Alzheimer’s amyloid-β peptide disturbs P2X7 receptor-mediated circadian oscillations of intracellular calcium

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Abstract

Recent data indicate that Alzheimer’s disease (AD) is associated with disturbances of the circadian rhythm in patients. We examined the effect of amyloid-β (Aβ) peptide, the main component of the senile plaques playing a critical role in the deregulation of calcium (Ca2+) homeostasis in AD, on the circadian oscillation of cytosolic calcium (Ca2+) levels in vitro. The experiments we carried out in human primary skin fibroblasts. This cell line was previously shown to exhibit circadian rhythms of clock genes. Moreover, the basic clock properties of these peripheral cells closely mimic those measured physiologically and behaviorally in human and do not change during aging. In this study we showed i) cytosolic Ca2+ oscillations depend on the activation of purinergic P2X7 receptors; and ii) these oscillations are abolished in the presence of Aβ. In total, our new findings may help to deepen our understanding of the molecular mechanisms involved in AD-related circadian alterations.

Key words: amyloid-β, Alzheimer’s disease, calcium, P2X7 receptors, circadian rhythms.

Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disorder that is characterized by progressive neuronal loss, mainly in the brain cortex and hippocampus [28]. One of the main neuropathological hallmarks of this disease is the deposition of extracellular senile plaques containing amyloid-β (Aβ) peptides, derived from processing of the amyloid-β precursor protein (APP) [3,18]. Many previous data point out to a critical role for deregulation of calcium (Ca2+) homeostasis in the pathogenesis of AD. Thus, the levels of intracellular Ca2+ concentration and calcium-regulated enzymes (e.g. calpains, proteases, phospholipases) were found to be elevated in animal models of AD [24] as well as the brains of AD patients [47,48]. The “calcium hypothesis” was further supported by demonstrations that, during brain aging, the molecular processes responsible for Ca2+ regulation were impaired. This involved the mechanisms of Ca2+ sequestration into its intracellular stores (endosomal-plasmic- reticulum (ER) and mitochondria) as well as Ca2+ influx into the cytoplasm by voltage-gated Ca2+ channels, ionotropic or metabotropic receptors [13,21,64]. In AD, disturbed Ca2+ homeostasis is not restricted...
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to neurons but represents a global phenomenon affecting virtually all cells in the brain. AD-related aberrant Ca\(^{2+}\) signaling in astrocytes and microglia probably contributes profoundly to an inflammatory response that, in turn, impacts neuronal Ca\(^{2+}\) homeostasis and brain function [8]. It has been shown that Aβ release induces intracellular calcium overload and activates intracellular calcium-dependent events, leading to a decrease in learning and memory as well as cognitive dysfunction [27,43,62]. Previous findings suggested that N-methyl-D-aspartate receptors (NMDARs) are the main mediators of enhanced Ca\(^{2+}\) entry evoked by Aβ [19,35]. However, more recent discoveries showed that the soluble form of Aβ oligomers induce their toxic effects by disrupting the integrity of the cell plasma membrane leading to uncontrolled fluxes of Ca\(^{2+}\) into the cells [66].

Moreover, recent data indicate that AD progression is associated with disturbances of the circadian rhythm in patients. Circadian rhythms govern a wide variety of physical, behavioral and metabolic processes that follow a roughly 24-hour cycle, responding primarily to the light/dark cycle. These are controlled by the circadian clock machinery, in which rhythm-generating mechanisms are encoded by a transcription-translation feedback loop. The mammalian circadian clock machinery is regulated by a central pacemaker in the suprachiasmatic nucleus (SCN) of the brain that synchronizes oscillators in peripheral tissue [16]. The entrained signals from SCN neurons are distributed through different target organs by efferent neural and humoral mechanisms, such as circulating melatonin, producing changes in metabolism, core body temperature, and sleep. Calcium ions are a potent second messenger coupling the clock gene oscillation and the rhythmic firing of action potential in SCN neurons [33,37,58,60]. Calcium mediates intracellular clock signals, such as entrainment processes [6,23,30], clock gene expression [33,37,45,55], and output signaling [2]. Moreover a topological specificity of the circadian Ca\(^{2+}\) rhythm in SCN was observed, suggesting that calcium plays a role in the hierarchical organization of rhythmicity in the central pacemaker [25].

The alterations of the SCN as well as in melatonin secretion are the major factors of circadian clock disruption [72]. Insomnia, nocturnal behavioral changes, sundowning syndrome and excessive daytime sleepiness are the common to circadian disturbances observed in AD patients as well as patients with mild cognitive impairment [7,17,67,68,70]. The studies in animal and humans demonstrated that Aβ level in the cerebrospinal fluid is modulated by sleep-wake cycles [5,36,40]. This raises the possibility that disturbances in the circadian rhythm causes brain Aβ accumulation over time, suggesting a causative rather than an associative link between sleep loss and Aβ accumulation [52]. However early-stage AD events such Aβ aggregation and disturbances in calcium homeostasis may also induce molecular changes that lead to circadian clock disruption. Therefore the aim of this study was to deepen our understanding of the molecular mechanisms involved in AD-related circadian clock alterations, by investigating i) the clock-dependent regulation of intracellular calcium levels in a peripheral tissue, and; ii) the effect of Aβ peptides on the changes of cytosolic calcium levels around the clock. For this purpose, we used primary cultures of human fibroblasts because: i) fibroblasts coming from AD patients present a disturbed Ca\(^{2+}\) homeostasis [31,39]; and ii) fibroblasts are a valuable in vitro model of peripheral oscillators [10,56,57].

Material and methods

Ethical permission

Prior ethical consent to the use of human skin tissues was given by the Ethical Committee of Basel, and informed written consent to participation in this study was obtained from all human subjects.

Materials

Human recombinant Aβ\(_{42}\) was purchased from Bachem (Germany). Coomassie Brilliant Blue G, DMSO, DMEM, Fetal Bovine Serum (FBS), Glutamax, Hank’s Balanced Salt Solution (HBSS), Horse Serum, Penicillin/Streptomycin, Pluronic® F-68, Thapsigargin and ATP were obtained from Sigma (St. Louis, MO, USA). Liberase was from Roche Diagnostics GmbH (Mannheim, Germany). Fluo-4 AM was obtained from Invitrogen (Thermofisher Scientific, Waltham, Massachusetts, USA).

Tissue isolation and fibroblast culture

Two cylindrical cutaneous biopsies (2 mm diameter) were taken from the buttocks of a healthy male subject. Fibroblasts were isolated from biopsies by
4-h digestion of tissue in DMEM/1% penicillin streptomycin/1% Glutamax (DMEMc)/20% FBS/87.5 ng/ml Liberase, and cultured in DMEMc/20% FBS.

Synchronization of cells and timetable to study cellular circadian rhythms

For all experiments, cells were seeded onto collagen-coated 48-well or 96-well dark plates at the density of 1.4×10⁵ cells per ml. Cells were synchronized by treatment with DMEM containing 50% Horse Serum for 2 hours at 37°C. After the synchronization, cells were washed with PBS and the medium was changed into DMEMc/2% FBS according to [1]. Experiments were performed every 4 hours, starting 4 h after synchronization and until 48 h.

Preparation of Aβ species and cell treatment

Aβ42 was dissolved in PBS to make stocks of 500 μM and stored at –80°C until use. Aging of the peptides was induced by shaking the diluted solution (50 μM) at 1000 rpm overnight at 37°C. The cells were treated after synchronization at a final concentration of 0.5 μM of Aβ42. In selected experiments, after measurement of basal Ca²⁺ levels, cells were treated either with a sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor (Thapsigargin; 10 nM), or with an agonist (ATP; 1 mM) of purinergic P2X7 receptors. The measurements were repeated immediately or after 5 min of incubation, respectively.

[Ca²⁺]i measurements

[Ca²⁺]i measurement was carried out using the fluorescent indicator Fluo-4 acetoxyethyl (AM) ester (200 μM stock solution in DMSO). At the specific time points after cell synchronization, fibroblasts were loaded with 4 μM Fluo-4 AM supplemented with 0.02% Pluronic® F-68 for 60 min at 37°C in a standard HBSS. The cells were washed 3 times with HBSS and, to ensure complete AM ester hydrolysis, kept for 30 min at 37°C in the dark. After a second washing step, the fluorescence was measured using Fluoroskan® counter at 485/520 nm. To study the involvement of purinergic receptors and endoplasmic reticulum (ER) stores on a cytosolic calcium level, cells were treated with an agonist (ATP; 1 mM) or an antagonist of purinergic P2X7 receptors (Coomassie Brilliant Blue G, 5 μM) for 1 min and a non-competitive SERCA inhibitor for 30 seconds, as described in figures’ captions, and the fluorescence of Fluo-4 was measured.

Statistical analysis

The results were expressed as mean values ± SEM. Differences between means were analyzed using Student’s two-tailed t test. P < 0.05 was considered statistically significant. Cosinor software analysis was used to evaluate and estimate the parameters of circadian rhythm (period plus mean, amplitude and acrophase).

Results

Since intracellular calcium was previously shown to exhibit a well-defined circadian rhythm in neuronal population of SCN [32], we first verified whether Ca²⁺ level exhibited a circadian rhythmicity in peripheral oscillators (Fig. 1). We used human primary fibroblasts since these cells are an excellent in vitro model of peripheral oscillators [10,56,57] and show a disturbed Ca²⁺ homeostasis in AD patients [31,39].

In the current study, fibroblasts presented changes in Ca²⁺ accumulation, with a peak 16 h post-synchronization (TP16) and a trough 28 h post-synchronization (TP28) (Fig. 1A). Under these conditions, relative Fluo-4 fluorescence in cell cultures was significantly different (p < 0.001) between the peak and the trough (Fig. 1D).

Considering the controlling mechanisms of cytosolic Ca²⁺ fluctuations, it is possible that receptor-mediated Ca²⁺ influx is involved in the regulation of circadian rhythm of [Ca²⁺]. Since primary human fibroblasts are electrically non-excitable and do not express voltage-gated Ca²⁺ channels, Ca²⁺ could be transported via purinergic P2X receptors, especially P2X7 subtype that is widely distributed in skin tissue [65]. Using the specific antagonist of P2X7 receptor—Brilliant Blue G (BBG), we showed that treatment with this compound abolished circadian oscillations
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Disturbances of the Ca^{2+} homeostasis have been demonstrated to be associated with Aβ neurotoxicity. Therefore, we investigated the effect of Aβ peptides on the circadian fluctuation of Ca^{2+} levels (Fig. 2). Our data showed that extracellular treatment with Aβ_{42} (aged peptide) completely abolished circadian oscillations of intracellular Ca^{2+} and impacted the levels of [Ca^{2+}] at

of [Ca^{2+}] (Fig. 1C). Thus, the significant difference between [Ca^{2+}] at TP16 and TP28 was not observed anymore (Fig. 1D).

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Fig. 1. Circadian oscillations of cytosolic calcium depend on activation of P2X7 receptors but not calcium uptake through SERCA. A) Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 μM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points (n = 5). B-C) Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 μM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points in presence of (B) thapsigargin (THAPS, 10 nM), inhibitor of SERCA, or (C) Coomassie Brilliant Blue G, antagonist of purinergic P2X7 receptors (BBG, 5 μM). D) Relative cytosolic calcium level at 16 hours post-shock (peak: TP16) and at 28 hours (trough: TP28) compared to non-treated cells (CTRL) (n = 3-5). The emitted fluorescence is linearly related to the cytosolic calcium content. *p < 0.05, **p < 0.001; Student’s two-tailed t test comparing single time points. Data are represented as average ± SEM.
peak and trough time points (Fig. 2A and 2C). Those Aβ-evoked disturbances were not mediated by deregulation of ER Ca\(^{2+}\) stores, because THAPS treatment did not restore rhythmic oscillations of [Ca\(^{2+}\)]\(_i\) (Supplementary Figure 3 online). Interestingly, treatment with adenosine triphosphate (ATP) abolished the negative effect of Aβ and restored oscillation of [Ca\(^{2+}\)]\(_i\) (Fig. 2B and 2C). These data suggest that Aβ peptides altered the P2X7 receptor-mediated circadian rhythm of [Ca\(^{2+}\)]\(_i\), leading to disturbances of calcium homeostasis.

Fig. 2. Cytosolic calcium oscillations are abolished in the presence of Aβ. A-B) Cytosolic calcium levels were evaluated by using fluorescent dye, Fluo-4 (4 μM), in synchronized human skin fibroblasts from 12 hours post-synchronization time point every 4 hours for 7 time points in presence of (A-B) Aβ\(_{42}\) (aged peptide) at 0.5 μM (n = 3). B) Aβ treated cells were co-treated with ATP (1 mM) to overcome the dampening due to Aβ presence (n = 3). C) Relative Cytosolic Calcium level at 16 hours post-shock (peak: TP16) and at 28 hours (trough: TP28) compared to non-treated cells (CTRL) (n = 3). Aβ treatment completely abolished differences in [Ca\(^{2+}\)]\(_i\) concentration between peak and trough time points while ATP treatment rescued the circadian oscillation of cytosolic calcium. The emitted fluorescence is linearly related to the cytosolic calcium content. **p < 0.01, ***p < 0.001; Student’s two-tailed t test comparing single time points. Data are represented as average ± SEM.
Discussion

Accumulating evidence has suggested that sleep disturbances may be early indicators of dementia and may actually precede the onset of cognitive symptoms in AD [53]. Moreover, the sleep–wake cycle was shown to be a critical regulator of Aβ release and loss of slow wave sleep resulted in higher cumulative levels of neuronal activity and higher Aβ concentration in CSF [12]. It was previously suggested that intracellular Ca²⁺ may be a coordinator of the circadian timing system and biochemical reactions due to its ubiquitous role as a metabolic regulator [4]. Therefore the disturbances in Ca²⁺ homeostasis observed in AD brains could be partly associated with deregulation of patients’ circadian rhythms. However the role of Ca²⁺ in regulating the clock function in pathophysiology is unknown. In this study, we showed for the first time that Alzheimer’s Aβ peptides could negatively influence circadian fluctuations of Ca²⁺ in peripheral oscillators. This subsequently may alter calcium-dependent molecular processes involved in circadian clock regulation in AD.

It was demonstrated that intracellular Ca²⁺ concentration exhibits circadian rhythms in pacemaker neurons of the SCN [15,38]. The oscillatory physiology of Ca²⁺ was shown to be regulated by the circadian fluctuations in the Ca²⁺ currents generated by voltage-dependent calcium channels (VDCC) [41,60]. Calcium fluctuations were also shown in astrocytes of SCN, but, unlike in neurons, they were regulated by the Ca²⁺ release from ER stores [11,20]. Our study extends previous results by showing the existence of daily fluctuations in cytosolic calcium in peripheral oscillators, the human skin fibroblasts. This cell line was previously shown to exhibit circadian rhythms of clock genes, and the clock properties of these peripheral cells closely mimic those measured physiologically and behaviorally in humans [10,57]. Therefore, skin fibroblasts are a good in vitro model for studying molecular mechanisms of circadian rhythms. Moreover, it was shown that aging does not alter the basic clock properties (period length, amplitude and phase) of fibroblasts [56]. We observed that calcium oscillations in fibroblasts correspond to the previously demonstrated changes in transcript levels of the clock genes Per2, Bmal1, Rev-Erb, and Cry1 in those cells [9]. Former data showed that Ca²⁺ mobilized from internal deposits modulates the molecular circadian clock of hepatic cells ex vivo, in a manner that did not depend on the entrainment cue (meal or light) [4]. This suggests that Ca²⁺ signaling is a key regulator of circadian rhythms in peripheral tissues in contrast to the central pacemaker mediating hierarchical organization of rhythmicity [25].

Calcium signaling in non-excitable cells is initiated by mobilization of Ca²⁺ from intracellular ER stores through IP3 and ryanodine receptors. In our experiments, inhibition of SERCA significantly elevated cytosolic Ca²⁺ level, but did not alter calcium fluctuations. A similar effect was observed in SCN astrocytes [11]. Besides, in SCN neurons, the expression of SERCA was shown to follow a circadian pattern [50]. Together, all these data suggested that ER stores are not necessary for controlling [Ca²⁺], daily oscillations in non-neuronal cells.

Previous data reported the crosstalk between circadian oscillation of intracellular Ca²⁺ and rhythmic extracellular ATP accumulation in SCN astrocytes [11]. Exogenous ATP was shown to be a mediator of intercellular communication in physiology and neurodegeneration, by acting on the cell surface receptors, including ligand-gated ion channels (P2X) and G-protein coupled (P2Y) receptor subtypes. It was demonstrated that ATP selectively promotes the expression of the clock gene Per1 through gene transactivation after stimulation of P2X7 purinergic receptors in microglial cells [54]. Moreover, the endogenous purinergic receptors were shown to determine the local clock activity in the urinary bladder cells [69]. Therefore, the ATP-signaling may also be involved in changes of Ca²⁺ fluctuations in peripheral oscillators. Indeed, our study demonstrated that the circadian rhythm of the calcium level in fibroblasts depend on activation of the ATP-binding receptor, P2X7.

In AD pathology, changes of neuronal Ca²⁺ concentration are responsible for the oxidative stress as well as altered metabolism of APP and overproduction of Aβ peptides. On the other hand, Aβ neurotoxicity has been associated with the disturbances of intracellular Ca²⁺ homeostasis in neurons as well as in glial cells. The studies using APP transgenic mouse models of AD identified significantly elevated numbers of neurites with overloaded cytosolic Ca²⁺ and this effect was positively correlated with the distance from Aβ plaques [43]. Many previous studies demonstrated impaired Ca²⁺ regulation in fibroblasts of AD patients [31,39]. Our study confirms those reports, by demonstrating that exposure
to Aβ_{42} (aged peptide) abolished Ca^{2+} fluctuations in the cytosol of fibroblasts.

A disruption of the Ca^{2+} regulation in the ER was previously shown to mediate signal-transduction alterations associated with AD [44]. Moreover, mutations that cause familial Alzheimer’s disease have been linked to disturbances in intracellular calcium signaling pathways [34]. Skin fibroblasts from humans that harbor a mutation in presenilin 1 (PS1-A246E) showed exaggerated Ca^{2+} release from IP3-gated stores compared to controls after treatment with bombesin and bradykinin [39]. The elevated Ca^{2+} release from the ER, evoked by activation of the IP3 [14] or ryanodine [61] receptors, was shown to increase Aβ level. Overexpression of SERCA was also shown to increase Aβ production [29]. Furthermore, ER is also a potential intracellular target for Aβ protein [26,71], which disrupts the function of the intracellular Ca^{2+} stores. In our study, thapsigargin treatment did not restore the physiological oscillations of [Ca^{2+}]_{i} that were significantly altered by Aβ. These data suggest that Aβ-mediated disruption of intracellular Ca^{2+} homeostasis may be evoked by an excess of calcium influx across the plasma membrane.

Furthermore, previous studies indicated that an altered activity of the purinergic P2X7 receptor mediates the pro-inflammatory processes in a transgenic AD model and in brains of AD patients [46,49,59]. The observation that Aβ may cause ATP release from microglia, and that P2X7 receptor is an obligate participant in microglia activation by Aβ, put the role of ATP and P2 receptors as a key event in neurodegeneration [42,63]. Recent data demonstrated that in vivo inhibition of P2X7 receptors significantly reduces the amyloid plaques formation in brain hippocampal structures through activation of α-secretase activity [51]. The mechanism of P2X7R-specific cleavage of APP was shown to be independent of ADAM9, -10, and -17 activity, but involved Erk1/2 and JNK phosphorylation [22]. In our study, we demonstrated that Aβ peptide significantly interferes with the P2X7-receptor mediated circadian oscillations of intracellular Ca^{2+}, however the mechanism underlying this phenomenon needs to be further investigated.

In summary, our data provide first evidence that Alzheimer’s Aβ_{42} peptides induce disturbances of P2X7 receptors mediated Ca^{2+} oscillation in peripheral oscillators. These findings may be therefore helpful for a better understanding of the circadian rhythms disruption related to AD.

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Disclosure

Authors report no conflict of interest.

References

11. Burkeen JF, Womac AD, Earnest DJ, Zoran MJ. Mitochondrial calcium signaling mediates rhythmic extracellular atp accumu-


