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Astrocytes' Role in Alzheimer's Disease Neurodegeneration

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Abstract

Central nervous system (CNS) astrocytes are glial cells performing crucial tasks encompassing energy metabolism, neurotransmission, ion and water stable levels, and immune defense and control local blood flow/oxygen levels. Arising from neural stem cells, astrocytes differentiate into subtypes that vary according to animal species. Human cerebral cortex astrocytes are sturdier and cytologically and functionally more complex, control wider domains, and spread calcium signals more quickly than their rodents' counterparts. They actively partake in CNS homeostasis maintenance and functioning by teaming up with their client neurons, other glial cell types, and cerebrovascular cells. Alterations of astrocytes' activities deeply impact on age-related chronic ailments like Alzheimer's disease (AD), the commonest senile dementia; AD involves the growing accumulation of amyloid-β peptides (Aβs) and hyperphosphorylated Tau proteins the astrocytes, and neurons supply following the interaction of their calcium-sensing receptors (CaSRs) with exogenous Aβs. The activated Aβ·CaSR signaling triggers a self-propagating mechanism that spreads the neuropathology among adjacent and far away astrocytes and their neuronal clients causing neurons' death. CaSR antagonists or calcilytics suppress these noxious effects in vitro. Hence, calcilytics are potential therapeutics that could halt the spread of AD neuropathology and safeguard the patients' neuronal viability, cognition, memory, and ultimately life.

Keywords: human, astrocyte, Alzheimer's disease, amyloid-β, tau protein, calcium-sensing receptor, calcilytics

1. Introduction

Between the 16th and 18th week of intrauterine life, a pool of stem cells of the neural plate generates every neural cell type, excepting microglia, in humans. Once differentiated, the astrocytes undergo a complex maturing process through which they acquire their specific morpho-functional

characteristics. When these processes achieve completion, human astrocytes account for up to 50%, if not more, of the entire CNS cell population. These cells are larger in size and endowed with more numerous branches than their much less abundant (<20% of all CNS cells) rodents' counterparts [1]. Being so plentiful, astrocytes have a relevant role in brain environment homeostasis maintenance [2, 3]. They metabolically sustain neurons, recycle neurotransmitters, affect synapse activity, control local blood flow, and partake in blood-brain barrier functional integrity (see for details [2–4] and below). Aging and CNS diseases, neurodegenerative ones included, can induce an activated or inflammatory or reactive condition in the astrocytes [5, 6].

2. Human astrocytes' varieties

As their designation indicates, astrocytes have a typical star-shaped morphology as they emit different numbers of cytoplasmic branches according to their subtype. Astrocytes of several subtypes dwell in the human CNS. Some of them display locational predilections, e.g., fibrous astrocytes for the white matter and protoplasmic astrocytes for the gray matter. More recently, it has been realized that the classical protoplasmic and fibrous astrocytes can be differentiated into several subtypes, some of which proper only of the human cerebral cortex. Such subtypes share a specific marker, the glial fibrillary acidic protein (GFAP), which is an intermediate filaments' constituent expressed by all the astrocytes cultured in vitro. However, in vivo only the fibrous astrocytes express GFAP in the white matter (see for Ref. [7]). Recent studies have singled out a novel marker expressed by both protoplasmic and fibrous astrocytes, the aldehyde dehydrogenase-1 family member L-1 (Aldh1L1) [1, 8, 9].

2.1. Radial astrocytes

Radial astrocytes are the first ones to appear in the course of embryogenesis. At that point in time, they aid neurons' migration by acting as scaffolds [10]. Later, they differentiate becoming *stellate astrocytes*. However, after birth, radial astrocytes persist as such in the cerebellum (Bergmann glia) and the retina (Müller glia).

2.2. Fibrous astrocytes

These white-matter-located astrocytes present very long and thin processes which mostly do not emit branches. The processes' terminal end-feet envelope the axonal nodes of Ranvier and also gets in touch with the walls of cerebral vessels. Most notably, fibrous astrocytes partake in the repair of injured brain tissue, especially at the spinal cord level [11].

2.3. Protoplasmic astrocytes

They are the most abundant astrocytic type. Their somata give out numerous (up to 200) long branches, which end up with leafy feet or end-feet in part touching the blood vessels' walls and in part enwrapping several thousands of synapses [1, 8]. Near the pia mater's inner surface, the astrocytes' end-feet cluster together forms the CNS *limiting peripheral membrane*.

The astrocytes' end-feet in contact with the outer wall of cerebral arteries and veins make up the *glia limitans*, a space in which the *glymphatic* drainage allows the influx and efflux of the brain interstitial fluid (lymph). Such fluxes are crucially assisted by the astrocytes' water-transporting aquaporin-4 channels. Via these paravascular pathways, nutrients reach the neurons and glial cells, while toxic metabolites and soluble amyloid-β peptides (sAβs) are removed from the CNS tissue [12]. In addition, the early connection between the endothelial cells of the brain's nascent blood vessels and the astrocytes derived from radial glia results in a tight interaction between the end-feet of mature astrocytes and the capillary endothelial cells which presides over the normal function of the blood-brain barrier (BBB) [13].

It is worth recalling here that both Golgi silver staining and GFAP immunolabeling of brain tissue sections make the astrocytes appear as star-like cells. However, the astrocytes are the possessors of a certain number of cytoplasmic branches these methods do not stain. Therefore, such methods do not reveal the astrocytes' true morphology as visible under the light and/or fluorescence microscope. Another concept of old is that during development the astrocytes' branches form an interdigitated scaffold permitting the organization of the neurons. Recently, it has become clear that independent and distinct astrocytic domains develop with no connection with similar neighboring domains within the hippocampus [14]. As abovementioned, the morpho-functional features of human protoplasmic and fibrous astrocytes differ from rodents' ones. For instance, the diameters of gray matter-located human protoplasmic astrocytes are 2.6-fold longer, and their GFAP-positive processes are 10-fold more abundant. A single protoplasmic astrocyte can control from 270,000 to 2.0 million synapses placed inside its spatial domain. Most important, the branches of a single astrocyte touch, envelop, and regulate not only a huge number of synapses but also the capillary vessels controlling the blood flow going to those same synapses. This organized structure has been interpreted as the indication of a control of synaptic activity by the astrocytes independently of neuronal activity. Although unable to transmit neural impulses, human astrocytes propagate calcium ion [Ca^{2+}] waves at speeds of up to 36 μm/s, i.e., 4–10-fold faster than rodents' astrocytes do [15–17].

2.4. Additional astrocytes' subtypes

Besides the above-described canonical kinds, several other astrocyte subtypes have been recognized. Emsley and Macklis [17] have used a combined approach consisting of S100β immunostaining, GFAP expression, and human GFAP promoter-prodded enhanced green fluorescent protein (eGFP) expression in transgenic mice, to identify within several subtypes of CNS astrocytes. The latter incorporate radial glia, protoplasmic astrocytes, fibrous astrocytes, ependymal glia, tanycytes, Bergmann glia, and velate glia. The cytoarchitectonics and functional requirements of their local placements mainly determine the morphological features, growth rates, and relative densities of these subtypes [17]. NG2 cells are an additional CNS glial cell type likely possessed of stem cell features and hence capable of giving raise to astrocytes, neurons, and oligodendrocytes (OLGs) during both intra- and extrauterine life. NG2 glial cells functionally interact with neurons at the level of synapses. Studies are under way to clarify the heterogeneity of NG2 glia [18].

2.5. Human cortex-specific astrocytic subtypes

At variance with other mammalian species, humans have developed two novel cerebral cortical astroglia subtypes: the *astrocytes with varicose projections* and the *interlaminar astrocytes*. The latter are plentiful in the cortical layer 1, whereas the former inhabit cortical layers 5 and 6. The somata of both subtypes give out prominent cytoplasmic branches. In the case of the astrocytes with varicose projections, such branches are up to 1 mm long and terminate on the cerebral vessels walls or in the neuropil. After twisting courses, the also lengthy branches of the interlaminar astrocytes end up like varicose projections in contact with vascular walls or in the neuropil. Hitherto, the specific roles of such recently identified cerebral cortical astrocytic subtypes are not understood. Anyhow, the lengthy processes of the human interlaminar astrocytes can propagate Ca^{2+} waves [15–17].

3. Astrocytes' physiology

In the past and still now, some scientists have been holding astrocytes as neuron-supporting and at the same time debris-scavenging cells protectively regulating the homeostasis of a microenvironment from which neurons derive the necessary nutrients [12, 19]. Astrocytes also control the workings of "tripartite synapses" by enveloping them with their branches, thus barring the diffusion of released neurotransmitters and preventing the firing activity of one neuron from altering that of adjacent neurons [20]. In addition, astrocytes' synaptic regulation does not influence only the tripartite synapses their branches envelop but also far away synapses via astrocytes' signals, a process named *lateral astrocyte synaptic regulation* [21]. Astrocytes can do this and also communicate with neighboring neurons, with which they form astrocyte-neuron gangs with a ratio of one "master" astrocyte and 20–30 "client" neurons [22] and adjust local blood flow by secreting various compounds called *gliotransmitters* [23]. Surges in intracellular Ca^{2+} levels drive the release of several gliotransmitters, comprising adenosine, ATP, D-serine, eicosanoids, glutamate, and TNF-α, which would adjust the activities of the astrocytes themselves, the far away synapses, and the surrounding cells [24].

Since astrocytes cannot be electrically excited, their plasma membranes do not propagate action potentials as instead neurons do. The membrane potential of astrocytes at rest has very low values, ranging from −85 to −90 mV. This is due to their intense expression of TREK-1 and TWIK-1 potassium ion [K^+] channels [25]. As recent lines of evidence show, astrocytes residing in separate brain areas express dissimilar types and levels of ion channels and hence are equipped with distinctive electrophysiological characteristics. The huge group of ion channels implicated is also differently expressed during astrocytes' developmental stages [26].

In addition, astrocytes express various kinds of metabotropic receptors, which are coupled to a number of intracellular second messenger systems. For example, astroglia are known to adjust neuronal excitability and synaptic transmission through the metabotropic glutamatergic receptor subtype 5 (mGluR5). The results of experiments using brain slices showed that in response to an assortment of neurotransmitters, comprising acetylcholine, adenosine, ATP, endocannabinoids, GABA, glutamate, norepinephrine, and prostaglandins, metabotropic receptors could raise the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) via phospholipase C (PLC)- and inositol (1,4,5)-triphosphate (IP3)-dependent activities [27].

4. Astrocytes and AD neuropathology

An aberrant reactivity of astrocytes is a telltale sign of chronic neurodegenerative ailments like AD and Parkinson's disease [1, 3, 5]. While AD advances an *astrogliosis* emerges as a sign of astrocytes' dysfunction. However, astrogliosis is a common event in all kinds of CNS injury or ailment and is marked by persistent scar-like structures made by proliferating and migrating reactive astrocytes [5, 11]. Two types of reactive astrocytes, the A1 and the A2, have been latterly identified, whose specific activities could result advantageous or detrimental according to the type of neuropathology considered. In fact, reactive astrocytes of the A2 type advance healing of ischemic injuries (e.g., stroke). Conversely, reactive astrocytes of the A1 type could either stop their physiological activities and next degenerate or become involved in detrimental activities [28]. Reportedly, astrocytes mediate Aβ neurotoxicity and Tau phosphorylation in primary cocultures with rat embryo neurons [29].

AD hits nearly 2% of the people of the Western world particularly after 60 years of age [30]. AD's clinical course can be dissected into (*a*) a quite protracted (~20–40 years) asymptomatic phase, the early diagnosis of which is hard to make because of the present lack of specific markers, (*b*) an amnestic minor cognitive impairment phase (aMCI; ~3–6 years) in which amnesia's severity progressively grows, and (*c*) a full-blown symptomatic phase (~6–8 years) typified by escalating losses of memory and cognitive abilities and ending up with patients' obit [31, 32]. Controversies still rage about the pathophysiological mechanisms promoting the opening and unforgiving progression of the sporadic or late-onset AD (SAD or LOAD) [33]. The neuropathology unhurriedly yet progressively destroys the neuronal networks. As shown by high-resolution fMRI studies, LOAD starts in the lateral entorhinal cortex of the hippocampus, the brain's memory-recording place, and next spreads along the extended projection circuits connecting the hippocampus with cognition-crucial wider and wider cognition-crucial areas of the upper cerebral cortex [34].

In the healthy brain, neurons produce and release at their synapses tiny amounts of non-toxic $A\beta_{42}$ monomers, the intra- and extracellular amounts of which remain at low (i.e., pM), physiological values owing to a set of removing mechanisms operated by several proteases, phagocytosis by microglia and astrocytes, and disposal into the blood circulation [35]. In aged brains, the ability to clear the Aβs from the CNS increasingly plummets likely because of local microcirculation problems. Consequently, as the *amyloid cascade hypothesis* posits, the accumulating $A\beta_{42}$ monomers start forming agglomerates first of toxic soluble oligomers ($A\beta_{42}$-os) and protofibrils [35] and next of insoluble, fibrils, and senile plaques, thereby driving the neuropathology progression [33, 36]. According to this *Aβs first hypothesis*, the hyperphosphorylated Tau (p-Tau) protein, the second main driver of AD, enters the stage some time later.

Conversely, as the *brainstem-Tau first hypothesis* posits, AD starts within a brainstem nucleus, the *locus coeruleus,* and its surroundings. There, presumably mutated accumulating p-Taues group into neurotoxic oligomers (p-Tau-os) which next steadily spread out across the cerebral cortex along lengthy and circuitous neural pathways that also reach the hippocampus, leaving as their aftermaths intra-neuronal neurofibrillary tangles (NFTs) [37–40]. Later, p-Tau-os can also prompt the production of $A\beta_{42}$ surpluses which too diffuse and accumulate intracerebrally [37, 39, 41]. A colocalization of Aβs and NFTs within the cytoplasm of human astrocytes can also occur [42]. Whatever is the temporal order of manifestation of the two main AD drivers [on

this topic, see also below], their joined toxic activities do speed up the occurrence of synapses loss, neuroinflammation, mitochondrial damage and dysfunction, oxidative stress, astrocytes' and microglia's reactivation, senile plaques' deposition, cerebral amyloid angiopathy (CAA), NFTs, and progressive oligodendroglia and neurons death—all hallmarks of AD's neuropathology—and therefore accelerate the clinical course of AD [37, 39].

Moreover, $A\beta_{42}$-os and $A\beta_{42}$ fibrillar aggregates bind various plasma membrane receptors, comprising the calcium-sensing receptors (CaSRs) and the receptors for advanced glycation end products (RAGEs) which can activate the astrocytes (see for further details [3, 11, 43, 44]). Such multiple receptor interactions with Aβs stir up astrocytes' JAK2 and MEK1/MEK2/ERK-1/ERK-2 signaling pathways stimulate the direct binding of STAT1 and HIF-1α/HIF-1β complexes to the *BACE1* and *VEGF-A* gene promoters and activate the microglia triggering synthesis and secretion of proinflammatory cytokines like IL-1β, TNF-α and INF-γ [37, 43, 44]. Such cytokines critically advance the formation of Aβ-os and fibrils [45, 46].

5. The CaSR

A highly conserved gene, the *CASR* is a member of family C of the G-protein-coupled receptors (GPCRs). Family C GPCRs do not share any DNA sequence homology with the members of other GPCR families. However, the CaSR exhibits topological and sequence homology to the metabotropic glutamate receptors (mGluRs) [47]. The CaSR protein has seven transmembrane α-helices (TM1–TM7) linked by extra- and intracellular loops altogether making the 7TM region. The CaSR protein has a massive (612 amino acids) extracellular N-terminal domain, the so-called Venus flytrap (VFT), and a much tinier intracellular C-terminal tail, which makes up the G-protein-binding domain [22, 48]. In their membrane-bound form, CaSRs form homodimers (CaSR/CaSR) or heterodimers (e.g., CaSR/mGluR) [22, 48]. CaSR dimers are put together at the endoplasmic reticulum (ER) and next are conveyed and fitted into the plasma membrane [49]. Once there, the CaSR senses minute changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$). However, the CaSR is not a ligand-discriminating receptor. Rather, it may be better described without changing its acronym as a *cation-sensing receptor*. In point of fact, its ligands can be distinguished in (*a*) VFT-binding CaSR-activating orthosteric ligands, comprising Ca^2, several di- and trivalent cations, aminoglycoside antibiotics, and the polyamine spermine, and (*b*) allosteric ligands which bind different sections of the 7TM domain, including aromatic L-α-amino acids, extracellular Na^+, and pharmacological *agonists* and *antagonists* (*see below*) [22]. The activation of CaSR encompasses a complex set of interactions among amino acids, Ca^{2+} and conceivably anions like PO_4^{3-} ions. Recently, Geng et al. [50] demonstrated that the CaSR can display an inactive state both in the absence and in the presence of Ca^{2+} ions and adopts the active state only when one L-amino acid, and one or more Ca^{2+} ions are bound to it. L-amino acids like L-Trp and Ca^{2+} ions are co-agonists of the CaSR, operating together to elicit the receptor's activation. Finally, it should be mentioned that in human adult astrocytes, CaSR expression increases in proliferatively quiescent cells with respect to actively growing ones, but is not affected by high or low levels of $[Ca^{2+}]_e$ [51].

Notably, being positively charged, both soluble or fibrillar Aβs specifically form complexes with the plasma membrane CaSRs. Subsequently, the Aβs·CaSR complexes coalesce into patches which are rapidly endocytosed and can be detected within EEA1-positive early endosomes in the cytoplasm (**Figure 1**) [54, 55]. However, it has not been ascertained whether Aβs' binding site[s] is [are] of the orthosteric or allosteric kind or both [22, 44].

Various species of G-proteins mediate CaSR's intracellular signaling by (*a*) activating a set of enzymes such as protein kinases (e.g., AKT, JNK, PKCs, and MAPKs like MEK/ERK) and lipid kinases (e.g., phospholipase A2, C, D), (*b*) triggering gene expression through transcription factors, (*c*) inhibiting adenylyl cyclase, and (*d*) inducing Ca^{2+} influx via TCPC6-encoded channels [56]. The relevant consequences are modifications to enzyme activities (e.g., proteases), cell proliferation, cell secretion, and/or cell death. In addition, CaSR-expressing neurons and all types of glial cells expressing the CaSR are liable to be harmed by the cytotoxic effects of CaSR-binding and CaSR-activating soluble Aβ oligomers [sAβ-os] and/or insoluble fibrillar Aβ (fAβ) aggregates [22, 44].

CaSR's expression occurs in every portion of the rat and human brain. By using the in situ hybridization method, Yano et al. [57] demonstrated that the CaSR is intensely expressed in several areas of the adult rat CNS. In relation to AD, we recall here that CaSR's expression abounds in the hippocampus especially at the level of the somata and axon terminals of the pyramidal neurons, suggesting the functional modulation of such cells by CaSR's signaling [47, 58]. Notably, the N-methyl-D-aspartate receptor (NMDAR) brain location is superimposable on the CaSR's. Both NMDARs and CaSRs play crucial roles in the induction of
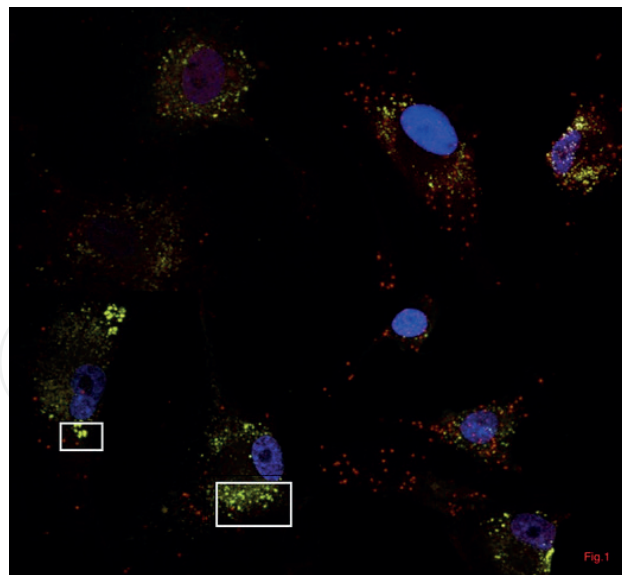
Figure 1. Cultured untransformed adult human astrocytes CaSRs bind Aβ$_{25-35}$ and internalize the Aβ$_{25-35}$·CaSR complexes within in EEA1-positive cytoplasmic early endosomes. Cells were incubated for 15 min at 37°C with Aβ$_{25-35}$ [5.0 μM] and next fixed and permeabilized using the in situ proximity ligation assay (PLA) (see for details [52, 53]), it is possible to specifically reveal the Aβ$_{25-35}$·CaSR complexes as *red dots*. Using an antibody against the EEA1 antigen marker, early endosomes [54] can be seen as *green dots*. The yellow fluorescence reveals the discrete colocalization of the Aβ$_{25-35}$·CaSR complexes with the EEA1-positive early endosomes. *Top panel* magnification, 600×. The other two panels are zoomed from the middle (middle panel) and left (bottom panel) rectangles in the top panels.

long-term potentiation (LTP) [59]. Typically, CaSR's expression occurs not only in neurons but also in human primary astrocytes, astrocytoma cell lines, oligodendroglia, and microglial cells [57]. Interestingly, total CaSR protein levels increase significantly though transiently in Aβ-exposed NAHAs [44]. Furthermore, the intensity of CaSR's immunoreactivity significantly increases with age in the hippocampus of 3xTg AD-model mice [60], particularly where Aβs and p-Taues also accumulate, a clear indication of the involvement of this receptor in AD pathophysiology in vivo (see also below).

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can vary widely under both normal and pathological conditions. The Ca^{2+} influx into cultured astrocytes is linear and normally increases only up to 1.8 mM, suggesting that CaSR signaling controls it [61, 62]. In the past, aberrations of cell surface and intracellular Ca^{2+}-controlling mechanisms were posited to happen in various neurodegenerative ailments, AD included [61–65]. Reportedly, exogenous $Aβ_{42}$ and its well-established proxy, $Aβ_{25–35}$, trigger $[Ca^{2+}]_i$ surges and oscillations which persist for hours in the neurons and astrocytes too and concur with the loss of the inner mitochondrial membrane potential. This in turn promotes the release of reactive oxygen species (ROS) and oxidative stress in both neurons and astrocytes. Coculturing such reactive astrocytes with neurons caused the neurons' death within 24 h unless the Aβ-elicited $[Ca^{2+}]_i$ surges were forestalled [61].

Recent findings from our laboratory lend credence to the view that the CaSR, one of the receptors astrocytes express, drives the pathogenic mechanisms of AD [48, 66–69].

6. Human cortical astrocytes, CaSRs, and AD promotion

It is time for us to zoom in on our preclinical model of cortical untransformed phenotypically stable, i.e., *normal* adult human astrocytes (NAHAs) obtained from temporal cortex surgical leftovers of motorbike accident victims with perforating skull trauma. After culturing and expanding the numbers of the NAHAs in vitro and next inducing them into proliferative quiescence, we have been investigating their metabolic responses to added exogenous Aβ-os or Aβ fibrils either in the presence or absence of a microglial cytokine mixture (i.e. IL-1β, TNF-α, and INF-γ) [44, 55, 70, 71].

As we recalled above, CaSR's expression takes place with dissimilar intensities, in every CNS cell type, astrocytes included [44, 57]. Recent studies have brought to light some of the physiological roles the CaSR plays in the human CNS, like modulation of neurons' dendrites and axons growth and of OLGs development [57, 72]. Using the NAHAs as our experimental system, we first demonstrated that exogenous $Aβ_{25–35}$—instigated CaSR signaling elicits the concurrent expression of nitric oxide synthase-2 (NOS-2) and of GTP cyclohydrolase-1 (GCH-1). GCH-1 makes the BH4 [tetrahydrobiopterin] cofactor that dimerizes and activates the NOS-2 moieties, thus allowing the synthesis of nitric oxide (NO) to occur [44, 73, 74]. Exogenous fibrillar Aβs also induce via direct CaSR signaling activation the cytoplasmic stabilization and nuclear translocation of the hypoxia-inducible HIF-1α•HIF-1β transcription complex in NAHAs. This elicits the vascular endothelial growth factor-A (*VEGF-A*) gene expression and the de novo synthesis of three splice protein variants (i.e., $VEGF-A_{121}$, $VEGF-A_{165}$, and $VEGF-A_{189}$) and the

secretion mainly of the VEGF-A$_{165}$ variant [55, 70]. A typical feature of AD is an overproduction and release of VEGF-A from neurons, glial cells, and cerebrovascular endothelium. Such VEGF-A surpluses are toxic for neurons, astrocytes, and endothelial cells, the constituents of the neurovascular units, and result in BBB's functional impairment (see for Refs. [75–78]). In vivo, an Aβ·CaSR-mediated VEGF-A$_{165}$ oversecretion from the human astrocytes' end-feet of their blood-vessel contacting processes could drive local surges of the blood flow in the hippocampus of aMCI stage patients [79, 80]. This event could be revealed as unexpected intensified blood oxygen level-dependent (BOLD) signals by means of high-resolution functional magnetic resonance imaging [fMRI] analysis [81, 82]. Hence, such BOLD signal is not due, as would be wrongly expected, to a presumptive hyperactivity of overtasked neurons in shrunken dentate gyrus/CA3 Aβ-damaged hippocampal areas of aMCI patients [80–82]. The increased VEGF-A release elicits a greater local vascular density via neoangiogenesis which augments blood oxygen delivery and BOLD signal intensity of these hippocampal areas once they are functionally activated. Yet, the progression of AD neuropathology destroys the overgrown local vessels, thus reducing the blood flow to the point that the fMRI-intensified BOLD signal vanishes. At any rate, this boosted BOLD signal at the hippocampal level of aMCI subjects is a harbinger of the impending symptomatic stage of AD [79].

However, the most exciting discoveries were subsequently made possible by the advent of very sensitive ELISA kits assaying Aβs. In untreated NAHAs, the metabolic processing of amyloid precursor holoprotein (APP) takes place along the nonamyloidogenic pathway [NAP] being mediated by the activity of the α-secretases (mainly ADAM10) and extracellularly sheds all the soluble sAPPα it produces. Notably, sAPPα is a neurotrophic and neuroprotective compound positively affecting neurons' functions and viability. Moreover, sAPPα synthesis precludes any Aβ$_{40/42}$ production from APP as it is cut from the middle amino acid sequence of Aβ$_{40/42}$. Therefore, NAP largely prevails over APP's amyloidogenic processing [AP] in the untreated astrocytes, which secrete only very low basal Aβ$_{40/42}$ amounts [71]. Conversely, adding fibrillar Aβ$_{25–35}$ by itself and hence stirring off Aβ$_{25–35}$·CaSR signaling remarkably reduces sAPPα's extracellular shedding while driving an overproduction and oversecretion of neurotoxic Aβ$_{42}$/Aβ$_{42}$-os owing to concurrent raises in the sequential activities of BACE-1 and γ-secretase. The further addition of a microglial cytokine mixture only accelerates but not increases the total amount of Aβ$_{42}$/Aβ$_{42}$-os secretion by the NAHAs despite a concurring APP overexpression [44, 71]. Thus, these events could start of self-sustaining vicious cycle of Aβ$_{42}$/Aβ$_{42}$-os spreading within the brain [37, 44]. The same Aβ·CaSR-induced signaling mechanism stimulates the secretion of neurotoxic Aβ$_{42}$/Aβ$_{42}$-os from human cortical postnatal HCN-1A neurons [44]. Thereafter, the neurons start dying slowly like they do in vivo [44]. Most important, we also gained preliminary evidence indicating that Tau and hyperphosphorylated (p)-Tau are both expressed by untreated NAHAs in culture and that their exposure to the usual Aβ$_{42}$ proxy, Aβ$_{25–35}$, significantly increases via Aβ·CaSR-induced signaling the activity of GSK-3β [83], the main Tau protein kinase [84, 85]. The upshot is an increased production of p-Tau/p-Tau-os which both accumulate inside the cells and are extracellularly released inside exosomes [83]. Novel lines of evidence suggest that extracellular vesicles, which comprise exosomes, play important physiological and pathological roles in the CNS [86]. The above mechanism could promote the concurrent diffusion of both p-Tau/p-Tau-os and Aβ$_{42}$-os, the two main AD drivers, within the brain, though the tauopathy's noxious effects will take longer to manifest [37, 83].
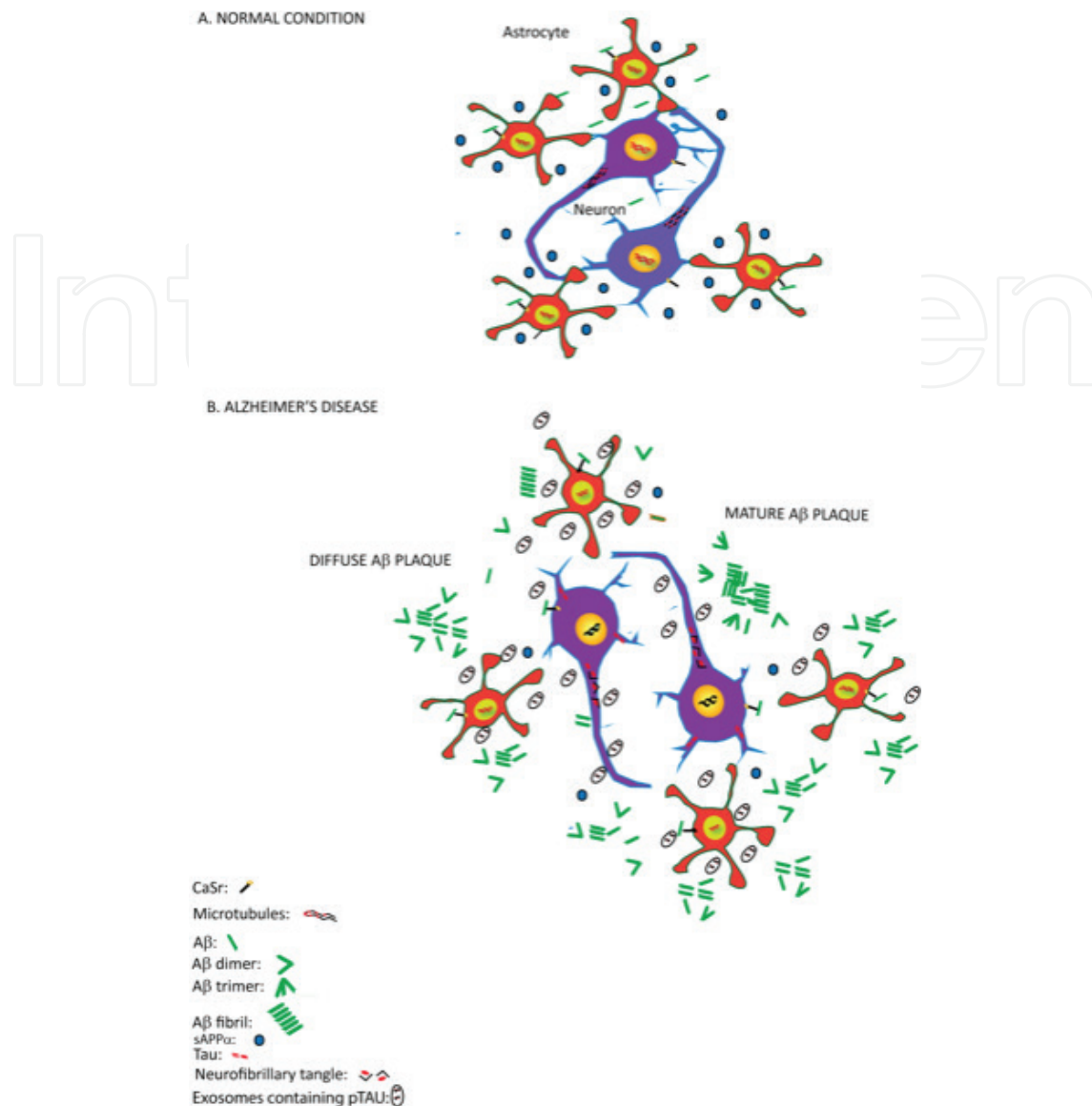
Figure 2. Cartoon depicting the calcilytic-suppressible differences between healthy and AD brain tissue brought about by Aβ-os·CaSR signaling. (A) Under healthy conditions, human astrocytes and neurons of the astrocyte-neuron gangs mutually interact to upkeep the environment's homeostasis. Via the prevailing nonamyloidogenic processing of APP the astrocytes release the neurotrophic and neuroprotective sAPPα, the synthesis of which obliterates any excess production and release of toxic Aβ-os and hence of Aβ fibrillar polymers. In parallel, multiple fully working clearance systems help keep the nontoxic but trophic $Aβ_{42}$ monomers at very low levels (not shown). The astrocytes release exosomes which enclose minimal amounts of p-Tau (not shown). No accumulation of Aβ-os or p-Tau-os occurs within the neurons and astrocytes. (B) Ongoing AD neuropathology involves several changes brought about by Aβ-os·CaSR signaling in the activated human astrocytes and neurons. The now prevailing amyloidogenic processing of APP leads to the overproduction of $Aβ_{42}$-os in both cell types at the expense of the NAP which severely curtails the extracellular shedding and beneficial activities of sAPPα. Thus $Aβ_{42}$-os both accumulate inside the cells and are oversecreted: this allows $Aβ_{42}$-os diffusion and interaction with the CaSRs of adjacent and far off neurons and astrocytes. This mechanism spreads the neuropathology promoting the progression of AD. Fibrillar polymers of oversecreted $Aβ_{42}$ also accumulate extracellularly giving raise to either diffuse or mature Aβ plaques, which can also release Aβ-os. Moreover, Aβ-os·CaSR signaling increases Tau protein phosphorylation by GSK-3β and the accumulation of p-Tau inside the astrocytes (and likely neurons). In the neurons, toxic p-Tau accumulates as NFTs, which cause severe dysfunctions. Moreover, the astrocytes release significantly increased amounts of p-Tau enclosed within membrane-bound exosomes, an activity which can aid a later emergence of the tauopathy. The Aβ-os·CaSR signaling also increases the production and release of NO and VEGF-A surpluses from the astrocytes [not shown]. Remarkably, administering a calcilytic-like NPS 2143 upkeeps the physiological condition shown in (A), thus disclosing its anti-AD therapeutic potential.

Given the relevance of the roles that the several upshots of the Aβ·CaSR-elicited signaling could have on the promotion of AD, we were enticed to test whether an allosteric highly specific CaSR antagonist [short-termed as *calcilytic*] could have any anti-AD therapeutic potential. Thus, we demonstrated that calcilytic NPS 2143 [87, 88] can persistently downregulate CaSR protein expression thus not only antagonizing but also curbing Aβ·CaSR signaling [44]. And the calcilytic enhances the proteolysis of endogenously amassing $Aβ_{42}$ by enhancing the 20S chymotrypsin-like activity of the proteasome. Moreover, NPS 2143 keeps down the Golgi/trans-Golgi network transport of endogenous $Aβ_{42}/Aβ_{42}$-os and, consequently, fully suppresses any oversecretion of the latter driven by the Aβ·CaSR signaling both in the case of NAHAs and of cortical human neurons [37, 44]. In addition, calcilytic NPS 2143 also wholly curbs the concurrent Aβ·CaSR signaling-elicited surplus production and secretion of NO and $VEGF\text{-}A_{165}$ from the NAHAs [37, 44, 55]. Conversely, the CaSR allosteric *agonist* or *calcimimetic* NPS R-568 enhances the surplus release of $Aβ_{42}/Aβ_{42}$-os, NO, and VEGF-A from the NAHAs confirming the positive involvement of the CaSR in these metabolic changes [44]. Also, NPS 2143 promotes APP and ADAM10 $α$-secretase translocation to the NAHAs plasma membrane, thereby restoring the extracellular shedding of neurotrophic and neuroprotective sAPP$α$ to nearly normal (i.e., untreated) levels. Yet, NPS 2143 does not change the concurrent APP's increased expression suggesting its promotion via mechanisms involving Aβs and other receptors but not the Aβ·CaSR signaling [76]. Notably, NPS 2143 remains beneficially effective even when a mixture of microglial proinflammatory cytokines is added to the Aβs treatment, indicating that a calcilytic could keep its beneficial effects even under AD-typical neuroinflammatory conditions [71]. Most important, NPS 2143 also fully suppresses the Aβ·CaSR-induced concurrent increase in GSK-3β activity and, consequently, the surges in both intracellular p-Tau/p-Tau-os levels and exosomal p-Tau/p-Tau-os release [83]. Extracellular vesicles, including exosomes, are likely to play both physiological and pathological roles in the CNS [86]. And, last but not least, adding NPS 2143 keeps the human cortical neurons *alive* and kicking notwithstanding the simultaneous presence of otherwise toxic levels of exogenous Aβs [44].

We wish to stress that these results could be gained by using untransformed human cortical adult astrocytes and postnatal neurons, *arguably the preclinical experimental models which at present are the closest one to human AD patients*. Our findings show that calcilytics terminate both stimulatory effects of pathological Aβ·CaSR signaling on $Aβ_{42}$ and p-Tau, the two AD's main drivers, surplus production and extracellular diffusion. On these grounds, we posit that such highly selective CaSR antagonists could effectively halt AD's progressive spread and preserve patients' cognition and life quality even when a neuroinflammation has already been ignited (**Figure 2**).

7. Conclusions and future perspectives

Mounting lines of evidence lend credence to the view that the human astrocytes—the characteristics of which remarkably differ from those of their rodent counterparts—play manifold roles in the molecular mechanisms associated with AD's pathophysiology. A growing accumulation of Aβs, p-Taues, NO, and VEGF-A hinges upon the signaling of Aβs·CaSR complexes. This initiates a self-spreading cascade of events which culminate in neuronal synaptic

disconnection, dysfunction, and death coupled with the oligodendrocyte dysfunction, axonal myelin sheaths damage and death, the activation of the microglia, the alteration of BBB permeability, and the expression of all the other neuropathological hallmarks of AD. The upshot is a concurrent degeneration of the gray and white matters. Thus, the CNS will keep shrinking; AD clinical symptoms will emerge and increase in intensity until the patients having lost their memories and cognitive abilities die. It is obvious that given their mounting numbers, the care of LOAD patients does heavily impact on their relatives and, for the huge costs of their assistance, on their National Health Services. In this disheartening scenario, the repurposing of highly specific CaSR antagonists or calcilytics as anti-AD therapeutics has the potential for shining a ray of hope.

Abbreviations

Aβs	amyloid-β peptides
AD	Alzheimer's disease
aMCI	amnestic minor cognitive impairment
APP	Aβ precursor protein
BBB	blood-brain barrier
BOLD	blood oxygen level-dependent
CaSR	calcium-sensing receptor
CNS	central nervous system
fMRI	functional magnetic resonance imaging
GPCRs	G-protein-coupled receptors
GSK-3β	glycogen synthase kinase-3β
LOAD	late-onset AD
NAHAs	normal (untransformed) adult human astrocytes
NFTs	neurofibrillary tangles
NO	nitric oxide
OLGs	oligodendrocytes
-os	oligomers
p-Tau	hyperphosphorylated Tau protein
VEGF	vascular endothelial growth factor

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