Review Article

Amyloid Oligomer Neurotoxicity, Calcium Dysregulation, and Lipid Rafts

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Amyloid proteins constitute a chemically heterogeneous group of proteins, which share some biophysical and biological characteristics, the principal of which are the high propensity to acquire an incorrect folding and the tendency to aggregate. A number of diseases are associated with misfolding and aggregation of proteins, although only in some of them—most notably Alzheimer's disease (AD) and transmissible spongiform encephalopathies (TSEs)—a pathogenetic link with misfolded proteins is now widely recognized. Lipid rafts (LRs) have been involved in the pathophysiology of diseases associated with protein misfolding at several levels, including aggregation of misfolded proteins, amyloidogenic processing, and neurotoxicity. Among the pathogenic misfolded proteins, the AD-related protein amyloid β (Aβ) is by far the most studied protein, and a large body of evidence has been gathered on the role played by LRs in Aβ pathogenicity. However, significant amount of data has also been collected for several other amyloid proteins, so that their ability to interact with LRs can be considered an additional, shared feature characterizing the amyloid protein family. In this paper, we will review the evidence on the role of LRs in the neurotoxicity of huntingtin, α-synuclein, prion protein, and calcitonin.

1. Introduction

Lipid Rafts (LRs) are highly dynamic, nanoscale domains of the plasma membrane, enriched in cholesterol and sphingolipids (Figure 1). They were originally defined on the basis of their resistance to solubilization in nonionic detergents, which allows their separation and isolation from the rest of the plasma membrane, using sucrose-density gradients [1]. Although their existence has initially been questioned [2, 3], it is now generally agreed that LRs are special membrane domains that act as platforms for the organization and interaction of proteins [4]. They are involved in several cell functions and play crucial roles in signal transduction, phagocytosis, protein sorting, and cell polarity. Besides the role in cell physiology, they are also involved in cell pathology. For example, certain pathogens, such as viruses and bacteria, as well as their toxins, interact with the host cells through LRs [5, 6]. In the pathogenicity of amyloid proteins, LRs have been implicated in amyloidogenesis, in the process of protein aggregation, in the mechanisms of interaction between the cell membrane and amyloid proteins, and in their neurotoxic effect. This paper will first provide an overview on the principal milestones in the history of amyloid proteins. After considering the mechanisms of neurotoxicity of misfolded proteins, it will then focus on the role played by LRs in the interaction between neuronal cells and four amyloid proteins: huntingtin (htt), α-synuclein (α-syn), prion protein (PrP), and calcitonin (CT).

2. The Amyloid Protein History: Breakthrough Discoveries

The history of amyloid proteins has been for a long time, with a few, though remarkable, exceptions, the history of Aβ. Aβ was first isolated in 1984 from brain blood vessels of AD patients and individuals with Down's syndrome
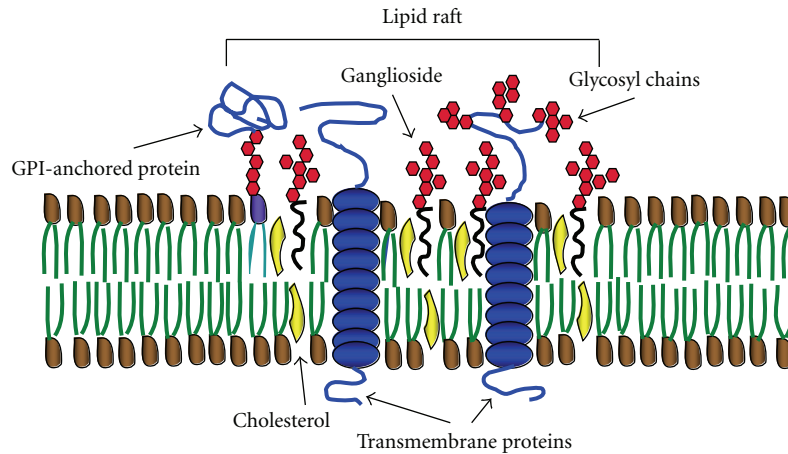
Figure 1: Schematic representation of an LR in the cell membrane, enriched in gangliosides and cholesterol. Glycosylated and non-glycosylated transmembrane proteins and GPI-anchored proteins are also sketched.

[7, 8]. In 1985, Colin Masters identified Aβ as the principal component of amyloid plaques, the hallmark of AD, and in collaboration with Konrad Beyreuther identified the amino acid composition, the molecular mass, and the NH2-terminal sequence of the peptide [9]. In addition, they recognized that the protein was identical to that described for the amyloid deposited in the congophilic angiopathy of AD and Down's syndrome [9]. In the same year, the first evidence was provided that prion proteins (PrP) assemble into filaments within the brain to form amyloid plaques into scrapie-infected hamsters [10]. The discovery of prions dated back to 1982, when the Nobel Prize winner Stanley B. Prusiner described them as novel proteinaceous infectious agents causing scrapie [11]. At the beginning of the 1990s, experiments on primary neuronal cultures showed that aggregated Aβ peptides were neurotoxic *in vitro*, suggesting a link between amyloid formation and neurodegeneration [12]. Since then, primary hippocampal cell cultures have been considered as an ideal cell culture model to study neurotoxic properties of amyloid proteins. In the same years, breakthrough discoveries on the genetics of AD (for a review, see [13]) led to formulate "the Aβ cascade hypothesis." According to this theory, the 1–42 and 1–40 Aβ peptides, deriving from the proteolytic cleavage of the amyloid β precursor protein, operated by the β and γ secretases, are the principal culprits in the development of AD [14, 15]. In the 1990s, the understanding of pathogenic mechanisms of AD dramatically advanced due to the introduction of transgenic animal models, which have provided invaluable insights into several aspects of AD pathophysiology (for a review, see [16]), although mice that precisely model all aspects of AD are not yet available [17]. In the same years, studies on other misfolded proteins started to accumulate. In 1993, the *HTT* gene, associated with Huntington's disease (HD), was identified [18]. It was shown that, in the mutated htt protein, a polyglutamine tract was abnormally expanded, leading to high aggregation propensity. In 1997, Spillantini et al. identified α-syn as the fibrillary component of Lewy bodies (LBs) in Parkinson's disease (PD) and dementia with LBs

[19]. In the same years, the first data suggesting a common neurotoxic mechanism of all amyloid proteins were provided [20]. Although aggregation was considered a critical process in the pathogenicity of Aβ from the beginning, it was not until the end of the 1990s that attention focused on the role of amyloid oligomers more than amyloid fibrils [21]. These studies also identified in the synapse a special target of soluble oligomer toxicity [22], providing a biological explanation to the well-known clinical-pathological observation that dementia in AD has a good correlation with the synapse loss, while the amyloid burden is a poor predictor of cognitive decline [23, 24]. Furthermore, they demonstrated that Aβ oligomers can impair long-term potentiation (LTP), an experimental form of synaptic plasticity resulting in long-lasting increase in the strength of synaptic transmission, which is the electrophysiological counterpart of learning and memory [25].

In the same period, attention shifted from the insoluble amyloid fibrils to the soluble oligomeric aggregates also for other amyloid proteins, which were found to be neurotoxic. They included both disease-associated proteins, such as islet amyloid polypeptide (IAPP), α-syn, PrP, and polyglutamine [26], and non-disease-associated proteins, such as HypF-N, a protein that is not associated with any amyloid disease but displays an aggregation-prone behavior [27]. These amyloid oligomers were found to form pores on model membranes with ion channel properties [28], a mechanism originally proposed for Aβ [29], and the induced Ca^{2+} dysregulation was proposed as a common pathogenetic mechanism through which all amyloid proteins lead to neurotoxicity [30].

One of the latest "coups de theatre" in the amyloid history is the observation that PrPC is a high-affinity cell-surface receptor for soluble Aβ oligomers on neurons and is a mediator of Aβ oligomers-induced synaptic dysfunction [31]. This hypothesis, however, has been challenged by several authors [32–34] and has become a highly controversial issue, still far from being settled [35].

3. Amyloid Proteins: A Large Family of Unrelated Proteins with Some Shared Features

Though differing in the amino acid sequences, amyloid proteins share the tendency to adopt an incorrect conformation (protein misfolding) and the propensity to aggregate. Until recently, there was a general agreement on the idea that only a limited number of proteins can undergo aggregation. However, it has been recently shown that the characteristics that enable a protein to become amyloid are present in almost all complex proteins and that the number of amyloid proteins is limited because the region promoting aggregation is generally hidden [36].

The process of aggregation is complex, depending on characteristics intrinsic to the protein and to environmental conditions and proceeds through several organization states, including dimers, trimers, tetramers, low molecular weight prefibrillar oligomers, and linear or annular protofibrils, to reach the final insoluble fibrillar structure, rich in β sheets. The term "amyloid" should more correctly refer to the mature fibrils, which deposit in tissues and are characterized by Congo red and Thioflavin T positivity. In some diseases, such as systemic amyloidosis, these deposits have a pathogenetic role, and the disease is caused by the deposition of mature fibrils. In neurodegenerative conditions associated with protein misfolding, however, it is now generally agreed that the pathogenic forms are not the mature fibrils but the intermediate, soluble oligomeric aggregates [21, 25, 37]. Oligomers of different amyloid proteins have a remarkable structural similarity, evident at Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM), showing an annular morphology with sizes ranging from 8 to 12 nm, a morphology sustaining the amyloid pore hypothesis (see below). In addition, conformation-specific antibodies have been raised, which cross-react with a number of chemically unrelated misfolded proteins, recognizing generic epitopes exposed in similar folding states of the different proteins [38].

Among the shared features, we believe that three characteristics deserve special attention: oligomeric aggregate pathogenicity, synaptotoxicity, and propagation of protein misfolding.

3.1. Pathogenicity of Oligomeric Aggregates. Besides AD, a role for pathogenic oligomeric amyloid species has been suggested for other protein misfolding diseases, most notably for PD, HD, and PrP diseases.

PD is the second most common neurodegenerative disease affecting aging populations, after AD. The characteristic symptoms of PD include rigidity, resting tremor, postural instability, and bradykinesia. The disease characteristically affects the substantia nigra, where dopaminergic neurons accumulate proteinaceous aggregates, referred to as LBs and degenerate. The majority of patients suffering from PD have a sporadic form of the disease, apparently with no genetic cause, while 5–10% of patients have mutations in a series of genes referred to as the PARK genes [39]. Among the proteins encoded by these genes, α-syn has been the object of considerable interest, since it constitutes the principal component of LBs [19]. Intracellular, α-syn-positive inclusions are also present in dementia with LBs and multiple system atrophy, which, together with PD, are collectively referred to as synucleinopathies [19]. α-syn shows a distinctive propensity to aggregate, a phenomenon associated with a conformational change from random coiled to predominantly β-pleated sheet [40, 41]. This characteristic is enhanced when α-syn is mutated or overexpressed, as in some familiar forms of PD and has been correlated to the pathogenesis of the disease [41, 42]. It is assumed that the aggregation process proceeds through progressive stages, from monomers, through partially folded intermediates, up to mature fibrils. As for Aβ, increasing evidence suggests that prefibrillar oligomers and protofibrils, rather than mature fibrils, are the pathogenic species in PD [41, 43]. Two mutations in the α-syn gene, linked to autosomal dominant early-onset PD, have been described to promote the formation of transient protofibrils at a higher rate than wild-type α-syn [42], although both wild-type and mutant α-syn have been shown to form pore-like structures in synthetic vesicles and model membranes [28, 44, 45]. The pore formation, inducing disruption of cellular ion homeostasis, may be responsible for the neurotoxic effect [44]. Although the question is still open [46], data obtained in three established model systems for PD, such as mammalian neurons, the nematode *Caenorhabditis elegans,* and *Drosophila melanogaster*, show a strong correlation between α-syn aggregates with impaired β-structure, neuronal toxicity, and behavioral defects [47], further sustaining a pathogenic role for α-syn oligomers in PD. Evidence on a role of phosphorylation in the oligomerization and neurotoxicity of α-syn has also been provided [48].

HD is a late-onset, autosomal dominant disorder clinically characterized by chorea, cognitive impairment, and psychiatric disorders. The mutation responsible for HD, an expanded CAG repeat sequence in the HD gene, leads to a polyglutamine expansion in the amino-terminal portion of the htt protein. Although the physiopathology of the disease has not been fully clarified, a role for protein misfolding is suggested by the observation that HD occurs when htt expands beyond around 35 glutamine residues, a modification that facilitates protein aggregation and the acquisition of β sheet structure [49]. In lymphoblasts from HD patients and medium spiny striatal neurons of the YAC72 HD mouse model, polyglutamine expansion in htt was accompanied by cytosolic and mitochondrial Ca^{2+} overload, triggering an apoptotic pathway [50, 51]. As for many other misfolded proteins, htt aggregation is a complex process advancing through a variety of different assemblies, eventually leading to the formation of insoluble inclusion bodies. The different aggregative intermediates have probably different biological activities. As described for Aβ and other misfolded proteins, the soluble aggregates, more than the insoluble inclusion bodies, are probably the neurotoxic species [26].

Prion diseases, also known as TSE, are progressive, mostly fatal neurodegenerative diseases. They include Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, kuru and fatal familial insomnia in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep,

and chronic wasting disease in deer and elk. The central pathogenic event in these diseases is the conversion of the PrPC, a normal cellular isoform, into the abnormal PrPSc (where Sc stands for "scrapie"). The conversion determines an increase in the β-sheet content of the protein and is accompanied by changes in biological and biochemical properties of PrPSc, such as increased resistance to proteases and propensity to form amyloid fibrils. The interaction between PrPC and pathogenic PrPSc is supposed to determine a template-induced, progressive deposition of new PrPSc, which accumulates in brain tissue as dense plaque-like amyloid deposits, perivascular deposits, or diffuse, non-fibrillary deposits, reminiscent of synaptotoxic oligomeric β amyloid aggregates. Although the deposition of amyloid plaques is a hallmark of prion diseases, recent studies suggest that, in analogy to Aβ and other amyloid proteins, the soluble oligomeric aggregates of PrPSc are the actual neurotoxic species. For example, prefibrillar oligomers are neurotoxic *in vitro* and *in vivo* [52], and soluble oligomeric species are most efficient in transmitting TSE [53]. It has also been proposed that the fibrillar form of PrP, which is typically observed at autopsy, may actually be neuroprotective [54].

3.2. Synaptotoxicity. Amyloid proteins are, by definition, neurotoxic. Neuronal cell damage induced by various amyloid proteins has remarkable analogies, especially considering one highly specific effect: synaptic dysfunction. Compromised synaptic function is a key event in the pathogenesis of AD. Quantitative evaluation of temporal and frontal cortical biopsies revealed a significant decrease in the density of synapses [55]. At autopsy, synapse loss, as demonstrated by decrease in synaptophysin immunolabeling, showed a clear-cut correlation with the severity of dementia [56]. Later on, it has been shown that synapse loss is an early event in the pathophysiology of the disease [56]. More subtle derangements of synaptic activity, induced by Aβ oligomers, precede synapse loss. The studies by Selkoe and collaborators have shown that natural Aβ oligomers, secreted from cultured cells, when injected in rat brain, potently inhibit LTP, enhance long-term depression (LTD), and impair the memory of learned behaviors in rats [25, 57, 58]. Similar results were obtained from Aβ dimers isolated from the brain of AD patients [59]. Evidence for a role of synaptic dysfunction in PD, HD, and PrP diseases has also been collected, showing modified synaptic activity as a consequence of the interaction with misfolded proteins. Synaptic dysfunction is an early symptom in α-syn-induced pathology [60, 61]. α-syn is localized at synapses, where it is involved in the modulation of synaptic transmission and neuronal plasticity [62], in the regulation of the size of different pools of synaptic vesicles [63], and in the SNARE complex assembly [64]. Recently, it has been demonstrated that α-syn directly regulates the dynamics of actin microfilaments, whose integrity is fundamental in synaptic vesicle mobilization, recycling, and exocytosis. This regulatory activity was profoundly altered in the A30P mutation, associated with familial PD [65]. Using paraffin-embedded tissue blot and protein aggregate filtration assays, it has been shown that the majority of α-syn oligomeric

aggregates are located at presynaptic terminals, suggesting an impact on synaptic function [66, 67]. This is also sustained by the observation that, in cultured neurons from brains of transgenic mice overexpressing human α-syn, excessive α-syn induced a decrease in other presynaptic proteins, leading to morphologic and functional changes of synapses [68].

Studies in animal models of HD have clearly shown that synaptic dysfunction precede neuronal loss [69–71]. Decreased pre- and postsynaptic markers and altered glutamate release were found at the corticostriatal synapse before the onset of motor symptoms [72]. Altered LTP and LTD were early electrophysiological signs of aberrant synaptic plasticity [73, 74]. In a *Drosophila* HD model, expanded full-length htt was observed to increase neurotransmitter release efficiency, leading to impairment of synaptic transmission and altered Ca^{2+} homeostasis [75].

In prion diseases, synaptic alterations are among the pathognomonic pathologic features, together with neuronal loss, spongiform change, astrocytosis, and deposition of amyloid aggregates. Immunocytochemical localization of PrPSc has a dot-like appearance around neuronal cell bodies and, along dendrites, reminiscent of synaptic protein localization in synapses [76, 77]. There is evidence that synaptic changes precede neuronal death [78–81], possibly sustained by mitochondrial dysfunction [82]. Mice with prion diseases can be cured at the stage of early synaptic dysfunction and impairments at neurophysiological, behavioural, and morphological levels are reversible [83]. Interestingly, the fact that reversible changes precede extensive accumulation of PrPSc deposits suggest that they may be caused by a transient neurotoxic species [84], in analogy with the effects of soluble oligomeric Aβ in AD.

3.3. Propagation of Protein Misfolding. In PrP diseases, the key molecular event is the conversion of the PrPC into the infectious PrPSc, which serves as template to produce further, aggregation-prone PrPSc. Emerging evidence seems to converge towards the theory that the ability to form auto-perpetuating amyloid aggregation is not exclusive to PrPSc. These findings suggest that several proteins, belonging to the amyloid family, accumulate and propagate through a nucleation-dependent aggregation, starting from what has been defined as an "amyloid seed," whose presence facilitates further oligomerization of the proteins [85].

One of these proteins is Aβ. Injection of brain extracts from human AD brains, but not from control age-matched patients, in transgenic mice overexpressing the AβPP, induced extensive Aβ deposition [86, 87]. The increased Aβ production did not occur when AD brain extracts were depleted of Aβ [87], suggesting that infused Aβ can act as an amyloid seed. The process has also been characterized *in vitro*, showing that homogenates from SHSY5Y cells, which had uptaken Aβ, were capable of seeding amyloid fibril growth [88]. Aggregation of α-syn is also a nucleation-dependent process. *In vitro* biophysical studies have shown that the process is accelerated by the presence of pre-aggregated protein [89]. This mechanism has also been demonstrated in cells, where α-syn aggregates, but not

monomers, can induce the formation of LB-like aggregates [90]. In addition, injection of preformed oligomeric aggregates in cells overexpressing α-syn determines the formation of highly filamentous intracellular, α-syn-positive inclusions [91]. Cross-seeding between different amyloid proteins has also been described. For example, pure α-syn and tau facilitate each other aggregation [92]. Amyloid fibril formation of α-syn is accelerated by preformed amyloid seeds of other amyloid proteins, such as *Escherichia coli* chaperonin GroES, hen lysozyme, and bovine insulin [93]. Susceptibility of different peptides toward cross-seeding is related to the intrinsic aggregation propensity of the peptides [94].

Cell-to-cell propagation of protein misfolding, characteristic of prion diseases, has been described for α-syn in cell cultures and animal models of PD [95]. Furthermore, it has been observed that transplanted neurons in PD patients develop in time LB and PD pathology, suggesting the propagation of α-syn aggregation from host cells to graft cells [96, 97]. This phenomenon may have important clinical implications. For example, efficacy of stem cell therapies in these diseases may be hampered by the risk of propagation of protein misfolding from the host cells to transplanted stem cells [98, 99].

4. Mechanisms of Amyloid Neurotoxicity: The Role of Ca^{2+} Dysregulation

Although there is now wide agreement on the role played by amyloid oligomers in neurotoxicity, the mechanisms through which they induce neuronal cell dysfunction and, eventually, cell death are not fully understood. A number of different possibilities have been explored, including mitochondrial dysfunction, lysosomal failure, and abnormal activation of signalling pathways. These mechanisms may or may not accompany a more general neuronal cell derangement, which is a common effect of the interaction of amyloid proteins with neuronal cells, Ca^{2+} homeostasis dysregulation.

In resting neurons, cytosolic Ca^{2+} concentration is maintained around 100 nanomolar, while the extracellular concentration is about 1 mM and that of intracellular Ca^{2+} stores, the endoplasmic reticulum (ER) and mitochondria, is between 100 and 500 μM. Ca^{2+} entry from the extracellular space occurs through ligand-gated, voltage-gated, and store-operated Ca^{2+} channels, while Ca^{2+} release from intracellular stores, mainly represented by the ER, are regulated by inositol trisphosphate receptors and ryanodine (RyR) receptors. Recently, it has been reported that presenilin I, an integral membrane protein whose mutations cause early-onset inherited AD, also functions as ER Ca^{2+} leak channel [100, 101]. The tight regulation of Ca^{2+} concentration gradient depends on the crucial roles played by Ca^{2+} ions in neuronal cell processes, including neurotransmitter release, generation of action potential, gene expression, synaptic plasticity, and neurite growth. In addition, excessive intracellular Ca^{2+} concentrations may activate a number of pathogenic responses, whose overall effects are modulation of membrane excitability and enzyme/kinase activity, induction of gene expression, formation of reactive oxygen/nitrogen species, mitochondrial dysfunction, and apoptosis/necrosis.

To explain the genesis of Ca^{2+} dysregulation in diseases associated to misfolding and aggregation of amyloid proteins, two main mechanisms have been postulated: the activation of preexisting ion channels and the formation of calcium-permeable amyloid pores.

4.1. Activation of Preexisting Ion Channels. Interaction with several Ca^{2+}-permeable channels has been described for amyloid proteins, potentially leading to an intracellular Ca^{2+} rise. As usual, most evidence derives from experiments on Aβ. The glutamatergic system has been thoroughly studied, on the basis of the role played by glutamate receptors in the excitotoxic neuronal cell damage, whose overstimulation leads to excessive intracellular Ca^{2+} rise [102]. Several *in vitro* studies showed that incubation of neuronal cultures with Aβ oligomers increased Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptors. The moderate-affinity, uncompetitive NMDA receptor antagonist memantine protects against Aβ oligomer toxicity by attenuating intracellular Ca^{2+} increase [103]. Currently, memantine is the only approved treatment for AD, besides acetylcholinesterase inhibitors, although the therapeutic efficacy is limited [104]. Aβ oligomers induce dynamin 1 degradation, which may endanger synaptic integrity. This effect is mediated by NMDA receptor activation [105]. Interactions of Aβ with other Ca^{2+} permeable channels have been documented, such as voltage-gated Ca^{2+} channels [106, 107]. An involvement of nicotinic acetylcholine [108–110], catecholamine [111], and serotonin receptors [112] has also been postulated in Ca^{2+} dysregulation following Aβ treatment.

Intracellular Ca^{2+} stores have also been implicated in Ca^{2+} dysregulation. When presenilin is mutated, its function as ER Ca^{2+} leak is disrupted, contributing to Ca^{2+} dysregulation [100, 101]. Exaggerated intracellular Ca^{2+} levels have also been put in relation to modulation of RyR receptors [113].

A role for calcium-permeable channel has been described for other amyloid proteins. Ca^{2+} influx via N-type voltage-dependent Ca^{2+} channels has been described following α-syn treatment in rat synaptosomes [114]. Overactivation of NMDA receptors, followed by an abnormal neuronal Ca^{2+} signaling, is believed to play a role in HD pathogenesis [50, 115, 116]. Activation of glutamate receptors has been described to be induced by HypF-N [117].

4.2. The Calcium-Permeable Pore Hypothesis. To explain Ca^{2+} dysregulation, a different mechanism has been hypothesized: amyloid oligomers may form nonselective calcium-permeable pores. This ability, originally described for Aβ [29], has also been described for other misfolded proteins and has been proposed as a common property of the amyloid protein family [28, 30]. Several pieces of evidence sustain this hypothesis. Cribbs et al. [118] showed that both D- and L-stereoisomers of truncated form of Aβ were neurotoxic *in vitro*. This observation argues against a role for specific ligand-receptor interaction in the mechanism of toxicity. Morphological studies at TEM and AFM levels have shown that oligomers of many amyloid proteins, such as Aβ and α-syn [44], serum amyloid A, amylin [28], and CT [119], have

a characteristic annular morphology, reminiscent of cation-permeable membrane pores [120]. Furthermore, TEM analysis has also revealed the presence of Aβ pore-like structures in the cell membrane of brains from AD patients but not from age-matched healthy patients [121]. Treatment of SH-SY5Y cells with a wide range of oligomeric, but not fibrillary, amyloid proteins, including Aβ, PrP, IAPP, polyglutamine, and lysozyme, induced increase in intracellular calcium. The increase could not be attributed to activation of endogenous Ca^{2+} channels, because the responses were unaffected by the potent endogenous Ca^{2+} channel blocker cobalt [30]. Electrophysiological recordings using model membranes showed heterogeneous single-channel conductances for several amyloid proteins [28]. Finally, it has been proposed that protein aggregates may mimic bacterial pore-forming toxin, which permeabilize membranes forming oligomeric pores characterized by β-sheet structure [122].

The different hypotheses are not mutually exclusive and can cooperate towards Ca^{2+} dysregulation. Recently, it has been proposed that amyloid oligomers may act at two steps, separated in time, a first, very rapid step, where Ca^{2+} increases due to glutamate receptor stimulation by the oligomers, followed by a second, delayed step, where oligomers permeabilize nonspecifically the cell membrane, possibly via the formation of amyloid pores [117].

5. Lipid Rafts and Amyloid Neurotoxicity

From the original description [123], the concept of LR has remarkably evolved. The introduction of high-resolution imaging techniques (for a review, see [124]) and the progress in lipidomics and proteomics methodologies have revealed that LRs have a highly heterogeneous composition and are characterized by an extremely dynamic structure. LRs can now be defined as nanoscale assemblies of sphingolipid, cholesterol, and proteins, fluctuating in a more fluid phospholipid matrix. By finely tuning lipid-lipid, protein-lipid, and protein-protein interactions, they can coalesce, forming more stable structures and providing functional platforms for crucial membrane activities, such as signaling and trafficking [124].

Considerable amount of data suggest the involvement of LRs in the interactions between amyloid proteins and cell membranes. Some crucial information has been obtained through the use of biophysical techniques on model membranes (for a review, see [125]), which will be briefly illustrated. Most of the work focused on the interactions between LRs and Aβ. However, compelling evidence has also been obtained for htt, α-syn, PrP, and CT, which will be reviewed here.

6. Using Model Membranes to Study Amyloid Proteins

6.1. Model Membranes. The use of model membrane systems has remarkably improved our knowledge on the biochemistry of amyloid proteins, providing information about the molecular mechanisms controlling aggregation, the structure of aggregates (oligomeric or fibrillar), and the interactions with cell membranes. Model membranes consist of mono- or bilayers of lipids that can be placed in contact with proteins of biological interest, such as the amyloid proteins. The monolayer model membranes are obtained by depositing at the water-air interface bidimensional molecular films composed of phospholipids, gangliosides, and cholesterol, with and without proteins (Languimur technique) [126, 127]. On these systems, thermodynamic measurements of compression at constant temperature (isotherms) provide useful information on the lipid mosaic phase (solid, liquid, or gaseous) and its modification due to the presence of proteins. Liposomes are vesicular structures, composed of bilayer model membranes. Mono- or bilayer membranes can be deposited onto solid substrates and studied with imaging techniques, such as Energy-Filtered TEM (EFTEM) or AFM, at nanometric resolution [128, 129]. In liposomes, which are suspended in a water solution, the conformation of proteins interacting or not with the lipid bilayer can be also investigated by Circular Dichroism Spectroscopy (CDS) [119, 130].

6.2. Imaging Techniques. EFTEM represents a powerful tool in the study of biological and nonbiological materials. The use of fast electrons (80–120 KeV) and magnetic lenses allows creating images of thin samples with horizontal resolution in the order of 0.4 nm. This is due to the small wavelength associated to electrons of this energy (about 0.005 nm). Using this technique, it is possible to investigate the quaternary structure and aggregation of misfolded proteins and their interaction with model membranes. In this case the image formation is obtained by negative staining with heavy metals such as tungsten and uranium. This technique allows obtaining horizontal resolution in the order of 1 nm. However, using microscopes equipped with energy filters, it is possible to improve the image quality and increase contrast even in unstained samples and perform spectroscopic studies of the transmitted electrons (Figure 2) [128, 131].

EFTEM can also be combined with immunolabeling techniques to identify proteins by using specific antibodies, conjugated with gold particles (Figure 3). This technique is particularly useful to investigate binding of amyloid proteins to lipid membranes [119].

In AFM, the surface of the sample to be analyzed is scanned by a very sharp tip. The interaction forces occurring between the tip and the atoms of the analyzed surface, in the order of nanonewtons, cause the deflection of the cantilever supporting the tip. Changes in the deflection of the cantilever, due to the morphology of the sample surface, are detected by the reflection of a laser beam. The microscope can operate in static or dynamic mode if the tip is at rest or oscillating vertically, respectively. The structural organization of liposomes or Langmuir films can be imaged by this technique after deposition onto flat substrates of mica, with a resolution up to 1 nm horizontally and 0.1 nm vertically. Morphologic changes induced in model membranes by the incorporation of pore-forming proteins, such as gramicidin A [132], or LR components, such as gangliosides [133], can be analyzed with this technique.
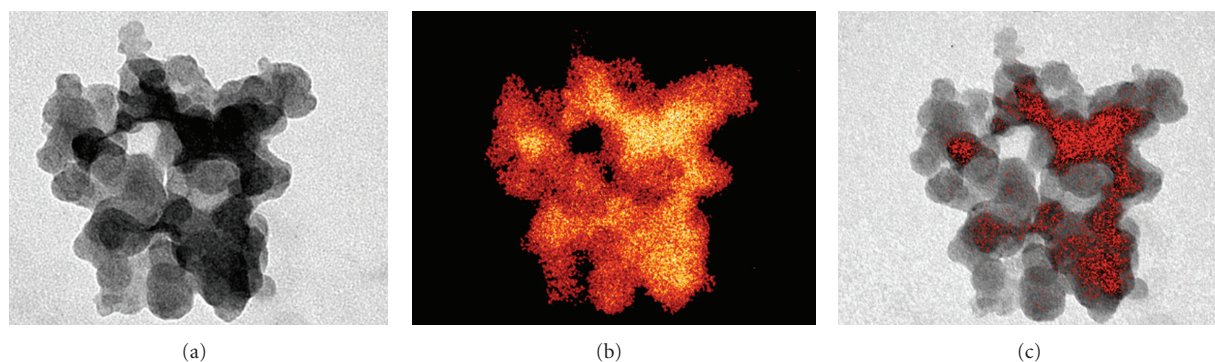
FIGURE 2: EFTEM micrograph of a cluster of liposomes (a). The ESI maps show a higher (b) and a lower (c) concentration of Cs entrapped in liposomes. Reproduced with permission from [131].
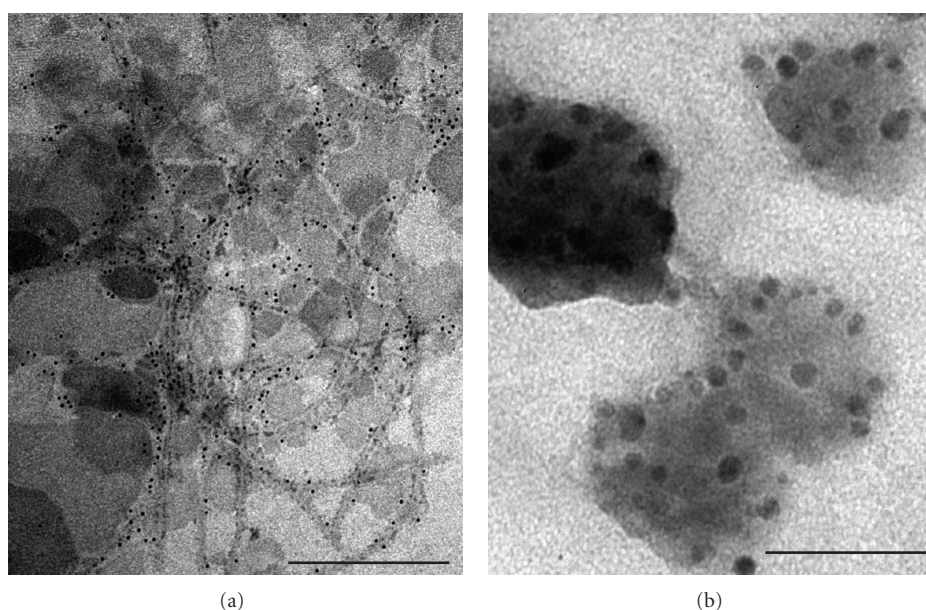

FIGURE 3: Immunogold EFTEM micrographs of sCT in mature fibres ((a) bar = 200 nm) and liposomes, where sCT is inserted in the lipid bilayer ((b) bar = 50 nm). Picture in (b) was reproduced with permission from [119].

6.3. CDS. CDS can be considered a special type of UV absorption spectroscopy and consists in the measure of the difference in the absorbance of left- and right-handed polarized light by optically active molecules, detected in a selected frequency range. This signal depends on the wavelength of the incident light. A dichroic spectrum can be obtained illuminating an optical active sample by light of increasing wavelength. The optical activity of a molecule depends, in the absence of magnetic field, on its chirality: in general, a molecule having an asymmetric charge distribution interacts in a different way with electromagnetic waves characterized by opposite circular polarization.

This type of spectroscopy is generally used to investigate the protein conformation and their change induced by the aggregation process or the interaction with model membranes [119, 132]. In the region of the near UV (250–350 nm), it is possible to obtain information about the tertiary structure of proteins. In this region the aromatic amino acid and the disulfide bonds are excited, giving rise to a dichroic signal depending on the overall three-dimensional structure of the protein. An important application of this technique is to analyze the "folded" state of the proteins; if this is "molten globule" or the protein is incorrectly folded, the near UV spectrum is practically flat. The near UV spectrum is sensitive to small variations of the tertiary structure, due to the interaction of the proteins with other molecules, such as lipids. In the far UV region (190–250 nm), the secondary structure of the proteins can be studied. In this region the chromophore, which is the protein component excited by the incident light, is the peptidic bond. A typical dichroic spectrum for each type of secondary structure exists, and the spectrum of a protein conformed with several secondary structures is formed by the convolution of the base spectra.

7. LRs and htt

Some observations suggest that LRs may be implicated in HD pathogenesis at different levels. From DNA microarray analysis conducted in striatal cells expressing wild-type or mutant htts, genes involved in cholesterol biosynthesis were found to be altered by mutant protein. Since in these cells mutant htt did not form aggregates or cause cell death, this pattern of gene expression may reflect early events in the pathogenetic mechanism [134]. Consistently, dysfunction in the cholesterol biosynthetic pathway was described in mice and cell culture models of HD [135]. In addition, abnormal expression of the genes encoding glycosyltransferases, an enzyme involved in the synthesis of gangliosides, were found in the striatum of the R6/1 transgenic mouse, an animal model of HD, and in postmortem caudate samples from human HD subjects [136]. These observations indicate a disruption in glycolipid metabolic pathways that may alter LR formation. Biochemical analysis of cell membranes from brains and primary neurons of wild-type and presymptomatic HD knockin mice showed that wild-type and mutant htt were recovered in LR-enriched membranes [137]. The association with LRs was stronger for mutant than wild-type htt. In addition, LR from HD mice had a higher content in glycogen synthase kinase 3-beta (GSK). Since GSK activation is involved in neuronal apoptosis, the authors speculate that accumulation of mutant htt and GSK in LRs may have a role in the mechanism of neurodegeneration in HD [137].

8. LRs and α-syn

Although the mechanisms correlating α-syn aggregates to PD pathogenesis remain unclear, there is substantial evidence that binding of α-syn aggregates to lipid membranes is a relevant factor. Oligomeric α-syn binds to model membranes inducing permeabilization of synthetic vesicles, which is considered a potentially cytotoxic event [138]. Dimeric aggregates of wild-type α-syn and its mutants, A53T and A30P, seem to bind to and disrupt lipid membranes more easily than monomeric forms [139], indicating that oligomeric forms are likely to be the pathogenic species. However, even monomeric α-syn can interact with model membranes, undergoing a conformational change from a random coil to an α-helical structure, which may facilitate aggregation [140]. The lipid components seem to have a relevant role in the interaction between α-syn and membranes. α-syn binds to GM1 ganglioside, which are enriched in LRs. This bound is attributed to specific interaction between α-syn and glycidic residues of GM1, such as sialic acid [141]. In addition, α-syn colocalizes with markers of LRs in Hela cell cultures [142]. In the neuronal cells, α-syn is localized in the synaptic terminals, as described above. LR disruption was found to abolish the synaptic localization of α-syn and redistribute it to different cell compartments [142]. Furthermore, association with synaptic LRs is also impaired in the A30P mutation, suggesting that the physiological role of α-syn, lost in the mutated protein, is mediated by LR interaction [142].

9. LRs and PrP

The role of LRs has been the object of considerable interest in prion infectivity. By using model membranes, it has been shown that recombinant forms of the PrPs bind to model LR membranes composed of phospholipids, cholesterol, and sphingomyelin, but not to zwitterionic PC lipids, an artificial model lacking LR components [143, 144]. Inhibitors of the synthesis of cholesterol, a major component of LRs, reduce prion formation *in vitro* [145, 146] and delay the progression of experimental infection [147]. A large body of evidence sustains that LRs are the site where conversion from PrPC to PrPSc takes place [148]. A crucial role has been detected for the PrPC glycosylphosphatidylinositol (GPI) anchor, a complex machinery that has several physiological roles, among which is the targeting of proteins to LRs. Through the GPI anchor, PrPC binds to cell membranes [149]. In absence of the GPI anchor, PrPC redistributes into non-raft regions of the plasma membrane, and the formation of PrPSc is reduced [150]. In addition, synthetic analogues of the GPI anchor [151] and its enzymatic modification [152] reduce the capacity of PrPSc to bind and replicate within neuronal cell lines or primary cortical neurons, suggesting that PrPSc conversion takes place in LR-like microenvironment, following targeting of PrPC to LRs. *In vivo* studies, however, have shown that the role of the GPI anchor is probably more complex than initially assumed. Enzymatic removal of the GPI anchor from PrPSc did not reduce prion infectivity [153], while, in scrapie-infected transgenic mice producing PrPC without a GPI anchor, a high amount of infectious PrPSc was produced, though in the absence of clinical symptoms [154]. However, when mice were engineered to express twofold more anchorless PrP, scrapie infection did induce a fatal disease [155].

10. LRs and CT

Calcitonin (CT), a 32-residue polypeptidic hormone secreted by the C cells of the thyroid gland, belongs to a family of structurally and functionally related regulatory hormones, which also includes amylin, adrenomedullin, and CT gene-related peptide. It plays an important role in Ca^{2+} regulation and bone metabolism. For its activity in reducing bone resorption, it is a therapeutic option in the treatment of osteoporosis. The amyloid nature of CT was unveiled when it was demonstrated that the protein is the principal component of the amyloid fibrils deposited in medullary carcinoma of the thyroid [156]. Later on, its ability to aggregate *in vitro* was studied as a factor limiting its efficacy as pharmaceutical agent [157–160]. The studies of Schubert and coworkers [20, 161] firstly showed that CT, in analogy with other amyloid proteins showing an aggregative behaviour, was toxic to cells in culture. These observations prompted investigators to use CT as a probe to study amyloid formation and neurotoxicity [162–165]. Salmon CT (sCT), which is neurotoxic as CT from other species [20, 159, 166], is characterized by a slower aggregation rate [166], and this
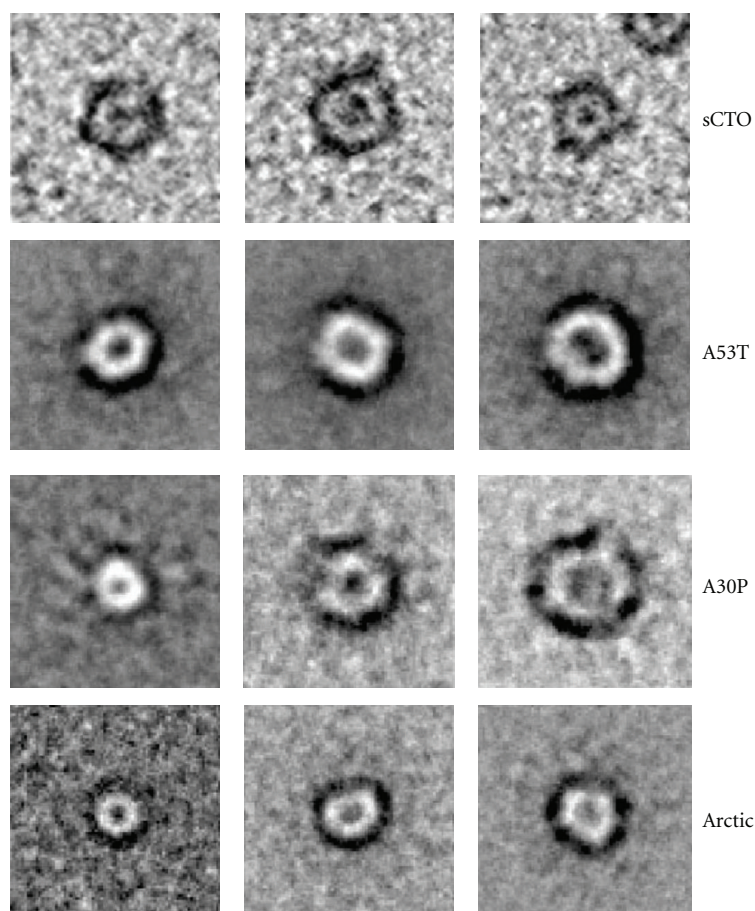
FIGURE 4: EFTEM of sCTOs. Annular sCTOs show remarkable morphologic similarities with amyloid pores of mutant α-syn (A53T and A30P) and Aβ (Arctic). TEM images of α-syn and Aβ were reproduced with permission from [169].

peculiarity is at the basis of its pharmacological use. We have studied the process of sCT oligomerization, focusing on the role of oxidation and time of aggregation [167, 168]. Recently, we showed that sCT oligomers (sCTOs) form Ca^{2+} permeable pores in liposomes [119], highly reminiscent of the ion channels formed by other amyloid proteins, such as Aβ and α-syn [169] (Figure 4). In addition, they damaged neuritic tree and synapses in hippocampal neurons, a behavior highly reminiscent of the effects induced by Aβ [170] (Figure 5).

We used sCT to investigate if a specific neuronal cell susceptibility to amyloid toxicity exists [170]. An issue that has seldom been addressed, in fact, is why misfolded proteins cause diseases so frequently in the CNS, in comparison to other systems or districts. Furthermore, several amyloid proteins, such as CT, are toxic to neuronal cells despite the fact that they are formed outside the CNS. An exception could be represented by amylin, an amyloid protein belonging to CT family. This amyloid protein is considered as a possible pathogenetic species in the development of diabetes, supposedly by damaging pancreatic beta cells, thus exerting a cytotoxic effect outside the brain [171]. If one considers, however, that pancreatic β cells share the same histogenesis

of neuronal cells, being neural crest-derived neuroendocrine cells, type I diabetes would not represent an exception. The reasons for this peculiar vulnerability are presently unknown, but several hypotheses may be formulated, which are not mutually exclusive. (1) Neurons may provide a particularly suitable environment for protein misfolding processes, or be more prone to dysfunctions of the machinery deputed to misfolded protein removal. (2) The abundant presence of calcium-permeable ion channels, activated by amyloid proteins, may render Ca^{2+} dysregulation a much more probable event in neurons than in other cell types. (3) Neuronal cells may be more sensitive to the toxic potential of amyloid proteins, a likely event due to the dramatic effects induced by Ca^{2+} dysregulation, as discussed above. (4) Finally, it may be speculated that neuronal cell membrane, due to its intrinsic characteristics, may be more prone to pore formation by oligomers. To address the latter hypothesis, we compared sCTO toxicity in mature, 14-day *in vitro* (DIV) or immature, 6 DIV, hippocampal neurons to that of cultured cells of different histogenesis: MG63 osteoblasts, NIH-3T3 fibroblasts (two immortalized cell lines), and primary astrocytic cultures from rat fetal brain [170].
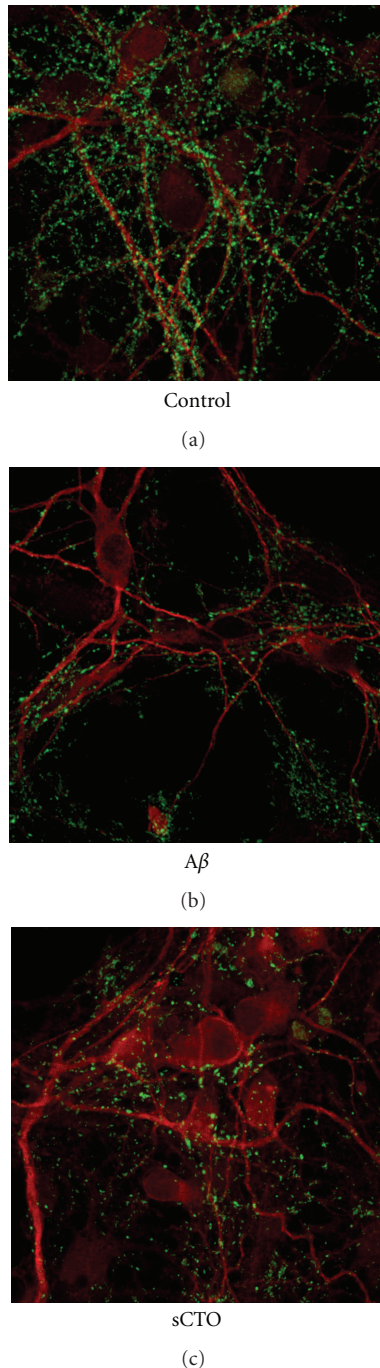
Control

(a)

Aβ

(b)

sCTO

(c)

Figure 5: sCTOs and Aβ oligomers similarly damage the neuritic tree and synapses in mature hippocampal neurons. After sCTO or Aβ treatments, the extension of the dendritic tree, immunolabeled for microtubule-associated protein 2 (red fluorescence), is evidently reduced, while the number of synapses, immunolabeled for synaptophysin (green fluorescence), is decreased.

Among the tested cell types, only mature hippocampal neurons responded to sCTOs with an intense and sustained rise in intracellular Ca^{2+} (Figure 6(a)) and an evident increase in apoptosis. This increase could be due to leakage of intracellular Ca^{2+} stores or sCTO-dependent stimulation

of preexisting Ca^{2+} channels, as previously proposed and discussed above. The use of thapsigargin, a specific sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump inhibitor, which depletes intracellular Ca^{2+} stores, showed that sCTO-induced Ca^{2+} rise was mostly due to an extracellular influx (Figure 6(b)). We then considered the activity of NMDA receptor, which, among glutamate receptors, is the one that has most frequently been considered to be involved in the toxicity of amyloid proteins, as already discussed. MK801, a specific NMDA receptor blocker, poorly affected sCTO-induced Ca^{2+} entry. Furthermore, pretreatment with an antibody against the subunit 1 of NMDA receptor (NR1), used to mask possible sites of interactions between sCTOs and the NMDA receptor, again failed to inhibit Ca^{2+} entry (Figure 6(b)). Thus, the different behavior of cell types to sCTO, in terms of Ca^{2+} rise, was conceivably unrelated to an activation of preexisting Ca^{2+} channels and pointed to the formation of calcium-permeable amyloid pores by CT oligomers. We reasoned that the neuronal plasma membrane has other distinctive characteristics, compared to other cell types, such as, for example, a rich content in LRs. This hypothesis was confirmed by our results, where mature neuronal cells showed a much more elevated content in LRs of the other cells types examined (Figure 6(c)). It has also been demonstrated that LRs increase in the plasma membrane during *in vitro* maturation in hippocampal neurons [172]. This could explain why immature neurons were insensitive to sCTO toxicity. Thus, content in LRs higher than the other cell types could render neurons more vulnerable to amyloid toxicity. To further corroborate this hypothesis, we manipulated LRs in mature neuronal cells in the attempt of modifying sCTO-induced intracellular Ca^{2+} entry. Pretreatment of neurons with an antibody against GM1, a ganglioside particularly abundant in LRs, completely suppressed sCTO-driven Ca^{2+} rise, without altering NMDA receptor activity (Figure 6(b)). Furthermore, LR disruption obtained by neuraminidase (NAA), which removes sialic acid from gangliosides, inhibited Ca^{2+} rise and protected against sCTO neurotoxicity, probably modifying the plasma membrane area susceptible to the insertion of the pore-like structures (Figure 6(d)). These results strongly support the conclusion that the intense and protracted Ca^{2+} dysregulation observed after sCTOs treatment is reliably due to the pore formation in a particularly suitable environment, that is, the LR-rich neuronal plasma membrane.

11. Conclusions

LRs are crucial sites in the cell membrane, where pivotal events in the physiology of the cell take place. However, they may also represent areas of fragility of the cell membrane, providing a way into potential cell hosts, such as pathogens and misfolded proteins. The high content in LRs of mature neuronal plasma membrane may render these cells particularly vulnerable to the cytotoxic attack of amyloid proteins and represent one of the reasons for the high vulnerability of CNS to misfolded protein diseases.
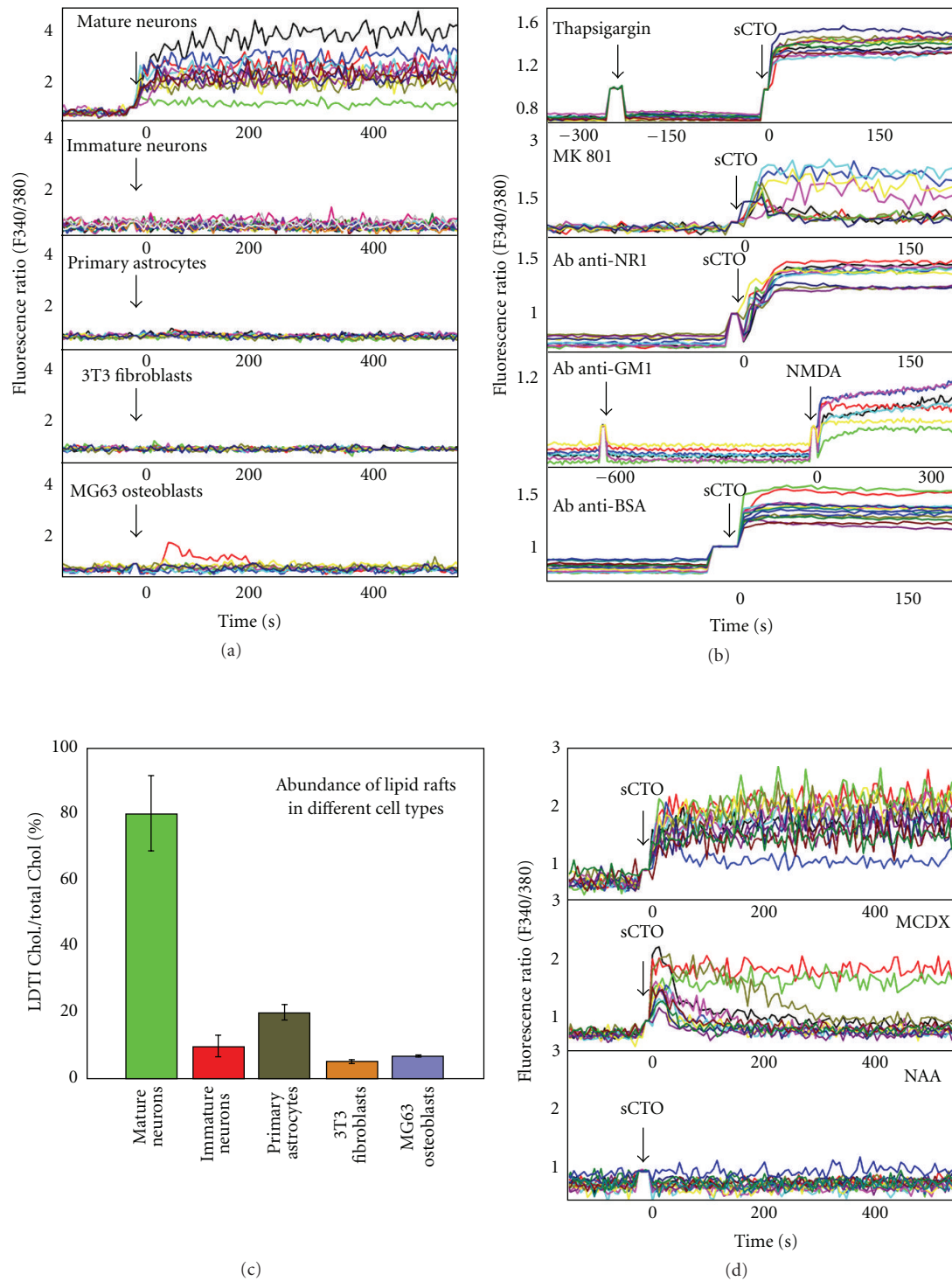
FIGURE 6: (a) sCTO induces increase in intracellular Ca^{2+} levels in mature hippocampal neurons, but not in immature neurons, primary astrocytes, 3T3 fibroblasts, and MG3 osteoblasts. Ca^{2+} levels were evaluated by optical fluorimetric recordings with Fura-2AM. (b) Depletion of intracellular Ca^{2+} stores with thapsigargin did not affect sCTO-induced Ca^{2+} rise, suggesting that it was mostly due to an extracellular Ca^{2+} influx. MK801, a specific NMDA inhibitor, as well as antibodies against NR1, failed to affect sCTO-driven Ca^{2+} influx, suggesting that the NMDA receptor was not involved. On the contrary, pretreatment with an antibody against the ganglioside GM1, aimed at blocking LRs, completely abolished sCTOs-induced Ca^{2+} increase. Pretreatment with anti-BSA IgGs, an unrelated antibody, did not affect sCTO response. (c) Measure of the weight ratio between cholesterol in LRs (LDTI) and total cholesterol indicates that plasma membrane of mature hippocampal neurons have a much higher content in LRs than the other cell types. (d) Pretreatment of hippocampal neurons with NAA totally suppressed sCTO-induced Ca^{2+} increase. Reproduced with permission and partially modified from [170].

Abbreviations

AD: Alzheimer's disease
$A\beta$: Amyloid β
AFM: Atomic force microscopy
CT: Calcitonin
CDS: Circular dichroism spectroscopy
DIV: Day *in vitro*
ER: Endoplasmic reticulum
EFTEM: Energy filtered TEM
GSK: Glycogen synthase kinase 3-beta
GPI: Glycosylphosphatidylinositol
htt: Huntingtin
HD: Huntington's disease
LDTI: Low-density, triton-insoluble
LBs: Lewy bodies
LRs: Lipid rafts
LTD: Long-term depression
LTP: Long-term potentiation
NAA: Neuraminidase
NR1: NMDA receptor subunit 1
NMDA: N-methyl-D-aspartate
PD: Parkinson's disease
PrP: Prion protein
RyR: Ryanodine
sCTOs: sCT oligomers
sCT: Salmon CT
TSEs: Transmissible spongiform encephalopathies
TEM: Transmission electron microscopy
α-syn: α-synuclein.

Acknowledgment

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