Chapter 17

The Role of Alzheimer Aβ Peptides in Ion Transport Across Cell Membranes

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Abstract: Accumulation of beta amyloid (Aβ) fibrils in senile plaques and cerebral blood vessel walls is characteristic of Alzheimer's disease (AD). We discuss several models that seek to explain the neurotoxic consequences, in particular the manner in which the neurotoxicity promotes cell dysfunction and cell death by an increase in cytosolic calcium ion concentration. To base correctly a new therapy on *in vitro* experiments, one must choose the right model mechanism.

Key words: Alzheimer's disease, Aβ peptide, ionophore, misfolding, β-sheets, calcium homeostasis, AMPA receptor.

1. INTRODUCTION

The pathology of Alzheimer's disease (AD) was first defined by Alois Alzheimer in 1907. He saw by light microscopy "senile plaques" (see Figure 1) and also "tangles" in stained sections from post-mortem brains of his patients. Much later the chemistry of the plaque components was described (Glenner and Wong, 1984; Sisodia *et al.*, 1990). It turned out that the plaques were composed of fibrils formed by the aggregation of short peptides, mostly 40 and 42 amino acids long, which had a perfectly normal amino acid sequence, common to both. They represented a part of a much larger transmembrane "amyloid precursor protein" (APP), commonly found but of unknown function. For a variety of reasons, some genetic, some unknown, these peptides are overproduced in Alzheimer's Disease. This matters, because the two overexpressed peptides, Aβ1-40 and Aβ1-42, once liberated from their parent APP, refold and aggregate spontaneously to a

neurotoxic form. They attack paritcular neurons in the CNS, causing them to become dysfunctional and eventually to die in large numbers. It should be remembered that the plaques are extracellular and exert their toxic effect from the outside. This distinguishes AD from other amyloidoses, such as Huntington's disease, for example, which are intracellular phenomena. A part of the consequent dysfunction is thought to be the hyperphosphorylation of the microtubule-asssociated protein tau (Ferreira *et al.*, 1997) which then aggregates to form the "tangles" that fill then neuronal cell body and produce dysfunctional neurons.

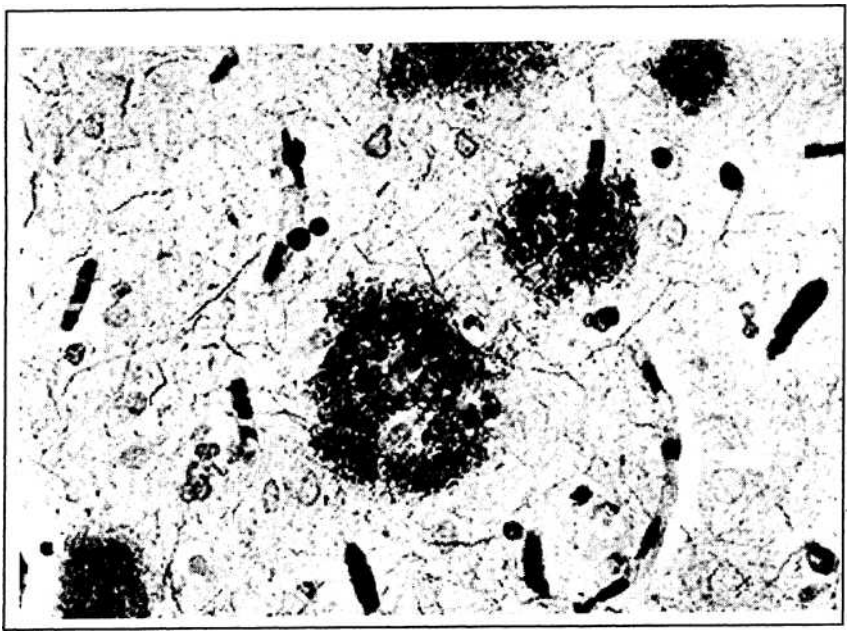

Figure 1. Plaques of Alzheimer's Disease (The Internet Pathology Laboratory). Stained section of an AD brain. The plaque is an accumulation of Aβ fibrils; note that the surrounding neuritis are also stained, indicating an interaction with plaque fibrils and neighboring neuritis.

The pathological over-production of this family of amyloid peptides is due to a variety of causes, some of which are genetically controlled, giving rise to the so-called Familial Alzheimer's disease, which are fortunately rare. Clearly, there are other causes, the mechanisms of which are not yet clearly understood. The accumulation of the Aβ1-42 peptide seems to be the most important phenomenon in causing disease, although this notion is somewhat controversial.

It is characteristic of both Aβ1-40 and Aβ1-42 that once released from the APP they rapidly and spontaneously refold to oligomeric aggregates that quickly form long fibrils with high β-sheet content. A currently popular view is that this "misfolding" produces a new conformation with new properties. Current research is devoted to understanding the molecular mechanism of Aβ neurotoxicity.

While these considerations place the emphasis on understanding the origin and pathological role of the Aβ peptides, it ignores the formation and function of the intracellular tangles. These fill the axons and somata of affected neurons and thereby interfere with axonal transport, synapse formation and signalling. It is unclear whether Aβ plaque formation and tau tangle formation are independent processes, or whether tangle formation is a downstream event that follows Aβ formation and Aβ aggregation, as this author believes. Further complicating this issue is the existence of a number of tau mutations that seem to favor tau hyperphosphorylation and that are associated with tangle formation and with severe dementia. The events leading to tangle formation will not be considered further in this review.

We here review the mechanisms of Aβ peptide toxicity that are currently under discussion. We must first devise a useful definition of "Aβ peptide toxicity". In many literature quotations this is taken to mean the induction of neuronal cell death by apoptosis or necrosis. These events occur in the central nervous system. Generally little notice is paid to the always present glial cells, their role in Alzheimer's and their fate. But there is a general gliosis associated with Alzheimer's, often a "reactive astrocytosis". Is there no glial cell death in AD? For the purposes of our discussions, and ignoring the uncertain fate of glial cells, we will assume that in AD Aβ peptide toxicity is sufficient to lead to the death of neurons, at least in the first instance.

Another important notion not usually mentioned is the important intermediate stage between initial peptide-neuron interaction and eventual cell death. It seems highly unlikely that a normal neuron "dies" instantly when attacked by the toxin. We clearly need a new definition of neuronal cell death. It seems more likely that there will be a series of stages when a neuron becomes gradually more and more dysfunctional. We have no idea how long this process might take or what its various steps will be. For the purposes of this review, we will define "Aβ peptide toxicity" as the mechanistic step that sends the neuron into the death process.

Although one's intuition is to find one single initiating mechanism, it is quite possible that there are two, perhaps three, quite different mechanisms that operate at different stages of Alzheimer's disease or that are found simultaneously in different brain cell types. The discussion that follows explores four different neurotoxic mechanisms below. The proposed

mechanisms all point to Aβ1-42 and Aβ1-40 as the best targets for therapeutic intervention, which makes it all the more important to understand these mechanisms.

1.1 Increased cytosolic [Ca^{2+}] causes cell dysfunction and cell death

While the action of aggregated Aβ peptides on neurons is the precipitating factor in causing internal [Ca^{2+}] to rise by the mechanisms discussed below, the real damage to the cell is done by the increased calcium itself. It triggers a number of important downstream effects. For example, Rao *et al.* (2004) write: "The endoplasmic reticulum (ER) regulates protein synthesis, protein folding and trafficking, cellular responses to stress and intracellular calcium (Ca^{2+}) levels. Alterations in Ca^{2+} homeostasis and accumulation of misfolded proteins in the ER cause ER stress that ultimately leads to apoptosis. Prolonged ER stress is linked to the pathogenesis of several different neurodegenerative disorders." Other examples of the role of Ca^{2+} levels is described by Agell *et al.* (2002) and Mattson *et al.* (1992) The literature is extensive enough to reinforce the notion that disturbance of the tightly controlled calcium homeostasis is pivotal in bringing about deleterious downstream effects in Alzheimer's disease.

2. Aβ PEPTIDES RAISE CYTOSOLIC Ca^{2+} CONCENTRATIONS BY AN UNKNOWN MECHANISM

Mattson *et al.* (1992) reported experiments using human cerebral cortical cell cultures to test the hypothesis that "β-amyloid can destabilize neuronal calcium regulation and render neurons more vulnerable to environmental stimuli that elevate intracellular calcium levels." The environmental stimuli used are glutamate and other excitatory amino acids. The β-amyloid peptides involved are Aβ1-38 and Aβ25-35, shorter versions of the main peptides now known to be involved in AD, Aβ1-40 and Aβ1-42. More recent experiments from other laboratories (*eg* Teplow, Ingram) indicate that the state of aggregation of the peptide is crucial in determining whether an aggregate is toxic to the neuron or not. Mattson and Furukawa (2003) in their chapter found that over a period of several days these peptides by themselves did not shorten survival times of the neurons, but they did exacerbate the killing effect of glutamate during the same time. The first time point recorded was 1 day. The relation of these findings to the pathology of AD is not obvious, since Aβ25-35 is not found in the disease

and Aβ1-38 is a minor product. Nevertheless these findings stimulated a large number of experiments.

Mattson and Furukawa (2003) summarize more than a decade of work from this group. Their ideas are centered on the notion that the Alzheimer Aβ peptides disturb cellular calcium homeostasis and cause oxidative changes in neuronal cell membranes. They see a large number of deleterious processes in AD as down-stream from this phenomenon. The initiating event is said to be that "during the process of self-aggregation, Aβ generates hydrogen peroxide and hydroxyl radicals and the production of these reactive oxygen species induces the peroxidation of lipids in the plasma membrane which can impair the function of membrane ion-motive ATPases (Na^+/K^+- and Ca^{2+}-ATPases, and glucose and glutamate transporters), resulting in membrane depolarization and a decrease in cellular ATP levels." Disruption of cellular calcium homeostasis follows. Evidence for these downstream cellular events is given in the summary and in the group of papers that are cited. These experiments involve substantial time delays from application of Aβ peptide and reading of the assay. The initiating event remains in doubt. Although the statement is made in Mattson and Furukawa that "During the process of self-aggregation, Aβ generates hydrogen peroxide and hydroxyl radical....." followed by the downstream events mentioned above, no direct evidence is given in the review or in the cited references to substantiate this important notion concerning initiation.

Behl *et al.* (1994) [*see also* Chapter 4] produced evidence that "the cytotoxic action of Aβ on neurons results from free radical damage to susceptible neurons". Using PC12 neuronal cells, B12 cells derived from rat brain tumors and CNS primary cultures they showed that Aβ causes the overproduction of H_2O_2, leading to cell death. The Aβ peptides used were only Aβ1-40 and Aβ25-35. (At the time of these experiments the importance of Aβ1-42 overproduction in the pathogenesis of AD was not generally appreciated.) Also, the question of whether the Alzheimer peptides are aggregated or not is not addressed in this early paper. Since the toxicity assays are done after considerable delay, one can assume that a measure of aggregation has taken place during the Aβ challenge. They show an increase in H_2O_2 production which could "lead directly to cell death, probably via hydroxyl radical induced oxidative damage". These authors demonstrate cytotoxicity in three ways: the MTT assay, thought to reflect early redox changes in the cell, LDH release, a sign of necrotic cell death, and the Trypan Blue assay which measures loss of membrane integrity. Oxidative damage is clearly demonstrated in these experiments, as is the role of various anti-oxidants in preventing cell death, as measured in their assays. There were striking time lapses before measurements were taken, ranging from several hours to 1 day. Therefore, all the biochemical events described or

postulated might be downstream from an initiating event or events. They remain important possibilities in the pathogenesis of AD.

The notion that Alzheimer pathogenesis is primarily due to or at least involves disturbance of calcium homeostasis is very appealing. In particular the impairment of long-term potentiation by Aβ peptides is intriguing, since it provides a mechanistic link between Aβ toxicity and the characteristic memory deficits of AD (Ferreira *et al.*, 1997).

Glabe (2004) recently pointed to the ability of Aβ peptide oligomers to effect ion transport across membranes. He writes "... We also examined the effect of soluble (*amyloid*) oligomers on membrane conductance. We found that soluble oligomers and not low MW species or fibrils from all types of amyloids tested specifically increase the conductivity of the membrane. The conductivity is not ion selective and does not appear to be due to pore or channel formation. This increase in conductivity could have a detrimental effect on a broad range of biological processes, ...". The actual mechanism remains to be determined.

3. Aβ PEPTIDES RAISE CYTOSOLIC Ca^{2+} CONCENTRATIONS BY INFLUX THROUGH AN IONOPHORE FORMED BY Aβ PEPTIDES

Starting with the early work of Arispe and colleagues (Arispe *et al.* 1993a,b; Durell *et al.*, 1994) the Alzheimer Aβ1-40 peptide has been reported to form calcium-specific ionophore "holes" in artificial membranes, allowing a Ca^{2+} current to flow down its electrochemical gradient. Some of these experiments have been repeated in membranes from a hypothalamic cell line. Models of possible ionophore configurations have been proposed based on certain assumptions of the α-helical content of Aβ1-40. The putative ionophores are not blocked by the usual antagonists of voltage-gated calcium channels (VGCCs), but are blocked by Al^{3+} and by high concentrations of $Tris^{+}$. At their simplest, such ionophores should be effective Ca^{2+}-channels in all cells, neuronal and non-neuronal alike. But Alzheimer's is highly regionalized and is especially active in killing neurons in particular brain nuclei.

Our early patch clamp experiments (Sanderson *et al.*, 1997) using hNT neuronal cells support such a mechanism for Ca^{2+} influx caused by the synthetic Aβ25-35 peptide. This is a short version of the naturally occurring Aβ1-40, Aβ1-42 peptides, but does not itself occur in AD or in normal brains. Models for the conformation of this peptide have been proposed, but curiously they postulate mostly α-helices in the aggregated Aβ25-35, even

though this is a peptide region generally assumed to be in β-sheet conformation. This question remains unresolved.

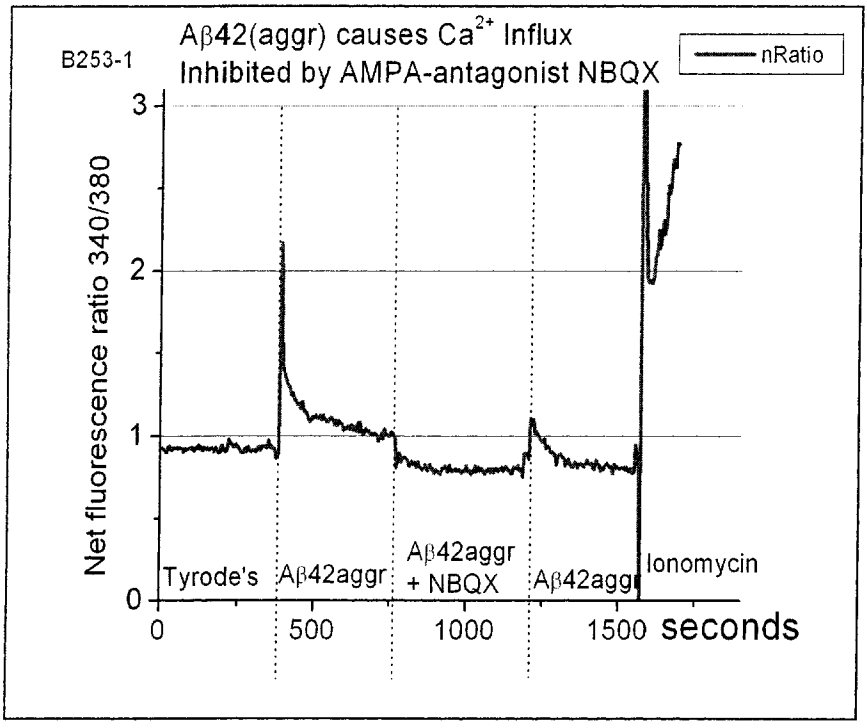

Figure 2. Aggregated 10 μM Aβ1-42 raises cytosolic Ca^{2+} in CATH.a cells. The AMPA antagonist NBQX completely block Aβ1-42 calcium influx into CATH.a cells, an effect that is partially reversible.

4. Aβ PEPTIDES RAISE CYTOSOLIC Ca^{2+} CONCENTRATIONS BY ACTIVATION OF LIGAND-ACTIVATED ION CHANNELS

Recent experiments from our laboratory (Blanchard *et al.*, 1997; 2000) have established a third mechanism of Aβ neurotoxicity that is active on neuronal cells in culture. This is an immediate and very early mechanism, producing a measurable influx of external Ca^{2+} ions within a few seconds.

Our proposal for this third mechanism states that aggregated Aβ1-42, and probably also Aβ1-40, interact specifically with Ca^{2+}-permeant α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptors on the surface of certain neurons, thereby allowing a dramatic immediate influx of external Ca^{2+} ions.

As can be seen in Figure 2, Ca^{2+} ions flow into the cell within seconds of application. This does not occur when the external Ca^{2+} concentration is zero, *i.e.* it is not due to release of Ca^{2+} ions from internal storage. It is completely inhibited by NBQX, a specific AMPA-receptor antagonist. Earlier experiments with CNQX, another AMPA antagonist, confirm this observation. These observations prove the direct and crucial involvement of Ca^{2+}-permeant AMPA receptors in this mechanism.

As was said earlier, both Aβ1-40 and Aβ1-42 self-aggregate and form cytotoxic aggregates that eventually lead to neuronal death (Blanc *et al.*, 1997; Yankner *et al.*, 1990). This process would account for the enormous neuronal loss in AD, affecting specific types of neurone that carry the specific AMPA-receptor involved.

Donut-like oligomers/aggregates of the Aβ1-42 peptide are very common at early incubation times at, pH7.4 (Blanchard *et al.*, 2000, and unpublished data). Certainly *in vitro* these donuts quickly change into protofibrils and then into fibrils. It is conceivable that in accordance with the ionophore mechanism, as described above, these donuts might insert into neuronal cell membranes and conduct Ca^{2+} influx as ionophores. In our hands, this is not the case. Aβ1-42 must be pre-incubated for at least 24 hours before the preparation will promote Ca^{2+}-ion influx. By this time they have turned into protofibrils and some fibrils. This transition into protofibrils and fibrils is likely to occur *in vivo,* as *in vitro*. Furthermore, donuts, the early oligomeric forms, do not contain β-sheet structures, as do protofibrils and fibrils. Such a scheme could explain the neurotoxicity (deleterious Ca^{2+}-ion influx) as well as the singular distribution of cell loss.

As seen in the electron microscope (e.g. by negative staining)

$(A\beta 1\text{-}42)_l \rightarrow (A\beta 1\text{-}42)_n \rightarrow (A\beta 1\text{-}42)_{protofibrils} \rightarrow (A\beta 1\text{-}42)_{fibrils}$

not visible "donuts"[1] ---------neurotoxic----------

Only certain neurons have the specific receptors that are sensitive to Aβ1-42 protofibrils and fibrils. The aggregated peptide $(A\beta 1\text{-}42)_{fibrils}$ apparently opens Ca^{2+}-permeant AMPA receptors, causing Ca^{2+}-influx only

[1] donut-like aggregates are very commonly observed at very early Aβ incubation times, at pH7.4 (*see also* Chapter 1)

in those neurons that have this type of receptor. This view would explain the nucleus-specific nature of Alzheimer's disease, since it is known that the distribution of, for example, a calcium-permeant AMPA-receptor is quite restricted.

It seems that AD pathology correlates with a much higher proportion of the Aβ1-42 species. The accumulated Aβ-peptides and their apparent mechanism of action are obvious targets for the development of therapeutic strategies (see Chapter 19).

5. Aβ PEPTIDES MODULATE SIGNALLING MECHANISMS

There are very interesting reports that the Alzheimer Aβ peptide can inhibit Long Term Potentiation (LTP) in brain slices (Wang *et al.*, 2002). Transgenic mice over-expressing APP gene show greatly diminished LTP *in vivo*, although regular transmission is normal (Chapman *et al.*, 1999). It is not known whether any of the above ionic transmembrane mechanisms are involved in LTP.

6. CONCLUSIONS

Three of the four models described agree that the end result of Aβ1-42 action on neuronal cells is a destabilization of cytosolic calcium homeostasis in the direction of increased $[Ca^{2+}]$. The mechanisms, however, differ with respect to neuronal specificity and the initiation of calcium dyshomeostasis. Only one, involving the action of fibrils of aggregated Aβ1-42 peptide on certain AMPA-receptors is effective within a few seconds.

Much remains to be done. At present, all the proposed mechanisms are based on *in vitro* experiments, yet we apply them to the *in vivo* situation. Currently that is the only thing that we can do. But there is hope! The direct photography of Alzheimer plaques in the lining mouse brain (Bacskai *et al.*, 2003) gives us hope that a refinement of that technique might make possible the detection of at least early protofibrils. That would substantiate part of the mechanism #4 above, since visualization is through dyes that specifically interact with β-sheet-containing structures. A time course, using such a technique, would be very helpful

It remains to be seen whether therapeutic candidates for treating Alzheimer's disease, that are based on *in vitro* findings, will be successful in animal models and in human trials.

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