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Multiscale analysis of the amyloid degradation toxicity hypothesis of Alzheimer's disease.

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Short title: Amyloid degradation toxicity hypothesis of Alzheimer's disease.

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia and affects millions of people around the world. Neuronal death in AD is initiated by the toxic action of oligomeric amyloid-β (Aβ) peptides. The formation of membrane channels by Aβ is a primary molecular action and does not require any other proteins. Channels are formed by short amyloid fragments faster and more frequently than by full-length peptides. The channel formation is dependent on an electrostatic interaction between a positively charged peptide and a negatively charged membrane. Negative membranes can be found in several locations of a cell – the inner leaflet of plasma membrane, mitochondria, and lysosomes, which all are well-known cellular targets in AD. Considering that the amyloid enters a cell by endocytosis and is exposed to lysosomal enzymes, we propose the a*myloid degradation toxicity hypothesis*.

Endopeptidases degrade the endocytosed peptide. Produced fragments form membrane channels, which can transfer various ions (including protons) and even relatively large compounds. The neutralization of lysosomal content inactivates enzymes, which fails the whole system of recycling cellular content, including autophagy. The permeabilization of lysosomes could also lead to cell death through necrotic and apoptotic mechanisms. We discuss several mechanisms that describe how amyloid degradation products reach plasma and mitochondrial membranes, and form membrane channels.

The pathogenesis of AD is discussed at various levels in a context of how the primary molecular mechanism of membrane channel formation could progress into the disease state. The discussion starts at the molecular level and extends to why the development of a disease takes years and is closely associated with aging. The proposed hypothesis offers an interpretation to several clinical observations such as the involvement of iron metabolism and an inverse association between developing Alzheimer's disease and cancer. Predictions about potential biomarkers and effectiveness of future treatments are discussed.

Keywords: Alzheimer’s disease; beta-amyloid; protease; membrane; membrane channel; lysosome; autophagy; mitochondrion

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Introductory general considerations

Fairy-tales, though far from true,
Teach good lads a thing or two
A. Pushkin (trans. by B. Deutsch)

The mechanisms of Alzheimer's disease have been under intense investigation in recent decades, but still, no comprehensive multiscale theory combining all levels of pathogenesis (molecular, biochemical, cellular, organismal, epidemiological) exists. Senile plaques are the best-known anatomical markers of the disease and consist mostly of beta-amyloid peptides of different lengths. Due to the similarity of the effects of these peptides, they will be referred to as "amyloid peptides" in this manuscript if their length is not critical to the discussion. Peptides are normally produced by neurons, and upon synthesis, they do not carry any conformation. However, they are prone to acquire secondary structure fixed by hydrogen bonds and to aggregate through a formation of pleated beta-sheets. The fixation of a conformation that allows for the peptide aggregation with other molecules is frequently called misfolding to stress that the product is considered detrimental for the host organism.

Senile plaques are not considered the reason of the disease by themselves, even though multiple studies describe that synaptic loss and neuritic dystrophy are greatest immediately adjacent to a plaque and lessens with increased distance from a plaque [1]. According to current views, the amyloid peptide is involved with neurodegeneration because it is toxic to cells. The toxicity can be observed *in vivo* by microinjecting the peptide into the brain, and *in vitro* by adding the peptide to cell cultures. It is established and accepted that the cytotoxicity of the beta-amyloid is mediated by oligomers, while monomers and fibrils are relatively non-toxic [2]. However, the biophysical or biochemical pathways between the formation of oligomers and eventual cell death remain not clear [3]. The absence of clarity explains why researchers have not identified any efficient way to stall the progression of the disease. It could be that such pathways lay outside of the current framework of Alzheimer's research.

Therefore, we have attempted an unorthodox approach - we started with the original biophysical data on the formation of membrane channel by amyloid peptide fragments. The major aim was not to review what is already known about amyloid channels, but rather to identify how these channels can be involved with the pathogenesis of Alzheimer's disease. Our goal was to find out what information is missing to confirm or exclude channel formation as a major target in the search for new treatments of AD. Like the assembly of mosaic typically starts with placing corner pieces, this data was combined and fit with peer-reviewed literature data. The result is our *amyloid degradation toxicity hypothesis*. Some speculations could look frivolous. Nevertheless, before they are disproven by experimental data, they are provided within a framework. Hence, the epigraph.

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1. Ion channel formation is a primary molecular action of amyloid fragments

Multiple biochemical pathways were hypothesized as the causes of neuronal death induced by amyloid. Numerous reviews mention the activation of apoptosis, an increase in oxidative stress, ion disturbances, immune system involvement, and more. However, all of these mechanisms do not name a specific molecular interaction that initiates biochemical and biophysical pathways leading to cell death. In pharmacological terms – no primary molecular action is identified. In general, and as is very typical for the toxicology of exogenous substances, it is reasonable to expect that amyloid requires some other protein (such as a receptor or ion channel) to be involved for exerting toxic action. Drugs can interact with multiple receptors, and as a result create a complex dose-dependence of pharmacological action. For example, yohimbine is a well-known antagonist of α_2-adrenoreceptors, but in doses frequently used in pharmacological studies, it is also an agonist of 5-HT_{1A} receptors; in higher concentrations, it can act on other receptors as well [4]. There are multiple studies in peer-reviewed journals that tested various molecular targets of amyloid, and these studies were extensively reviewed [5, 6]. However, amyloid provides an alternative mechanism that does not require the presence of any other macromolecular protein structure [7].

In early 1990s, several groups independently demonstrated that when lipid membranes are exposed to either amyloid protein itself (40-42 amino acid-long) or its short fragment ($A\beta_{25-35}$), it is possible to detect the formation of ion channels using traditional electrophysiological techniques [8-25]. Ion channels were reproducibly formed if the peptide was mixed with lipids during the formation of membranes. Alternatively, if the peptide was added to already-formed lipid membranes, the ion channels appeared very fast - within minutes at most. Since the first discoveries, amyloid channel theory has become one of the major theories explaining the development of Alzheimer's disease [9, 12, 16, 19, 26-29]. Nevertheless, critical questions about potential role of amyloid channels in the pathogenesis of AD summarized 15 years ago in the live discussion at Alzheimer Research Forum [30] remain unanswered.

Amyloid-formed channels are quite different from typical specialized ion channels, such as sodium or potassium channels. Amyloid channels had multiple conductance states and demonstrated high- and low-frequency transitioning between these states. Also, there was a significant variability in the conductance between experiments. However, the most unique feature of these channels was the absolute values of their conductance – single channel can have a conductance up to several nanosiemens, while typical ion channels (such as sodium channels) are characterized by a conductance measured in picosiemens [15]. All features pointed to multiunit supramolecular structures resembling transmembrane barrels formed by multiple peptide molecules, as was first modeled by

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Durell et al. [18]. The need of aggregation appears to be in line with the role of oligomers in the Aβ toxicity [31, 32].

Most of the studies demonstrating channel formation by beta-amyloid were done on two experimental models – black lipid bilayer (lipid bilayer formed over a small hole in a Teflon membrane) and patch (lipid bilayer formed over the micron-size opening of a glass pipette). Both techniques can provide a trove of information about the biophysical aspects of the formed channel, but it could be difficult to estimate the biological significance of the phenomenon since it is impossible to quantify the number of formed channels in a living system, such as a suspension of cells.

In an attempt to measure the extent of channel formation in experimental conditions and to create a technique that is more suitable for high-throughput applications, one of the approaches tested was to observe the transmembrane transfer of ions in liposomes [23]. In this study, liposomes were prepared with various phospholipids in a buffer containing fluorescent probes sensitive to various ions – calcium (Indo-1), sodium (SBFI), and potassium (BBFI). After the liposomes were formed, the extravesicular probe was removed. Undamaged phospholipid membranes are not permeant to sodium, potassium, and calcium; therefore, manipulations of extravesicular ion concentrations do not affect the florescence of the intravesicular probe. However, if membranes were made permeable to some ion by using corresponding ionophore, the fluorescence increased after the addition of this ion to the medium. This phenomenon of increasing fluorescence after an addition of ionophore was used as a positive control. When $A\beta_{1\text{-}40}$ was added to a suspension of liposomes made of phosphatidylserine with an enclosed fluorescent probe, the intensity of the fluorescence increased as liposomes become permeable to ions. In accordance with previous electrophysiological data, phosphatidylserine liposomes were efficiently permeabilized by $A\beta_{1\text{-}40}$ to all the tested ions – calcium, sodium, and potassium. Permeabilization was also observed in liposomes made of phosphatidylinositol, another negatively charged phospholipid. In contrast, liposomes made of phosphatidylcholine – a neutral phospholipid - were not sensitive to the effects of amyloid. Importantly, the peptide with a reverse sequence ($A\beta_{40\text{-}1}$) did not affect the permeability of membranes.

Therefore, earlier studies demonstrated that 1) amyloid peptides are able to create ion channels in membranes, and 2) permeabilization can be observed only in negatively charged membranes.

However, some questions remained unanswered. First, the data on the channel creation were not confirmed by all laboratories. Sokolov et al. [33] reported that a full-length peptide increased conductance without creating channels. In the discussion, the authors mentioned that in some experiments they observed channel-like behavior, but it was only in a very small number of experiments [33]. Secondly, it is not clear how to interpret the need of the negative charge of membrane. In case of the short fragment $A\beta_{25\text{-}35}$, such a need is relatively obvious: the peptide carries a positive charge, so it

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interacts electrostatically with a negatively charged membrane, but not with a neutral membrane. Unfortunately, this mechanism is not obvious for longer peptides, because both $A\beta_{1-40}$ and $A\beta_{1-42}$ are acidic at a neutral pH. It is possible to speculate, though, that it is due to a 3D distribution of charge in a channel-forming oligomer, because charges are distributed unevenly in an amyloid polypeptide chain. It is quite possible that only the C-terminal part of $A\beta_{1-42}$ corresponding to $A\beta_{25-35}$ forms a channel. In this case, the channel itself would be positively charged and would interact with a negative membrane, while the rest of the supramolecular complex can carry any other charge. However, this speculation is looking unlikely considering “Occam’s razor principle” and a possibility of a potentially simpler interpretation that we will discuss below. Finally, we looked for a way to estimate the biological significance of channel-forming activity. An observation of only single channels in electrophysiological experiments described by Arispe and others suggests that the channels are formed in low numbers. This contradicts the acute toxic action of the peptide in cell cultures with thousands of cells.

In an attempt to address these three questions, we developed a flow cytometric technique to observe and quantitatively describe the formation of channels in liposomes [34]. Advancing the idea of using enclosed ion-sensitive fluorescent probes, we measured the permeability of thousands of individual liposomes. In short, liposomes are made with the enclosed calcium-sensitive probe Fluo-3, and the extravesicular probe is washed out. Liposomes are too small to be detected through the light scatter, as is typical in flow cytometric applications. To detect liposomes in the flow, we added a fluorescent lipid-soluble dye to the lipids. When calcium is added to the undamaged liposomes, the enclosed probe does not fluoresce because lipid bilayer membranes are not permeable to calcium. The addition of calcium ionophore ionomycin to liposomes in the presence of free calcium increases the fluorescence of intravesicular Fluo-3 and serves a positive control.

Using this independent experimental method, we confirmed that amyloid peptides in fact can permeabilize lipid membranes [34]. This permeabilization occurs when negatively charged membranes are exposed to the positively charged amyloid fragment $A\beta_{25-35}$, which is known to aggregate through the creation of beta-sheets. A similar peptide without a positive charge, $A\beta_{22-35}$, did not create a noticeable number of channels. Also, $A\beta_{25-35}$ did not permeabilize liposomes made of neutral phosphatidylcholine. The difference of effects between phospholipids and fragments with a different charge confirms the role of electrostatic interactions.

However, in contrast to most of the previous studies, the full-length peptide $A\beta_{1-42}$ was ineffective in the permeabilization of tested membranes in our experiments [35]. This discrepancy is critical for understanding the toxicity of beta-amyloid and can be addressed by a comparison of the technical details of various studies. As was mentioned, electrophysiological observations essentially demonstrated only single formed channels, despite test system containing billions of billions of peptide

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molecules. To form a single channel, minimal contamination would be sufficient – even 0.1% of a contaminant would provide trillions of fragments. Considering that the variety of fragments able to form channels is still not known, even nanoimpurities cannot be excluded as a potential explanation of the minimal number of channels demonstrated in electrophysiological experiments.

To extend this logic, the reliance of channel formation on impurities in the studies of the full-length peptide can explain why, in some laboratories, the formation of channels was not reproducible; their peptide synthesis could be either more pure, or just have a different set of peptide impurities - specifically, those that did not form channels. That said, based on the ability of amyloid peptide to non-specific action on lipid bilayers, it is reasonable to accept that high concentrations of peptide can permeabilize membranes without forming ion channels *per se,* as was shown by Sokolov et al. [33] and Sciacca et al. [36]. Interestingly, non-channel-dependent changes of conductance were also electrostatic-dependent [33]. Full length peptide, which carries a net negative charge, affected the conductance in neutral PC membranes much more than in negative PS membranes, which is exactly opposite to the ability to form ion channels.

A direct comparison between $A\beta_{25-35}$ and full-length $A\beta_{1-40}$ or $A\beta_{1-42}$ was previously made only by Mirzabekov et al, who showed that only the fragment was able to create channels in reasonably low concentrations [11]. Interestingly, in this study, channels were formed by a short fragment in a neutral PC membrane, but the peptide was added to lipids during the preparation of membranes, so the formation of channels was not dependent on the incorporation. This observation allows to hypothesize that electrostatic interactions are required for the interaction of peptide with the membrane, but not for the functionality of the channel. In this study, however, high concentrations of full-length peptide (above 100 μM) created channels.

To conclude, short fragments appeared incomparably much more potent than full-length amyloid peptides in the permeabilization of membranes. Importantly, the flowmetry technique allows us to estimate the number of channel-forming units in the solutions of peptide and, potentially, in biological samples [34]. This number appears extremely small compared to the total amount of peptide (it takes at least 10^{12} molecules of peptide to form one channel). The low ratio of channels created by the peptide does not allow to study channel formation using the typical methods of protein biology such as spectroscopy or NMR, the signal of interest from a peptide in the form of channel would be negligible compared to the peptide in other conformations. Nevertheless, even though the proportion of channels is small, when the peptide was added in micromolar concentrations, we still observed thousands of permeabilized vesicles. This finding directly demonstrates that the phenomenon is sufficiently powerful to be the mechanism of cellular toxicity.

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This channel formation is not dependent on any other proteins and, therefore, is a primary molecular mechanism. The word “primary” in this context does not mean that it is the verified major reason of AD, but rather that nothing precedes this initial molecular interaction. Once this interaction occurs, it results in a cascade of sequential biochemical and cellular reactions. It can be one of many other primary actions. It may not even be relevant to many effects; however, as described below, the existing data points to the potential extreme importance of this phenomenon.

2. Potential consequences of amyloid channel formation in cellular membranes

2.1. Estimation of size exclusion for amyloid channels

As was mentioned above, the most dramatic feature of amyloid channels is their conductance – the absolute numbers of conductance are at least two orders larger than typical sodium channels [15]. The conductance is a reflection of the pore size. It is reasonable that due to an extremely large pore, the channel is not selective because various molecules can fit inside and pass through a membrane. Since amyloid channels were first described, it is believed that they are made by multiple copies of peptide that form a barrel-like structure, which themselves are made of or at least include beta-sheets [18]. Interestingly, such a structure makes amyloid channels similar to the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane, which is one of most well-known porins. VDAC is also made by multiple copies of peptide that are arranged in a barrel [37]. The conductance of VDAC channel is in the same nanosiemens range as the amyloid channel [10, 15, 24, 38]. VDAC has a diameter of 2.5 nm and allows macromolecules of up to 4-6 kDa to pass [37, 39, 40]. The size exclusion of VDAC is determined by the complex shape of the pore, which is not a cylinder with the mentioned diameter. Unlike VDAC, which has voltage-dependence and functional selectivity [37], amyloid channel could be less selective. Therefore, the size of the pore can have a more direct correlation with the size exclusion. If that’s the case, up to a 2.5 nm pore corresponding to this conductivity [24] would be able to accommodate a passage of a 50 kDa molecule [41]. Even with some level of selectivity that will be decreasing the effective size exclusion, such a permeability will be sufficient for the transfer of large molecules, such as amyloid molecules/oligomers and cytochrome c.

2.2. Amyloid channels destroy barrier function of membrane in cells and organelles

As was already mentioned above, the most dramatic feature of amyloid channels is their conductance. The appearance of non-controllable transmembrane pores characterized by such a conductance essentially disrupts the main biological function of the membrane – the creation of a barrier between compartments.

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In one of the first manuscripts describing amyloid channels, Arispe et al [15] stressed that the creation of even a single channel will dramatically affect cellular ion homeostasis in a matter of seconds. They based their calculations on a cell with a diameter of 25 microns. It is obvious that the same channel in a neuronal axon will have absolutely devastating consequences, making the function of a cellular branch impossible. As will be discussed below, amyloid channels can be formed not only in plasma membranes, but also in membranes of cellular organelles. From that perspective, the formation of a single membrane channel - for example, in an inner membrane of the mitochondrion - will definitely affect a function of the organelle and, probably, kill the organelle itself as a result.

There are no direct studies demonstrating that amyloid channels pass protons. However, such ability should be considered because of non-specificity of membrane permeabilization by amyloid. In electrophysiological experiments amyloid channels passed all studied cations – sodium, potassium, calcium, cesium, and lithium [17]. Exposure of cells to Aβ permeabilized lysosomes, which start leaking membrane-impermeant anionic fluorescent dye Lucifer Yellow [42]. Importantly, permeabilization of plasma membranes to calcium and protons was synchronous when the fluxes of both ions were recorded simultaneously [43]. Due to the importance of the proton gradient to various cellular functions and low absolute values of proton concentrations, the concentration of protons will be used here as an example.

The individual volume of each mitochondrion is small – less than 0.1 μm^3 [44]. The pH of the mitochondrial matrix is 0.9 higher than the pH of the intermembrane space (which is ~7.6), which is close to the pH of cytosol [45]. At pH~8, the concentration of protons is 10^{-8} Eq/L. Even with the largest estimate of the volume of an individual mitochondrion at 0.1 $\mu m^3 = 10^{-16}$ L, it contains approximately 10^{-24} Eq. or, on average, less than one free proton per mitochondrion. Quite obviously, most of the protons for the gradient are bound by buffers such as taurine [46], but even if a buffer has a concentration of 1 mM, the number of available protons in the same volume is in the range of $6*10^4$ (10^{-19} Eq * $6*10^{23}$ protons/Eq). In electrophysiological studies, the conductance of one nanosiemens means that when a voltage of one millivolt (just one millivolt!) is applied, an individual amyloid channel with such a conductance provides an electrical current of one pA. Considering that 1 pA =10^{-12} Coulombs/sec and 1 proton is $1.6*10^{-19}$ Coulombs, each channel with a 1 nS conductance transfers $6*10^6$ ions/second for each applied mV. With the inner mitochondrial membrane potential exceeding 100 mV [47], just one functional amyloid channel is more than sufficient to kill the function of a single mitochondrion completely. Channels are stable for at least multiple minutes, so the incorporation of one channel into a mitochondrion is a death sentence for this organelle.

In organelles that do not have significant membrane potential, the transmembrane diffusion of substances will be slower. The ability of a channel to pass ions due to a diffusion and dissipate the

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concentration gradient (without transmembrane potential driving the transfer) can be estimated from currents created in asymmetrical systems. At zero potential across the bilayer, a net positive current is observed and is still measured in pA [17], meaning that the equilibration of gradients across an organelle's membrane will be achieved within seconds at most. If the membrane loses the ability to provide a barrier for even macromolecular complexes due to an appearance of a single amyloid channel, the fate of organelles or cells can be predestined.

3. Cellular targets for amyloid channels formation

The affinity of amyloid channel-forming units to negatively charged membranes is a key feature of the toxicity mediated by the formation of non-selective ion channels. In short, almost all electrophysiological experiments demonstrating amyloid ion channel formation were performed in membranes prepared from negatively charged lipids (see the Section 1). Accordingly, liposomes were permeabilized only if they were made with a significant ratio of acidic phospholipids [23, 34]. Therefore, it appears logical to identify subcellular locations where negatively charged membranes are known to exist.

Considering that amyloid plaques are extracellular and there is an ion disturbance on a cellular level, the plasma membrane is the first target to consider. Phosphatidylcholine, a major component of cellular membranes, is a neutral lipid. Its negative charge of a phosphate is countered by a positive charge of a nitrogen in a choline residue. Plasma membranes contain other lipids, such as acidic phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI), with PS being prevalent. There are no known biologically relevant cationic lipids; therefore, the average charge of the plasma membrane is, in fact, negative.

In most experimental models, the toxicity of amyloid is tested by adding exogenous peptide, and the association of extracellular plaques with the disease argues in favor of such an approach. However, not all patients exhibit such plaques, and many genetic models of AD develop significant neuronal deficits without any histological protein deposits. Also, the addition of already fibrillated Aβ to cells affects cell survival much less than soluble peptide fraction [48]. Therefore, it is quite possible that the plaques are just a consequence of peptide production, but not an actual cause of the disease [31, 49], though senile plaques could be a reservoir for the oligomeric peptide [50]. Considering that the peptide is synthesized intracellularly and is excreted from the cell, one of the possibilities for the source of the toxic peptide would be an intracellularly synthesized one. Finally, cells take beta-amyloid by endocytosis and can accumulate it intracellularly in dystrophic neurons. Altogether, it appears reasonable to search for targets that are alternatives to plasma membranes – specifically, intracellular targets.

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Mitochondrial and lysosomal dysfunction are considered hallmarks of AD [51-58]. It is difficult to consider it a coincidence that both lysosomal and mitochondrial membranes (mostly inner, but also outer mitochondrial membrane) carry a significant negative charge due to the presence of phospholipids that are specific to each organelle and are intricately involved in active biochemical pathways. The summary of the potential cellular targets for beta-amyloid is shown in Fig.1. Blue dots on the lipids identify where negative charges are located.

3.1. Plasma membrane

Plasma membrane as a target. Despite acidic phospholipids being present in plasma membranes, healthy cells do not have PS in the outer leaflet of the lipid bilayer, with special biochemical mechanisms responsible for keeping PS only in the inner leaflet [59, 60]. PS appears in the outer leaflet during apoptosis, and it is considered a signal that prompts phagocytes to engulf apoptotic cells [61]. The targeting of early apoptotic cells by amyloid can explain a toxic synergism between excitatory amino acids and amyloid [62]. There are threshold concentrations of amyloid and ibotenic acid below which they are not decreasing cell culture viability if applied alone. However, the mixture of both in the same concentrations is cytotoxic. It can be hypothesized that a low concentration of ibotenate that is not sufficient to kill the cell would allow for the appearance of PS in the outer leaflet of plasma membrane, possibly short-term. However, such an appearance can be enough for amyloid to incorporate into the cell membrane as an ion channel and induce cytotoxicity - an outcome that does not occur without ibotenate.

However, the exposure of presumably healthy cells to amyloid results in a significant drop of cellular viability [63, 64], despite healthy cells do not have a negative charge in the outer leaflet; this negative charge is needed for extracellular amyloid to form channels in the plasma membrane. If cytotoxicity requires apoptotic changes, such as an appearance of PS in the outer leaflet of plasma membranes, this theory falls short of being an obvious mechanistic interpretation. It should be mentioned, though, that the suppression of cellular viability in cell cultures requires a long incubation (usually overnight). It is quite possible that each cell has short periods of PS presence in the outer leaflet, so many cells become valid targets for beta-amyloid at some point if the exposure lasts over the extended period.

An alternative explanation of the toxicity of amyloid on healthy cells could be the presence of lipid rafts – portions of the membrane that, due to the spontaneous heterogeneity of membranes, are not dissolved by non-ionic detergents. In cellular membranes, lipid rafts are enriched with cholesterol (not charged) and gangliosides (carry a negative charge). As a cluster, lipid rafts are carrying a net negative charge [65]. Lipid rafts disproportionally attract membrane depositions of Aβ [66, 67], and this spatial preference was hypothesized to have a role in both the aggregation of peptide [68-70] and the

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preferable location of membrane-permeabilizing pores [67, 71]. The role of lipid rafts in Aβ toxicity was observed experimentally [72]. This potential target attracts attention by associating amyloid toxicity with cholesterol, since AD is linked with cholesterol disturbances [73].

Consequences of channel formation in plasma membranes. The incorporation of an amyloid channel into the cellular membrane can result in a significant ion disturbance. Considering that ion channels are non-specific, this disturbance will affect the transmembrane distribution of virtually all of the ions, including sodium, potassium, and calcium. Opening the channels should result in the equilibration of concentrations of various ions between the intra- and extracellular space.

The permeabilization of membranes to calcium after exposure to Aβ was described at both the membrane and cellular levels [14, 16, 17, 23, 43, 74, 75]. Cells usually have a very low cytosolic concentration of calcium. Any increase of intracellular calcium prompts the evacuation of the added amounts. In mammalian cells, the mitochondria serve as one of main reservoirs where an excess of calcium can be pumped by active transport [76]. Calcium homeostasis is especially important in neurons, where calcium directly participates in neuromediators release.

It is not difficult to link the permeabilization of the plasma membrane to calcium with mitochondrial damage and cell death. An increase of intracellular calcium, which is induced by exposure to the oligomeric forms of amyloid and the formation of non-specific ion channels in the plasma membrane, would require a removal of calcium to preserve the normal function of cell. If calcium is transferred to the mitochondria for a prolonged time, calcium overload would eventually activate various pathological processes, such as a mitochondrial membrane permeability transition, which in turn leads to apoptosis and necrosis [77].

Despite most of the interest to the amyloid channel formation in plasma membranes was attracted to a disturbance of calcium homeostasis, such selective attention is not justified by existing knowledge about these channels. Firstly, the channels are not selective and pass various cations. The permeability of channels to ions that are most critical for neurons are not equal, specifically $P_{Ca^{2+}} = P_{K^+} > P_{Na^+}$ [17]. However, the permeability to calcium is only 30% higher than to sodium [17]. In another study, the ratio was higher $P_{Ca^{2+}} : P_{K^+} : P_{Na^+} : P_{Cl^-} = 5.4 : 1.6 : 1.4 : 1$ [11]. However, the difference still does not justify the exclusivity of interest to calcium. In fact, disturbances of the intracellular concentrations of major cations induced by the application of Aβ mirror each other – once the cellular membrane opens for one ion, others start flowing across [43]. Secondly, as was estimated in the previous sections, the gigantic conductance of channels would result in a complete loss of the barrier function of the membrane, even if one functional unit will appear per cell, and a such loss would be applicable to all ions. The formed channels in the artificial membranes are functionally stable for extended periods of time. Potentially, in cells, a fast protein turnover is a survival solution for this cellular problem: in experiments described by

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Abramov et al [43], Fura-2-loaded astrocytes demonstrated calcium oscillations on a scale of minutes with a considerable variability of delays before responses were observed, of amplitude of oscillations, and of patterning. The time course of these oscillations allows to suggest that if ion disturbances were in fact induced by plasma membrane channels, live cells inactivated channels much faster than it would be expected from a spontaneous closure based on the studies in model systems. In the same experiments, confocal imaging revealed that the signal originated from a focal initiating point, spreading progressively but decrementing through the cell. The oscillations were dependent on external calcium, but not on the intracellular reserves, which is consistent with the appearance and disappearance of single membrane channels with a large conductance. Kagan suggested that a single pore would pose a significant but not necessarily a lethal leak [30], but the outcome is clearly dependent on how long the pore stays functional. If the recovery of such channels is in fact controlled by protein turnover, slowing the said turnover in aging would result in the longer presence of a functioning channel after it is formed, and can explain the higher potential of irreversible damage to the cell physiology by a formed channel(s) in aging cells. Essentially, if the formed channel is quickly cleared in a young cell, the cell survives. In contrast, in aged cell, a prolonged channel opening results in irreversible changes and death.

The problem with finding the relevance of this specific observation with Alzheimer's disease pathogenesis is that the authors did not detect calcium channel formation in cultured neurons, but only in astrocytes that were serving as a nutritional support for a culture of hippocampal neurons [43].

Another problem with formation of channels in plasma membranes as a pathophysiological path to neuronal death is the time course of channel formation and cell death. In most experimental settings, the channels are formed in artificial membranes almost immediately, especially when a short $A\beta_{25-35}$ is used [23, 34]. However, ionic responses in cells are not immediate – it still takes more than five minutes for calcium waves to start after an addition of $A\beta_{25-35}$ in a high concentration 50 μM [43] with the delay being negatively correlated with the concentration of peptide [25]. The absence of negative charges in the outer leaflet of the plasma membrane can explain this discrepancy, since the peptide needs to reach the cytosol first, but there is no known direct transport system for amyloid across the plasma membrane.

An even more critical deficiency of attempting to interpret the development of AD through channel formation in plasma membranes is that it does not explain decreased brain metabolism in AD, which is a well-described phenomenon and an established diagnostic tool [78-81]. Importantly, imaging metabolic activity provides a better diagnostic value than the imaging of plaque appearance [82]. Therefore, while it is tempting to focus on plasma membranes, which in experimental settings are

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usually first exposed to the exogenously added amyloid, it is critical to study alternative targets for amyloid. It appears that the intracellular targets can provide an alternative toxicity pathway for amyloid.

Route of peptide delivery to this target. The outer leaflet of the plasma membrane is directly exposed to an extracellular peptide. In the case of an apoptotic cell, which carries acidic phospholipids in this outer leaflet, beta-amyloid would have a clear and close target. However, healthy cells, which should not have a negative charge in the outer leaflet, also die when exposed to the exogenous peptide. Short-lived leakages of acidic phospholipids into the outer leaflet and the presence of lipid rafts could be potential explanations, but otherwise, in order to consider the plasma membrane as a place for channel formation by beta-amyloid, the peptide needs to first be taken by the cell. A major mechanism of amyloid uptake is endocytosis [83]; therefore, the taken peptide needs to exit the endocytic vesicle before getting access to the inner leaflet of the plasma membrane. Such speculation readily explains why amyloid toxicity requires significant time to develop, and also why inhibitors of endocytosis can effectively decrease amyloid toxicity in cell cultures [84, 85].

3.2. Mitochondria

Mitochondrion as a target. Mitochondrial dysfunction is considered one of the hallmarks of AD [52, 86-89]. Considering that the mitochondria are a source of energy for the cell, it is not a surprise, at first glance, that a decrease of total brain metabolism is one of the biomarkers of Alzheimer's [78-81]. However, the link between the two phenomena does not appear straightforward, as in most scenarios of biochemical damage to the mitochondrion, the damaged organelle continues to oxidase substrates, but loses the ability to store released energy in the form of ATP. In contrast, decreased brain metabolism requires having a smaller number of mitochondria.

Each mitochondrion is surrounded by two membranes. The outer mitochondrial membrane (OMM) is permeable to dissolved substances; in contrast, the inner mitochondrial membrane (IMM) is not permeable to ions, including protons and calcium. The most important biological function of the mitochondrion – respiration – actually occurs at the inner membrane. The active transport of protons across the membrane by oxidative phosphorylation creates a pH gradient, which is then used to generate ATP. An addition of amyloid to cellular cultures results in the decrease of mitochondrial voltage recorded using fluorescent probes [90].

Lipids of IMM have up to a quarter of cardiolipin (1,3-bis(sn-3'-phosphatidyl)-sn-glycerol) and several percent of phosphatidylserine and/or phosphatidylinositol [91]. It is considered that cardiolipin is critical for the regulation of pH changes relevant to proton transport across this membrane. In total, up to one third of IMM lipids are negatively charged. Therefore, IMM represents a clear target for ion channel-forming amyloid.

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Consequences of channel formation in mitochondrial membranes. If an amyloid would form an ion channel in the IMM, it would not only allow for the entry of calcium, but also would dissipate any other gradients, including the proton gradient and electrical transmembrane potential. As the main function of the mitochondrion is to be the powerhouse of a cell – which requires an electrochemical proton gradient - ion channel formation will be essentially equal to the uncoupling of mitochondrial oxidative phosphorylation. As soon as the mitochondrion is not able to produce ATP, the cell loses the source of energy.

By itself, the acute uncoupling of phosphorylation does not decrease, but can rather even increase oxygen consumption [92]. The oxidation of substrates continues, but no ATP is synthesized, and all energy is converted to a heat. One could hypothesize that the dysfunctional mitochondrion will not continue to burn resources forever, and that after some time, it will die. However, there is no such dramatic thing as “the death of a mitochondrion” inspiring the likes of Jacques Louis David paintings. Mitochondria can undergo fission, fusion, or autophagy [93]. Recycling a damaged mitochondrion and producing a new functional one as a replacement can restore cellular function. Similarly, mitochondrial fission can separate the damaged region of an organelle, which can then be removed by mitophagy [94]. Traditionally, a mitophagy was considered a random process; however, accumulating data seem to point at the idea that it is a selective process targeting damaged mitochondria [95], with a rising interest in developing treatments aiming to activate mitophagy [96, 97] as a mitochondrial quality control mechanism [94]. The decrease of mitochondrial membrane potential is considered one of the main triggers of mitophagy [94, 98]; therefore, channel formation, which would also results in the dissipation of IMM potential, would be a signal for recycling the damaged organelle.

It is important to consider that the use of fluorescent dyes accumulating in the mitochondria due to the voltage on the IMM as a marker of this voltage has a substantial flaw. Usually, it is assumed that an increase or decrease in fluorescence intensity reflects the voltage. In manuscripts, the decrease of fluorescent signal is usually interpreted as a decrease of mitochondrial voltage with an (unsaid) assumption that it characterizes all mitochondria. However, there is no way to distinguish two scenarios – one where only some mitochondria completely lose voltage, versus where all mitochondria lose some voltage. The creation of a single ion channel in the IMM of a mitochondrion would most likely completely dissipate the voltage in this specific mitochondrion without affecting the other. Such dissipation in some organelles would decrease the total fluorescence of the mitochondrially accumulated dye per cell. In such a scenario, the decrease will be the average of a complete loss in some organelles with an unaffected status of the rest.

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To summarize, the creation of ion channels in the inner mitochondrial membranes would result in a disappearance of the functional mitochondria. Paired with insufficient mitochondrial biogenesis, such a disappearance explains the decreased metabolism of brain tissue affected by Alzheimer's disease.

What will happen when amyloid channels are formed in the outer mitochondrial membrane? One of the obvious consequences would be the depolarization of the OMM. Such a depolarization will fix VDAC channels in the open state, making them permeable to NAD and other small molecules [37]. However, despite the voltage-dependence of VDAC being in its name (voltage dependent anion-selective channels), the importance of voltage-dependence for cell function is not clear. While VDAC is believed to be involved in the formation of the mitochondrial permeability transition pore, which in turn induces the release of cytochrome c and consequent apoptosis, short-circuiting the membrane to fix VDAC in an open state by itself would not increase the permeability of the OMM for larger molecules such as cytochrome c.

Unfortunately, it appears that amyloid channels themselves can provide permeability sufficient for large molecules. If large enough, amyloid channels can be formed in the OMM (see the discussion in previous sections). This could open the OMM to both the transfer of amyloid to the IMM, and more importantly, the release of intramitochondrial components such as cytochrome c and other apoptogenic factors.

Route of peptide delivery to this target. The effect of channel formation in the IMM looks most catastrophic – an immediate loss of power generation in this organelle with an accompanying dramatic production of reactive oxygen species. Therefore, it is tempting to start with this target and analyze how an amyloid can reach the IMM. The outer membrane is considered permeable to various compounds with relatively low molecular weights [99]. The permeability of OMM to calcium, salts, and ATP is mediated by a voltage-dependent anion channel (VDAC,) and the potential involvement of VDAC in Alzheimer's disease pathophysiology was extensively reviewed [100, 101]. However, the molecular cut-off of VDAC is estimated at 4000 Da [37], which is not sufficient to translocate $A\beta_{1-40}$ (MW 4330), even as a monomer, or the bigger $A\beta_{1-42}$. Correspondingly, there is no evidence of VDAC transferring Aβ, despite some data demonstrating that VDAC can bind beta-amyloid [102-104]. It is worth noting, though, that some studies show the cut-off being up to 6000 Da [39, 40]. Interestingly, this cut-off allows for the passage of $A\beta_{25-35}$ (MW 1060), or even trimers of this fragment, but the presence of a positive charge and peptide structure does not allow for reliable predictions. Unfortunately, data concerning whether VDAC can in fact translocate short amyloid fragments across OMM were not found in peer-reviewed publications.

Larger molecules also can be transported across the outer mitochondrial membrane through specialized protein transporters called the translocases of the outer membrane (TOM). It is well

established that Aβ is imported into mitochondria via the TOM import machinery [105, 106]. However, despite full-length Aβ being clearly transported into the mitochondria, data demonstrating the fast depolarization of the IMM after the exposure of the mitochondria to Aβ is missing in available literature. Probably, even if monomeric Aβ is transferred across the OMM by a translocase, small amounts of amyloid is degraded in the intermembrane space by proteases [106], and cannot form ion channels, since channel-forming requires oligomerization and time. Another open question is: can TOM transfer amyloid fragments, and, if so, will such a transfer be sufficient to create membrane-damaging oligomers in the intermembrane space?

An overview of the role of the permeability of the OMM in allowing channel-forming units to enter the mitochondrion, so they can make ion channels in the IMM and make the mitochondrion dysfunctional, is not looking promising. This prompted the consideration of an alternative scenario. While the IMM is clearly negatively charged due to the presence of cardiolipin and a significant concentration of acidic phospholipids, the OMM also carries some negative charge. Firstly, cardiolipin is present in the OMM – there is significantly less cardiolipin in the OMM than in the IMM, but it still can comprise up to 10% of the total lipids [107]. In turn, phosphatidylserine appears in the IMM by trafficking through the OMM [108], and, therefore, is present in the OMM. In addition, the OMM contains phosphatidic acid as a product of cardiolipin metabolism [109]. As shown by Alarcon et al [23], having 5% of negatively charged lipids is sufficient for allowing amyloid to permeabilize membranes. Clearly, the OMM contains more than 5% of negatively charged lipids. Therefore, to conclude, the outer mitochondrial membrane can be a target of amyloid for ion channel formation. The biological importance of channel formation in the OMM is dependent on the ability of amyloid channels to pass apoptogenic factors such as cytochrome c, which have molecular weights of 10+kDa.

Finally, there is a recently described phenomenon of “kiss-and-run” interactions between endosomes/lysosomes and mitochondria [110]. Amyloid, which is taken by endocytosis, can be directly delivered into the intermembrane mitochondrial space by such an interaction. This will be analyzed in more details in the section reviewing lysosome-induced mitochondrial damage.

3.3. Lysosomes

Lysosomes as a target. The dramatically increased presence of autophagic vacuoles is one of the features of Alzheimer’s disease [111]. This anatomical phenomenon may suggest that AD results in the lysosomal pathology. However, the appearance of non-functional lysosomes can be the first step of the disease.

The lysosomal membrane contains a significant ratio of bis(monoacylglycero)phosphate [112]. The lysosomal content is highly acidic, with pH estimated in the range of 3 to 5 [113-115]. Amino and

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carboxy groups become protonated with a lowering pH, with the former gaining a positive charge and the latter losing a negative one. Therefore, by moving to an acidic environment, all biological compounds become more positively charged, so negatively charged membranes can become neutral. This concern does not complicate the analysis, though. Phosphatidylserine has an isoelectric point at 1.2-1.5 [116, 117], above which PS has an overall negative charge. However, pK for one of the carboxyl groups is such that it is protonated between pH 3 and 5 [116, 118], which means that in more acidic lysosomes PS has a lesser negative charge. In contrast, due to an absence of amino groups, bis(monoacylglycero)phosphate is negatively charged at the acidic pH, and this charge is considered a key to bis(monoacylglycero)phosphates role in the degradation of lipids [119]. Due to a significant presence of this lipid, the total charge of the lysosomal membrane is negative at all biologically possible pH values.

The negative charge of the lysosomal membrane makes it another potential target of channel formation. Much more importantly, these organelles are unique in the cellular machinery due to their role in protein degradation. Membrane channels are created by amyloid fragments, but not a full-length peptide [35]. Therefore, the main function makes lysosomes a primary suspect in the initiation of the biochemical pathway leading to cellular death. This organelle first produces an amyloid fragment, and lysosomal membrane has a key characteristic required to incorporate channel-forming units and form membrane channels.

Consequences of channel formation in lysosomes. Typically, when ion channels are considered, ions of metals, such as sodium or calcium, are coming to mind. The role of various ion channels in lysosomal functions was recently reviewed [120], but the most vital function of this organelle – protein digestion and recycling - is dependent on a different ion. Lysosomal proteases are mostly acidic hydrolases and require actively supported acidification of lysosomal lumen. As in the case of mitochondrial membranes, a permeabilization of membranes to protons is critical for lysosomes.

As soon as any channel-forming unit is inserted into the lysosomal membrane, the intralysosomal pH equilibrates with the cytoplasm, and digestion stops. The lysosome becomes inactive and requires utilization. The lysosomal membrane is probably exposed to the highest concentrations of amyloid due to the way in which amyloid is utilized in tissues. More importantly, short amyloid fragments are more prone to form membrane channels compared with a full-length peptides [10]. Essentially, as soon as the lysosome starts digesting amyloid, the products can break the functionality of this organelle, which still contains non-digested peptide.

The presence of dysfunctional lysosomes can be the least of the potential troubles resulting from amyloid channels compared with the consequences of lysosomal permeabilization. The content of the lysosome can escape due to its destruction, but it should be considered that the size of this non-

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specific cation channel can allow for the passage of relatively large molecules, such as amyloid fragments themselves (the 11-aminoacid fragment $A\beta_{25-35}$ has MW of 1060 and is also a cation at a neutral and acidic pH). If such fragments leak into the cytoplasm, they can produce more channel-forming units, with other membranes being next possible targets. Another component leaking from a permeabilized lysosome could be the enzymes themselves. Leaked lysosomal enzymes can destruct intracellular structures directly, but, more importantly, can also activate a biochemical cascade of apoptosis [121-123].

Route of peptide delivery to this target. Extracellular amyloid is captured by cells through endocytosis. The endocytic vesicle fuses with lysosomes for the processing of trapped content. Lysosomes have a highly acidic content to keep acidic peptidases active. These peptidases digest endocytosed amyloid creating shorter fragments, including those that can form membrane channels.

3.4. Schematic of amyloid metabolism and intracellular targets

Amyloid metabolism links all the potential cellular targets for amyloid membrane channel formation (Fig.2). Extracellular beta-amyloid is taken by endocytosis and is digested by lysosomal enzymes as a part of normal cell physiology. The formed fragments permeabilize the lysosomal membrane. While amyloid channels are relatively non-specific and can carry various cations, the dissipation of proton gradient across the lysosomal membrane is the major pathophysiological consequence of channel formation. Permeabilized lysosomes can release fragments into the cytoplasm, so they can reach other cellular targets. If channels are formed in the plasma membrane, a major pathophysiological consequence would be the permeabilization of the membrane to sodium and potassium, which would prevent normal neuronal transmission. Influx of calcium from extracellular space into the cytoplasm can lead to calcium overload and, eventually, to cell death. Alternatively, the fragments can reach the mitochondria. If the fragments can pass the outer mitochondrial membrane and form channels in the inner membrane, the dissipation of proton gradient would result in the loss of ATP production. Opening mitochondrial calcium storage would disrupt cytoplasmic calcium control. The dissipation of IMM potential is a signal to recycle the failed mitochondrion, and, in a normally functioning cell, the new one replaces it. Failed autophagy in AD prevents normal recycling.

4. *Amyloid degradation toxicity hypothesis* of Alzheimer's disease

4.1. Fragments vs full-length peptide

As was extensively discussed in Section 1, short fragments of beta-amyloid are much more effective in the permeabilization of lipid membranes than a full-length peptide (either 40- or 42-amino

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acid long). In our own experiments, using a flow cytometry technique that allows for the estimation of the number of channel-forming units in the sample, we could not find proof that the full-length peptide $A\beta_{1–42}$ creates any channels [35]. In high non-physiological concentrations, a full-length peptide can increase membrane permeability through non-specific mechanisms [33], but permeabilization due to membrane channel formation is mediated by shorter fragments such as $A\beta_{25–35}$.

This conclusion is important for the hypothesis summarized below. The identification of the potential cellular targets with negative membranes does not answer a commonsense question - how can a full-length peptide reach these targets? With a shift of our attention towards degradation products, the answer becomes easier to find, as it will be discussed below.

4.2. Lysosomes are likely first targets due to a natural pathway of amyloid metabolism

When cells are exposed to the beta-amyloid, it is accumulated intracellularly [124]. The internalization of amyloid can involve lipid rafts as a binding site at the membrane [125]. The process of the internalization of Aβ occurs through endocytosis [83, 126, 127]. In general, endosomes merge with lysosomes to digest the taken extracellular content [128, 129].

This mechanism readily explains how the amyloid peptide accumulates in lysosomes [130]. However, the reason for which AD is associated with an accumulation of undigested peptide in dysfunctional lysosomes, for some reason, remains unexplained. The facts known about amyloid channel formation provide an explanation in the form of a very simple hypothetical sequence of molecular events, which can be called the *amyloid degradation toxicity hypothesis*.

The endocytic vesicle containing the amyloid peptide merges with the lysosome. Acidic peptidases degrade the peptide. The degradation occurs not only through exopeptidases, but also through endopeptidases, so various short fragments are formed. In normally functioning cells, short fragments are not accumulating, but are rather degraded further by exopeptidases. However, if short fragments accumulate for any reason, they can form membrane channels. These channels are not ion-specific and can most likely transfer even relatively large compounds, both charged and uncharged. Among the various ions, which become equilibrated between the interior of the lysosome and the cytosol, protons are most critical for lysosomal function. Peptidases become inactive in a neutral environment. The content of the lysosome cannot be degraded, but the lysosome is not eliminated until cargo is digested. Dysfunctional lysosomes carrying undigested peptides accumulate intracellularly.

Essentially, according to the *amyloid degradation toxicity hypothesis*, lysosomal failure through a proteolysis inhibition is induced by the deacidification of lysosomal content. It was long noted that dystrophic swellings induced by lysosomal proteolysis inhibition resemble those in AD brains and in mouse models of AD [131]. In experiments, lysosomal proteolysis can be disrupted by either a direct

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cathepsin inhibition or a suppression of lysosomal acidification. The inhibition of proteolysis in lysosomes slows the axonal transport of autolysosomes, late endosomes, and lysosomes, and causes their selective accumulation within dystrophic axonal swellings, despite the axonal transport system being preserved [132]. Importantly, when experimental lysosomal proteolysis is reversed, autophagic substrates are cleared and the axonal dystrophy is reversed [132]. Unfortunately, in the case of the deacidification of lysosomes by amyloid channels, it is difficult to imagine how the reversal can be achieved conceptually.

A critical role of lysosomal deacidification in neurodegeneration is also supported by studies of presenilin 1 [56, 57]. Along with participation in the processing of amyloid protein, this protein is also a chaperone essential for the delivery of v-ATPase to lysosomes, which in turn is responsible for lysosome acidification and protease activation. Mutations of presenilin 1 are the most common cause of early onset familial AD. In various experimental models involving presenilin dysfunction, lysosomes demonstrate an increased pH, decreased activity of cathepsins *in situ* and *in vitro*, and delayed clearance of autophagy substrates.

Functional impairment of lysosomes would result in autophagy failure - this is one of the major characteristics of Alzheimer's disease [56, 57, 133-136]. There are three subtypes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy [137]. Chaperone-mediated and micro-autophagy are, most likely, irrelevant to this topic of amyloid-induced toxicity – these two are the mechanisms of transferring the cytoplasmic content into the lysosomes for digestion. In contrast, macroautophagy is the one of interest. Essentially, this is a manner of recycling damaged organelles by destructing them with enzymes. Therefore, it is critical to examine how amyloid can impair this process.

In brief, macroautophagy involves the direct sequestration of whole areas of the cytosol by a membrane that seals to form a double membrane vesicle or autophagosome. A fusion between autophagosomes and lysosomes mediates the delivery of the autophagic cargo into the lysosomal lumen [128, 138].

How can amyloid metabolism affect the degradation of mitochondria if amyloid is delivered by endosomes, while mitochondrial recycling comes from autophagy? In fact, lysosomes degrade the content that is delivered to them by both endocytic and autophagic routes. The system of lysosomal degradation is frequently called the endosomal-lysosomal network (ELN) [139]. This term emphasizes that these pathways merge. Even further, in neurons, endosomes frequently fuse with autophagosomes (creating amphisomes) before their fusion with lysosomes. As a result, the damage to lysosomes induced by amyloid will prevent the normal degradation of both endocytosis- and autophagy-originated lysosomal cargo.

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It is notable that dystrophic swellings induced by lysosomal proteolysis inhibition appear in dendrites. Historically, it was considered that lysosomes are formed in the neuronal soma, while autophagosomes are created where needed, including axons and dendrites of neurons, and are carried to the soma for processing [140]. However, autophagosomes can fuse with lysosomes while transported along microtubules to the cell body, or autophagy can be carried out completely at the cell's periphery [141]. The dynamic of immunochemical markers also supports the notion that neurite dystrophy evolves from dysfunctions of pre-autophagosomes [142].

The permeabilization of lysosomes by amyloid and even the leakage of lysosomal content can be visualized. Ji et al., 2002 allowed cells to accumulate membrane-impermeant Lucifer Yellow, which enters the cell through endocytosis [143]. In untreated cells, fluorescent objects were observed as small, circumscribed vesicular structures resembling intact lysosomes creating a punctate pattern of fluorescence. After treatment with $A\beta_{1-42}$, however, it was readily apparent that cells displayed a diffuse intracellular pattern of fluorescence. In these experiments, the incubation time with amyloid was 20 hours [143], a period sufficient enough for an endocytic uptake and processing of the exogenously added peptide. On a side note, apoE4-transfected cells showed the most marked lysosomal leakage [143], which correlates with apolipoprotein E4 (apoE4) being the most prevalent genetic risk factor of AD [144]. Such observations confirm that an exposure to amyloid peptide makes lysosomes permeable to relatively large compounds such as Lucifer Yellow (MW 444).

While typically endocytic vesicles merge with lysosomes, their path could be different. For example, zinc is known to induce endocytosis in cells, but in cells treated with zinc, immunofluorescent labeling shows that most formed endocytic vesicles end up with a Golgi compartment, while compartments labelled with Lysotracker dye are clear of endocytic material [145]. A treatment with zinc, in this case, would redirect the uptake of the peptide to locations where a different set of degradation enzymes is responsible for the recycling. It is possible that no harmful degradation products are generated, and this allows for an alternative interpretation of how zinc prevents neuronal death induced by amyloid [146], in addition to the potential ability of Zn^{2+} to block amyloid ion channels [13].

The *amyloid degradation toxicity hypothesis* is finally identifying a potential link behind the previously hypothesized role of lysosomes in the development of Alzheimer's disease [147]. The accumulation of non-digested amyloid protein by lysosomes [111] is not a consequence of AD, but rather a major part of the disease pathogenesis.

Lysosomal inefficiency in AD can prompt considering an activation of autophagy as a way to treat neurodegeneration [148]. However, increased autophagy induction, associated with a neurodegeneration, had also been described [56]. The synthesis of many components of the lysosome is upregulated at the transcriptional and translational levels in the AD brain tissue and AD mouse

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models. Considering that the degradation process is not efficient, it is reasonable to suggest that such an induction is a compensatory phenomenon. Unfortunately, an increased number of lysosomes results in an increased number of lysosomes degrading amyloid. As a result, more lysosomes will become dysfunctional, so the activation of autophagy could promote neurodegeneration instead of ameliorating it. Alternatively, the prevention of autophagy can decrease the number of lysosomes damaged by degraded amyloid. In fact, an inhibition of endocytosis prevents mitochondrial damage in cells exposed to exogenous Aβ [90]. At the same time, however, the inhibition of autophagy will result in a slowing normal turnover of intracellular structures and the improper removal of damaged organelles, with an overall result of promoting neurodegeneration. Quite clearly, the secret of finding the treatment is in discovering some specific way to inhibit “bad” autophagy, while finding ways to clear failed organelles and restore the functionality of this repair system.

4.3. Lysosome-induced mitochondrial dysfunction

In our initial considerations of potential cellular damage by the created amyloid channels, the mitochondria appeared to be a promising research target. The inner mitochondrial membrane has a lot of negatively charged lipids. The insertion of non-specific channel into the IMM would result in the dissipation of the pH gradient, so a mitochondrion with even a single channel is a non-functional one. Considering that channels are relatively stable structures, the only way to restore cellular respiration will be to recycle the damaged mitochondrion and produce a new one as a replacement. It is important that mitophagy can be directly activated by the depolarization of the IMM [149]; therefore, the membrane-damaging action of intracellular amyloid should result in decreased number of mitochondria, which would fit with a concept of hypometabolism typical for AD. Mitochondrial fission and growth require the appearance of additional space, which will be in limited supply due to ineffective lysosomes which are themselves damaged by amyloid. In this scenario mitochondrial dysfunction is the result of direct channel-forming action in mitochondrial membranes, which is aggravated by lysosomal failure.

However, a deeper dive into the biophysics of intracellular function (see the section about targeting and delivery of amyloid to specific organelles) revealed that it would be very difficult for amyloid to reach the IMM. Simultaneously, the creation of non-specific channels for ions and compounds with low molecular weight in the outer mitochondrial membrane is unlikely to result in anything significant, because the OMM already has such channels – VDAC. Therefore, the idea of the mitochondria being the primary target for the incorporation of membrane amyloid channels loses scientific attractiveness. Nevertheless, this general idea still needs to be tested to be rejected.

However, mitochondrial dysfunction still can be a consequence of amyloid membrane channel formation. Lysosomes are most likely the first victim in the pathogenetic pathway. Intracellular space

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becomes clogged by non-functional mitochondria and lysosomes. The replacement of damaged mitochondria is delayed. In this scenario, Aβ does not affect mitochondria directly, but the overall mitochondrial function is suffering due to the failed quality control of the mitochondria by lysosomes.

It is worth considering that much evidence points to the notion that lysosomal disorders can in fact lead to mitochondrial dysfunction. Lysosomal storage disorders, which are induced by mutations of specific enzymes and are therefore limited in expression exclusively to lysosomes, are characterized by mitochondrial dysfunction, which by itself appears to be a pathogenetic factor in these diseases [150, 151]. In a simplified interpretation, lysosomes are responsible for mitophagy. Impaired mitophagy results in an accumulation of impaired mitochondria. Along with the production of reactive oxygen species, which can further damage lysosomes, dysfunctional mitochondria do not produce sufficient ATP to keep the ATP-dependent proton transport mechanism and the pH gradient needed for proper lysosomal function. The impairment of lysosomes in Alzheimer's disease initiates a vicious cycle: the damage to lysosomes prevents the normal function of mitochondria, which, in a dysfunctional state, further damages lysosomes.

The concept of the failed clearance of damaged cellular components as a cornerstone of neurodegeneration can be applied to various cellular components - organelles and molecular complexes. For example, tauopathy in AD is associated with impaired clearance of tau aggregates [139], and can be another consequence of lysosomal deficiency, which by itself stems from amyloid-induced membrane permeability.

However, one more mechanism of interaction between lysosomes and mitochondria, which is neither well-known nor well-studied, can be relevant to AD. Typically, in the recycling process, the interaction between the organelle and lysosomes starts with covering this organelle with a double membrane (isolation membrane or phagophore), which originates in the endoplasmic reticulum [152]. A complete sequestration of the cytoplasm and organelle inside the vesicle formed by a double membrane produces an autophagosome. The outer membrane of the autophagosome fuses with the membrane of lysosome. The resulting vesicle has an outer membrane consisting of a lysosomal membrane and outer membrane of autophagosome, while the inner membrane of the autophagosome covers the cargo for digestion. The intermembrane space contains lysosomal content, including lipases, along with all other catabolic enzymes. After the digestion of internal membrane covering cargo, lysosomal enzymes recycle the cargo.

This process relies on the fusion of the lysosomal membrane with an outer membrane of the other organelle, which has double membranes. Therefore, it is tempting to inquire whether there is any possibility that lysosomes can interact with an outer membrane of mitochondria, fuse with them, and thus deliver lysosomal content into the intermembrane mitochondrial space, allowing amyloid

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degradation products to directly target the IMM. In fact, interactions of this kind are actually described in research literature and were initially considered in context of iron homeostasis [153].

The final step of heme synthesis, which is the addition of Fe^{2+} by ferrochelatase, occurs in the mitochondria [154]. For this reaction, mitochondria require an intense uptake of iron, which is especially critical for erythropoetic cells. That explains why it was studied almost exclusively in these specific cells. The problem for any living organism is that free iron is extremely toxic for cells as it induces lipid peroxidation, so all iron needs to be bound to carriers such as transferrin. Iron is mostly taken from the extracellular space by the endocytosis of transferrin-bound iron, using transferrin receptors. Ferric ion (Fe^{3+}) taken up by endocytosis as a part of the transferrin receptor (TFR)-transferrin (TF) complex is reduced to ferrous iron (Fe^{2+}) by a metalloreductase. Iron is dissociated from the transferrin in endocytic vesicles due to acidification.

One of the several mechanisms for transporting iron into the mitochondria includes “kiss and run” [155]. When an endosome is actively transported by intracellular transport mechanisms close to a mitochondrion, it is possible to observe a short interruption of the movement. The stop is accompanied by a transfer of endosomal content into the intermembrane space of the mitochondrion allowing direct transfer of dissociated iron from the endosome to the mitochondria without entering the cytosol [153]. The transfer occurs through diffusion, so it is reasonable to assume that there is an exchange of other content as well.

The “kiss and run” mechanism provides a functional iron transfer from endosomes to the mitochondria [156]. This mechanism was considered as functional in erythroid cells due to their high intensity of heme synthesis and the need for efficient iron uptake by mitochondria [155], but it was recently demonstrated in non-erythroid cells as well [156]. While most initial studies were focused on endosomes (not lysosomes), more recent publications directly refer to the interaction between mitochondria and lysosomes [157, 158].

The involvement of an endosome-mitochondrial interaction into iron transport is especially intriguing considering an accumulation of epidemiological data linking iron pathology and AD [159-161]. The promoting role of iron on acute Aβ toxicity *in vitro* was demonstrated by Shubert&Chevion [162]. Along with other multiple pathways potentially linking iron with amyloid disorders, the involvement of iron with the control of interactions between the endosome-lysosome system and mitochondria is worthy of attention. The interaction between two organelles is very short [156] and it is possible that the exchange of content is not complete. An extension of the interaction could simply mean an increase of the probability of the transfer of large molecular complexes from one organelle to another. Higher iron concentrations require a longer time to empty iron storage, which was found to be a signal for

disconnection in erythroid cells [155]. Increased local iron concentration, therefore, will promote such transfer explaining experiments *in vitro* [162].

Also, the interaction between the endosome and the mitochondrion is dependent on the transferrin receptor, and in cells lacking functional transferrin receptors the interaction is significantly longer [156]. The control of iron transport is known for negative feedback. Increased systemic transferrin could be associated with less density of transferrin receptors, and thus result in a longer interaction between two organelles. This negative correlation would support clinical observations that, despite baseline plasma transferrin levels being not correlated with an AD diagnosis in patients, higher levels of blood transferrin are associated with a steeper cognitive decline in patients with AD [163]. This is in line with the concept that iron, by itself, is not an etiological factor of AD, but that it clearly can affect the development or progression of AD.

Interestingly, cholesterol is another molecule that enters cells by receptor-mediated endocytosis and reaches the mitochondria [110]. The involvement of the interaction between endosomes and mitochondria into the cholesterol metabolism could be another reason why cholesterol is involved in the development of AD [73, 164].

There are some alternative pathways that could be involved in the direct delivery of extracellular amyloid into the mitochondria. Typically, internalized molecules are delivered to early endosomes, which fuse with lysosomes. However, it was reported that styryl pyridinium FM dyes can traffic to the mitochondria directly from the plasma membrane by clathrin-mediated endocytosis, but with a mechanism that is different from the classical endocytic pathway [165]. The phenomenon was demonstrated in astrocytes cultured from newborn Sprague-Dawley rats, so more studies are needed to prove its relevance to amyloid accumulation in the mitochondria, which by itself is a well-established fact [105, 166]. Whatever transport mechanism is involved, it allows either for intramitochondrial peptidases to degrade endocytosed peptide and produce channel-forming fragments or for the fragments formed in the endolysosomes to have direct access to the IMM and form channels in the negatively charged lipid bilayer.

4.4. Pathways from a lysosomal dysfunction to a cell death

Lysosomal dysfunction can lead to cell death through multiple pathways.

Two of the most obvious mechanisms are necrosis and apoptosis, induced by proteases entering the cytosol after the permeabilization of the lysosomal membrane [121]. Cytosol contains inhibitors that guard against the undesirable leakage of enzymes [167], but it is believed that this protection can be overpowered by the amount of leaked enzyme. Despite an acidic pH (actively supported in the lysosomal lumen) is critical for the optimal activity of proteolytic enzymes, released proteases can

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remain active in the neutral pH of cytosol, at least for some time [168]. A complete breakdown of lysosomes and a release of their content into the cytoplasm results in unregulated necrosis [169]. With molecular weights in the range between 20-50 kDa, cathepsins are unlikely to pass through all the amyloid channels (see the discussion on the molecular weight cut-off of the channels above). However, the channels are not uniform in size, with larger channels being less frequent [10]. The upper limit of molecular weight cut-off is unknown. Importantly, acidic intralysosomal content would promote the formation of larger channels: the distribution of channels formed by $A\beta_{25-35}$ in artificial membranes was shifted to significantly higher conductances with the acidification of the medium from 7.4 to 5.0 [10].

A complete breakdown of lysosomes in a living cell is unlikely; however, a more limited release of lysosomal enzymes can activate the caspases in the signaling pathway. The lysosomal pathway of apoptosis was extensively reviewed [121-123, 170]. It can be activated by various factors releasing proteases from the lysosomes into the cytosol [121]. In order to participate in the apoptotic process, the enzymes need to be translocated to the cytosol. A formation of pores was implicated in a process called lysosomal permeabilization, along with other potential mechanisms such as the generation of reactive oxygen species.

Cathepsin B, which is one of the most stable acidic peptidases at a neutral pH, was demonstrated to be essential in various models of apoptosis [167]. However, other less stable proteases were also shown to participate in an initiation of apoptotic mechanisms. Released proteases can directly cleave and activate caspases [171]. Another programmatic cascade to avoid necrosis in favor of apoptosis is initiated by cathepsin B through an activation of pro-apoptotic and a degradation of anti-apoptotic protein families [169, 172] which promote a release of cytochrome C from the mitochondria [173]. Independent of the biochemical route leading to activation, once caspases are activated, apoptosis takes its course along well-established pathways.

Necrosis and apoptosis induced by the leakage of lysosomal enzymes into the cytoplasm would be fast-acting mechanisms of cell death. In contrast, amyloid can induce slow-developing cell dysfunction, eventually leading to cell death through the damage to the lysosomal apparatus due to its role in cell quality control. Neuronal autophagy is constitutively active and very efficient. If the processing of autophagic vesicles occurs properly, even a significant increase to the rate of autophagy is unlikely to produce an overwhelming number of un-degraded autophagic vacuoles - a phenomenon which is observed in many neurodegenerative diseases [137]. It can be counterintuitive that an inhibition, rather than a stimulation, of macroautophagy increases neuronal survival in some pathological conditions displaying a high content of neuronal autophagic vacuoles, such as degeneration caused by protein misfolding [140, 174-176].

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However, this paradox resolves when we accept the hypothesis that an accumulation of autophagosomes is not a consequence of their increased formation, but rather impairment of their maturation, processing, and elimination [56, 111]. Cellular death is the result of an accumulation of un-processed vacuoles; therefore, by slowing the formation of autophagosomes, it is possible to decrease the number of space-occupying intracellular objects that disrupt normal cellular physiology. In that sense, it is another example of similarity with neurodegeneration induced by lysosomal storage diseases [140].

One of the major consequences of lysosomal dysfunction is the breaking of normal vesicular traffic through neuronal extensions due to the accumulation of autophagosomes in dystrophic neurites. By itself, this is not necessarily a death sentence for a neuron but can explain the functional failure of neurons to receive and process signaling. It is likely that dystrophy will be spread across multiple areas of the same cell, so the recovery of one area would not recover cell function. At the same time, a potential reversibility of such changes can explain the existence of lucid periods in patients with AD. Also, it gives a hope of finding a cure, at least for patients in the early stages of clinical progression.

4.5. What happens with lysosomes damaged by amyloid

The concept that an acidic environment is critical for the proper function of lysosomal enzymes is a cornerstone of our model. As was mentioned above, the permeabilization of lysosomal membranes by amyloid channels would neutralize lysosomal content, so acidic peptidases will become inactive. Slowing the degradation of cargo down will result in an accumulation of non-degraded amyloid, as well as of lysosomes carrying content. A closer look, though, reveals some interesting details.

Cathepsins (and especially cathepsin B) have both endopeptidase and exopeptidase activity. A complete degradation of peptides requires exopeptidase action. In contrast, endopeptidases produce peptides fragments. The neutralization of the reaction medium suppresses mostly exopeptidase activity [167]. Most likely, due to the remaining endopeptidase activity, the neutralization of the intralysosomal space will essentially shift degradation to the production of fragments. In an acidic environment, these fragments will be degraded efficiently by exopeptidases, but it is reasonable to hypothesize that without exopeptidase action, fragments will accumulate. As it was speculated above, the fragments are smaller than the molecular weight cut-off of the largest amyloid channels, and, therefore, can exit from damaged lysosomes into the cytoplasm. In this scenario, the damaged lysosome, instead of being a plant recycling the amyloid into useful amino acids, becomes a factory producing toxic amyloid degradation products. These toxic products will be delivered into the cytoplasm and can reach potential targets – not only mitochondria (where the effect would depend dramatically on the ability to reach the

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inner membrane), but also the inner leaflet of plasma membrane, which is readily negatively charged. This is an alternative pathway linking lysosomal damage with neuronal death.

Such scenario is not observed in every cell. In the cells that do not die in this acute phase of amyloid invasion, a preservation of some (endo)peptidase activity should allow for the continuous degradation of intralysosomal proteins to small oligomers which would not be recognizable as original macromolecules. The degradation will, however, not end with the most elemental components. Nevertheless, the brains of AD patients and animals with AD models contain cells with dystrophic lysosomes that carry relatively preserved cargo. If we are to assume that a lysosome with a channel still contains functional enzymes, why do cells end up with undigested intralysosomal content? And what is a mechanism of the degradation cessation inside of the affected lysosomes?

The channels could be still the answer. Healthy cells contain inhibitors of cathepsins, which are most likely needed to protect cells from enzymatic leaks of various genesis [170]. Intracellular inhibitors are relatively small molecules (such as stefins, Type I cystatins) with a molecular weight of 10-12 kDa [170]. Large enough channels could allow these to enter lysosomes and inhibit peptidases, and, as a result, to preserve the remaining proteins, including amyloid, in an undigested form. From this point of view, increased concentrations of naturally present inhibitors would slow down the destruction of autophagosomes with non-digested proteins. Therefore, higher concentrations of natively present inhibitors would be promoting the development of Alzheimer's disease (see Fig.3, middle column of events).

There is an important alternative interaction between cathepsin inhibitors and lysosomal permeabilization. Considering that it is a biological function of these inhibitors to protect a cell from leaking lysosomal enzymes, higher concentration of them should protect the cell from necrosis and apoptosis induced by the peptidases leaking from permeabilized lysosomes. From that point of view, higher concentration of inhibitors would be a protective factor against the development of Alzheimer's disease (Fig.3, left column).

Complicating the analysis of the biological landscape, at least some cathepsin inhibitors (such as cystatin C) can bind Aβ and inhibit Aβ aggregation [177].

This speculation is worthy of consideration, since cathepsin inhibitors are of a special interest to oncology due to the association of cathepsins with the ability of cancers to develop and spread [178, 179]. Most of the practical interest is directed towards the use of pharmaceutical inhibitors to suppress the activity associated with an increased production of cathepsins during tumorigenesis. From this point of view, an increased activity of endogenous inhibitors can correlate with a lower frequency of cancerogenesis. The pathogenetic sequence is included in the schematic found in the right column of Fig.3.

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In the recent decade, there have been observations that patients with Alzheimer's disease have a lower incidence of cancer. Furthermore, patients with a history of cancer have a lower prevalence of AD, as was found in multiple studies [180, 181]. This negative correlation occurs despite a surprising similarity in risk-factors for both diseases [182]. With more studies published, this negative correlation appears to reflect some innate biological connection, and is not the result of better diagnostics, involved treatment, or bias in patients' enrollment into studies. Considering this notion, an important question must be raised: could endogenous protease inhibitors be the link?

Returning to the discussion of the outcomes of the damaged lysosome in the cell. Based on the complexity of the involved mechanisms [129, 141], it is reasonable to hypothesize that, in a healthy cell, the vacuole will remain inactive until cargo is reasonably disintegrated. If the cargo will not be degraded, the vacuole will stay, and, as a result, the number of vacuoles will increase. However, let's assume that with the passage of time, the vacuole may become active. Since it was created by a fusion of the autophagosome and lysosome, it carries the membranes and proteins from both. If the intracellular machinery considers it a lysosome and it will fuse with another autophagosome, then the produced vacuole will still be dysfunctional. The new oversized vacuole will be filled with a content that cannot be degraded. Alternatively, if it is considered an autophagosome, then a functional lysosome can fuse with it. In this case, the fused vesicle will still carry an amyloid pore. As was estimated in one of the previous sections, even one pore is sufficient to prevent the acidification of lysosome of any biologically-relevant size. As a result, the formed organelle will remain dysfunctional as well. However, reports do not describe dysfunctional non-mature phagocytic vacuoles of extreme sizes [111, 183], so it is unlikely that such growth in fact occurs.

The absence of uncontrolled vacuole growth means that at least one of the several options is possible. The first option is that vacuoles somehow finish processing the cargo. The processing requires a dramatically extended period, but eventually finishes. One of possible mechanisms is channel disintegration, followed by a restoration of normal function. The second option is that there is a way to process the failed autophagosomes through a normal autophagy. An "isolation membrane" sequesters failed autophagosome inside of a bigger autophagosome. It is reasonable to theorize that such sequestration requires extra space and can only be efficiently done in the soma. Therefore, the axons and dendrites of neurons are at a disadvantage. This explains why cellular extensions demonstrate dystrophic swellings early in the development of the disease [56]. Finally, it is possible that failed vacuoles remain forever. The cell accumulates the debris until normal physiological processes become impossible. Among various organelles, the mitochondria may be the most sensitive to such space restrictions due to a need for intense repair through mitophagy, followed by mitochondrial fission and growth. This also explains mitochondrial deficiency as one of the major biomarkers of AD.

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In any case, the most important question is: how, if at all possible, can the dysfunctional lysosomes be cleared from the cell? The only reliable way to degrade such an organelle would be to form a new phagosome around it. Hypothetically, finding a way to induce such directional repair could be a cure for early stages of AD.

4.6. pH-dependency in the *amyloid degradation toxicity hypothesis*

The interaction between the channel-forming unit and membrane is electrostatic. Considering that the content of the lysosome is acidic, it is important to consider what charge do peptides and lipids hold at various pH.

The isoelectric point for $A\beta_{25-35}$ is above 10, so at any pH below 8 it carries a positive charge. In turn, the isoelectric point for both $A\beta_{1-40}$ and $A\beta_{1-42}$ is just above 5. This means that at a neutral pH, they carry a negative charge but i become positively charged in a typical lysosomal content, mostly due to the neutralization of acidic amino acids in the N-terminal part of the peptides. If we are to assume a purely electrostatic interaction, then even if full-length channel-forming units exist, they will not be able to incorporate into a negatively charged lipid bilayer at a neutral pH (as we found [34] but can start doing this at a pH below 5.

One of the arguments supporting the electrostatic nature of the interaction between the channel-forming unit and lipid membrane is that unlike $A\beta_{25-35}$, $A\beta_{22-35}$ does not form channels in a model system at a neutral pH [34]. It is believed that the additional negatively charged aminoacids in positions 22 and 23 (glutamate and aspartate, correspondingly) prevent electrostatic interaction. However, the pK_a for the carboxylic group in acidic aminoacids are 4.3 (glutamic acid) and 3.7 (aspartic acid). Therefore, acidification below pH 5 results in the increased number of fragments which will have an ability to form channels due to positive charge and damage lysosomal membrane.

In contrast, as was discussed above, lysosomal membranes are negatively charged at all pH values. Still, due to the ionization of phosphatidylserine that occurs around pH 4 [118], an increased intralysosomal pH will increase the charge density on the membrane. As a result, the formation of channels will be promoted by the deacidification of lysosomes.

In short, acidic intralysosomal content provides a better environment for channel formation in lysosomal membranes, including the involvement of a wider range of peptide fragments being able to incorporate into the membrane due to a positive charge (up to full-length peptides). However, the deacidification of lysosomes would also result in conditions favorable to channel formation. This favorability has multiple mechanisms, such as the lower activity of exopeptidases digesting channel-forming fragments, as well as an increased density of the negative charge on membranes due to the ionization of acidic phospholipids. The deacidification of lysosomes was previously suggested as a

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factor aggravating lysosomal dysfunction in AD [184]. Here, we hypothesize several molecular mechanisms that make the deacidification an integral component of AD pathophysiology.

In the context of deacidification, proton pump inhibitors (PPIs) need to be mentioned. PPIs are used to reduce gastric acid secretion, and are well tolerated in short term regimens [185]. Due to an absence of clearly identified side effects of their chronic use - as long as several years [185] - this class of drugs is sold over-the-counter in many countries, including the USA. However, after several decades of widespread use, observations suggest that that proton pump inhibitors can lead to serious consequences [186, 187]. Epidemiologic observations point to a link between PPI use and the development of dementia [188]. A causative connection was never established, and the observed hazard ratio is not extreme (1.44 in the study [188]).

Is there any reason to link proton pump inhibitors to AD pathogenesis? In fact, yes. Their intended target is the H,K-ATPase of parietal cells, so that the inhibition of this exchanger prevents the secretion of protons into the stomach lumen. The target in the gastric epithelium is in fact predominant, but the drugs have a good bioavailability. Due to covalent binding to the ATPases, their inhibitory effects last much longer than their plasma half-life [189]. PPIs pass the blood-brain barrier and inhibit not only the P-type ATPases (H+/K+-ATPase is a member of this protein family), but also the vacuolar H+-ATPase (v-ATPase). That opens up an alternative applications for these drugs, such as cancer chemotherapy [190]. The inhibitory effect on the v-ATPase explains why exposure to PPI results in the deacidification of lysosomes and the inhibition of lysosomal enzymes [191]. The inhibition of lysosomal enzymes, in turn, can increase the accumulation of amyloid [184] and would explain why PPI worsen lysosomal dysfunction.

However, the *amyloid degradation toxicity hypothesis* offers a mechanism that can pose PPIs as potential initiators of neuronal death, leading to dementia. By deacidifying autophagosomes, PPIs would increase the probability of amyloid to exert its primary toxic action through the formation of membrane channels.

4.7. *Amyloid degradation toxicity hypothesis* summary

Amyloid channels easily explain the primary damage, but several major problems are not addressed by classic theory. 1). Why does an incorporation of channels require negative membrane if full-length Aβ is also negative? Also, $A\beta_{1\text{-}42}$ is not effective in channel creation 2). How can channels be formed in the plasma membrane if it does not have negative phospholipids in the outer leaflet? 3). How does targeting plasma membrane explain the dysfunction of autophagy/lysosomes and decreased metabolism? 4). If it is not a plasma membrane, which negative intracellular membranes are targeted, and how is peptide delivered there? 5). What is the mechanism of lysosomal permeabilization?

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The *amyloid degradation toxicity hypothesis* was developed to answer these questions and can be summarized in the form of a very simple hypothetical sequence of molecular events (Fig.4). The endocytic vesicle containing the amyloid peptide is merged with a lysosome. Endopeptidases form various short fragments which are mostly degraded further by acidic exopeptidases. Short fragments can form non-specific membrane channels. If a channel is formed in the lysosomal membrane, it allows pH gradient to dissipate. Even a single channel allows for the dissipation of the proton gradient across a membrane of a particular organelle, so peptidases become inactive in a neutral environment.

The etiology of the disease is the formation of fragments that form membrane channels. The formation of an amyloid membrane channel is the core event in the *amyloid degradation toxicity hypothesis*. The appearance of dysfunctional lysosome is the core consequence of this event. In normally functioning lysosomes, peptide fragments do not form channels because they are further digested into smaller fragments before aggregating into channels. However, due to the stochastic nature of the formation of single channels, even in a system with the effective digestion of fragments, there is a chance of channel formation.

In this concept, cell death is not an automatic consequence of a failed lysosome. There are several potential pathways leading to cell death. One of the most well-studied ones is the leakage of lysosomal enzymes (2, Fig.4), which either directly digest cellular content (necrosis) or activate cytoplasmic caspases to induce apoptosis-mediated cell death. The second pathway could involve leakage of the channel-forming peptides (1, Fig.4). The fragments are formed either during the initial degradation or due to residual activity of endopeptidases while neutralization selectively deactivate endopeptidases. A likely target for such leaked fragments could be the internal leaflet of the plasma membrane. The activation of these two pathways leads to the relatively fast demise of the cell.

In contrast, the third major pathway is long-developing, and would require an accumulation of multiple failed lysosomes over extended periods. Here, the content of the lysosome is not degraded anymore, but the lysosome cannot be eliminated until a cargo is digested. The dysfunctional lysosome carrying undigested content accumulates intracellularly. Increasing the number of failed lysosomes prevents the normal recycling of other failing organelles, with the mitochondria being most sensitive to the loss of cellular quality control (3, Fig.4). The improper inactivation of failed mitochondria results in keeping damaged mitochondria that continue to metabolize nutrients without the production of ATP, thus the production of ROS, but also in the prevention of the normal appearance of healthy new mitochondria, thus leading to hypometabolism typical for AD.

There are potentially alternative mechanisms that are not included in the schematic. One of them is the direct delivery of intralysosomally produced fragments into the intramembrane space of the

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mitochondria during the “kiss-and-run” interaction between the endosome-lysosomal system and mitochondria.

It is important to consider that fast and slow mechanisms are not exclusive of each other. Slow mechanisms could be responsible for the functional failure of neurons, leading to the inability of neuronal branches to function. Despite functional failure, some of cells stay alive, which gives them a chance to restore the normal function through the recovery of intracellular damage. In this scheme of cellular events, the critical question is what happens with failed lysosomes. If, once failed, they stay inside neurons forever, is it possible to find a way to digest and recycle them? And if, vice versa, there exist natural mechanisms for recycling failed lysosomes, can we improve this process to normalize the cellular physiology disturbed by AD pathophysiology before cells die due to irreversible damage?

At the same time, fast mechanisms can work independently of slow ones. If this is the case, the accumulation of autophagic vacuoles would still be a marker of the disease, but actual neuronal death would mostly occur from fast mechanisms. It would be important if the accumulation of slow changes (such as vacuoles) would result in neuronal dysfunction, and if it actually correlates with cognitive impairment. In such studies, the need to estimate the effects on neurites/axons separately from the changes in neuronal soma would create an additional level of difficulty.

5. Neuronal death is a stochastic phenomenon

Age is considered the most important risk factor for Alzheimer’s disease. The effect of aging can be detected even in cell cultures: $A\beta_{1-42}$ had no effect on young cultured hippocampal neurons, but induced apoptosis in mature neurons [192]. It is reasonable to hypothesize that aged cells have some differences from young cells. One of these differences could be a mechanism of mitophagy. While typical mitophagy requires the ATG32 protein [193], aged cells demonstrated an additional ATG32-independent mechanism of mitochondrial quality control [194, 195].

Why does it take many years to develop Alzheimer’s disease, even in early-onset forms of the disease? And why is the late-onset form associated with advanced ages? The progression of AD correlates with the disappearance of biochemical markers of neuronal transmission. While recent studies suggest that cognitive impairment could precede the loss of synaptic markers [196], brain atrophy and a loss of neurons are part of AD pathology, at least in advanced stages. The lack of understanding of the mechanisms underlying the timeline of neuronal damage could be a major obstacle in the way of finding effective methods of prevention or treatment.

In the conceptual framework of *amyloid degradation toxicity hypothesis*, an attempt to answer such questions will include the consideration that cellular death due to the presence of amyloid can be divided into two relatively independent events – lysosomal damage due to a formation of a membrane

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channel, followed by the progression through the pathways leading to cell death. Factors determining both steps are most likely relatively independent. There are factors determining the probability of producing a channel in each lysosome; it would depend on the concentration of peptide, activity of enzymes producing and digesting fragments, as well as conditions favoring or opposing the formation of channels instead of less-toxic aggregates. The event consisting of the primary lysosomal damage is relatively fast and occurs within hours after the peptide is taken by endocytosis. Therefore, the progression of the disease would mostly depend on the frequency of the primary events, and how these primary events translate into cell death. As it was discussed above, cell death after lysosomal damage can occur through slow and fast mechanisms. The type of mechanism that is actually involved in each case would determine the interpretation of the disease development timeline.

Experimental approaches to studying the involvement of amyloid in the death of neurons are mostly concentrated on the effects of exogenous amyloid on cell survival. This approach is based on exposure to massive concentrations, which results in the cell death within a matter of hours. Can any involved mechanisms be relevant to the actual disease that develops over years? This discordance in timescale is obvious, but there is little that can be done about it experimentally. Considering that multiple experimental facts are known, it is reasonable to turn to modeling. Keeping the ion channel formation mechanism as a primary pathophysiological mechanism of AD in mind, several conceptual models are proposed below. However, it all originates from a critical question of how effective channel formation is.

5.1. There is very small chance of damage to each lysosome.

It is possible to estimate the potential frequency of channel formation in cells. Exposure of a cell in the culture to exogenous amyloid results in the accumulation of peptide inside endocytic vesicles, which fuse with lysosomes. Using a fluorescently labeled monomeric peptide (1 μM), it was estimated that over a period of 8 h, each cell accumulates 400,000 molecules of $A\beta_{1–40}$ or 800,000 molecules of $A\beta_{1–42}$ which are almost exclusively associated with vesicles labeled by a lysosomal tracker [83]. The degradation of the peptide is not immediate - cells retain around 40% of the labelled peptide after 24 h.

It is important to note that unlike in the study [143], the internalized fluorescent label was observed as a punctate pattern, without clear diffusion into the cytoplasm. The difference could be due to the properties of the used label – the low molecular weight of Lucifer Yellow (MW 444) vs. that of amyloid peptides (MW >4000). As was discussed above, the size cutoff of amyloid channels could be less than 4000 Da. Channels are large enough to leak compounds with a low molecular weight as in the study [143], but still keep macromolecules as in other experiments [83].

In our *in vitro* experiments with artificial membranes, we found that channels are created with an extremely low probability [34, 35]. Despite the presence of trillions of dissolved peptide molecules, only

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thousands of liposomes become permeable. The ratio of formed channels to the number of peptide molecules in the solution was in the range of $1:10^{13}$ for low micromolar concentrations. If channels would in fact be formed with the same probability in cells, we would never observe them. However, it is possible that the probability is actually much higher. We speculate that channel formation is a two-step process – first, the channel-forming unit appears from the dissolved peptide molecules, and then this unit incorporates itself into the membrane. Due to the high concentration of intralysosomal amyloid, which was estimated as 60-100 μM [83], the process of channel formation can be non-linearly dependent on the concentration. Next, the channel-forming fragments are produced by peptidases, so freshly formed fragments could be positioned closely and spatially arranged in favor of forming a specific oligomeric structure. Finally, the internal leaflet of the lysosomal membrane can provide favorable conditions for an incorporation of the formed channel, in contrast to experiments using artificial membranes.

To observe a significant drop in the number of surviving cells in an experiment, a high concentration of added amyloid is used. Nevertheless, even after an exposure to Aβ concentrations a high as 20 μM, not all lysosomes, which are clearly filled with the taken amyloid peptide, become permeant [143]. Considering that it is unlikely that the appearance of each damaged lysosome results in the death of the host neuron, it is obvious that in the actual brain, the death of each neuron cannot be predicted, but occurs with some very low probability. However, over many years, this small probability results in a significant cumulative decrease of the neuronal population.

It could be speculated that the dependence of cell survival on the concentration of Aβ is different for different mechanisms involved in cell death. In any biologically relevant scenario, the probability that a lysosome is damaged at a given moment is very low. After one particular lysosome is damaged, the cell is unlikely to die. Significant drop of cell survival occurs due to long exposure and eventual multiple events of lysosomal permeabilization. If the accumulation of dysfunctional organelles due to an absence of proper recycling is a major reason of neuronal death, then it would be reasonable to expect that the probability of cell death will be non-linearly dependent on the concentration of peptide due to the cumulative effect from each damaged lysosome. The size of the channel will not be critical, as any channel will be able to pass protons. In contrast, if cell death would be dependent on an activation of apoptosis by leaking lysosomal content, then each leak will be an independent event. In this case, the concentration dependence could be linear. Also, leaks will be dependent on the creation of not just any channel, but channels large enough to pass macromolecular complexes relevant to the initiation of apoptosis. Quite obviously, actual biological systems can make such dependencies more complex by adding multiple factors.

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5.2. Factors determining lysosomal damage frequency.

While the probability of damage to each lysosome involved in the degradation of amyloid consumed by the endocytic mechanism is not high, it is also not constant. The amount of amyloid inside each endosome depends on the concentration of the extracellular peptide. It is reasonable to hypothesize that a higher dose of peptide per endosome would result in the greater production of fragments. Endosomes contain not only the peptide, which is dissolved in the liquid, but also the peptide which is membrane-bound. Along with liquid absorption, membrane binding explains why estimates of the intralysosomal concentrations of amyloid exceed the concentration of peptide in the incubation medium. In cells incubated in 1 μM solutions of either $A\beta_{1–40}$ or $A\beta_{1–42}$, the intralysosomal concentrations of the peptides after 8 h of exposure were 60 and 140 μM, correspondingly [143].

While fibrils of extracellular amyloid are considered inactive, the aggregation is reversible. Otherwise, it is impossible to explain how the brain can be cleared of amyloid plaques by some treatments, such as anti-amyloid antibodies [197]. The effect of various chemical compounds on the aggregation of amyloid into fibrils was studied and published in literally thousands of manuscripts, due to the relative simplicity of observing the formation of fibrils using fluorescent probes. In contrast, the ability to disaggregate already-formed fibrils has attracted much less attention. However, fibrils made of beta-sheet structures can be effectively dissolved using well-known pharmacological agents such as antibiotic rifampicin in therapeutically relevant concentrations [198], though this study was done on α-synuclein. Therefore, it should be considered that the presence of plaque can create a source of amyloid. The concept of the plaque being the source of amyloid can explain why neuronal damage is centered around plaques.

This same concept can explain why recent anti-amyloid treatments appeared ineffective in patients. If endocytosed amyloid is bound to an antibody, and digestion could still produce the same fragments, then the process of dissolving plaques can in fact provide more substrate for endopeptidases. Similarly, a reservoir of Aβ in the plaque makes an inhibition of synthesis *de novo* (using BACE1 inhibitors) not sufficient enough to modify local concentration of amyloid in the vicinity of the existing plaques. Insignificant decrease of local amyloid does not prevent a progression of the disease.

The conceptual framework of the *amyloid degradation toxicity hypothesis* presents an interesting perspective on the role of oligomers in toxicity. In experimental settings, the oligomers of a full-length peptide are more toxic than monomers. If oligomers of a full-length peptide are taken by endocytosis, they will be exposed to lysosomal enzymes. We could not find studies comparing the digestion of monomers and oligomers by peptidases, though scientific logic dictates that the spatial requirements of the catalytic cleavage could place limitations on the activity of enzymes towards oligomers. If endopeptidases can still cleave oligomeric aggregates as long as the cleavage site is exposed to the

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enzyme active site, it is quite possible that exopeptidases lose access to terminal amino acids due to the presence of parallel strands. If that is the case, then oligomers will be cleaved preferentially into longer fragments, which will remain unprocessed. Even further, fragments produced from an oligomer could already be arranged into a beta-sheet structure ready to form a barrel and incorporate itself into a membrane as a channel.

The next variable defining the probability of lysosomal damage is the actual activity of peptidases able to form channels, and the balance between endo- and exopeptidase activity. While the degradation of beta-amyloid occurs at or near maximal capacity [199], the activity of degradation enzymes varies over a lifetime, with different enzymes having different changes [200]. Changes in the intralysosomal pH associated with aging could be an additional factor defining the status of enzymes, and should be considered in the interpretation of the age-dependent onset of AD [184].

The formation of membrane channels by degradation products of amyloid taken through endocytosis is not cell death and does not constitute the disease itself. The development of the disease, and its progression, requires the cellular consequences of lysosomal permeabilization. As was discussed, these consequences could develop through slow and fast mechanisms. Accordingly, the progression of cellular damage is discussed below as “evolutionary” (fast mechanisms) and “catastrophic” (slow mechanisms) scenarios.

5.3. Scenario of the “evolutionary” progression of amyloid-induced cellular damage.

An accumulation of failed autophagosomes, which occupy intracellular space but do not provide any functional benefit, prevents the normal physiological processes from occurring inside the cell. If such a vacuole appears in the neuronal appendage that has much smaller physical dimensions, it also affects axonal and dentritic transport, which are critical for the trophic processes supporting the normal synaptic function. The repair of mitochondria can occur at the periphery, without a return to the soma [201]. If this is the case, the total number of organelles balancing local energy supply and demand is relatively small, so a noticeable drop in the number can be irreplaceable for a specific part of neuronal appendage. However, those who have ever dealt with local anesthesia know that if a nerve is blocked at one point, it loses its function all the way distal to this point. The sensitivity of extended neuronal appendages to a problem at any part of a neuron is the reason why synaptic transmission and survival can suffer before neuronal death occurs. The problems associated with the recycling of failed mitochondria in general readily explain both the decreased metabolic activity and increased production of damaging reactive oxygen species.

However, when cellular damage occurs, normally it is repaired. As long as the repair rate exceeds the rate of damage, the cell is able to survive and potentially recover. If damage exceeds the repair for

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a sufficient amount of time, the damage becomes irreversible, and the cell dies. Cellular damage - in a wide sense - would be dependent on the number of endo-lysosomes damaged by amyloid. The accumulation of plaques as a reservoir of the peptide, age-related lysosomal decline, and deacidification would lead to a rate of damage that increases with age. At the same time, the number of mitochondria is decreasing over time [149], and this can be a reflection of a decreased reparation rate, rather than a higher rate of mitochondrial damage *per se*. Therefore, the rate of damage increases over time, while the rate of repair decreases. The time point when these two trends cross marks the disease's on-set. It is clear that each cell in this case is essentially independent of all others, so there is an inherent variability in the times at which neurons reach the point of no return. In this scenario, the markers of dysfunction should appear not only before an onset of symptoms (which are believed to be well compensated for a long time, even when neuronal death is already happening), but also long before the first neuron dies from the biochemical changes related to the disease. Also, an evolutionary scenario provides a good chance of finding a way to reverse the accumulated damage.

5.4. Scenario of a "catastrophic" progression of amyloid-induced cellular damage.

In a catastrophic scenario, cell death is also initiated by lysosomal permeabilization. However, it occurs through fast acting mechanisms such as necrosis, due to the direct action of the leaked cathepsins and necrosis or through an activation of caspases and sequential apoptosis. Fast acting mechanisms are normally prevented by intracellular protection such as cytoplasmic cathepsin inhibitors; therefore, after each lysosomal leak, cell death occurs with low probability. The disease progresses faster either due to a dramatically increased frequency of primary events (lysosomal permeabilizations and leaks), or due to changes to the said probability. One of the most obvious potential mechanisms leading to the increased probability of cell death after an enzymatic leak would be a low concentration of cytoplasmic cathepsin inhibitors. The absence of inhibitors would allow the leaked enzyme to act in the cytoplasm. Looking back to Fig.3 - comparing a potential role of endogenous protease inhibitors in AD and cancer - this option is shown in the left column, resulting in a positive correlation between the two conditions. Strikingly, clinical practice demonstrates the opposite. However, the whole concept discussed in relation to Fig.3 is based on the hypothesis that any correlations between AD and cancer involve cathepsin inhibitors as a significant part of the mechanism.

In the simplest picture of the catastrophic scenario, biomarkers would not precede cell death, despite it being possible to identify informative biochemical changes before the development of symptoms (for example, decreasing inhibitor concentrations). Finding such markers will not allow for the reversal of damage, as neurons are already dead.

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The evolutionary and catastrophic scenarios are not exclusive of each other. There are no reasons to reject a hypothesis stating that intracellular damage will be accumulating, can be detected before neurons are dying, and even be responsible for symptoms, but that neuronal death *per se* occurs mostly due to fast acting mechanisms. Furthermore, there is no reason to reject a hypothesis that there is a continuum of conditions with various involvement of both scenarios.

General considerations as conclusions

The presented hypothesis allows us to generate several critical testable predictions for the development of future treatments for AD.

Firstly, and most importantly, the key pathophysiological processes of AD development occur inside the cell. Therefore, the blood-brain barrier could be not the most critical obstacle in the way of the delivery of any pharmacological agent to the target. It is relatively easy to go around this by relying on intracranial delivery - at least in a proof-of-concept - but crossing the plasma membrane can be much more difficult to arrange for an effective pharmacological agent.

Secondly, treatments that are designed to close the channel will be efficient *in vivo* only if such an inhibition is nearly complete. The channel is so potent for a small organelle that its partial closure does not terminate its damaging action for the mitochondrial or lysosomal functions. If the channel is already formed, only a near-complete closure can keep the function of the organelle.

Next, considering that the death of a specific neuron is dependent on the rate of mitochondrial synthesis, any treatment that can affect this rate would be affecting the probability of neuronal death. If some interaction increases mitochondrial synthesis, it will decrease the chance of neuronal death, and, in the case of AD, will delay disease onset and progression. With this in mind, it becomes clear why multiple studies demonstrated an association between exercise and protection from AD [202]. Exercise improves mitochondrial function - not only in skeletal muscles [203] - and attenuates age-related mitochondrial dysfunction in skeletal muscles [204]. It would be no surprise if it could do so in the brain, too. In relevance to AD, it actually increases mitochondrial biogenesis [205].

Is it possible that pharmacological treatments can bring about a similar positive effect? Yes, probably. Various compounds can mimic the effect of exercise or have an alternative pathway to activate mitochondrial improvement [206]. Similar pathways can be, and should be, studied in the neurons. For example, it was shown that quercetin can increase mitochondrial biogenesis in the brain [207], while the same compound protected against AD in animal models [208, 209].

It is obvious that even if a breakthrough in identifying specific mechanisms that are responsible for neuronal death in Alzheimer’s disease will occur, it will still require tedious work to identify, develop, and test treatments affecting these mechanisms.

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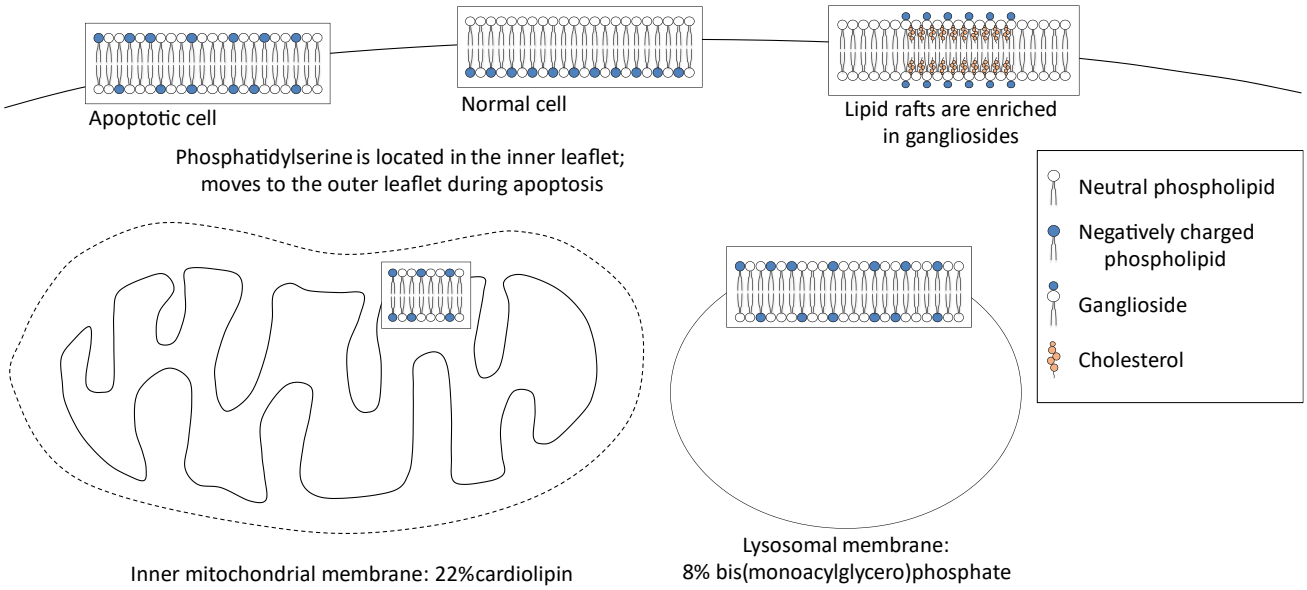

Figure 1. The localization of cellular membranes with a negative charge.

Plasma membranes of healthy cells contain negatively charged phospholipids (mostly phosphatidylserine) in the inner leaflet. In apoptotic cells negative charges appear in the outer leaflet. Lipid rafts are rich in gangliosides. In organelles, the negative charge of membranes is biochemically required in the inner mitochondrial membranes and lysosomes.

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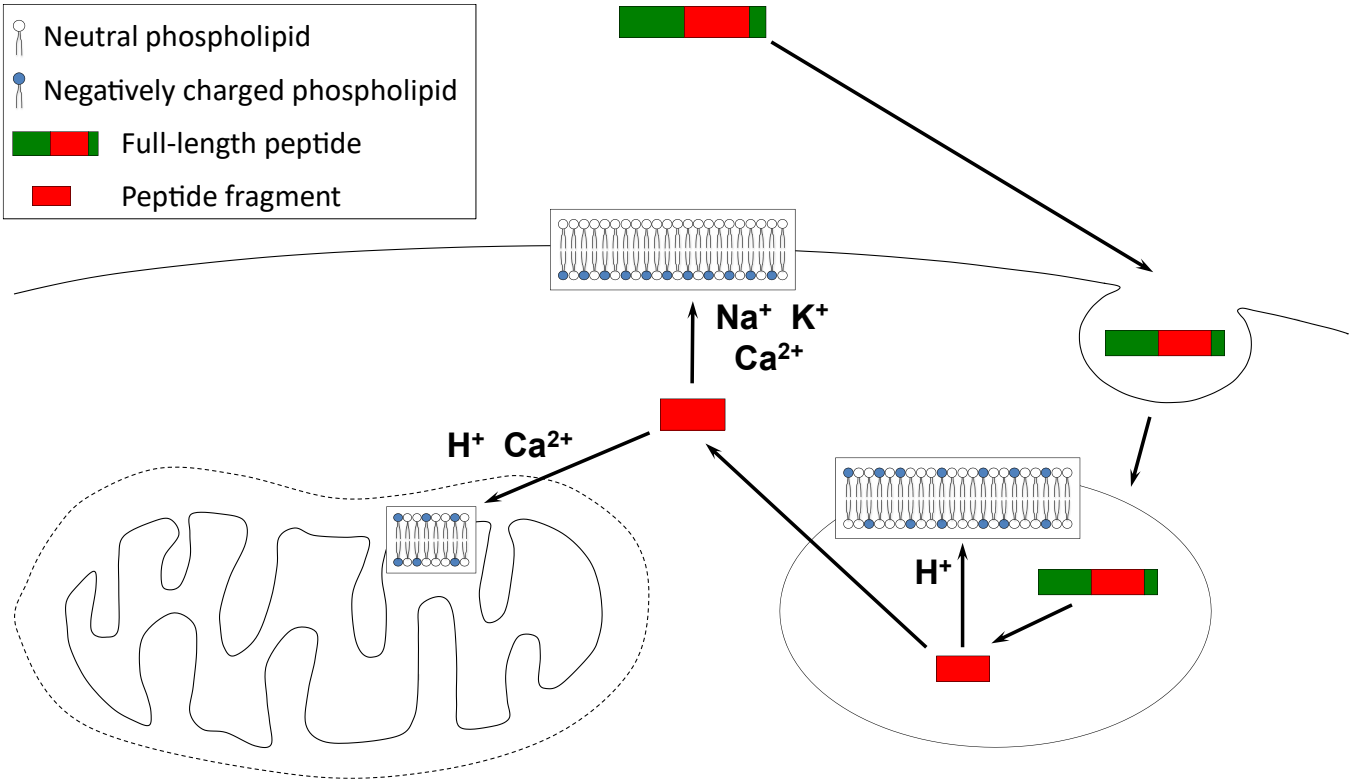

Figure 2. The pathway of beta-amyloid metabolism and critical ion gradients which can be dissipated by membrane channels in various cellular membranes of a healthy cell.

The schematic assumes that amyloid fragments can exit to the cytoplasm through permeabilized membranes of lysosomes as well as reach the inner mitochondrial membrane.

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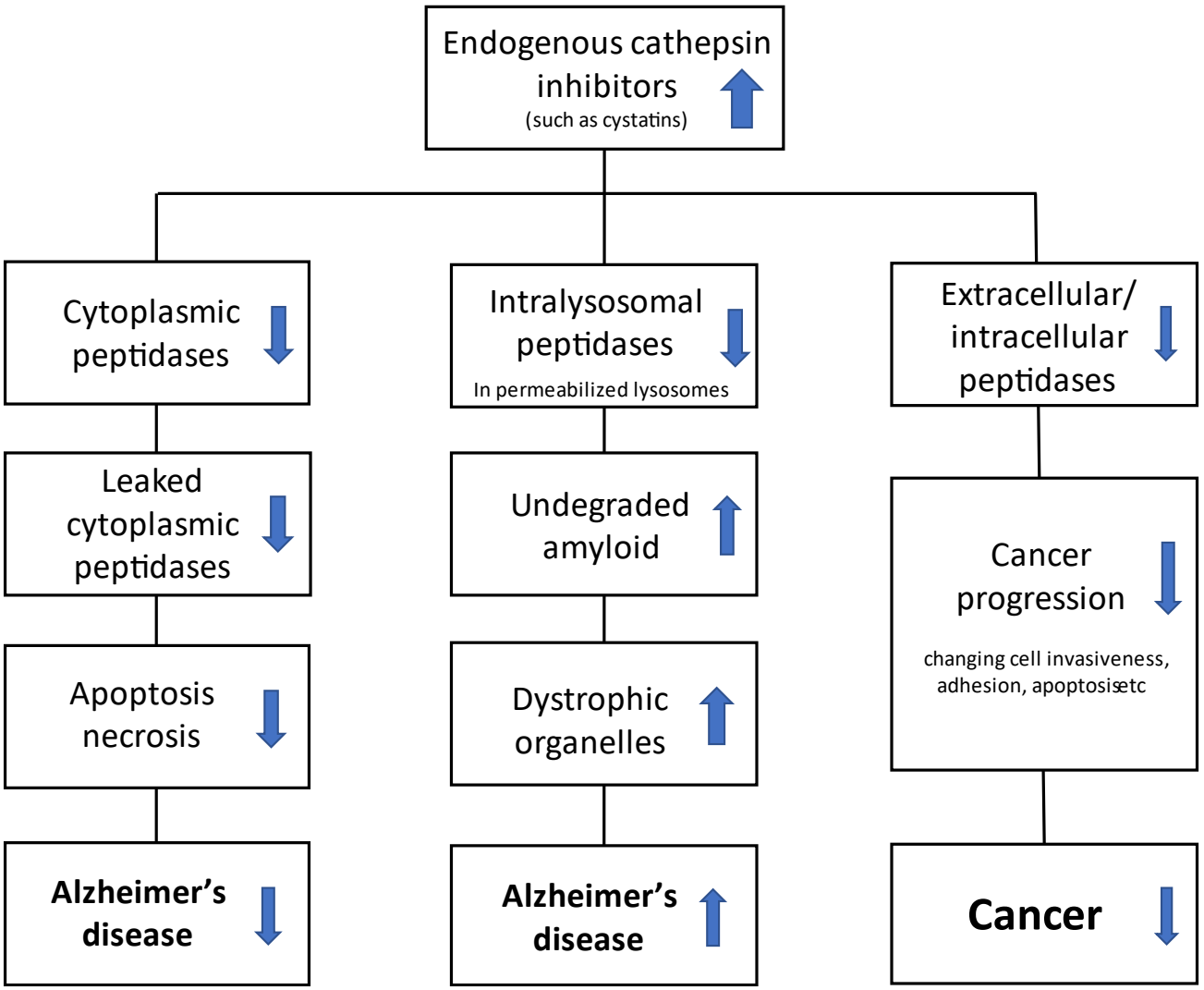

Figure 3. The correlations between Alzheimer's disease and cancer mediated by the activity of endogenous protease inhibitors.

The schematic uses increased inhibitor activity as an example. Arrows describe the change of activity or an intensity of the process.

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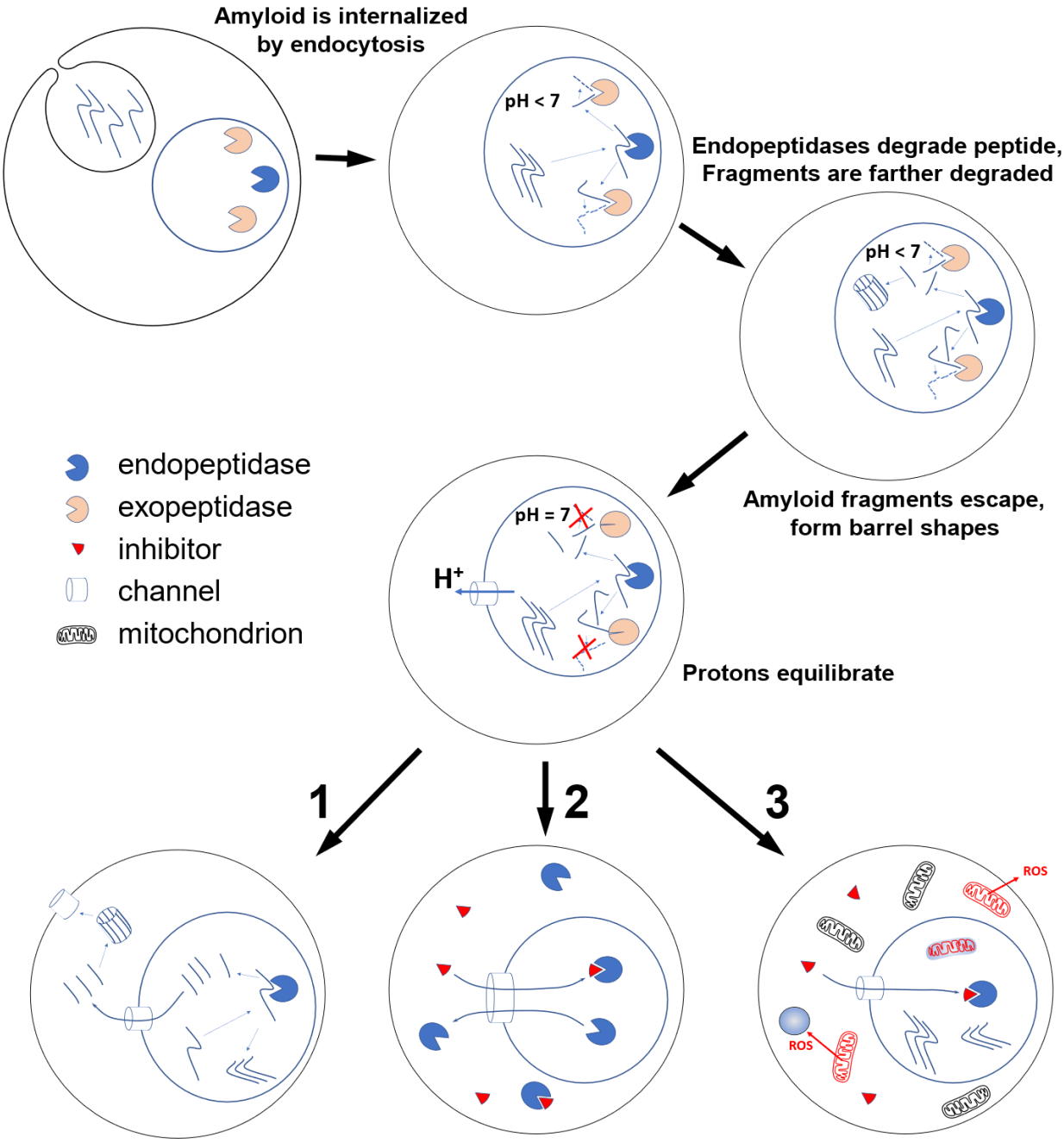

Figure 4. *Amyloid degradation toxicity hypothesis*.

The endocytic vesicle containing the amyloid peptide is merged with a lysosome. Endopeptidases produce various short fragments which are mostly degraded by acidic exopeptidases. Short fragments can form non-selective membrane channels dissipating pH gradient. The neutralization inhibits acidic proteases. Lysosomal failure leads to a cell death through several pathways. 1. Channel-forming peptides leak to a cytoplasm through the permeabilized membrane and target other membranes including plasma membrane. 2. Lysosomal enzymes leak to the cytoplasm and cause necrosis or activate apoptosis. 3. Dysfunctional lysosomes accumulate, while recycling of organelles fails. Damaged mitochondria are not recycled and produce reactive oxygen species damaging other organelles.