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Review

Amyloid-β and Synaptic Vesicle Dynamics: A Cacophonic Orchestra

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Abstract. It is now more than two decades since amyloid-β (Aβ), the proteolytic product of the amyloid-β protein precursor (AβPP), was first demonstrated to be a normal and soluble product of neuronal metabolism. To date, despite a growing body of evidence suggests its regulatory role on synaptic function, the exact cellular and molecular pathways involved in Aβ-driven synaptic effects remain elusive. This review provides an overview of the mounting evidence showing Aβ-mediated effects on presynaptic functions and neurotransmitter release from axon terminals, focusing on its interaction with synaptic vesicle cycle. Indeed, Aβ peptides have been found to interact with key presynaptic scaffold proteins and kinases affecting the consequential steps of the synaptic vesicle dynamics (e.g., synaptic vesicles exocytosis, endocytosis, and trafficking). Defects in the fine-tuning of synaptic vesicle cycle by Aβ and deregulation of key molecules and kinases, which orchestrate synaptic vesicle availability, may alter synaptic homeostasis, possibly contributing to synaptic loss and cognitive decline. Elucidating the presynaptic mechanisms by which Aβ regulate synaptic transmission is fundamental for a deeper comprehension of the biology of presynaptic terminals as well as of Aβ-driven early synaptic defects occurring in prodromal stage of AD. Moreover, a better understating of Aβ involvement in cellular signal pathways may allow to set up more effective therapeutic interventions by detecting relevant molecular mechanisms, whose imbalance might ultimately lead to synaptic impairment in AD.

Keywords: Amyloid-β, intracellular signaling, neurotransmitter release, presynaptic function, SNARE complex, synaptic vesicle cycle

Alzheimer's disease (AD) is a chronic neurodegenerative disorder, whose prominent neuropathological features are the progressive extracellular deposition of amyloid plaques, the intracellular neurofibrillary tangles, and the loss of synapses and neurons [1]. Among the distinctive neuropathological hallmarks of AD, the extent of synaptic loss has been reported as a quantitative neuropathological correlate of memory deficit and cognitive decline observed in AD patients [2]. Such evidence suggests a causal role for dwindling synaptic integrity in the etiology of AD [3] and raises a central question in AD research concerning the role played by synaptic damage. However, the molecular mechanisms underlying such synaptic dysfunction remain largely unknown.

Clinical studies, alongside animal models, have widely demonstrated the importance of amyloid-β (Aβ) [4], a 4-kDa peptide derived from the sequential proteolysis of the amyloid-β protein precursor (AβPP) by β- and γ-secretase, in the progression of AD. Besides its widely-investigated role as the main pathogenic marker responsible for neurodegenerative processes, significant advances have been

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made over recent years to understand whether Aβ might be an important synaptic regulator affecting age-related synaptic changes. Accordingly, Aβ has been shown to induce several functional and morphological synaptic changes. Intriguingly, these defects in synaptic activity are recognized as one of the earliest event in AD, preceding the deposition of Aβ plaques into the brain [5]. Such evidence has emphasized the need to refocus the experimental approach to Aβ-induced neurotoxicity from frank neurodegeneration to earlier structural and functional perturbations of synaptic homeostasis triggered by Aβ [6]. A great effort has been directed toward evaluating Aβ-driven effects on synaptic activity, in conditions not resulting in neurotoxicity. A highly heterogeneous amount of data, ranging from an Aβ-driven increase in spontaneous synaptic activity [7] to a lack of effect on synaptic transmission [8] or even its depression [9, 10], has been produced. Such contrasting data have been mainly related to crucial factors affecting the outcome of the experiments, such as the different variants, concentrations, and aggregation forms of Aβ peptides in the different experimental settings [11, 12], as well as to the extreme supplier-to-supplier and batch-to-batch variability of synthetic Aβ peptides [13]. Indeed, Aβ has been demonstrated to exhibit a biphasic action, i.e., neuromodulatory/neuroprotective versus neurotoxic, depending on its concentration and aggregation status [14, 15]. Low concentrations (picomolar-low nanomolar) of Aβ peptides positively modulate neurotransmission and memory, whereas, higher concentrations (high nanomolar-low micromolar) exhibit a neurotoxic and detrimental action on synaptic plasticity and memory. In addition, the complex dynamic balance existing between the different species of Aβ (i.e., monomers, oligomers, protofibrils, and fibrils) contribute to the widespread and controversial literature on Aβ-driven synaptic effects [16], further challenging consistent interpretation of the experimental data.

Aβ AS POTENTIAL MODULATOR OF PRESYNAPTIC ACTIVITY

Kamenetz and colleagues demonstrated for the first time that, in healthy brain, neuronal activity directly promotes the production and the secretion of Aβ peptides into the extracellular space, and that, in turn, Aβ downregulates excitatory synaptic transmission [9, 10]. This negative feedback loop, wherein neuronal activity promotes Aβ production and Aβ depresses synaptic activity, may provide a physiological homeostatic mechanism preventing the overexcitation of brain circuits [9]. However, in normal brain, extracellular concentrations of endogenous Aβ peptides have been estimated to low picomolar levels, far lower than the concentrations used in the mentioned studies demonstrating Aβ-mediated synaptic depression [10, 15]. This observation prompted extensive research to investigate the impact of lower concentrations of Aβ, which are likely to approximate the endogenous level of the peptide. Several lines of evidence converge to indicate that Aβ peptides at pM concentrations act as a positive endogenous regulator of neurotransmission at presynaptic terminals [14, 15, 17]. Abramov et al. demonstrated that the inhibition of extracellular Aβ degradation and the subsequent increase in endogenous levels of Aβ peptides enhanced both the release probability of synaptic vesicles and neuronal activity in rodent hippocampal culture [11]. However, the specific Aβ isoform and conformation responsible for this synaptic effect cannot be identified [11]. These acute effects mediated by the inhibition of Aβ clearance increased spontaneous excitatory postsynaptic currents without affecting inhibitory currents. Such effect was specifically presynaptic and dependent on firing rates, with lower facilitation observed in hippocampal neurons showing higher firing rates [11]. Furthermore, the exposure of rodent neuronal cultures to picomolar amounts of $A\beta_{40}$ monomers and dimers enhanced presynaptic release probability via AβPP-AβPP interactions at excitatory hippocampal synapses [18]. $A\beta_{40}$ monomers and dimers have been found to bind to AβPP, increasing the fraction of AβPP homodimers at the plasma membrane and inducing activity-dependent AβPP-AβPP conformational changes [18]. In turn, AβPP homodimer activation triggers structural rearrangements within the presynaptic AβPP/G_0 protein signaling complex, enhancing calcium (Ca^{2+}) build-up and, consequently, synaptic vesicle exocytosis and glutamate release [18]. These findings suggest that AβPP homodimer may act as a presynaptic $A\beta_{40}$ receptor that translates local changes in the extracellular levels of Aβ peptides to modulation of synaptic release probability, maintaining basal neurotransmitter release under physiological conditions. Such a positive modulatory action of endogenous Aβ peptides on synaptic transmission has been fur-

ther supported indirectly by the observation that mice deficient for AβPP [19], PS1 (Presenilin 1) [20], or BACE1 (Beta-site AβPP-cleaving enzyme 1) [21] displayed evident defects in synaptic transmission.

According to experimental data suggesting a modulatory action of Aβ peptides on synaptic transmission, Puzzo et al. demonstrated that the exposure of hippocampal neurons to high picomolar-low nanomolar concentrations of synthetic $A\beta_{42}$ oligomers markedly increased synaptic transmission, whereas higher concentrations (high nanomolar-low micromolar) of $A\beta_{42}$ induced the well-established synaptic depression [15]. The facilitator effect of low Aβ concentrations on excitatory transmission did not affect postsynaptic N-methyl-d-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). This effect was sensitive to α-bungarotoxin, a selective antagonist of α7-nicotinic acetylcholine receptor (α7-nAChR), thus implying that functional α7-nAChR are required for $A\beta_{42}$-mediated facilitator effect [14, 15]. This observation is consistent with data from literature reporting high-affinity binding of Aβ to α7-nAChR [22] and enhanced Ca^{2+} build-up through α7-nAChR at presynaptic nerve endings of hippocampal synaptosomes upon application of picomolar $A\beta_{42}$ [23]. Under normal conditions, picomolar concentrations of Aβ, released by synaptic activity during vesicle exocytosis [10], positively stimulate α7-nAChR, whose activation enhances Ca^{2+} influx into the presynaptic terminals and neurotransmitter release boosting synaptic plasticity [15]. In line with this hypothesis, blocking or removing α7-nAChRs both decreased Aβ secretion and blocked Aβ-induced facilitation [24]. Instead, nanomolar concentrations of Aβ have been found to inactivate α7-nAChRs.

Recently, Gulisano et al. corroborated such evidence demonstrating that, in rodent CA1 pyramidal neurons, the extracellular administration of 200 pM oligomeric $A\beta_{42}$ induced, via α7-nAChRs, an increase of miniature EPSCs (excitatory postsynaptic currents) frequency and a decrease of paired pulse facilitation [17]. Such $A\beta_{42}$-induced effects were associated with an enhanced number of docked vesicles at presynaptic terminals, thus indicating that picomolar concentrations of $A\beta_{42}$ stimulate neurotransmitter release at presynaptic level [17]. Notably, intracellular application of 6E10, an antibody raised against human $A\beta_{42}$, did not hinder the effects induced by extracellular $A\beta_{42}$, which were conversely prevented by the extracellular application of 6E10 [17].

Overall, these findings strongly support a potential relationship between concentration of Aβ peptides and synaptic transmission, wherein low concentrations (high picomolar-low nanomolar) of Aβ peptides play a positive modulatory role upon neurotransmission [14, 15], abnormally low levels decrease presynaptic efficacy [19–21] and high concentrations (high nanomolar-low micromolar) induce detrimental effects depressing synaptic transmission, mainly by postsynaptic mechanisms including enhanced internalization or desensitization of postsynaptic glutamate receptors and downstream signaling [9, 25, 26].

Moreover, the time of exposure to $A\beta_{42}$ picomolar concentration significantly affects synaptic activity. Koppensteiner et al. examined the time course of synaptic changes in mouse hippocampal neurons exposed to picomolar concentration (200 pM) of $A\beta_{42}$. They demonstrated that $A\beta_{42}$ exerted opposite effects depending also on the time of exposure, with short exposures in the range of minutes enhancing synaptic potentiation in hippocampal cultures and slices and increasing synaptic plasticity as well as memory in mice, and longer exposures lasting several hours decreasing them [27]. In addition, the prolonged exposure to picomolar concentrations of $A\beta_{42}$ has been found to induce microstructural changes at the synapse including an increase in the basal frequency of spontaneous neurotransmitter release and in the basal number of functional presynaptic release sites, as well as a redistribution of synaptic proteins such as the vesicle-associated proteins synapsin I and synaptophysin [27].

The clues gained from recent studies in modulation of presynaptic functions by Aβ highlight a significant variety of mechanisms and functional outcomes. Elucidating the intracellular mechanisms underlying synaptic alterations in early AD represents a keystone to uncover AD pathobiology and to define the associated early behavioral sings and/or therapeutic interventions, able to block factors that fuel the progression of AD and to slow down and, ultimately, even prevent the onset of irreparable intracellular damage leading to synaptic loss and cognitive decline [1]. Indeed, the observed changes in synaptic activity and associated neurotransmission may be at the basis of the onset of psychiatric symptoms during the early phases of the disease (e.g., anxiety, changes in mood) in absence of the usual warning symptoms (e.g., memory loss).

THE EFFECTS OF Aβ ON SYNAPTIC VESICLE DYNAMICS

Most of presynaptic functions has been reported to directly or indirectly converge on the synaptic vesicle cycle, whose different steps collaborate to allow rapid, regulated and repeated rounds of neurotransmitter release (reviewed by [28]). In recent years, a major goal in neurobiology has been to gain insight into the tightly coordinated membrane-fusion machinery that mediates synaptic vesicle cycle, characterized by sequential steps.

Data from literature showed that Aβ peptides directly interfere with key presynaptic proteins regulating different steps of the synaptic vesicle cycle and, consequently, influencing neurotransmitter release and neurotransmission between functionally related neurons [29, 30]. Aβ has been found to interact with presynaptic proteins mediating synaptic vesicles docking and fusion, necessary for a regulated exocytosis, as well as synaptic vesicles recycling and recovery (illustrated in Fig. 1) (reviewed by [31, 32]). In addition, the regulation of synaptic vesicle cycle and, subsequently, of neurotransmitter release by Aβ has been suggested to be, at least in part, mediated by Aβ interactions with specific protein kinases and phosphatases controlling the consequential steps of the synaptic vesicle cycle. It can be postulated that Aβ by influencing the fine-tuning of synaptic vesicle cycling may transiently influence synaptic homeostasis. Such alteration triggered by Aβ may not be restricted to the immediate period. A series of transient modifications by Aβ may generate long-lasting and, then, permanent alterations at synapse, by possibly catalyzing a linear progression from synaptic dysfunction to neuronal degeneration. It is therefore essential to deeper understand Aβ involvement in intraneuronal pathways to identify new drug targets and to set up more precise therapeutic interventions targeting the most relevant molecular mechanisms leading to AD.

In the following sections, we will dissect this remarkably complex scenario in a reductionist fashion, providing an overview of the evidence

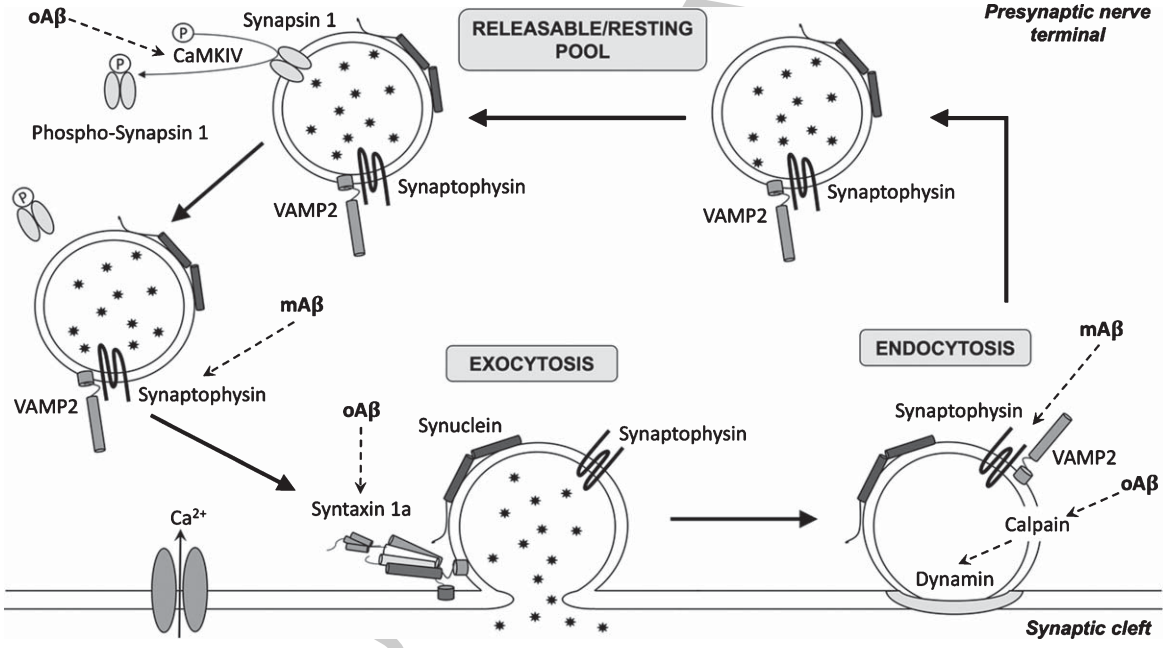

Fig. 1. Aβ interplay with synaptic vesicle dynamics. Monomeric Aβ (mAβ) directly competes with Synaptobrevin/VAMP2 for binding to Synaptophysin, stimulating the formation of the fusion pore complex, followed by neurotransmitter release. Moreover, oligomeric Aβ (oAβ) exerts an inhibitory effect on SNARE-mediated exocytosis by binding to the SNARE motif region (SynH3) of Syntaxin 1a. In addition, oAβ decreases dynamin-1 levels by increasing its cleavage by calpain, thus impairing endocytosis of synaptic vesicles. mAβ has been also hypothesized to prevent synaptophysin from triggering synaptic vesicle endocytosis through its interaction with synaptobrevin/VAMP2. Finally, oAβ enhances the levels of phosphorylated Synapsin I, by activating CaMKIV, thus increasing the availability of synaptic vesicles to dock to the active zone and to allow neurotransmitter release. It should be noted that Aβ, in addition to the direct actions here represented, may affect neurotransmitter release also indirectly through the modulation of various kinases (see text).

demonstrating Aβ involvement in synaptic vesicle exocytosis, endocytosis and recycling (illustrated in Table 1), focusing on its interplay with key presynaptic proteins and kinases.

Regulation of synaptic vesicle exocytosis

Exocytosis of synaptic vesicles is mediated by a conserved array of membrane proteins, commonly known as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors) [33]. These proteins include synaptobrevin/VAMP (vesicle-associated membrane protein), which is located on the membrane of synaptic vesicles (v-SNARE), syntaxin 1 and SNAP 25, which are predominantly localized at the synaptic plasma membrane (t-SNARE). In neurons, Synaptobrevin-2/VAMP2 has been found to bind to syntaxin 1a and SNAP-25, located on the presynaptic membrane, thereby assembling a tight stoichiometric complex that catalyzes membrane fusion for exocytosis [34]. Fusion-competent conformations of SNARE proteins are maintained by chaperone complexes composed by CSPα (Cysteine string protein α), Hsc70 (Heat shock cognate 70). and SGT (small glutamine-rich tetratricopeptide repeat protein) and by non-enzymatically acting synuclein chaperones. The folding/refolding of SNARE proteins is regulated by several synaptic modulators, such as α-synuclein (α-syn) [35]. After fusion, the disassembly of SNARE complexes is mediated by ATPase *N*-ethylmaleimide-sensitive factor (NSF) and its cofactors SNAPs [36]. Disassembled t-SNAREs are immediately available to participate in subsequent vesicle docking and fusion reactions, whereas v-SNAREs have to be recycled to the donor membrane before engaging in productive SNARE complex assembly [33].

Sharma et al. demonstrated for the first time that, in neurodegenerative diseases including AD, the membrane-fusion machinery is strongly altered [37, 38] and that the level of SNARE complex assembly, necessary for driving synaptic vesicle fusion at the presynaptic active zone, is substantially decreased in postmortem brains of AD patients [39]. The authors suggest a potential involvement of Aβ as hindering of SNARE-mediated fusion of synaptic vesicle. In line with such hypothesis, Yang et al. demonstrated by biochemical assay *in vitro* that both $A\beta_{42}$ monomers and oligomers are capable to specifically bind to the SNARE motif region (SynH3) of syntaxin 1a [30], which forms a four-helix bundle necessary for membrane fusion [40, 41]. However, only oligomeric form of Aβ (10 μM) has been found to exert an inhibitory effect on the SNARE complex assembly and SNARE-mediated exocytosis in AβPP-PS1 mice. In particular, oligomeric form of Aβ inhibits the fusion step between docking and lipid mixing by binding to the SNARE motif of syntaxin-1a, without changing the expression of SNARE proteins [30]. This study identifies a potential molecular mechanism by which intracellular Aβ oligomers hinder SNARE-mediated exocytosis, possibly leading to synaptic dysfunctions occurring in AD. Otherwise, Aβ monomers failed to exhibit any inhibitory effects on SNARE complex assembly or SNARE-mediated exocytosis, despite their proved capability to bind to syntaxin-1a. Impairments of synaptic vesicle docking by monomeric and oligomeric form of Aβ have not been observed. Such evidence suggests a differential sensitivity of synaptic vesicle docking and fusion to Aβ. A possible explanation is that the steric hindrance of Aβ oligomers inhibits the "zippering" of SNARE proteins into the *cis*-SNARE complex, but not influences their partial assembly into the *trans*-SNARE complex required for docking. Future investigations are needed to better examine how Aβ differentially influences the docking and fusion of synaptic vesicle at presynaptic terminals. Moreover, another issue to fully elucidate concerns the presence of intraneuronal Aβ accumulations, whose occurrence and relevance in AD have been a matter of controversial scientific debate. First reports showing that Aβ is initially deposited in neurons before occurring in the extracellular space date back roughly 20 years [42]. More recently, intracellular $A\beta_{42}$ accumulations have been identified in basal forebrain cholinergic neurons in adult human brain explants, and increases in the prevalence of intermediate and large oligomeric assembly states are related to both aging and AD [43]. Such early accumulation of $A\beta_{42}$ seems to be a selective feature of basal forebrain cholinergic neurons when compared with cortex, and not due to differences in AβPP expression [43]. Accordingly, studies with transgenic animal models of AD have further supported the presence of intraneuronal Aβ before the appearance of extracellular deposits [44, 45]. Observations concerning an intracellular activity of Aβ are also present in *in vitro* models. Even if data are not at the synaptic level there is evidence that $A\beta_{40}$ and $A\beta_{42}$ at pM and nM concentrations are able to interfere with the pathways regulating the maintenance of genomic integrity, thus resulting in the comparison of dysfunctional cells [46].

Table 1
Regulation of synaptic vesicle cycle by amyloid-β

Exocytosis				
Molecular species of Aβ	Aggregation status and concentration/time of exposure	Observed effect on synaptic vesicle cycle	*In vitro* and *in vivo* model	Reference
Aβ	Oligomers (1–20 nM)	Oligomeric form of Aβ has been found to exert an inhibitory effect on SNARE-mediated exocytosis by binding to the SNARE motif region (SynH3) of Syntaxin 1a, thus specifically inhibiting the fusion step between docking and lipid mixing. Instead, the ability of synaptic vesicles to dock to the target membrane has not been affected.	*In vitro* single-vesicle content-mixing assay	[30]
$A\beta_{42}$	Monomers (50 nM/20 min)	At presynaptic terminals, $A\beta_{42}$ has been demonstrated to directly compete with Synaptobrevin/VAMP2 for binding to Synaptophysin, thus hindering the formation of Synaptophysin/VAMP complex and, subsequently, inducing the formation of the fusion pore complex followed by neurotransmitter release.	Primary cultures of rat CA3-CA1 hippocampal neurons	[29]
Endocytosis				
Molecular species of Aβ	Aggregation status and concentration/time of exposure	Observed effect on synaptic vesicle cycle	*In vitro* and *in vivo* model	Reference
Aβ	Soluble oligomers (2 μM)	Aβ oligomers have been found to decrease dynamin-1 levels by increasing its cleavage by calpain, thus impairing endocytosis of synaptic vesicle. Abnormally accumulated amphiphysin at synaptic membrane, following Aβ oligomers application, has also been observed.	Cultured rat hippocampal neurons	[66]
$A\beta_{42}$	Monomers (50 nM/20 min)	At synapse, $A\beta_{42}$ has been shown to compete with synaptobrevin/VAMP2 for binding to synaptophysin, which is known to regulate the retrieval kinetics of VAMP2 during endocytosis. $A\beta_{42}$ has been hypothesized to prevent synaptophysin from triggering synaptic vesicle endocytosis through its interaction with synaptobrevin/VAMP2.	Primary cultures of rat CA3-CA1 hippocampal neurons	[29]
Aβ	Monomers and oligomers (200 nM/2h; 200 nM/72h)	Preparation containing both synthetic Aβ oligomers and monomers reduced the efficacy of synaptic vesicle recycling. 72-h treatment with Aβ oligomers induced more severe defects in synaptic vesicle endocytosis. Notably, preparation containing only Aβ monomers did not impaired synaptic vesicle endocytosis.	Rat hippocampal neurons	[69]
$A\beta_{40}$ and $A\beta_{42}$	Increased levels of endogenous	Increased levels of endogenous $A\beta_{42}$ and $A\beta_{40}$ have been found to increase activity-driven recycling of synaptic vesicles in both excitatory and inhibitory synapses, as shown by quantification of synaptotagmin 1 antibody uptake, strongly supporting the involvement of endogenous Aβ peptides in the modulation of basal synaptic vesicle recycling.	Rat cortical and hippocampal neurons	[76]

(*Continued*)

Table 1
(*Continued*)

	$A\beta_{42}$ and $A\beta_{40}$ (1.6- and 1.2-fold compared to controls)/ Synthetic	1-h exposure to 200 pM $A\beta_{40}$ and $A\beta_{42}$ caused an increase in synaptic vesicle recycling.		
	$A\beta_{42}$ and $A\beta_{40}$ (200 pM/1h; 1 μM/1h)	1-h treatment with 1 μM Aβ has been found to decrease it. The endogenous Aβ-driven modulation of synaptic vesicle recycling has been hypothesized to rely on CDK5 and calcineurin signaling pathway downstream of α7-nAChR.		
Intra-synaptic trafficking and distribution among vesicle pools				
Molecular species of Aβ	Aggregation status and concentration/time of exposure	Observed effect on synaptic vesicle cycle	*In vitro* and *in vivo* model	Reference
Aβ	Soluble oligomers (200 nM/2h)	Aβ oligomers have been found to alter the recycling/resting pool ratio by expanding the resting fraction at the expense of the recycling fraction, without altering the average total number of synaptic vesicles. Such Aβ-driven effect on pool size was mediated by the activation of CDK5, the main kinase involved in the regulation of synaptic vesicle pool size (Kim and Ryan, 2010).	Cultured rat hippocampal neurons	[69]
$A\beta_{42}$	Soluble oligomers (300 nM/30 min)	At presynaptic terminals, $A\beta_{42}$ enhanced the levels of phosphorylated Synapsin I at Ser^9, thus increasing the availability of synaptic vesicles to dock to the active zone and to allow glutamate release.	Primary rat hippocampal neurons	[79]
$A\beta_{42}$	Soluble oligomers (200 nM)	$A\beta_{42}$ markedly enhanced the levels of phosphorylated Synapsin at Ser^9 by activating CaMKIV. As a result, Synapsin I has been found to disassembly either from synaptic vesicles and actin.	Rat hippocampal neurons	[80]

Another interplay between Aβ and a synaptic vesicle-associated protein has been reported by Russel et al. This study demonstrated that, in rat CA3-CA1 hippocampal neurons, the acute application of low concentrations (50 nM) of $A\beta_{42}$ was followed by its internalization and localization to presynaptic terminals, where the peptide interacted with Synaptophysin-1, a glycoprotein that binds VAMP2 [29]. At the cell soma, the interaction between Synaptophysin-1 and VAMP2 has been found to regulate the transport of this latter from the Golgi to the synapse, whereas, at presynaptic compartment, to control the availability of VAMP2 to participate to the assembly of SNARE complex, necessary for regulated exocytosis [47]. $A\beta_{42}$ has been demonstrated to directly compete with VAMP2 for binding Synaptophysin-1 at synaptic contacts and to prevent the formation of Synaptophysin/VAMP2 complex. As a result, $A\beta_{42}$ contributed to the formation of the fusion pore complex, resulting in the expansion of the primed synaptic vesicle pool, followed by neurotransmitter release [29]. Consistently, the enhancement of single-shock fEPSPs (field excitatory post-synaptic potential) by $A\beta_{42}$ at synapses further suggest an increased availability of releasable synaptic vesicles in hippocampal slices [29]. To prove that the enhancement of fEPSPs is not an artefact of the synthetic peptide, hippocampal slices were incubated with cell derived oligomers providing similar results.

Despite these data, a full comprehension of the intracellular mechanism through which Aβ influences the SNARE-mediated priming and fusion of synaptic vesicles and, subsequently, the release of neurotransmitter from presynaptic terminals is still under debate. Data from literature suggest that post-translational modifications, such as phosphorylation, of SNARE and accessory proteins by protein kinases at specific sites might represent a key regulatory mechanism that tightly modulates the exocytosis of synaptic vesicles and, consequently, neurotransmitter release from presynaptic terminals [41]. Moreover, taking into account that Aβ affects protein kinase transduction machinery [48, 49], it could be hypothesized that Aβ influences the phosphorylation of

SNARE and accessory protein and, subsequently, the assembly of SNARE complex by interacting with the transduction machinery of protein kinases. Within this context, experimental results demonstrated that low concentrations of Aβ inhibit the *in vivo* dopamine release in the rat nucleus accumbens (NAc) and counteract *in vitro* the muscarinic receptor-activated dopamine release from dopaminergic terminals by impairing protein kinase C (PKC) transduction machinery [50]. This hypothesis is further supported by *in vitro* results showing that the t-ACPD-induced PKC-mediated release of DA, elicited by the presynaptic metabotropic glutamate receptors (mGluRs) located on striatal nerve endings, was completely antagonized by $A\beta_{40}$ [51]. Such an action has also been demonstrated on signaling cascades downstream mGluRs, where 1 μM Aβ has been reported to impair mGluRs regulation of the γ-amminobutirric acid (GABA) transmission by inhibiting PKC transduction machinery in prefrontal cortical neurons [48]. Accordingly, Zhong et al. showed that Aβ impairs the muscarinic regulation of GABA transmission in prefrontal cortex, acting on the transduction machinery downstream muscarinic receptors and, particularly, inhibiting PKC [49]. Altogether, all these data point PKC as one of the potential substrates for Aβ inhibitory actions, a view which is also supported by data on a reduced PKC activity/content in tissues derived from AD patients [52, 53]. Interestingly, PKC has been demonstrated to serve a key role in post-translational modifications of SNARE and accessory proteins. The activation of PKC has been observed to enhance the exocytosis of synaptic vesicles by phosphorylating SNARE proteins including SNAP-25, Munc-18, and synaptotagmin [54–56]. In particular, phosphorylation of SNAP-25 at Ser^{187} in the SNARE domain [57] has been associated to an increased exocytosis of synaptic vesicles [58]. Katayama et al. recently demonstrated that knock-in (KI) mice deficient in the phosphorylation by replacing Ser^{187} of SNAP-25 with Ala exhibit an accumulation of synaptic vesicles in enlarged presynaptic terminals and a decreased efficacy of basal synaptic transmission at hippocampal CA1 synapses [59]. Moreover, Gao et al. found that phosphorylation of SNAP-25 by PKC regulates the exocytosis of synaptic vesicles and, consequently, noradrenaline release in PC12 cells, by affecting the SNARE complex assembly [60]. Phosphorylation of SNAP-25 at Ser^{187} by PKC has been found to increase the amount of bound VAMP-2. Such a finding suggests that Ser^{187}-phosphorylation may either upregulate v-SNARE (VAMP-2) binding to pre-existing t-SNARE (SNAP-25) or increase the stability of ternary SNARE complex, thereby promoting SNARE complex assembly and enhancing Ca^{2+}-dependent exocytosis. Phosphorylation of SNAP-25 at Ser^{187} by PKC has also been found to enhance Ca^{2+}-dependent release of dopamine and acetylcholine in PC12 cells [57].

Altogether, the involvement of PKC in the regulation of SNARE complex formation and experimental results demonstrating Aβ-induced impairment of PKC transduction machinery support the hypothesis that Aβ may also affect the exocytosis of synaptic vesicle by acting on protein kinases. Notably, Lee et al. first demonstrated that Aβ can modulate PKC activity by inhibiting PKC phosphorylation in a dose-dependent manner in cell-free *in vitro* condition [61], thus suggesting a direct interaction between Aβ and PKC. However, further investigations are needed to define Aβ-driven direct and indirect modulatory effects on PKC activity and to reveal the exact action mechanism underlying Aβ regulation of PKC activity.

At a first glance, the emerging role of the direct monomeric Aβ protein interaction with synaptic proteins seems to point to a putative facilitatory role on synaptic release machinery. It is not easy to predict what will be the consequences of a disease-associated excess Aβ production and oligomer formation. It can be postulated that, at preliminary step, synapses will face the upregulation of a reinforcing mechanism, leading to an excess of signaling which may contribute, for example, to excitotoxicity. With time and Aβ oligomer accumulation the picture may change.

Regulation of endocytosis

In neurons, synaptic vesicle endocytosis is controlled by a wide array of regulatory and adaptor proteins including epsin, AP-2 (adaptor protein-2), AP-180 (adaptor protein-180), and dynamin [62]. This latter is a GTPase synaptic protein, highly enriched in presynaptic terminals and involved in synaptic vesicle endocytosis and recovery. It promotes fission, pinching off, and recycling of synaptic vesicles, allowing them to reenter the synaptic vesicle pool to be refilled for future release [63, 64] and its levels and function are regulated by its cleavage by calpain. A decrease in dynamin levels due to its cleavage by calpain has been observed to inhibit synaptic vesicle endocytosis and, subsequently, their refill with neurotransmitters [65]. Interestingly, $A\beta_{42}$

has been reported to affect synaptic vesicle recycling acting on dynamin-1. Kelly et al. demonstrated that, in rat stimulated hippocampal neurons, high concentration (2 μM) of $A\beta_{42}$ soluble oligomers impair synaptic vesicle endocytosis and that such disruption was, at least in part, dependent on dynamin-1 depletion induced by calpain activation [66, 67]. However, further investigations are required to examine the specific action mechanism by which Aβ soluble oligomers stimulate calpain activation and to evaluate the functional consequences of Aβ-mediated dynamin-1 depletion in neurons.

Furthermore, $A\beta_{42}$ at nanomolar concentrations (50 nM) has been demonstrated to compete with VAMP2 for binding to Synaptophysin-1 at the synapse [29], which is known to regulate the retrieval kinetics of VAMP2 during endocytosis [68]. $A\beta_{42}$ has been postulated to hinder the ability of Synaptophysin-1 to initiate synaptic vesicle endocytosis via its interaction with VAMP2 [29]. Such hypothesis implies that Aβ peptides may act as a negative regulator of synaptic vesicle endocytosis after fusion and is consistent with data from literature demonstrating the Aβ-driven disruption of endocytosis and depletion of synaptic vesicles [66, 69].

Among these data, a work by Park et al. demonstrated that acute exposure (2 h) of rat stimulated hippocampal neurons to nanomolar concentrations (200 nM) of synthetic Aβ oligomers and monomers transiently reduced the efficacy of synaptic vesicle endocytosis [69]. When Aβ oligomer-containing medium was replaced with control medium after 2 h of exposure, endocytosis recovered to normal levels, indicating that Aβ oligomers-induced effects on endocytosis are transient and not permanent. Prolonged treatment (72 h) of neurons with the same concentration of Aβ oligomers has been shown to induce more severe defects, compared to acute treatment, in synaptic vesicle endocytosis, thus demonstrating that the extent of Aβ-induced endocytic damage also depends on the time of exposure [69]. Interestingly, defects in synaptic vesicle endocytosis were not observed when hippocampal neurons were exposed to the same preparation containing only Aβ monomers. Such result provides evidence that endocytosis was impaired by Aβ oligomers, even at low concentration, and not by monomers, thus suggesting that the aggregation states of Aβ peptides may be a key factor in Aβ-driven effects on synaptic vesicle endocytosis. Notably, PIPkinase-γ (phosphatidylinositol-4-phosphate-5-kinase type I-γ) overexpression, which is known to increase PtdIns(4,5)P_2 (phosphatidylinositol-4,5-bisphosphate) levels, completely prevented the Aβ-induced defects in endocytosis in rat stimulated hippocampal neurons [69]. Accordingly, Berman et al. found that Aβ oligomers induced a reduction in PtdIns(4,5)P_2 levels via phospholipase C (PLC). In addition, PtdIns(4,5)P2 has been demonstrated to affect clathrin-mediated endocytic processes by binding several endocytic components, including AP-2, AP-180, dynamin, and epsin, thus playing an key role in recruiting these molecules to sites of endocytosis [70–74]. Collectively, these findings support the hypothesis that PIPkinase-γ overexpression compensates for the Aβ oligomers-induced decrease in PtdIns(4,5)P_2 levels, whose abnormally reduced or increased levels have been linked to defects in synaptic vesicle endocytosis [75].

Furthermore, Lazarevic et al. tested the effects on synaptic vesicle recycling of increased extracellular concentrations of $A\beta_{42}$ and $A\beta_{40}$ (1.6 and 1.2 fold, respectively), induced by the inhibition of the Aβ-degrading enzyme neprilysin, in rat cortical and hippocampal neurons cultures [76]. Enhanced levels of $A\beta_{40}$ and $A\beta_{42}$ have been found to increase the activity-driven synaptic vesicles recycling in both excitatory and inhibitory synapses, as shown by quantification of synaptotagmin 1 antibody uptake. Such effect was completely prevented by chelation of extracellular Aβ using 4G8 antibody, thus confirming that changes in synaptic vesicle recycling rely on the concentrations of the endogenously secreted Aβ peptides. In line with this evidence, treatment either with β-secretase or γ-secretase inhibitors led to a significant decrease in synaptic vesicle recycling, strongly supporting the involvement of endogenous Aβ peptides in the modulation of basal synaptic vesicle recycling. Moreover, 1-h exposure to picomolar (200 pM) concentrations of synthetic $A\beta_{40}$ and $A\beta_{42}$ induced a significant enhancement in synaptotagmin 1 antibody uptake; whereas, 1-h treatment with 1 μM Aβ has been found to decrease it. Collectively, these experimental results are consistent with the hypothesis of an hormetic effect of Aβ peptides, with low concentration (high picomolar) potentiating synaptic vesicle recycling and high (high nanomolar-low micromolar) exhibiting the opposite effect in the same experimental setting.

Furthermore, the effects on depolarization-driven synaptic vesicle recycling, induced both by inhibition of Aβ degradation and application of 200 pM $A\beta_{42}$, have been demonstrated to be fully inhibited by pretreatment with α-bungarotoxin, thus suggesting

the involvement of functional α7-nAChR in Aβ-mediated regulation of presynaptic functions [76]. While the effect of 200 pM $A\beta_{40}$ and $A\beta_{42}$ was completely prevented by pharmacological interference with α7-nAChR, the effect of 1 μM $A\beta_{42}$ was not hindered by the blockage of these receptors, suggesting that, at higher concentrations, $A\beta_{42}$ may act through different action mechanisms.

Moreover, the endogenous Aβ-driven modulation of synaptic vesicle recycling has been hypothesized to rely on calpain-cyclin dependent kinase 5 (CDK5) and calcineurin signaling pathway downstream of α7-nAChR. A CDK5 and calcineurin activity assay confirmed that cells treated with neprilysin inhibitor and 200 pM $A\beta_{42}$ showed significant decrease in CDK5 activity, without changes in total protein levels; on the other hand, a phosphatase activity assay revealed significantly higher calcineurin activity [76]. Such results indicate that balancing the activity of CDK5 and calcineurin may play a role in Aβ-driven modulation of recycling. However, further investigations are needed to better characterize the specific intracellular mechanism through which Aβ regulates this step of synaptic vesicle cycle. To date, only few studies investigated Aβ-driven effects on synaptic vesicle recycling and the potential underlying intracellular mechanism.

Overall, the defects of endocytosis elicited by Aβ oligomers, as well as monomers, may aggravate the synaptic derangement as the disease progresses. The impairment of endocytosis might alter the ability of the synapse to sustain neurotransmitter release, particularly at the level of nerve terminals discharging at high rate, leading to their dysfunction.

Regulation of recycling/resting pool ratio

The synaptic vesicle pool constitutes a recycling pool, including a ready releasable pool, which is docked at the active zone and ready for immediate release, and a reserve pool, a reservoir to refill vesicles after depletion, and a resting pool that does not normally participate in the synaptic vesicle recycling. Park et al. (2013) observed that the acute treatment (2 h) of cultured rat hippocampal neurons with nanomolar concentrations (200 nM) of soluble Aβ oligomers altered the recycling/resting pool ratio by expanding the resting fraction at the expense of the recycling fraction [69]. The average total number of synaptic vesicles has not been altered. Pretreatment of Aβ oligomers with 6E10 antibody blocked the effect on recycling/resting pool, thus indicating that alteration of the recycling/resting pool ratio relies on Aβ oligomers. In addition, they suggested that the observed effects of Aβ oligomers on pool size are mediated by the activation of CDK5 pathway. Consistently, the CDK5 inhibitor roscovitine and the calpain inhibitor III have been observed to restore the recycling and resting pool near to control levels. Such evidence demonstrates that CDK5 mediates Aβ oligomers-induced alterations of the recycling/resting pool size, an observation consistent with data from literature pointing CDK5 as the main kinase involved in the regulation of synaptic vesicle pool size [76, 77].

Moreover, two independent studies by Marsh et al. and Park et al. recently proved that soluble $A\beta_{42}$ oligomers interfere with Synapsin I, a presynaptic adaptor phosphoprotein, that, under resting conditions, tethers synaptic vesicles to the cytoskeletal network clustering them in the resting pool, by interacting both with synaptic vesicles and the actin cytoskeleton [78–80]. Activity-dependent phosphorylation of Synapsin I at Ser^9 within a small N-terminal lipid-binding domain by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) induces its transient disassembly from synaptic vesicles [81] and stimulates the release of synaptic vesicles from the resting pool, enabling their participating in neurotransmitter release. Aβ has been reported to affect the phosphorylation/dephosphorylation dynamics of Synapsin I [79, 80]. Marsh et al. demonstrated that the acute exposure (30 min) of primary rat hippocampal neurons to nanomolar concentrations (300 nM) of $A\beta_{42}$ oligomers enhanced the levels of phosphorylated Synapsin I at Ser^9 after neuronal activity at presynaptic terminals [79]. While in neurons exposed to scrambled $A\beta_{42}$ peptide, the enhanced levels of phosphorylated Synapsin I at Ser^9 have not been detected, confirming that the effect is mediated by $A\beta_{42}$ oligomers. The prolonged phosphorylation of Synapsin I has been found to prevent Synapsin I from tethering synaptic vesicles to the reserve pool after depolarization, thus increasing the availability of synaptic vesicles to dock to the active zone and, consequently, to allow glutamate release from presynaptic terminals [79]. Such hypothesis is consistent with several reports showing that $A\beta_{42}$ oligomers affect glutamate release in a concentration and time dependent manner [11, 15, 29, 82]. Interestingly, the levels of phosphorylated Synapsin I at Ser^9 are increased in postmortem tissue from AD patients [83]. Accordingly, Park et al., using a

live-cell imaging technique to monitor synaptic vesicle trafficking, demonstrated that the exposure of rat hippocampal neurons to nanomolar concentrations (200 nM) of soluble $A\beta_{42}$ oligomers markedly enhances the levels of phosphorylated Synapsin at Ser^9 by activating CaMKIV. As a result, Synapsin I has been found to disassembly either from synaptic vesicles and actin, subsequently inhibiting the intersynaptic vesicular trafficking along the axon [80]. However, it is still unclear how soluble $A\beta_{42}$ oligomers increase intracellular Ca^{2+} that is critical for the phosphorylation-dependent dissociation of Synapsin-synaptic vesicles-actin ternary complex. Recently, soluble $A\beta_{42}$ oligomers have been demonstrated to increase intracellular Ca^{2+} both enhancing extracellular Ca^{2+} influx and Ca^{2+} release from mitochondria [84].

CONCLUDING REMARKS

Despite the intense effort directed to develop novel therapeutic interventions for the treatment of AD, to date no drugs are yet available to significantly benefit people affected by AD and the few approved drugs so far can only be used for symptomatic treatment of the disease, but not to prevent or reverse it. The main strategy for the development of drugs counteracting AD has been to reduce Aβ accumulation due to its overproduction and/or defective clearance. However, the proved ineffectiveness shown by such approaches, specifically targeting the production or clearance of Aβ peptides, has sparked an intense debate in the scientific community concerning the validity of the amyloid cascade hypothesis. Nevertheless, the neuronal dysfunction caused by Aβ accumulation is still recognized as a significant factor contributing to the progression AD that cannot be discounted [3]. The failure of several clinical trials to meet the desired endpoints highlights the necessity to refocus the experimental approach from frank neurodegeneration on early pathogenic alterations that may cause or contribute to AD. Defective synaptic activity and loss of synapses are the earliest event in AD that precedes the accumulation of Aβ plaques in the brain and clinical outcomes of the disease [5]. In particular, it emerges from the previous paragraphs that during progression of the disease two phenomena may lead the transition from physiology to pathology. At the beginning the increasing concentrations of Aβ monomers may lead to synaptic reinforcement through fusion stimulation and endocytosis inhibition. With further increase of Aβ and the onset of aggregation phenomena the exocytosis inhibition may prevail leading to the impairment of nerve terminals, mainly of those discharging at a high frequency rate, accompanied by an inhibition of the release leading to a more generalized synaptic failure. In all phases, additional intracellular signaling effects exerted through an action of Aβ on kinases may add further complexity in an area-dependent manner. Hence, a deeper understanding of the mechanisms through which Aβ peptides affect synaptic activity and, in particular, synaptic vesicle dynamics orchestrating neurotransmitter release, is needed to elucidate Aβ functions and might be a starting point to understand the early phases and manifestation of the disease as well to design new neurotransmitter/synaptic based strategies to correct these symptoms.

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