

Pore-Forming Neurotoxin-Like Mechanism for A β Oligomer-Induced Synaptic Failure

Luis G. Aguayo, Jorge Parodi, Fernando J. Sepúlveda, and Carlos Opazo

Abstract Cortical and hippocampal synapse densities are reduced in Alzheimer's disease (AD), and this strongly correlates with memory dysfunction. It is now believed that these changes in neuronal networking occur at the onset of AD and may lead to the neuronal loss displayed in later stages of the disease, which is characterized by severe cognitive and behavioral impairments. Mounting evidence indicates that amyloid- β (A β) oligomers are responsible for synaptic disconnections and neuronal death. One of the main consequences of A β oligomers interaction with neurons is an increase in intracellular Ca²⁺ concentration that could, when large enough, cause a marked alteration in ionic homeostasis. It has also been postulated that Ca²⁺ influx occurs when A β oligomers induce the opening of Ca²⁺ channels or the disruption of the plasma membrane. We recently found that the effects of A β oligomers on synaptic transmission are similar to pore-forming toxins, such as α -latrotoxin, a neurotoxin from the black widow spider. Here, we discuss evidence supporting a neurotoxin-like mechanism for the effects induced by A β oligomers on neuronal membranes, which could explain the alterations in the functionality of synapses in the central nervous system in AD that leads to major neurodegeneration with time of exposure to A β oligomers.

Abbreviations A β : amyloid- β peptide, AD: Alzheimer's disease; α -LTX: α -latrotoxin; A β PP: amyloid- β protein precursor; Ca²⁺: calcium; LTP: long-term potentiation; pS: picoSiemen

1 Increase in Soluble A β Oligomers is a Key Factor for Alzheimer's Disease Onset

One of the main histopathological features of Alzheimer's disease (AD) is the presence of extracellular proteinaceous deposits in the brain, identified as senile plaques [1], which are enriched in amyloid- β (A β) peptide oligomers. It is widely

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accepted that AD onset can be initially triggered by interaction of A β oligomers with the brain parenchyma [1, 2]. However, the specific biochemical/structural characteristics of the A β oligomers that induce the neurotoxicity observed in AD have not been thoroughly identified, but it has been suggested that soluble A β oligomers (ranging from 17 to 56 kDa) [2, 3] are key determinants for neurotoxicity, including synaptic failure, observed in AD [2]. In agreement with this, the levels of soluble A β oligomers appear to correlate well with the severity of AD dysfunction [2, 4]. Interestingly, these soluble oligomers produced synaptic toxicity as expressed by inhibition of hippocampal long-term potentiation (LTP) *in vivo* and alterations in complex animal learning behaviors [2, 5]. These data strongly suggest that AD onset, probably associated to a mild synaptic dysfunction, occurs before amyloid plaque formation. However, the *mechanism (pre- or postsynaptic) by which A β oligomers cause synaptic dysfunction is largely unknown*.

Additionally, previous studies have shown that A β oligomers affect neuronal morphology and survival, and produce axonal and dendritic dystrophy [6]. These alterations seem to occur following amyloid deposition in the brain, indicating that accumulation of A β oligomers precedes the alterations in neuritic morphology [7]. Thus, these studies provide evidence suggesting a *decrease in neuronal networking in AD as a product of A β oligomers accumulation*. How this A β oligomers accumulation produces such a strong disruption in synaptic transmission in the central nervous system is currently under active investigation with the aim of discovering therapeutic targets and disease-modifying treatments.

2 Early Synaptic Alterations Precede AD Onset

It has been postulated that alterations in synaptic plasticity might be the primary failure responsible for the cognitive dysfunction in AD [8]. However, the scope and strength of studies supporting this challenging suggestion is only now being considered at the cellular and molecular level. It is currently known that synaptic transmission in the brain can be altered by specific and nonspecific mechanisms at pre- or postsynaptic sites.

In the case of A β , studies in hippocampal neurons treated with synthetic A β oligomers showed that it reduced the number of synaptic contacts and various pre- and postsynaptic proteins, thus suggesting extensive alterations in neuronal connectivity [9, 10]. In agreement, transgenic mice models overexpressing amyloid- β protein precursor (A β PP) showed a marked reduction in synaptophysin levels [11]. Interestingly, it was reported that synapse loss was highly correlated to neurological deficits observed in mild-to-severe stages of AD, supporting a direct link between cognitive functions and neurotransmission [12]. Furthermore, it was shown that early changes in synaptic morphology and markers such as synaptophysin correlate better to disease progression, suggesting that synaptic components are the most probable targets for the early neurotoxic actions of A β oligomers. Specifically, several proteins having well-defined functions in synaptic vesicle endocytosis,

including AP2, AP180, dynamin, and synaptotagmin, have been reported to be extensively altered in AD [13].

How A β oligomers are able to produce this myriad of effects on synaptic proteins is unknown, but it is possible that these changes have a common triggering membrane mechanism. Nevertheless, these findings suggest that AD is associated with failure in the cellular machinery responsible for synaptic release and recycling. Additionally, alterations in synaptic proteins produced by the action of A β oligomers can explain its functional impact in models of cellular learning and memory, such as LTP [5]. *Here, we are proposing that A β oligomers affect synaptic transmission through its channel-forming properties (see below).* If A β oligomers cause synaptic transmission failure by pore formation, the following steps must occur for this mechanism to be demonstrated: (1) interaction of A β oligomers with neuronal membranes, (2) pore formation, (3) increase in intracellular calcium, (4) sustained increase in vesicular release, and (5) vesicular depletion (synaptic failure). We have found that A β oligomers produce several of these cellular events, as described below.

3 Calcium and Synaptic Dysfunction in AD

There is a current growing body of evidence suggesting the existence of a dysfunction in intracellular Ca²⁺ homeostasis in AD [14]. Prefibrillar A β oligomers have been shown to elevate Ca²⁺ in neurons. This increase in intracellular Ca²⁺ can follow receptor activation [15], modulation of voltage-activated Ca²⁺ channels [16], and influx via nonselective cations or by pore/channels formed by A β oligomers [17].

Analysis of the peptide secondary structure suggests the possibility of ion channel formation induced by membrane-bound A β oligomers [18]. The A β -pore/channel hypothesis was first proposed by Rojas and collaborators at the NIH using artificial membranes. They demonstrated the formation of pores with A β_{1-40} that were highly cation-selective, allowing permeation of Ca²⁺, Na⁺, and Cs⁺ [19]. These early studies in synthetic membranes were validated in membranes from hypothalamic cell lines [20]. Interestingly, cholesterol levels favored the formation of A β channels in artificial and hypothalamic membranes [20, 21]. Single channel measurements showed that the behavior of the A β_{1-40} -induced channels were exceptionally complex, in addition to their strong dependency on Cs⁺ concentration and variability of single channel conductance (50–500 pS) [21]. Also, it was found that Zn²⁺, known to bind A β in solution [22], blocked ion current flow [21], suggesting that the A β amyloid pore can be a pharmacological target. All together, the data suggest that A β oligomers do not form a unique, well-behaved type of ion channel, but they contribute to the formation of a complex multiple family of conducting pores [23]. Interestingly, using an “oligomer-enriched” form of A β , an increase was shown in lipid bilayer conductance, in the absence of unitary events, adding to the complex behavior of the peptide in the membrane [24].

In conclusion, it is evident that A β oligomers are able to increase the conductance in artificial membranes, but this has not been demonstrated in biologically relevant cell (neuron) membranes.

4 Proposed Neurotoxin-Like Mechanism for A β Oligomer-Induced Synaptic Failure

The cellular and molecular mechanisms that induce AD are largely unknown and deter development of effective disease-preventing/modifying therapies. The most accepted working hypothesis of AD is that excess of A β oligomers either (1) bind to membrane receptors affecting their functions [25], (2) interfere with signaling cascades [26], or (3) directly disrupt neuronal membranes causing pore formation thus leading to alterations in ionic homeostasis [21]. Although the latter is an attractive hypothesis because it could explain several effects of A β oligomers on brain synapses, it has not been documented to occur in brain neuronal membranes and this could be due to the high complexity of biological membranes, such as heterogeneity in native ion channels and receptors.

In an attempt to elucidate the mechanism by which A β oligomers induce synaptotoxicity, we undertook an experimental approach to characterize how A β oligomer affect synaptic transmission and compared these effects with those produced by neurotoxins known to form membrane pores. We found that the effects of A β oligomers, although at higher concentrations (nM vs pM), were very similar to those of pore-forming α -latrotoxin (α -LTX, 130 kDa) allowing us to suggest that its neurotoxicity was dependent on pore formation within the cell membrane. For example, similar to α -LTX [27, 28], we found that A β oligomers directly increased membrane conductance and intracellular calcium causing an early increase and a delayed failure in synaptic release (Fig. 1b).

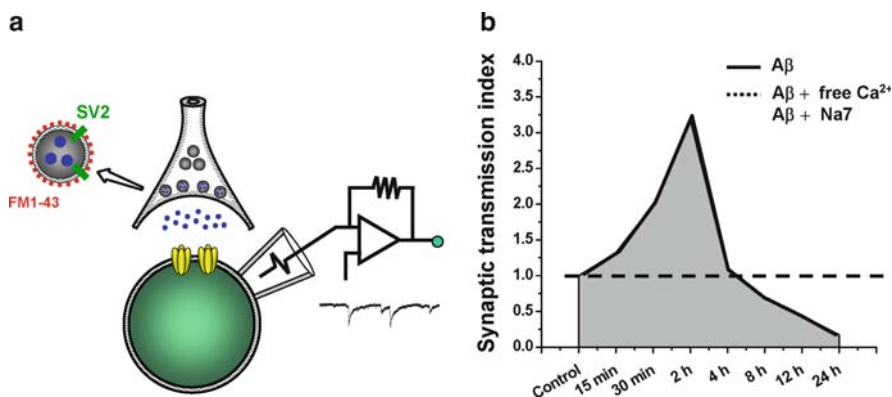


Fig. 1 Effects of A β_{1-40} oligomers on synaptic activity of hippocampal neurons. **a** The scheme illustrates pre- and postsynaptic components of a central synapse. The vesicles are released in a calcium-dependent fashion. Presynaptic activity can be determined by the presence of vesicular proteins (SV2) or by the staining of synaptic vesicles with fluorescent probes such as FM1-43 (red dots). The postsynaptic membrane currents associated to the vesicular release and postsynaptic receptor density can be analyzed using the patch clamp technique. **b** Time-dependent biphasic effect of 500 nM A β oligomers on synaptic transmission in hippocampal neurons. The effects of A β oligomers were blocked by lowering extracellular calcium or by adding Na7 (broken line). The values were obtained from three independent experiments. (See Color Plates)

It has been recognized for several years that α -LTX can alter membrane permeability generating nonselective ionic pores [29]. Therefore, the mechanism for toxicity depends on attachment to the cell membrane and disruption of ionic permeability [27]. The majority of studies with α -LTX strongly support the idea that the main increase in intracellular calcium results from Ca^{2+} entry through nonselective cation channels formed by membrane-bound toxins [30]. Once bound to neuromuscular junction membranes, α -LTX forms oligomeric structures that stimulate exhaustive release of neurotransmitters [29]. One of the most distinguishing features of α -LTX on the synapse is that it produces a marked vesicular depletion [31]. Interestingly, we found that A β oligomers were able to mimic all of these effects in hippocampal neurons. For example, when examining the effects of nanomolar concentrations of A β_{1-40} oligomers on the spontaneous synaptic activity in living hippocampal neurons, using patch clamp and fluorometric imaging (Fluo-3 and FM1-43) (Fig. 1a), we found that the effects of low concentrations of A β oligomers on synaptic transmission were biphasic, with a rapid facilitation followed by a delayed failure (Fig. 1b). We also found that the delayed synaptic failure correlated nicely with a decrease in several presynaptic proteins, such as SV2 (Fig. 2a and b). These results indicate that A β oligomers were able to produce a significant loss of connectivity in central neurons participating in learning and memory, which is in agreement with the idea that cognitive alterations in AD are associated to a synaptic failure [8, 12]. More importantly, blockade of the A β pore with a small peptide, previously shown to inhibit A β oligomers-induced increase on membrane permeability [32], protected the hippocampal neurons from synaptotoxicity, maintaining high levels of SV2 associated to neurotransmitter vesicles in the presence of A β oligomers (Figs. 1b and 2).

We propose that future studies of this membrane phenomenon will reveal that the target of A β oligomers are not another protein, *but will show that A β oligomers themselves are the cellular target thereby explaining the failure of pharmacological*

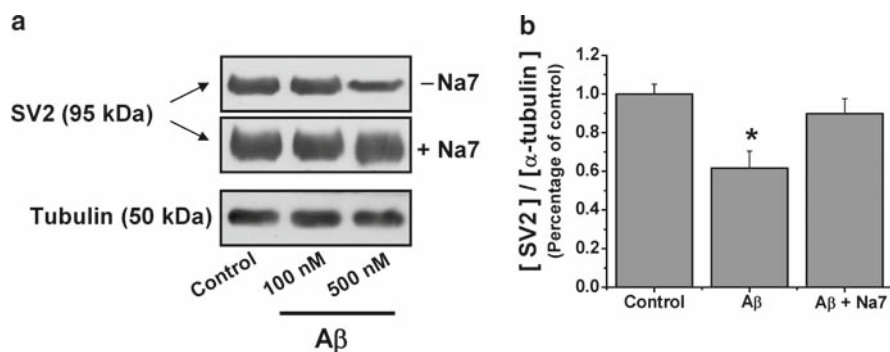


Fig. 2 The effect of A β_{1-40} oligomers on vesicular SV2 level was blocked by Na7. **a** Western blots for SV2 obtained from hippocampal neurons incubated in the absence (control) or presence of A β_{1-40} oligomers (100 and 500 nM) during 24 h. **b** Quantification of SV2 levels in the absence or presence of Na7 (200 nM). Note that Na7 blocked the reduction in SV2 induced by A β oligomers.

agents to modify the course of AD. Additionally, these studies should provide a new rationale for the development of drugs that block the A β pore and possibly interfere with AD onset.

Our data indicates that the effects of A β oligomers on intracellular Ca²⁺ play a key role in the alterations on synaptic transmission induced by the peptide, which is in agreement with previous studies involving this divalent cation on A β oligomers effects. For example, we found that the effects of A β oligomers on intracellular calcium and its associated synaptic transmission were largely attenuated by reducing the influx of calcium either by removal of this cation or by pharmacological means (Figs. 1b and 2). Thus, it is possible to conclude that the synaptic effects of A β oligomers are calcium-dependent [14] and able to be modulated.

Several questions concerning the mechanisms for A β oligomers insertion and perturbation of neuronal membranes should be resolved in future studies. For example, although formation of α -LTX pores seems to be mostly independent of membrane receptors, some membrane proteins could facilitate pore insertion [29]. Equivalent mechanisms may be true for the interaction of A β oligomers with neuronal membranes. Studies of the features of α -LTX using cryoelectron microscopy demonstrate that α -LTX penetrates the cell membrane and forms pores having a large diameter (10–25 Å) that facilitates the release of several neurotransmitters (e.g., norepinephrin, glutamate, and gamma amino butyric acid) by a nonvesicular efflux mechanism [29]. Such data is not available for A β -induced pores, but functional studies have indicated that A β pores are able to carry divalent cations [19, 33]. The inner diameters of A β pores, estimated by atomic force microscopy and molecular dynamics analysis [34, 35], have a similar range (15–20 Å). Therefore, they might permit the nonvesicular efflux of several metabolites, generating important changes in the metabolic cellular state. Because of the remarkable similarities in the mechanism of action between these pore-forming neurotoxins and A β oligomers (Table 1), we postulate that a pore-forming mechanism might explain how A β oligomers induce the synaptic dysfunction and neurodegeneration in AD (Fig. 3). We propose that A β oligomers might also bind to postsynaptic membranes causing their remodeling, but with a slower time course.

Table 1 Comparison between α -LTX- and A β oligomers-induced pores

	¹ α -LTX	² A β	References
MW of monomer	130 kDa	4 kDa	[² 1, ¹ 29]
Proposed number of monomers/pore	4	≥ 12 and < 24	[¹ 29, ² 35]
Channel conductance (approximated)	Multiple levels, 100–300 pS	Multiple levels, 50–500 pS	[² 21, ¹ 27]
Estimated inner pore diameter	10–25 Å	15–27 Å	[¹ 29, ² 35]
Main cations transported	Ca ²⁺ , Na ⁺	Ca ²⁺ , Na ⁺ , Cs ⁺	[² 19, ¹ 27]
Effective protein concentration	< 1 –10 nM	100–500 nM	[¹ 27, ² 33]
Onset of synaptic action	20 min	30 min	[¹ 28, ² 33]
Acute enhancement of vesicular release	yes	yes	[¹ 31, ² 33]
Delayed vesicular depletion	yes	yes	[¹ 31, ² 33]

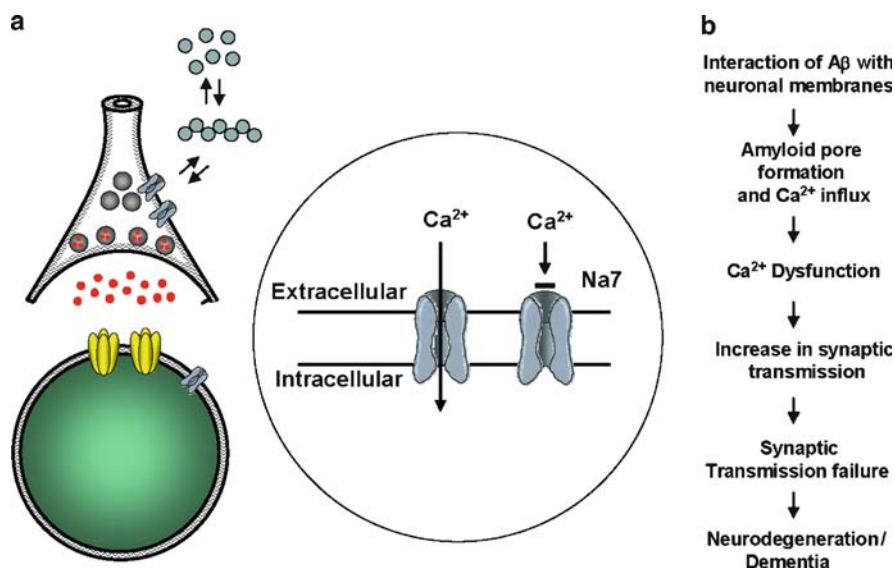


Fig. 3 Proposed hypothesis that explains the effect of A β oligomers on synaptic transmission. **a** A β oligomers bind to the membrane inducing the formation of pores in the pre- and postsynaptic membranes. The pores allow calcium to enter the cell and this event can be blocked by Na7. **b** Proposed series of events that leads to AD. Its initiation depends on oligomerization and membrane perturbations that lead to a calcium dysfunction and alterations in synaptic transmission. (See Color Plates)

5 Conclusions

We are currently studying the mechanisms that can explain the changes in intracellular calcium and synaptotoxicity following A β oligomers application to brain neurons by testing: (1) voltage-dependent calcium channels, (2) *N*-methyl D-aspartate (NMDA) receptors, and (3) membrane conductance following A β pore or channel formation. From this data, we expect to learn how A β oligomers inhibits synaptic transmission in brain neurons involved with learning and memory, with the aim of stopping or better still, reversing this process. The most interesting feature of A β oligomers on synaptic transmission is its biphasic action which leads to a strong synaptic failure that we interpret as a process of synaptic depletion. Interestingly, when the early effects of A β oligomers were blocked with low extracellular calcium, cadmium, or ruthenium red, the neurons did not show synaptic inhibition suggesting that the delayed inhibition was similar to the synaptic depletion induced by α -LTX.

According to previous and present evidence, three principal steps are involved in the neurotoxin-like mechanism for the action of A β oligomers to induce the early synaptic effects needed to trigger AD onset. First, A β oligomers have to bind to neuronal cell membranes, pre- and postsynaptic, long enough to ensure A β -A β

interactions into the membrane. Second, A β forms oligomers into the cell membrane to allow the formation of a pore. Third, a sustained flow of cations (Ca²⁺, Na⁺) through the A β pores initiates the modification of synaptic activity, which leads to remodeling synaptic morphology. *We postulate that understanding the precise mechanism for each of these steps will greatly facilitate a pharmacological therapy urgently needed for the world-wide population affected by AD.*

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