from the extracellular environment via the membrane if internal Ca^{2+} stores are depleted (Chiang et al., 2012). Here, LPS was continually supplied for 3min to mimic the acute inflammatory process. In both subject groups, LPS-induced cytosolic Ca^{2+} increased slowly, and then after a short plateau phase, it decreased slowly and then more rapidly. The data indicate LPS-mediated activation of monocytes from ALS patients causes lower intracellular Ca^{2+} than controls. This result implies that immune activation may participate in the observed Ca^{2+} transient in monocytes of ALS patients and controls. Abnormal Ca^{2+} hemostasis has been described previously as an aspect of immune disorders (Garcia et al., 1999, Feske, 2007). Ca^{2+} disturbance causes cell injury and can initiate apoptotic pathways by inflammasome-driven activation of caspase 1 (Lamkanfi and Dixit, 2011). Therefore, Ca^{2+} dysregulation may be both the outcome and the cause of chronic inflammatory alteration during long-term disease progression correlated with the pathological features of monocytes in ALS patients.

The data presented here shows that Ca^{2+} transients started to decline in the middle of LPS stimulation and reached baseline in 3 min. This is not consistent with the outcome reported by Kenneth et.el (McLeish et al., 1989) in which monocytes require longer (more than 5 min) incubation for LPS–induced elevation of cytosolic Ca^{2+}, despite testing cells with different concentrations of LPS. Significantly lower Ca^{2+} amplitude were found in patients compared to controls. Although many factors can contribute to ER uptake of Ca^{2+}, the amount of releasable Ca^{2+} from storage and extracellular matrix requires further clarification. Monocytes from ALS patients exhibit a lower capability to buffer the LPS-stimulated increase in Ca^{2+}, similar to the response of ATP described above. Thus, it is conceivable that abnormal ATP/LPS-induced Ca^{2+} levels could function as ionic signals in ALS and may be indicative of immune interactions in neurodegenerative disease.

Ca^{2+} is a key component of physiological and pathological changes in various responses to insult or toxic injury and can promote repair. In addition to its fundamental role in monocytic activation, Ca^{2+} signaling also regulates neuroinflammation. Previous observation of long-term Ca^{2+} disturbances in monocytes demonstrated genetic changes due to activation of Ca^{2+}-dependent pathways, including the IKK-NF-kB pathway, ERK (1/2) pathway, JNK pathway, and p38 pathway (Guha and Mackman, 2001). These pathways may therefore also be involved in the pathogenesis of ALS. The removal of inflammatory factors and maintaining functional Ca2+ dynamic balance in monocytes may be potential therapeutic targets for patients with ALS.

Various neuroinflammation-related processes can cause proinflammatory cytokines release from monocytes, including interleukin-1α (IL-1α), IL-1β, IL-18, and TNF-α, through the activated P2XR pathway. ATP can be released by damaged cells and mediated by upregulated purinergic signaling to have effects on metabolism and cytotoxicity. Accumulation of ATP can act as a chemotactic factor to promote mature IL-1β release and Ca^{2+} influx via P2X7R (Ferrari et al., 2006). P2X4R is an important receptor in inflammasome activation which participates in the early stage of inflammatory injury. Both P2X4R and P2X7R receptors can interact with the LPS-induced pathway and can accelerate cytokine production and immune responses. At the same time, LPS can modulate P2X7R activity to inhibit ATP-stimulated Ca^{2+} influx and alleviate ATP-induced apoptosis (Leiva-Salcedo et al., 2011). Functional

interaction between P2X4R and P2X7R was shown in immune cells and activation of both receptors has been directly linked to IL-1β production. A burst release of IL-1β depends on caspase-1-related activation, TLR-induced NFkB pathway activation, and activation of nucleotide-binding oligomerization domain receptors (NLRP3) (Gong et al., 2010). Interestingly, Ca^{2+} plays a very complex role in the activation of NLRP3 in monocytes. ATP-related NLRP3 activation by P2X7R requires a basal level of Ca^{2+}, but a high level of extracellular Ca^{2+} may inhibit NLRP3 activation (Lee et al., 2012). Amplified NLRP3 activation can modify ER Ca^{2+} uptake through the SERCA receptor, which may attenuate ER stress and Ca^{2+} overload. Such Ca^{2+} mobilization is also involved in mitochondrial dysregulation and ROS production (Leiva-Salcedo et al., 2011). Therefore, there is potential crosstalk between calcium mobilization, the purinergic receptor, and inflammatory activation. As described above, consistent Ca^{2+} dysregulation was observed in monocytes from ALS patients, which suggested that ATP and LPS-induced Ca^{2+} influx are strongly related to the purinergic receptor in the physiological roles and pathological mechanisms of ALS.

As pointed above, consistent Ca^{2+} dysregulation was observed in monocytes from ALS patients, which suggested that ATP and LPS-induced Ca^{2+} influx are strongly related to purinergic receptor in physiological roles and pathological mechanisms of ALS.

4.3 The regulation of ER in monocytes from ALS

This study also interested in the role of ER Ca^{2+} stores in monocytes from ALS patients. Calcium imbalance can result from dysfunction in the calcium buffering system, damage of intracellular Ca^{2+} stores, activity of Ca^{2+} channels, or activation of the Ca^{2+}-sensing receptor. ER acts as the main intracellular Ca^{2+} store and Ca^{2+} storage is dependent on the activity of Ca^{2+} channels that allow Ca2+ transport and the production of Ca^{2+} binding proteins (Taylor et al., 2010).

Thapsigargin is extracted from the root of thapsia garganica L and is widely used for studies of Ca^{2+} signalling in ER. Thapsgargin were applied to control luminal Ca^{2+} uptake by inhibition of SERCAs. A dramatic increase in cytosolic Ca^{2+} was observed in monocytes from both ALS patients and control subjects. This increase in Ca^{2+} is consistent with previous studies on monocytes from healthy controls (Tran et al., 2001). Surprisingly, thapsigargin-induced Ca^{2+} elevation in monocytes from ALS patients was extremely high compared to controls. This is in contrast to the results

from ATP and LPS stimulation. Do SERCAs contain different functional isoforms in the ER to cope with Ca^{2+}? The SERCA family includes 3 SERCA gene types: SERCA1 and SERCA2a are only expressed prominently in muscles and SERCA2b and SERCA3 are widely expressed in immune cells, and may be irreversiblely blocked by thapsgargin at nanomolar concentrations (Treiman et al., 1998). In monocytes, stimulating with thapsigargin above 100nM can induce a rapid and complete emptying of Ca^{2+} stores (Stone et al., 2000). Could other receptors expressed on the ER cause this change? Intracellular Ca^{2+}-related receptors generally include RyR and IP3R. However, RyR is differently expressed in various cells. RyR1 is less prevalent in T cells and monocytes, RyR2 is only detected in CD3 T cells, and RyR3 is not expressed in PBMCs. In addition, gene profiling of monocytes indicate that RyR expression is age-related, showing a decrease in both human and mouse with age (Stone et al., 2000). In this study, the ages of the enrolled subjects ranged from 48 to 70 years. This may explain the failure of the RyR in monocytes to mobilize Ca^{2+}. Furthermore, thapsigargin does not increase the production of IP_3 during the increase in intracellular Ca^{2+} (Putney, 1990, Treiman et al., 1998). Thus, neither RyR nor IP3 contribute to this mediation.

Thapsigargin not only acts as an inhibitor of SERCA but also acts as an activator of inflammatory response and an inducer of ER stress (Ali et al., 1985). These experiments demonstrate that intracellular Ca^{2+} can be used as an indicator of the cell stress response and immune reaction. Thapsigargin-induced Ca^{2+} elevation in monocytes can activate the Ca^{2+} related immune response and the related Ca^{2+} receptors may enhance their permeability. Therefore, this study suggests that other potential mechanisms may involve ER luminal Ca^{2+} mobilization of monocytes in ALS patients.

Free Ca^{2+} in the lumen of ER must be combined with special proteins. Calnexin is the main Ca^{2+}-binding protein that binds Ca^{2+} at two sites and cooperates with SERCA to deal with Ca^{2+} oscillation and restoration. In this study, calnexin was found to be highly expressed in monocytes from ALS patients compared to controls. In the investigation of structure of Ca^{2+}-binding proteins, calnexin binds Ca^{2+} affected by a continuous increase in free Ca^{2+} (Schrag et al., 2001). On the one hand, calnexin serves as a calcium buffer that helps relieve Ca^{2+} burden and activates the UPR (Michalak et al., 2002). On the other hand, calnexin acts as an important ER-resident

chaperone directly prevent protein misfolding and aggregation (Guerin et al., 2008). Under pathological conditions, adaptive UPR commonly mediates the activation of protein degradation pathways and increases the ability of ER to handle the accumulation of misfolded or unfolded protein. The expression of molecular chaperones in the ER such as calnexin and calreticulin can monitor Ca^{2+} binding to maintain Ca^{2+} homeostasis. However, if the ER stress is beyond control, the accumulation of inappropriate proteins can directly activate the apoptotic pathway due to poor handling of UPR (Fu et al., 2010).

In the present study, cyclopiazonic acid (CPA) and thapsigargin were applied in monocytes for 6h to induce ER stress. Calnexin was expressed at higher levels in ALS patients compared to in the healthy controls. The ER in monocytes from the healthy control patients may be able to correct un/mis-folded protein via UPR. In thapsigargin-treated monocytes, the expression of calnexin was higher than in the CPA treated cells. This may be because CPA is a reversible inhibitor and thapsigargin is a completely irreversible stressor that leads to ER Ca^{2+} depletion, organelle damage, and cell death. The study correlates the finding from thapsigargin-induced Ca^{2+} release with calnexin expression. These results demonstrated that Ca^{2+}-binding protein plays an essential role in Ca^{2+} homeostasis to cope with Ca^{2+} dysregulation of monocytes in patients with ALS.

5 Conclusion and clinical potential

This study suggests Ca^{2+} is a key component in ALS. Here determined that Ca^{2+} dysregulation in ALS is not only present in MNs but also in immune cells. Monocytes from patients with ALS seem to lose the functional modulation of Ca^{2+} homeostasis.

Two common monocyte activators, LPS and ATP, were used to elicit the cytosolic Ca^{2+} response. Dose-dependent activation of ATP was shown. This process appeared linked to both P2X4R and P2X7R and related to Ca^{2+} homeostasis. These results indicate crosstalk between immune response and the purinergic pathway. The intimate relationship between purinergic activation and Ca^{2+} signaling suggests that the altered sensitivity of P2X4/7R is involved in functional dysregulation of monocytes in patients with ALS.

Events that cause Ca^{2+} imbalance are crucial in the pathogenesis of ALS. Intracellular Ca^{2+} stores and buffer capacity in monocytes were measured. The

results suggest that accumulation of Ca^{2+}-binding protein in monocytes from ALS patients can act as dynamic buffers by binding to overloaded Ca^{2+} in the ER lumen. This further strengthened the point that the surrounding environment of MNs such as immune cells is an important pathogenic factor that cannot be ruled out. One of the limitations of this study is that patients were not divided into groups by different stages of the disease. Clinical heterogeneity and strict rules about enrollment are the main reasons behind this limitation.

Overall, the discovery of Ca^{2+} signaling dysregulation in monocytes from patients with ALS presents increased opportunities for understanding the pathogenesis of ALS. Based on the immune-modulated mechanism of neurodegeneration, measurement of Ca^{2+} levels can be used to detect and complement the therapeutic potentials of ALS.

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Annex

Acknowledgement

My deepest gratitude is to my advisor, Dr. Julian Grosskreutz. He provided me with guidance, assistance, and expertise in my doctoral work. He has always been there to listen and give advice.

I am grateful to Dr. Beatrice Stubendorff and Dr. Tino Prell for their insightful comments and constructive criticisms at different stages of my research and they helped me focused on ideas.

I am thankful to Vedrana Tadic and Dr. Milan Stojiljkovic for their encouragement and practical advice. I am also thankful to Chuangqiang Zhang, Thanh Tu Le and Saikata Sengupta for reading my manuscript, commenting on my views and helping me enrich my ideas.

Lastly, I warmly appreciate the love, support, and understanding of my family and friends.

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

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