● PERSPECTIVE

Targeting amyloid precursor protein shuttling and processing - long before amyloid beta formation

Targeting early steps in amyloid-beta production: Alzheimer's disease (AD) has a long history as the "amyloid deposit" disorder. Many disorders are now known to be caused by protein β-sheet misfolding and aggregation (*e.g.*, Parkinson's disease: α-synuclein; Huntington's disease: Huntingtin; spongiform encephalopathy: prion protein) (Rambaran and Serpell, 2008). Commonly, the family of amyloid mental disorders often have multiple aggregating proteins with most having one or two highlighted. For example in AD, amyloid beta (Aβ) extracellular aggregates in senile plaques (SP) are the most obvious postmortem observation along with intracellular tau tangles. However, AD patients often have accumulation of TDP43 and α-synuclein (in Lewy bodies) (Josephs et al., 2014). While reducing Aβ remains the main AD target, there has been a recent shift from targeting the late forming amyloid plaques, to earlier steps in production of Aβ. Recent work has suggested that small oligomers of Aβ could be the neurotoxic compounds with structures on the order of Aβ octomers forming pores in neuronal membranes causing cell death (Arbor et al., 2016). Under this paradigm, the larger extracellular amyloid plaques could actually be neuroprotective *via* a mechanism of sequestering the more deleterious Aβ monomers. In addition to not being neurotoxic (wrong marker physically) the amyloid plaques present late in disease after neuronal death has occurred (wrong marker temporally). While the field continues to target the already existing Aβ in AD patients, the effort to target even the production of Aβ has been reinvigorated. Possible targets include the initial production of amyloid precursor protein (APP), APP insertion in membrane lipid rafts, APP shuttling to early endosome compartments, and processing of APP. The molecular pathways causing this shift away from targeting amyloid plaques as well as therapeutics will be the content of this perspective.

APP processing: APP processing can occur in two ways, termed amyloidogenic (harmful) or non-amyloidogenic (beneficial), based on the resulting cleaved peptide length and function. Initially APP is cleaved by either α-secretase (non-amyloidogenic and protective) or β-secretase (amyloidogenic and harmful) followed by γ-secretase cleavage (**Figure 1**). Many targets for AD have been discovered from analysis of familial Alzheimer's disease (FAD), also called early-onset familial Alzheimer's disease (EOFAD), which is an autosomal dominant condition only occurring in ~1% of the general population. However, searching Online Mendelian Inheritance in Man (www.omim.org) show families with known FAD can have single protein genetic defects, such as presenillin-1 (PSEN1) detected as frequently as 56% of the time. While deleterious PSEN mutations in FAD only increase γ-secretase cleavage of APP into Aβ, other genetic predispositions have been found to increase Aβ through increased APP production, decreased α-secretase activity, or increased β-secretase activity. The most common initial cleavage of APP is done by α-secretases within the ADAM (a disintegrin metalloproteinase domain) family of proteins, which leads to the non-amylodogenic pathway. The α-secretase cleavage is non-amyloidogenic because it cleaves APP close to the outer membrane at a spot that is in the middle of the Aβ sequence thereby resulting in no Aβ production while producing non-harmful sAPPα, p3, and AICD (**Figure 1**). ADAM9, ADAM17, ADAM19, and ADAM10 have been shown to process APP with the latter being the most abundant in the brain. Two mutations in ADAM10 have recently been linked to late-onset AD (Suh et al., 2013), and α-secretase agonists are being investigated as AD therapeutics and have even been shown to increase sAPPα in phase 3 clinical trials.

The processing of APP to create Aβ cannot actually happen at the plasma membrane. There are two routes in which APP can be cleaved into Aβ (**Figure 1**). Normally, APP is shuttled to the plasma membrane (PM) from the trans-golgi network (TGN) *via* Sorl1 (sortilin-related receptor, L (DLR class) A repeats containing) where it aggregates in lipid rafts high in cholesterol and is processed first by α-secretase and then by γ-secretase in the non-amyloidogenic pathway. One of the amyloidogenic paths involves the shuttling of APP in the plasma membrane, which had evaded α-secretase cleavage, out of the membrane seemingly through a clathrin coated endocytosis mechanism. This clathrin coat curvature and formation is enhanced by phosphatidylinositol binding clathrin assembly protein (PICALM) and CD2-associated protein (CD2AP) while Myc box-dependent-interacting protein 1 (BIN1) aids protein shuttling *via* cytoskeleton rearrangement, all of which have known genetic variants that are linked to AD. APP moves along microtubules to acidic early endosome organelles where β-secretase and then γ-secretase sequentially cleave APP, releasing sAPPβ followed by Aβ and AICD respectively. β-Secretase (also known as β-site APP cleaving enzyme, BACE) performs the first APP digestion on the road to forming Aβ because it cleaves APP at a point that becomes the N-terminus of Aβ. The second route APP takes to enter the amyloidogenic pathway is more direct, going straight from the TGN to endosomes *via* AP4 (adaptor related protein complex 4). Once Aβ is produced and in the extracellular space it can aggregate with the help of chaperone clusterin (Clu) to form amyloid plaques or β-barrel pores which lyse neurons. There are at least three Aβ clearance pathways being investigated. The primary Aβ clearance pathway appears to be transcytosis across the blood-brain barrier (BBB) by Aβ binding ApoE which then docks to cell surface receptor lipoprotein receptor-related protein 1 (LRP1) and undergoes clathrin-mediated endocytosis with the help of BIN1 and PICALM. This export mechanism is supported by the fact that there is an ApoE-ε4 variant that is the single greatest risk factor for AD, as well as an ApoE-ε2 variant, which is protective against AD. People that are homozygous for apoE-ε4 are a staggering 15 fold more likely to develop AD. Levels of ApoE have also been shown to be down-regulated by γ-secretase cleavage of APP forming AICD which binds directly to the LRP1 promoter inhibiting it, which is also known as apolipoprotein e receptor (APOeR), and regulates cholesterol and ApoE levels in the brain. Aβ has been shown to bind to ApoE-ε4 and increase the oligomerization/fibrillization of Aβ (Koffie et al., 2012), and indeed blocking the Aβ ApoE-ε4 interaction has shown beneficial effects (Liu et al., 2014). ApoE

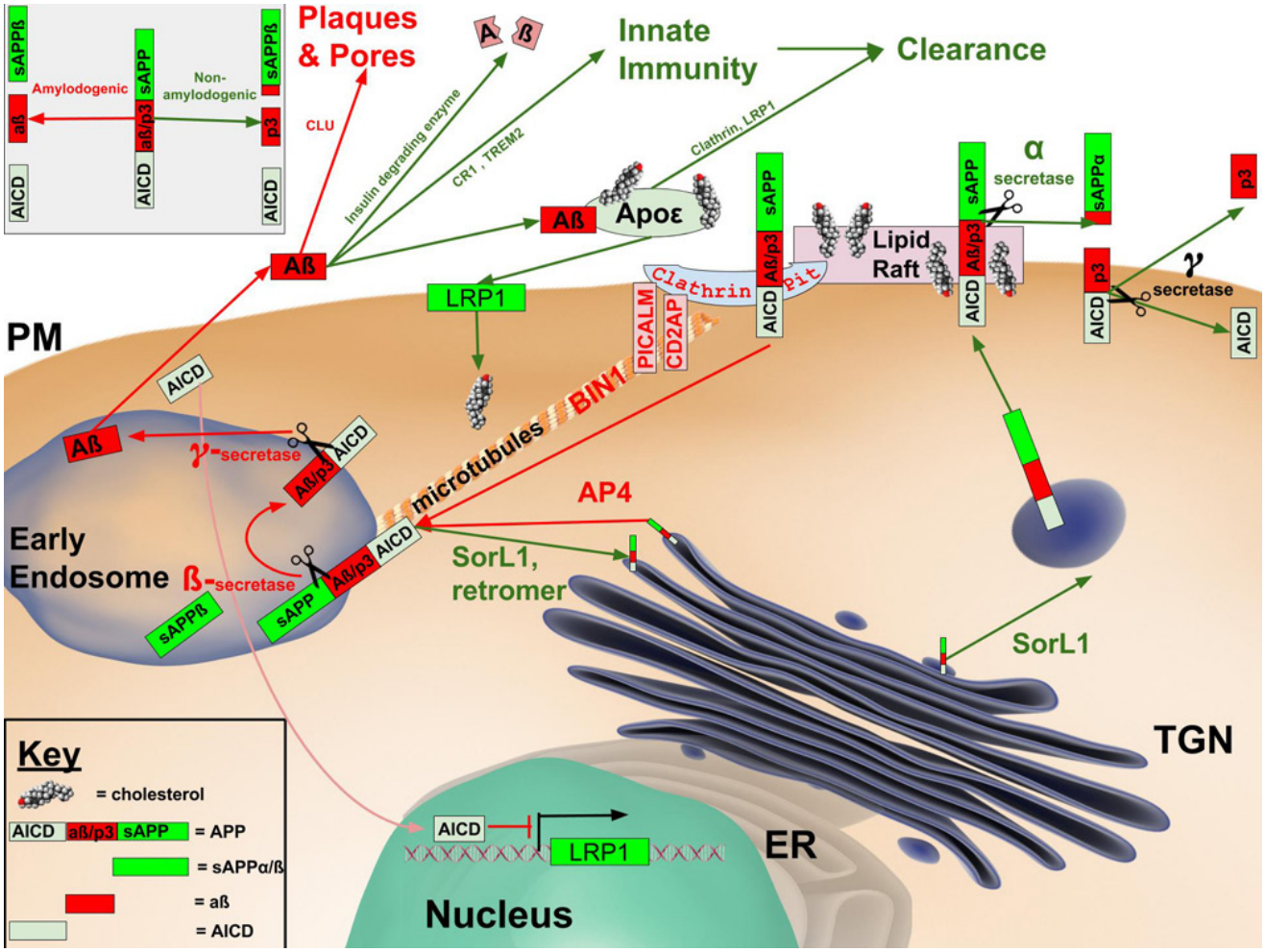

Figure 1 Amyloid precursor protein (APP) processing.
APP preferred processing is the non-amyloidogenic pathway which digests with α-secretase followed γ-secretase and takes place at the plasma membrane (PM). APP can be endocytosed back to endosomes or sent directly there from the trans-golgi network (TGN), either of which begins the amyloidogenic pathway *via* β-secretase and then γ-secretase cleavage leading to Aβ production. Once in extracellular space Aβ can form the plaques and deleterious Aβ pores or be cleared *via* ApoE binding to shuttle across the blood-brain barrier, proteolytically digested, or removed by the innate immune response. Macromolecules and molecular pathways which increase and decrease Aβ are shown in red and green respectively. AICD: Activiation-induced cell death; Apoε: apolipoprotein ε; ER: endoplasmic reticulum; LRP1: lipoprotein receptor-related protein 1; SorL1: sortilin-related receptor 1.

clearly plays an important role in AD, binding cholesterol, Aβ, and LRP1 (Bohm et al., 2015). While the disrupting APP endocytosis is being investigated therapeutically, there are potentially deleterious effect from such inhibition. There are overlapping molecular players which endocytose APP deleteriously back into neurons and go on to be cleaved and produce Aβ. It has been found that decreasing phosphatidylinositol binding clathrin assembly protein (PICALM), which is involved in endocytosis, causes a decrease in intracellular APP and secreted sAPPβ. On the other hand, ApoE binding to Aβ can cause Aβ to be taken past the BBB lowering the brains amyloid burden. It is generally seen that LRP1 beneficially increases Aβ export from the brain whereas the receptor for advanced glycation endproducts (RAGE) deleteriously increases import of Aβ into the brain, perhaps by degrading the gap junction at the BBB. Aβ can also be degraded in neuronal extracellular space *via* insulin degrading enzyme. Lastly the misfolded amyloid initiates the innate immune response *via* compliment receptor 1 (CR1) and triggering receptor expressed on myeloid cells 2 (TREM2) which may initially help clearance of Aβ but later hurt the neurons *via* the inflammasome (**Figure 1**). It should be emphasized that there have been disappointing results with both β-secretase and γ-secretase inhibitors, which has been intellectually confounding.

Current Alzheimer's disease therapeutics and clinical trials: There are still many therapeutics in development aimed at binding Aβ for removal. For example, the antibodies Crenezumab, Aducanumab, Gantenerumab, and Bapineuzumab all bind fibrillar Aβ and are in current phase 3 trials, with Bapineuzumab also binding soluble Aβ (Arbor et al., 2016). Aducanumab gained significant press as recently as September 2016, when early results were published in *Nature* showing a 10 mg/kg dose resulted in nearly amyloid free scans after 12 months, and saw an even more impressive slowing of clinical decline (both dose and time dependent) as measured by the Mini Mental State Examination (MMSE) and the Clinical Dementia Rating scale Sum of Boxes (CDR-SB) (Sevigny et al., 2016). Solanezumab, also in a current phase 3 clinical trial, is more unique in that it only binds to soluble Aβ which should make it a better therapeutic with the newer view of 2–8 mer being the harmful Aβ size. Despite failure in past β-secretase clinical trials there are multiple

current clinical trials still aimed at inhibiting β-secretase due to the scientific basis discussed in this perspective. The β-secretase inhibitors E2609, AZD3293, MK-8931 are recruiting for a phase 2, phase 3, and phase 3 studies respectively (with MK-8931 also having a phase 2/3 trial already active). Acitretin, a retinoid currently used safely for long-term treatment of psoriasis in the elderly, has recently been tested as a α-secretase agonist in a phase 3 European Union clinical trial (EudraCT No 2009-011881-27) and shown to increase the non-amyloidogenic sAPPα. Tretinoin, an all-trans retinoic acid (another pharmaceutical form of vitamin A) displays similar anti-amyloidogenic effects *in vitro*, but the synthetic retinoid acitretin is of greater interest because tretinoin is toxic in humans.

Peptides have also been developed to bind Aβ to be used either as therapeutics or as probes for early detection. With the consensus in the field shifting towards the Aβ fibrils not being the harmful molecular structures, the peptide design has shifted to having selectivity for certain Aβ structures. Peptides which specifically bind aggregated $Aβ_{1–42}$, the most common deleterious length of Aβ, have been developed. Some of these agents have shown the ability to cross the BBB and inhibit amyloid fiber formation (Bartnik et al., 2010; Larbanoix et al., 2011). A 15-amino acid peptide was developed and shown to bind soluble Aβ, reducing the formation of soluble $Aβ_{42}$ oligomers, while sequestering the Aβ by increasing the level of insoluble aggregates (Barr et al., 2016). There is also a vaccine for AD currently in a phase 2 clinical study, CAD106, which is an Aβ mimic that produces antibodies to Aβ however the trial is not slated to be completed until 2023. Lastly, there are multiple clinical trials currently aimed at the lipid Aβ link either through interactions with ApoE or lowering cholesterol content of lipid rafts (statins were an already approved class of compounds use for this research early on). Some older investigations aimed at altering cholesterol levels thought to act by disrupting lipid raftsare now being reinvestigated to see if their effects were actually due to better Aβ clearance through cholesterol binding of ApoE and Aβ. ApoE transcription is induced by peroxisome proliferator-activated receptorgamma (PPARγ) along with retinoid X receptors (RXRs) which can form heterodimers, and RXR agonists are now viewed as potential AD therapies. Bexarotene is an RXR agonist which was found to decrease amyloid plaques in mice by an amazing 50% in 72 hours in an apoE-dependent manner (Cramer et al., 2012).

Conclusion: The increased understanding of the spatial and sequential processing of APP has retargeted the fields attention to a pre-Aβ aim of attack. Therapeutics targeting processing of APP and therefore initial creation of Aβ should be easier to rationally develop because the number of conformations Aβ adopts has been shown to be extremely diverse, and therefore the level of structural knowledge needed to design unique Aβ binders seems exhaustive. The clathrin endocytosis of APP has sprung up as a newer target and the ApoE efflux of Aβ from the brain continues to be studied. While the increased Aβ processing and influx/efflux pathway knowledge is promising there are still large gaps in our understanding which should cause optimism about near term novel therapeutics to be tempered. For example, the natural *in vivo* function of APP remains elusive and the exact level of LRP1 mediated influx into neurons in different parts of the brain verse efflux past the blood brain barrier needs to continue to be investigated.

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