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Calcium-Sensing Receptors of Human Astrocyte-Neuron Teams: Amyloid-β-Driven Mediators and Therapeutic Targets of Alzheimer's Disease

I. Dal Prà[1], A. Chiarini[1], R. Pacchiana[1], E. Gardenal[1], B. Chakravarthy[2], J. F. Whitfield[2] and U. Armato[1,*]

[1]Histology & Embryology Section, Department of Life & Reproduction Sciences, University of Verona Medical School, Verona, Venetia, Italy; [2]National Research Council of Canada, Ottawa, Ontario, Canada

Abstract. It is generally assumed that the neuropathology of sporadic (late-onset or nonfamilial) Alzheimer's disease (AD) is driven by the overproduction and spreading of first Amyloid-β_{x-42} ($A\beta_{42}$) and later hyperphosphorylated (hp)-Tau oligomeric "infectious seeds". Hitherto, only neurons were held to make and spread both oligomer types; astrocytes would just remove debris. However, we have recently shown that exogenous fibrillar or soluble Aβ peptides specifically bind and activate the Ca^{2+}-sensing receptors (CaSRs) of untransformed human cortical adult astrocytes and postnatal neurons cultured *in vitro* driving them to produce, accrue, and secrete surplus endogenous $A\beta_{42}$. While the Aβ-exposed neurons start dying, astrocytes survive and keep oversecreting $A\beta_{42}$, nitric oxide (NO), and vascular endothelial growth factor (VEGF)-A. Thus astrocytes help neurons' demise. Moreover, we have found that a highly selective allosteric CaSR agonist ("*calcimimetic*"), NPS R-568, mimics the just mentioned neurotoxic actions triggered by Aβ•CaSR signaling. Contrariwise, and most important, NPS 2143, a highly selective allosteric CaSR antagonist ("*calcilytic*"), fully suppresses all the Aβ•CaSR signaling-driven noxious actions. Altogether our findings suggest that the progression of AD neuropathology is promoted by unceasingly repeating cycles of accruing exogenous $A\beta_{42}$ oligomers interacting with the CaSRs of swelling numbers of astrocyte-neuron teams thereby recruiting them to overrelease additional $A\beta_{42}$ oligomers, VEGF-A, and NO. Calcilytics would beneficially break such Aβ/CaSR-driven vicious cycles and hence halt or at least slow the otherwise unstoppable spreading of AD neuropathology.

Keywords: Alzheimer's disease, amyloid-beta oligomers, astrocyte-neuron teams, calcium-sensing receptor, calcilytics, calcimimetics.

PREAMBLE

Previous reviews from our Group have covered either the origins and diffusion of the "infectious Aβ and Tau seeds" advancing Alzheimer's disease (AD) or more restricted views on the links between the calcium-sensing receptor (CaSR) and the overproduction of nitric oxide (NO) by cortical normofunctioning adult human astrocytes (NAHAs) exposed to mixtures of microglial proinflammatory cytokines and/or to Amyloid (A)β peptides (Aβs) [1-4]. The present work is based on recently gained evidences [5,6] revealing the manifold critical roles the Aβ-binding CaSRs of human astrocyte-neuron teams play to promote AD progression and the possibility of effectively interfering with the pathological consequences of Aβ•CaSR signaling through the administration of selective allosteric CaSR antagonists ("*calcilytics*").

ALZHEIMER'S DISEASE SLOW CIRCUIT-BREAKING MARCH THROUGH THE BRAIN

Late-onset (sporadic or nonfamilial) Alzheimer's disease (AD) patients (about 95% of all AD cases) are older than 65 years of age, whereas the infrequent younger cases (*e.g.*, in their 40's) suffer from the early onset (or familial, *i.e.* genetic) form of AD [7,8]. AD most likely subclinically starts its ~30-year-long march through the brain in neurons of the parahippocampal region and the layer II of entorhinal cortex [8-10]. The neuropathology begins when such neurons groups increasingly lose their ability to rid themselves (*via* the action of proteases such as neprilysin or expulsion into the extracellular space and out of the brain) of the non-toxic Amyloid-β_{x-42} ($A\beta_{42}$) peptide monomers they produce during their physiological activity [11]. The accumulating $A\beta_{42}$ monomers rapidly aggregate into neurotoxic oligomers [12]. The $A\beta_{42}$ oligomers start the full cytopathological cycle leading to the production of as well neurotoxic hyperphosphorylated (hp)-Tau oligomers, which impair axonal transport and cause synaptic failure by damaging dendritic spines [13,14]. Alternatively, the hp-Tau oligomers can by themselves trigger the hp-Tau production and/or hp-Tau "infection" [9,15]. Thus, the long march of the AD neuropathology begins when $A\beta_{42}$ and hp-Tau oligomers, both "AD infectious seeds", are released from "infected" entorhinal neurons and disseminated to "infect" neurons of the dentate gyrus and the CA3 region of the memory-encoding and -retrieving hippocampal formation (reviewed in [1]). The neuropathology driven by the two kinds of "AD infectious seeds" slowly yet progressively spreads disconnecting neuronal circuits along the pathways going to

*Address correspondence to this author at the Histology & Embryology Section, Department of Life & Reproduction Sciences, University of Verona Medical School, Verona, Venetia, Italy; Tel/Fax: 0039-045-8027159; E-mail: uarmato@gmail.com

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the brain's upper areas of the cognition machinery. It leaves in its wake neurons jammed by neurofibrillary tangles (NFTs) and vulnerable to being further damaged if not killed by proinflammatory cytokines released from a microglia activated by a trail of extracellular $A\beta_{42}$ fibrillar deposits and their associated oligomers and of "ghost" NFTs [8,14,15]. Because of the limbic sites of origin and subsequent upward trajectory of the AD neuropathology, it is a person's increasingly failing memory and growing mental confusion that eventually attract the clinician's attention.

ASTROCYTE-NEURON TEAMWORKS AND AD

The conventional view of AD pathology is a "*neuronocentric*" one, in which the neurons are the producers, transmitters, and at the same time the victims of the $A\beta_{42}$ and hp-Tau toxic oligomeric "seeds" [16]. But what about the astrocytes, of which in the AD-targeted regions there are least as many as, or maybe even several fold more than, neurons [17-19]? Astrocytes have traditionally been regarded as neuron-supporting cells and/or janitorial bystanders working with the activated microglia to sweep up neuronal debris [20]. But by experimentally using cultures of functionally normal adult human astrocytes (NAHAs) isolated from the cerebral cortex we have recently gained evidences that send a new message, that is "*along with the neurons, the Aβ oligomer-exposed astrocytes become makers and spreaders of neurotoxic "AD infectious seeds" thereby remarkably advancing the transmission of the AD neuropathology"* [5,21] (Fig. **1**).

It is well established that each astrocyte can embrace several "client" neurons with its mobile processes to form a working team [22-24]. For example, a single human astrocyte domain in the hippocampus can contain from 27 x 10^4 to 2.0 x 10^6 synapses *vs*. the 2.0 x 10^4 - 12 x 10^4 synapses of a rodent astrocyte domain [25-28]. Thus, human cortical astrocytes have from 1.4-to-16.7-fold more chances to directly trade with their neuron partners a variety of factors influencing, amongst other processes, memory formation and retrieval. And to add to the impact of astrocytes on neuronal networks, several astrocytes may embrace the synapses of a single neuron. Under normal conditions, astrocytes induce synapses and stabilize their neuron partners' synapses by sweeping up glutamate and K^+ spillovers and by modulating signal transmission *via* so-called "*gliotransmitters*" [22-24,29-31]. Regional astrocyte heterogeneity may be crucial to refine neural circuits postnatally, *e.g.*, *via* the release of Semaphorin 3a (Sema3a) in the spinal cord [30,31]. An important consequence of this astrocyte-neuron physical and functional embrace is the astrocytes' ability to promote or reduce the release of neurotransmitters into the synapses they envelop with the Ca^{2+} they expel or take up during their Ca^{2+} waves [32]. Moreover, the working astrocyte-neuron teams are fueled with glucose and oxygen when signalers secreted from the astrocytes' vessel-touching end-feet open the arteriolar "taps" and increase the local blood flow [33].

But this constructive astrocyte-neuronal teamwork in the circuits of the cortical cognitive regions can become disruptive if aging or mutant neurons start producing, accruing, and releasing a surplus of Aβ peptides (Aβs). When neurons and the brain tissue become increasingly unable to get rid of the accumulating Aβ monomers, toxic Aβ oligomers are generated that trigger the formation, oligomerization, and release of pathological hp-Tau oligomers. Thus, neurons are likely to spill out both kinds of "AD infectious seeds" onto their astrocyte team partners [13] with consequences that are now beginning to emerge (Fig. **1**).

An example of the destructive potential of an $A\beta_{42}$-attacked astrocyte-neuron team is provided by Talantova *et al*. [34]. $A\beta_{42}$ oligomers released from a neuron can bind to its astrocyte partner's α-7-nicotinic acetylcholine receptors (α-7-nAChRs). Next, the signals from these receptors induce the astrocyte to exocytose the glutamate spillover from the neuronal synapses it has been sweeping up. The astrocyte-released glutamate activates the partner neuron's *extrasynaptic* glutamate N-methyl-D-aspartate receptors (NMDARs). This in turn triggers intraneuronal Ca^{2+} surges inducing a cascade of events that include dysfunctional mitochondria pumping out reactive oxygen species (ROS). In sequence, ROS inflict oxidative damage that destroys neuronal synapses thereby cutting communications between the client neurons of the astrocyte's network [14,34].

Amyotrophic lateral sclerosis (ALS) may be another example of astrocytes actually being in the driver's seat of a major motor neuron disease. Re *et al.* [35] set up a model system in which astrocytes isolated from sporadic ALS patients are co-cultured with human embryonic motor neuron stem cells. They found that the ALS astrocytes secreted neurotoxic factors that killed the motor neurons *via* necroptosis. In other words, as stated by Pirooznia *et al*. [36], the motor neurons in sporadic ALS may be surrounded by killer astrocytes, which therefore should be the targets of therapeutic efforts. Thus, it seems that astrocytes might be the sole drivers of sporadic ALS, while both astrocytes and neurons are the co-drivers of AD.

In addition, astrocytes can express enough prion protein (PrP^C) to support progressive neurodegeneration in prion disease [37] and are also likely to act essential parts in neurodevelopmental diseases such as Rett syndrome and fragile X mental retardation [30].

Some of the roles astrocytes play in AD were brought to light by our demonstration that when cultured NAHAs isolated from the cerebral cortex were exposed to exogenous Aβs, both their β-secretase (BACE1) and γ-secretase (γ-S) activities were stimulated to produce increased amounts of $A\beta_{42}$ out of the endogenous amyloid precursor protein (APP) [5,21]. The astrocytes then intracellularly accrued and oversecreted the endogenous oligomerizing $A\beta_{42}$ monomers into the culture medium together with a surplus of nitric oxide (NO) and vascular endothelial growth factor (VEGF)-A [5,6,38]. If *in vivo* surplus Aβ oligomers, VEGF-A, and NO are released from the astrocytes' end-feet, they will also impair the supply of oxygen and glucose by distorting and eventually destroying the local vasculature [39,40]. Thus can start the astrocyte-neuronal engine that widely disseminates cognition-destroying neurotoxic "AD infectious seeds" [13-15]. And notably, while the AD pathology is driven by the toxic secretions from both team members, it appears that human astrocytes are far stronger and abler to survive than

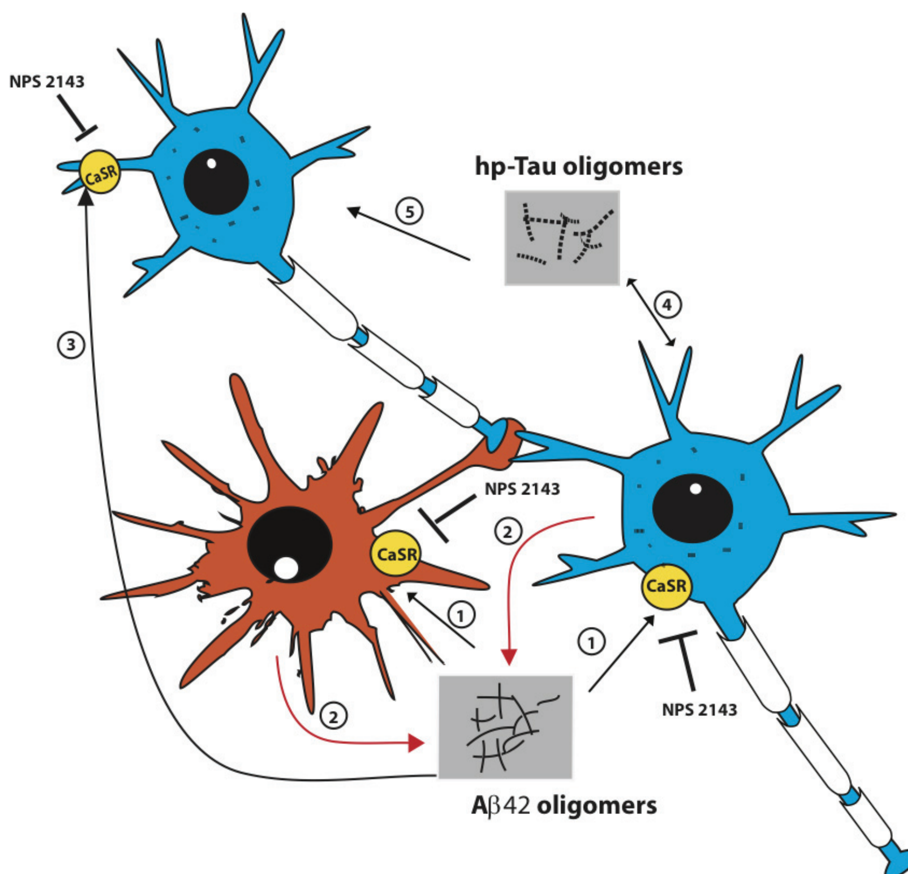

Fig. (1). The interactions between exogenous Aβs and the CaSRs located on the plasma membrane of astrocyte-neuron teams advance the extracellular release and spread of $A\beta_{42}$ and hp-Tau oligomers, the "AD infectious seeds". Two neurons and one astrocyte of the same team are schematically depicted here. The neuropathology begins when for aging-related causes $A\beta_{42}$ monomers accumulate in the extracellular space, oligomerize, and bind the CaSRs inserted in the plasma membranes of both cell types (1). The engendered Aβ•CaSR signaling induces the intracellular accrual (not shown) and oversecretion of *de novo* produced $A\beta_{42}$ monomers from both neurons and astrocytes (2). The $A\beta_{42}$ monomers oligomerize, spread, bind, and activate the CaSRs of a further neuron of the team (3). By doing this the $A\beta_{42}$ oligomers cause the additional release of surplus $A\beta_{42}$ moieties from this and other neurons (not shown). These vicious cycles can be unceasingly repeated and hence recruit ever-increasing numbers of astrocyte-neuron teams and thus inexorably advance the progression of AD. Hp-Tau oligomers formation is also triggered in the $A\beta_{42}$-exposed neurons by still unclear mechanisms that might be at least partially driven by pathological Aβ•CaSR signaling (4). Secreted hp-Tau oligomers are next either taken up by the secreting cells or transferred to contiguous neurons (and maybe astrocytes too) to hinder microtubule functions and help destroy synaptic spines (5). Once produced, hp-Tau oligomers are also capable of an independent self-induction and spreading. A highly selective allosteric CaSR antagonist (calcilytic) like NPS 2143 can completely suppress the manifold noxious effects driven by pathological $A\beta_{42}$•CaSR signaling both in neurons and in astrocytes thereby restoring conditions close if not identical to physiological ones [5,6]. Other relevant effects of the pathological $A\beta_{42}$•CaSR signaling, like the surplus production and secretion of NO and VEGF-A from the astrocytes, which are also suppressed by calcilytic NPS 2143 [6,38], and the extracellular accrual of $A\beta_{42}$ oligomers, which activate microglia, damage oligodendrocytes, and cause cerebral amyloid angiopathy, have been omitted from the picture for the sake of clarity.

their teams' client neurons because they appear to be unaffected by their several neurotoxic secretions. Consequently, as the astrocytes can keep producing and releasing their neurotoxins, they become the chief murderers of the neurons [5,6].

Thus, our *in vitro* observations strongly suggest that $A\beta_{42}$ released from aging or mutant human neurons partaking in astrocyte-neuron teams *in vivo* would likely stimulate the teams' astrocytes to make and secrete additional $A\beta_{42}$ oligomers. These would spread and recruit even more astrocyte-neuron teams to produce and secrete surplus $A\beta_{42}$ oligomers. The involved neurons would also synthesize and release hp-Tau oligomers (whether the astrocytes too play any part in this process remains to be ascertained). Therefore, both kinds of "AD seeds" would advance the inexorable progression of AD [5,13].

Here, the obvious question that arises is how to stop or at least significantly slow AD's development. Ideally, inhibiting the overproduction of the pivotal $A\beta_{42}$ and its toxic oligomers by both the astrocytes and neurons would halt the disease. But preventing the astrocytes and their client neuron partners from oversecreting their endogenous $A\beta_{42}$ oligomers could also effectively hinder AD spreading and progression (Fig. **1**).

Therefore, we set out to answer this question through the experimental use of NAHAs and human cortical postnatal (HCN-1A) neurons. This allowed us to find a way to stop the exogenous Aβ-treated NAHAs and neurons from oversecreting their endogenous $A\beta_{42}$ and concurrently the NAHAs from releasing toxic amounts of NO and VEGF-A [5,6,38]. The clue that led us to this discovery was the knowledge that Aβ oligomers bind several membrane receptors harbored by both astrocytes and neurons, namely those for ApoE, insulin, NMDA, cellular prion protein, advanced glycation end products, and in addition the p75 neurotropin receptor, α-7-nAChR, frizzled receptor, formyl peptide receptor-like 1, and the CaSR (see [5] for references). The CaSR was of particular interest to us, as we had previously shown that it is involved in the overproduction of NO by NAHAs exposed to a mixture of proinflammatory cytokines or to $A\beta_{25\text{-}35}$, an established proxy of $A\beta_{42}$ [5,41]. But before proceeding further it is worth recalling here the salient features of the CaSR to get a better glimpse of the complex roles it plays in AD.

STRUCTURE, SIGNALING, AGONISTS AND ANTAGONISTS OF THE CaSR

Structure

The CaSR belongs to family 3 of G protein-coupled receptors (GPCRs), also named seven transmembrane spanning receptors (7TMRs), and plays regulatory roles in Ca^{2+} homeostasis and cellular signaling [42]. It was first cloned from the bovine and later the human parathyroid gland [43,44]. In humans, the CaSR's structure is comprised of an aminoterminal extracellular domain (ECD; 612 amino acids (aa)), seven α-helical transmembrane domains (TMD with TM1-TM7; 250 aa), which are typical of the GPCRs, and an intracellular domain with a carboxyterminal tail (ICD; 216 aa). A cysteine rich domain (CysRD) links the ECD with TM1 and is important for signals transmission to the TMD upon binding of a ligand (Fig. **2**). The ECD's surface is glycosylated and can form CaSR•CaSR homeodimers *via* a disulphide-link involving Cys^{129} and Cys^{131} [45,46] or CaSR•mGluR1α heterodimers. The conformational arrangement of each monomer, determined by molecular modeling, indicates that the CaSR's ECD has a bilobed Venus flytrap (VFT)-like structure. The orthosteric Ca^{2+}-binding site is located between the two lobes of the VFT [46] (Fig. **2**). The cleft formed by the orthosteric Ca^{2+}-binding site is thought to be open when the agonist is missing and closed after binding the Ca^{2+} or any other orthosteric (type I) agonist. A second putative Ca^{2+}-binding site is placed in the TMD, because an ECD-lacking CaSR still responds to a Ca^{2+} signal [46]. A binding site for aromatic L-amino acids is located near the orthosteric Ca^{2+}-binding site. Several putative binding pockets are located in the seven extracellular loops of the TMD [47]. Ca^{2+} binding triggers changes in the conformational structure of the TMD and ICD that allow the ICD's carboxyterminal tail to interact with various G proteins (*e.g.*, $G_{11\alpha}$, $G_{i/o}$, $G_{q\alpha}$), which in turn mediate the activation/inhibition of manifold signaling pathways [48].

Signaling

CaSR signaling is triggered and modulated by several factors and the kind(s) of response(s) it evokes depend(s) on the cell type considered and the pathways involved. Different ligands besides Ca^{2+} trigger CaSR's activity, such as divalent and trivalent cations (*e.g.*, Mg^{2+}, Sr^{2+}, La^{3+}, Gd^{3+}), polycations (*e.g.*, polylysine, polyarginine), polyamines (spermine, spermidine, protamine), and aminoglycoside antibiotics (tobramycin, neomycin, gentamycin) [49-51]. These type I agonists bind the orthosteric Ca^{2+}-binding site even in concert with the Ca^{2+} itself. The levels of these agonists vary in the tissues expressing the CaSR; hence the signaling differs accordingly. In tissues where the Ca^{2+} abounds, like the bone, CaSR's activity is affected by the constitutive presence of high Ca^{2+} levels. On the other hand, the gastrointestinal mucosa cells are exposed to changeable Ca^{2+} concentrations depending on the variable composition of meals and their content of other cations [52,53]. Multivalent cationic proteins like Aβs also bind the CaSR [6,38,54,55] (Fig. **3B**). Moreover, CaSR's signaling activity is modulated by the extracellular pH and ionic strength, both of which alter the EC_{50} for Ca^{2+}, as they do in the kidney tubules [56,57]. CaSR signaling is affected also by factors modifying its level of mRNA and protein expression at a given Ca^{2+} concentration, *e.g.*, cellular proliferation or mitotic quiescence, and agents like interleukin (IL)-1β, IL-6, vitamin D3, and Ca^{2+} itself [39,58-60]. On the other hand, stromal derived factor (SDF)-1 and macrophage chemotactic protein (MCP)-1 and some ligands (see below) regulate CaSR's traffic from the endoplasmic reticulum to the plasma membrane [61]. Various studies have identified a panoply of G-protein-mediated CaSR signal transducing pathways besides the TRPC6-encoded Ca^{2+} channels. They include: (A) the inhibition of adenylyl cyclase preventing the synthesis of cyclic AMP (cAMP) [62]; (B) the activation of lipid kinases including several phospholipases with the production of *(i)* inositol triphosphate (IP3) eliciting the release of Ca^{2+} from intracellular stores [48]; and of *(ii)* diacyl glycerol (DAG) activating conventional protein kinase C (PKC) isoforms that phosphorylate at Ser^{133} the CREB transcription factor [63] and CaSR's Thr^{888} in the ICD that has been recognized as a critical negative regulator of CaSR signaling [64]; and (C) the stimulation of other protein kinases, *e.g.*, AKT, and filamin-regulated mitogen-activated protein kinases (MAPKs), including MEK, ERK, and JNK [41,55,56]. In turn, the various second messengers involved activate downstream-placed signaling cascades, which are likely to amplify the noxious effects of a pathological Aβ•CaSR signaling [41,48,65] (Fig. **2**).

CaSR's Allosteric Agonists (Calcimimetics) and Antagonists (Calcilytics)

The allosteric or type II and agonists and antagonists bind the CaSR at sites different from the orthosteric one and sensitize the receptor to activation by the Ca^{2+} in opposite directions. The best characterized classes of type II CaSR *agonists* are aromatic L-amino acids and two highly selective and CaSR-specific synthetic polyalkylamines, *i.e.* NPS R-568 and Cinacalcet HCl, which were named "*calcimimetics"* because

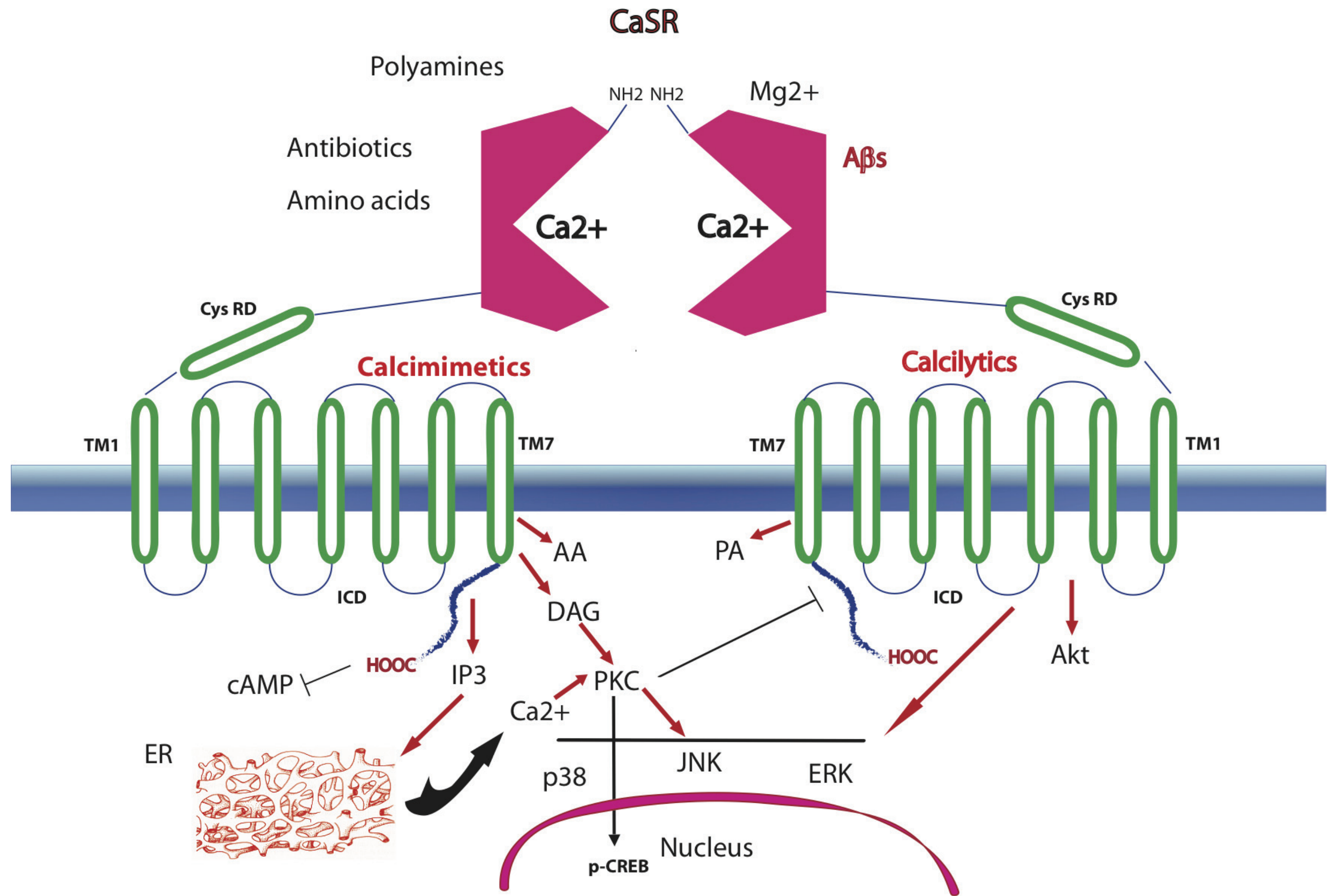

Fig. (2). A CaSR homeodimer inserted in the plasma membrane. Each extracellular domain (ECD) has a bilobed Venus fly-trap (VFT; enclosing between its lobes the orthosteric binding site of the Ca^{2+} or of divalent or trivalent cations, aminoglycoside antibiotics and polyamines. A cysteine-rich domain (CysRD) connects the VFT with the first (TM1) of the seven transmembrane helices, being important for signal transmission. Aromatic L-α-amino acids, calcimimetics, and calcilytics are among the allosteric (type II) CaSR modulators and bind overlapping but not identical sites of the receptor, *i.e.* calcilytics between TM3 and TM5, and calcilytics and calcimimetics between TM6 and TM7. The last transmembrane helix (TM7) is joined to the intracellular domain (ICD). Ligand binding changes the CaSR conformation (not shown), and causes the interaction of the ICD's tail with various G-proteins (not shown), which then activate/inhibit several signaling pathways (only a few of them are depicted here; see the text for more details and relevant references). By phosphorylating the ICD tail Ca^{2+}/diacyl glycerol (DAG)-activated protein kinase Cs (PKCs) attenuate the signaling of the ligand bound CaSR. AA, arachidonic acid; Akt, protein kinase B; cAMP, cyclic AMP; p-CREB, phosphorylated cAMP response element-binding protein; ER, endoplasmic reticulum; IP3, inositol triphosphate; p38, JNK, and ERK are members of the mitogen-activated protein kinases (MAPKs); PA, phospholipase A.

their effects mimic those elicited by extracellular Ca^{2+} [66,67]. Conversely, highly selective synthetic allosteric *antagonists* or "*calcilytics*", *i.e.* NPS 89636 and NPS 2143, diminish the CaSR response to Ca^{2+} and other type I agonists [66,67]. Indeed, *calcilytics*, which increase PTH secretion, and *calcimimetics*, which decrease PTH secretion, were first developed by NPS Pharmaceuticals [66,67]. As revealed by the results of point mutation studies, these quite interesting pharmacological agents bind to partially overlapping but not identical sites of the CaSR, the calcilytics between TM3 and TM5, and both the calcimimetics and calcilytics between TM6 and TM7 [68,69]. Calcimimetics are right now used to treat primary or secondary hyperparathyroidism conditions and to rescue loss-of function CaSR mutants. Conversely, calcilytics were initially meant (but till now not clinically used) to treat osteoporosis and may mitigate the effects of gain-of-function CaSR mutants [66-73]. Finally, it is worth recalling that the CaSR exhibits several distinct conformational states, each of which is induced and stabilized by a different ligand, allosteric agonists and antagonists included, and is linked to a particular set of intracellular signaling pathways—a property defined "*ligand-biased signaling*" [74]. And, it is the specific cell type considered that determines the preferential activation of a particular set of CaSR signaling pathways by the same ligand [75].

HUMAN ASTROCYTES, CaSR, AND AD

In recent years, a growing body of novel evidences has led to realize that human cortical astrocytes substantially differ from rodent ones. The former are much bulkier, emit

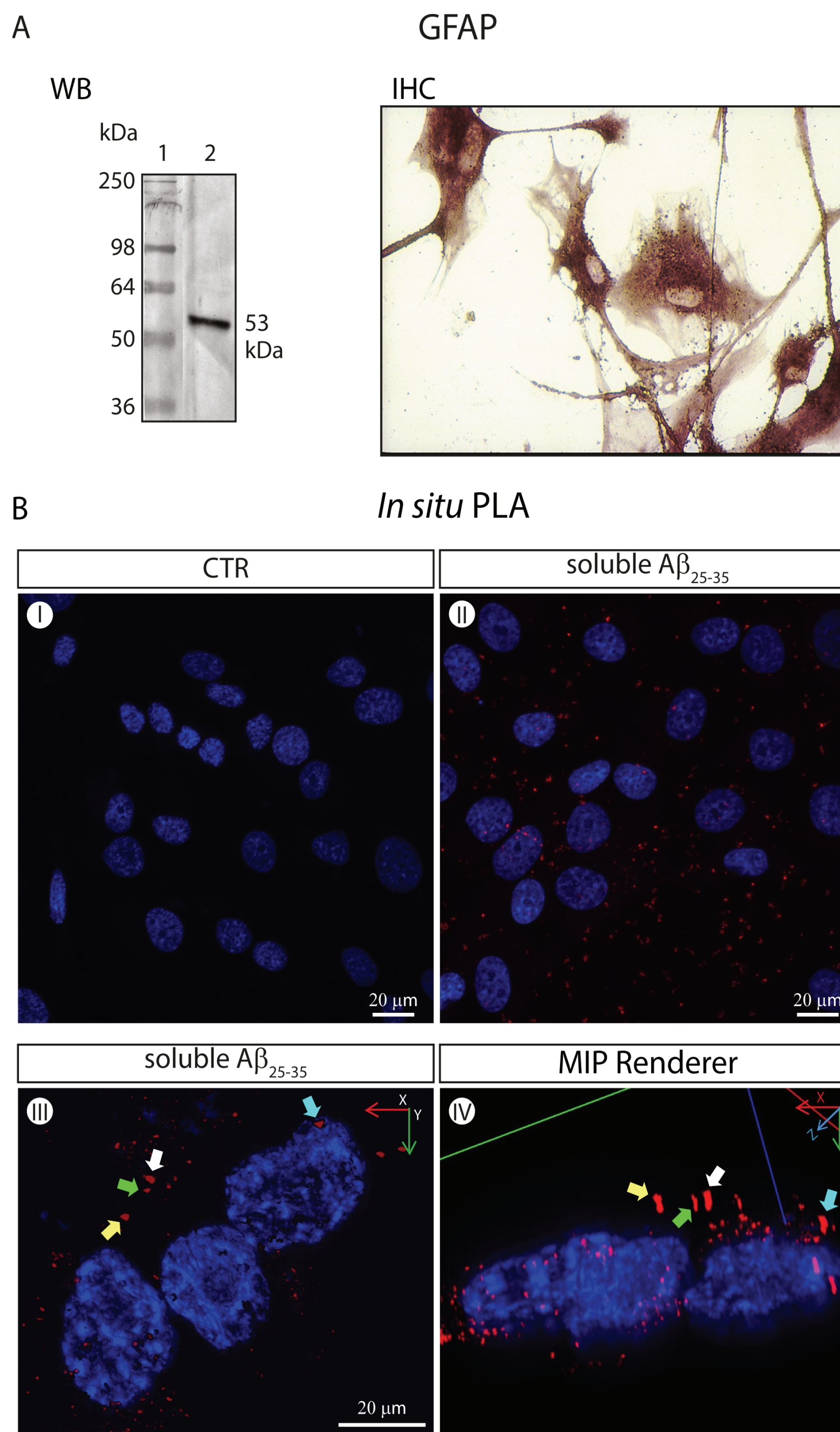

Fig. (3). The CaSRs of GFAP-expressing NAHAs form specific complexes with soluble A$\beta_{25\text{-}35}$ moieties visualized *via* the *in situ* PLA (*is*PLA) method. (**A**). Untransformed proliferatively quiescent NAHAs set in culture strongly express the GFAP marker. *WB*. GFAP Western immunoblotting was carried out according to Chiarini *et al.* [108] on NAHAs protein lysates using an anti-human GFAP rabbit polyclonal antiserum followed by an alkaline phosphatase (AP)-conjugated secondary antibody. Lane 1, molecular weight markers. Lane 2, specific GFAP protein band (53 kDa). *IHC.* GFAP immunohistochemistry on NAHAS using an anti-human GFAP mouse IgG1 followed by an AP-conjugated secondary antibody (for technical details see [108]). (**B**) Imaging of specific A$\beta_{25\text{-}35}$•CaSR complexes *via* the *in situ* Proximity Ligation Assay (*is*PLA) according to Söderberg *et al.* [105] and 3D digital renderings. Proliferatively quiescent NAHAs were exposed for 1 h at 37 °C to soluble *biotinylated* A$\beta_{25\text{-}35}$ (5.0 mM, Anaspec, CA, USA) dissolved in complete growth medium prior to fixation in 4 % v/v paraformaldehyde (PFA). Notably, *after fixation NAHAs were not permeabilized, which restricted the antigen-antibody interactions to the outside of their plasma membrane*. A mouse anti-CaSR monoclonal and a rabbit anti-biotin polyclonal were the two primary antibodies. The *PLUS* and *MINUS is*PLA probes were donkey anti-rabbit IgG (heavy + light chains) and donkey anti-mouse IgG (heavy + light chains),

Fig. (3). contd....

respectively (Olink Bioscience, Uppsala, Sweden). Once challenged with antibodies and *is*PLA probes, the samples were sequentially incubated with the *Duolink Hybridization Solution, Ligation Mix*, and *Amplification Mix* (all from Olink Bioscience) to produce single-stranded rolling-circle amplification products that were hybridized to oligonucleotide probes labeled with the red fluorophore *Tye624* (λ_{ex} = 594 nm and λ_{em} = 624 nm) by incubating with the *Duolink Detection Reagents* (Olink Bioscience). Next, nuclear DNA was stained with 1.0 µg ml^{-1} 4′,6-diamidino-2-phenylindole dihydrochloride (*DAPI*, Sigma-Aldrich). Pictures were acquired under a Leica TCS SP5 AOBS Laser confocal microscope (Leica-Microsystems, Wezlar, Germany). A 40X/1.25 NA oil-immersion objective (HCX PL APO 40x 1.25 OIL UV, Leica-Microsystem) was used. The *Maximum Intensity Projection* (*MIP*) renderings of original *is*PLA pictures were obtained using the Image Pro Plus 3D Viewer (Image-Pro Plus ™, version 7.0, Media Cybernetics, Bethesda, MD). (I) Untreated (*i.e.* not exposed to $A\beta_{25-35}$) NAHAS show no specific *is*PLA signal or background. Only the DAPI-stained nuclei are detectable. (II) $A\beta_{25-35}$-incubated NAHAs exhibit *is*PLA signals (*red dots*) corresponding to specific $A\beta_{25-35}$●CaSR complexes that are in part aggregating in patches. Nuclear DNA is blue (*DAPI*). (III) A higher magnification of $A\beta_{25-35}$-exposed adult human cortical astrocytes showing specific $A\beta_{25-35}$●CaSR complexes (*in red*) visualized *via* the *is*PLA method. Nuclear DNA is blue (*DAPI*). The complexes have started aggregating in patches, an event preceding their internalization. Some of the patches have been purposedly marked by differently colored arrows to allow the identification of the same patches in a different projection shown in panel (IV). (IV) The 3D MIP-rendering of the picture shown in panel (III) seen in an oblique lateral projection. The colored arrows indicate the same specific $A\beta_{25-35}$●CaSR complexes (*in red*) as in panel (III). The location of the $A\beta_{25-35}$●CaSR complexes at the periphery of the cytoplasm can be appreciated here somewhat better than in the top-bottom 2D image in panel (III).

10-fold more primary processes, exhibit novel morphological subtypes, *e.g.*, the interlaminar one, control broader synaptic domains, and are capable of performing more complex and intense functional tasks, *e.g.*, faster Ca^{2+} waves propagation, than the latter [22,24,76-83]. The new evolutionary features acquired by the astrocytes have significantly impacted on human brain physiology. This is indirectly confirmed by recent findings of Han *et al.* [84], who showed that human astrocytes engrafted on the brains of mice increase the learning abilities and activity-dependent plasticity of the animals. Obviously, astrocytes' evolutionary changes have also impacted on human neurodegenerative diseases, AD included. Perhaps, this is the reason why animal AD models fail to fully reproduce the human disease and why pharmacological findings gained in animal AD models cannot be successfully translated to human clinical settings [85-89]. Various authors had previously shown that astrocytes are able to engulf and degrade exogenously accruing Aβs [90,91]. However, more recent findings prove that astrocytes can also significantly contribute to the Aβs overload of an AD brain. In fact, unstimulated (control) adult human cortical astrocytes exhibit a discrete level of activity of β-site APP-cleaving enzyme 1/β-secretase (BACE1/β-S) and γ-secretase (γ-S), and under chronic stress or during AD or after an exposure to exogenous Aβs both these enzymatic activities surge remarkably thereby leading to the *de novo* production of larger amounts of Aβs [5,21,92].

From a neuropathological standpoint, a diffuse astrogliosis is detected in both AD-model animals [92] and postmortem human AD brains [93]. Generally, the astrocytes become hypertrophied, keep their spatial domains, and overexpress S100β and GFAP proteins while partially losing their complement of glutamate metabolizing enzymes [80,83,92]. Notably, an activation of the human astrocytes can be detected *via* Positron Emission Tomography (PET) after administering the inflammation-revealing tracer (11)C-D-deprenyl to mild cognitive impairment (MCI) patients, being more intense in them than in patients at later AD stages and in healthy individuals [94]. Alterations in astrocyte signaling as revealed by intercellular Ca^{2+} waves and synchronous hyperactivity were reported to occur in transgenic AD-model animals [95]. In addition, Aβ-exposed astrocytes exhibited increases in their intracellular Ca^{2+} levels, yet at variance with neurons they did not die [5,96]. Hence, as a Ca^{2+} dyshomeostasis befalls in the activated astrocytes of AD brains (reviewed in [97-99]), what role(s) would play the CaSRs jutting from the plasma membranes of the astrocytes and of their client neurons?

The CaSR is expressed ubiquitously in the brain, though more intensely in some regions, *e.g.*, the hippocampus, than in others [100]. In primary cultures of rat embryo brains, neurons and oligodendrocyte progenitor cells expressed CaSR's mRNA more intensely than astrocytes did. In addition, CaSR expression tended to decline from postnatal to adult age likely in relation to oligodendrocyte and astrocyte differentiation [101]. Chattopadhyay *et al.* [102] were the first to show that a functional CaSR is expressed in cultured human embryo astrocytes, besides human astrocytoma and meningioma cells. On their own part, Dal Prà *et al.* [41] showed that NAHAs cultured from surgical left-overs of the cerebral cortex also express a functional CaSR, at lower levels while proliferating and at higher ones while mitotically quiescent, levels that were little affected by the actual Ca^{2+} concentration in the medium.

In tissues like the brain, where the cells are not involved in the maintenance of systemic Ca^{2+} homeostasis, the extracellular Ca^{2+} acts as the first messenger to regulate through the CaSR a variety of cellular functions; *(i)* during the developmental stages, in which controls proliferation, migration, and differentiation of oligodendrocytes and neurons; *(ii)* in the postnatal life, in which modulates neurotransmission and synaptic plasticity [51,82,103]; and *(iii)* in the course of diseases affecting the central nervous system [104].

Concerning AD, Ye *et al.* [54] showed that exogenous Aβs bind and activate the CaSR causing the opening of a Ca^{2+}-permeable non-selective cation channel that elicited a sustained surge of intracellular Ca^{2+} in cultures of hippocampal pyramidal neurons from wild-type mice and

rats with resultant neuronal dysfunction. By contrast, exogenous Aβs could not evoke these intracellular Ca^{2+} surges in $CaSR^{-/-}$ mice [54]. In addition, exogenous Aβs activated the same cationic channel in human embryo kidney cells overexpressing the CaSR (HEK293-CaSR), but failed to do so in wild-type HEK293 cells [54]. Moreover, using a sensitive luciferase-reporter gene assay, Conley *et al*. [55] demonstrated that in CaSR-transfected Cos1 cells exogenous $A\beta_{1-42}$ activated CaSR signaling in a dose-dependent fashion. Recently, by applying to GFAP-expressing NAHAs in cultures (Fig. **3A**) the *in situ* Proximity Ligation Assay (*is*PLA) method, which reveals through a fluorescent sharp signal the very close (≤30 nm) and definite interaction between two proteins [105], we showed that soluble Aβ oligomers do *specifically* bind the CaSRs at the plasma membrane [5,6] (Fig. **3B**) to be subsequently endocytosed (unpublished observations). That exogenous Aβs not only *bind* but also *activate* the human astrocytes' CaSR is supported by our findings that *(i)* either soluble or fibrillar $A\beta_{25-35}$, an established $A\beta_{42}$ functional surrogate [5,106], stimulates the excess production, accrual, and release of endogenous $A\beta_{42}$ in the NAHAs and in human cortical postnatal HCN-1A neurons in parallel with the astrocytes' surplus synthesis and secretion of NO and VEGF-A [5,38]; *(ii)* a highly selective *calcimimetic*, NPS R-568 also stimulates the synthesis, accrual, and release of surplus $A\beta_{42}$ besides of NO and VEGF-A from the NAHAs thereby mimicking the effects of exogenous Aβs [5,6,38]; *(iii)* conversely, *in the presence of exogenous Aβs*, calcilytic NPS 2143 effectively blocks the oversecretion of $A\beta_{42}$ by the cortical astrocytes and neurons, and of NO and VEGF-A by the NAHAs [5,6,38]; and *(iv)* last but not least, calcilytic NPS 2143 fully preserves the viability of the Aβs-exposed human cortical postnatal HCN-1A neurons, which would otherwise have progressively died [5]. Therefore, the extracellularly accruing $A\beta_{42}$ oligomers do bind and activate the CaSRs of both members of the astrocyte-neuron teams. Thus, they excite the further release and spreading of a set of neurotoxins, including $A\beta_{42}$, NO, and VEGF-A, which would advance AD progression. Importantly, the addition of a calcilytic agent like NPS 2143 fully prevents these noxious effects driven by the pathological Aβ•CaSR signaling [5].

Interestingly, an exposure of the NAHAs to exogenous Aβ oligomers also induced by 48 hours a significant albeit transient increase in astrocytes' total CaSR proteins; under the same respect calcimimetic NPS R-568 was ineffective, calcilytic NPS 2143 given by itself caused an early, but transient decrease of the total CaSR protein, whereas in the presence of exogenous Aβs NPS 2143 elicited an intense and persistent fall of the total CaSR levels and likely of the Aβ•CaSR signaling intensity [5]. These are examples of alterations of the CaSR life cycle according to the bound agonist/antagonist that up- or downregulate CaSR availability and consequently its signaling intensity occurring in the NAHAs. Breitwieser [107] designated this recently identified mechanism as "*agonist-driven insertional signaling (ADIS)"* and our just mentioned findings support her suggestion that ADIS is likely to have therapeutic relevance. In addition, together with the lysosomes the proteasome helps reduce the availability of the total CaSR and the intracellular $A\beta_{42}$ accrual in the NAHAs since its 20S chymotrypsin-like activity is remarkably though transiently increased by calcilytic NPS 2143 in the presence of exogenous Aβs [5].

CONCLUSIONS

Our view that the CaSRs of human astrocyte-neuron teams play specific and relevant roles in the spreading and progression of the AD neuropathology is supported not only by their just mentioned ability to form specific complexes with Aβ oligomers [6], but also by the opposite effects of CaSR's highly selective synthetic allosteric agonists or antagonists [5,6,38]. Therefore, the suggestion of using allosteric CaSR antagonists (calcilytics) like NPS 2143 or similarly acting agents to hinder or at least remarkably slow the otherwise inexorable progression of AD stems from the experimental results gained through the use of cultured NAHAs and human cortical HCN-1A neurons [5,6,38,41]. Given the destructive effects of the disease on cortical neurons, it would be advisable to try CaSR antagonists, just like any other anti-AD candidate drug, on MCI or early post-MCI cases to assess their real therapeutic potential.

AUTHORS' CONTRIBUTIONS

All the authors contributed to the drafting of the manuscript and to the drawing of the figures. All the authors read and approved the final manuscript.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

aa	=	amino acid
$A\beta_{42}$	=	amyloid-β_{x-42}
Aβs	=	amyloid-β peptides
AD	=	Alzheimer's disease
ALS	=	amyotrophic lateral sclerosis
CaSR	=	Ca^{2+}-sensing receptor
ECD	=	extracellular domain
hp	=	hyperphosphorylated
ICD	=	intracellular domain
MCI	=	mild cognitive impairment
MIP	=	Maximum Intensity Projection
NAHA	=	normal adult human astrocyte
NMDAR	=	N-methyl-D-aspartate receptor
α-7-nAChR	=	α-7-nicotinic acetylcholine receptor
NO	=	nitric oxide
ROS	=	reactive oxygen species

TMD = transmembrane domain

VEGF = vascular endothelial growth factor

VFT = Venus fly-trap

REFERENCES

[1] Dal Prà, I.; Chiarini, A.; Gui, L.; Chakravarthy, B.; Pacchiana, R.; Gardenal, E.; Whitfield, J. F.; Armato, U. Do astrocytes collaborate with neurons in spreading the "infectious" Aβ and Tau drivers of Alzheimer's disease? *Neuroscientist*, **2014**, doi: 10.1177/ 1073858414529828.

[2] Armato, U.; Bonafini, C.; Chakravarthy, B.; Pacchiana, R.; Chiarini, A.; Whitfield, J. F.; Dal Prà, I. The calcium-sensing receptor: A novel Alzheimer's disease crucial target? *J. Neurol. Sci.* **2012**, *322*, 137-140. dx.doi.org/10.1016/j.jns.2012.07.031

[3] Chiarini, A.; Dal Prà, I., Marconi, M.; Chakravarthy, B.; Whitfield, J. F.; Armato, U. Calcium-sensing receptor (CaSR) in human brain's pathophysiology: roles in late-onset Alzheimer's disease (LOAD). *Curr. Pharm. Biotechnol.*, **2009**, *10*, 317-326. dx.doi.org/ 10.2174/138920109787847501

[4] Dal Prà, I.; Chiarini, A.; Pacchiana, R.; Chakravarthy, B.; Whitfield, J. F.; Armato, U. Emerging concepts of how β-amyloid proteins and pro-inflammatory cytokines might collaborate to produce an 'Alzheimer brain'. *Mol. Med. Rep.,* **2008**, *1*(2), 173-178. dx.doi.org/10.3892/mmr.1.2.173

[5] Armato, U.; Chiarini, A.; Chakravarthy, B.; Chioffi, F.; Pacchiana, R.; Colarusso, E.; Whitfield, J. F.; Dal Prà, I. Calcium-sensing receptor antagonist (calcilytic) NPS 2143 specifically blocks the increased secretion of endogenous Aβ42 prompted by exogenous fibrillary or soluble Aβ25-35 in human cortical astrocytes and neurons-Therapeutic relevance to Alzheimer's disease. *Biochim. Biophys. Acta*, **2013**, *1832*, 1634-1652. dx.doi.org/10.1016/j.bbadis.2013.04.020

[6] Dal Prà, I.; Armato, U.; Chioffi, F.; Pacchiana, R.; Whitfield, J. F.; Chakravarthy, B.; Gui, L.; Chiarini, A. The Aβ peptides-activated calcium-sensing receptor stimulates the production and secretion of vascular endothelial growth factor-A by normoxic adult human cortical astrocytes. *Neuromolecular Med.*, **2014**, doi: 10.1007/ s12017-014-8315-9. doi: 10.1007/s12017-014-8315-9

[7] Selkoe, D. J. Alzheimer's disease. *Cold Spring Harb. Perspect. Biol.*, **2011**, *3*(7), pii: a004457. doi:10.1101/ cshperspect.a004457. doi:10.1101/ cshperspect.a004457

[8] Braak, H.; Thal, D. R.; Ghebremedhin, E.; Del Tredici, K. Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. *J. Neuropathol. Exp.. Neurol.*, **2011**, *70*, 960-969. dx.doi.org/10.1097/nen.0b013e318232a379

[9] Braak, H.; Del Tredici, K. Where, when, and in what form does sporadic Alzheimer's disease begin? *Curr. Opin. Neurol.*, **2012**, *25*, 708-714. dx.doi.org/10.1097/wco.0b013e32835a3432

[10] Attems, J.; Thal, D. R.; Jellinger, K. A. The relationship between subcortical Tau pathology and Alzheimer's disease. *Biochem. Soc. Trans.*, **2012**, *40*, 711-715. dx.doi.org/10.1042/bst20120034

[11] Pluta, R. Unresolved questions concerning etiology of Alzheimer's disease: hypometabolism. *Nutrition*, **2011**, *27*(1), 1-2. doi: 10.1016/ j.nut.2010.07.010.

[12] Masters, C. L.; Selkoe, D. J. Biochemistry of amyloid β-protein and amyloid deposits in Alzheimer disease. *Cold Spring Harb. Perspect. Med.*, **2012**, *2*(6), a006262. doi:10.1101/cshperspect.a006262.

[13] Ittner, L. M.; Götz, J. Amyloid-β and Tau−a toxic *pas de deux* in Alzheimer's disease. *Nat. Rev. Neurosci.*, **2011**, *12*, 65-72. dx.doi. org/10.1038/nrn2967

[14] Klein, W. L. Synaptotoxic amyloid-β oligomers: A molecular basis for the cause, diagnosis, and treatment of Alzheimer's disease? *J. Alzheimer's Dis.*, **2013**, *33*, Suppl 1, S49-65. PMID: 22785404

[15] Ward, S. M.; Himmelstein, D. S.; Lancia, J. K.; Binder, L. I. Tau oligomers and Tau toxicity in neurodegenerative disease. *Biochem. Soc. Trans.*, **2012**, *40*, 667-671. dx.doi.org/10.1042/bst20120134

[16] Bero, A. W.; Yan, P.; Roh, J. H.; Cirrito, J. R.; Stewart, F. R.; Raichle, M. E.; Lee, J. M.; Holtzman, D. M. Neuronal activity regulates the regional vulnerability to amyloid-β deposition. *Nat. Neurosci.*, **2011**, *14*, 750-756. dx.doi.org/10.1038/nn.2801

[17] Araque, A.; Navarrete, M. Glial cells in neuronal network function. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, **2010**, *365*, 2375-2381. dx.doi.org/10.1098/rstb.2009.0313

[18] Giaume, C.; Koulakoff, A.; Roux, L.; Holcman, D.; Rouach, N. Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat. Rev. Neurosci.*, **2010**, *11*, 87-99. dx.doi.org/10.1038/ nrn2757

[19] Halassa, M. M.; Haydon, P. G. Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu. Rev. Physiol.*, **2010**, *72,* 335-355. dx.doi.org/10.1146/annurev-physiol-021909-135843

[20] Nagele, R. G.; Wegiel, J.; Venkataraman, V.; Imaki, H.; Wang, K. C.; Wegiel, J. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol. Aging*, **2004**, *25*, 663-674. dx.doi.org/10.1016/j.neurobiolaging.2004.01.007

[21] Dal Prà, I.; Whitfileld, J. F.; Pacchiana, R.; Bonafini, C.; Talacchi, A.; Chakravarthy, B.; Armato, U.; Chiarini, A. The amyloid-β(42) proxy, amyloid-β(25-35), induces normal human cerebral astrocytes to produce amyloid-β(42). *J. Alzheimers Dis.*, **2011**, *24*(2), 335-347. PMID: 21258151

[22] Verkhratsky, A.; Butt, A. *Glial Neurobiology, A textbook*; JohnWiley & Sons, Chichester, **2007**. dx.doi.org/10.1002/hup.954

[23] Kettenmann, H.; Ransom, B. R. *Neuroglia*; 3rd Edition, Oxford University Press, New York, **2013.** dx.doi.org/10.1126/science. 1222381

[24] Tsai, H. H.; Li, H.; Fuentealba, L. C.; Molofsky, A. V.; Taveira-Marques, R.; Zhuang, H.; Tenney, A.; Murnen, A. T.; Fancy, S. P.; Merkle, F.; Kessaris, N.; Alvarez-Buylla, A.; Richardson, W. D.; Rowitch, D. H. Regional astrocyte allocation regulates CNS synaptogenesis and repair. *Science*, **2012**, *337*(6092), 358-362. dx.doi.org/10.1126/science.1222381

[25] Bushong, E. A.; Martone, M. E.; Jones, Y. Z.; Ellisman, M. H. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J. Neurosci.*, **2002**, *22*, 183-192. PMID: 11756501

[26] Ogata, K.; Kosaka, T. Structural and quantitative analysis of astrocytes in the mouse hippocampus. *Neuroscience*, **2002**, *113*(1), 221-233. dx.doi.org/10.1016/s0306-4522(02)00041-6

[27] Chvátal, A.; Anderová, M.; Kirchhoff, F. Three-dimensional confocal morphometry−A new approach for studying dynamic changes in cell morphology in brain slices. *J. Anat.*, **2007**, *210*(6), 671-683. dx.doi.org/10.1111/j.1469-7580.2007.00724.x

[28] Halassa, M. M.; Fellin, T.; Takano, H.; Dong, J. H.; Haydon, P. G. Synaptic islands defined by the territory of a single astrocyte. *J. Neurosci*., **2007**, *27*(24), 6473-6477. dx.doi.org/10.1523/jneurosci. 1419-07.2007

[29] Stevens, B. Neuron-astrocyte signaling in the development and plasticity of neural circuits. *Neurosignals*, **2008**, *16*(4), 278-288. dx.doi.org/10.1159/000123038

[30] Molofsky, A. V.; Krencik, R.; Ullian, E. M.; Tsai, H. H.; Deneen, B.; Richardson, W. D.; Barres, B. A.; Rowitch, D. H. Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev.*, **2012**, *26*(9), 891-907. Erratum in: *Genes Dev.*, **2012**, *26*(13), 1508. dx.doi.org/10.1101/gad.188326.112

[31] Molofsky, A. V.; Kelley, K. W.; Tsai, H. H.; Redmond, S. A.; Chang, S. M.; Madireddy, L.; Chan, J. R.; Baranzini, S. E.; Ullian, E. M.; Rowitch, D. H. Astrocyte-encoded positional cues maintain sensorimotor circuit integrity. *Nature*, **2014**, *509*(7499), 189-194. dx.doi.org/10.1038/nature13161

[32] Antanitus, D. S. A theory of cortical neuron-astrocyte interaction. *Neuroscientist*, **1998**, *4*, 154-159. dx.doi.org/10.1177/1073858498 00400310

[33] Lovick, T. A.; Brown, L. A.; Key, B. J. Neuronal activity-related coupling in cortical arterioles: involvement of astrocyte-derived factors. *Exp. Physiol.*, **2005**, *90*(1), 131-140. dx.doi.org/10.1113/ expphysiol.2004.028811

[34] Talantova, M.; Sanz-Blasco, S.; Zhang, X.; Xia, P.; Akhtar, M. W.; Okamoto, S.; Dziewczapolski, G.; Nakamura, T.; Cao, G.; Pratt, A. E.; Kang, Y. J.; Tu, S.; Molokanova, E.; McKercher, S. R.; Hires, S. A.; Sason, H.; Stouffer, D. G.; Buczynski, M. W.; Solomon, J. P.; Michael, S.; Powers, E. T.; Kelly, J. W.; Roberts, A.; Tong, G.; Fang-Newmeyer, T.; Parker, J.; Holland, E. A.; Zhang, D.; Nakanishi, N.; Chen, H. S.; Wolosker, H.; Wang, Y.; Parsons, L. H.; Ambasudhan, R.; Masliah, E.; Heinemann, S. F.; Piña-Crespo, J. C.; Lipton, S. A. Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. *Proc. Natl. Acad. Sci. U S A*, **2013**, *110*(27), E2518-E2527. Correction in:

Proc. Natl. Acad. Sci. U S A, *110*(33), 13691. dx.doi.org/10.1073/pnas.1313546110

[35] Re, D. B.; Le Verche, V.; Yu, C.; Amoroso, M. W.; Politi, K. A.; Phani, S.; Ikiz, B.; Hoffmann, L.; Koolen, M.; Nagata, T.; Papadimitriou, D.; Nagy, P.; Mitsumoto, H.; Kariya, S.; Wichterle, H.; Henderson, C.E.; Przedborski, S. Necroptosis drives motor neuron death in models of both sporadic and familial ALS. *Neuron*, **2014**, *81*, 1001-1008. dx.doi.org/10.1016/j.neuron.2014.01.011

[36] Pirooznia, S. K.; Dawson, V. L.; Dawson, T. M. Motor neuron death in ALS: programmed by astrocytes? *Neuron*, **2014**, *81*, 961-963. dx.doi.org/10.1016/j.neuron.2014.02.024

[37] Raeber, A. J.; Race, R. E.; Brandner, S.; Priola, S. A.; Sailer, A.; Bessen, R. A.; Mucke, L.; Manson, J.; Aguzzi, A.; Oldstone, M. B.; Weissmann, C.; Chesebro, B. Astrocyte-specific expression of hamster prion protein (PrP) renders PrP knockout mice susceptible to hamster scrapie. *EMBO J*., **1997**, *16*(20), 6057-6065. dx.doi.org/10.1093/emboj/16.20.6057

[38] Chiarini, A.; Whitfield, J. F.; Bonafini, C.; Chakravarthy, B.; Armato, U.; Dal Prà, I. Amyloid-β(25-35), an amyloid-β(1-42) surrogate, and proinflammatory cytokines stimulate VEGF-A secretion by cultured, early passage, normoxic adult human cerebral astrocytes. *J. Alzheimers Dis*., **2010**, *21*(3), 915-926. PMID: 20634577

[39] Meyer, E. P.; Ulmann-Schuler, A.; Staufenbiel, M.; Krucker, T. Altered morphology and 3D architecture of brain vasculature in a mouse model for Alzheimer's disease. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*, 3587-3592. dx.doi.org/10.1073/pnas.0709788105

[40] Jantaratnotal, N.; Ryu, J. K.; Schwab, C.; McGeer, P. L.; McLarnon, J.G. Comparison of vascular perturbations in an Aβ-injected animal model and in AD brain. *Int. J. Alzheimers Dis.*, **2011**, *2011*, 918280. dx.doi.org/10.4061/2011/918280

[41] Dal Prà, I.; Chiarini, A.; Nemeth, E.F.; Armato, U.; Whitfield, J. F. Roles of Ca2+ and the Ca2+-sensing receptor (CaSR) in the expression of inducible NOS (nitric oxide synthase)-2 and its BH4 (tetrahydrobiopterin)-dependent activation in cytokine-stimulated adult human astrocytes. *J. Cell. Biochem.*, **2005**, *96*(2), 428-438. dx.doi.org/10.1002/jcb.20511

[42] Bräuner-Osborne, H.; Wellendorph, P.; Jensen, A. A. Structure, pharmacology and therapeutic prospects of family C G-protein coupled receptors. *Curr. Drug Targets*, **2007**, *8*(1), 169-184. dx.doi.org/10.2174/138945007779315614

[43] Brown, E. M.; Gamba, G.; Riccardi, D.; Lombardi, M.; Butters, R.; Kifor, O.; Sun, A.; Hediger, M. A.; Lytton, J.; Hebert, S. C. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*, **1993**, *366*(6455), 575-580. dx.doi.org/10.1038/366575a0

[44] Huang, Y.; Zhou, Y.; Yang, W.; Butters, R.; Lee, H. W.; Li, S.; Castiblanco, A.; Brown, E. M.;Yang, J. J. Identification and dissection of Ca(2+)-binding sites in the extracellular domain of Ca(2+)-sensing receptor. *J. Biol. Chem.*, **2007**, *282*(26), 19000-19010. dx.doi.org/10.1074/jbc.m701096200

[45] Garrett, J. E.; Capuano, I. V.; Hammerland, L.G.; Hung, B. C.; Brown, E. M.; Hebert, S. C.; Nemeth, E. F.; Fuller, F. Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J. Biol. Chem.*, **1995**, *270*(21), 12919-12925. dx.doi.org/10.1074/jbc.270.21.12919

[46] Hu, J.; Spiegel, A. M. Structure and function of the human calcium-sensing receptor: insights from natural and engineered mutations and allosteric modulators. *J. Cell. Mol. Med.*, **2007**, *11*(5), 908-922. dx.doi.org/10.1111/j.1582-4934.2007.00096.x

[47] Hu, J.; Reyes-Cruz, G.; Chen, W.; Jacobson, K. A.; Spiegel, A. M. Identification of acidic residues in the extracellular loops of the seven-transmembrane domain of the human Ca2+ receptor critical for response to Ca2+ and a positive allosteric modulator. *J. Biol. Chem.*, **2002**, *277*(48), 46622-46631. dx.doi.org/10.1074/jbc.m207100200

[48] Conigrave, A. D.; Ward, D. T. Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best Pract. Res. Clin. Endocrinol. Metab*., **2013**, *27*(3), 315-331. dx.doi.org/10.1016/j.beem.2013.05.010

[49] Brown, E. M., Fuleihan, G. El-H.; Chen, C. J.; Kifor, O. A comparison of the effects of divalent and trivalent cations on parathyroid hormone release, 3',5'-cyclic-adenosine monophosphate accumulation, and the levels of inositol phosphates in bovine parathyroid cells. *Endocrinology*, **1990**, *127*(3), 1064-1071. dx.doi.org/10.1210/endo-127-3-1064

[50] Brown, E. M.; Katz, C.; Butters, R.; Kifor, O. Polyarginine, polylysine, and protamine mimic the effects of high extracellular calcium concentrations on dispersed bovine parathyroid cells. *J. Bone Miner. Res*., **1991**, *6*(11), 1217-1225. dx.doi.org/10.1002/jbmr.5650061112

[51] Chakravarti, B.; Chattopadhyay, N.; Brown, E. M. Signaling through the extracellular calcium-sensing receptor (CaSR). *Adv. Exp. Med. Biol*., **2012**, *740*,103-142. dx.doi.org/10.1007/978-94-007-2888-2_5

[52] Hebert, S. C., Cheng, S., Geibel, J. Functions and roles of the extracellular Ca2+-sensing receptor in the gastrointestinal tract. *Cell Calcium,* **2004**, *35*(3), 239-247. dx.doi.org/10.1016/j.ceca.2003.10.015

[53] Brennan, S. C., Davies, T. S., Schepelmann, M., Riccardi, D. Emerging roles of the extracellular calcium-sensing receptor in nutrient sensing: control of taste modulation and intestinal hormone secretion. *Br. J. Nutr.,* **2014**, Jan 2, 1-7. [Epub ahead of print]. dx.doi.org/10.1017/s0007114513002250

[54] Ye, C.; Ho-Pao, C. L., Kanazirska, M.; Quinn, S.; Rogers, K.; Seidman, C. E.;Seidman, J. G.; Brown, E. M.; Vassilev, P. M. Amyloid-beta proteins activate Ca(2+)-permeable channels through calcium-sensing receptors. *J. Neurosci. Res.*, **1997**, *47*, 547-554. dx.doi.org/10.1002/(sici)1097-4547(19970301)47:5<547::aid-jnr10>3.0.co;2-v

[55] Conley, Y. P.; Mukherjee, A.; Kammerer, C.; DeKosky, S. T.; Kamboh, M. I.; Finegold, D. N.; Ferrell, R. E. Evidence supporting a role for the calcium-sensing receptor in Alzheimer disease. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.*, **2009**, *150B*(5), 703-709. dx.doi.org/10.1002/ajmg.b.30896

[56] Quinn, S. J.; Bai, M.; Brown, E. M. pH Sensing by the calcium-sensing receptor. *J. Biol. Chem*., **2004**, *279*(36), 37241-37249. dx.doi.org/10.1074/jbc.m404520200

[57] Quinn, S. J., Kifor, O.; Trivedi, S.; Diaz, R.; Vassilev, P.; Brown, E. Sodium and ionic strength sensing by the calcium receptor. *J. Biol. Chem.*, **1998**, *273*(31), 19579-19586. dx.doi.org/10.1074/jbc.273.31.19579

[58] Canaff, L.; Hendy, G. N. Human calcium-sensing receptor gene. Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1,25-dihydroxyvitamin D. *J. Biol. Chem.*, **2002**, *277*(33), 30337-30350. dx.doi.org/10.1074/jbc.m201804200

[59] Canaff, L.; Hendy, G. N. Calcium-sensing receptor gene transcription is up-regulated by the proinflammatory cytokine, interleukin-1beta. Role of the NF-kappaB pathway and kappaB elements. *J. Biol. Chem.*, **2005**, *280*(14), 14177-14188. dx.doi.org/10.1074/jbc.m408587200

[60] Canaff, L.; Zhou, X.; Hendy, G. N. The proinflammatory cytokine, interleukin-6 up-regulates calcium-sensing receptor gene transcription *via* Stat1/3 and Sp1/3. *J. Biol. Chem.*, **2008**, *283*(20), 13586-13600. dx.doi.org/10.1074/jbc.m708087200

[61] Breitwieser, G. E. The calcium sensing receptor life cycle: trafficking, cell surface expression, and degradation. *Best Pract. Res. Clin. Endocrinol. Metab.* **2013**, *27*(3), 303-313. dx.doi.org/10.1016/j.beem.2013.03.003

[62] Chang, W.; Pratt, S.; Chen, T. H.; Nemeth, E.; Huang, Z.; Shoback, D. Coupling of calcium receptors to inositol phosphate and cyclic AMP generation in mammalian cells and *Xenopus laevis* oocytes and immunodetection of receptor protein by region-specific antipeptide antisera. *J. Bone Miner. Res.,* **1998**, *13*(4), 570-580. dx.doi.org/10.1359/jbmr.1998.13.4.570

[63] Avlani, V. A.; Ma, W.; Mun, H. C.; Leach, K.; Delbridge, L.; Christopoulos, A.; Conigrave, A. D. Calcium-sensing receptor-dependent activation of CREB phosphorylation in HEK293 cells and human parathyroid cells. *Am. J. Physiol. Endocrinol. Metab.*, **2013**, *304*(10), E1097-E1104. dx.doi.org/10.1152/ajpendo.00054.2013

[64] Lazarus S, Pretorius CJ, Khafagi F, Campion KL, Brennan SC, Conigrave AD, Brown EM, Ward DT. A novel mutation of the primary protein kinase C phosphorylation site in the calcium-sensing receptor causes autosomal dominant hypocalcemia. *Eur. J. Endocrinol*., **2011**, *164*(3), 429-35. dx.doi.org/10.1530/eje-10-0907

[65] Chiarini, A.; Dal Prà, I.; Gottardo, R.; Bortolotti, F.; Whitfield, J. F.; Armato, U. BH(4) (tetrahydrobiopterin)-dependent activation, but not the expression, of inducible NOS (nitric oxide synthase)-2 in proinflammatory cytokine-stimulated, cultured normal human

astrocytes is mediated by MEK-ERK kinases. *J. Cell. Biochem.*, **2005**, *94*(4), 731-743. dx.doi.org/10.1002/jcb.20334

[66] Nemeth, E. F. Calcimimetic and calcilytic drugs: just for parathyroid cells? *Cell Calcium*, **2004**, *35*(3), 283-289. dx.doi.org/10.1016/j.ceca.2003.10.020

[67] Nemeth, E. F. Allosteric modulators of the extracellular calcium receptor. *Drug Discov. Today Technol*., **2013**, *10*(2), e277-e284. dx.doi.org/10.1016/j.ddtec.2012.11.002

[68] Miedlich, S. U.; Gama, L.; Seuwen, K.; Wolf, R. M.; Breitwieser, G. E. Homology modeling of the transmembrane domain of the human calcium sensing receptor and localization of an allosteric binding site. *J. Biol. Chem.*, **2004**, *279*(8), 7254-7263. dx.doi.org/10.1074/jbc.m307191200

[69] Petrel, C.; Kessler, A.; Dauban, P.; Dodd, R. H.; Rognan, D.; Ruat, M. Positive and negative allosteric modulators of the Ca2+-sensing receptor interact within overlapping but not identical binding sites in the transmembrane domain. *J. Biol. Chem.*, **2004**, *279*(18), 18990-18997. dx.doi.org/10.1074/jbc.m400724200

[70] Steddon, S. J.; Cunningham, J. Calcimimetics and calcilytics-fooling the calcium receptor. *Lancet*, **2005**, *365*(9478), 2237-2239. dx.doi.org/10.1016/s0140-6736(05)66782-7

[71] Letz, S.; Rus, R.; Haag, C.; Dörr, H. G.; Schnabel, D.; Möhlig, M.; Schulze, E.; Frank-Raue, K.; Raue, F.; Mayr, B.; Schöfl, C. Novel activating mutations of the calcium-sensing receptor: the calcilytic NPS-2143 mitigates excessive signal transduction of mutant receptors. *J. Clin. Endocrinol. Metab.*, **2010**, *95*(10), E229-E233. dx.doi.org/10.1210/jc.2010-0651

[72] White, E.; McKenna, J.; Cavanaugh, A.; Breitwieser, G. E. Pharmacochaperone-mediated rescue of calcium-sensing receptor loss-of-function mutants. *Mol. Endocrinol.*, **2009**, *23*(7), 1115-1123. dx.doi.org/10.1210/me.2009-0041

[73] Park, S. Y.; Mun, H. C.; Eom, Y. S.; Baek, H. L.; Jung, T. S.; Kim, C. H.; Hong, S.; Lee, S. Identification and characterization of D410E, a novel mutation in the loop 3 domain of CASR, in autosomal dominant hypocalcemia and a therapeutic approach using a novel calcilytic, AXT914. *Clin. Endocrinol. (Oxf)*, **2013**, *78*(5), 687-693. dx.doi.org/10.1111/cen.12056

[74] Davey, A. E.; Leach, K.; Valant, C.; Conigrave, A. D.; Sexton, P. M.; Christopoulos, A. Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology*, **2012**, *153*(3), 1232-1241. dx.doi.org/10.1210/en.2011-1426

[75] Leach, K.; Sexton, P. M.; Christopoulos, A.; Conigrave, A. D. Engendering biased signalling from the calcium-sensing receptor for the pharmacotherapy of diverse disorders. *Br. J. Pharmacol*., **2014**, *171*(5), 1142-1155. dx.doi.org/10.1111/bph.12420

[76] Sherwood, C. C.; Stimpson, C. D., Raghanti, M. A.; Wildman, D. E.; Uddin, M.; Grossman, L. I.; Goodman, M.; Redmond, J. C.; Bonar, C. J.; Erwin, J. M.; Hof, P. R.. Evolution of increased glia-neuron ratios in the human frontal cortex. *Proc. Natl. Acad. Sci. USA*, **2006**, *103*, 13606-13611. dx.doi.org/10.1073/pnas.0605843103

[77] Oberheim, N. A.; Wang, X.; Goldman, S.; Nedergaard, M. Astrocytic complexity distinguishes the human brain. *Trends Neurosci*., **2006**, *29*(10), 547-553. dx.doi.org/10.1016/j.tins.2006.08.004

[78] Oberheim, N. A.; Takano, T.; Han, X.; He, W.; Lin, J. H.; Wang, F.; Xu, Q.; Wyatt, J. D.; Pilcher, W.; Ojemann, J. G.; Ransom, B. R.; Goldman, S. A.; Nedergaard, M. Uniquely hominid features of adult human astrocytes. *J. Neurosci.* **2009**, *29*(10), 3276-3287. dx.doi.org/10.1523/jneurosci.4707-08.2009

[79] Oberheim, N. A.; Goldman, S. A.; Nedergaard, M. Heterogeneity of astrocytic form and function. *Methods Mol. Biol.*, **2012**, *814*, 23-45. dx.doi.org/10.1007/978-1-61779-452-0_3

[80] Verkhratsky, A.; Rodríguez, J. J.; Parpura, V. Astroglia in neurological diseases. *Future Neurol.*, **2013**, *8*(2), 149-158. dx.doi.org/10.2217/fnl.12.90

[81] Heneka, M. T.; Rodríguez, J. J.; Verkhratsky, A. Neuroglia in neurodegeneration. *Brain Res. Rev*., **2010**, *63*(1-2), 189-211. dx.doi.org/10.1016/j.brainresrev.2009.11.004

[82] Ruat, M.; Traiffort, E. Roles of the calcium sensing receptor in the central nervous system. *Best Pract. Res.Clin. Endocrinol. Metab.,* **2013**, *27*(3), 429-442. dx.doi.org/10.1016/j.beem.2013.03.001

[83] Verkhratsky, A.; Olabarria, M.; Noristani, H. N.; Yeh, C. Y.; Rodriguez, J. J. Astrocytes in Alzheimer's disease. *Neurotherapeutics,* **2010**, *7*(4), 399-412. dx.doi.org/10.1016/j.nurt.2010.05.017

[84] Han, X.; Chen, M.; Wang, F.; Windrem, M.; Wang, S.; Shanz, S.; Xu, Q.; Oberheim, N.A.; Bekar, L.; Betstadt, S.; Silva, A. J.; Takano, T.; Goldman, S. A.; Nedergaard, M. Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. *Cell Stem Cell*, **2013**, *12*(3), 342-353. dx.doi.org/10.1016/j.stem.2012.12.015

[85] Apelt, J.; Schliebs, R. Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain Res.*, **2001**, *894*(1), 21-30. dx.doi.org/10.1016/s0006-8993(00)03176-0

[86] Bacskai, B.J.; Kajdasz, S. T.; McLellan, M. E.; Games, D.; Seubert, P.; Schenk, D.; Hyman, B. T. Non-Fc-mediated mechanisms are involved in clearance of amyloid-beta *in vivo* by immunotherapy. *J. Neurosci.*, **2002**, *22*(18), 7873-7878. PMID:12223540

[87] Koistinaho, M.; Lin, S.; Wu, X.; Esterman, M.; Koger, D.; Hanson, J.; Higgs, R.; Liu, F.; Malkani, S.; Bales, K. R.; Paul, S. M. Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides. *Nat. Med.*, **2004**, 10(7), 719-726. dx.doi.org/10.1038/nm1058

[88] Pihlaja, R.; Koistinaho, J.; Malm, T.; Sikkilä, H.; Vainio, S.; Koistinaho, M. Transplanted astrocytes internalize deposited beta-amyloid peptides in a transgenic mouse model of Alzheimer's disease. *Glia*, **2008**, *56*(2), 154-163. dx.doi.org/10.1002/glia.20599

[89] Wyss-Coray, T.; Loike, J.D.; Brionne, T. C.; Lu, E.; Anankov, R.; Yan, F.; Silverstein, S. C.; Husemann, J. Adult mouse astrocytes degrade amyloid-beta *in vitro* and in situ. *Nat. Med.*, **2003**, *9*(4), 453-457. dx.doi.org/10.1038/nm838

[90] Heneka, M. T.; Sastre, M.; Dumitrescu-Ozimek, L.; Dewachter, I.; Walter, J.; Klockgether, T.; Van Leuven, F. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. *J. Neuroinflam.*, **2005**, *2*(1), 22. doi:10.1186/1742-2094-2-22

[91] Rossner, S.; Lange-Dohna, C.; Zeitschel, U.; Perez-Polo, J. R. Alzheimer's disease beta-secretase BACE1 is not a neurospecific enzyme. *J. Neurochem.*, **2005**, *92*(2), 226–234. dx.doi.org/10.1111/j.1471-4159.2004.02857.x

[92] Perez, J. L.; Carrero, I.; Gonzalo, P.; Arevalo-Serrano, J.; Sanz-Anquela, J. M.; Ortega, J.; Rodriguez, M.; Gonzalo-Ruiz, A. Soluble oligomeric forms of beta-amyloid (Abeta) peptide stimulate Abeta production *via* astrogliosis in the rat brain. *Exp. Neurol*., **2010**, *223*(2), 410-421. dx.doi.org/10.1016/j.expneurol.2009.10.013

[93] Overmyer, M.; Helisalmi, S.; Soininen, H.; Laakso, M.; Riekkinen P. Sr.; Alafuzoff, I. Astrogliosis and the ApoE genotype. an immunohistochemical study of postmortem human brain tissue. *Dement Geriatr Cogn Disord.,* **1999**, *10*(4), 252-257. dx.doi.org/10.1159/000017128

[94] Nordberg, A. Astroglia and microglia imaging markers in the progression of Alzheimer's disease. *13th Intl. Geneva/Springfield Symposium on Advances in Alzheimer Therapy*. **2014**, Abstract Book, p. 65. dx.doi.org/10.1016/j.neurobiolaging.2014.01.098

[95] Kuchibhotla, K. V.; Lattarulo, C. R.; Hyman, B. T.; Bacskai, B. J. Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. *Science*, **2009**, *323*(5918), 1211-1215. dx.doi.org/10.1126/science.1169096

[96] Abramov, A. Y.; Canevari, L.; Duchen, M. R. Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. *J. Neurosci.*, **2003**, *23*, 5088-5095. PMID: 12832532

[97] Bezprozvanny, I., Mattson, M. P. Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci.,* **2008**, *31*(9), 454-463. dx.doi.org/10.1016/j.tins.2008.06.005

[98] Mattson, M. P., Chan, S. L. Neuronal and glial calcium signaling in Alzheimer's disease. *Cell Calcium*, **2003**, *34*(4-5), 385-397. dx.doi.org/10.1016/s0143-4160(03)00128-3

[99] Berridge, M. J. Calcium regulation of neural rhythms, memory and Alzheimer's disease. *J. Physiol*., **2014**, *592*(Pt 2), 281-293. dx.doi.org/10.1113/jphysiol.2013.257527

[100] Yano, S.; Brown, E. M.; Chattopadhyay, N. Calcium-sensing receptor in the brain. *Cell Calcium*, **2004**, *35*(3), 257-264. dx.doi.org/10.1016/j.ceca.2003.10.008

[101] Chattopadhyay, N.; Espinosa-Jeffrey, A.; Tfelt-Hansen, J.; Yano, S.; Bandyopadhyay, S.; Brown, E. M.; de Vellis, J. Calcium receptor expression and function in oligodendrocyte commitment and lineage progression: potential impact on reduced myelin basic protein

in CaR-null mice. *J. Neurosci. Res*., **2008**, *86*(10), 2159-2167. dx.doi.org/10.1002/jnr.21662

[102] Chattopadhyay, N.; Evliyaoglu, C.; Heese, O.; Carroll, R.; Sanders, J.; Black, P.; Brown, E. M. Regulation of secretion of PTHrP by Ca(2+)-sensing receptor in human astrocytes, astrocytomas, and meningiomas. *Am. J. Physiol. Cell Physiol.*, **2000**, *279*(3), C691-C699. PMID: 10942719

[103] Bandyopadhyay, S.; Tfelt-Hansen, J.; Chattopadhyay, N. Diverse roles of extracellular calcium-sensing receptor in the central nervous system. *J. Neurosci. Res*., **2010**, *88*(10), 2073-2082. dx.doi.org/10.1002/jnr.22391

[104] Ward, B. K.; Magno, A. L.; Walsh, J. P.; Ratajczak, T. The role of the calcium-sensing receptor in human disease. *Clin. Biochem.*, **2012**, *45*(12), 943-953. dx.doi.org/10.1016/j.clinbiochem.2012.03.034

[105] Söderberg, O.; Gullberg, M.; Jarvius, M.; Ridderstråle, K.; Leuchowius, K-J.; Jarvius, J.; Wester, K.; Hydbring, P.; Bahram, F.; Larsson, L. G.; Landegren, U. Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Methods*, **2006**, *3*, 995-1000. dx.doi.org/10.1038/nmeth947

[106] Kaminsky, Y. G., Marlatt, M. W., Smith, M. A., Kosenko, E. A. Subcellular and metabolic examination of amyloid-beta peptides in Alzheimer disease pathogenesis: evidence for Abeta(25-35). *Exp. Neurol.,* **2010**, *221*(1), 26-37. dx.doi.org/10.1016/j.expneurol.2009.09.005

[107] Breitwieser, G. E. The intimate link between calcium sensing receptor trafficking and signaling: Implications for disorders of calcium homeostasis. *Mol. Endocrinol.*, **2012**, *26*, 1482-1495. dx.doi.org/10.1210/me.2011-1370

[108] Chiarini, A.; Dal Prà, I.; Menapace, L.; Pacchiana, R.; Whitfield, J. F.; Armato, U. Soluble amyloid beta-peptide and myelin basic protein strongly stimulate, alone and in synergism with combined proinflammatory cytokines, the expression of functional nitric oxide synthase-2 in normal adult human astrocytes. *Int. J. Mol. Med.*, **2005**, *16*(5), 801-807. dx.doi.org/10.3892/ijmm.16.5.801

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