



# Family C G-Protein-Coupled Receptors in Alzheimer's Disease and Therapeutic Implications

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Alzheimer's disease (AD), particularly its sporadic or late-onset form (SAD/LOAD), is the most prevalent (96–98% of cases) neurodegenerative dementia in aged people. AD's neuropathology hallmarks are intrabrain accumulation of amyloid- $\beta$  peptides (A $\beta$ s) and of hyperphosphorylated Tau (p-Tau) proteins, diffuse neuroinflammation, and progressive death of neurons and oligodendrocytes. Mounting evidences suggest that family C G-protein-coupled receptors (GPCRs), which include  $\gamma$ -aminobutyric acid B receptors (GABA<sub>B</sub>Rs), metabotropic glutamate receptors (mGluR1-8), and the calcium-sensing receptor (CaSR), are involved in many neurotransmitter systems that dysfunction in AD. This review updates the available knowledge about the roles of GPCRs, particularly but not exclusively those expressed by brain astrocytes, in SAD/LOAD onset and progression, taking stock of their respective mechanisms of action and of their potential as anti-AD therapeutic targets. In particular, GABA<sub>B</sub>Rs prevent A $\beta$ s synthesis and neuronal hyperexcitability and group I mGluRs play important pathogenetic roles in transgenic AD-model animals. Moreover, the specific binding of A $\beta$ s to the CaSRs of human cortical astrocytes and neurons cultured *in vitro* engenders a pathological signaling that crucially promotes the surplus synthesis and release of A $\beta$ s and hyperphosphorylated Tau proteins, and also of nitric oxide, vascular endothelial growth factor-A, and proinflammatory agents. Concurrently, A $\beta$ s•CaSR signaling hinders the release of soluble (s)APP- $\alpha$  peptide, a neurotrophic agent and GABA<sub>B</sub>R1a agonist. Altogether these effects progressively kill human cortical neurons *in vitro* and likely also *in vivo*. Several CaSR's negative allosteric modulators suppress all the noxious effects elicited by A $\beta$ s•CaSR signaling in human cortical astrocytes and neurons thus safeguarding neurons' viability *in vitro* and raising hopes about their potential therapeutic benefits in AD patients. Further basic and clinical investigations on these hot topics are needed taking always heed that activation of the several brain family C GPCRs may elicit divergent upshots according to the models studied.

**Keywords:** Alzheimer's disease, G-protein-coupled receptors, amyloid-beta, calcium-sensing receptor, GABA<sub>B</sub> receptors, metabotropic glutamate receptors

## INTRODUCTION

Alzheimer's disease (AD), particularly its sporadic or late-onset form (SAD/LOAD), is by far the most prevalent cause of senile dementia in humans (Alzheimer's Association, 2018). Typically, multiple neurotoxic factors accumulate in the AD brain, such as soluble amyloid- $\beta$  oligomers (sA $\beta$ -os) and insoluble A $\beta$  fibrils (fA $\beta$ s), the latter aggregating into senile plaques (Gouras et al., 2015); hyperphosphorylated soluble Tau oligomers (p-Tau-os) that collect into insoluble neurofibrillary tangles (NFTs) (Bloom, 2014); overproduced reactive oxygen species (ROS) (Butterfield and Boyd-Kimball, 2018); nitric oxide (NO); vascular endothelial growth factor-A (VEGF-A), and proinflammatory agents (Dal Prà et al., 2014a; Chiarini et al., 2016). Altogether, these neurotoxins cause a spreading neuroinflammation, progressive synaptic losses, and cortical human neurons and oligodendrocytes deaths with the consequent breaking up of neural circuits. The clinical counterparts of AD neuropathology are steadily worsening losses of memories and cognitive abilities, which inexorably lead to patients' demise (Dal Prà et al., 2015a; Dal Prà et al., 2015b; Calsolaro and Edison, 2016).

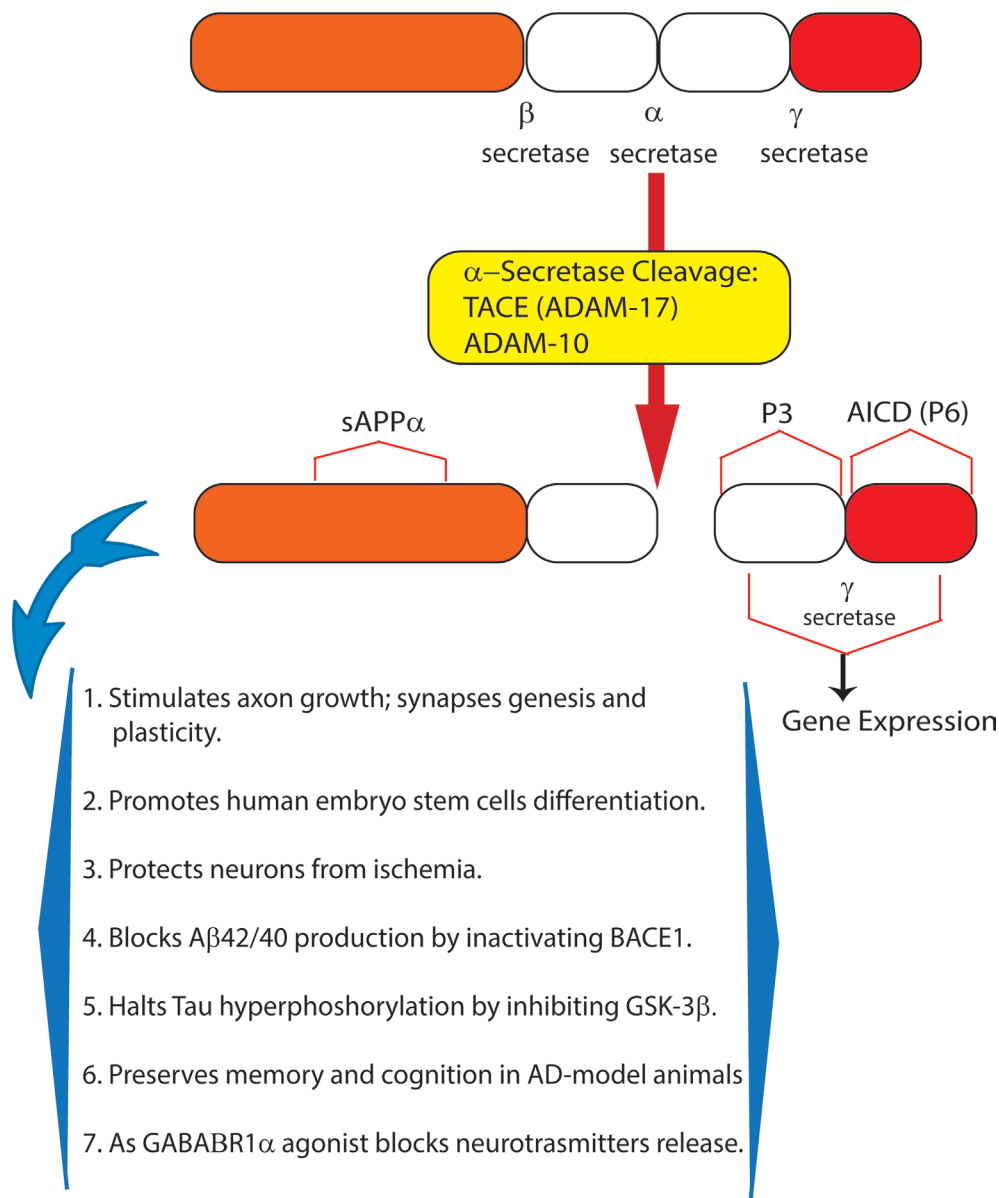
Amyloid precursor protein (APP), a multifunctional protein widely expressed in the central nervous system (CNS), represents the source of the neurotoxic sA $\beta$ -os and fA $\beta$ s that progressively accumulate in AD brains. Transmembrane APP holoprotein can undergo alternative enzymatic handling: (i) *nonamyloidogenic processing (NAP)* by  $\alpha$ -secretases that leads to the production of the soluble (s)APP- $\alpha$  while obstructing A $\beta$ s synthesis (Chiarini et al., 2017b; Rice et al., 2019) (**Figure 1**); and (ii) *amyloidogenic processing (AP)* by  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase liberating A $\beta$ s (**Figure 2**). Notably, sAPP- $\alpha$ 's physiological roles are multifaceted, and to-date only partly understood. The available evidence reveals that sAPP- $\alpha$  promotes the neural differentiation of human embryo stem cells (Freude et al., 2011) and protects hippocampal neurons from the harm due to ischemia (Smith-Swintosky et al., 1994), glucose deficiency (Furukawa et al., 1996), brain trauma, and excitotoxicity (Mattson et al., 1993; Goodman and Mattson, 1994). In addition, sAPP- $\alpha$  complexes with and inhibits the activity of BACE1/ $\beta$ -secretase protein thus hindering any excess production of toxic A $\beta_{42}$ /A $\beta_{42}$ -os (Stein and Johnson, 2003; Obregon et al., 2012; Peters-Libeu et al., 2015). Moreover, sAPP- $\alpha$  stimulates axonal outgrowth (Ohsawa et al., 1997), synaptogenesis, and synaptic plasticity (Hick et al., 2015; Habib et al., 2016). Remarkably, sAPP- $\alpha$  also curbs the activity of glycogen synthase kinase (GSK)-3 $\beta$  and the hyperphosphorylation and overrelease of neurotoxic p-Tau/p-Tau-os, the main components of NFTs (Deng et al., 2015). And an increased activity of GSK-3 $\beta$  has been linked to hyperphosphorylation of Tau in the brains of AD patients. Typically, in AD Tau is phosphorylated at over 30 serine/threonine residues by various protein kinases, including GSK-3 $\beta$  (Pei et al., 1999). The D1 and D6a domains of sAPP- $\alpha$  are the locations of its neuroprotective and neurotrophic activities since they stimulate axons outgrowth when added as separate fragments to *in vitro* hippocampal neurons (Jin et al., 1994; Qiu et al., 1995; Ohsawa et al., 1997). In keeping with such findings, sAPP- $\alpha$  upholds cognition and memory integrity in animal models of physiological aging and of AD (Roch et al., 1994;

Meziane et al., 1998; Bour et al., 2004; Ring et al., 2007; Corrigan et al., 2012; Xiong et al., 2016) (**Figure 1**).

SAD/LOAD, which comprises ~98–96% of the cases, starts from neuronal nests in the layer II of the lateral entorhinal cortex (LEC) in the temporal lobe (Khan et al., 2014) where small ischemic areas may occur in aged subjects (Ishimaru et al., 1996). Thence, in the course of 20–40 years (*asymptomatic stage*) SAD/LOAD silently spreads to wider and wider upper cerebral cortex areas, particularly to those involved in storage and retrieval of memories and in handling complex cognitive activities (Khan et al., 2014). When the unremitting attrition depletes the cortical human neurons' functional reserve, SAD/LOAD's first clinical symptoms start manifesting as amnesias. This marks the onset of the *amnestic minor cognitive impairment* or *aMCI stage* that lasts 3–5 years while its symptoms progressively worsen. Eventually, the *full symptomatic stage* takes over, whose exacerbating symptoms include permanent losses of short-term (first) and long-term (later) memories, changes in personality and behavior, loss of the several language-related abilities, failure to cope with daily tasks and needs, motor problems, cognitive shortfalls, dementia, and eventually death. However, it is still hard to diagnose the earliest asymptomatic stage of AD because specific biomarkers are few and the highly neurotoxic, synapse-destroying sA $\beta_{42}$ -os are hardly detectable when senile plaques and NFTs are still absent (Selkoe, 2008a; Selkoe, 2008b; Ferreira and Klein, 2011; Klein, 2013; Dal Prà et al., 2015a). Even so, the ghostly sA $\beta_{42}$ -os eventually cause a noticeable accumulation of A $\beta_{42}$  as fibrils and senile plaques, and of p-Tau-os as NFTs (Medeiros et al., 2013). Presently, the diagnosis of SAD/LOAD is based upon detecting brain deposits of insoluble A $\beta$ s (senile plaques) *via* PET imaging and specific changes in A $\beta_{42}$ /A $\beta_{40}$  and Tau/p-Tau ratios values in the cerebrospinal fluid (CSF), which are deemed to be pathognomonic (McKhann 2011). PET imaging can also detect the brain accumulation of NFTs (Hall et al., 2017). The quest of blood biomarkers of AD is still ongoing with some preliminary promising results (Nabers et al., 2018; Nakamura et al., 2018; Palmqvist et al., 2019).

Presently, no drug therapy modifies or mitigates AD's relentless course (Jessen et al., 2014). This unsatisfactory situation still lingers because of various reasons. First, SAD/LOAD pathogenesis remains unclear and, hence, an open to speculation topic. Second, animal models closely mirroring human SAD/LOAD are as yet not available (Ameen-Ali et al., 2017). Transgenic (*tg*) animal (mostly rodent) AD-models only partially and imperfectly emulate the early-onset familial (EOF) AD variety, which comprises at most 2–4% of AD cases. It is well established that EOFAD results from mutations in the amyloid precursor protein (*APP*) or presenilin1 (*PSEN1*) or presenilin2 (*PSEN2*) genes. These mutations drive a constitutive, diffuse intrabrain overproduction and overload of sA $\beta$ -os, insoluble A $\beta$  fibrils, and hence A $\beta$ -heaped senile plaques and concurrently of p-Tau-os and NFTs. Conversely, no genetic mutations underlie SAD/LOAD pathogenesis, although *APOE* (Huang and Mahley, 2014) and *TREM2* (Gao et al., 2017) gene variants could increase AD proclivity. While A $\beta$ -os appear as the first main AD drivers, in such *tg* AD-model animals p-Tau accumulates as NFTs later and only when a mutated *MAPT* transgene is inserted

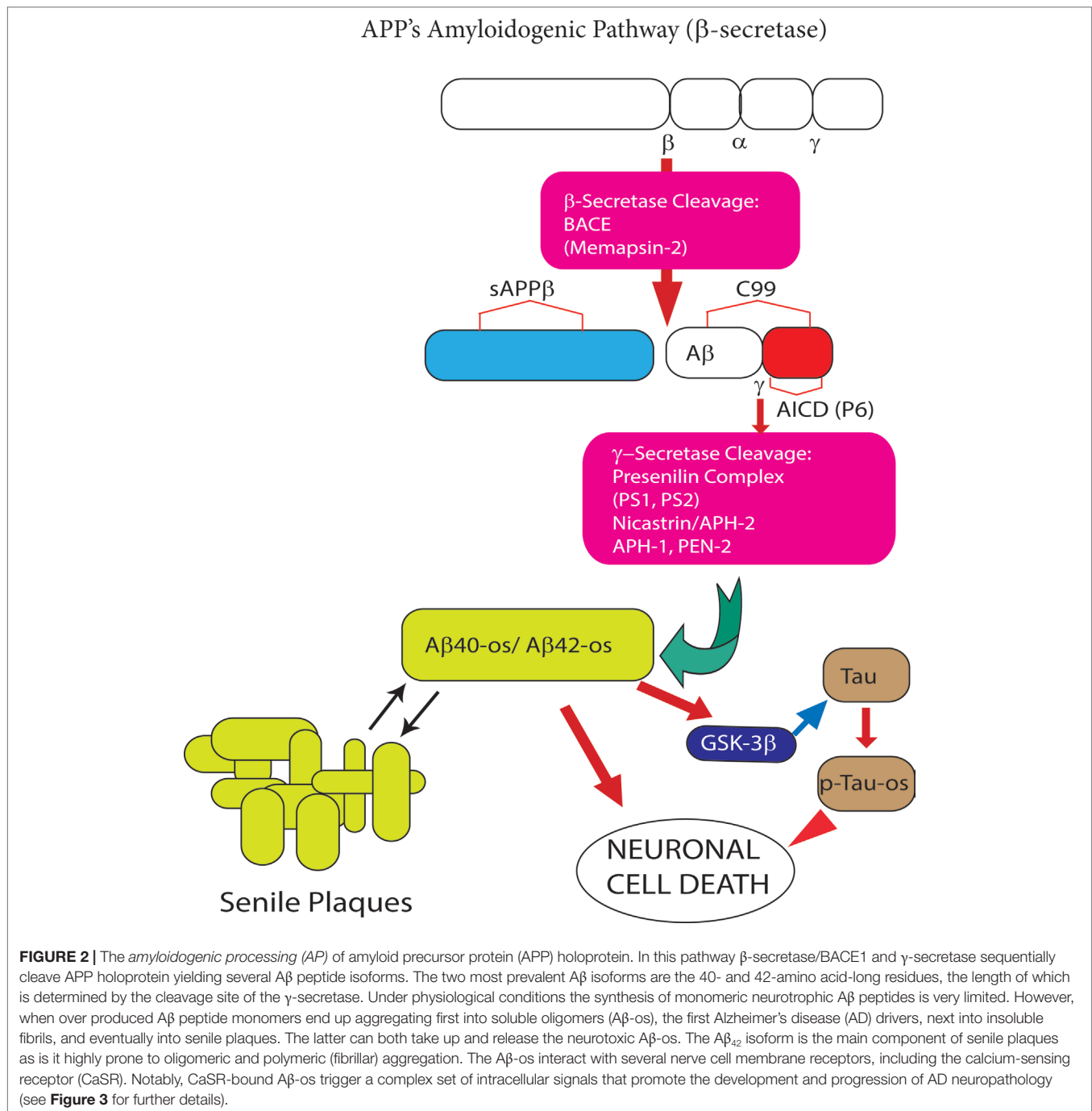
## APP's Nonamyloidogenic Pathway ( $\alpha$ -secretase)



**FIGURE 1 |** The *nonamyloidogenic processing (NAP)* of amyloid precursor protein (APP) holoprotein. By itself, APP holoprotein is not neurotoxic and is cleaved at three different locations by  $\alpha$ - or  $\beta$ - and/or  $\gamma$ -secretase. Proteolytic cleavage by  $\alpha$ -secretase represents the *NAP* of membrane-inserted APP holoprotein. *NAP* occurs just within the amino acid sequence of A $\beta$ <sub>42</sub>, whose synthesis it consequently obstructs. Thus,  $\alpha$ -secretase activity (mostly due to ADAM10) sheds from APP holoprotein the soluble (s)APP- $\alpha$  peptide, whose multiple neurotrophic and neuroprotective effects are summarized in this figure. Recent evidence indicates that as a GABA<sub>B</sub>1aR agonist sAPP- $\alpha$  also constitutively moderates neuronal excitability thus preventing neurons' harm. In summary, APP holoprotein's *NAP* hinders the development of AD and preserves neuronal viability, trophism, and function.

too (Oddo et al., 2003; Cohen et al., 2013). *Third*, AD-model rodent brains substantially differ under several cytological, structural, and functional standpoints from the human brain. Some differences are obvious, such as brain size and weight, the cerebral cortex limited extent, the largely prevailing primitive olfactory cortex, and so on. Other differences are more subtle,

but still exceedingly important, as they critically regard a genetic homology of only 80% and structural and functional features of cortical neurons and neuroglial cells--e.g. the total absence of some human cortical astroglial subtypes from rodent brain cortices--and the dissimilar extension of the astrocytes' domains, and the unlike reactions (e.g. Ca<sup>2+</sup> fluxes) of each neural cell



type once exposed to AD-driving neurotoxins. As a revealing example, in *tg* AD-model animals, the cortical astrocytes die sooner than neurons, whereas cortical neurons die earlier than astrocytes in human AD brains. Altogether, such a complex set of divergences has suggested that *tg* rodent AD-model animals may not be the ultimate means to identify therapeutic approaches benefiting human AD patients (Ransohoff, 2018). Hitherto, all drugs that resulted advantageous when given to *tg* AD-model animals have failed to act as beneficial therapeutics in human AD patients (Cummings, 2017). It should be noted that this is

a currently recognized general problem affecting the quest for and successful trial of novel drugs preclinically tested in animal models of other human diseases besides AD (Gabrielczyk, 2019). Unquestionably, novel rodent and non-human primate (NHP) AD models are under development (Podlisny et al., 1991; Sasaguri et al., 2017) but their potential worth for human AD therapeutic research remains to be assessed.

So, are there acceptable alternatives to AD-model animals? Given the just mentioned species-related differences, one should take stock of untransformed human neural cells making

up “preclinical AD models in Petri dishes”. An example of this kind may be neural cells differentiated from human induced pluripotent stem cells (iPSCs) isolated from normal subjects and/or from EOFAD and SAD/LOAD patients and set into 2D or 3D cultures (Kim et al., 2015). Human iPSC models are easier to handle than NHP models and may also be integrated into mouse AD models (Espuny-Camacho et al., 2017).

Still another suitable preclinical human AD model *in vitro* consists of untransformed cortical adult human astrocytes and/or neurons. Human astrocytes from the temporal lobe cerebral cortex exhibit a stable (“locked-in”) differentiated phenotype and give reproducible responses when exposed to fAβs and/or sAβs. The experimental exploitation of the latter cells has revealed that exogenous Aβs specifically bind the calcium-sensing receptor (CaSR) (Dal Prà et al., 2014a; Dal Prà et al., 2014b; Dal Prà et al., 2015b), a member of family C G-protein coupled receptors (GPCR), and activate a pathological signaling that could drive human LOAD/SAD onset and progression and also worsen EOFAD's course. These findings have clearly pointed out to a class of therapeutic agents, the CaSR negative allosteric modulators (NAMs), which effectively block all such AD's pathogenetic mechanisms in untransformed cortical human neurons and astrocytes *in vitro* and could stop the progression of AD neuropathology in the patients (Armato et al., 2013; Chiarini et al., 2017a; Chiarini et al., 2017b).

Family C GPCRs also include the metabotropic glutamate (mGlu) and GABA<sub>A/B</sub> receptors (Bräuner-Osborne et al., 2007; Urwyler, 2011). Results of studies in animal model suggest that mGlu-Rs and GABA-Rs might also be involved in AD pathophysiology because AD concurs with alterations of glutamatergic transmission (Caraci et al., 2018). Therefore, we discuss here the roles of family C GPCRs in AD progression and hence their potential relevance to human AD therapy.

## FAMILY C G-PROTEIN-COUPLED RECEPTORS

In general, GPCRs are among the most numerous groups of transmembrane proteins of the mammalian genome. To date, about 800 of these proteins have been identified in humans (Fredriksson et al., 2003). The relevance of their manifold functions has made them therapeutically attractive as shown by the fact that they are the targets of ~34% of United States Food and Drug Administration-approved drugs (Hauser et al., 2017). Currently, GPCRs are distinguished into six classes (families A–F) (Table 1) based upon amino acid sequence homologies, elected signal transduction pathways, and pharmacological outlines.

Details concerning the structure of family C GPCRs are known for the mGluRs (Kunishima et al., 2000; Tsuchiya et al., 2002; Muto et al., 2007; Doré et al., 2014; Wu et al., 2014), GABA<sub>B</sub>R (Geng et al., 2013), and CaSR (Gama et al., 2001; Geng et al., 2016) extracellular domains (ECDs), and for the mGluRs and CaSR transmembrane domains (Doré et al., 2014). Family C GPCRs share a common general structure characterized by a huge bilobed N-terminal extracellular domain (ECD) or

**TABLE 1 |** G-Protein-coupled receptors (GPCRs) Families.

Family A	rhodopsin-like receptors
Family B	secretin receptors
Family C	γ-aminobutyric acid B (GABA <sub>B</sub> ) receptors; metabotropic glutamate receptors (mGluR1–8); calcium-sensing receptor CaSR; taste receptors 1–3; *V2 pheromone receptors; *GPRC6A receptor; **various orphan receptors.**
Family D	fungal mating pheromone receptors
Family E	cyclic AMP receptors
Family F	frizzled/smoothened receptors

\*These receptors abound in rodents but are absent from humans (Niswender and Conn 2010; Alexander et al., 2017).

\*\*Not considered in this review.

“Venus Flytrap” (VFT) (Fredriksson et al., 2003; Lagerström and Schlöth, 2008; Rosenbaum et al., 2009). A cysteine-rich region (CR) links the ECD/VFT to the 7TM domain including seven transmembrane helical hydrophobic regions (TM1–TM7) connected extracellularly by three loops (ECL1–ECL3) and intracellularly by three loops (ICL1–ICL3). The CR domain is extant in all family C GPCRs save for GABA<sub>B</sub>Rs. Finally, the 7TM domain is linked to the intracellular C-terminal domain (ICD), whose tail interacts with G proteins to activate downstream signaling pathways.

Family C GPCRs function as mandatory dimers (El Moustaine et al., 2012) joined by a disulfide bond topping the two VFTs. GPCRs can be formed into homodimers or into heterodimers with other members of the same group or family (Goudet et al., 2005; Doumazane et al., 2011; Kammermeier, 2012; Sevastyanova and Kammermeier, 2014) or with extraneous GPCRs (Ciruela et al., 2001; Gama et al., 2001; Cabello et al., 2009; Kniazefz et al., 2011). The orthosteric ligands bind the pockets placed in the slit between the two VFT's lobes causing the active closure of both slits (closed-closed conformation) or of only one slit (open-closed conformation) (Parnot and Kobilka, 2004). Conformational changes inside the VFT domains are conveyed through the cysteine-rich and 7TM regions to the ICD domain to regulate G-proteins binding and activate intracellular signaling (Rondard et al., 2006).

The family C GPCRs subtype specificity of orthosteric agonists and antagonists varies because the amino acid sequence of the VFT binding pocket may or may not be extensively conserved. Group III mGluRs are an example of the former case, as their orthosteric agonists and antagonists are possessed of a mostly unchanging broad-spectrum activity (MacInnes et al., 2004; Austin et al., 2010). To overcome this obstacle to therapeutic applications, an active quest has been and still is going on for drugs that bind on particular amino acid sequences defined as allosteric sites or pockets (e.g. on the ECL1–ECL3 of the 7TM domain) and placed well outside the VFT-inside orthosteric site. A number of allosteric sites located within the 7TM domain were identified by investigations using site-directed mutagenesis and allosteric modulator cocrystal methods (Gregory et al., 2012; Doré et al., 2014; Wu et al., 2014; Christopher et al., 2015). These novel drugs selectively modify the receptor signaling triggered by an orthosteric ligand acting as either positive allosteric modulators or PAMs or negative allosteric modulators or NAMs (Engers and

Lindsley, 2013). PAMs favor whereas NAMs hinder the binding affinity/activity (the so-called cooperativity) of orthosteric ligands. Finally, neutral allosteric ligands (NALs) bind their sequence on a receptor but do not change the cooperativity of its orthosteric ligand (Wootten et al., 2013; Christopoulos, 2014). The otherwise steady structural conformation of orthosteric ligand-bound GPCRs undergoes transient changes, which impact on the interactions with G proteins or other transducers, when the receptors also bind allosteric modulators (Canals et al., 2011). Lipophilic PAMs and NAMs cross the blood–brain barrier (BBB) (Ritzén et al., 2005). However, extremely lipophilic PAMs and NAMs may exhibit a lesser ability to reach the neural cells while their unwanted side effects may become stronger (Goudet et al., 2012). Present-day methods used to evaluate allosteric interactions, i.e. functional and radio-ligand binding assays, need proper probes to reveal the receptor's response and fully disclose the allosteric ligand's properties (Price et al., 2005; Hellyer et al., 2018). The affinity and effectiveness of allosteric modulators can be quantified *via* the allosterism's operational model that associates the allosteric ternary complex model (ATCM) with Black–Leff's operational model of pharmacological agonism (Black and Leff, 1983; Ehlert, 1988; Leach et al., 2007; May et al., 2007). The latter allows to quantify the modulator's effectiveness and its impact on orthosteric agonist affinity vs. efficacy (Black and Leff, 1983; Leach et al., 2007; Keov et al., 2011; Gregory et al., 2012). Classically, NAMs and PAMs are effective only when the natural orthosteric ligands (or probes) are present (Conn et al., 2009). At times, surrogate orthosteric probes are required for functional assays. Probes of different chemical nature may affect the cooperativity in opposite directions or leave it unchanged (Koole et al., 2010; Valant et al., 2012; Sengmany et al., 2017). Remarkably, allosteric modulators evoke saturable effects, i.e. no further activity obtains when they reach saturating doses (May et al., 2007; Klein, 2013). Specific PAMs and NAMs may or sometimes may not elicit the internalization of their receptors, and hence may or may not desensitize the cells to corresponding orthosteric ligands (Conn et al., 2009). Some PAMs and NAMs are of a mixed type acting as both orthosteric agonists and PAMs, while others act as both agonists for one receptor subtype and as antagonists for another receptor subtype. Preferred PAMs or NAMs should not permanently activate or inactivate the involved receptors because this could elicit harmful effects (Célanire and Campo, 2012). Clearly, receptor subtype-specific PAMs and NAMs are indispensable tools for basic and preclinical pharmacological research and, when beneficial, might be transformed into drugs apt for clinical trials. For example, one chemokine receptor-5 NAM, i.e. Maraviroc, successfully reached the clinical use to treat late-stage HIV disease (Dorr et al., 2005).

Here, a cautionary note is in order about the interspecies translatability of experimental results related to family C GPCRs. In fact, brain locations of the same family C GPCRs can significantly vary between animal species (e.g. mouse vs. rat) and between animals and humans. Such species-related divergences could explain the inconsistent observations one may make when experimenting with more than one animal species. This is a further drawback to translating the beneficial effects evoked by orthosteric agonists/antagonists or allosteric PAMs/NAMs in animal models

of diseases into beneficial therapeutic upshots in disease-matching human patients. Moreover, one should not overlook another caveat concerning the extrapolation to postnatal life of results gained by administering PAMs or NAMs to animal embryo–fetal cellular models. In fact, during development mGluRs expression undergoes divergent changes in distinct types of cells. Finally, on a positive note, the combined administration of orthosteric agonists or antagonists with NAMs or PAMs can provide additive or synergistic neuroprotection, which might have future therapeutic applications (Vernon et al., 2005; Bennouar et al., 2013).

## GABA<sub>B</sub>Rs, APP, Aβs, AND AD

There are two classes of GABA<sub>B</sub>Rs, i.e. GABA<sub>A</sub>Rs and GABA<sub>B</sub>Rs. While GABA<sub>A</sub>Rs are fast-acting ionotropic receptors functioning as ligand-gated ion channels, GABA<sub>B</sub>Rs are metabotropic family C GPCRs, whose structure comprises the subunits R1a, R1b, and R2 (Chang and Shoback, 2004). Receptor dimerization or oligomerization is obligatory for GABA<sub>B</sub>Rs as it impacts on function. Only GABA<sub>B</sub> R1a/R2 or R1b/R2 heterodimers do reach the cell surface, bind GABA to R1 subunit, and activate G-protein-mediated intracellular signals *via* R2 subunit (White et al., 1998; Marshall et al., 1999; Margeta-Mitrovic et al., 2001). Of note, GABA<sub>B</sub>R1 and R2 subunits form heterodimers also with the CaSR (this topic will be further discussed below). Two “*sushi domains* (SDs)” abut from the N-terminus of GABA<sub>B</sub>R1a VFT, which are required for the receptor's trafficking to the cell surface and for its presynaptic inhibitory activity (Gassmann and Bettler, 2012; Hannan et al., 2012). Adaptor proteins link the GABA<sub>B</sub>R1a SD1 domains to axoplasmic kinesin-1 motors. Recently, proteomic methods permitted to identify three adaptor proteins playing this role, i.e. APP, adherence-junction associated protein 1 (AJAP-1), and PILRα-associated neural protein (PIANP). Thus, axonal trafficking cargo vesicles carry at least three distinct types of GABA<sub>B</sub>R1a/adaptor protein/kinesin-1 complexes. It is noteworthy that the formation of any of such GABA<sub>B</sub>R1a/APP/kinesin-1 complexes obstructs the amyloidogenic processing (AP) of the involved APP molecules into Aβ<sub>42</sub>/Aβ<sub>42</sub>-os, the AD main drivers. This could be a novel AD-preventing mechanism involving GABA<sub>B</sub>R1a axonal trafficking (Dinamarca et al., 2019).

Neurons express GABA<sub>B</sub>Rs on both presynaptic and postsynaptic membranes. Neuronal activity controls GABA<sub>B</sub>Rs presynaptic expression (Guetg et al., 2010; Terunuma et al., 2010; Orts-Del'Immagine and Pugh, 2018). Conversely, model animals of AD (Chu et al., 1987; Iwakiri et al., 2005) and of various other diseases, like chronic epilepsy (Thompson et al., 2006–2007), fragile X syndrome (Kang et al., 2017), and Parkinson disease (Borgkvist et al., 2015), downgrade GABA<sub>B</sub>Rs expression and inhibitory function causing neuronal hyperexcitability. Human astrocytes express both GABA<sub>A</sub>Rs and GABA<sub>B</sub>Rs and constitutively synthesize and release GABA, therefore being GABAergic cells. GABA release from human astrocytes is dose-dependently increased by glutamate or by NMDAR coagonists like D-serine and glycine. Conversely, inhibitors of kainic acid receptors and of NMDARs decrease GABA release from human astrocytes. Interestingly, the administration of exogenous

GABA suppresses the proinflammatory responses of activated astrocytes and microglia to noxious stimuli (Lee et al., 2011a; Lee et al., 2011b).

The GABA<sub>B</sub>Rs classical ligand is  $\gamma$ -aminobutyric acid (GABA), which is the chief inhibitory neurotransmitter of the mature central nervous system (CNS) of vertebrates. GABA-releasing (GABAergic) neurons are ubiquitous in the CNS. Nearly half of all CNS synapses express some GABA<sub>B</sub>Rs and thus respond to GABA (Li et al., 2016). The signaling activated by the GABA•GABA<sub>B</sub>R complexes inhibits the release of neurotransmitters from the targeted postsynaptic neurons (Benes and Berretta, 2001; Bettler et al., 2004). But the signaling activated by the presynaptic GABA•GABA<sub>B</sub>R complexes of the GABAergic neurons blocks their further GABA release thus acting as a physiological self-controlling mechanism exerting an indirect excitatory effect on postsynaptic neurons. An astrocyte  $\rightarrow$  neurons metabolic cycle upkeeps the neuronal stores of GABA (Losi et al., 2014; Mederos et al., 2018). The released GABA moieties are taken up by both pre- and postsynaptic neurons and by the astrocytes enveloping tripartite synapses. In their mitochondria, the astrocytes convert the uptaken GABA into glutamine and forward the glutamine to adjoining neurons. In presynaptic neurons, two sequentially acting enzymes synthesize GABA from glutamine: first, glutaminase, which converts glutamine into glutamate; and second, glutamic acid decarboxylase (GAD), which transforms glutamate into GABA. Next, the vesicular transporter of GABA (VGAT) transfers it into cargo/synaptic vesicles, which release GABA into the synaptic cleft after the neuron's membrane depolarization. GABA signaling regulates several physiological aspects of CNS activity, like neurogenesis, neuronal development, sexual maturation, circadian rhythms, motor functions, learning, and memory (Petroff, 2002). GABAergic interneurons are the chief inhibitory neurons in the CNS. Abundant reports in the literature stress how dysfunctions of the activity of GABAergic interneurons disrupt the glutamatergic excitatory/GABAergic inhibitory (E/I) balance in neural circuits of the cerebral cortex, hippocampus, and subcortical structures (e.g. amygdala) causing declining cognitive capabilities and worsening memory losses. Therefore, it is generally held that E/I imbalance significantly impacts on the pathogenesis of AD (Govindpani et al., 2017; Villette and Dutar, 2017) and of various other CNS disorders, such as major depression (Fee et al., 2017), schizophrenia, autism's spectrum, bipolar disorder (Benes and Berretta, 2001; Lehmann et al., 2012; Gao and Penzes, 2015; Xu and Wong, 2018), and anxiety (Babaev et al., 2018).

Unfortunately, the results of postmortem studies on AD patients did not throw much light onto GABA<sub>B</sub>Rs role(s) in AD and this for good reasons--one being the wide-ranging variability of the patients' terminal lifetime. Different alterations of biological parameters--such as expression of RNA, proteins, and enzymes--can be induced by co-morbidities, medications, aging, and leading death causes and are all directly linked to the duration and severity of the full-blown or symptomatic AD phase (Govindpani et al., 2017). Therefore, it is not surprising that divergent findings abound concerning GABA<sub>B</sub>Rs' possible role(s) in AD. Thus, recent postmortem studies of human brains

and of *tg* AD-model animals reported that GABAergic neurons and GABA<sub>B</sub>Rs were unaffected by AD neuropathology (Li et al., 2016). Conversely, earlier studies had conveyed the opinion that GABA<sub>B</sub>Rs signaling undergoes profound changes in AD (Takahashi et al., 2010). Significantly lowered GABA levels were detected in the temporal cortex of AD patients (Gueli and Taibi, 2013) and in cerebrospinal fluid (CSF) samples from both AD patients and the cognitively normal elderly (Grouselle et al., 1998). Conversely, raised GABA levels turned up in the hippocampus and CSF samples of AD patients and were ascribed to the impairment of synaptic plasticity (Jo et al., 2014). These studies also noted that the reactive astrocytes surrounding A $\beta$ s senile plaques overproduced GABA *via* monoamine oxidase-B (MAO-B) activity and abnormally released it through the bestrophin-1 channels. Under physiological conditions, bestrophin-1 channels are mostly localized at the microdomains of hippocampal astrocytes nearby glutamatergic synapses and mediate glutamate release. A switch from the glutamate-releasing normal astrocyte to the reactive astrocyte releasing GABA *via* bestrophin-1 channels is a common phenomenon occurring in various pathological conditions coupled with astrogliosis, such as traumatic brain injury, neuroinflammation, neurodegeneration, and hypoxic and ischemic insults. In AD, bestrophin-1 channels are redistributed to the soma and the processes of hippocampal reactive GABA-containing astrocytes. Bestrophin-1 channels-mediated GABA release from reactive astrocytes hinders synaptic plasticity and transmission and spatial memory by reducing dentate granule cell excitability (Oh and Lee, 2017). It was claimed that suppressing GABA overproduction by monoamine oxidase-B (MAO-B) or GABA overrelease through bestrophin 1 channels from the dentate gyrus reactive astrocytes fully restored learning and memory in AD-model mice (Jo et al., 2014). However, the long-term administration of selegiline, an irreversible MAO-B inhibitor, did not improve AD neuropathology in a clinical trial (Park et al., 2019). To explain this unforeseen upshot, the authors suggested that multiple factors, like age, sex, and differences in brain regions could impact on the GABA release from astrocytes and neurons and should not be ignored when planning therapeutic drug attempts. Indeed, different brain regions of Tg2576 (human APP695 plus the Swedish double mutation K670N, M671L) AD-model mice released dissimilar amounts of GABA in relation to their actual age and sex. Cortical GABA levels were higher in older than 6 months female than male mice; however, at a more advanced age, this difference vanished in the parietal cortex but became more pronounced in the prefrontal cortex. Moreover, at 12–18 months of age, hippocampal levels of GABA were lower in female than in male mice. Altogether, these data revealed that with advancing age the functional disruption of GABA signaling turns out to be more intense in AD-model female mice (Hsiao et al., 1996). Conversely, under or up to 9 months of age, hippocampal GABA levels were higher in female than in male mice, likely because the former enjoyed the protective activity of estrogens (Roy et al., 2018). By extrapolating these data from animals to humans one can infer that a single therapeutic strategy addressing GABA<sub>B</sub>Rs modulation might not be so easily feasible in AD. In fact, any drug inhibiting the GABA<sub>B</sub>Rs residing in one brain region might

exacerbate the dysregulation of GABAergic signaling in other brain areas.

Hence, the role(s) of GABA/GABA<sub>B</sub>Rs signaling in the pathogenesis of AD has(ve) hitherto remained unclear if not confusing (Govindpani et al., 2017). However, the recent studies we mention just below performed on wild-type and AD-model animals have thrown some more light on the contribution of GABAergic remodeling to the pathogenesis of both early and late stages of AD.

First, we recall here that the  $\epsilon 4$  allele of apolipoprotein E (APOE) is the main known genetic risk factor for LOAD/SAD. Notably, in the brains of aged APOE $\epsilon 4$  mice, an attenuation of GABAergic inhibitory inputs on associated excitatory neurons drives a specific neuronal hyperactivity phenotype. Hence, an APOE $\epsilon 4$ -driven hippocampal neuronal excitatory hyperactivity might be among the causative factors underlying the increased risk of AD among APOE $\epsilon 4$  carriers (Nuriel et al., 2017). In addition, several AD-mouse models exhibit an early and marked neuronal hyperactivity in the hippocampus (Busche et al., 2012; Busche et al., 2015). Moreover, functional magnetic resonance imaging (fMRI) studies have revealed that humans with mild cognitive impairment (MCI), as well as presymptomatic carriers of EOAD mutations show enhanced neuronal activity in the same brain region, the hippocampus (Quiroz et al., 2010; Haberman et al., 2017). Therefore, when the increase in brain activity takes place early in the pathogenetic process it may be rightly considered a driving factor in AD development.

Second, according to a recent report, A $\beta$ s, AD's main drivers, are intensely degraded by endothelin-converting enzyme-2 (ECE-2) and neprilysin (NEP) in the somatostatinergic and parvalbuminergic synapses of GABAergic interneurons residing in the neocortex and hippocampus. These observations support the view that under physiological conditions A $\beta$ s may partake in the regulation of interneurons' inhibitory signaling in AD-relevant brain areas (Pacheco-Quinto et al., 2016). However, it must be stressed here that a reduction of A $\beta$ s catabolism at the synapses of these two distinct populations of GABAergic neurons is not the unique GPCRs-mediated mechanism favoring A $\beta$ s accumulation in AD brains.

Third, the exciting results of very recent studies have revealed that GABA<sub>B</sub>R1a receptors bind three novel orthosteric agonists besides GABA, i.e. soluble (s)APP- $\alpha$ , sAPP- $\beta$ , and sAPP- $\eta$  proteins. The  $\alpha$ -,  $\beta$ -, and  $\eta$ -secretases, respectively, shed them from the extracellular domain of APP into the brain environment. Next, each of these peptides can bind GABA<sub>B</sub>R1a receptors and block the release of neurotransmitters from hippocampal presynaptic excitatory axonal terminals thus silencing synaptic transmission. Most interesting, a 17-mer peptide of the ExD flexible portion of sAPP- $\alpha$ , which binds the extracellular Sushi1 domain of the GABA<sub>B</sub>R1a could replicate the squelching effect on neurotransmission brought about by the whole sAPP- $\alpha$  molecule. These results explain, at least in part, the synaptic dysfunction affecting some APP-overexpressing AD-model animals. Moreover, they suggest that this 17-mer peptide could therapeutically counteract the excitatory hyperactivity of neuronal synaptic function brought about by A $\beta$ s (Rice et al., 2019; Tang, 2019).

## mGLURs AND AD

The seven-transmembrane-spanning mGluRs physiologically control synaptic transmission and neuronal excitability in the CNS and influence behavioral output processes. These receptors are assigned to three groups according to their G-protein coupling and signal transduction pathways. Group I encompasses mGluR1 and mGluR5; group II includes mGluR2 and mGluR3; and group III embraces mGluR4, mGluR6, mGluR7, and mGluR8. In general, group I receptors are coupled to the phospholipase (PL) C/InsP<sub>3</sub>/Ca<sup>2+</sup> release cascade, whereas groups II and III receptors are linked up to the adenylyl cyclase/cyclic AMP/PKA release cascade (Niswender and Conn, 2010). Initial studies performed with agonists (or antagonists), which bind the intragroup-shared extracellular orthosteric sites, indicated that activation of group II or group III mGluRs brought about neuroprotection, whereas activation of group I mGluRs elicited either neuroprotection or neurotoxicity according to experimental models and conditions employed (Nicoletti et al., 1999; Bruno et al., 2001). More recent studies using PAMs or NAMs, which are receptor subtype-specific, brought to light a somewhat different picture (see for references: Gregory and Conn, 2015). Indeed, the allosteric modulation of mGluRs is a major area of interest for Basic and Clinical Pharmacology (Stansley and Conn, 2019). In the CNS, mGluRs are involved in the regulation of glutamate uptake, cell proliferation, neurotrophic support, and proinflammatory responses. Accordingly, the potential therapeutic spectrum of mGluRs allosteric modulators embraces AD, and also covers PD, stress, anxiety, autism, depression, and schizophrenia (Stansley and Conn, 2019).

### Group I mGluRs (-1 and -5)

Group I mGluR1 and mGluR5 are expressed at postsynaptic membranes, couple to G $\alpha_q$ , and positively modulate neuronal excitability through the interaction with scaffolding proteins such as Homer or Shank. The consequent activation of phospholipase C leads to an increase in [Ca<sup>2+</sup>]<sub>i</sub>. Activation of group I mGluRs may set off a multiplicity of neurons' and astrocytes' signaling pathways variously modulating synaptic plasticity and, likely, synaptic protein synthesis (D'Antoni et al., 2014). These transduction mechanisms form a highly complex network including polyphosphoinositide hydrolysis, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phospholipase D, phospholipase A2, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), and the endocannabinoid 2-arachidonoylglycerol synthesis. Activation of ERK and mTOR by group I mGluRs is especially linked to *de novo* protein synthesis in neurons, a process that underlies long-term changes in activity-dependent synaptic plasticity. Group I mGluRs also enhance postsynaptic excitability thus exacerbating neuronal damage (Nicoletti et al., 1996). It is also noteworthy to recall here that in preclinical studies *antagonists* of mGluR1 and mGluR5 exhibited anxiolytic-like properties just as did *agonists* of group II/III mGluRs (Stachowicz et al., 2007).

By interacting with NMDARs, mGluR1 and mGluR5 regulate neuronal developmental plasticity. The interaction between group



I mGluRs and NMDARs is reciprocal (Alagarsamy et al., 1999). Moreover, the expression of these receptors is developmentally regulated (Nicoletti et al., 1986; Schoepp and Johnson, 1989; Minakami et al., 1995; Romano et al., 1996; Casabona et al., 1997). Group I mGluRs are cross-linked with NMDARs through a chain of anchoring proteins (Tu et al., 1999), and their activation amplifies NMDA currents (Aniksztejn et al., 1995; Awad et al., 2000; Pisani et al., 2001; Skeberdis et al., 2001; Heidinger et al., 2002; Kotecha and MacDonald, 2003). In addition, activation of mGluR1 accelerates NMDARs trafficking (Lan et al., 2001). The NMDA component of long-term potentiation (LTP) is abolished in mice lacking mGluR5 (Jia et al., 1998). In cultured neurons and developing brains the interaction between mGluR5 and NMDAR is amplified by EphrinB2 (Calò et al., 2005), a ligand for EphB receptor tyrosine kinases playing a role in activity-dependent synaptic plasticity in the CNS (Slack et al., 2008).

In the developing brain, mGluR5 contributes to the functional maturation of astrocytes since mGluR5 ablation leads to serious deficits in arborization of astroglial processes and in the expression of glutamate transporters (Morel et al., 2014; Verkhatsky and Nedergaard, 2018).

To date, the roles of group I mGluRs in the pathogenesis of AD are poorly understood and the object of controversy.

*In vitro* cultured fetal (E15) Sprague–Dawley rat neurons expressed mGluR1 whereas neonatal astrocytes did not. These findings limited to neurons the investigation of an alleged neuroprotective effect of the mGluR1/R5 agonist, 3,5-dihydroxyphenylglycine (DHPG), against A $\beta$  neurotoxicity, which was instead suppressed by the mGluR1 antagonist JNJ 16259685 (3,4-dihydro-2H-pyrano(2,3-b)quinolin-7-yl)-(cis-4-methoxy-cyclohexyl)-methanone). Interestingly, estrogen- $\alpha$  receptors (E- $\alpha$ Rs) could activate the same neuroprotection against A $\beta$  toxicity and cell survival pathways as mGluR1 did. Indeed, E- $\alpha$ Rs and mGluR1 were colocalized in cultured cortical neurons and were interdependent in activating the PI3K/Akt pathway that favors cell survival in pure neuronal cultures (Spampinato et al., 2012).

As regards mGluR5s, they are expressed by both astrocytes and neurons in all the CNS areas, signal through Gq protein (Vanzulli and Butt, 2015), and partake in synaptic plasticity, assembly of neuronal circuitry, and neuronal viability (Ballester-Rosado et al., 2010; Purgert et al., 2014).

Data gained from both *in vitro* and animal models suggest that the synaptic dysfunction of mGluR5s might favor the development of AD (Kumar et al., 2015). mGluR5s are overexpressed by astrocytes as a reactive response induced by stimulation with growth factors (i.e. FGF, EGF, and TGF- $\beta$ 1) or by exposure to soluble A $\beta$  oligomers (A $\beta$ -os) *in vitro* (Casley et al., 2009; Grolla et al., 2013; Lim et al., 2013). A $\beta$ -os exposure also raises the expression of type I InsP3Rs, which are placed downstream from mGluR5, and strengthens Ca<sup>2+</sup> responses mediated through the mGluR5/InsP<sub>3</sub>R cascade in hippocampal astrocytes (Grolla et al., 2013). Notably, astrocytes surrounding A $\beta$  senile plaques overexpressed mGluR5, which was associated with Ca<sup>2+</sup> signaling dysregulation and abnormal ATP release in APP<sub>swE</sub>/PS1 transgenic AD-model mice (Shrivastava et al., 2013). Reportedly, A $\beta$ -os exposure caused an excessive clustering

and widely reduced diffusion of A $\beta$ -os/mGluR5 complexes on the plasma membrane of *in vitro* rat embryo astrocytes. These effects were coupled with an augmented Ca<sup>2+</sup> influx altogether damaging synapses (Renner et al., 2010; Shrivastava et al., 2013). Activation of mGluR5s by the allosteric agonist DHPG increased ATP release from A $\beta$ -os-exposed astrocytes, which delayed mGluR5 diffusion in cultures of astrocytes plus/minus neurons *in vitro* (Renner et al., 2010)—an effect mGluR5's selective antagonist MPEP counteracted thus preventing A $\beta$ -os/mGluR5-driven synaptotoxicity (Shrivastava et al., 2013).

Interestingly, proinflammatory cytokines like interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) downregulated the expression of mGluR5 while upregulating that of mGluR3 in cortical astrocytes isolated from the hSOD1(G93A) rat model of amyotrophic lateral sclerosis (entailing like AD an intense neuroinflammation) and cultured *in vitro*. These findings suggested the existence of a protective antiexcitotoxic adaptive mechanism (Berger et al., 2012). In fact, the mGluR5 selective antagonist MPEP hampered the astrocytes' secretion of two proinflammatory cytokines, IL-6 and IL-8 (Shah et al., 2012). Therefore, the activation of astrocytes' mGluR5 advances the release of proinflammatory cytokines, which then downregulate mGluR5 expression. This indicates that under physiological conditions a reciprocal feed-back mechanism controls the expression levels of mGluR5 in astrocytes and in microglia too (Berger et al., 2012). This mechanism might be offset by the A $\beta$ -os-forced overexpression of mGluR5 in AD, thus potentiating the release of toxic amounts of proinflammatory cytokines and glutamate. Next, the latter increases the production/release of p-Tau-os and of NO and the activity of apoptotic caspase-3 (Talanta et al., 2013; Lee et al., 2014).

Another noteworthy study showed that the activation of mGluR5 stimulated the  $\alpha$ -secretase-mediated extracellular shedding of neurotrophic and neuroprotective sAPP- $\alpha$  (Sokol et al., 2011), also an agonist of GABA<sub>B</sub>R1a receptors (Rice et al., 2019). But mGluR5 forms complexes with the Homer proteins that interact with and activate NMDARs (Tu et al., 1999; Awad et al., 2000; Attucci et al., 2001; Moutin et al., 2012). A $\beta$ <sub>1-42</sub>-os can bind mGluR5s and enhance their clustering together, causing mGluR5 signaling overactivation, intracellular Ca<sup>2+</sup> accumulation, impaired calcium homeostasis, and synaptic disruptions (Renner et al., 2010; Zhang et al., 2015b). In greater detail, mGluR5s act as coreceptors for A $\beta$ -os bound to prion protein (PrP<sup>C</sup>). Next PrP<sup>C</sup> activates the mGluR5, which elicits the loss of synapses through *Fyn* tyrosine kinase activation and eukaryotic elongation factor 2 (eEF2) phosphorylation (Um et al., 2013). *Fyn* phosphorylates NR2A and NR2B subunits of NMDA receptors thus altering the receptors' intracellular trafficking that is essential for synaptic plasticity. Moreover, interactions between *Fyn* tyrosine kinase and Tau proteins play a role in regulating the synapse function and the postsynaptic toxic signaling pathways driven by A $\beta$ -type excitotoxicity, causing the loss of dendritic spines. Notably, A $\beta$ -os exposure also induces the eEF2 phosphorylation by eEF2 kinase that is known to associate with mGluR5. A $\beta$ -os-induced impairment of LTP is dependent on eEF2 phosphorylation that is increased in brains from both *tg* AD-model mice and AD patient autopsies (Nygaard, 2018).

Altogether these data support the view that mGluR5 activation by specific PAMs facilitates excitotoxic mechanisms causing the death of neurons (Parmentier-Batteur et al., 2014), whereas mGluR5-specific NAMs act neuroprotectively in AD-model animals (Bruno et al., 2017). Administering MPEP, a mGluR5 selective antagonist, prevents this synaptic loss in *tg* AD-model mice (Rammes et al., 2011; Hu et al., 2014; Kumar et al., 2015). In addition, deletion of mGluR5 prevents memory loss in AD-model mice (Hamilton et al., 2014). But to gain these benefits there is a price to pay, which is the negative impact of mGluR5 selective antagonists on activity-dependent synaptic plasticity mechanisms in brain regions that are not affected by AD (Bruno et al., 2017). Of course, the translatability of these interesting results to human AD patients remains a topic worth exploring.

### Group II mGluRs (-2 and -3)

Group II mGluR2 and mGluR3 are mostly localized presynaptically. Depending on the nature of the ligand, mGluR2s signal *via* Gi/o or Gq11 proteins (González-Maeso et al., 2008; Fribourg et al., 2011) and negatively modulate neuronal excitability (Conn and Pin, 1997). Thus, activation of group II mGluRs is endowed with potential neuroprotective properties as it may curtail glutamatergic signaling and mitigate neuronal hyperexcitability (Nicoletti et al., 1996). Stimulation of group II mGluRs inhibits adenylyl cyclase (AC), activates K<sup>+</sup> channels, and blocks presynaptic voltage-gated calcium channels, thus hindering intracellular Ca<sup>2+</sup> fluxes and synaptic neurotransmitters release (Benarroch, 2008; Niswender and Conn, 2010). Groups II mGluRs also team with the MAPK and PI3K pathways to confer neuroprotection (D'Onofrio et al., 2001). As mentioned also below, neuroprotection is mediated by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) released through astrocytes' mGluR3 signaling. TGF- $\beta$ 1 binds and activates its membrane receptors coupled with serine/threonine kinase activity thereby inducing the *Smad* signaling cascade. It also synergistically operates with other neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) (Caraci et al., 2011b). In the rodents' thalamus, the selective activation of mGluR2 modulates the inhibition at synapse level of sensory neurons functionally linked to information processing, attention, and cognition (Copeland et al., 2017). Conversely, the selective activation of mGluR2 increases the incidence of neuronal deaths *in vitro* (Corti et al., 2007; Caraci et al., 2011b). Accordingly, a mGluR2-specific NAM hindered the death of ischemia-sensitive neurons in the hippocampal CA1 area, whereas a mGluR2-specific PAM promoted the death both of ischemia-sensitive CA1 neurons and of ischemia-resistant CA3 neurons (Motolese et al., 2015). Recent investigations have revealed the formation of intragroup and intergroup heterodimers between different mGluRs (Doumazane et al., 2011; Rondard et al., 2011; Kammermeier, 2012). New allosteric modulators capable of differentiating homodimers from heterodimers have disclosed the assembly of mGluR2/mGluR4 heterodimers in corticostriatal fibers (Yin et al., 2014).

The mGluR3, whose activation inhibits AC activity and hence cyclic AMP production, is the most abundant astroglial receptor along all the lifetime (Sun et al., 2013). Mounting evidence indicates that mGluR3 upkeeps synaptic homeostasis, including synaptic plasticity and synaptogenesis (see for references: Durand et al., 2013). In addition, activated mGluR3 plays major neuroprotective roles in AD and other neuropathologic conditions. Once added to pure cultures of newborn rat astrocytes, the orthosteric agonists LY379268 or LY354740 specifically activated mGluR3 (rodent astrocytes do not express mGluR2), promoting the production and release of TGF- $\beta$  and of GDNF (see also above and Caraci et al., 2011a). The same agonists increased the expression of  $\alpha$ -secretase, whose activity is essential for APP's physiological NAP. The upshot is an amplified extracellular shedding of the neurotrophic and neuroprotective and GABA<sub>B</sub>1a agonist sAPP- $\alpha$  (Figure 1) (Bruno et al., 1997; Bruno et al., 1998; Corti et al., 2007; Battaglia et al., 2009; Di Liberto et al., 2010; Battaglia et al., 2015; Rice et al., 2019). Moreover, an indirect role for mGluR3 in AD is denoted by the progressive decrease with aging in mGluR2 and mGluR3 expression and, consequently, in their antiamyloidogenic action in hippocampal astrocytes from PDAPP-J20 AD-model mice (Durand et al., 2014). In subsequent studies, the same authors showed that the LY379268-elicited activation of astrocytes' and neurons' mGluR3 suppressed or mitigated the A $\beta$ -driven neurotoxicity and death of both neurons and astrocytes. In both cell types, agonist-activated mGluR3 increased the shedding of neuroprotective sAPP- $\alpha$  and the expression of BDNF. In addition, LY379268-activated mGluR3s induced astroglia- and microglia-mediated phagocytosis and removal of A $\beta$ s from the extracellular environment. Finally, mGluR3 orthosteric agonists LY379268 or LY404039 suppressed the nitric oxide (NO)-induced death of cultured rat astrocytes *via* the inhibition of AC, which reduced intracellular cAMP levels, the activation of Akt, and the formation of antiapoptotic p65 and c-Rel complexes of the NF- $\kappa$ B family (Durand et al., 2011; Durand et al., 2017).

Conversely, Caraci et al. (2011a) showed that mGluR2 and mGluR3 enhanced neurotoxicity in pure cultures of rat brain neurons challenged with A $\beta$ <sub>1-42</sub> or with its neurotoxic fragment A $\beta$ <sub>25-35</sub>. However, if the neurons were cocultured with the astrocytes, the activation of mGluR2 and mGluR3 brought about neuroprotective effects through the release of TGF- $\beta$ <sub>1</sub> from the astrocytes. TGF- $\beta$ <sub>1</sub> is a well-known agent endowed with neuroprotective and anti-inflammatory activities (see also above) in experimental AD-models (Chen et al., 2015) as it also stimulates microglia to scavenge A $\beta$ s (Tichauer and von Bernhardt, 2012).

### Group III mGluRs (-4, -6, -7, and -8)

Group III mGluRs (-4, -6, -7, and -8) are mainly localized presynaptically, couple to Gai/o, and negatively modulate neuronal excitability (Conn and Pin, 1997). They are likely to act as autoreceptors on glutamatergic synaptic terminals and as heteroreceptors on GABAergic and other neurotransmitter terminals (Cartmell and Schoepp, 2000; Ferraguti and Shigemoto, 2006). Group III mGluRs stimulation results in AC inhibition, K<sup>+</sup> channels

activation, and block of presynaptic voltage-gated calcium channels, thus decreasing  $\text{Ca}^{2+}$  flow into cells and neurotransmitters release from synapses (Benarroch, 2008; Niswender et al., 2008). Therefore, their activation elicits potential neuroprotective effects that dampen glutamatergic signaling and inhibit neurotransmitters release thereby mitigating neuronal excitability (Nicoletti et al., 1996). As a particularity, activated mGluR7 stimulates protein kinase C (PKC) or phospholipase C resulting in the inhibition of neuronal calcium channels (Perroy et al., 2000; Pelkey et al., 2007). Brain expression of mGluR4, -7, and -8 is proper of cortical and hippocampal neurons and of synapses located in the basal nuclei (striatum, pallidum), subthalamic nucleus, and substantia nigra (both pars compacta and pars reticularis) (Bruno et al., 1996; Faden et al., 1997; Hovelsø et al., 2012). Instead, mGluR6 expression is exclusive of the retina (Nakajima et al., 1993). The main subcellular location of mGluR7 is at the central area of presynaptic terminals just where the membrane coalesces with synaptic vesicles: this suggests its involvement in the modulation of neurotransmitter release. Conversely, mGluR4 and mGluR8 are placed at the periphery of presynaptic terminals (Shigemoto et al., 1997; Schoepp, 2001; Palucha and Pilc, 2007). Group III mGluRs also cooperate with MAPK and PI3K signaling pathways to impart neuroprotection (Iacovelli et al., 2002). Recently, these receptors have become the focus of therapeutic attempts because they (i) can modulate defective neurotransmission yielding symptomatic improvements through the neuroprotective hindering of multiple neurodegenerative mechanisms and (ii) have more favorable safety and tolerability profiles (Hovelsø et al., 2012). Activation of group III mGluRs by glutamate and/or other agonists is neuroprotective as it inhibits glutamate release from neurons' presynaptic terminals and from microglia, thus mitigating excitotoxicity; concurrently, astrocytes intensify the uptake of glutamate and microglia increase neurotrophic factors synthesis (see Williams and Dexter, 2014 for an in-depth review on this topic).

Rather few studies exist about the effects on a neurodegenerative disease like AD exerted by group III mGluRs activation *via* broad spectrum agonists and PAMs or inactivation *via* NAMs. Copani et al. (1995) reported that broad-spectrum group III mGluRs agonists L-serine-O-phosphate (L-SOP) and L-2-amino-4-phosphono-butanoate (L-AP4) could lessen the apoptotic death rate of neurons exposed to A $\beta$ s. The authors suggested that such agonists would exert neuroprotective effects in AD. Similarly, group III agonist RS-PPG, which activates preferentially mGluR8 and likely also mGluR4, exerted neuroprotective actions on neurons exposed to harmful hypoxic or hypoglycemic conditions (Bruno et al., 2000; Sabelhaus et al., 2000). Notably, acute hypoxia can induce neurons to overproduce lethal amounts of A $\beta$ s *via* a mechanism involving another family C GPCR, the CaSR (Kim et al., 2014; Bai et al., 2015). Besides, PHCCC, a specific mGluR4 PAM, and also a partial antagonist of group I mGluRs, protected cultured cortical mouse neurons against the A $\beta$ s-elicited cytotoxicity and NMDAR excitotoxicity (Maj et al., 2003).

But what about the astrocytes? Under basal conditions, rodent (rat and mouse) astrocytes in primary cultures express mGluR4, but neither mGluR7 nor mGluR8 (Phillips et al., 1998; Janssens and Lesage, 2001). However, mGluR8 is expressed

by reactive astrocytes adjacent to chronic inflammatory lesions (Geurts et al., 2005). Besong et al. (2002) provided evidence that broad spectrum orthosteric agonists activating group III mGluRs, like L-AP4, 4-phosphonophenylglycine (4-PPG), or L-SOP hindered the expression and secretion of the proinflammatory chemokine RANTES in astrocyte cultures. These beneficial effects of the mGluR4 broad spectrum agonists were counteracted by pretreating the astrocytes cultures with the selective group III mGluRs NAM (R,S)- $\alpha$ -methyl-serine-O-phosphate or with pertussis toxin. Altogether, these findings suggest that such agonists might mitigate neuroinflammation in conditions like AD, multiple sclerosis, and experimental allergic encephalomyelitis.

Extracellular glutamate homeostasis, which is essential for physiological glutamatergic neurotransmission and excitotoxicity prevention, depends on the activity of astrocytes' transporters like GLT-1 and GLAST (Anderson and Swanson, 2000). Neuroinflammatory conditions associated with a neurodegenerative disease like AD or experimental treatments (e.g. with LPS, MPTP, etc.) reduce astrocytes' GLT-1- and GLAST-mediated glutamate uptake due to a fall in endogenous antioxidant glutathione (GSH) activity. Broad spectrum group III mGluRs agonists rescue GSH normal levels and restore astrocytes' GLT-1- and GLAST-mediated glutamate uptake alleviating neuronal excitotoxicity (Yao et al., 2005; Zhou et al., 2006; Foran and Trotti, 2009). Thus, activation of astrocytes' group III mGluR3 and mGluR5 and also of group II mGluRs by broad spectrum agonists increases GLT-1- and GLAST protein expression and glutamate uptake activity as the signaling of both groups likely involves Gi/o, MAPKs, and PI3K pathways (Aronica et al., 2003; Beller et al., 2011; Williams and Dexter, 2014). The activation of group III mGluRs by wide spectrum agonists also curtails the release of proinflammatory cytokines from activated microglia (Combs et al., 2000). Wide spectrum group III mGluRs agonists also hinder proinflammatory cytokines release, RANTES included, from the astrocytes exposed to neurotoxic agents (Mennicken et al., 1999; Besong et al., 2002), thereby helping mitigate neuroinflammation and reduce neuronal demise. Therefore, one might surmise that the effects of these agonists on astrocytes and microglia would likely impact on the course of AD and perhaps also of other neurodegenerative diseases.

## CALCIUM SENSING RECEPTOR

The CaSR is a (poly)cationic receptor, as its evolutionary history shows (Riccardi and Kemp, 2012). This is why CaSR's preferred yet not unique orthosteric agonist is  $\text{Ca}^{2+}$ . A CR region, necessary for receptor activation (Huang et al., 2011; Hendy et al., 2013) connects CaSR's huge (~612 amino acids) ECD, the bilobed (LB1 and LB2) VFT to the 7TM domain whose seven transmembrane  $\alpha$ -helices (TM1-TM7) are joined by three extracellular and three intracellular loops. Two domains of the CASR's intracellular C-terminal tail are necessary for CaSR expression at the cell surface and its composite signaling functions *via* G-proteins (see below). The VFT contains the binding pockets for the

orthosteric (type I) agonists (Hendy et al., 2013), which besides extracellular  $\text{Ca}^{2+}$  (Hofer and Brown, 2003), include various divalent and trivalent cations, polyamines, aminoglycoside antibiotics, and cationic polypeptides (Silve et al., 2005; Saidak et al., 2009; Magno et al., 2011; Zhang et al., 2015a). The CaSRs of human cortical astrocytes also specifically bind A $\beta$ s, likely at the VFTs (Dal Prà et al., 2014a; Dal Prà et al., 2014b; Dal Prà et al., 2015b). Moreover, X-ray crystallography studies (Geng et al., 2016) have revealed that in the resting state the 3D structure of CaSR's ECD exhibits an open conformation kept up by  $\text{PO}_4^{3-}$  anions. Independently of the presence or absence of  $\text{Ca}^{2+}$  ions, CaSR activation occurs when an L- $\alpha$ -amino acid closes the VFT groove, triggering the formation of a new homodimer interface between the membrane-proximal LB2 and the CR domains.  $\text{Ca}^{2+}$  ions stabilize the active state to fully activate the receptor. Indeed, CaSR's ECD is endowed with four  $\text{Ca}^{2+}$ -binding sites, of which the  $\text{Ca}^{2+}$  ion at site #4 stabilizes, upon orthosteric agonist binding, the CaSR homodimer's active conformation (Geng et al., 2016). Importantly, orthosteric agonists also induce the dissociation of inhibitory  $\text{PO}_4^{3-}$  anions from the arginine residues acting as their relatively weak binding sites. Thus, the CaSR-inactivating action of bound  $\text{PO}_4^{3-}$  anions is overturned (Quinn et al., 1997; Cheng et al., 2004; Geng et al., 2016). As the other GPCRs do, CaSR swings between conformation-varying active and inactive states (Rosenbaum et al., 2011). The changes in conformation due to activation include a rearrangement of the 7TM and ICD domains. The CaSR's 7TM helical domains can modulate signal transduction. The 7TM's intracellular loops 2 and 3 are crucially involved in the activation of downstream effectors (Goolam et al., 2014). Besides, various CaSR's 7TM sites bind allosteric (type II) ligands. The latter include both the aromatic L- $\alpha$ -amino acids and the highly selective *allosteric agonists* or *PAMs*, short-termed *calcimimetics*, and *allosteric antagonists* or *NAMs*, short-termed *calcilytics* (Nemeth, 2002). As will be discussed later, these pharmacological agents offer exciting perspectives in the field of clinical therapeutics. In response to orthosteric ligand binding, the CaSR's ICD tails interact with  $\text{G}_s$  or  $\text{G}_{q/11}$  or  $\text{G}_{12/13}$ , or  $\text{G}_{i/o}$ , proteins (Chang et al., 2001; Hofer and Brown, 2003; Conigrave and Ward, 2013), and with  $\beta$ -arrestin 1/2 (Thomsen et al., 2012). Such interactions turn on several signaling pathways (Saidak et al., 2009; Magno et al., 2011), which underlie the receptor's complex actions and comprise: (i) second messenger-producing enzymes (e.g., AC); (ii) phospholipases A2, C, and D; (iii) protein kinases (e.g. PKCs, MAPKs, AKT); (iv)  $\text{Ca}^{2+}$  influx *via* TRPC6-encoded receptor-operated channels; and (v) transcription factors (reviewed in Zhang et al., 2015a). Moreover, the intracellular adaptor-related protein complex (AP2) binds the CaSR's ICD promoting the receptor's clathrin-mediated endocytosis (Nesbit et al., 2013). Finally, CaSR's ICD ubiquitylation and phosphorylation modulate the receptor's recycling, degradation, and desensitization (Zhuang et al., 2012; Breitwieser 2013).

In general, the CaSR preserves systemic  $\text{Ca}^{2+}$  homeostasis by promptly sensing any changes in the extracellular calcium concentration  $[\text{Ca}^{2+}]_e$  and, accordingly, by modulating the amounts of parathyroid hormone (PTH) released from parathyroid glands as well as the reabsorption of  $\text{Ca}^{2+}$  from

kidneys and its deposition in bones (Hofer and Brown, 2003). Dysfunctions of the CaSR severely alter systemic  $\text{Ca}^{2+}$  homeostasis (Brown, 2007; Hendy et al., 2009). Gain-of-function CaSR mutations result in autosomal dominant hypocalcemia, whereas loss-of-function CaSR mutations cause severe neonatal primary hyperparathyroidism (Hendy et al., 2009; Ward et al., 2012; Hannan et al., 2018).

But, what about the CaSR in the brain? All types of brain neural and cerebrovascular cells express the CaSR, with particular intensity in the hippocampus, an AD-relevant area (Chattopadhyay, 2000; Yano et al., 2004; Noh et al., 2015). Dal Prà et al. (2005) showed that untransformed astrocytes isolated from the adult human temporal cortex and cultured *in vitro* express functional CaSRs, less intensely when proliferating but more strongly when mitotically quiescent. Notably, changes in the growth medium  $[\text{Ca}^{2+}]_e$  did not impact on CaSR expression levels by adult human astrocytes. But preservation of systemic  $\text{Ca}^{2+}$  homeostasis is not the CaSR's main task in the brain. In fact, fluctuations in  $[\text{Ca}^{2+}]_e$  physiologically modulate, *via* corresponding adaptations of CaSR signaling, a variety of neural cells activities like CaSR's L-amino acid sensing (Conigrave and Hampson, 2006),  $\text{K}^+$  fluxes (Chattopadhyay et al., 1999), proliferation, differentiation, migration of both neurons and oligodendrocytes during growth, and synaptic plasticity and neurotransmission during postnatal life (Bandyopadhyay et al., 2010; Riccardi et al., 2013; Ruat and Traiffort, 2013; Kim et al., 2014; Noh et al., 2015; Tharmalingam et al., 2016).

Remarkably, CNS diseases, such as AD and ischemia/hypoxia/stroke, change the CaSR's expression levels and hence alter the cellular processes CaSR signaling regulates (Armato et al., 2013; Dal Prà et al., 2014a; Dal Prà et al., 2014b; Bai et al., 2015; Dal Prà et al., 2015b). The first hint that the CaSR might play a role in AD pathogenesis stemmed from the observation that A $\beta$ s-elicited peaks of cytosolic  $[\text{Ca}^{2+}]_i$  had a killing effect on hippocampal neurons (Brorson et al., 1995). A second clue was the opening of  $\text{Ca}^{2+}$ -permeable nonselective cation channels (NSCCs) by fibrillar A $\beta_{1-40}$  or A $\beta_{25-35}$  in hippocampal neurons of wild type (WT) *CaSR*<sup>+/+</sup> rats; notably, this effect could not be replicated in *CaSR*<sup>-/-</sup> rats. The authors speculated that A $\beta$ s might bind the CaSR because they have, just like polyamines, orderly spaced arrays of positive charges (Ye et al., 1997).

In this regard, the specific formation of plasma membrane A $\beta$ s•CaSR complexes and their subsequent endocytosis in cultured cortical untransformed adult human astrocytes could be proven by using the *in situ* proximity ligation assay (isPLA), which reveals the specific formation of stable complexes between two molecules placed within a 30 nm range (Dal Prà et al., 2014a; Dal Prà et al., 2014b; Dal Prà et al., 2015b). The latter results implied that since all types of human neural and cerebrovascular cells express the CaSR, they are vulnerable to the neurotoxic effects driven by pathological A $\beta$ •CaSR signaling (Chiarini et al., 2016). However, it remains to be ascertained whether at the level of CaSR•GABA<sub>B</sub>R1 heterodimers of human cortical astrocytes and neurons A $\beta$ s•GABA<sub>B</sub>R1 complexes also form and what their functional roles would be under both physiological and pathological conditions: topics worth investigating further.

Moreover, a genetic analysis study on cohorts of 435 healthy controls and 692 SAD patients showed that an intron 4 polymorphic dinucleotide repeat marker of the *CASR* gene associated with an AD susceptibility, while three nonsynonymous SNPs of exon 7 were linked with an AD propensity only in non-*APOEε4* allele carriers. Hence, variations in the *CASR* gene sequence may impact on SAD susceptibility especially in subjects having no *APOEε4* allele (Conley et al., 2009).

The *CASR* gene P1 and P2 promoters regulate its transcription by binding several transcription factors, including SP1, SP3, STAT1, STAT3, CREB, and NFκB, which concurrently control the expression of other AD-related genes (see for details and references: Chiarini et al., 2016). Therefore, the transcription factors regulation of CaSR expression is tightly linked to the pathophysiology of AD.

It is well known that Aβ<sub>42</sub>-os simultaneously bind to several other CNS cells surface receptors besides the CaSR (see for details and references: Chiarini et al., 2016). Therefore, Aβ<sub>42</sub>-os•CaSR signaling triggers a throng of cellular responses some authors include in the so called “calcium dyshomeostasis”, such as toxic ROS overrelease from mitochondria, and intracellular Ca<sup>2+</sup> surges *via* NMDARs' activation driving further mitochondrial ROS releases (Kam et al., 2014; Jarosz-Griffiths et al., 2016).

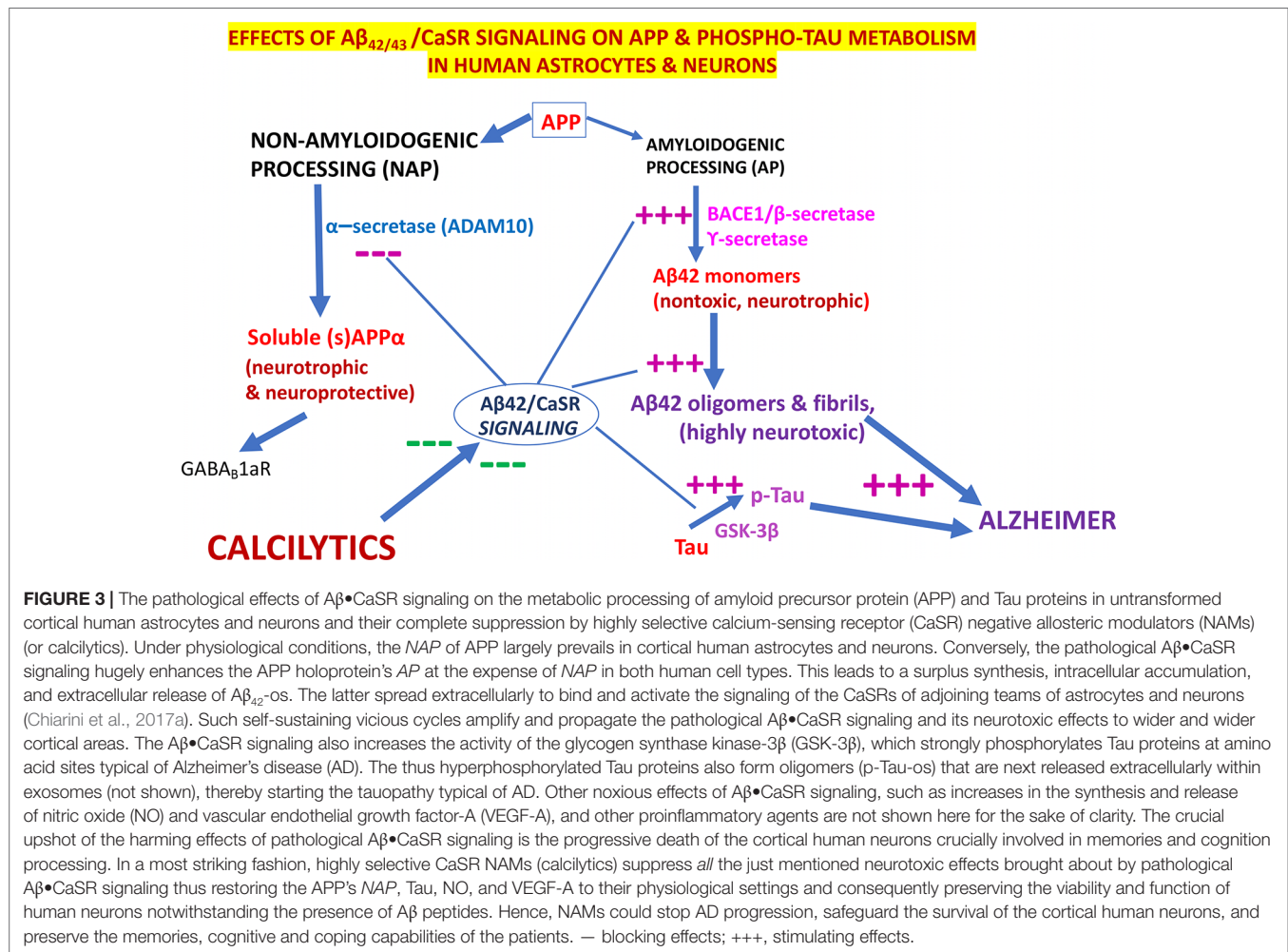
However, it must be stressed here that the pathological Aβ<sub>42</sub>-os•CaSR signaling performs much more AD-specific upstream feats than those just mentioned. In fact, it drives the overproduction and overrelease of Aβ<sub>42</sub>-os and p-Tau-os, the two main AD culprits, from human cortical neurons and astrocytes. Moreover, it also induces the production and release of surpluses of other neurotoxic agents, such as NO and VEGF-A, and likely others more, from the adult human cortical astrocytes. Additionally, the pathological Aβ<sub>42</sub>-os•CaSR signaling profoundly suppresses sAPP-α extracellular shedding from human astrocytes and neurons (Chiarini et al., 2016; Chiarini et al., 2017a; Chiarini et al., 2017b). These Aβ-os-elicited noxious effects associate with concurrent upsurges in the expression of APP, BACE1, and CaSR proteins. Remarkably, the crucial upshot of all the mentioned effects of Aβ<sub>42</sub>-os•CaSR signaling is the death of human cortical neurons both *in vitro* (Armato et al., 2013) and in the *in vivo* brain. In the latter, the progressive disconnections of neural circuits—a cause of advancing cognitive decline—and a chronic diffuse reactive neuroinflammation eventually lead to full blown or symptomatic AD (Crimins et al., 2013; Kaye and Lasagna-Reeves, 2013; Medeiros et al., 2013).

Moreover, a study using *3xTg* AD-model mice showed that the amount of brain CaSR immunoreactivity progressively increased with age, particularly in areas where Aβ<sub>42</sub> fibrils accumulate most, such as the hippocampi. Thus, local fibrillar Aβ<sub>42</sub> buildup and CaSR expression raise in parallel in both Aβ-exposed human cortical neurons and astrocytes cultured *in vitro* and in the hippocampi of *3xTg* AD-model mice (Armato et al., 2013; Chiarini et al., 2016; Gardenal et al., 2017). This soaring expression of neural cells' CaSRs associates with a declining expression of inhibitory GABA<sub>B</sub>1as (Chang et al., 2007; Kim et al., 2014).

Whereas GABA<sub>B</sub> and taste receptors obligatorily function as heterodimers (Jones et al., 1998; Nelson et al. 2002), mGluRs

and CaSR function both as disulfide-linked homodimers (Zhang et al., 2001; Pidasheva et al., 2006) and as CaSR/GABA<sub>B</sub>Rs, CaSR/mGlu1aR and CaSR/mGlu5R heterodimers (Gama et al., 2001). Ectopic overexpression and coimmunoprecipitation studies revealed that CaSR/GABA<sub>B</sub>R1a heterodimers do affect CaSR protein expression in opposing ways. The total and cell surface expression and signaling of the CaSRs were suppressed by coexpressing GABA<sub>B</sub>R1as, being instead increased (i) by co-expressing GABA<sub>B</sub>2Rs; (ii) by knocking out GABA<sub>B</sub>R1a in mouse brains; and (iii) by deleting GABA<sub>B</sub>R1a in cultured hippocampal neurons. The GABA<sub>B</sub>Rs and CaSRs form heterodimers as soon as they are synthesized, since these protein complexes are already detectable around the cells' nuclei and in the endoplasmic reticulum. In such early complexes GABA<sub>B</sub>Rs bind an immature form of the CaSR. Clearly, GABA<sub>B</sub>R1a and GABA<sub>B</sub>R2 subunits compete for the CaSRs. The CaSR/GABA<sub>B</sub>R heterodimers appear to have altered pharmacological properties with respect to the prevailing CaSR homodimers. Results gained using (i) the GABA<sub>B</sub>Rs agonists baclofen and GABA, (ii) the GABA<sub>B</sub>R1a antagonist CGP-3548, and (iii) GABA<sub>B</sub>R1a expression knockdown in cultured mouse growth plate chondrocytes indicated that GABA<sub>B</sub>R1a can elicit both CaSR-independent and CaSR-mediated actions. However, divergent results gained from different experimental models suggested that an endogenous expression or a targeted overexpression of one or more of these receptors, coexisting differences in ligands and in their relative quantities and in downstream intracellular signaling pathways could elicit unlike upshots under various physiological and/or pathological conditions (Gama et al., 2001; Chang et al., 2007). During the initial phases of disease progression in AD-model animals, the decline of GABA<sub>B</sub>R1s' availability, which concurs with CaSRs overexpression, induced a *neuronal hyperactivity* in hippocampal and cerebrocortical circuits, whose upshot was functional impairment (Busche and Konnerth 2015). The mechanism(s) underlying this loss of neuronal working capability remain(s) unclear: an overconsumption of O<sub>2</sub> on the part of the hyperactive neurons might be a contributory factor. Nothing is so far known about the existence and pathophysiological roles of CaSR heterodimers in cortical human untransformed astrocytes and neurons. Therefore, to-date the impacts (if any) the CaSR/GABA<sub>B</sub>Rs and CaSR/mGluRs heterodimers might exert on human AD's course and on anti-AD therapeutic approaches remain to be assessed.

Notably, in cortical adult human astrocytes the pathological Aβ•CaSR signaling heavily affects the APP holoprotein metabolism significantly deflecting it from its physiological *NAP* (Figure 3). APP's *NAP* typically obstructs the *de novo* production of Aβ<sub>42</sub>s/Aβ<sub>42</sub>-os since the α-secretases (mainly ADAM 10) cut the APP molecule just within the Aβ<sub>42</sub> amino acid sequence (Kuhn et al., 2010) (Figures 1 and 3). Notably, APP's *NAP* prevails over APP's *AP* in untreated (control) cortical adult human astrocytes, which directly shed all the sAPP-α they produce into the environment while secreting only tiny amounts of monomeric Aβ<sub>42</sub> (Chiarini et al., 2017b). Hence, it has been posited that by constitutively releasing substantial amounts of sAPP-α, which is an agonist of GABA<sub>B</sub>R1as (Rice et al., 2019), human astrocytes could continually abate any noxious neuronal hyperexcitability.



Under the same basal conditions, CaSR signaling is only modulated by extracellular cations levels, particularly by the  $[Ca^{2+}]_e$ . On the other hand, a dramatic change in this mechanism occurs when increased quantities of exogenous A $\beta$ s bind the human astrocytes' (and neurons') CaSRs and activate their pathological signaling that strongly promotes APP's *AP* over APP's *NAP* (Figure 3). This leads to an excess production, accumulation, and secretion of neurotoxic A $\beta$ <sub>42</sub>/A $\beta$ <sub>42</sub>-os from the cortical astrocytes and from the neurons in which an alike APP's *AP* mechanism operates (Armato et al., 2013). Concurrently, the astrocytes' and neurons' intense extracellular shedding of sAPP- $\alpha$  is curtailed by ~70%, while sAPP- $\alpha$  abnormally accumulates within the cells (Chiarini et al., 2017a). On the basis of such results the authors posited that an ongoing A $\beta$ •CaSR signaling that would spread in vicious cycles from teams to teams of "master" astrocytes' and "client" neurons could cause a substantial loss of the neurotrophic and neuroprotective effects otherwise brought about by extracellularly shed sAPP- $\alpha$ , including its agonistic action on GABA<sub>B</sub>1aR, thereby favoring a harmful neuronal hyperexcitability. In addition, the A $\beta$ <sub>42</sub>/A $\beta$ <sub>42</sub>-os-exposed human astrocytes and neurons could simultaneously release increasing amounts of neurotoxic A $\beta$ <sub>42</sub>-os (Armato et al., 2013), p-Tau-os

(within exosomes) (Chiarini et al., 2017a), NO, VEGF-A (Dal Prà et al., 2005; Chiarini et al., 2010; Dal Prà et al., 2014a; Dal Prà et al., 2014b; Chiarini et al., 2016), and likely other noxious agents. Therefore, it would not be surprising that under such dire circumstances cortical human neurons keep losing synapses and consequently die. Interestingly, in line with the just mentioned findings, CSF levels of sAPP- $\alpha$  significantly decrease in LOAD/SAD patients (Lewczuk et al., 2010, which indirectly confirms the substantial fall of its extracellular shedding from human astrocytes (Chiarini et al., 2017b).

### CaSR NAMs as Potential Anti-AD Therapeutics

As mentioned above, several PAMs and NAMs of the CaSR are available. L- $\alpha$ -amino acids with an aromatic ring and positively charged amino groups (NH<sup>3+</sup>) are naturally occurring CaSR PAMs (Lee et al., 2007). Synthetic phenylalkylamine CaSR PAMs ("calcimimetics"; e.g. AMG 416, Cinacalcet, and NPS R-568) having two-to-four aromatic rings and NH<sup>3+</sup> groups have been synthesized. PAMs augment the CaSR's sensitivity to activation by  $[Ca^{2+}]_e$  and hence lower the EC<sub>50</sub> for  $[Ca^{2+}]_e$ .

Notably, three CaSR PAMs, i.e. Evocalcet, Etelcalcitide, and Cinacalcet, have successfully reached the clinical use to mitigate primary and secondary hyperparathyroidism and tumor-elicited hypercalcemias (Nemeth and Goodman, 2016).

CaSR NAMs are small amino-alcohol molecules like NPS 2143 (Nemeth et al., 2001), Calhex 231 (Kessler et al., 2006), NPSP795 (Gafni et al., 2015), or quinazolinones like ATF936 and AXT914 (Wildler et al., 2010). CaSR NAMs right-shift the  $[Ca^{2+}]_e$  response curve decreasing the CaSR sensitivity to  $[Ca^{2+}]_e$  and thus increasing the  $EC_{50}$  for  $[Ca^{2+}]_e$  (Ferry et al., 1997; Huang and Breitwieser, 2007). As previously anticipated, both PAMs and NAMs bind the 7TM domain of the CaSR. The CaSR binding pockets of NAMs and PAMs partially overlap but are not identical. NAMs bind between the TM3 and TM5 loops, whereas both PAMs and NAMs attach between the TM6 and TM7 loops (Petrel et al., 2003; Petrel et al., 2004; Miedlich et al., 2004). It has been shown that point mutated residues of the 7TM helices (i.e. Phe<sup>668</sup>, Phe<sup>684</sup>, Trp<sup>818</sup>, Phe<sup>821</sup>, Glu<sup>837</sup>, and Ile<sup>841</sup>) lessen the antagonism of CaSR NAM NPS 2143 (Petrel et al., 2003; Petrel et al., 2004; Miedlich et al., 2004). CaSR's allosteric agonism and antagonism are modulated *via* the involvement of distinct amino acids and mechanisms (see for further details Leach et al., 2016; Keller et al., 2018). The identification of synthetic allosteric modulators of the CaSR has prompted searches for their therapeutic applications in diseases in which the CaSR signaling is dysfunctional (Hannan et al., 2018). However, hitherto the therapeutic potentials of both PAMs and NAMs of the CaSR have been only modestly exploited (Nemeth, 2013; Saidak et al., 2009; Widler, 2011; Ward et al., 2012). Like other GPCRs, CaSRs exhibit the "ligand-biased signaling" feature, i.e. in a certain type of cell a signaling pathway may be steadily picked up over the others according to the specific ligand involved (Leach et al., 2015). Interestingly, NAMs and PAMs too can induce this biased signaling, which in future might allow to therapeutically target a specific cell type over others (see for details: Davey et al., 2012; Leach et al., 2015; Hannan et al., 2018).

Let's zero in on an important NAMs' feature: they enhance parathyroid hormone (PTH) secretion from the parathyroid glands and increase blood calcium levels (calcemia) (Nemeth, 2004; Nemeth, 2013). Several phase II clinical trials were undertaken to assess CaSR NAMs potential therapeutic efficacy in women with postmenopausal osteoporosis based on the assumption that the released PTH would stimulate osteogenic processes. However, these trials failed because NAMs induced a several hour-lasting oversecretion of PTH that stimulated both osteogenic and osteolytic processes in the osteoporotic bones. These failures prompted to search for new CaSR NAMs inducing a lesser and shorter-lasting PTH release (Nemeth, 2013; Riccardi and Kemp, 2012; Davey et al., 2012; Ward et al., 2012). The same failures also demoted CaSR NAMs from the drugs potentially beneficial in humans even because of the modest hypercalcemia (hyperparathyroidism) they induced. However, attempts were performed to treat hypoparathyroidism and autosomal dominant hypocalcemia (ADH) driven by gain-of-function CaSR mutations with CaSR NAMs (Nemeth, 2013; White et al., 2009; Letz et al., 2010; Park et al., 2013; Nemeth and Goodman, 2016). The use of CaSR NAMs was also considered in cases of

breast and prostate carcinomas to prevent bone metastases, which are established through a CaSR-mediated signaling (Liao et al., 2006; Mihai et al., 2006). Other potential therapeutic uses of CaSR NAMs have included asthma attacks (Yarova et al., 2015); pulmonary artery idiopathic hypertension (Yamamura et al., 2012; Yamamura et al., 2015); stroke (Kim et al., 2014); and, last but not least, LOAD/SAD and EOFAD (Armato et al., 2013; Chiarini et al., 2016; Chiarini et al., 2017a; Chiarini et al., 2017b; Chiarini et al., 2017c).

The use of CaSR NAMs as therapeutics in SAD/LOAD and EOFAD is supported by the results gained from preclinical AD models "in Petri dishes" made up by untransformed human cortical astrocytes and/or neurons. In fact, a 30-min administration of a CaSR NAM, be it NPS 2143 or NPS 89696, completely suppressed all the above-mentioned neurotoxic responses evoked by the pathological  $A\beta$ •CaSR signaling (Chiarini et al., 2010; Armato et al., 2013; Dal Prà et al., 2014b; Dal Prà et al., 2015a; Chiarini et al., 2016; Chiarini et al., 2017c). Therefore, the authors posited that *in vivo* administered CaSR NAMs would (A) preserve the shedding of neurotrophic and neuroprotective and GABA<sub>B</sub>R1a agonist sAPP- $\alpha$  from the plasma membranes of astrocytes and likely neurons, thereby (i) obstructing the amyloidogenesis from APP and hence the cerebral accumulation of neurotoxic soluble  $A\beta_{42}$ -os and fibrillar  $A\beta_{42}$  polymers, and (ii) abating the noxious neuronal hyperexcitability *via* sAPP- $\alpha$ •GABA<sub>B</sub>R1a signaling; (B) suppress the surplus synthesis and exosomal intrabrain dissemination of neurotoxic p-Tau-os and the consequent hypertoxic effects elicited by combined actions of the  $A\beta_{42}$ -os/p-Tau-os duet (Ittner and Götz, 2011; Chiarini et al., 2017a); (C) reduce the increased synthesis and secretion of neurotoxic amounts of NO, VEGF-A, and likely other neurotoxic agents; (D) suppress any other harmful effects elicited by  $A\beta$ •CaSR signaling in oligodendrocytes, microglia, cerebrovascular cells of any kind; and (E) safeguard the blood-brain barrier (BBB) functional integrity. The above *in vitro* results also indicate that NAM efficacy persists notwithstanding a continued presence of soluble  $A\beta$ -os, fibrillar  $A\beta$ s, and p-Tau-os (Chiarini et al., 2017a; Chiarini et al., 2017b; Chiarini et al., 2017c). Therefore, it is likely that CaSR NAMs could safeguard *in vivo*, as they do *in vitro* (Armato et al., 2013), the viability and functions of the cortical human neurons preserving the integrity of critical cognition-essential upper cerebral cortical regions (Choi et al., 2013; Lee et al., 2013; Barateiro et al., 2016). In brief, CaSR NAMs would uphold the patients' ability to record and recover memories and to deal with their daily needs. Most important, the relatively cheap to synthesize CaSR NAMs appears to be the so far unique class of anti-AD therapeutics capable of concurrently targeting the multiple noxious effects triggered by pathological  $A\beta$ s•CaSR signaling in human neurons, astrocytes, and the other brain cell types (Chiarini et al., 2010; Dal Prà et al., 2011; Armato et al., 2013; Dal Prà et al., 2014b; Dal Prà et al., 2015a; Chiarini et al., 2016).

Ischemic neuronal injury is known to locally generate  $A\beta$ s surpluses (Ishimaru et al., 1996). More recent studies showed that the intraventricular administration of CaSR NAMs did decrease the death of neurons in the cortical *penumbra* zone of animal models of ischemia/hypoxia/stroke by effectively suppressing the concurrent *acute* increase in the local  $A\beta$ -os production (Kim et al.,

2014; Bai et al., 2015). These results further strengthen the idea that pathological A $\beta$ •CaSR signaling is crucially involved in both acute (ischemia/stroke) and chronic (AD) conditions causing neuronal death (Armato et al., 2013; Dal Prà et al., 2015a; Chiarini et al., 2016).

*Fyn* kinase inhibitor Saracatinib (AZD0530) and NMDAR inhibitors Memantine and Nitromemantine were endorsed as drugs to counteract the neurotoxicity driven by the extracellular accumulation of A $\beta_{42}$ -os (Kaufman et al., 2015). However, CaSR NAMs act on A $\beta$ •CaSR signaling well upstream of *Fyn* and NMDARs. In addition, CaSR NAMs obstruct any cytotoxic effects and likely also any impediments to proliferation and differentiation of neural stem cells in the dentate gyrus subgranular zone (Unger et al., 2016).

Concerning their most salient pharmacological characteristics, because of their lipid-soluble chemical structures and limited numbers of electrical charges, CaSR NAMs traverse the BBB. They can be administered by any route: hitherto the oral route has been the preferred one for clinical trials. Rodents could endure NAM NPS 2143 administration with no serious off-target effects being reported (Nemeth, 2002; Kim et al., 2014). During the failed phase I and phase II clinical trials assessing NAMs antiosteoporosis activity, human subjects also satisfactorily tolerated the administration of novel NPS 2143 derivatives, which affected PTH release less intensely (no record was taken concerning any brain-related effects). In general, the safety data collected from the clinical trials of CaSR NAMs did not record any major side-effect. Obviously, the calcemia levels had to be checked periodically due to NAM-elicited increases in plasma PTH levels (Nemeth and Shoback, 2013).

## CONCLUSIONS

This survey--necessarily short given the huge amount of literature concerning this verily fascinating topic--leads us to several closing considerations. First and foremost, a lot of data from AD-model animals had to be forcibly mentioned because analogous human data are not available. Therefore, there is still quite a lot to discover and learn about the physiological roles of family C GPRs particularly in relation to human CNS and other viscera. Second, it is undeniable that some of these GPCRs could play central roles on human AD. Our work has been mainly, but not exclusively, based upon the experimental exploitation of human cortical astrocyte cultures and has focused on the pivotal role pathological A $\beta$ •CaSR signaling exerts on the onset and progression of AD

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and on the potentially beneficial therapeutic effects CaSR NAMs could exert in LOAD/SAD patients. The interactions of CaSR heterodimers with other family C GPCRs, e.g. GABA $_B$ Rs and group I mGluRs, still constitute a mostly unexplored field of endeavor and their impact on AD onset and progression (if any) needs to be clarified. Notably, even in the gene mutations-driven EOFAD, CaSR NAMs could bring to bear mitigating and life-lengthening upshots by suppressing the additional aggravating consequences brought about by the concurrent A $\beta$ •CaSR signaling adding up to those stemming from the mutated genes. Third, any possible AD-promoting effects of CaSR PAMs (calcimimetics) in humans should be thoroughly investigated since in our preclinical *in vitro* AD model PAM NPS R-568 significantly increased A $\beta_{42}$ /A $\beta_{42}$ -os release from untransformed human adult cortical astrocytes (Armato et al., 2013). Fourth, we wish to add a last comment about CaSR NAMs as candidate therapeutics for human AD. For reasons pertaining to normal physiology, CaSR NAMs failed their initial task as antiosteoporosis therapeutics (Nemeth, 2004; Nemeth, 2013; Nemeth and Shoback, 2013; Nemeth and Goodman, 2016). Moreover, the induction of a mild hypercalcemia by CaSR NAMs has been a bit too much stressed as “hyperparathyroidism” creating a prejudice against their use in humans. Nevertheless, one should remember that no drug is devoid of unwanted and/or off-target effects: the chemotherapeutics administered to oncological patients are a striking example of this. Therefore, CaSR NAMs' rather slight off-target effects, chiefly the mild controllable hypercalcemia, should be objectively weighed against CaSR NAMs' crucial capability of averting the worsening loss of memories and cognitive abilities, including recognition of the self, and the later unavoidable demise AD would inexorably deliver.

## AUTHOR CONTRIBUTIONS

All the authors i.e. IDP, UA, and AC, equally contributed to the bibliographic searches and to the conception, discussion, and writing of the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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