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Calcium-sensing receptor antagonist (calcilytic) NPS 2143 specifically blocks the increased secretion of endogenous $A\beta_{42}$ prompted by exogenous fibrillary or soluble $A\beta_{25-35}$ in human cortical astrocytes and neurons—Therapeutic relevance to Alzheimer's disease

Ubaldo Armato [a,*], Anna Chiarini [a], Balu Chakravarthy [b], Franco Chioffi [c], Raffaella Pacchiana [a], Enzo Colarusso [c], James F. Whitfield [b], Ilaria Dal Prà [a]

[a] Histology and Embryology Section, Department of Life and Reproduction Sciences, University of Verona Medical School, 8 Strada Le Grazie, I-37134, Verona, Italy
[b] National Research Council of Canada, Montreal Road 51, Ottawa, Ontario, K1A-0N6, Canada
[c] Neurosurgery Unit, St. Chiara Hospital, Largo Medaglie D'Oro 9, I-38122, Trento, Italy

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ABSTRACT

The "*amyloid-β (Aβ) hypothesis*" posits that accumulating Aβ peptides (Aβs) produced by neurons cause Alzheimer's disease (AD). However, the Aβs contribution by the more numerous astrocytes remains undetermined. Previously we showed that fibrillar (f)$A\beta_{25-35}$, an $A\beta_{42}$ proxy, evokes a surplus endogenous $A\beta_{42}$ production/accumulation in cortical adult human astrocytes. Here, by using immunocytochemistry, immunoblotting, enzymatic assays, and highly sensitive sandwich ELISA kits, we investigated the effects of $fA\beta_{25-35}$ and soluble (s)$A\beta_{25-35}$ on $A\beta_{42}$ and $A\beta_{40}$ accumulation/secretion by human cortical astrocytes and HCN-1A neurons and, since the calcium-sensing receptor (CaSR) binds Aβs, their modulation by NPS 2143, a CaSR allosteric antagonist (calcilytic). The $fA\beta_{25-35}$-exposed astrocytes and surviving neurons produced, accumulated, and secreted increased amounts of $A\beta_{42}$, while $A\beta_{40}$ also accrued but its secretion was unchanged. Accordingly, secreted $A\beta_{42}/A\beta_{40}$ ratio values rose for astrocytes and neurons. While slightly enhancing $A\beta_{40}$ secretion by $fA\beta_{25-35}$-treated astrocytes, NPS 2143 specifically suppressed the $fA\beta_{25-35}$-elicited surges of endogenous $A\beta_{42}$ secretion by astrocytes and neurons. Therefore, NPS 2143 addition always kept $A\beta_{42}/A\beta_{40}$ values to baseline or lower levels. Mechanistically, NPS 2143 decreased total CaSR protein complement, transiently raised proteasomal chymotrypsin activity, and blocked excess NO production without affecting the ongoing increases in BACE1/β-secretase and γ-secretase activity in $fA\beta_{25-35}$-treated astrocytes. Compared to $fA\beta_{25-35}$, $sA\beta_{25-35}$ also stimulated $A\beta_{42}$ secretion by astrocytes and neurons and NPS 2143 specifically and wholly suppressed this effect. Therefore, since NPS 2143 thwarts any Aβ/CaSR-induced surplus secretion of endogenous $A\beta_{42}$ and hence further vicious cycles of Aβ self-induction/secretion/spreading, calcilytics might effectively prevent/stop the progression to full-blown AD.

1. Introduction

Human brain's healthy neurons constitutively make and during synaptic activity release the amyloid beta peptides (Aβs), which are cleavage products of the sequential activities of BACE1/β-secretase (β-S) and γ-S enzymes on the transmembrane Aβ precursor protein (APP) [1]. Two of the produced Aβ isoforms prevail, *i.e.* $A\beta_{40}$ (90%) and $A\beta_{42}$ (10%), the ratio of which changes in favor of $A\beta_{42}$ in Alzheimer's disease (AD) [2,3]. At its C-terminus $A\beta_{40}$ lacks two hydrophobic amino acids, Ala and Ile, which confer to $A\beta_{42}$ its greater aggregation, higher resistance to proteolysis, and more intense cytotoxicity [2,3]. Various mechanisms, which fail in AD, keep the extracellularly released Aβs at physiological (~pM) levels, including degradation by various proteases, ingestion by microglia, and transport into the blood via the α_2-macroglobulin receptor/low density

Abbreviations: Aβ, amyloid-β; Aβs, Aβ peptides; AD, Alzheimer's disease; AβDPs, Aβ-degrading proteases; APP, Aβ precursor protein; BACE 1, β-site APP-cleaving enzyme 1; β-S, β-secretase; CaSR, calcium-sensing receptor; fAβ, fibrillated Aβ; γ-S, γ-secretase; GCH-1, GTP cyclohydrolase-1; GPCRs, G-protein-coupled receptors; HCN, human cortical neurons; IFN-γ, interferon-γ; IL-1β, interleukin-1β; sAβ, soluble Aβ; LRP1, lipoprotein receptor-related protein 1; LOAD, late-onset AD; NFTs, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; $p75^{NTR}$, p75 neurotrophin receptor; RAGE, receptor for advanced glycation endproducts; NOS, nitric oxide synthase; TGN, *trans*-Golgi network; TNF-α, tumor necrosis factor-α

* Corresponding author. Tel./fax: +39 045 8027159.
E-mail addresses: uarmato@hotmail.com (U. Armato), anchiari@gmail.com (A. Chiarini), balu.chakravarthy@nrc-cnrc.gc.ca (B. Chakravarthy), franco.chioffi@apss.tn.it (F. Chioffi), raffaella.pacchiana@libero.it (R. Pacchiana), enzo.colarusso@apss.tn.it (E. Colarusso), pthosteo@rogers.com (J.F. Whitfield), ippdalpra@gmail.com (I. Dal Prà).

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lipoprotein receptor-related protein 1 (LRP1) (see for references [4]). At physiological pM concentrations Aβs preserve neuronal viability, hippocampal synaptic plasticity, and memory recording ability [5,6]. Since BACE1/β-S is more intensely expressed in neurons than in glial cells (*e.g.* astrocytes), brain neurons are deemed to be the main source of Aβs [1,2,7].

Conversely, pathological increases in brain tissue Aβs levels and the deposition of Aβ-containing senile plaques in the neuropil together with neurofibrillary tangles and chronic neuroinflammation are the main hallmarks of AD [2,4,8]. The intra- and extracellular accrual of Aβs may be due to *(i)* an Aβs overproduction driven by mutations of APP or presenilin 1 (PSEN1) or presenilin 2 (PSEN2) genes in the rare early-onset familial AD (EOFAD) or *(ii)* an ageing-related failure of Aβ-clearing mechanisms in the common late-onset AD (LOAD) [2,4,8]. During the slow (~5-to-15 years) progression of AD, accumulating Aβs target all types of nerve and cerebrovascular cells and elicit complex harmful effects, such as loss of synapses, NFTs, neurotoxicity, neurodegeneration, cerebrovascular damage, and proinflammatory astrocytes' and microglia's activation, all of which concur with a progressive loss of the patient's cognitive functions [1,4,9–11]. Aβ-activated microglia phagocytize fibrillar (f) and soluble (s)Aβs, favor Aβs fibrillation inside dense core senile plaques, and release, besides proinflammatory cytokines, nitric oxide (NO) and reactive oxygen species (ROS) leading to the formation of highly toxic peroxynitrite ($ONOO^-$) [2,4,9–11]. But astrocytes' roles in AD development should not be neglected as they are 5-to-10-fold more numerous than neurons, form wide gap-junctionally interconnected networks, are essential components of tripartite synapses, and tightly "cradle" neurons insulating them from external "chemical noise" while exchanging with them various physiologically essential compounds (reviewed in [12–14]). Such a physical and functional intimacy means that astrocytes may also be vulnerable to a direct attack by neuron-released and/or extracellularly accumulating Aβ oligomers and fibrils. So how might the astrocytes respond to these? According to one view, astrocytes are cellular janitors that sweep up the Aβs, which they take up via LRP1, LRP2/Megalin, and other receptors, accumulate, enzymatically degrade, and in dying release favouring the formation of smaller, GFAP-rich, cortical Aβ senile plaques [15]. Although initially beneficial, the astrocytes' engagement in clearing excess amounts of Aβs ends up competing with and hence impairing their neuron-supporting functions [12–17]. Moreover, $Aβ_{25–35}$-activated murine astrocytes produce and secrete apoptogenic prostate apoptosis response-4 (PAR-4) protein and ceramide-loaded exosomes ("apoxosomes") perhaps to kill, and thus protect themselves from, excess Aβs-releasing neurons [18]. Notwithstanding their huge numbers, the contribution of astrocytes' own Aβs production to the development of AD has received comparatively little consideration; but, were astrocytes' Aβs production even modestly intensified, it would hugely increase brain's Aβs load [19]. Blasko et al. [20] reported that human astrocytes produce $Aβ_{40}$ and $Aβ_{42}$ when stimulated by interferon (IFN)-γ + tumor necrosis factor (TNF)-α or interleukin (IL)-1β, all cytokines released from the Aβ-activated microglia within senile plaques [9–11]. Recently, Zhao et al. [21] reported that a TNF-α + IFN-γ mixture or $sAβ_{42}$ or $fAβ_{42}$ increased endogenous BACE1 and APP levels, and $Aβ_{40}$ secretion by activated primary astrocytes isolated from the cerebral cortex of neonatal mouse pups; they suggested that a feed-forward mechanisms would drive Aβ production in rodent astrocytes under neuroinflammatory conditions. And we showed that adding $fAβ_{25–35}$, a proxy retaining the main physical and biological features of $Aβ_{42}$ [22], stimulated untransformed adult human astrocytes isolated from fragments of the temporal lobe cerebral cortex to synthesize and accumulate large amounts of endogenous $Aβ_{42}$ and its oligomers [23]. The molecular mechanism(s) underlying this exogenous Aβ ⇒ endogenous Aβ induction in astrocytes remained to be elucidated: the interaction of exogenous Aβs with some component(s) located at the outer surface of the cells' plasma membrane seemed to be likely. It was also to be established whether this Aβ self-inducing feed-forward mechanism might operate in human neurons too, although this was reported to occur in cultures of dissociated rat cortical neurons and mouse hippocampal slices [24].

Several reports indicate that both sAβ oligomers and fAβ aggregates bind/activate a number of cell membrane receptors, like p75 neurotrophin receptor ($p75^{NTR}$) [25], Frizzled receptor [26], insulin receptor [27], N-methyl D-aspartate (NMDA) glutamate receptor [28], α7 nicotinic acetylcholine receptor (α7nAChR) [29], apolipoprotein E (APOE) receptors [30] scavenger receptor for advanced glycation endproducts (RAGE) [31], formyl peptide receptor-like 1 (FPRL1/2) [32], cellular prion protein (PrP^c) acting as an Aβ oligomer receptor [33], and the calcium-sensing receptor (CaSR) [34]. As none of such receptors exclusively binds Aβs, the existence and/or specificity of several of these Aβ/receptor interactions has been the object of controversy. But in some cases, the internalization of Aβ/receptor complexes resulted in intracellular accumulation of Aβs (reviewed in [35]). In other instances, specific Aβ/receptor complexes were formed, but could not be rapidly internalized due to the fibrils' mechanical hindrance: yet, the resulting persistent aberrant signalling from the Aβ-bound receptors profoundly altered neurons' critical functions with toxic and/or deadly outcomes, as for instance in SK-N-BE neuroblastoma cells engineered to over-express the *whole* $p75^{NTR}$ molecule [25]. In previous work, we showed that NPS 89686, a CaSR allosteric antagonist (calcilytic) [36], suppressed the MEK/ERK-mediated nitric oxide (NO) overproduction in adult human astrocytes exposed to a mixture of three cytokines (IFN-γ, TNF-α, and IL-1β) or to $sAβ_{40}$ [37,38] or $fAβ_{25–35}$ or $fAβ_{1–42}$ (unpublished; see the Results section) thereby indicating the occurrence of a specific Aβ/CaSR interaction. Therefore, we undertook to further investigate the roles of the CaSR in AD development.

The CaSR belongs to family C of G-protein-coupled receptors (GPCRs) (reviewed in [39]). Structurally, the CaSR's huge (~612 amino acids) extracellular N-terminal, the Venus Flytrap (VFT) domain, is joined via a cysteine-rich region to seven transmembrane α-helices (TM1–TM7) joined by extra- and intracellular loops (the 7TM region), while two domains of the intracellular C-terminal tail are crucial for CaSR's expression at the cell surface and its complex signalling activities [39]. Once inserted in the plasma membrane, the CaSR forms homodimers (CaSR/CaSR) or heterodimers (CaSR/mGluR1α) via disulfide-bonds and non-covalent interactions, even though it may function as a monomer [40]. CaSR dimerization also occurs at the endoplasmic reticulum (ER) being needed for its insertion into the plasma membrane [41]. The two huge VFT lobes of each CaSR homodimer bind orthosteric (type I) agonists like Ca^{2+} (the physiological ligand), several other divalent and trivalent cations, positively charged aminoglycoside antibiotics, and polyamines [39,42]. Extracellular Na^+ concentration and aromatic L-α-amino acids are allosteric (type II) CaSR agonists and bind various 7TM sites [43]. CaSR promptly detects and responds to changes in extracellular calcium concentration ($[Ca^{2+}]_e$) [44]. The binding of any orthosteric type I agonist turns the CaSR signalling on via a rearrangement the 7TM region, thereby letting the C-tails interact with various species of G proteins and activate a variety of intracellular signalling pathways involving second messenger-producing enzymes (e.g. adenylyl cyclase), lipid kinases (e.g. phospholipase C, D, A2), protein kinases (e.g. PKCs, MAPKs, AKT), Ca^{2+} influxes through TRPC6-encoded receptor-operated channels, and transcription factors (reviewed in [39,44]). Like other GPCRs, the CaSR exhibits the "*ligand-biased signalling*" feature, *i.e.* according to the ligand involved a specific CaSR signalling pathway may be preferred over the others [45]. Modulation of the CaSR's signalling has been related to gene expression control, MAPK (MEK/ERK and JNK) activations, cell proliferation, and apoptosis [44–46]. Most important, various phenylalkylamines endowed with aromatic rings and NH_3^+ groups have been synthesized and shown to act either as allosteric agonists (calcimimetics, e.g. NPS R-568) or antagonistis (calcilytics, e.g. NPS 89636, NPS 2143, etc.) as they are able to shift to the right or left, respectively, the CaSR's response curve to $[Ca^{2+}]_e$ changes (see for references

[36,46–48]). These CaSR allosteric modulators, which bind to a site in the 7TM region, have attracted great interest for their therapeutic potential in human ailments caused by CaSR mutations or malfunctioning, *e.g.* calcimimetics in various forms of hyperparathyroidism and calcilytics (possibly) in osteoporosis [47,48]. CaSR allosteric modulators may also induce a "*ligand-biased signalling*": in some models calcimimetics NPS R-568 and calcilytic NPS 2143 were reported to elicit a preference for intracellular calcium mobilization rather than MEK/ERK1/2 phosphorylation [45].

Notably, the CaSR is expressed all over in the brain, in which plays important physiological roles [49]. A role for the CaSR in AD pathogenesis was suggested first by the Aβ-induced degeneration of hippocampal neurons apparently linked to surges in cytosolic $[Ca^{2+}]_i$ [50], and then by the fAβ$_{25–35}$- and fAβ$_{1–40}$-elicited activation of Ca^{2+}-permeable non-selective cation channels increasing the $[Ca^{2+}]_i$ in the hippocampal neurons of wild type (WT) CaSR$^{+/+}$ rats but *not* of CaSR$^{-/-}$ rats. It should be recalled in this regard that, like polyamines, Aβs are endowed with orderly spaced arrays of positive charges [33]. Given the CaSR ubiquitous brain expression [49], not only hippocampal neurons but all types of human nervous cells and even cerebrovascular cells might be vulnerable to any cytotoxic effect elicited via a pathological Aβ/CaSR signalling. Hence, in this work we have investigated whether the exogenous Aβ-driven overproduction and accrual of endogenous Aβ$_{42}$ previously observed in human cortical astrocytes [23] might also occur in cultured human cortical HCN-1A neurons [51]; and whether exogenous f- or sAβs might also elicit an excess secretion of Aβ$_{42}$ and/or Aβ$_{40}$ on the part of either neurons or astrocytes or both. Concurrently, we have tested the hypothesis that, besides preventing NO overproduction induced by a mixture of three proinflammatory cytokines (IFN-γ, TNF-α, and IL-1β) [37] or fAβ$_{25–35}$ or fAβ$_{1–42}$ (see the Results section), a CaSR allosteric antagonist (in this instance, NPS 2143 [48]), might beneficially break the vicious cycle of exogenous Aβs inducing the production, accrual, and maybe secretion of endogenous Aβs by human cortical astrocytes and/or HCN-1A neurons. The findings herein reported reveal that the Aβ/CasR interaction elicits an excess secretion of Aβ$_{42}$ by both human astrocytes and HCN-1A neurons, and that CaSR antagonist NPS 2143 specifically suppresses the surplus release of endogenous Aβ$_{42}$ by the Aβ$_{25–35}$-exposed human astrocytes and HCN-1A neurons. Therefore, our findings denote for the first time a possible therapeutic benefit of this calcilytic agent in preventing vicious cycles of Aβs self-induction, over-secretion, and spreading, thereby halting the progression towards full-blown AD.

2. Materials and methods

2.1. Cell cultures

Untransformed human adult astrocytes were isolated from normal temporal cerebral cortex, cultured, and propagated as previously described [23]. Briefly, small fragments of temporal lobe cortices from five patients who had undergone perforating head injuries due to motorcycle accidents were dipped into MCDB 153 medium (Sigma-Aldrich, Milan, Italy), put into a Dewar flask at 4 °C and carried to the tissue culture laboratory. There they were further cut into tiny pieces, the cells in which were released by a mild treatment with 0.25% (w/v) trypsin (Eurobio, Les Ulis Cedex, France) in Hank's Basal Salt Solution (BSS; Eurobio) at 18 °C and triturated with a series of Pasteur pipettes of decreasing bores. The isolated cells were planted in culture flasks (BD Biosciences, Le Pont de Claix, France) containing a medium consisting of 89% (v/v) of a 1:1 mixture of DMEM/F-12 medium (AppliChem GmbH, Darmstadt, Germany), 10% (v/v) heat-inactivated (at 56 °C for 30 min) foetal bovine serum (FBS; BioWhittaker Europe, Belgium) and 1% (v/v) of a penicillin–streptomycin solution (Eurobio) and fibroblast growth factor-2 (FGF-2; 10 ng ml^{-1}, Sigma). This complete medium was replaced every 2–3 days. When the primary cultures reached 70% confluence (1–4 weeks), the cells were detached with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Eurobio) in Hank's BSS, split 1:4 and planted in new flasks. After the third subculture a homogeneous population of astrocytes obtained and FGF-2 addition was no longer needed. In these pure cultures, the cells only expressed astrocyte-specific markers such as glial fibrillary acid protein (GFAP) and glutamine synthase (GS). None of the cells expressed neuronal (enolase), oligodendrocytes (galactocerebroside), microglia (CD-68), or endothelial cells (factor VIII) markers. These astrocytes proliferated slowly in serum-enriched DMEM (AppliChem) medium and were by now phenotypically "locked-in". But the serum remained essential as its withdrawal triggered astrocytes' apoptosis. The proliferatively quiescent astrocyte in confluent cultures started cycling again when subcultured. At least 15–18 subcultures could be obtained over 2.5 years from a piece of normal cerebral cortex.

Human cortical nontumorigenic clonal strain, HCN-1A cell line (Catalog No. CRL-10442), originally derived from postnatal cortical tissue removed from a patient undergoing hemispherectomy for unilateral megalencephaly with intractable seizures [51], was obtained frozen from ATCC Cultures and Products via its partnership with LGC Standards S.r.l. (Sesto San Giovanni, Italy). Rapidly thawed 5×10^5 HCN-1A neurons (passage 14) were grown on 9 × 22-mm glass cover slips or 30-mm Petri dishes in 2–4 ml ATCC-formulated Dulbecco's modified Eagle's medium (DMEM) fortified with 10% FBS, 4.5 g L^{-1} glucose, 2.2 g L^{-1} $NaHCO_3$, and a pH of 7.35. This complete medium was half-replaced on alternate days. At 70% confluence (1–4 weeks after seeding), the cells were detached with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Eurobio) in Hank's BSS, split 1:3 or 1:2 and planted in cover slips or dishes. The already sluggish growth rate of HCN-1A cells (five-to-ten days for each cell duplication) tended to decelerate further at each subculture. Cell proliferation stopped at 80–90% confluence when ~50% of the neurons had grown multiple cytoplasmic processes of various lengths. HCN-1A neurons do not express GFAP, but express markers like neuron-specific enolase, neurofilament protein, and neurotransmitters like somatostatin (SST), GABA, glutamate, cholecystokinin-8 (CCK-8), and vasointestinal peptide (VIP) [51]. The experimental treatments and processing of HCN-1A neurons were alike those described for the astrocytes.

2.2. Cell numbers

Astrocytes' and HCN-1A neurons' numerical densities in the treated and untreated culture specimens were assessed under a phase contrast IM35™ inverted microscope (Zeiss, Germany) by counting, at 500× magnification, all of the nuclei present in 12 randomly chosen fields (each 29.400 μm^2 in area) per single dish. For each determination at least three separates per culture and four different cultures were examined. To avoid bias, the specimens were coded and counted by two researchers who were unaware of the key code, and the two results thus obtained for each sample group were pooled [52].

2.3. Aβ peptides

Aβ$_{25–35}$ and Aβ$_{1–42}$ (Bachem) were dissolved at 1.5 mM in PBS. Fibrillogenesis by Aβ$_{25–35}$ and Aβ$_{1–42}$ was (fast minutes) at room temperature and was checked *via* thioflavin-T tests before the experiments ([23] and unpublished data). Non fibrillar sAβ$_{25–35}$ was dissolved in DMSO at 4.0 mM (stock solution). The reversemer peptide Aβ$_{35–25}$ (Bachem) was dissolved in the same way as Aβ$_{25–35}$, but did not form fibrils.

2.4. Experimental protocol

Since normally astrocytes and neurons do not proliferate in the adult human brain, we employed them once they had reached mitotic quiescence. At experimental "0 h", some of such cultures served as

untreated controls while others had 20 μM of either fAβ_{25-35} or fAβ_{1-42} or reversemer Aβ_{35-25} added to their medium. The dose we used for the fAβs had been found to be optimal in previous studies [23,25,53]. sAβ_{25-35} was given daily in 2 μM doses dissolved in fresh medium. It must be stressed here that Aβ_{25-35}, an Aβ_{42} proxy, was used to specifically assess the production and secretion of the *endogenous* Aβ_{42} and Aβ_{40} peptides by the astrocytes and HCN-1A neurons (see also the Discussion section).

The CaSR allosteric antagonist (calcilytic) NPS 2143 hydrochloride (2-chloro-6-[(2R)-3-1,1-dimethyl-2-(2-naphtyl)ethylamino-2-hydroxy-propoxy]-benzonitrile hydrochloride; Tocris Bioscience, UK) [37,47,48] was dissolved in DMSO and next diluted in the growth medium at a final concentration of 100 nM. The CaSR allosteric agonist (calcimimetic) NPS R-568 ([(R)-N-(3-methoxy-(ÿ-phenylethyl)-3-(2-chlorophenyl)-1-propylamine hydrochloride], Tocris) [47] was also dissolved in DMSO and used at a final concentration of 1.0 μM. Starting at experimental "0 h" time and every 24 h thereafter astrocytes and HCN-1A neurons were first exposed for 30 min to either NPS 2143 or NPS R-568 dissolved in fresh medium; thereafter, fresh (at 0.5 h) or the previously cell-conditioned (at 24.5 and 48.5 h) media were added again to the cultures. In one set of experiments, MEK1/MEK2 inhibitor U0126 (10 μM; Tocris) was added for 30 min and next washed out just before fAβ_{25-35} addition to be administered again at 24 h and washed out at 24.5 h. Cultures and cell-conditioned media were sampled at 24 hourly intervals. Phosphoramidon (10 μM; Sigma), an inhibitor of thermolysin and other proteases, was added to the media at "0 h" experimental time.

2.5. Western immunoblotting (WB)

At selected time points, control and treated adult human astrocytes were scraped into cold PBS, sedimented at 200 ×*g* for 10 min, and homogenized in T–PER™ tissue protein extraction reagent (Pierce-Celbio, Milan, Italy) containing a complete EDTA-free protease inhibitor cocktail (Roche, Milan). The protein contents of the samples were assayed according to Bradford using BSA as standard. Equal amounts (10–30 μg) of protein from the samples were heat-denatured for 10 min at 70 °C in an appropriate volume of 1× NuPAGE LDS Sample Buffer supplemented with 1× NuPAGE Reducing Agent (Invitrogen, Milan). The samples were next loaded on NuPAGE Novex 4–12% Bis–Tris polyacrylamide gel (Invitrogen) (for CaSR and Aβ_{42} detection). After electrophoresis in NuPAGE MES SDS Running Buffers using the Xcell SureLock™ Mini*Cell (Invitrogen) (50-min runtime at 200 V constant), proteins were blotted onto nitrocellulose membranes (0.2 μm; Pall Life Sciences, Milan). To ensure efficient and reproducible binding to the membrane, transfer proceeded under low power conditions (30 V constant) for 1 h in 1× NuPAGE transfer buffer containing 10% methanol. Immunoblots were performed using the SNAP i.d. protein detection system (Merck-Millipore, Milan). The membranes were probed with: *(i)* the specific mouse monoclonal 12F4 antibody against Aβ_{42} raised using as immunogen a peptide corresponding to the C-terminal amino acids 37–42 of Aβ_{42} coupled to Keyhole limpet hemocyanin (KLH), an *in vivo* immunostimulant, through the addition of an N-terminal cysteine (Merck-Millipore); before use it was diluted 1:300 according to the seller's documentation; 12F4 antibody cross-reacts with neither Aβ_{1-43} nor Aβ_{1-40} nor Aβ_{25-35} [23]; *(ii)* the specific mouse monoclonal HL1499 antibody against the CaSR (Sigma), raised using as an *in vivo* immunogen a peptide corresponding to amino-acids 15–29 of the CaSR extracellular N-terminus; it was used at a final 3.0 μg ml^{-1} as previously detailed [23]; *(iii)* mouse monoclonal 5C3 anti-Aβ_{40} antibody (Acris, Germany), raised using as an immunogen a C-terminal peptide conjugated with KLH, does not cross-react with either Aβ_{42} or Aβ_{25-35} as we ourselves confirmed via gel electrophoresis and immunoblotting (not shown); 5C3 antibody was used at 1.0 μg ml^{-1}; *(iv)* an antibody against lamin B1 (C-20, Santa Cruz Biotechnology, Germany) was used at 1.0 μg ml^{-1} to assess the loading controls. The integrated intensities of the bands specific for each protein of interest were assessed using the Sigmagel™ software package (Jandel Corp., Erkrath, Germany).

2.6. Fluorescence microscopy

Control and treated HCN-1A neurons were fixed for 20 min with 4% paraformaldehyde (PAF) at room temperature. Next, cells were washed with Tris buffered saline (TBS—0.05 M L^{-1} Tris base, 9 g/L NaCl, pH 8.4), four times for 5-min each time, and then permeabilized with 0.05% Triton X-100 in TBS. To saturate unspecific binding sites, cells were incubated for 1 h at room temperature with a blocking solution containing 2.0% FBS, 2.0% BSA, and 0.02% Triton X-100 in TBS (TBS-TX). Immunofluorescence staining for Aβ_{42} and Aβ_{40} was done with the respective mouse monoclonals: anti-Aβ_{42} (8G7, 1.0 μg ml^{-1}; Acris), and anti-Aβ_{40} (5C3; 5.0 μg ml^{-1}; Acris). After three washes with TBS-TX (four changes in 10 min), the cells were incubated with the secondary antibody, chicken anti-mouse Alexa Fluor 488 used at 1.0 μg ml^{-1} (Molecular Probes, Invitrogen). This was followed by 10 min incubation with 1.0 ng ml^{-1} of 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) to stain DNA. Coverslips were mounted in anti-bleaching medium (Dabco, Sigma-Aldrich). The negative control procedure omitted the primary antibody. Confocal microscopy analysis was performed on a Leica TCS SP5 AOBS confocal microscope (Leica-Microsystems) equipped with violet (405 nm laser), blue (Argon, 488 nm), orange (543 nm), and red (633 nm, HeNe laser) excitation laser line. A 63×/1.40 NA oil-immersion objective (PL APO 63×, Leica-Microsystem, Germany) was employed for the specimens' analysis.

2.7. Assays of secretases (S) activities

Untreated and treated adult human astrocytes were harvested with a cell scraper, pelleted, and washed once in ice-cold PBS. They were next re-suspended in 20 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM EGTA, and a complete protease inhibitor mixture (Roche), and homogenized using a Dounce homogenizer. Cell lysates were centrifuged at 800 ×*g* for 10 min to remove nuclei and cell debris and the resulting supernatant was stored at −80 °C until needed. The respective activities of β-site APP-cleaving enzyme (BACE1)/β-S and of γ-S were measured in cell lysates (30–50 μg) and the results were expressed as specific activity (means ± SEMs of ΔF μg^{-1} protein pertaining to each experimental group) (for further details see [23]). To establish the specificity of the observed γ-S activity additional parallel control assays were carried out using the γ-S inhibitor XII (Z–IL–CHO) (8.5 μM; Calbiochem-Millipore).

2.8. Assays of proteasomal proteolytic activities

They were performed according to Beyette et al. [54]. Astrocytes' lysates (10 μg) were incubated at 37 °C with the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC; 50 μM), t-Butoxycarbonyl-Leu-Arg-Arg-AMC (Boc–LRR-AMC; 100 μM), and Benzyloxycarbonyl-Leu-Leu-Glu-βNaphthylamide (Z–LLE-βNA; 400 μM) (Enzo Life Sciences AG, Switzerland). The results were expressed as specific activity (means ± SEMs of ΔF μg^{-1} protein pertaining to each experimental group).

2.9. Assay of NO released into cell-conditioned growth media

The secreted amount of NO was deduced from the concentrations of NO's two stable oxidation products, NO_2^- and NO_3^-, in the media. The fluorometric method we used is based upon the reaction of NO_2^- with 2,3-diamino-naphthalene (DAN; Sigma-Aldrich) to form the fluorescent 1-(H)-naphthotriazole [37,38,55]. Nitrates in the medium were reduced

to NO_2^- by incubation for 30 min with nitrate reductase (0.1 U ml^{-1}; Roche, Milan) in the presence of 10 mM FAD and 100 mM NADPH. Any residual NADPH was then oxidized with lactate dehydrogenase (10 U ml^{-1}) in the presence of 10 mM sodium pyruvate. The total NO_2^- concentration was then determined in 50 μl samples of the culture supernatants that were brought up to 100 μl with doubly deionized water. Freshly prepared DAN (10 ml of a 0.05 mg ml^{-1} solution in 0.62 M HCl) was added to these diluted samples, and after a 10 min incubation at 20 °C in the dark, the reaction was stopped with 5 μl of 2.8 N NaOH. The amount of 1-(H)-naphthotriazole formed was measured fluorometrically at excitation and emission wavelengths of 365 nm and 450 nm, respectively. Samples of fresh culture medium were used as blanks to correct for background NO_2^- and NO_3^- concentrations in the medium (~7 μM). NO_2^- concentrations were calculated from a standard curve using $NaNO_2$.

2.10. Enzyme-linked immunosorbant assays (ELISAs) of secreted $A\beta_{42}$ and $A\beta_{40}$ in cell-conditioned growth media samples

Quantifications of $A\beta_{42}$ and $A\beta_{40}$ released into in cell-conditioned growth media samples were carried out by means of specific Human/Rat ELISA Kits, High-Sensitive (both from Wako, Japan). The $A\beta_{42}$ kit has a dynamic range of 0.1–20.0 pmol L^{-1} and a sensitivity of 0.024 pmol L^{-1}. This kit uses the monoclonal antibody BNT77, the epitope of which is $A\beta_{11-28}$ and the monoclonal antibody BC05, which specifically detects the C-terminal portion of $A\beta_{x-42}$. The Wako $A\beta_{42}$ ELISA kit is 700-fold more sensitive than the SIG-38956 ELISA kit (Covance) we previously used [23]. The $A\beta_{40}$ kit has a dynamic range of 1.0–100 pmol L^{-1} and a sensitivity of 0.049 pmol L^{-1}. This kit uses the monoclonal antibody BNT77 (*v. supra*) and the monoclonal antibody BA27, which specifically detects the C-terminal portion of $A\beta_{x-40}$. Briefly, the astrocytes' and HCN-1A neurons' conditioned media samples were added with a protease inhibitor cocktail (Roche) and centrifuged for 10 min at 13,000 rpm to remove any cellular debris. Supernatants were tested in triplicate according to the manufacturer's protocol.

2.11. Statistical analysis

The data were analyzed using SigmaStat 3.5™ Advisory Statistics for Scientists (Systat Software). For immunoblotting, bands' densitometric data were normalized to corresponding loading control (lamin B1) bands and next analyzed by one-way ANOVA. When the ANOVA's upshot was significant ($p < 0.05$), *post hoc* Holm–Sidak's test was used for comparisons vs. 0 h (untreated controls) values. Null hypotheses were rejected when $p > 0.05$.

3. Results

3.1. Total CaSR protein expression is modulated by exogenous $fA\beta_{25-35}$ + calcilytic NPS 2143 or by NPS 2143 alone but is not changed by calcimimetic NPS R-568 alone in adult human astrocytes

Since the CaSR is expressed by adult human astrocytes [37,38] and is an Aβs target [34,38], we asked whether a $fA\beta_{25-35}$-triggered CaSR signalling may contribute to the $fA\beta_{25-35}$-elicited stimulation of endogenous $A\beta_{42}$ production, and intracellular accrual we previously reported [23]. As a first step, we checked whether, as suggested by the work of Huang and Breitweiser [56], a calcilytic by itself or added to $fA\beta_{25-35}$ or a calcimimetic alone might affect total CaSR protein expression.

The CaSR protein was discretely expressed in the untreated astrocytes, and its total amount transiently increased peaking at 48 h

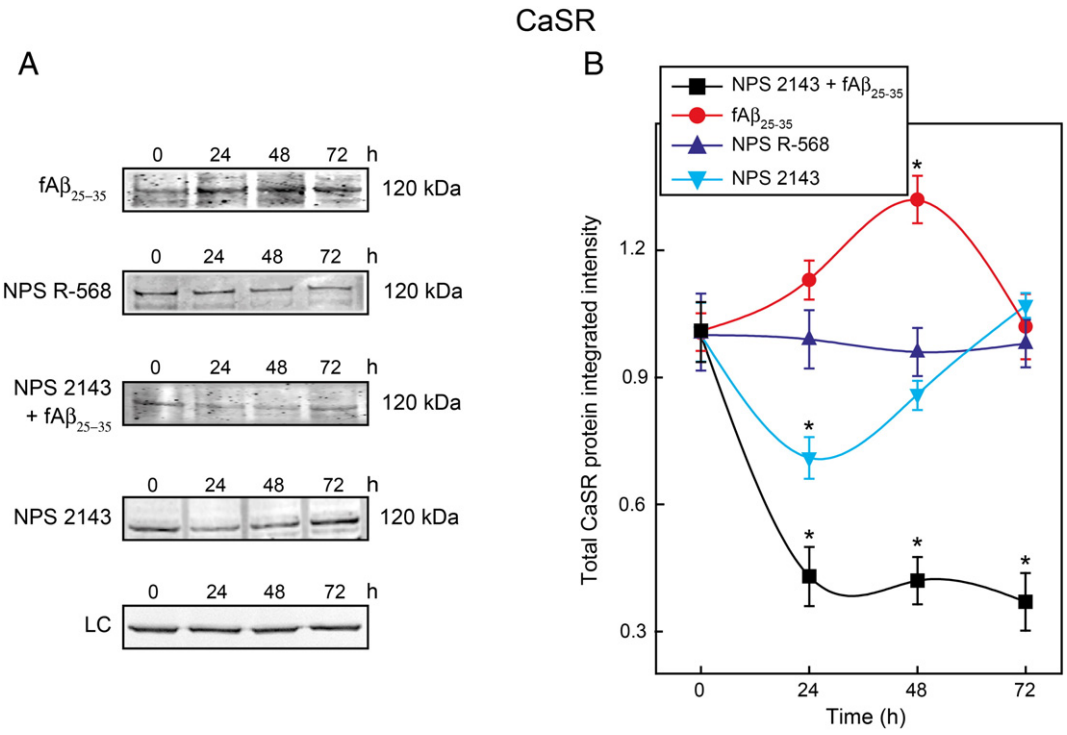

Fig. 1. The total CaSR protein complement of cultured astrocytes is transiently increased by the exposure to exogenous $fA\beta_{25-35}$, yet quickly and then persistently decreased by $fA\beta_{25-35}$ + NPS 2143, only momentarily reduced by NPS 2143 alone, and totally unaffected by NPS R-568. (A) Typical immunoblots showing the changes in total CaSR protein *vs.* untreated (0 h) controls according to the experimental treatments (displayed at the top). LC, loading control (*i.e.*, lamin B1). (B) Densitometric assessments of the specific CaSR protein bands in the immunoblots for each time point and experimental treatment. Points in the curves are means ± SEMs of 4 separate experiments with 0 h values normalized to 1.0. *ANOVA*: $fA\beta_{25-35}$: $F = 9.459$, $p = 0.002$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.009$. $fA\beta_{25-35}$ + NPS 2143: $F = 26.807$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.0000157$ at least at each marked point. NPS 2143: $F = 9.833$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.00176$ at least at each marked point. NPS R-568: $F = 0.125$, $p = 0.944$.

(+34%, $p < 0.05$) following an exposure to fAβ_{25-35} (20 μM). By contrast, the CaSR total protein levels fell rapidly and then were kept steadily reduced between 24 h and 72 h (−60/−65%, $p < 0.001$) in the NPS 2143 + fAβ_{35-25}-exposed astrocytes. Conversely, a transient decrease (−30%, $p < 0.05$) of the total CaSR protein was induced at 24 h in astrocytes treated with NPS 2143 alone (Fig. 1A,B). Finally, the calcimimetic NPS R-568 given by itself did not change the basal CaSR total protein levels in the astrocytes (Fig. 1A,B).

Thus, such observations revealed a specific Aβ/CaSR interaction that could be significantly reduced by NPS 2143, which would cause the CaSR to shift into a proteasomal protease-targeting conformation [37,47,48]. Therefore, we felt entitled to further investigate the effects of fAβ_{25-35} + NPS 2143 on the metabolism of the two main Aβ species in the adult human astrocytes.

3.2. Stimulation of both secretases' activities by exogenous fAβ_{25-35} is not hindered adding NPS 2143 in adult human astrocytes

We previously reported that the activities of both the Aβ-generating secretases were significantly ($p < 0.001$) stimulated by exogenous fAβ_{25-35} but not by its reversemer Aβ_{35-25} [23]. Thus, we first confirmed such results and next assessed the effects of adding NPS 2143. Thus, while exogenous fAβ_{25-35}, increased both BACE1/β-S and γ-S activities (Fig. 2A,B), in the fAβ_{25-35} + NPS 2143-exposed astrocytes the time-related γ-S activity curve rose just like it did when fAβ_{25-35} was given alone, thereby suggesting that it bore no relation to Aβ/CaSR signalling (Fig. 2A,B). Conversely, adding NPS 2143 delayed from 24 h to 48 h the surge of BACE-1/β-S activity otherwise elicited by fAβ_{25-35} (Fig. 2A,B). But, notwithstanding this delay, the *total* stimulated BACE-1/β-S activity over the first 72 h, as measured by the area under the curve, increased marginally (+14.4%, $p = 0.045$) in the NPS 2143 + fAβ_{25-35}-treated with respect to the fAβ_{25-35} alone-exposed astrocytes. Hence, the deep and persistent negative modulation of the total CaSR protein expression by NPS 2143 + fAβ_{25-35} (Fig. 1A,B) did not curtail the Aβ_{25-35}-induced increases in the activity of each secretase and hence the intensified endogenous production of Aβs.

3.3. Calcilytic NPS 2143 specifically suppresses the fAβ_{25-35}-driven surplus intracellular accrual and secretion of endogenous Aβ_{42} by adult human astrocytes

Just as we previously observed [23], densitometric analysis of the several specific oligomeric Aβ_{42} bands in the immunoblots showed that between 0 h and 48 h the total intracellular levels of endogenous Aβ_{42} peptides more than tripled in the fAβ_{25-35} (20 μM)-treated astrocytes *vs.* the 0 h (untreated) controls (Fig. 3A,B). In sharp contrast, when the astrocytes were treated with NPS 2143 + fAβ_{25-35} no significant ($p > 0.05$) intracellular accrual of Aβ_{42} obtained at any time point examined (Fig. 3A,B). On the other hand, when given each by itself, the calcilytic NPS 2143 and the calcimimetic NPS R-568, did not change ($p > 0.05$) between 0 h and 72 h the astrocytes' basal intracellular levels of Aβ_{42} (Fig. 3A,B).

To better gauge the stimulatory effects of exogenously administered fAβ_{25-35} on the *de novo* production of *endogenous* Aβ_{42} (and of Aβ_{40}: see Section 3.4), we reckoned its release into the medium. Thus, between 0 h and 72 h the fAβ_{25-35} alone-driven intracellular accrual of endogenous Aβ_{42} went together with an increasing secretion of Aβ_{42} that stayed well above the basal values (Fig. 3A–C). At variance with previous results [23], in this work we reproducibly detected this Aβ_{42} secretion through the use of a very highly sensitive and specific Aβ_{42} ELISA kit (see for details the Materials and methods section). In sharp contrast, adding NPS 2143 to fAβ_{25-35} treatment steadily kept the secreted amount of endogenous Aβ_{42} at basal (*i.e.* control) levels (Fig. 3C). On the other hand, calcimimetic NPS R-568 given alone significantly raised the secreted amount of Aβ_{42} without changing its basal intracellular contents (Fig. 3A,B,C). Therefore, calcilytic NPS 2143 fully suppressed the fAβ_{25-35}-induced huge intracellular accumulation and surplus secretion

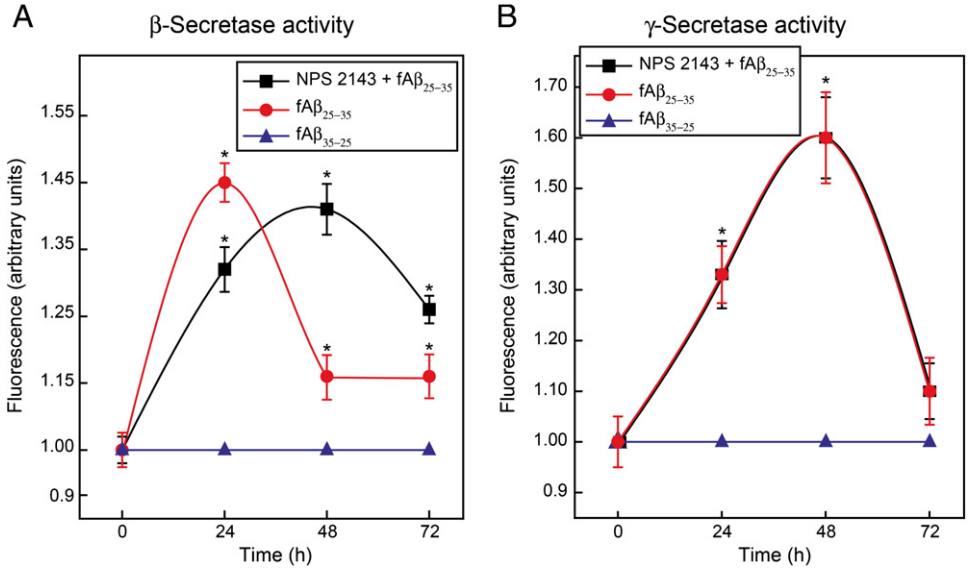

Fig. 2. The activities of BACE1/β-S and of γ-S surge in adult human astrocytes treated with fAβ_{25-35} and the addition of NPS 2143 only delays the peak of BACE1/β-S activity. Conversely, reversemer Aβ_{35-25} is ineffective on the basal activity of either secretase. (A) The BACE1/β-S peak activity shifts from 24 h to 48 h in astrocytes exposed to NPS 2143 + fAβ_{25-35} with respect to that induced by fAβ_{25-35} alone. Notably, the total (0–72 h) activity of BACE1/β-S as measured by the area under the curve is marginally greater (+14.4%, $p = 0.045$) in astrocytes exposed to NPS 2143 + fAβ_{25-35} *vs.* fAβ_{25-35} by itself. Points on the curves are means ± SEMs of 4 separate experiments with 0 h values normalized as 1.0. *ANOVA*: fAβ_{25-35}, $F = 27.913$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls) *, $p < 0.03$ at least for each time point. NPS 2143 + fAβ_{25-35}: $F = 22.899$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.005$ at least for each point. Reversemer Aβ_{35-25}: $F = 0.048$, $p = 0.986$. (B) Astrocytes' γ-S activity similarly peaks by 48 h of exposure to fAβ_{25-35} + NPS 2143. Points on the curves are means ± SEMs of 4 separate experiments with 0 h values normalized as 1.0. *ANOVA*: fAβ_{25-35} + NPS 2143: $F = 35{,}896$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.007$ at least for each point. Reversemer Aβ_{35-25}: $F = 0.007$, $p = 0.999$.

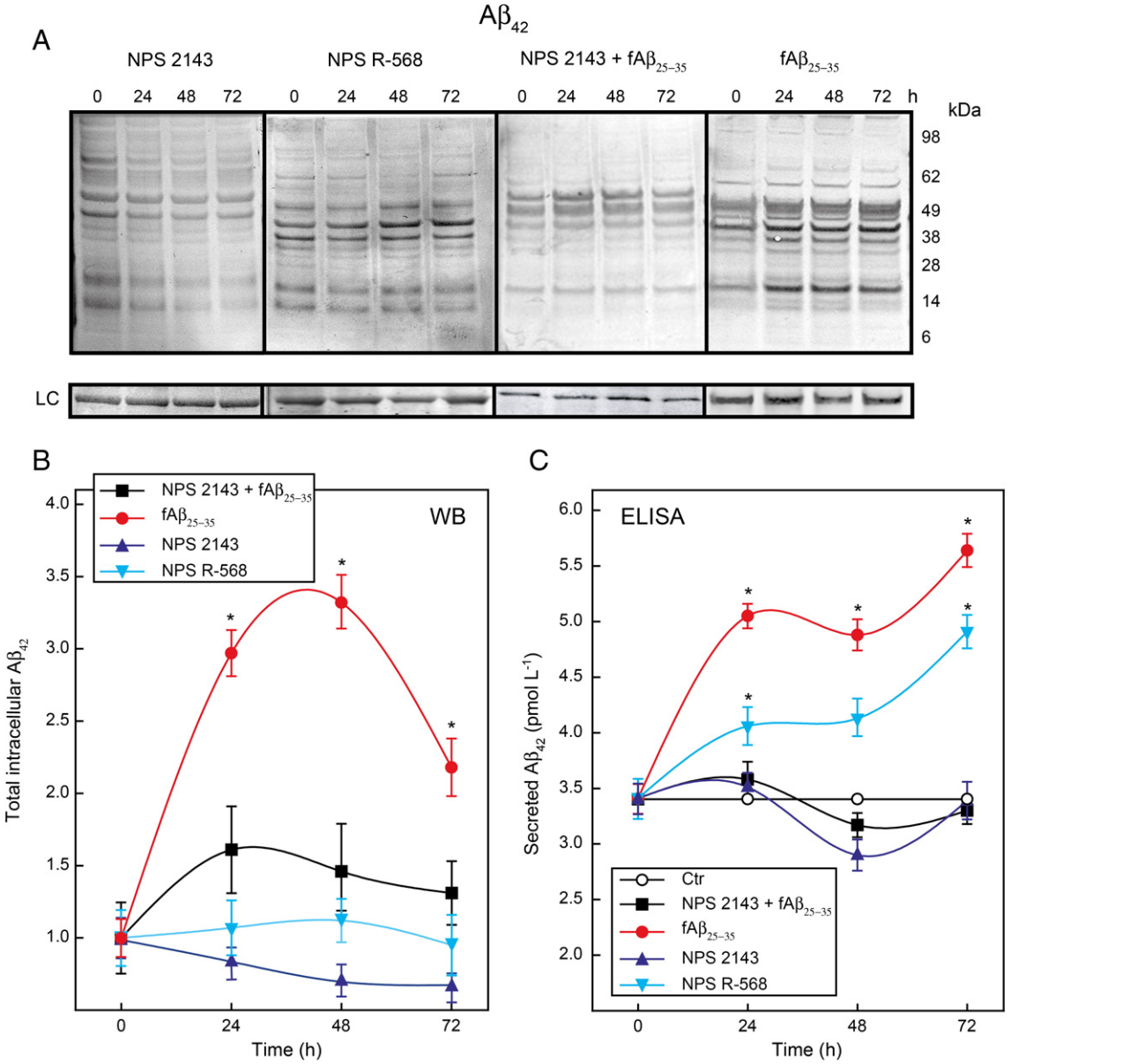

Fig. 3. Changes in the adult human astrocytes' intracellular and secreted amounts of endogenous $A\beta_{42}$ according to the several experimental treatments. (A) Typical immunoblots revealing the changes in intracellularly accrued endogenous $A\beta_{42}$ peptides/oligomers *vs.* untreated (0 h) controls according to the experimental treatments (displayed at the top). Note the denser bands elicited by $fA\beta_{25-35}$ by itself. Conversely, reversemer $A\beta_{35-25}$ is totally ineffective as previously demonstrated [23] (but not shown here). LC, loading control (*i.e.*, lamin B1). (B) Densitometric assessments of the whole sets of intracellular $A\beta_{42}$ bands in the immunoblots for each time point and experimental treatment. Points in the curves are means ± SEMs of 4 separate experiments, with 0 h values normalized as 1.0. *ANOVA*: $fA\beta_{25-35}$, $F = 16.574$, $p < 0.001$. Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.006$ at least, at each time point. $fA\beta_{25-35}$ + NPS 2143: $F = 0.952$, $p = 0.446$. NPS 2143: $F = 3.222$, $p = 0.162$. NPS R-568, $F = 1.149$, $p = 0.360$. WB, Western immunoblots. (C) The secreted amounts of endogenous $A\beta_{42}$ peptides is increased by the exposure to $fA\beta_{25-35}$ or to the calcimimetic agent NPS R-568, but does not change when NPS 2143 is associated with $fA\beta_{25-35}$ or given by itself. The various cell-conditioned media samples were processed and assayed with a highly sensitive ELISA kit as indicated in the Materials and methods section. Points on the curves are means ± SEMs of 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$: $F = 35.152$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.0000242$ at least, at each point. $fA\beta_{25-35}$ + NPS 2143: $F = 0.820$, $p = 0.507$; NPS 2143: $F = 3.558$, $p = 0.048$; NPS R-568: $F = 12.836$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.0223$ at least at each time point.

of endogenous $A\beta_{42}$ by the human adult astrocytes. Conversely, the calcimimetic NPS R-568 by itself simply raised the $A\beta_{42}$ secretion rate.

3.4. Calcilytic NPS 2143 slightly reduces the accrual but discretely increases the secretion of endogenous Aβ40 in fAβ25–35-exposed adult human astrocytes

$A\beta_{40}$ is the other main peptide isoform produced by the sequential activities of BACE-1/β-S and γ-S [1–3]. This warranted the parallel investigation of $A\beta_{40}$ metabolism in the NPS 2143 + $fA\beta_{25-35}$-exposed astrocytes. Thus, between 24 h and 48 h the intracellular levels of $A\beta_{40}$ were almost doubled ($p < 0.001$ *vs.* 0 h) in the $fA\beta_{25-35}$ alone-treated astrocytes and then started falling (Fig. 4A,B). At variance with what we observed with $A\beta_{42}$, adding NPS 2143 to $fA\beta_{25-35}$ only partially decreased at 24 h and 72 h the $fA\beta_{25-35}$-induced intracellular accrual of $A\beta_{40}$ (Fig. 4A,B). In fact, the *total* (0 h–72 h) rises in $A\beta_{40}$ intracellular accumulation *vs.* basal (untreated) levels, as assessed by the areas under the respective curves, were +73.4% in the $fA\beta_{25-35}$-exposed astrocytes, and +51.3% in the NPS 2143 + $fA\beta_{25-35}$-treated astrocytes, respectively ($p < 0.001$ in both instances *vs.* 0 h controls). On the other hand, preliminary evidence also indicated that when given alone NPS 2143 did not change the basal intracellular levels of $A\beta_{40}$ (not shown).

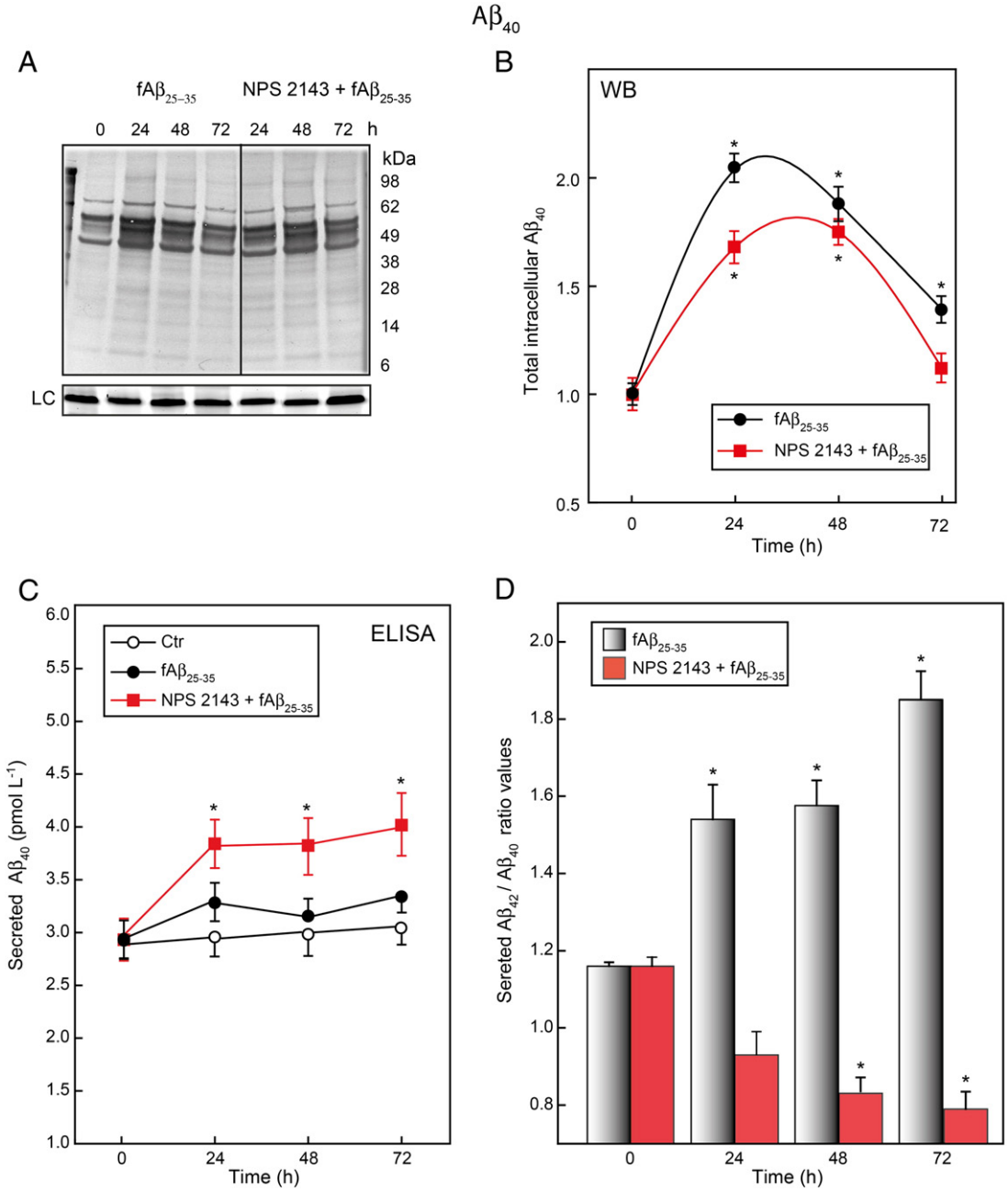

Fig. 4. Changes in the intracellular and secreted amounts of endogenous $A\beta_{40}$ peptides by adult human astrocytes' treated with NPS 2143 ± $fA\beta_{25-35}$. (A) Typical immunoblots revealing the increases in intracellularly accrued endogenous $A\beta_{40}$ peptides/oligomers *vs.* untreated (0 h) controls according to the experimental treatment (displayed at the top). Note the denser bands elicited by the exposure to $fA\beta_{25-35}$ by itself. Adding NPS 2143 to the $fA\beta_{25-35}$ only slightly reduces the intracellular accrual of $A\beta_{40}$ peptides during the time lag considered. LC, loading control (lamin B1). (B) Densitometric evaluations of the whole sets of intracellular $A\beta_{40}$ bands in the immunoblots for each time point and experimental treatment. Points in the curves are means ± SEMs of 4 separate experiments, with 0 h values normalized as 1.0. *ANOVA*: $fA\beta_{25-35}$, $F = 39.074$, $p < 0.001$. Holm–Sidak's test *vs.* 0 h (controls):*, $p < 0.003$ at least, at each time point considered. $fA\beta_{25-35}$ + NPS 2143: $F = 29.331$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): $p < 003$ at least at each point. (C) The secretion of endogenous $A\beta_{40}$ peptides by the astrocytes is increased by an exposure to $fA\beta_{25-35}$ + NPS 2143, whereas is left unchanged by $fA\beta_{25-35}$ by itself. The cell-conditioned media samples were processed and assayed with a highly sensitive and specific ELISA kit as indicated in the Materials and methods section. Points on the curves are means ± SEMs of 4–8 separate experiments. *ANOVA*: $fA\beta_{25-35}$: $F = 0.888$, $p = 0.446$; NPS 2143 + $fA\beta_{25-35}$: $F = 5.076$, $p = 0.012$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.0171$ at least, at each point. $fA\beta_{25-35}$ vs. NPS 2143 + $fA\beta_{25-35}$, $F = 3.813$, $p < 0.008$; Holm–Sidak's test *vs.* 0 h (controls): $p < 0.003$ at least at each time point. (D) Secreted $A\beta_{42}/A\beta_{40}$ ratio values significantly shift in favour of $A\beta_{42}$ in the growth media of $fA\beta_{25-35}$-treated astrocytes, but are kept at values significantly lower than control (0 h) ones in the media of NPS 2143 + $fA\beta_{25-35}$-exposed astrocytes. Bars are means ± SEMs of at least 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$, $F = 22.445$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.0002$ at least at each point. $fA\beta_{25-35}$ + NPS 2143: $F = 9.958$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.006$ at least at each point.

Untreated adult human astrocytes steadily secreted $A\beta_{40}$ in slightly lower amounts than $A\beta_{42}$'s. But, again at variance with $A\beta_{42}$, in the growth media of the $fA\beta_{25-35}$-treated astrocytes the quantities of $A\beta_{40}$ secreted between 0 h and 72 h were not significantly changed ($p > 0.05$) *vs.* untreated controls. However, adding NPS 2143 to $fA\beta_{25-35}$ increased by about 25% ($p < 0.05$) the secreted amounts of $A\beta_{40}$, (Fig. 4C) whereas under this respect NPS 2143 by itself was ineffective (data not plotted).

It is well established that increases in $A\beta_{42}/A\beta_{40}$ ratio values are held as important markers of AD [1–3] Thus, an exposure to $fA\beta_{25-35}$ by itself

steadily raised between 0 h and 72 h the endogenous $A\beta_{42}/A\beta_{40}$ ratio values from 1.16 to 1.86 ($p < 0.001$) in the astrocyte-conditioned growth media (Fig. 4D). However, during the same time lag, adding NPS 2143 to the $fA\beta_{25-35}$ treatment kept the $A\beta_{42}/A\beta_{40}$ ratio values always below the starting value (0.79 at 72 h, $p < 0.001$) (Fig. 4D). Hence, NPS 2143 prevented from unduly escalating the secreted $A\beta_{42}/A\beta_{40}$ ratio values in the conditioned media of adult human astrocytes treated with $fA\beta_{25-35}$.

3.5. Astrocytes' proteasomal chymotrypsin-like activity is changed by exogenous $fA\beta_{25-35}$ + NPS 2143

Intracellular $A\beta_{42}$ and $A\beta_{40}$ oligomers have been reported to selectively inhibit the proteasome and hence to promote their own intracellular accumulation [57]. Hence, we asked whether exogenous $fA\beta_{25-35}$ while stimulating the activities of BACE1/β-S and γ-S would concurrently inhibit proteasomal enzymatic activities in the adult human astrocytes. Our results showed that the three enzymatic activities of the proteasome were differently affected in the $fA\beta_{25-35}$ alone-exposed cells. The caspase-like and trypsin-like activity levels did not change between 0 and 72 h ($p > 0.05$, data not plotted). Conversely, the chymotrypsin-like (20S) activity increased slowly peaking by 72 h (+39.9%, $p < 0.001$) (Fig. 5). But in the NPS 2143 + $fA\beta_{25-35}$-exposed astrocytes, an earlier sharp surge in the chymotrypsin-like (20S) activity obtained, which peaked by 24 h (+59.3%, $p < 0.001$) when CaSR's total protein had reached its lowest level and the intracellular $A\beta_{42}$'s and $A\beta_{40}$'s levels were still on the rise (cf. Figs. 1B, 3B, and 4B). Thereafter this activity fell reaching by 72 h levels significantly lower (−34%, $p < 0.01$) than at 0 h (Fig. 5). On the contrary, adding NPS 2143 to the $fA\beta_{25-35}$ treatment did not change between 24 h and 72 h the proteasomal trypsin-like activity while marginally lessening the proteasomal caspase-like activity (data not shown). Notably, the human proteasomal chymotrypsin-like (h20S) activity was reported to degrade small intracellular $A\beta_{42}$ oligomers acting as its own competitive substrates [58]. However, according to our findings, this enhanced chymotrypsin-like proteolytic action was restricted to the first 24–36 h in the NPS 2143 + $fA\beta_{25-35}$-treated astrocytes. Therefore, the later remarkable reductions in the intracellular levels of endogenous $A\beta_{42}$ and $A\beta_{40}$ we observed (cf. Figs. 3 and 4) may have been caused by other Aβ-degrading proteases (AβDPs) [59].

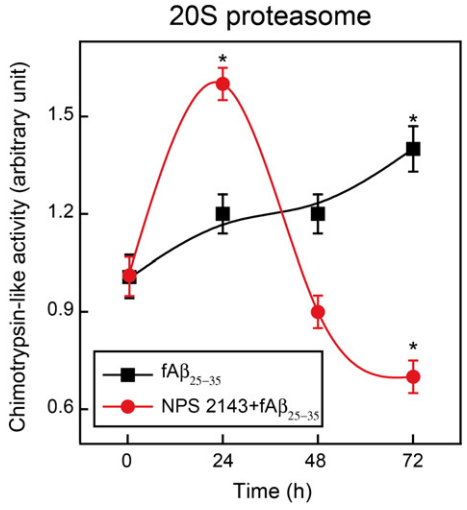

Fig. 5. The proteasomal chymotrypsin-like (20 S) activity marginally increases during the first 48 h but rises significantly by 72 h in $fA\beta_{25-35}$-exposed adult human astrocytes. Conversely, the $fA\beta_{25-35}$ + NPS 2143 treatment triggers a sharp peak of 20S activity at 24 h followed by a drop to sub-basal levels by 72 h. The chymotrypsin-like (20 S) activity was assayed on whole protein extracts from astrocytes as described in the Materials and methods section. Points on the curves are means ± SEMs of 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$: $F = 6.539$, $p = 0.007$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.000747$. $fA\beta_{25-35}$ + NPS 2143: $F = 45.514$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.00310$ at the two points involved.

3.6. Exogenous fAβs-driven increases in astrocytes' NO release are suppressed by NPS 2143 or by U0126 and rescued by adding tetrahydrobiopterin (BH4)

A clue to one of the other AβDPs involved is the ability of $fA\beta_{25-35}$ and of $fA\beta_{1-42}$, but not of reversemer $A\beta_{35-25}$, to stimulate the production and release, from 48 h onwards, of likewise huge amounts of NO by the adult human astrocytes (Fig. 6). But, adding NPS 2143 or the MEK inhibitor U0126 to the $fA\beta_{25-35}$ or $fA\beta_{1-42}$ treatment fully suppressed this intensified NO release (Fig. 6). Then again, adding exogenous BH4 to NPS 2143 or U0126 + $fA\beta_{25-35}$ or $fA\beta_{1-42}$ treatment fully rescued the excess release of NO from inhibition (Fig. 6). These results indicate the involvement of Aβ/CaSR interactions and downstream MEK/ERK signalling in the increased production of BH4, the cofactor needed to dimerize and activate NOS-2, by the GTP cyclohydrolase-1 (GCH-1) [60,61]. Such findings are relevant to intracellular $A\beta_{42}$ and $A\beta_{40}$ degradation because NO overproduction is known to block the Aβ-degrading IDE, whereas effectively preventing NO overproduction preserves IDE's activity [62].

3.7. $sA\beta_{25-35}$-driven surplus secretion of endogenous $A\beta_{42}$ by astrocytes is specifically blocked by NPS 2143 whereas a discretely increased secretion of $A\beta_{40}$ obtains with $sA\beta_{25-35}$ + NPS 2143

At present, $sA\beta_{42}$ oligomers are held as perhaps the most important cell damaging forms of Aβs [1–3]. Therefore, we were enticed to test the effects of $sA\beta_{25-35}$ + NPS 2143 on $A\beta_{42}$ and $A\beta_{40}$ secretion by the adult

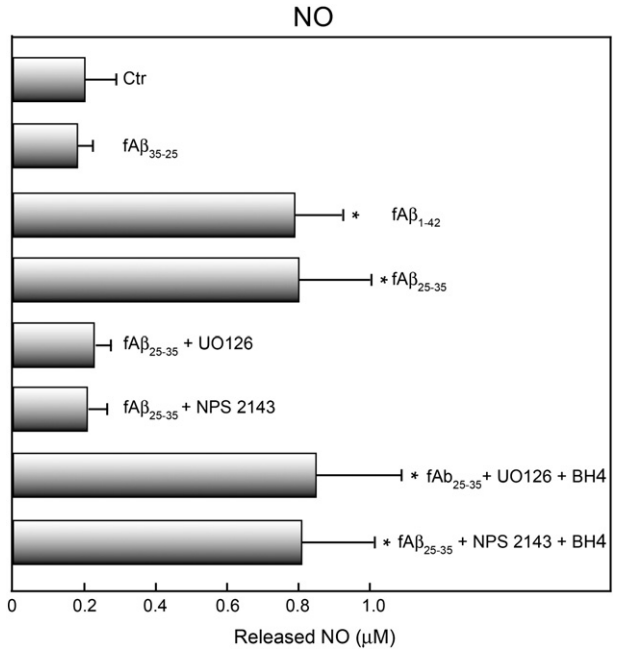

Fig. 6. The release of NO by adult human astrocytes is similarly increased by the exposure to $A\beta_{25-35}$ or $A\beta_{1-42}$ (both in fibrillar form) but is totally prevented by the concurrent addition of calcilytic NPS 2143 or of U0126 (a MEK/ERK inhibitor). Conversely, reversemer $fA\beta_{35-25}$ is completely ineffective. The addition of BH4, a cofactor dimerising and activating NOS-2, overcomes the NPS 2143- or of U0126-elicited blocks of BH4 production by GCH1 and hence of NO over-production and release. NO levels in the respective growth media samples were assessed as detailed in the Materials and methods section. Bars are means ± SEMs of 4 distinct experiments. *ANOVA*: $F = 5.065$, $p = 0.002$; Holm–Sidak's test *vs.* CTR (0 h controls): *, $p < 0.00964$ at least for each marked bar.

human astrocytes. Within 24 h and 48 h the amount of secreted endogenous $A\beta_{42}$ increased by about 2.45-fold ($p < 0.001$) in the $sA\beta_{25-35}$ alone-exposed astrocytes, to start slowly falling thereafter. Most remarkably, this $A\beta_{42}$ over-secretion was totally suppressed by adding NPS 2143 to the $sA\beta_{25-35}$ treatment (Fig. 7A). Conversely, between 48 h and 72 h $sA\beta_{25-35}$ alone discretely increased (+25%, $p < 0.01$) the amount of $A\beta_{40}$ secreted by the adult human astrocytes, and adding NPS 2143 to $sA\beta_{25-35}$ elicited between 0 h and 48 h a nearly steady

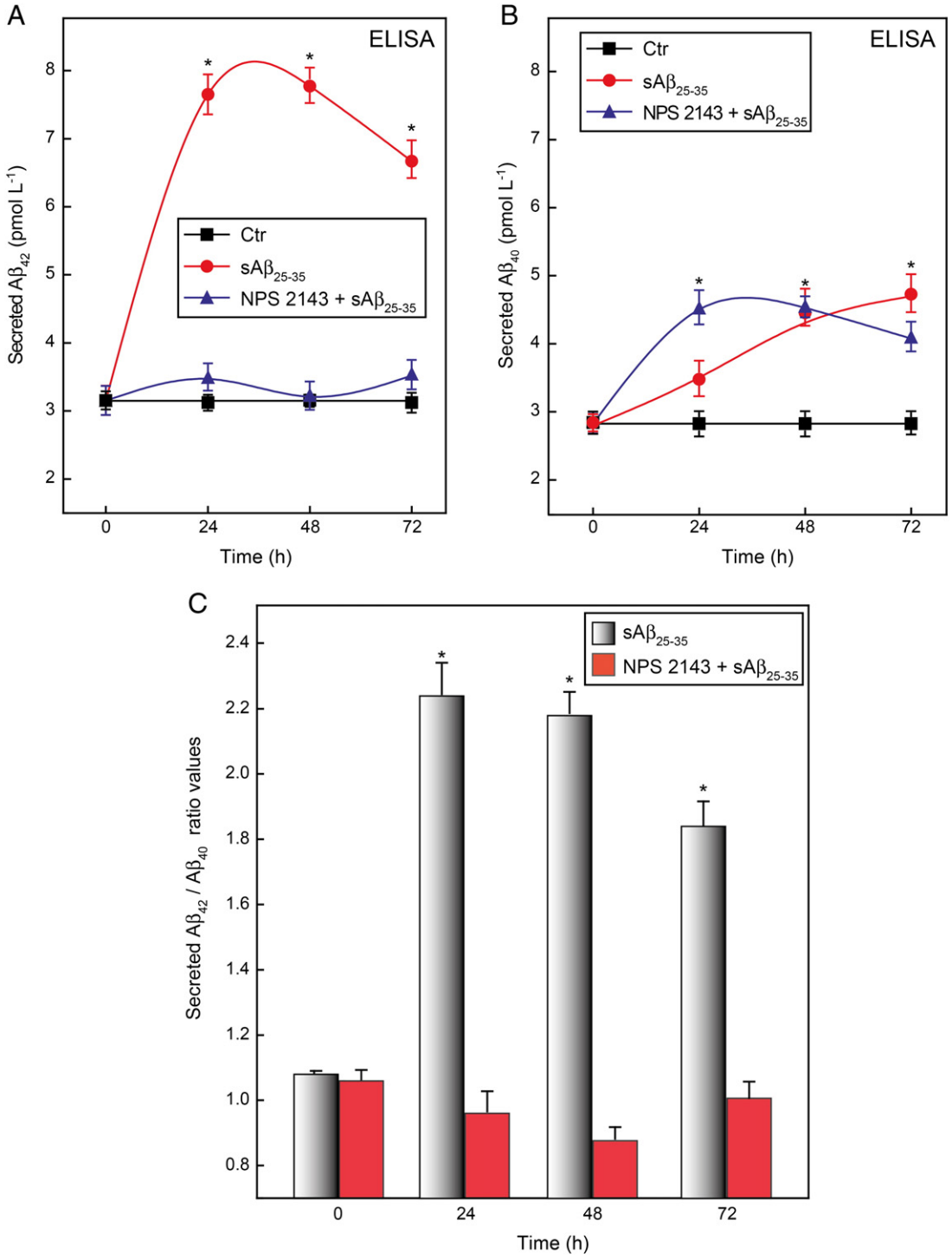

Fig. 7. Changes in the amounts of $A\beta_{42}$ and $A\beta_{40}$—and in the corresponding $A\beta_{42}/A\beta_{40}$ ratio values—secreted by adult human astrocytes after treatment with $sA\beta_{25-35}$ + NPS 2143 (for experimental details see the Materials and methods section). (A) The secretion of $A\beta_{42}$ is significantly increased following a daily exposure to $sA\beta_{25-35}$ (2.0 μM), but this massive surge is totally blotted out by the daily pre-treatment with NPS 2143 (100 nM). Points in the curves are means ± SEMs of 4–8 separate experiments. *ANOVA*: $sA\beta_{25-35}$, $F = 89.384$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.0001$ at least; $sA\beta_{25-35}$ + NPS 2143: $F = 0.695$, $p = 0.568$. (B) The secreted amounts of $A\beta_{40}$ are much less increased by an exposure to $sA\beta_{25-35}$ ± NPS 2143. Points in the curves are means ± SEMs of 4–8 separate experiments. *ANOVA*: $sA\beta_{25-35}$, $F = 12.707$, $p < 0.001$; $sA\beta_{25-35}$ + NPS 2143: $F = 11.306$, $p < 0.001$; for both treatments, Holm–Sidak's test *vs.* 0 h (controls): *, $p < 0.004$ at least. (C) Secreted $A\beta_{42}/A\beta_{40}$ ratio values are significantly raised by $sA\beta_{25-35}$ alone but kept close to or somewhat lower than control (0 h) values by adding NPS 2143 to $sA\beta_{25-35}$ treatment. *ANOVA*: $sA\beta_{25-35}$, $F = 83.114$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls) $A\beta_{42}/A\beta_{40}$ ratio values: *, $p = 0.00001$ at least; $sA\beta_{25-35}$ + NPS 2143: $F = 1.687$, $p = 0.187$.

increase in the secreted amounts of $A\beta_{40}$ (Fig. 7B). Because of the just detailed changes in $A\beta_{42}$ and $A\beta_{40}$ released amounts, the $A\beta_{42}/A\beta_{40}$ ratio values also changed remarkably: adding $sA\beta_{25-35}$ alone increased the $A\beta_{42}/A\beta_{40}$ ratio values from 2.0-to-1.7-fold between 24 h and 72 h ($p < 0.0001$ at least in all instances), whereas adding NPS 2143 + $sA\beta_{25-35}$ steadily kept the $A\beta_{42}/A\beta_{40}$ ratio values below or close to control (0 h) ones ($p > 0.05$ at all time points) (Fig. 7C). Therefore, $sA\beta_{25-35}$ was more effective than $fA\beta_{25-35}$ in triggering the CaSR-mediated $A\beta_{42}$ secretion and adding NPS 2143 specifically and totally curtailed the $A\beta_{42}$ oversecretion. In addition, $sA\beta_{25-35}$ discretely increased $A\beta_{40}$ secretion whereas $fA\beta_{25-35}$ did not (*cf.* Figs. 4C and 7B). On its own part, NPS 2143 modestly enhanced astrocytes' $A\beta_{40}$ secretion when added to the $fA\beta_{25-35}$ or $sA\beta_{25-35}$ (Fig. 7B). Consequently, adding NPS 2143 kept $A\beta_{42}/A\beta_{40}$ ratio values below starting ones independently of the involvement of $sA\beta_{25-35}$ or $fA\beta_{25-35}$.

3.8. Exogenous $fA\beta_{25-35}$ and $sA\beta_{25-35}$-driven death of human cortical HCN-1A neurons is fully prevented by NPS 2143

Since $fA\beta_{25-35}$ (20 μM) [25] and $fA\beta_{40}$ (25–50 μM) [63] have proven to be neurocytotoxic, we asked whether the cell death-inducing activity of Aβs might be hindered by NPS 2143. As expected, $fA\beta_{25-35}$ by itself caused a significant fraction of the HCN-1A neurons to die and disintegrate between 0 h and 72 h, whereas it did not impact at all on astrocytes' survival (Fig. 8A,B). Super-imposable results, *i.e.* fractional neuronal death and no effect on astrocytes viability, obtained when either cell type was daily exposed to $sA\beta_{25-35}$ (2 μM; data not plotted). Remarkably, adding NPS 2143 fully prevented this f- or $sA\beta_{25-35}$-elicited death of a fraction of the HCN-1A neurons (Fig. 8A and data not shown). On the other hand, NPS 2143 by itself exerted no effect on the survival of otherwise untreated HCN-1A neurons or astrocytes or of $fA\beta_{25-35}$- or $sA\beta_{25-35}$-treated astrocytes (Fig. 8A,B and data not plotted). Hence, NPS 2143 had the noteworthy beneficial property of preserving neuronal survival in the presence of $fA\beta_{25-35}$ or $sA\beta_{25-35}$.

3.9. Calcilytic NPS 2143 specifically suppresses the $fA\beta_{25-35}$-driven surplus secretion of endogenous $A\beta_{42}$ by HCN-1A neurons

Proliferatively quiescent, untreated HCN-1A neurons were polymorphic cells endowed with cytoplasmic branches of variable length (Fig. 9A, panels Ctr). But, the HCN-1A neurons surviving an exposure to $fA\beta_{25-35}$ exhibited somata of increased sizes endowed with more numerous, longer, and thicker cytoplasmic processes (Fig. 9A, *panel* $fA\beta_{25-35}$). Immunocytochemical analysis showed that the anti-$A\beta_{42}$ antibody decorated a restricted number of cytoplasmic round bodies (vesicles) in the untreated HCN-1A neurons (Fig. 9A,B, *panels Ctr*). However, in the 48 h $fA\beta_{25-35}$-exposed neurons the anti-$A\beta_{42}$ antibody decorated increased numbers of vesicles that had spread throughout the somata and the entire length of their branches (Fig. 9A, *panel* $fA\beta_{25-35}$ *with inserts 1–3* magnified on the right). Moreover, $fA\beta_{25-35}$-treated HCN-1A neurons released significantly higher amounts of $A\beta_{42}$ into the growth medium than did their untreated (0 h) counterparts (Fig. 9C). Treating the HCN-1A neurons with $fA\beta_{25-35}$ + NPS 2143 restricted the $A\beta_{42}$-positive vesicles to the peripheral portions of the somata, leaving a perinuclear zone, probably corresponding to the site of the Golgi apparatus/*trans*-Golgi network (TGN) and inner endosomes, nearly completely free of $A\beta_{42}$-positive vesicles just as mostly free of such vesicles also were the cellular processes (Fig. 9A and B, *panel NPS 2143* + $fA\beta_{25-35}$ *with inserts 1–3* magnified on the right). Most important, adding

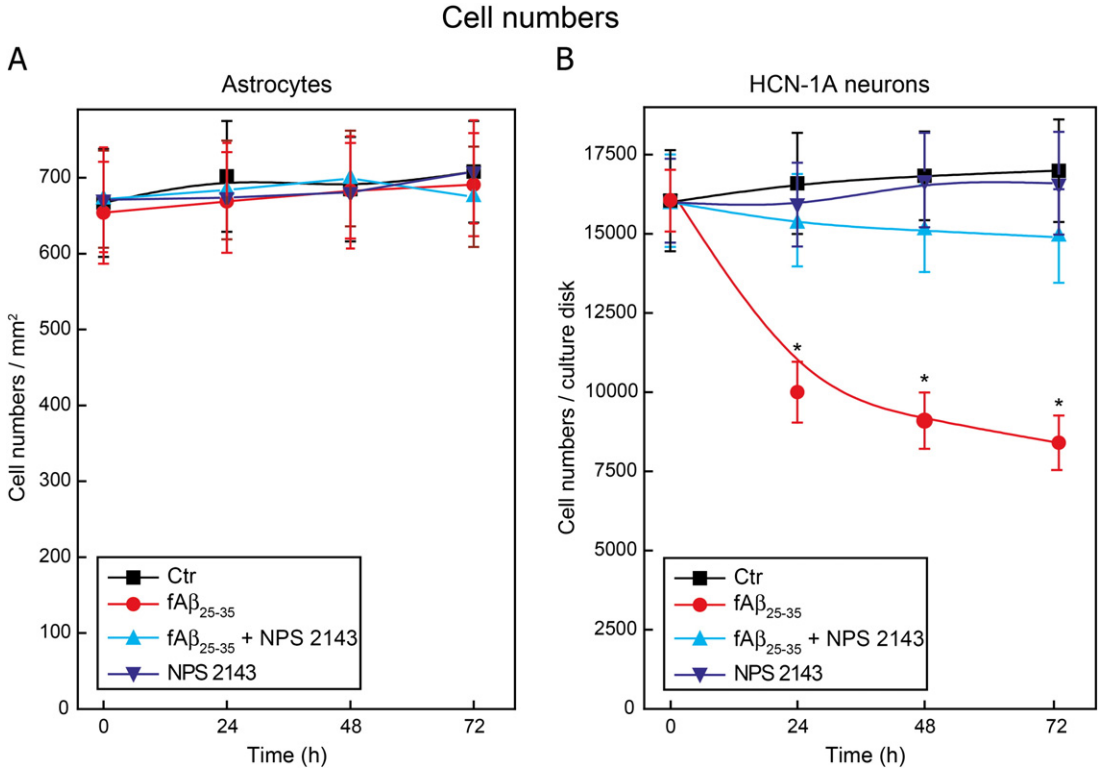

Fig. 8. The time-related numbers/mm^2 or culture disk of proliferatively quiescent normal human cerebral cortex astrocytes and HCN-1As neurons exposed to $fA\beta_{25-35}$ + NPS 2143 or to NPS 2143 alone. (A) During the time lag considered astrocytes' survival was not affected by exogenous $fA\beta_{25-35}$ + NPS 2143 or by NPS 2143 given alone. Points in the curves are means ± SEMs of 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$, $F = 0.0436$, $p = 0.979$; $fA\beta_{25-35}$ + NPS 2143: $F = 0.0612$, $p = 0.979$; NPS 2143: $F = 0.0805$, $p = 0.969$. (B) The early deadly effect of an exposure to $fA\beta_{25-35}$ on a fraction of the HCN-1A neurons is fully prevented by adding NPS 2143, whereas NPS 2143 by itself does not impinge on viable neurons' numbers. Points in the curves are means ± SEMs of 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$, $F = 10.626$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (control) HCN-1A neurons numbers: *, $p < 0.001$ at least. $fA\beta_{25-35}$ + NPS 2143: $F = 0.172$, $p = 0.914$. NPS 2143: $F = 0.0584$, $p = 0.981$.

NPS 2143 to fAβ_{25-35} treatment prevented the surge of endogenous Aβ_{42} secretion otherwise caused by fAβ_{25-35} by itself, thereby holding the secreted amounts of endogenous Aβ_{42} at starting (0 h) values (Fig. 9C). Therefore, NPS 2143 significantly altered the intracellular distribution of the Aβ_{42}-positive vesicles and thereby kept the secretion rates of endogenous Aβ_{42} at control levels (Fig. 9C).

Immunofluorescence analysis showed that a few irregularly shaped bodies were decorated by the anti-Aβ_{40} antibody particularly in the perinuclear cytoplasm of untreated HCN-1A neurons (Fig. 10, *panel Ctr*). A 48 h exposure to fAβ_{25-35} increased the numbers of the Aβ_{40}-positive bodies which spread to the whole somata and their processes (Fig. 10, *panel fAβ_{25-35}*). Adding NPS 2143 to fAβ_{25-35} did not change 48 h later the intracellular distribution of Aβ_{40}-positive bodies with respect to fAβ_{25-35} by itself (Fig. 10, *panel NPS 2143 + fAβ_{25-35}*). Finally, the secreted amounts of Aβ_{40} did not differ in the growth media of untreated controls *vs.* NPS 2143 + fAβ_{25-35}-exposed neurons (Fig. 10, *panel ELISA*). Thus, at variance with Aβ_{42} secretion, Aβ_{40} release was not affected by adding NPS 2143 to fAβ_{25-35} treatment.

3.10. Calcilytic NPS 2143 specifically suppresses the sAβ_{25-35}-driven secretion of endogenous Aβ_{42} by HCN-1A neurons

Finally, we tested the effects of sAβ_{25-35} + NPS 2143 on the HCN-1A neurons. Adding sAβ_{25-35} increased 48 h later the secreted amount (+46%, $p < 0.001$) of endogenous Aβ_{42} (Fig. 11A). But in the growth media samples of neurons treated with NPS 2143 + sAβ_{25-35} the secreted amount of endogenous Aβ_{42} was at control levels (Fig. 11A). Notably, the untreated HCN-1A neurons secreted a 7-fold higher amount of endogenous Aβ_{40} than of Aβ_{42}, and Aβ_{40} secreted basal amounts were not significantly changed by adding sAβ_{25-35} + NPS 2143 (Fig. 11A,B). As a consequence, the Aβ_{42}/Aβ_{40} ratio value increased (+36%, $p < 0.001$) with respect to controls following the addition of sAβ_{25-35} alone, but was brought back close to starting value when NPS 2143 was added to sAβ_{25-35}. Hence, sAβ_{25-35} increased Aβ_{42} secreted amounts, but adding the CaSR antagonist NPS 2143 specifically and wholly annulled this sAβ_{25-35}'s stimulatory effect.

4. Discussion

4.1. The Aβ_{42} proxy Aβ_{25-35}

As in previous works [23,25,53], here we challenged untransformed cortical adult human astrocytes and HCN-1A neurons with Aβ_{25-35}, an Aβ_{42} proxy endowed with the aggregation-essential function key Met^{35} [64]. This allowed us to easily assay the endogenously produced and secreted Aβ_{42} and Aβ_{40} via very highly specific and sensitive ELISA kits. Abundant lines of evidence indicate that Aβ_{25-35} holds all the crucial properties of Aβ_{1-42}, like fibril formation, neurotropism, induction of free radical generation, neurotoxicity, and the ability to interact with various proteins, apolipoprotein E (apoE) included [23,25,65–68]. Notably, fAβ_{25-35} is actually produced in human AD brains but never in normal brains [22,69,70]. Reportedly, fAβ_{25-35} and fAβ_{1-42} elicit identical functional (*e.g.*, $p75^{NTR}$ induction) and cytotoxic (*e.g.*, apoptogenesis) effects in a panoply of experimental models [25,65–71], which justifies the widespread experimental use of Aβ_{25-35} for AD-related studies. We could validate the full biopathological interchangeability between fAβ_{25-35} and fAβ_{1-42} in cultured untransformed adult human astrocytes with respect to the induction of NO overproduction/release, the triggering of the intranuclear translocation of HIF-1α transcription factor, and of the consequent synthesis/secretion of various VEGF-A isoforms ([53] and unpublished results). The dose of fAβ_{25-35} used here was found to be the most effective one in previous studies [23,25,53]. Although at first sight it might be considered a high bolus, it should be taken stock of the fact that, at variance with sAβs, only the Aβ molecules arrayed at the surface of the fibrils are allowed to interact with components of the cell plasma membranes and they correspond to a tiny fraction of the total.

4.2. The CaSR, fAβ_{25-35}, calcilytic NPS 2143, and calcimimetic NPS R-568

CaSR expression is a feature of all types of nerve cells in the mammalian brain [44,49]. In cultured adult human astrocytes CaSR expression is independent of the actual $[Ca^{2+}]_e$, but is conditioned by the proliferative state, being significantly higher in mitotic quiescence than active cell division [37]. Previously, the Aβ-evoked degeneration of hippocampal neurons was related to raises in cytosolic $[Ca^{2+}]_i$ [50], and the activation of Ca^{2+}-permeable non-selective cation channels by fAβ_{25-35} and fAβ_{1-40} was observed only in wild type (WT) $CaSR^{+/+}$ rats but *not* $CaSR^{-/-}$ rats [33]. Changes in CaSR expression and degradation are among the mechanisms regulating intracellular CaSR signalling. The present results show that the astrocytes' *total* CaSR protein amount was transitorily increased (peak at 48 h) by an exposure to fAβ_{25-35} alone, was unchanged by the CaSR agonist NPS R-568 by itself, was fleetingly decreased (with a through at 24 h) by the CaSR antagonist NPS 2143, and most important of all, was quickly and permanently downregulated by coupling NPS 2143 with fAβ_{25-35}. Breitweiser et al. [56] reported that NPS 2143 promoted CaSR's stabilization in an "inactive" CaSR conformation at a checkpoint located in the endoplasmic reticulum (ER); the "inactive" CaSR would then be polyubiquitinylated by dorfin, an E3 ligase, and next degraded by the proteasome [56]. This mechanism was probably operating when NPS 2143 was given by itself and the fall in total CaSR protein was transient. But the persistent decrease of the total CaSR protein elicited by the fAβ_{25-35} + NPS 2143 is the likely upshot of a more complex mechanism as under such circumstances the surge of proteasomal chymotrypsin activity occurred only during the first 48 h. Hence, some other mechanism (receptor endocytosis, lysosomal digestion or else?) might have been involved and its identification requires further studies. The intense and persistent CaSR downregulation in the NPS 2143 plus fAβ_{25-35}-treated astrocytes would curtail the impact of the Aβ-driven CaSR signalling on Aβs metabolism and secretion and on NO over-production (see below and [37]). Conversely, CaSR allosteric agonist NPS R-568 did not change total CaSR protein levels in the adult human astrocytes. Then again, Breitweiser et al. [56] reported that NPS R-568 steadied the CaSR's protein thereby increasing its plasma membrane and total cellular levels *via* an enhanced cotraslational stabilization of its "active" conformation at the ER [56]. These differing results may be explained by the use by Breitweiser et al. of CaSR over-expressing cells that were exposed to NPS R-568 for lengthy periods, whereas we used untransformed adult human astrocytes that were only briefly treated with the calcimimetic. In any event, our results stress the specific impact of the Aβ/CaSR signalling on the intracellular accrual and secretion of endogenous Aβ_{42} and its quite distinct modulation by a calcilytic or a calcimimetic in the adult human astrocytes.

4.3. Aβ-driven CaSR signalling induces both Aβ_{42} and Aβ_{40} intracellular accrual and increases Aβ_{42} secretion by both astrocytes and HCN-1A neurons

According to the debated "*amyloid hypothesis*", LOAD results from the progressive intracellular and extracellular build-up of neurotoxic Aβs presumably due to an ageing-related failure of their clearance [1–4]. Yet, the mechanisms underlying Aβs accumulation in progressively broader areas of AD brains and the respective contributions to it by the various types of resident cells are still poorly defined [72]. The concurrent binding/signalling-activating interactions between sAβ oligomers and/or fAβ aggregates and various receptors at the plasma membrane level, including the CaSR [25–34], are believed to alter critical cellular functions and jeopardize neurons' viability. Previously, we showed that a specific interaction between the extracellular domain of $p75^{NTR}$ and exogenous Aβ_{25-35} or Aβ_{1-42} was necessary

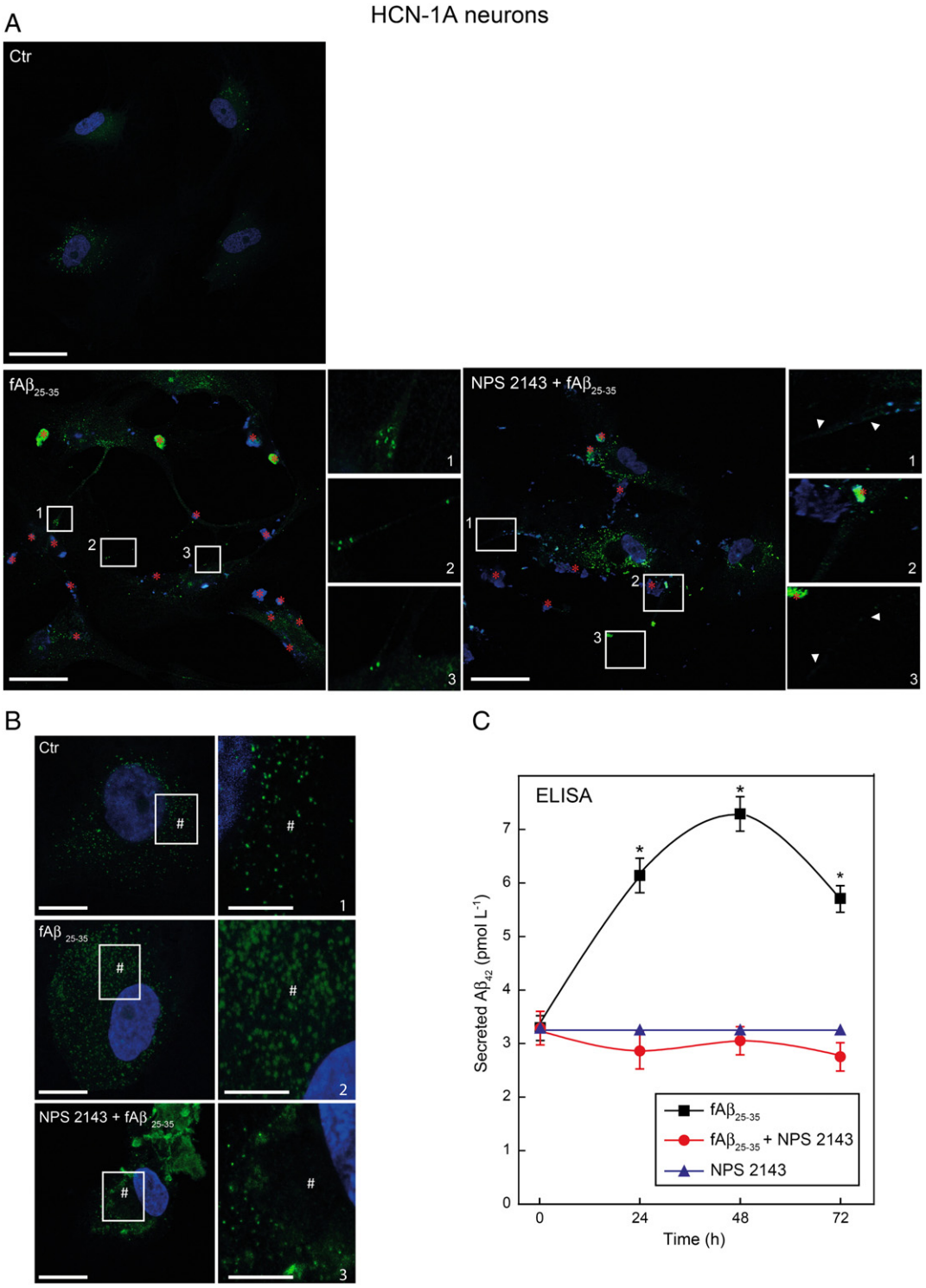

Fig. 9. The changes in cytological features and intracellular distribution and secreted amounts of $A\beta_{42}$ in HCN-1A neurons exposed for 48 h to $fA\beta_{25-35}$ + NPS 2143 *vs.* 0 h untreated controls. These are merged pictures: DAPI-stained nuclei are blue; endogenous $A\beta_{42}$ is yellow-green; blue clumps of extracellular $fA\beta_{25-35}$ are marked by a red *. (A) *Top panel (Ctr)*. Untreated HCN-1A neurons appear as sparse sizeable cells endowed with few short processes. Discrete numbers of cytoplasmic bodies containing endogenous $A\beta_{42}$ are seen mostly in the perinuclear cytoplasm being rare within the thereby poorly visible cellular processes. *Lower left panel ($fA\beta_{25-35}$)*. The HCN-1A neurons that have survived a 48 h exposure to $fA\beta_{25-35}$ exhibit somata of increased in size and processes of augmented numbers and length. The endogenous $A\beta_{42}$-containing bodies have grown very abundant and are evenly distributed inside the somata and processes; such bodies also collect at the tips of some processes (*insets 1–3* are shown at a higher magnification on the right). *Lower right panel ($fA\beta_{25-35}$ + NPS 2143)*. In the NPS 2143 + $fA\beta_{25-35}$-treated neurons the endogenous $A\beta_{42}$-positive bodies were confined within the peripheral cytoplasm of the somata, but were absent from a perinuclear area likely occupied by Golgi apparatus/TGN network and inner endosomes and also from the cellular processes, which consequently had become hardly visible (*insets 1–3* are shown at a higher magnification on the right, in which the *arrowheads* indicate the actual course of otherwise feebly visible cellular processes). Bar, 50 μm. Red * indicates aggregates of $fA\beta_{25-35}$. (B) In untreated (*Ctr*) and $fA\beta_{25-35}$-exposed HCN-1A neurons endogenous $A\beta_{42}$-positive bodies, although differing in numbers, are evenly distributed in the perinuclear cytoplasm. Conversely, in $fA\beta_{25-35}$ + NPS 2143-exposed HCN-1A neurons a large perinuclear area, likely corresponding to the Golgi/TGN network and inner endosomal compartment, is devoid of $A\beta_{42}$-positive bodies, just as are the cellular processes. Corresponding *insets 1–3*, indicated with #, are shown at a higher magnification on the right. (C) Secretion of endogenous $A\beta_{42}$ is increased by the exposure of HCN-1A neurons to $fA\beta_{25-35}$, whereas it is kept at starting (basal) levels by treating with NPS 2143 + $fA\beta_{25-35}$ or with NPS 2143 alone. Experiments were carried out and secreted $A\beta_{42}$ was assayed as described in the Materials and methods section. Points in the curves are means ± SEMs of 4 separate experiments. *ANOVA*: $fA\beta_{25-35}$: $F = 13.845$, $p < 0.001$; Holm–Sidak's test *vs.* 0 h (controls): *, $p = 0.0106$ at least. $fA\beta_{25-35}$ + NPS 2143: $F = 1.188$, $p = 0.356$. NPS 2143: $F = 0.164$, $p = 0.918$.

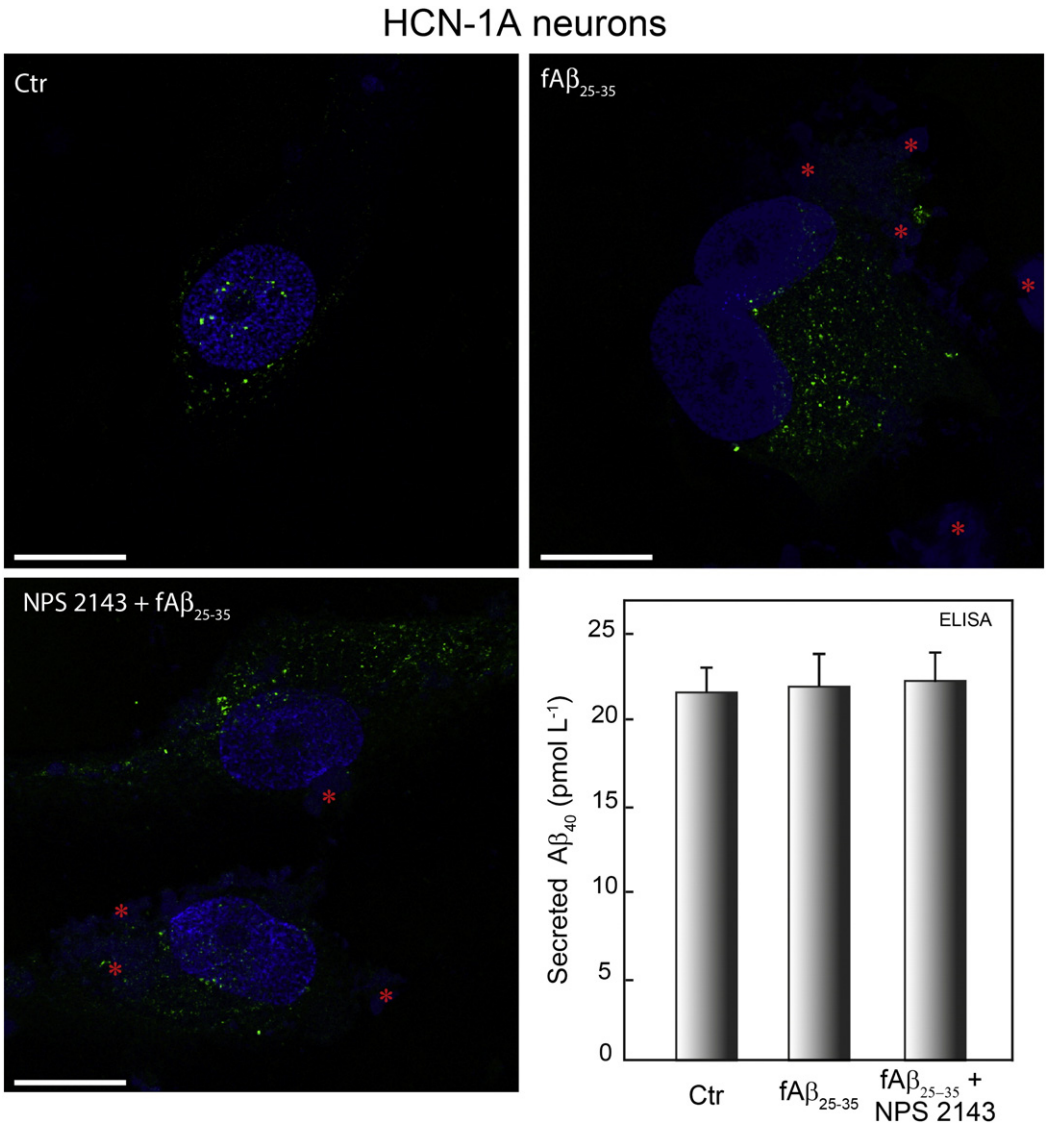

Fig. 10. The intracellular distribution and secreted amounts of $A\beta_{40}$ are not changed by a 48 h exposure of HCN-1A neurons to $fA\beta_{25-35}$ by itself or with NPS 2143. *Panel Ctr*. Only a discrete number of $A\beta_{40}$-positive bodies are detectable within the perinuclear cytoplasm of an untreated (control) HCN-1A neuron. *Panel* $fA\beta_{25-35}$. Anti $A\beta_{40}$-antibody decorated bodies have increased in numbers 48 h after an exposure to $fA\beta_{25-35}$, and evenly occupy the whole cytoplasm of the neurons revealing an intracellular accrual of the peptide. *Panel NPS 2143 +* $fA\beta_{25-35}$. The picture is very similar to that of the $fA\beta_{25-35}$ alone panel indicating that NPS 2143 does not affect intracellular accrual and distribution of the $A\beta_{40}$-positive bodies. Red asterisks (*) indicate $fA\beta_{25-35}$ clumps that emit a blue fluorescence and may mask part of the neurons' bodies. Bar, 20 μm. *Panel ELISA*. The secreted amounts of $A\beta_{40}$ are similar in the growth media of untreated, $fA\beta_{25-35}$ and NPS 2143 + $fA\beta_{25-35}$-treated neurons. Experiments were carried out and secreted $A\beta_{40}$ was assayed using a specific ELISA kit as described in the Materials and methods section. Bars are means ± SEMs of 4 separate experiments. *ANOVA*: $F = 0.070$, $p = 0.932$.

for the induction of human SK-B-NE neuroblastoma cells death [25]. In the present study, we show through the use of NPS 2143, a CaSR allosteric antagonist [36,47,48], that a specific Aβ/CaSR interaction drives the intracellular excess accumulation of both $A\beta_{42}$ and $A\beta_{40}$ and a hugely increased secretion of $A\beta_{42}$ by both human cortical astrocytes and neurons, and moreover the death of a fraction of such neurons. Intraneuronal accumulation of $A\beta_{42}$, which preferentially occurs in late endosomes and particularly in multivesicular bodies (MVBs) [73], typically precedes the appearance of NFTs and the deposition of senile Aβ plaques in the neuropil, and has been linked to the pathogenesis of human AD [1–3]. Intracellular and extracellular accruals of soluble Aβ oligomers induce the loss of synapses and the clinical manifestation of AD [1–3]. And although AD symptoms may not tightly correlate with senile plaque burden, one should not overlook the neurotoxic properties of extra- and intracellular insoluble fAβ aggregates [74].

In keeping with our previous report [23], the APP cleaving and $A\beta_{40}$- and $A\beta_{42}$-producing activities of BACE-1/β-S and γ-S were significantly stimulated by an exposure of adult human astrocytes to exogenous $fA\beta_{25-35}$. Under this respect, the $fA\beta_{25-35}$-driven CaSR signalling might have quickened only the happening of the BACE-1/β-S peak activity, as suggested by its 24 h delay caused by adding the CaSR allosteric antagonist NPS 2143 to the $fA\beta_{25-35}$ treatment. Conversely, the γ–S activity was totally independent of $fA\beta_{25-35}$-evoked CaSR signalling, as indicated by the ineffectiveness of NPS 2143 in this regard. Several mechanisms are known to control BACE-1/β-S activity, such as mRNA transcription and protein translation, both of which are increased by an exposure to $fA\beta_{25-35}$ in adult human astrocytes [23], posttranslational protein modification(s), complexing with other proteins, subcellular and membrane microdomain distribution, and availability of a number of substrates in addition to APP [75]. Even the signalling triggered by the interactions of exogenous Aβs with several other membrane receptors [25–33], besides the CaSR [34], might influence BACE-1/β-S and γ-S activity levels. Further studies, some of which are under way in our laboratory, will help reveal these complex interacting mechanisms in the adult human astrocytes.

Most important, an increased accumulation and secretion of endogenous $A\beta_{42}$ and $A\beta_{40}$ was mediated by the Aβ-driven CaSR signalling as revealed by its specific suppression by CaSR antagonist NPS 2143 [36,47,48] by mechanisms not hindering the enhanced BACE-1/β-S and γ-S activity. Indeed, initially a massive decrease of the total CaSR protein and an as yet insignificant intracellular accumulation of endogenous $A\beta_{42}$ coincided with a transient surge of the proteasome's chymotrypsin-like (20S) activity in the $fA\beta_{25-35}$ + NPS 2143-exposed astrocytes. But, concurrently and/or at later times, other mechanisms started operating to keep both the total CaSR protein and endogenously overproduced $A\beta_{42}$ at low levels within the same cells. A delayed, slow activation of the proteasome's chymotrypsin-like (20S) activity might have contributed to the later post-peak fall of endogenous $A\beta_{42}$ levels observed in both the previous [23] and present study in the astrocytes exposed to $fA\beta_{25-35}$ alone. Although the proteasome is sited in the cytosol, endoluminally produced $A\beta_{42}$ is known to passively diffuse from ER lumen into the cytosol, where it is degraded by both the proteasome and IDE [35]. Several other $A\beta_{42}$-degrading proteases (AβDPs) can powerfully contribute to the control of intracellular $A\beta_{42}$ levels so that the net $A\beta_{42}$ levels result from the balance between production and degradation rates. Moreover, $A\beta_{42}$ proteolysis is negatively, and $A\beta_{42}$ accumulation positively, influenced by ageing, endogenous inhibitors, pharmacological blockers, and oxidative damage (see for references: [59]). The finding that NPS 2143 only partially reduced the intracellular accrual of $A\beta_{40}$ suggests that the disposal mechanism(s) of $A\beta_{40}$ differ(s) from those of $A\beta_{42}$ in astrocytes and remain to be identified in their details.

It is relevant to recall here that human astrocytes partake to the inflammatory component of AD (reviewed in [15]). Our results demonstrate that, besides a cytokine mixture [37,61], exogenously added $sA\beta_{40}$ [38], $fA\beta_{1-42}$, and $fA\beta_{25-35}$ (present results) quite similarly stimulate, *via* the parallel induction of GCH-1 and NOS-2, the overproduction/release of NO by adult human astrocytes [60]. Increased production of NO by inducible NOS-2 has been shown not only to cause NO- and peroxynitrite-mediated neurotoxicity [38,61], but even to reduce IDE, but not neprilysin, activity in APP/PS1 transgenic AD-model mice [62]. Most important, they also reveal that calcilytics like NPS 89626 or NPS 2143, just as the MEK inhibitor U0126 ([61] and present results) block the increase in BH4 synthesis by GCH-1 and, hence, NO overproduction by a BH4-dimerized/activated NOS-2. Incidentally, U0126 and other MEK inhibitors have been reported to decrease Aβ generation and secretion through a secretase-independent drop of the AβPP C-terminal fragment (β-CTF) levels in APP_{Swe}-expressing human SH-SY5Y neuroblastoma cells [76]. We are currently investigating whether this mechanism might also operate in adult human astrocytes and postnatal neurons. In summary, the beneficial effects of NPS 2143 in $fA\beta_{25-35}$-exposed adult human astrocytes are manifold, including a suppression of the excess intracellular accumulation and of the surplus (but not basal) secretion of endogenous $A\beta_{42}$ and of their cytotoxic consequences, a prevention of the noxious effects of NO overproduction/release, and the preservation of the Aβ-cleaving activity of IDE and of other AβDPs.

Under physiological conditions, *in vivo* neurons produce $A\beta_{40}$ more abundantly than $A\beta_{42}$ [1–3] and the secreted amount of $A\beta_{40}$ is about 10-fold that of $A\beta_{42}$; moreover, their respective oligomers form via different routes [77,78]. Because of its high secretion rate $A\beta_{40}$ was reported to be poorly detectable within normal human, rat, and mouse brain neurons [73]. At physiological (pM) levels $A\beta_{40}$ acts as a neurogenic factor [79], though it becomes cytotoxic at pathological concentrations *in vitro* [35,63]. Yet, under morbid conditions *in vivo*, like the early onset familial AD cases and in transgenic animal AD-models, the $A\beta_{42}/A\beta_{40}$ ratio value shifts in favor of $A\beta_{42}$ and small changes of such values are believed to enhance the peptides' neurotoxicity [1–3]. Most important, $A\beta_{42}$ peptides form fibrils faster than $A\beta_{40}$; hence, $A\beta_{42}$ is deemed to be the most neurotoxic species [1–3]. Consequently, it was important to define how the respective production and secretion of $A\beta_{40}$ and of $A\beta_{42}$ behaved when cultured human adult astrocytes or postnatal HCN-1A neurons were challenged with $A\beta_{25-35}$ + NPS 2143. According to an early report, similar proportions of secreted $A\beta_{40}$ and $A\beta_{42}$ were released by cultured brain rat cells of various types [80]. Our present results show that both Aβ isoforms kept being produced and secreted *in vitro* by either cell type: in the untreated astrocytes $A\beta_{40}$ and $A\beta_{42}$ were produced and secreted in closely similar amounts, whereas HCN-1A neurons secreted 7-fold more $A\beta_{40}$ than

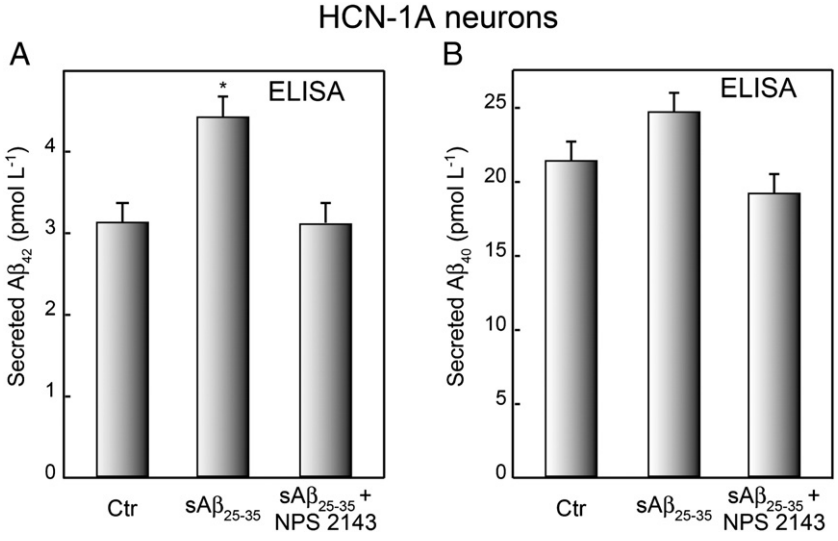

Fig. 11. Effects of a 48 h treatment with soluble (s)$A\beta_{25-35}$ + NPS 2143 on the secreted amounts of $A\beta_{42}$ and $A\beta_{40}$ peptides by HCN-1A neurons. (A) The secreted amount of $A\beta_{42}$ is significantly raised by a daily administration of $sA\beta_{25-35}$ (2 μM), but this stimulatory effect of $sA\beta_{25-35}$ is totally abolished by a co-treatment with NPS 2143. Experiments were performed and secreted amount of $A\beta_{42}$ peptides assayed in the growth media samples by a highly sensitive and specific ELISA kit as detailed in the Materials and methods section. Bars are means ± SEMs of 3 separate experiments. *ANOVA*: $F = 9.646$, $p = 0.013$; Holm–Sidak's test *vs.* control (Ctr) HCN-1A neurons: *, $p < 0.001$. (B) The basal amount of $A\beta_{40}$ secreted by the HCN-1A neurons, which is 7-fold higher than that of $A\beta_{42}$, is not significantly changed by treating the cells with $sA\beta_{25-35}$ + NPS 2143. Experiments were performed and $A\beta_{42}$ assayed in the growth media samples by a specific and highly sensitive ELISA kit as detailed in the Materials and methods section. Bars are means ± SEMs of 3 separate experiments. *ANOVA*: $F = 4.021$, $p = 0.078$.

$A\beta_{42}$. However, in both the f- and $sA\beta_{25-35}$-treated astrocytes and HCN-1A neurons the secreted amount of $A\beta_{42}$ increased remarkably, while that of $A\beta_{40}$ did not change in the neurons but rose a little in the astrocytes. Therefore, in both instances the $A\beta_{42}/A\beta_{40}$ ratio values shifted neatly in favor of $A\beta_{42}$. Here, we wish to stress that the CaSR allosteric antagonist (calcilytic) NPS 2143 *specifically suppressed the $A\beta_{25-35}$-elicited surplus secretion of $A\beta_{42}$* by both neurons and astrocytes. These findings strongly indicate that the metabolism of $A\beta_{40}$ significantly diverges from that of $A\beta_{42}$ in the adult human astrocytes and HCN-1A neurons, as it happens in other cellular models [81]. Importantly, NPS 2143 did not hinder the basal low secretion rate of $A\beta_{42}$, thereby preserving its beneficial physiological effects [5,6].

In keeping with other reports [23,82,83], on a 72 h basis $fA\beta_{25-35}$ and $sA\beta_{25-35}$ induced the death of a discrete fraction of the HCN-1A neurons whereas they were innocuous for the astrocytes. Remarkably, adding NPS 2143 preserved the viability of all the $fA\beta_{25-35}$ or $sA\beta_{25-35}$-exposed HCN-1A neurons. Hence, by negatively modulating Aβ/CaSR signalling NPS 2143 exerts a valuable protective effect on the survival of $A\beta_{25-35}$-treated human cortical neurons in part by preventing an excess accrual and secretion of the over-produced endogenous $A\beta_{42}$. Notably, immunocytochemical observations showed that the distribution of the $A\beta_{42}$-containing vesicles in the HCN-1A neurons changed according to the treatment considered. The $A\beta_{42}$-positive vesicles became more abundant within the somata and the whole length of their processes in the $fA\beta_{25-35}$-exposed neurons. In the $fA\beta_{25-35}$ + NPS 2143-treated neurons the $A\beta_{42}$-positive vesicles were found at the periphery of the somata, but were missing from a perinuclear region likely occupied by Golgi apparatus/TGN network and inner endosomes. Because of this, the $A\beta_{42}$-positive vesicles were largely missing even from the entire length of the neuronal processes. Reportedly, in rat embryo (17 days) primary neuronal cultures, $A\beta_{42}$ and $A\beta_{40}$ are mostly made within the TGN network and next packaged inside post-TGN secretory vesicles; yet, $A\beta_{42}$ is also made and kept in an insoluble condition within the ER and the TGN [84]. Aβ production after endocytosis of APP also happens in early endosomes, where BACE1/β-S and γ-S cleavages take place [85]. Thence, Aβ is retrotransported to late endosomes/multivesicular bodies (MVBs) [86]. When Aβ production is raised, a small fraction (1%) of $A\beta_{42}$ accumulates in flotillin-1-positive endosomes/MVBs. Following MVBs fusion with the plasma membrane, the inner vesicles of MVBs and their associated $A\beta_{42}$ moieties are released extracellularly as exosomes [86,87]. But a pool of $A\beta_{42}$ is released not bound to the exosomes [88]. Our results suggest that by antagonising Aβ/CaSR signalling NPS 2143 might hinder the subcellular TGN- and endosome-related intracellular transport and distribution of endogenous $A\beta_{42}$ bound to endosomal vesicles/MVBs, which are essential for an excess secretion of the $A\beta_{42}$ peptides. Evidence from Busciglio et al. [19] indicated that Golgi processing is needed for the secretion of Aβs. Further studies will clarify in their fine molecular details how calcilytic compounds specifically hinder the processes underlying endogenous $A\beta_{42}$ over-secretion by exogenous Aβ-exposed human neurons.

4.4. AD progression via exogenous Aβ-induced endogenous $A\beta_{42}$ release by neurons and astrocytes

So, what might be the import of our present observations on AD onset and progression? Do they signal novel therapeutic approaches to this unforgiving ailment? If these observations made in cultured human adult astrocytes and postnatal neurons reflect *in vivo* brain reality, they raise the possibility of the operation of a significant astrocyte-assisted Aβ-induced increase in the Aβs load of the AD brain. Extracellular Aβ was previously reported to upregulate intracellular Aβ in APP overexpressing cells [89]. And cultured foetal human neurons and astrocytes have the ability to produce/secrete surplus Aβ peptides [90]. It is virtually certain that the Aβs released by neurons once locally accumulated over a basal threshold due to failing removal mechanisms [4] would impact on their cradling astrocytes. Initial reactions of astrocytes to Aβs accumulation in triple transgenic mice (3xTg-AD) harboring mutated APP_{Swe}, $1PS1_{M146V}$ and tau_{P301L} would consist in cell degeneration/atrophy thereby contributing to synaptic loss [91,92]. Later on, rodent astrocytes become activated, phagocyte and degrade accumulated Aβs, and start expressing BACE1/β-S thereby becoming possible Aβ producers [93]. It must be recalled here that adult human astrocytes do have unique features with respect to their rodent counterparts, being not only the most represented brain cell type [16] but even having ~10-fold more primary processes and covering from 16-fold to 100-fold more synapses (see for references [92–94]). As shown by our findings, the contact with $fA\beta_{25-35}$ does not induce adult human astrocytes to degenerate, though it kills a fraction of the HCN-1A neurons. The cultured human astrocytes basally express BACE1/β-S, and intensify this expression after exposure to $fA\beta_{25-35}$ [23]. It would be expected from the present relatively fast responses of the human astrocytes and HCN-1A neurons to externally added f- or $sA\beta_{25-35}$ that the endogenous $A\beta_{42}$ peptides increasingly released from the neurons in AD brains could directly stimulate the astrocytes they tightly associate with to make, accumulate, and secrete mounting amounts of $A\beta_{42}$ peptides [10]. Once secreted such peptides would directly impact onto the spines and synapses of the astrocyte-cradled neurons [6] and by diffusion induce neighboring neurons and astrocytes to produce and release further amounts of Aβs. This might start a self-sustained spreading of oligomeric $A\beta_{42}$ peptides through astrocytes ⟺ neurons, neurons ⟺ neurons, and astrocytes ⟺ astrocytes along established trajectories in the AD-developing brains [94–97]. Thus, the present results together with the toxic spread of oligomeric $A\beta_{42}$ peptides from neuron-to-neuron demonstrated by Nath et al. [94] suggest that a self-sustaining and self-amplifying Aβs spread would initially drive the decades-long, but gradually accelerating, diffusion of synapse-disconnecting oligomeric Aβs through neocortical networks and the reciprocally interlinked entorhinal cortex and hippocampi [94–98]. Indeed, such a feed-forward mechanism of Aβ self-induction and excess release by the astrocytes and neurons might have helped spread the β-amyloidosis throughout the brains of young APP23 AD-transgenic mice and marmoset monkeys [99–101] during four months after injection into their hippocampi and overlying neocortices of stainless steel wires coated with a dried Aβ-containing brain extract from aged APP23 mice. Moreover, the surplus $A\beta_{42}$ released by both astrocytes and neurons would activate microglia, which would in turn secrete proinflammatory cytokines capable of further enhancing the production and release of $A\beta_{42}$ and $A\beta_{40}$, besides VEGF-A isoforms and NO by the astrocytes, thereby reinforcing a vicious cycle [20,21,37,38,53,60]. Therefore, LOAD may arise from both an age-related failure of the Aβ-clearing brain mechanisms and the locally accumulating exogenous Aβ-induced overproduction, accrual, secretion, and spreading of endogenous Aβ oligomers by both neurons and astrocytes reinforced by neuroinflammatory mechanisms mediated by activated astrocytes and microglia.

4.5. Conclusions and potential therapeutic impact on AD

The present results clearly indicate that Aβs accumulating in the extracellular spaces can stimulate via CaSR signalling both human neurons and astrocytes to make and secrete much higher amounts of endogenous $A\beta_{42}$ peptides, besides other compounds, like the VEGF-A isoforms and NO, released by astrocytes ([37,53] and present results). The upshots of these activities would be *(i)* the loss of synapses, *(ii)* the killing of neurons and, maybe, of their associated astrocytes, and *(iii)* the local diffusion of newly produced and secreted Aβs driving adjacent neurons and astrocytes to produce and release further surplus amounts of endogenous $A\beta_{42}$. By this means, the cycles of "Aβ self-induction" and consequent Aβ excess production

and release would keep going on slowly, nearly at a subliminal level, until a lethal endpoint is reached. This self-sustained spreading of Aβs and its neuropathological and cognitive consequences would explain the inexorable though relatively slow progression of AD. The present findings indicate that CaSR allosteric antagonists would halt the development of this process and hence the progression to full AD by specifically and effectively preventing any excess (but not basal) endogenous $A\beta_{42}$ release by both neurons and astrocytes. We are well aware that extrapolating our present results to *in vivo* settings requires wariness and self-restraint. However, our findings were gained from untransformed *human* cerebral cortical adult astrocytes and postnatal neurons. A similar self-inducing activity of exogenous Aβs has been observed in cultured rat embryo/neonatal neurons [24]. Altogether, these findings strengthen the idea that the production of endogenous Aβs can be induced by the exposure to locally increased levels of Aβs in both neurons and astrocytes if not in other nerve cell types too. Hence, we may envision that a proper therapeutic use of NPS 2143 or other calcilytic compound, besides anabolically improving age-related osteoporosis [36,48], would prevent the Aβ-elicited overproduction and over-secretion of Aβs and hence obstruct the otherwise inexorable path to full blown AD, thereby preserving nerve cells viability and functions and with them the patients' higher cognitive functions. These preliminary results have enticed us to plan further studies with transgenic AD-model mice to be treated with NPS 2143 to assess both biochemical and behavioral end points that would determine the full value of NPS 2143 as a beneficial AD therapeutic drug.

Authors' contributions

UA, AC, and IDP designed the research project and drafted the manuscript; AC, RP, BC, and IDP set up the cultures and carried out the experiments; FC and EC provided the essential cortical tissue fragments; UA and RP collected and statistically analyzed the data; JFW helped collect the literature and draft the paper. All authors read and approved the final manuscript.

Competing interests

None.

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