The effect of the amyloid-beta peptide on the fluidity of cell membranes : a study by fluorescence correlation spectroscopy

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**THE EFFECT OF THE AMYLOID-BETA
PEPTIDE ON THE FLUIDITY OF CELL
MEMBRANES: A STUDY BY FLUORESCENCE
CORRELATION SPECTROSCOPY**

NG WEI QING JUSTIN
SCHOOL OF BIOLOGICAL SCIENCES

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Common Abbreviations

A β	Amyloid-beta
AD	Alzheimer's disease
BSA	Bovine serum albumin
Chol	Cholesterol
CtxB	Cholera toxin subunit B
DiI/DiI-C18	1,1'-dioctadecyl-3,3,3,3'-
tetramethylindocarbocyanine	
DOPC	Dioleoyl-phosphatidylcholine
DPPC	Dipalmitoyl-phosphatidylcholine
DPBS	Dulbecco's phosphate buffered saline
FCS	Fluorescence correlation spectroscopy
GFP-GPI	Glycophosphatidyl inositol linked-GFP
GM1	Monosialoganglioside GM1
HBSS	Hanks' balanced salt solution
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
ITIR-FCS	Imaging Total Internal Reflectance-FCS
MCI	Mild cognitive impairment
PI	Propidium iodide
Rho-PE/TR-PE	Rhodamine/Texas Red-
phosphatidylethanolamine	
SM	Sphingomyelin
SPB	Supported planar (lipid) bilayer

Abstract

Alzheimer's disease (AD) is an age-related neurodegenerative disease which affects up to 80% of patients presenting dementia. One of the key factors thought lead to AD is the aggregation of the amyloid-beta ($A\beta$) in susceptible brain regions, namely the cerebral cortex and hippocampus. $A\beta$ is thought to interact with the cell membrane, perturbing its structure and organization, thus changing the fluidity and normal function of the cell membrane, leading to detrimental effects on the cells. Although there have been several studies done regarding this topic, the results have been conflicting and thus inconclusive, likely due to the drawbacks and biases introduced by techniques used in those studies. We therefore sought to find a conclusive answer to the question of whether $A\beta$ affects cell plasma membrane fluidity. To this end, we used imaging TIRF-fluorescence correlation spectroscopy (ITIR-FCS) to make measurements of fluidity of one bulk membrane probe, DiI, and two ordered/raft-type membrane probes, a transiently expressed GPI-linked GFP and cholera toxin subunit B, on the membranes of living cells that were exposed to $A\beta$. Three cell lines – SH-SY5Y human neuroblastoma, U87 human glioma and Neuro-2a mouse neuroblastoma – were selected for our study based on their origin in brain tissue and previous use in $A\beta$ -related studies. We found that $A\beta$ did not change the fluidity of bulk and raft-type membranes of these cells, even though the concentration of $A\beta$ administered was sufficient to trigger acute programmed cell death (apoptosis). The reason for this negative response is yet unclear, and we do not rule out the possibility that $A\beta$ exposure may induce changes in membrane fluidity under other conditions, such as *in vivo* and in AD patients, through complex mechanisms such as altered lipid metabolism. We also note the following limitations of our study – 1) because there was no solution-based assaying of the $A\beta$ content, the exact composition of the $A\beta$ solution is unknown; 2) the number and type of membrane probes used is not totally comprehensive; 3) there may be changes to the membrane that are too subtle or complex to be measured by standard ITIR-FCS and 4) our method of $A\beta$ treatment represents an acute, high-dose exposure of cells to $A\beta$ and may not be representative of what happens in the natural AD disease progression. While our observations do not agree with several earlier studies, to our knowledge, ours is the first study examining the effect of $A\beta$ on membrane fluidity of living cells. We thus believe and conclude that within the parameters examined in this study, we have shown that exposing cells to $A\beta$ does not result in changes in

membrane fluidity in the short term, even at A β concentrations that are sufficient to kill the cells. This information may be of help to researchers planning studies investigating the effects of A β on the cell membrane.

The Effect of the Amyloid-Beta Peptide on Cell Plasma Membrane Fluidity

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that mainly affects individuals above the age of 60. Although it has been observed and studied for several decades, its exact disease pathology is still not totally clear. One of the main factors thought to result in the onset of AD is the amyloid-beta ($A\beta$) peptide, as it is commonly found in the brains of AD patients, as well as in the cerebrospinal fluid of individuals presenting mild cognitive impairment (MCI). One of the ways $A\beta$ is thought to impact neuronal cells is in cell membrane fluidity, where $A\beta$ perturbs membrane fluidity, resulting in abnormal cell function and disease states. There has been almost two decades of research in this aspect, yet it is still inconclusive whether $A\beta$ increases or decreases neuronal cell membrane fluidity and several studies have been published with opposing conclusions. To our knowledge, none of the studies done thus far in this area has observed the fluidity of the plasma membrane of living cells. Most of them use artificial lipid membranes, which may not be ideal models of the living cell membranes, or cell membrane extracts, which may have altered properties compared to native cell membranes. In our study, we attempt to conclusively answer the question of whether $A\beta$ affects cell membrane fluidity by making measurements on the cell membrane of living cells, using methods that are minimally invasive to the cell. In the first part of this thesis, I discuss the research history of $A\beta$ and how it is thought to be involved in AD pathology. Next, I review various methodologies and findings from previous studies pertaining to $A\beta$ effects on the cell membrane as well as cell membrane fluidity. Following that, I present the case for using fluorescence correlation spectroscopy as an ideally suited method to study the membrane fluidity in living cells. I then detail the methods used in our study and the experimental results obtained. Finally, I discuss the significance of the results and its impact on our understanding of how $A\beta$ affects cell membrane fluidity as well as other broader implications.

1.1. A brief history of the Amyloid-Beta peptide

The $A\beta$ peptide is a short, self-aggregating peptide thought to be a key causative agent in the pathology of Alzheimer's disease. Originally named A4, it is usually 39-

43 amino acids in length and was first purified and characterized in a groundbreaking study by Colin Masters and Konrad Beyreuther in 1985. They isolated and purified the protein from amyloid plaque cores from AD patients as well as from patients with Down's syndrome (DS), as the latter exhibited similar symptoms to that of the former. In their experiments, Masters and Beyreuther found that the amyloid plaque cores (APCs) were resistant to denaturation by guanidine hydrochloride and urea, and the protein isolated from these plaque cores was about 40 amino acids in length, 4.5 kDa in molecular weight, and able to form various types of aggregates ranging from dimers to tetramers as pH changes (Masters et al., 1985a). In that study, they also partially sequenced the peptide and found that the peptides from both AD and DS patients were essentially the same, which was an important clue in the next step of finding out the origin of the A4 peptide. At this time, it was known that AD patients presented three major pathologic symptoms in their brains: intracellular neurofibrillary tangles (NFTs), extracellular amyloid plaques and vascular damage in the brain that was termed "amyloid congophilic angioplasty (ACA)", according to the observation that these amyloid deposits could be stained with the Congo red dye. In the same year, Masters and Beyreuther published a study proving the neuronal origin of A4 by showing that it was a component of NFTs found within the neurons in the brains of AD patients (Masters et al., 1985b). The next important discovery made by the pair was the localization of the coding sequence for the A4 peptide to chromosome 21, and the finding that this sequence was part of a gene that coded for a larger transmembrane protein which they thought may be a cell-surface receptor (Kang et al., 1987). In that study, they also identified the cleavage sites of the precursor protein that would generate the A4 fragment. Together with the earlier studies, this much was known about the A4 peptide by this time: it was an approximately 42-43 amino-acid, 4.5 kDa peptide that was generated by proteolytic cleavage of a larger transmembrane precursor protein; the first 28 amino acids of the A4 fragment were part of the extracellular domain of the precursor protein and the subsequent 14-15 amino acids were in the transmembrane region of the precursor protein (see Fig. 1); this A4 fragment was able to spontaneously self-aggregate to form dimers, trimers, tetramers and other larger aggregates; and the A4 fragment was found in NFTs, APCs and ACA, all of which were pathological symptoms found in the brains of Alzheimer's disease patients. This concluded the first wave of research conducted on this small but intriguing peptide that was implicated in a major human disease.

Alzheimer's disease, first described by Alois Alzheimer in 1907, in an article titled "On an Unusual Illness of the Cerebral Cortex", is a disease that mainly affects the aged (original article in German, translation by Stelzmann et al. (Alzheimer et al., 1995)). In the paper, Alzheimer reported a case study of a 51-year old female patient in an insane asylum who exhibited various neurotic symptoms, such as mood swings, paranoia, confusion, impaired memory and cognitive decline, but not a decline in motor function. The patient eventually died after 4.5 years of illness. In the postmortem, it was found that severe atrophy had occurred in the brain, especially in the cortical regions. Abnormal fibrillar structures were observed within neurons and tiny foci was also observable in the cortex, which was attributed to the "deposition of a special substance", most likely the neurofibrillary tangles and amyloid plaques that have come to be the hallmarks of Alzheimer's disease. According to reports in 2009/10 by Alzheimer's disease International, It is estimated that up to 80% of all dementia cases are comprised of Alzheimer's disease, making it the single largest cause of dementia. Worldwide, dementia currently afflicts approximately 36 million people, or about 5% - 7% of all people aged 60 and above. As life expectancies grow, the number of people suffering from dementias worldwide is estimated to hit 65.8 million by the year 2030 and more than 100 million by 2050 (Abbott, 2011). The economic burden alone caused by dementia is enormous – an estimated US\$604 billion in 2010 alone, attributed to direct costs such as institutional care and indirect costs such as unpaid care rendered by family members (Abbott, 2011) – not to mention the psychological burden experienced by caregivers and family members of patients suffering from dementia. Because of its prevalence, the finding that amyloid plaques were almost always present in people older than 80 years of age and even in some cognitively normal people who had died in their fifties, senile dementia, as it was called before it was identified as the same disease that Alois Alzheimer had described, was once thought to be an inevitable outcome of aging (Abbott, 2011; Schnabel, 2011). Nevertheless, people have set out to understand the disease progression and pathology of Alzheimer's disease, in the hope that there may one day be therapies, both preventive and curative for those afflicted by it.

1.2. A β and Alzheimer's disease

In the attempt to understand the link between amyloid-beta and Alzheimer's disease, many mechanisms have been proposed. The earliest of these is also the most straightforward – that excessive amounts of A β in the brain leads to the formation of neurotoxic fibrils and plaques, thereby causing cell death and the ensuing symptoms of AD. This mechanism was called the “amyloid hypothesis” and was supported by the early-known fact that A β could self-aggregate and that A β was found in both neurofibrillary tangles and amyloid plaques in brain autopsies of AD patients (Schnabel, 2011). This theory became more complicated, however, when it was found that in individuals patients presenting a heritable form of AD, or familial AD, it was not the total production of A β but rather the relative over-production of the 42-amino acid variant of A β that caused early-onset AD (Schnabel, 2011). One early study conducted by Peter Lansbury and colleagues showed that different length-variants of A β and A β fragments had different solubility and aggregation potential

Figure 1. Origin of the Amyloid-beta Peptide

Amyloid-beta (A β) is generated by proteolytic cleavage of the transmembrane amyloid precursor protein (APP). There are various ways in which APP can be processed (Peters et al., 2009). α -secretase cleaves APP extracellularly to generate a soluble fragment, sAPP α , and precludes the formation of A β as the α -cleavage site is within the A β sequence. Sequential cleavage by β - and γ -secretase yields the A β peptide fragment. α -, β - and γ -secretase are all transmembrane or membrane-associated proteins and γ -secretase exists as a multi-protein complex. Roles have been proposed for each of the cleavage products, including the c-terminal APP-intracellular domain fragment (AICD), that range from the neurotrophic to the neurotoxic. It is still uncertain how proteolytic processing of APP is modulated and what exactly are the physiological roles of each of the cleavage products, including A β .

(Jarrett et al., 1993). Specifically, the 42- and 43-amino acid variants were more prone to aggregation and less soluble than the 39- and 40- amino-acid variants. These variants differed in the length of their c-terminal regions. Other studies revealed that amyloid plaques and fibrils were composed predominantly of the A β (1-42) variant rather than the shorter A β (1-40), in cases of familial and sporadic AD, as well as in Down's syndrome patients (Mann et al., 1996; Schnabel, 2011). At this relatively early point in A β research, it was already evident that not all A β peptides are equal, although how this was related to pathological effects of A β was still unclear. Concurrently, researchers studying the production and presence of A β in humans found that A β could be produced by a variety of cell types (Busciglio et al., 1993; Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992), and that A β was detectable in the bodily fluids of not just AD patients but also in healthy individuals and at comparable levels (Seubert et al., 1992; Shoji et al., 1992). These findings complicated matters, as it seemed to suggest that the mere presence of amyloid-beta was not sufficient to cause disease, even though amyloid-beta had always been present in the NFTs and amyloid plaques in AD brains. One

Figure 2. Synthetic A β variants common used in scientific research

The A β peptide contains both extracellular and transmembrane regions of its precursor protein, APP. The C-terminal region is thought to be important for peptide aggregation (Jarrett et al., 1993). Although several of the commonly used A β variants used in research differ by only a few amino acids, this difference seems enough to significantly alter the peptide activity. “Full-length” can refer to any peptide between A β (1-38) to A β (1-43), although A β (1-40) and A β (1-42) are the most studied variants. Particularly, A β (1-42) is known to be more amyloidogenic and found more commonly in amyloid plaques. In a study by Bruce Yankner and his colleagues, the minimal sequence of the peptide with comparable activity to full-length peptide in E18 rat hippocampal neurons is A β (25-35) (Yankner et al., 1990). In their study, the fragments A β (1-28), (1-16), (17-28), (34-42) had no activity compared to the full length peptide. Therefore, it seems that a short stretch of the extracellular region, together with some hydrophobic region of the peptide is required for peptide activity in cells.

notable fact was that the antibody used by Shoji et al. in their study was specific to A β (1-40). Seubert et al. on the other hand used an N-terminal-specific antibody in their study, therefore it was not clear whether the A β variant detected in their study was the shorter and supposedly less toxic A β (1-40) or the more amyloidogenic A β (1-42).

At the same time, as new knowledge was being discovered about the different variants of A β and how that correlated with Alzheimer's disease progression and pathology, other studies were being conducted to understand the molecular basis of A β toxicity to neurons. Early work done by Bruce Yankner and his colleagues using E18 rat embryonic hippocampal neurons suggested that A β (1-40) functioned as a kind of neuropeptide similar to the tachykinins (Yankner et al., 1990). In their study, they found that 20 μ M of A β (1-40) could induce neurotoxicity in mature rat hippocampal neurons, and suggested that this could be due to inhibition of cellular adhesion. In the same study, they also identified amino acids 25-35 of the peptide as the active region, having comparable activity to the full length A β (1-40) peptide. Another study by Charles Glabe and associates, also done using rat hippocampal neurons, this time using the longer A β (1-42) variant, suggested that peptide aggregation was crucial for cytotoxic effects (Pike et al., 1991). Mark Mattson and his associates, however, using A β (25-35) and A β (1-38), via experimenting with glutamate-induced neurotoxicity in human cortical neurons, concluded that the neurotoxicity of A β in human neurons had more to do with the dysregulation of calcium homeostasis (Mattson et al., 1992). This was significant, because it suggested a possible link between the A β peptide and the pathological symptom of progressive memory impairment in AD patients. Subsequently, studies by Nelson Arispe and his associates with A β (1-40) and artificial lipid membranes suggested that A β was able to form cation pores in those membranes, making those membranes permeable to Ca²⁺ (Arispe et al., 1993a; Arispe et al., 1993b). Thus, by the mid-1990s, a picture was emerging regarding the method by which A β caused neurotoxicity, and this was not necessarily in tandem with plaque or fibril formation.

1.3. Effects of aggregation on A β activity in cells and organisms

In most of the studies done on A β up to the mid-1990s, researchers had not put special emphasis on the variant and aggregation state of the A β used, although earlier research had shown that peptide aggregation depended on pH (Masters et al., 1985a), and that different length variants of A β had different aggregation potentials (Jarrett et al., 1993). Research mainly focused on A β (1-40), which was the most abundant form of A β protein in the brain (Näslund et al., 1994), and A β (1-42), which was known to be more aggregation-prone and more common in the neurofibrillary tangles and amyloid plaques seen in AD patients (Mann et al., 1996; Oda et al., 1995),

but other fragments of varying lengths were also used (Avdulov et al., 1997; Mattson et al., 1992; Müller et al., 1995; Yankner et al., 1990). The peptides used were solubilized in a variety of solvents, including pure water, various buffers and pure DMSO, or a combination of methods. Peptides were also not deliberately aged, typically being used immediately after solubilizing and dilution to a suitable concentration, and in some cases, stored as aliquots after solubilization. Already in 1991, Charles Glabe and his colleagues showed that pre-aggregated A β (1-42) could cause neurotoxicity to rat hippocampal neurons but the unaggregated peptide could not (Pike et al., 1991). In their experiment, A β (1-42) was dissolved in deionized water and aged by incubation for 2 – 4 days at 37°C. No further treatment was rendered to the peptide. The SDS-PAGE analysis showed the presence of monomers and what appears to be dimers and tetramers in the aged sample, but only monomer in the fresh sample. The aggregates were also resistant to heat treatment and 2-mercaptoethanol treatment. It is unclear whether larger fibrillar aggregates were present in the solution eventually added to the cells, as they do not show up on the PAGE gel, although the researchers reported observing precipitates in the cell culture of cells treated with aged peptide. Larger aggregates may have been excluded from the PAGE gel, if they were present. By the mid- to late 1990s, findings had emerged that specifically linked the aggregation state of A β to its binding and neurotoxicity on cells in *in-vitro* settings (Good and Murphy, 1995; Lambert et al., 1998; Oda et al., 1995). Crucially, William Klein and his team showed that aggregated yet non-fibrillar forms of amyloid-beta were themselves neurotoxic to mouse brain slice cultures (Lambert et al., 1998), bringing a potential answer to the emerging conundrum by the 1990s that amyloid plaques did not correlate particularly well with disease progression in Alzheimer's disease (Dickson and Yen, 1989; Schnabel, 2011; Terry et al., 1991). Klein's team called these A β oligomers "A β -derived diffusible ligands" (ADDLs), based on the observation that these oligomers could diffuse through layers of filters to reach the brain slice cultures. Two other pieces of information reported by Klein and his colleagues were firstly, that brain slices from mice with a double-knockout of the Fyn kinase protein were resistant to ADDL neurotoxicity, and secondly, that ADDLs inhibited long-term potentiation. The former finding was particularly prescient – it was known by then that Fyn kinase could be upregulated by A β (Zhang et al., 1996), and that the microtubule-associated protein tau was hyperphosphorylated in AD; it was later found that Fyn could also phosphorylate tau (Lee et al., 2004). An A β -Fyn-tau relationship has now become

one of the major proposed mechanisms of A β -mediated neurotoxicity (Ittner and Götze, 2011). The latter finding of Klein and his colleagues was important because it corroborated with the fact that AD patients experienced impaired memory formation; since a non-fibrillar form of A β could inhibit LTP, it might help to explain why AD progression does not correlate well with the presence of amyloid plaques.

Following the earlier studies in the 1990s that A β oligomers themselves had cellular activity and were not simply an intermediate of amyloid fibril formation, several further studies were done which shed greater light on the biochemical effects these A β species could have on cells. By the early 2000s, it was quite certain that A β oligomers were not only neurotoxic but they even seemed to be more toxic and had greater activity than both unaggregated and fibrillar forms of A β . One such study by William Stine and Mary Jo LaDu convincingly showed that this was the case, and they further showed that the neurotoxic potential of A β (1-40) was lower than A β (1-42), whether it was unaggregated, oligomeric or fibrillar (Dahlgren et al., 2002). By this time, enough interest had also developed in the study of the ADDL-type of A β oligomers that a sort of standard protocol for the generation of these oligomers had been developed in the field (Klein, 2002). In the years that followed, further study on aggregated but non-fibrillar forms of A β has seen in the types of oligomers mushrooming into a rather bewildering myriad of types, ranging from the small, such as dimers, trimers and tetramers, to the large, such as amylospheroids and membrane pore complexes (Benilova et al., 2012). Despite this, there seems to be a coalescing in the neurotoxic effects of these A β species. Generally, non-fibrillar A β aggregates cause neurotoxicity via impairment of long-term potentiation, calcium dysregulation, effects on the cell membrane such as membrane permeabilization and perturbation of membrane fluidity, aberrant intracellular cell signaling, mitochondrial damage and oxidative stress (Benilova et al., 2012; Demuro et al., 2005; Deshpande et al., 2006; Glabe and Kaye, 2006; Klein, 2002; Lee et al., 2015; Sakono and Zako, 2010). Importantly, the presence of these oligomers also correlates with the disease progression of Alzheimer's disease (Lesne, 2014).

Given the large range of morphologically different A β species shown to have cytotoxic effects on neurons and other cell types, it is difficult to pinpoint a specific link between any particular mechanism and a corresponding A β species. Furthermore, it is hard to separate specific types of A β aggregates from each other,

as several different aggregates may concurrently form in a single preparation of A β . Indeed, Benilova, Karran and Strooper suggest in a recent review of the topic that quite possibly a mixture of different types of oligomers exert non-specific cytotoxic effects on cells (Benilova et al., 2012). A few discoveries beginning in the late 1990s started to support the idea that A β might elicit cytotoxic effects on the neuron in a non-specific way that does not involve receptor-ligand interactions. One such finding was that of Carl Cotman's group that both D- and L-enantiomers of A β had the ability to form fibrils with similar structures and cytotoxic effects (Cribbs et al., 1997). Cribbs and his colleagues used circular dichroism (CD) spectroscopy to show that D- and L-enantiomers of A β (1-42) and A β (25-35) had similar structures, and when applied to D18 rat embryonic hippocampal neurons, they had identical dose-dependent responses between 1 μ M and 25 μ M. Electron microscopy allowed them to inspect the fibrils visually, and corroborated with the CD data that the structures were similar. Another finding was that of Steven Estus and Sarah Wright that amylin fibrils could trigger apoptosis in cultured primary rat cortical neurons via gene activation that was similar to that observed in A β -induced neurotoxicity (Tucker et al., 1998). Amylin is a 37 amino-acid peptide that can be found in amyloid deposits in the pancreas of non-insulin dependent diabetics. Estus and Wright had previously studied the gene expression pattern in rat cortical neurons during A β -induced apoptosis (Estus et al., 1997). Because amylin has a low sequence similarity to A β but could also form similar beta-pleated sheet structures as A β did, and was found to be toxic to rat hippocampal neurons, they sought to study whether amylin-induced apoptosis in neurons followed a similar pathway as for A β . Their results showed that similar to A β -induced apoptosis, treatment of E18 rat cortical neurons with amylin triggered an increase in *c-Jun*, *c-fos* and *transin* within 12 hours, an observation that was virtually identical to their earlier study with A β (1-40) aggregates. Additionally, they found in the same study that the *cox-2* and *I κ B* genes were upregulated, implying a role of oxidative stress in amylin-induced apoptosis. A third finding was that by Campioni et al. using the bacterial self-aggregating peptide, HypF-N, that differently formed aggregates that were similar in morphology could have different effects in terms of cytotoxicity (Campioni et al., 2010). In their study, Campioni and his colleagues showed that the reason for the difference in cytotoxicity of the differently formed aggregates lay in the surface hydrophobicity of the aggregates – cytotoxic HypF-N aggregates had a larger hydrophobic surface than the morphologically similar but non-cytotoxic aggregates. Working with human

neuroblastoma (SH-SY5Y) cells, they also showed that only the cytotoxic aggregates were internalized by the cell even though both could bind to the cell membrane, and that cells treated with cytotoxic aggregates had increased intracellular Ca^{2+} levels. These and other related studies strongly suggest that $\text{A}\beta$ may cause neurotoxicity not via specific ligand-receptor interactions but perhaps by perturbation firstly of the cell membrane and subsequently internal cell functions such as intracellular signaling or gene regulation, due to the structural properties of aggregated $\text{A}\beta$ (Benilova et al., 2012; Glabe and Kayed, 2006).

1.4. $\text{A}\beta$ and lipid membranes

One key area of inspection whereby researchers try to understand the mechanisms of $\text{A}\beta$ toxicity has been at the cell membrane. Following the earlier studies which characterized the $\text{A}\beta$ variants (Jarrett et al., 1993) and identification of $\text{A}\beta(25-35)$ as the minimal sequence for peptide activity (Mattson et al., 1992), research quickly followed that linked $\text{A}\beta$ with effects on the cell membrane. Many types of model membranes were used to study $\text{A}\beta$ effects on membranes, ranging from synthetic lipid bilayers and liposomes, to biological membrane extracts. Results generally fell within two main trends – firstly, that $\text{A}\beta$ permeabilized membranes, leading to destabilization of intracellular Ca^{2+} homeostasis, or secondly, that $\text{A}\beta$ caused changes in membrane fluidity and concomitantly, membrane function (Arispe et al., 1993a; Arispe et al., 1993b; Bacia et al., 2004; Müller et al., 1995). Also emergent beginning from the early 2000s, along with the understanding that different types of $\text{A}\beta$ aggregates could have similar effects on cells, was the aspect of studying specifically aggregated $\text{A}\beta$ on the cell membrane (Cecchi et al., 2008; Glabe and Kayed, 2006; Kremer et al., 2000). The plasma membrane is the first point of contact of the cell and its extracellular environment. It does not simply function as a barrier between the inside and outside of the cell, but also serves as a site for signal and mechano-transduction. For a neuron, some specific functions that the plasma membrane facilitates are the transmission of synaptic potentials along the axons and dendrites, regulation and maintenance of neurotransmitter receptors structurally and biochemically through lipid-protein interactions, which in turn affect neurotransmitter activity, as well as the control of ionic channels and other membrane-bound enzymes and proteins. Unsurprisingly, the neuronal cell membrane had been a topic of keen study in the field of $\text{A}\beta$ research.

1.5. A β effects on and interaction with artificial lipid membranes

One method whereby researchers have attempted to address the question of how A β affects structure and fluidity of membranes has been the use of synthetic lipid membranes. Such membranes include membrane monolayers, liposomes and supported lipid bilayers. One early study by Nelson Arispe and his associates involved the use of A β (1-40) and a POPE lipid membrane on the tip of a patch pipette in a CaCl₂ solution. The researchers found that when A β was added into the solution together with the lipid membranes, the conductance of the membrane was changed. This was attributed to cation channels being formed in the membrane, allowing for Ca²⁺ to pass through, supporting what was then known as the “ion channel hypothesis for amyloid neurotoxicity”. Arispe continued his studies with the A β peptide and later on reported also that cholesterol levels in lipid membranes could modulate the interaction and incorporation of A β into lipid membranes, to the extent of reducing cytotoxicity in PC12 pheochromocytoma cells (Arispe and Doh, 2002). Some other research suggested that A β (1-40) could interact with positively charged headgroups of certain lipids, such as POPG and DPPG, via electrostatic interactions (Lin et al., 2009; Terzi et al., 1997). Lin and his colleagues also showed that this A β interaction caused cholesterol in the membranes to become clustered and fluidity of the membranes to be reduced (Lin et al., 2009). Research by Ari Gafni and Duncan Steel suggests that the membrane insertion and permeabilization of lipid membranes by the A β peptide are distinct processes, the former being related to electrostatic interactions and the latter being affected by membrane fluidity (Wong et al., 2009), which is modulated by the membrane lipid composition.

In addition to studies on how the A β peptide perturbs lipid membranes, there has also been interest in how the lipid membrane affects the processing and maturing of the A β peptide. Tashima and his colleagues demonstrated using liposomes with approximately the same lipid composition of the human cerebral cortex, that the presence of cholesterol and GM1 were important for the aggregation and release of A β from the membrane. Tashima et al. suggested that in a healthily functioning brain, A β would be released in a form necessary for its intended physiological function; however, as the lipid composition of the brain membrane changes with age, it could promote aggregation and pathogenicity of A β (Tashima et al., 2004). Indeed, it seems that certain specific lipids such as GM1 and cholesterol play a key role in

modulating the effects of A β on lipid membranes. In support of this theory, Kenji Sasahara and his colleagues recently showed using a combination of supported planar bilayers and fluorescence recovery after photobleaching (FRAP), that addition of initially monomeric A β (1-42) caused a reduction in the fluidity of the membrane, which was reversed when the A β was removed from the membrane. This decrease of membrane fluidity was not observed when the membrane did not contain GM1 (Sasahara et al., 2013). The SPBs used in their experiment contained DOPC/DOPS/SM/Chol (0.4/0.1/0.25/0.25 proportion), and GM1 was added in amounts of 1.3%, 2.5% and 3.8%. This membrane composition mimics the composition of lipid rafts, which are enriched in SM and cholesterol. In the study, they also showed that A β aggregates formed and remained bound to these lipid membranes and that the ganglioside GM1 was included in the aggregates, as detected by the GM1-binding cholera toxin subunit B protein (CtxB). Additionally, the fluorescent lipid probe used in determining the membrane fluidity, rhodamine-PE, was reduced in the regions where the A β aggregates were located. A model was proposed whereby membrane binding of initially monomeric A β leads to a nucleation event that induces the phase separation of raft lipids, in this case GM1, eventually resulting in formation of lipid-peptide aggregates which are then released from the membrane. In another experiment, Andrey Filippov and Oleg Antzutkin showed that A β (1-40) decreased membrane fluidity of SPBs containing a 1:1 ratio of DOPC/SM and that these changes in membrane fluidity varies according to cholesterol content of the bilayers (Filippov and Antzutkin, 2013). Interestingly, they found that at high (33%) membrane cholesterol content, A β decreased the fluidity of both liquid ordered and liquid disordered phases of the membrane. They suggested that the reason for such an observation is that the concentration of cholesterol was reduced in both phases; in other words, A β caused membrane cholesterol to somehow be sequestered into a solid phase. Cholesterol plays a dual role in modulating membrane fluidity – it intercalates into the hydrophobic region of the membranes, interacting with the long acyl tails of the lipids, reducing order in highly-packed lipid domains such as lipid rafts, thus increasing fluidity in these domains, and increasing order in more liquid domains of the lipid membranes, limiting the extent to which they can freely diffuse laterally. Therefore, a reduction in total membrane cholesterol could result in a reduction of bulk membrane fluidity. To sum up, experiments using artificial lipid membranes have allowed us to glean several useful and important aspects pertaining to the biophysics and biochemistry of A β

with respect to lipid membranes. Chiefly, A β seems to either form cation channels in the membrane and/or perturb membrane fluidity via some interaction with lipid membranes that is yet to be totally understood.

1.6. A β effects on biological membranes

Although artificial lipid membranes are useful tools in understanding A β interaction with membranes, they can only serve as approximates to actual cell plasma membranes. The plasma membranes of living cells are exceedingly complex. Apart from the broad range of lipids present, there are also membrane proteins as well as the underlying cytoskeleton that contributes to membrane dynamics (Vereb et al., 2003). Therefore, in parallel with the studies using artificial lipid membranes, much research has also been done using actual biological membranes. As early as in 1995, about 10 years after the isolation and identification of the A β peptide, Eckert, Müller and their associates reported that A β could decrease the membrane fluidity of cellular membranes. They tested the effects of A β (25-35) and A β (1-40), on the fluidity of membranes from human lymphocytes, and parts of the rat brain including the cerebral cortex and hippocampus, areas which are greatly affected by death loss of neurons in patients with Alzheimer's disease. In the next few years, Eckert and Müller went on to show that A β had a caused decrease in the fluidity of membranes from cells in the human frontal and parietal lobe (Muller et al., 1998) and that this effect extended to that of synaptosomal plasma membranes and mitochondrial from mouse neurons (Eckert et al., 2001). The membranes used in these studies were purified membrane extracts, and the method for determining membrane fluidity was fluorescence anisotropy of the fluorescent hydrocarbon, diphenylhexatriene (DPH), and its polar derivative, TMA-DPH. The principle of fluorescence anisotropy is where light emitted by a fluorophore has unequal intensities according to its axial orientation relative to the light source when exposed to polarized light. Orientation of the fluorescent molecule in this case, DPH, is dependent on its vibrational and rotational movements, which within the hydrophobic core of a lipid bilayer is dependent on the order of the lipid acyl tails, since DPH interacts with the acyl tails of the lipids. Fluorescence anisotropy of TMA-DPH operates on the same principle, except that because of the polar TMA group, the molecule is located at the surface of the lipid bilayer instead of inside the hydrophobic core. Therefore, fluorescence

anisotropy of DPH and TMA-DPH is used to measure viscosity of membranes within the hydrophobic core and at the surface respectively.

The studies conducted by Eckert and Müller showed that the more neurotoxic species of A β , such as A β (1-42), had greater ability to *decrease* membrane fluidity and that aged membranes were more sensitive to these effects, possibly due to increased cholesterol content. However, conflicting findings have also been reported. W. G. Wood and his associates showed that A β caused an *increase* in the fluidity of both lipids in the membrane-at-large (bulk fluidity) and lipids that were associated with transmembrane proteins (annular fluidity), in the synaptosomal plasma membranes (SPM) of rats (Avdulov et al., 1997). Even more puzzling was that Wood and his colleagues used the very same peptides as was used in Müller's 1995 study, yet they reported what was basically an opposite effect. Furthermore, in this study, Wood and his team also measured the fluorescence anisotropy of DPH in the rat SPM treated with either A β (1-40) or A β (25-35) and the reported no significant differences between the treated samples and controls, as opposed to Eckert and Müller's report that those peptides reduced the fluidity of the cell membranes from rat cerebral cortex and hippocampus, also using DPH as a reporter of fluidity. In Avdulov and Wood's study, the emission spectra of pyrene excimers were used as the main determinant of membrane fluidity. Pyrene is a molecule formed by four fused benzene rings and has a planar structure, allowing it to intercalate with the hydrophobic lipid acyl tails. When excited, the emission wavelength of pyrene depends on whether it is in a monomeric or excimer form the latter of which forms when two pyrene molecules are spatially close enough to each other. The viscosity of the surrounding environment, in this case the hydrophobic core of the lipid membrane, affects the probability of excimers forming, thus the fluorescence intensity ratio of excimer/monomer, F_e / F_m , is used as an indicator of fluidity, with a higher F_e / F_m ratio (more excimers) indicating greater fluidity. Measurement of annular fluidity was achieved by using an excitation wavelength of 286 nm, utilizing fluorescent energy transfer from tryptophan residues of membrane proteins to excite pyrene molecules, which limits the excitation of pyrene to only pyrene molecules in close association with membrane proteins. The "usual" pyrene excitation wavelength of 334 nm was used to measure bulk fluidity as most of the pyrene in the membrane would be excited, not just those in close association with membrane proteins. Later on, Wood and his colleagues also reported that A β -induced increase in bulk and

annular fluidity of the rat SPM was dependent on the brain region. (Chochina et al., 2001). Specifically, A β increased fluidity of membranes isolated from the hippocampus and cerebral cortex, whereas membranes isolated from the cerebellum were not affected. The researchers attributed this to the difference in cholesterol content of the membranes in different brain regions.

Moving on from membrane fluidity, Ari Gafni and associates observed in their study that A β (1-40) oligomers bound to and remained immobilized on live cell membranes. (Johnson et al., 2011). They treated human neuroblastoma SH-SY5Y cells with both wild-type and fluorescently labeled A β (1-40), at the low A β concentration of 50 nM as it would be more physiologically relevant. They found that at this A β concentration and with living cells, A β (1-40) did not cause massive calcium leakage in the cells, unlike observations reported in earlier studies, but only sporadic, minimal calcium leakage. They suggested that this could be because in the other studies, either high ($> 10^{-6}$ M) concentrations of A β or membrane destabilizing solvents were used. In another study, Suman Nag and Sudipta Maiti used fluorescence correlation spectroscopy (FCS) and confocal fluorescence imaging to show that A β (1-40) oligomers bind to PC12 pheochromocytoma cells at low (150 nM and 350 nM) concentrations (Nag et al., 2010). In summary, current knowledge suggests that A β binds to biological membranes, and one of the effects is the perturbation of cell membrane fluidity or membrane permeability. Notably, the studies related to membrane fluidity were done using extracted biological membranes, while the studies related to A β membrane binding were done using live cells. To our knowledge, no study has been done thus far to observe whether A β perturbs the membrane fluidity of living cells.

1.7. Membrane lipids modulate both production of A β and its effects on cells

Thus far, the discussion has been focused on how A β affects cell health and in particular, the cell membrane. However, it seems that not only does A β exert certain effects on cells; early on in the study of A β and Alzheimer's disease, researchers realized that there were cell- and organism-related factors that influenced the generation and cytotoxicity of A β , both *in vitro* and *in vivo*. There have been several studies linking membrane lipids, such as cholesterol and other classes of fatty acids to both A β production and A β effects on cells (Ariga et al., 2011; Di Paolo and Kim,

2011; Diaz et al., 2012; Grosgen et al., 2010; Howland et al., 1998; Simons et al., 2001; Simons et al., 1998). A major role of cholesterol in cell function is the ability of cholesterol to modulate membrane fluidity, and thus, membrane structure. Given that firstly, the amyloid precursor protein is a transmembrane protein, secondly, the α -, β -, and γ -secretase enzymes that process APP are all membrane associated enzyme complexes (Yang et al., 2014), and thirdly, that aggregated A β is expected to interact with cell membranes due to its hydrophobicity and perhaps being a ligand for one or more membrane-bound receptors, it is not surprising that lipids, including cholesterol, might have a big role to play in the relationship between A β and cell function. One of the most studied effects between lipids and A β is *how lipids affect the production of A β* . In a highly-cited study, Kai Simons, Konrad Beyreuther, Bart De Strooper and their colleagues showed that by reducing cholesterol content in rat hippocampal neurons, they could reduce the amount of A β produced, although production of the non-amyloidogenic, α -secretase generated proteolytic product of APP, APP_{sec}, was not affected. In their study, they reduced membrane cholesterol of the cells by first treating the cells with lovastatin, which blocks the enzymatic action of HMG-CoA reductase and inhibits cholesterol synthesis, followed by further treatment with methyl- β -cyclodextrin, which binds to and sequesters cholesterol. This resulted in a ~70% reduction in cellular cholesterol compared to untreated control cells, and a concomitant reduction in A β production. They further showed that by replenishing cholesterol to the cells, A β production could be restored, indicating that cellular cholesterol alone was sufficient to modulate A β production. Many studies have showed a positive correlation between cholesterol levels and A β production (Simons et al., 2001). However, there has also been some research that suggests an opposite effect – that higher cholesterol levels may instead reduce A β production. Earlier in the same year that Kai Simons' study was published, Dorothy Flood and a team of researchers from Cephalon Inc. published a study showing that by increasing dietary cholesterol, transgenic mice expressing the mutated APP gene for familial AD produced less secreted APP derivatives, including A β (1-40) and A β (1-42) but did not affect the levels of APP in the cell (Howland et al., 1998). The reduction in the production of secreted APP was correlated with increased serum cholesterol and increased serum and brain ApoE. This suggested that firstly, cholesterol metabolism was implicated and secondly, the target was proteolytic processing of APP. Since cholesterol metabolism changes with age, this presented a

possible reason for the abnormal production of A β and pathologic consequences such as Alzheimer's disease.

In addition to the production of A β , there has also been investigation on how lipids modulate A β *cytotoxicity* to cells and organisms. A team led by Monica Bucciantini and Massimo Stefani studied the effects of the amyloidogenic bacterial peptide, HypF-N on 10 different cell lines and found that mitochondrial damage and cellular toxicity was negatively correlated with membrane cholesterol levels (Cecchi et al., 2005). They attributed the effect to membrane destabilization by the peptide, leading to intracellular Ca²⁺ dysregulation and increased oxidative stress. Thierry Pillot and his colleagues reported that increased membrane cholesterol levels could protect rat cortical neurons against cytotoxicity from A β (1-40) oligomers and also that increased cholesterol content prevented A β -mediated aggregation of unilamellar liposomes (Sponne et al., 2004). Similarly, Nelson Arispe and Michael Doh showed by lowering cholesterol levels, PC12 cells were more susceptible to A β cytotoxicity. Increasing cholesterol content could also decrease A β -mediated aggregation of phosphatidylserine liposomes (Arispe and Doh, 2002). They also linked the effect to the dysregulation of intracellular Ca²⁺, proposing that higher cholesterol content makes the membranes more rigid, thereby preventing association and insertion of A β to the membrane. Both A β (1-40) and A β (1-42) were used in their study, and had similar effects. Increased membrane cholesterol is thought to be a cause the neuronal cell membrane to become less fluid in aged individuals (Yehuda et al., 2002). While Alzheimer's disease is a generally age-related disease, it is not clear if there is any correlation between cholesterol-related changes in the membrane fluidity and A β -related pathological states. It is also currently unclear whether an increase or decrease of cholesterol is protective or detrimental against A β pathology, or whether there is a difference in effect of cholesterol at the cell level versus the organism level. What seems clear is that cholesterol levels modulate the production of A β , as well as how aggregated A β interacts with cells.

Apart from cholesterol, it seems that other lipids also have a role to play in modulating the effects of A β on cells. One of these types of lipids are the gangliosides – lipids with a sphingolipid backbone and containing one or more sialic acid conjugated to their head groups. Gangliosides are especially enriched in the brain, playing important roles in neuronal maturation and function (Yu et al., 2011).

Up to 10-12% of the lipids in the neuronal cell membrane are gangliosides, typically in the membrane outer leaflet (Stillwell, 2013). Gangliosides, especially GM1, have been associated with A β -mediated cytotoxicity and A β aggregation (Ariga et al., 2011; Wakabayashi and Matsuzaki, 2007; Wakabayashi et al., 2005). More recently, a study done by Raquel Marin and his associates using transgenic mouse models of familial Alzheimer's disease suggested that in aged mice, the raft-type domains of cell membranes from the frontal cortices were slightly more ordered i.e. lower in fluidity compared to that of wild-type mice (Diaz et al., 2012). The method used in that study was also measurement of DPH and TMA-DPH anisotropy. In the study, Marin and associates showed that the reduction in fluidity of the raft domains has to do with cholesterol, sphingomyelin, phosphatidylcholine as well as the poly-unsaturated fatty acids (PUFA). Significantly, they found that lipid rafts from aged transgenic mice exhibited lower fluidities than wild-type individuals of the same age, although lipid analyses showed that the raft cholesterol content in the former was actually lower, and a lower cholesterol content would be expected to result in increase in membrane fluidity. In addition to a lower cholesterol content, the rafts from the transgenic animals also had higher levels of saturated fatty acids, and lower levels of unsaturated fatty acids, long-chain PUFA and omega-3 and -6 fatty acids compared to wild-type animals of the same age. The researchers concluded that the although lipids that promote higher order of packing in the membrane causes an increase in membrane viscosity, the levels of PUFA in the membrane is the key factor in controlling the net effect on viscosity. This is in agreement with current understanding in the field (Yehuda et al., 2002). Another class of lipids, the gangliosides, have also been implicated in modulating A β effects on cells. As can be seen, it seems that membrane lipids, especially cholesterol, play important roles in the complex interactions between A β and cells. Not only can lipids affect the production of A β , such as in the case of cholesterol and GM1, they also play a role in how the produced A β eventually affects the cells, such as in the case of poly-unsaturated fatty acids. Because of inherent differences in the membrane lipid content of cells in different tissue types, this may represent a paradigm explaining why A β -associated lesions are found mainly in the brain cortex and hippocampal regions and not the other cells.

1.8. Elucidating a clear picture of A β interactions with biological lipid membranes

The plasma membrane is the first point of contact of the cell and its extracellular environment. It does not simply function as a barrier between the inside and outside of the cell, but also serves as a site for signal and mechano-transduction. This membrane exists in the form of a membrane bilayer formed by a mixture of various lipids and proteins (Fig. 3). Cholesterol is interspersed in the phospholipid bilayer and helps to modulate the fluidity of the bilayer. This “sea” of lipids is not completely homogenous but rather it exists in a state of dynamic equilibrium, approximately described by the fluid-mosaic model proposed by S. J. Singer and G. L. Nicolson in 1972. The intracellular and extracellular face of the bilayer are different in composition, which is effected and maintained by various enzymes. For example, the phospholipid phosphatidylserine is almost never found on the extracellular leaflet of the plasma membrane, except in the case of cell death. This lipid environment is crucial to the maintenance and function of several membrane-bound and transmembrane proteins, such as cell surface receptors, ion channels; Giorgio Lenaz has written an excellent review about such effects (Lenaz, 1987). More detail regarding the cell plasma membrane, its makeup and its functions can also be found in the comprehensive and easy-to-read textbook “An Introduction to Biological Membranes; From Bilayers to Rafts” by William Stillwell (Stillwell, 2013).

Membrane fluidity is a crucial parameter in determining membrane structure and is intrinsically influenced by the lipid composition of the membrane. Membrane fluidity can be seen as a function of the order of the membrane in terms of the interaction and packing of the lipids – areas of higher order tend to have lower fluidity as measured by the lateral diffusion of lipids within the membrane and vice versa. Transient membrane microdomains form via phase separation according to the availability and spatial distribution of various lipids. For example, transient microdomains that are enriched in sphingomyelin and cholesterol have been found to exist in the cell membrane and are required for certain cell functions (Morris et al., 2004). The formation and lifetimes of these membrane microdomains is crucial for the certain functions, such as receptor clustering, signal transduction and neurotransmitter receptor maintenance, and these processes depend very much on the fluidity of the cell plasma membrane. Indeed, it has been observed that the

neuronal plasma membrane is more rigid in aged individuals (Yehuda et al., 2002) as the membrane lipid content changes, and also that membrane cholesterol and ceramide content are altered in aged individuals, both in humans and in mice, normal and diseased (AD) (Cutler et al., 2004), due to altered lipid metabolism.

1.9. Important factors to consider in studying A β

Although there have been numerous and varied studies regarding A β interaction with lipid membranes over the past 20 years or so, there are still many ambiguities and unanswered questions in our current understanding. It is especially confounding that conflicting results have been reported regarding two key effects that A β is thought to have on membranes, namely the formation of pores and thereby permeabilizing the membrane, and the change in fluidity of the membrane, whether an increase or decrease. Both membrane integrity and membrane fluidity are key areas in the normal function of the cell plasma membrane. The reason for such conflict maybe in the varied way that the studies have been conducted. Eckert, Müller and Wood have written in a 2010 review that “*The most parsimonious explanation for the reported differences in A β effects using fluorescence of DPH or TMA-DPH and pyrene is that the fluorescent probes are report [sic] on the behavior of A β in different membrane compartments as a function of the location and of the lifetime of the fluorescence probes. Moreover, differences in the results of the aforementioned studies may have also occurred as a consequence of variations in peptide structure and dissimilarities in physico-chemical properties of artificial versus biological membranes.*” (Eckert et al., 2010). Even fluorescently labeled lipids or lipid analogues, while having the advantage of being relatively similar to lipids in the native biological membranes and thus able to integrate into the lipid membrane alongside the other membrane lipids, are subject to various factors such as hydrophobic interactions that may result in different phase partitioning than expected, thereby introducing bias into the membrane domains that they are intended to probe (Sezgin et al., 2012). The method of preparation and type of the lipid membranes used in studying A β may also influence the type of results that are obtained. We know, for example, that native biological membranes are far more complex than what can be achieved with synthetic lipid membranes. The study conducted by Marin and associates related the overall membrane viscosity of membrane rafts to an interplay between the levels of cholesterol, saturated and unsaturated fatty acids, PUFAs and omega fatty acids

(Diaz et al., 2012). Indeed, the actual contribution of each class and type of lipid to membrane viscosity is surely several degrees more complex *in vivo*. In view of this, the method of preparation and/or treatment of the lipid membranes in the study must be carefully considered in the interpretation of any observations. It is quite possible that the process of membrane isolation and purification, as well as the presence of any kind of hydrophobic or amphiphatic solvents in treatments of the membrane can

Figure 3. The cell plasma membrane and some typical membrane lipids

The cell plasma membrane is a "soup" consisting of several proteins dissolved in a sea of lipids. The amphiphatic nature of membrane lipids causes them to arrange themselves in an orientation where the charged head groups face outwards and their acyl tails form a hydrophobic "core" region. Both proteins and lipids within the membrane are mobile, which is crucial for their proper function. Membrane microdomains form via phase separation, typically driven by hydrophobic interactions between the acyl tails of the membrane lipids, together with cholesterol interspersed within the hydrophobic core (not depicted in this schematic). These microdomains can function as transient but stable "islands" within the fluid membrane, allowing for temporary clustering of several membrane proteins or lipids, or a combination of both, thus promoting certain functions, for example, receptor activation or signal transduction. Membrane microdomains are too small to be resolved via typical light -based microscopy, perhaps ranging between tens to a couple hundreds of nanometers in diameter. The lipids themselves are not inert with respect to cell function – several of them act as membrane receptors, such as the ganglioside GM1, or signaling molecules, such as the well-known phosphatidylinositol 4,5-bisphosphate-diacylglycerol second-messenger system. Therefore, the integrity and homeostasis of the cell plasma membrane is critical for normal cell function. Lipid drawings were obtained from Avanti Polar Lipids.

introduce biases or artefacts in the observations. Because of this, it is advantageous if one can use living biological membranes in studying A β . To that end, several types of cells have commonly been used, ranging from non-neuronal cells such as human T-lymphocytes, HEK293 and the PC12 pheochromocytoma cell lines, to cells of neuronal origin such as the Neuro2A and SH-SY5Y neuroblastoma cell lines originating from mice and human respectively, and finally to actual neurons, including neuronal cell lines such as HCN-1 and primary neurons, usually from the cerebral cortex or the hippocampus as those are the brain regions most affected in Alzheimer's disease pathology. With regard to cell type, although it may be superficially acceptable to use any cell for studies of A β on biological membranes, it should be considered that A β pathology does not affect all cell types, even in cases of familial AD, wherein the gene encoding APP and/or other processing factors such as presenilin-1 (PSEN1) are mutated. Even for neuron-related cell types, sensitivity to A β neurotoxicity can vary according to its degree of differentiation (Lambert and Stevens, 1994). Different cells types have different lipid compositions in the cell membranes; indeed, even something as specific as ganglioside expression pattern can differ in differentiated cells compared to their undifferentiated precursors (Liour et al., 2000; Rebhan et al., 1994). Changes in lipid composition has been shown in to be key in modulating A β effect on lipid membranes, as well as in modulating the aggregation state of A β , as mentioned in several studies listed in the preceding sections. Taken together, these factors suggest that in order to truly understand the dynamics of A β on living biological membranes, one has to give careful thought into selecting the type of membrane system to be used. Because of the current ambiguity of how the A β peptide affects cell membrane fluidity, we have decided to resolve this question by using an optimal set up to study A β interaction with the cell membrane; that is, the use of living cell membrane and a method of observation that is minimally invasive to the cells, itself introducing minimal effects to the cell membrane.

1.10. Confocal fluorescence correlation spectroscopy and membrane fluidity

Fluorescence correlation spectroscopy (FCS) is a technique that relies on the movement of mobile fluorescent particles within an acquisition area of known size to calculate the dynamics of the movement of that particle. In FCS, one records the fluorescence intensity of a fixed acquisition area on the sample over a certain time,

T. Individual fluorescent particles, for example a dye molecule or a fluorescently-labeled protein, moves in and out of this acquisition area, contributing to fluctuations in the observed fluorescence intensity that is recorded as a fluorescence intensity trace. Because FCS relies on observation of fluctuations in the fluorescence intensity, the average number of observed molecules must be sufficiently small so that small changes in the intensity due to a single molecule leaving or entering the acquisition area can be detected, that is, a good signal-to-noise ratio can be obtained. This is typically achieved by two means – confocal optics and low concentration of fluorescent probes. In a confocal setup, the excitation laser beam is focused to a tiny, diffraction limited spot in the sample. Assuming the intensity profile of the light beam follows a Gaussian distribution, the shape of the confocal volume is approximately a 3-dimensional Gaussian function, an ellipsoid elongated along the beam axis, and with the radius s and half-length u . Considering a cylinder 2 μm in height and 0.4 μm radius, the volume of the cylinder is 1 femtoliter, which is the typical observed volume in confocal optics (Fig. 4). In a confocal instrument, s and u are also commonly referred to as ω and z . In practice, the confocal volume does not have a clearly defined surface, and the lengths s and u refer to the distances where the profile decreases to e^{-2} of its maximum intensity, I_0 . In a sample with a fluorophore concentration of 10 nM, 1 fL will contain on average 6 particles. In practice, however, the actual confocal volume does not have clearly defined edges and is defined by a Gaussian distribution instead. Fluorescence emission from excited particles in this confocal volume is detected and fluorescence from outside this volume is rejected via the use of a confocal pinhole, thereby improving the signal-noise ratio. Because of the very low number of fluorescing particles observed, sensitive detectors, such as an avalanche photodiode, are required to pick up the emission signal.

A typical setup for confocal FCS is shown in Fig. 5. Although the observed volume, in other words the acquisition volume, will always contain 6 particles on average, the detected fluorescence intensity will fluctuate over time as particles freely diffuse in and out of this volume. These fluctuations are recorded over a certain acquisition time, T , as an intensity trace. This intensity trace is auto-correlated over the acquisition time T , in other words, compared with itself, over time. For a certain delay time, τ , if fluorescence intensity signal at time $t+\tau$, is similar to that at time t , there will be a high degree of correlation, and if the intensity is not similar, there will

Figure 4. The confocal detection volume

Confocal optics uses lenses to focus an excitation beam, usually provided by a laser, to a point. The size of this volume is diffraction limited and is usually on the order of 10^{-15} . Combined with the use of a pinhole which is placed in the light path to block out light that does not originate from the focal volume, one is able to achieve a very high signal-to-noise ratio. This allows the FCS technique to be actualized, as the principle of FCS requires that very few fluorescing molecules be observed, in order that fluorescence fluctuations arising from a single molecule entering or exiting the observation area is distinguishable. Thus, FCS is a single-molecule sensitive technique; however, it is unlike some other single-molecule microscopy methods, such as single particle tracking, in the sense that it does not allow for the resolution and tracking of any one individual molecule, but rather it provides aggregate information on an ensemble of molecules, for example, average diffusion time and average particle count of a fluorescent dye in solution.

be a low degree of correlation. The degree of correlation decays as time τ increases and can be represented by an auto-correlation function, $G(\tau)$:

$$G(\tau) = \frac{\langle \delta F(0) \delta F(\tau) \rangle}{\langle F \rangle^2} \quad (\text{Equation 1.1})$$

In this expression, $G(\tau)$ is the auto-correlation function (ACF) with respect to the decay time τ , normalized by the square of the average intensity. $\delta F(0)$ refers to the average fluorescence fluctuation at $T = 0$, $\delta F(\tau)$ refers to the average fluorescence fluctuation at $T = \tau$. The correlation function for 3-dimensional diffusion is given by:

$$G(\tau) = G(0) \left(1 + \frac{4D\tau}{s^2}\right)^{-1} \left(1 + \frac{4D\tau}{u^2}\right)^{-1/2} \quad (\text{Equation 1.2})$$

Where τ is the delay time, $G(0)$ is the amplitude of the function at $\tau=0$ and τ_D is the diffusion time of the molecule. The expression on the right of the equation contains a component that accounts for diffusion in the xy-plane (containing the term s^2) and a component that accounts for diffusion in the z-axis (containing the term u^2). FCS can also be used to study lateral diffusion of particles within a plane, such as molecules diffusing on or within a lipid membrane. The correlation function for 2-dimensional diffusion is given as:

$$G(\tau) = G(0) \left(1 + \frac{4D\tau}{s^2}\right)^{-1} \quad (\text{Equation 1.3})$$

Figure 5. Typical confocal FCS instrumentation

In the simplest setup, a 1-particle diffusion experiment, the sample is illuminated by a laser beam focused to a diffraction-limited spot. Fluorescence emission from particles in the confocal volume, or observed volume, is collected selectively by the means of a pinhole, which blocks light coming from regions outside of the observed volume. The signal is amplified by an avalanche photodiode detector and correlated by a dedicated hardware correlator, allowing for real-time generation of an auto-correlation function (ACF). The ACF can be then fitted, using a software, with theoretical models of diffusion, such as 3-dimensional diffusion, or 2-dimensional, planar diffusion, accounting for factors such as fluorophore triplet state, multiple diffusing species and so on. The average diffusion time, τ_D , is given by the time τ at which the curve decays to half of its maximum, $G(0)$. The signal intensity trace and fits are actual data obtained by our system.

This equation is naturally similar to Equation 1.2, except that it lacks the component accounting for diffusion in the z-axis. By fitting the measured ACF with theoretical models of diffusion, we can obtain a diffusion time, τ_D of the fluorescing particle, which is basically the average time it spends in the acquisition area. Since we know the size of the acquisition area, we can subsequently calculate the diffusion coefficient of the particle, D , which is measured by area per unit time, through the equation:

$$\begin{aligned}\tau_D &= s^2 / 4D \\ D &= s^2 / 4\tau_D\end{aligned}\quad (\text{Equation 1.4})$$

Where τ_D is the translational diffusion time of the observed molecule, s is the radius of the illumination beam at its focal point and D is the diffusion coefficient. τ_D is represented as units of time, typically in milliseconds and is instrument-dependent as different beam widths may result in different transition times for a particular molecule. The diffusion coefficient, D , is represented as area per unit time, for example as square micrometers per second, and should remain the same for a particular molecule even when measured with different instruments, as long as the physical conditions of the experiment, for example, ambient temperature, solution

viscosity and so on, remain the same. In addition to the diffusion coefficient, other useful information can be obtained from an FCS measurement. One of these is the average number of molecules, N , observed for each measurement. This is given by the inverse of the ACF at time $\tau=0$, in other words, the y-intercept of the ACF:

$$G(0) = \frac{1}{N} \quad (\text{Equation 1.5})$$

As can be seen from this equation, the lower the average number of molecules, the greater the amplitude of the ACF. This also explains why a low number of particles is important when making FCS measurements. Because the τ_D of a molecule is the point where the ACF decays to half its maximum value, if the ACF has too low of an amplitude, it will be difficult to calculate the diffusion time of the molecule.

To understand the contributing factors in the translation diffusion of a molecule, we can observe the equation for the translational diffusion coefficient of a molecule, which is given by:

$$D = \frac{kT}{6\pi\eta R} \quad (\text{Equation 1.6})$$

Where k is the Boltzmann constant, T is the temperature in Kelvins, η is the viscosity of the solvent and R is the hydrodynamic radius of the molecule. The hydrodynamic radius, is related to its size in molecular weight MW , and is given by the equation,

$$V = MW\bar{v} = \frac{4}{3}\pi R^3$$

$$R = \left(\frac{3MW\bar{v}}{4\pi}\right)^{1/3} \quad (\text{Equation 1.7})$$

where $\bar{v}$ is the specific gravity of the molecule, and V is the volume of the molecule. Looking at equations 1.6 and 1.7, we see that the diffusion coefficient, D , is largely dependent on the temperature of the system and viscosity of the environment, and to a lesser extent on the molecular weight of the molecule. A change in apparent MW of a membrane probe could happen as a result of the probe molecule binding to a receptor on the membrane, such as a lipid or a protein, and a change in viscosity may happen in scenarios where the membrane viscosity changes, or when a membrane-bound probe transits from one membrane phase to another phase that has a different viscosity. To observe molecules diffusing on a membrane, the confocal observation volume can be focused on the membrane of a cell, as illustrated in Fig. 6.

Figure 6. Confocal FCS measurement on a cell membrane

The confocal observation volume, represented by the blue oval, can be focused on the membrane in order to observe membrane-bound molecules. The cell membrane is on average between 3-10 nm thick, which means that the observation volume is relatively huge, therefore the observation volume will contain in part the cytosol as well as the extracellular environment, which is typically the cell culture medium or buffer. In such experiments, steps must be taken to reduce signal from fluorescent molecules that are not on the membrane. This can be achieved by proper washing off of unbound probe and minimizing the time elapsed between the staining and observation steps so that minimal internalization of the probe has occurred. Care must also be taken during the calculation of diffusion times of the molecule to factor in the possible presence non-membrane bound probe. Picture not drawn to scale.

In addition to the single-molecule sensitivity of FCS, other aspects of the technique make it ideal for studying membrane diffusion. Firstly, only a low concentration of probe molecules is needed, which means the effects of the probe itself on the cell and on the cell membrane are lowered. Secondly, only a low laser power is needed, which means lower phototoxicity and heating effects when the technique is applied to living cells. In addition to the diffusion time of a molecule, one other useful piece of information that can be gleaned from FCS measurements is the aggregation state of the fluorescent molecule. Even though low- n aggregates of a fluorescent molecule will not significantly change the observed diffusion time of the molecule because any effect due to an increase in MW will be reduced to its cube root (see Equation 1.7), the acquired intensity trace contains information about the brightness of the observed molecule. Therefore, if one knows accurately the stoichiometry of fluorescent labeling of a molecule, such as a fluorescently-tagged peptide, one may observe aggregates and calculate the number of monomers present in the aggregate. For this reason, FCS can be utilized to study aggregation dynamics of the A β peptide. Greater detail about the FCS and its myriad applications can be found in several excellent book chapters written by J.R. Lackowicz (Lackowicz, 2006), Xianke Shi and Thorsten Wohland (Shi and Wohland, 2010).

Because of the factors stated above, FCS should be a useful tool in the studying membrane diffusion (Sengupta et al., 2002). Despite this, there has been a dearth in the number of studies using FCS as a method of studying membrane fluidity or other membrane-related perturbations in the area of A β . A current search of the PubMed database with the search terms “amyloid-beta”, “fluorescence correlation

spectroscopy” and “membrane” yielded only 6 results, of which only 1 was a study conducted using FCS on A β interaction with cell membrane. In that study, Widening the PubMed search by dropping the term “membrane”, yielded a further 19 reports that used FCS in studies of A β . Of these, 3 were related to the use of FCS as a screening method to detect A β aggregates in the brain and the remaining 16 were related to the dynamics of A β aggregation. Considering that the combination of the search term “amyloid-beta” with the terms “membrane” and “aggregation” yielded 5118 and 3515 results respectively, this means that FCS was utilized in far less than 1% of all A β membrane or aggregation related research, despite the great interest in how A β affects the cell membrane. With regard to the fluidity of A β on the cell membrane, Aladdin Pramanik and his colleagues used confocal FCS to probe the interaction of rhodamine-labeled A β (1-40) and human cortical neurons from the HCN-2 cell line. They detected 3 species of A β with diffusion times, τ_D of 0.1 ms, 1.1 ms and 5.9 ms, and deduced that the fastest-diffusing species was that of unbound A β , while the two slower-diffusing species belonged to membrane-bound A β . (Hossain et al., 2007). Similarly, Nag and Maiti’s study used FCS to measure the diffusion times of membrane bound-A β using PC12 cells and nanomolar concentrations of A β (Nag et al., 2010). Although the diffusion time for unbound A β reported in their study (126 μ s or 0.126 ms) is comparable to that reported in Aladdin Pramanik’s the diffusion time for membrane bound-A β oligomers (200 μ s or 0.2ms) however was 5 and 30 times slower than that of the second and fastest diffusing species respectively in Pramanik’s study. Both studies used N-terminal labeled A β (1-40) with a lysine linker between the fluorophore and the N-terminal aspartate residue of the A β peptide, although Pramanik used rhodamine and Maiti used fluorescein. In both cases, FCS was used due to its advantage in measuring very low concentrations of fluorophores (5 nM in Pramanik’s study and 150/350 nM in Maiti’s study). Both Pramanik’s and Maiti’s studies shed some insight into the biophysical properties of A β when bound to cells, however, to our knowledge, there still has not been any study examining how A β affects *plasma membrane fluidity in living cells*.

1.11. Total Internal Reflectance-based Fluorescence Correlation Spectroscopy

Confocal microscopy has been the mainstay instrumentation in FCS because of the advantages that it offers in the form of a high signal-to-noise ratio due to the

combination of the focusing power of high NA lenses, effectively making the observation volume a singular point in the sample, and the confocal pinhole, which rejects out-of-focus light. One drawback of confocal FCS for use in measuring membrane diffusion, however, is that the sampling rate is low, since only one singular spot (the confocal focal volume) can be observed at a time. For molecules diffusing in a homogenous environment, such as fluorescent dye molecules in an aqueous solution, a relatively small number of data points in a sample may suffice, as one does not expect the diffusion coefficients of molecules to vary very much, if at all, at different spatial locations in the sample. For example, the diffusion coefficient of Atto-488 dye in a droplet of water on a glass coverslip should be the same regardless of whether the observation volume is just above the surface of the coverslip, in the middle of the droplet, or close to the water-air interface. This can be true in a homogenous two-dimensional system as well, such as molecules of fluorescently tagged lipid, such as rhodamine-phosphatidylethanolamine (Rho-PE) diffusing in a supported planar bilayer composed by only a single type of lipid, for example, the saturated fatty acid DPPC. However, when the environment or the sample is heterogeneous, a single or small number of measurements will not be sufficient to give accurate information on the diffusion of probe molecules. In a phase-separating artificial membrane, for example a supported bilayer made from DOPC, sphingomyelin and cholesterol, measurements at individual points on the membrane may not return similar results due to phase separation of the membrane. When it comes to living cells, the situation becomes even more complicated, because a population of cells is itself heterogeneous, meaning that there will be variability in the fluidity of the cell membrane from cell to cell, in addition to the membrane heterogeneity of each individual cell. Furthermore, the cell membrane is highly dynamic and changing even when cells are at a resting state, meaning that separate membrane phases continually form and dissolve in the cell membrane. To address population heterogeneity, that is the variability of membrane fluidity from cell to cell, the only way is to measure diffusion on a sufficiently large number of cells. To address heterogeneity of the membrane of each individual cell, one would need to measure several spots on the same cell. Together, this represents a formidable challenge for the researcher attempting to measure membrane diffusion in living cells. In our acquisition method for confocal FCS, an average of 4 readings of 45s each are taken at a single spot on the membrane, for a total time needed of 3 min for measuring 1 spot. If 10 spots are measured on each cell, and 10 cells are measured

in a sample, it will already take 5 hours to complete the required measurements for a single sample, assuming there is zero-time lag between each measurement. In reality the time taken is much greater because care has to be taken to properly focus the observation volume for each spot, and acquired data must be saved in between measurements. Other than it being physically challenging, another problem with this approach is that the effects one intends to observe, such as changes in membrane fluidity upon addition of a certain chemical, may not persist over such a long period of observation time. For example, in a theoretical experiment, treatment of cells by Compound A causes a rapid but transient increase in cell membrane fluidity. However Compound A is quickly internalized and broken down by the cells. Within 1h, cell membrane fluidity recovers to pre-treatment values. This is only about sufficient time to complete measurements on about 2 cells. Therefore, for one condition, that is, treatment of cells with Compound A, the researcher has to prepare 5 samples and 5 treatments in order to get complete measurements on 10 cells. Even if the researcher doing this experiment had the endurance of a robot, the amount time and materials required may be a concern. This problem can be alleviated if there were a method of sampling many spots on the membrane of a single cell. Such a technique has indeed been developed by the mid-late 2000s.

The combination of the FCS principle with the use of total internal reflectance fluorescence microscopy (TIRFM) has allowed the development of a technique, called “imaging total internal reflectance-fluorescence correlation spectroscopy” (ITIR-FCS), that is ideally suited for studying membrane diffusion (Guo et al., 2008; Kannan et al., 2007; Sankaran et al., 2009). The principle of TIRFM is to use an excitation beam that is totally internally-reflected at the glass/medium interface of the sample to generate an evanescent light field along the plane of the interface that is used to illuminate the sample (Fig. 7). Using this method of illumination, a broad field of the sample can be illuminated, allowing for a 2-D region to be sampled in a single acquisition run, even up to the entire basal cell membrane. This evanescent field decays exponentially with distance and can penetrate typically no more than ~100 nm from the surface of the glass/medium interface. This introduces an inherent depth limit in the fluorescence image taken using such a setup, and is advantageous for studying fluorescence on membranes resting on a flat glass surface because it limits the probability of stray, non-membrane-bound fluorescent probes contributing to the fluorescence intensity signal measured from the area of acquisition. Instead of

measuring fluorescence fluctuations at a single point as is done confocal FCS, the data collected in TIRF-based FCS is in the form of a *time-stack of images* using an electron-multiplying charge-coupled device (EM-CCD) camera. At each point in time, each pixel in the image has a certain brightness due to the fluorescent probe molecules that are being imaged. Because the molecules are mobile and diffuse across the membrane bilayer, the brightness of each pixel will fluctuate with time, thus auto-correlation function can be calculated for each pixel in the image using appropriate software (Sankaran et al., 2010).

Figure 7. Total Internal Reflectance Fluorescence Microscopy (TIRFM)

In TIRFM, instead of focusing the illumination light beam to a point, a tilting mirror is used to change the angle of incidence, θ_i , of the excitation laser beam to the coverslip-sample interface (see also Fig. 7). When θ_i is greater than the critical angle, the light beam is totally reflected and no light is transmitted across the coverslip-sample interface. However, an evanescent field is generated along the glass-sample interface which decays exponentially with distance, limiting the depth of field to about 10^{-8} meters from the surface of the interface. Picture not drawn to scale.

The geometry of the observed area in ITIR-FCS is like that for 2-dimensional diffusion in confocal FCS, and because the pixel dimensions are known, the coefficient diffusion of the molecule can be calculated according to Equation 1.3. This immediately increases throughput in terms of the number of points measured on the cell as an image of any size, limited only by the maximum number of pixels the camera has, can be designated as the acquisition area, otherwise referred to as the region of interest (ROI) (Fig. 8). For example, for the human neuroblastoma cell line SH-SY5Y, a region of interest on the cell membrane of typically 21 x 21 pixels

is selected, giving a total of 441 measurement points on the membrane. Using ITIR-FCS, Thorsten Wohland and Aseem Mishra were able to probe the effects of human amylin, or human islet amyloid polypeptide (hIAPP), on artificial and live cell membranes (Bag et al., 2013b). ITIR-FCS data also allows for a type of post-hoc analysis called the FCS diffusion law analysis. The FCS diffusion laws were first proposed by Pierre-Francois Lenne and Didier Marguet in 2005 as a method of probing the membrane on a submicron scale, in other words, sizes that are smaller than the diffraction limit for visible light (Wawrezynieck et al., 2005). FCS diffusion law analysis is based on the principle that a lateral diffusion time, τ_D , of a molecule freely diffusing in a homogeneous environment should scale proportionately with varying sizes of the observed area, where s^2 is the effective observation area. If the environment of the diffusing molecule were homogeneous, D would be expected to remain the same no matter what s^2 is, therefore the ratio s^2/D is expected to scale linearly, and s^2/D plotted against s^2 should produce a line that cuts the y-intercept at approximately $s^2 = 0$ (Fig. 9a). By studying the diffusion behavior of fluorescent GM1 ganglioside and transferrin receptor on living cells, Lenne and Marguet determined that for molecules confined by permeable barriers, such as lipids within a lipid raft, the y-intercept will be positive (Fig. 9b, “Raft Partitioning”). This is because at $s^2 <$ the size of the raft domain, the molecule will appear to be diffusing homogeneously. At $s^2 >$ the size of the raft domain, observed molecules will start to exhibit heterogeneous translational diffusion times, as they pass s^2 . Consider a theoretical molecule with a diffusion time, τ_D , of 250 ms in an observed area of $1 \mu\text{m}^2$. The calculated diffusion coefficient, D , of this molecule per equation 1.4 would be:

$$D = s^2 / 4\tau_D$$

$$D = \frac{(1\mu\text{m})^2}{[4(0.25 \text{ s})]} = 1 \mu\text{m}^2\text{s}^{-1}$$

from within the more ordered raft phase to the surrounding, more fluid membrane phase and vice versa. Because the environment outside of the raft is less ordered and more fluid, the molecule diffuses faster. This means that plotting s^2/D against s^2 for values of s^2 greater than the size of the raft domain, the gradient of the curve will be gentler than for s^2 less than or equal to the size of the raft domain (Fig. 9b, green line versus red line). Because of technical limitations thus far in optical microscopy, an s^2 equal or less than the size of a raft domain, typically no larger than 300 nm in

Figure 8. ITIR-FCS instrumentation and data analysis

In ITIR-FCS, an EM-CCD is used to acquire the image that will be used for later analysis. This allows an image of a region of interest to be captured at once, greatly improving sampling rate vs. confocal scanning microscopy. This allows for a better overview of the diffusivity of the probe on the observed membrane, as one is able to see a distribution of diffusion coefficients, as well as the mean and median diffusion coefficients.

diameter, is unachievable. Therefore, for molecules that diffuse heterogeneously because they transit between raft and non-raft domains, one can only plot the line for $s^2 >$ the raft size and when extrapolating this line to the $s^2 = 0$, will always get a positive y-intercept (Fig. 9b, green line). For molecules confined by impermeable barriers, such as the cytoskeletal meshwork, the y-intercept will be negative (Fig. 9b, “Meshwork”). The reason for this is somewhat opposite to the situation of raft partitioning. Because of impermeable barriers, the only way a molecule can pass over the barrier is via “hopping”, which is a kinetically slower process than diffusion. Thus, for $s^2 >$ the size of the area confined by the meshwork, the gradient of the plot of s^2/D against s^2 will be steeper. Again, due to physical limits of optical microscopy, one can only plot the line for $s^2 >$ the size of the mesh-delineated area and when extrapolated, the line will always give a y-intercept of < 0 (Fig. 9b, blue line). This technique can account for sub-micron membrane microdomains such as raft domains, which cannot be observed visually.

(a)

s^2	τ_D	D	s^2/D
0.01	0.025	1	0.01
0.5	0.125	1	0.5
1	0.25	1	1
4	1	1	4
9	2.25	1	9

Plot of s^2/D versus s^2 for a freely diffusing molecule

(b)

Figure 9. FCS Diffusion Law analysis

(a) Theoretical s^2/D plot for a freely diffusing molecule. (b) Experimental values for molecules experiencing free, raft-type and meshwork-limited diffusion. A_{eff} = effective observation area, equivalent to s^2 . Figure 9b is adapted from Nirmalya Bag and Thorsten Wohland (Bag and Wohland, 2014).

1.12. FCS as an ideal method for probing A β effects on membrane fluidity

Ever since A β was identified as a potential causative agent of Alzheimer's disease, it has been closely studied and much has understood about its biophysical and biochemical properties. With respect to the cell membrane fluidity, even though several studies have been conducted in the past 20 years, we do not yet have a conclusive answer. The reason for this could be because of the difference in the way membrane fluidity was studied. As detailed in sections 1.4 – 1.7, previous studies mainly utilized artificial membranes or extracted lipid membranes in their studies, which may not be an optimal scenario. In the case of artificial lipid membranes, the much simpler organizational structure in terms of the types of lipids present may result in observations that do not accurately mirror that of actual living cell membranes. In the case of extracted cell membranes, the detergents and handling procedures used in the extraction and purification of the membranes may themselves perturb the membrane behavior and introduce biased results. About the method of measuring fluidity, most of the earlier studies and even several recent studies use fluorescence anisotropy of DPH and TMA-DPH or measuring pyrene excimer formation (detailed in section 1.6). These molecules are good reporters of membrane fluidity; however, a drawback is that they are exogenous chemicals and also, they only can report on bulk or annular fluidity of lipids in the membrane. The advantages of fluorescence correlation spectroscopy over these techniques is that first, it can be conducted on living cells with minimal intrusion. The instrumentation for FCS, whether it is using a confocal microscope or a TIRF microscope, is amenable to live cell experiments via the use of a stage-mounted environmental chamber. The use of such a setup also allows measurements to be made immediately following treatments to the cells/membrane, if necessary. Secondly, the laser power used for FCS is typically very low, in the range of 5 to several 10s of μ W, because only a small amount of probe (5 – 100 nM) is required. This gives a dual advantage; a low laser power minimizes light-/heat-induced damage to cells and a low concentration of probe minimizes the effect of the probes themselves on the cells, as well as on membrane behavior, which is applicable even to artificial membranes. Indeed, cells that have been experimented on using FCS can often be returned to culture without noticeable deterioration (our own observations). As such, we have decided to use FCS to provide a conclusive study on how A β affects cell membrane fluidity.

1.13. Rationale, Hypothesis and Approaches

Cell membrane fluidity underpins cell membrane function and its homeostasis is crucial to cell health. Apart from normal functioning of cell membrane components, such as membrane proteins, membrane fluidity could also modulate the entry of exogenous molecules to the cell. Although there have been several studies conducted to determine whether and how amyloid-beta affects cell membrane fluidity, a conclusive answer has not been found, the likely reason for this is that different methodologies introduce various biases into the experiments, as noted by Eckert, Muller and Wood in their extensive review on the subject (Eckert et al., 2010). Additionally, several of such studies, measurements were not conducted on living cells; instead, either artificial membranes (Filippov and Antzutkin, 2013; Sasahara et al., 2013) or extracted cell membranes were used (Chochina et al., 2001; Muller et al., 1998; Müller et al., 1995). Against this backdrop, I hypothesize that the actual situation, whether amyloid-beta does affect cell membrane fluidity, may be different in *living* cells, and my study seeks to conclusively determine this. To achieve this, I will be measuring membrane fluidity of living cells exposed to A β (1-42) using fluorescence correlation spectroscopy (FCS). The cell type used will be the SH-SY5Y cell line, which is human and neuronal in origin and known to be sensitive to A β -induced cellular damage (Lambert and Stevens, 1994; Lee et al., 2015; Qu et al., 2011). The U87 human glioma and Neuro-2a mouse neuroblastoma cell lines will also be used, to provide a more comprehensive view of the effects of A β on living cell membranes of cells originating from brain tissue. Although some preliminary cell membrane fluidity measurements employ the “classical” confocal-FCS method, the mainstay of this study will be imaging-TIR FCS, which will allow for real-time, live cell measurements with coverage of a substantial area of the cell membrane. Finally, A β (1-42) will be used because it is the more amyloidogenic A β variant and is more strongly linked to AD pathology. An oligomer pre-aggregation step will be done for the A β because research in the past 15 years have increasingly shown that small, soluble A β aggregates may be the ones responsible for causing detrimental effects to neuronal cells (Klein et al., 2001; Malchiodi-Albedi et al., 2011; Sakono and Zako, 2010). This setup should allow us to sensitively measure the cell membrane fluidity of living cells exposed to A β . To our knowledge, our study is the first to study the effects of A β on the plasma membrane fluidity by direct measurement of the membrane fluidity in living cells.

2. Materials and Methods

All buffers and cell culture medium used were phenol red-free, except EMEM with NEAA.

2.1. A β preparation and aggregation

Synthetic A β (1-42) was purchased from Anaspec (Fremont, CA) and prepared per the protocol adopted from William Klein and W. B. Stine and colleagues (Klein, 2002; Stine et al., 2011). Hexafluoroisopropanol (HFIP) (Sigma, St. Louis, MO) was first chilled on ice. Chilled HFIP was added directly using a micropipette to a vial of lyophilized A β (1-42) to a final concentration of 1 mM. The vial was agitated to make sure all the powder was washed into the HFIP, obtaining a cloudy mixture which was then transferred to a microcentrifuge tube. The mixture was then incubated for 1h at room temperature, during which the peptide completely dissolved and the solution turned clear and colorless. After 1h, the tube was transferred onto ice for another 5-10min before aliquots were made. Aliquots of the A β solution was transferred to microcentrifuge tubes and left overnight in the fume hood with the caps open for the HFIP to evaporate. The next morning, the tubes were centrifuged for 10min in a SpeedVac vacuum centrifuge lyophilizer (2000 rpm, 1 mbar) to remove all traces of HFIP. This is important as HFIP may affect cell membranes and cell viability. A thin, clear film of A β peptide could be observed at the bottom of the microcentrifuge tube. The dry peptide was then stored at -80°C until further use. All procedures involving HFIP were done in a fume hood as HFIP is toxic and highly volatile.

To prepare small globular A β aggregates, the dry peptide was first retrieved from -80°C and placed on ice for gradual warming up. Because A β has a high propensity to self-aggregate, the following steps were done as swiftly as possible to minimize undesired aggregation of A β . Anhydrous DMSO (ThermoFisher, Waltham, MA) was added to the tube to dissolve the peptide to a concentration of 5 mM, followed by sonication for 10min in a water bath sonicator. Pure DMSO-dissolved A β solutions must not be placed on ice as it will freeze, which may cause undesired effects to the A β . Chilled, supplement-free DMEM/F12 or Neurobasal medium (ThermoFisher, Waltham, MA) were added to the tube and pipetted to mix and dilute

the peptide to a final reaction concentration of 100 μ M. Tubes were immediately placed on ice and transferred to the cold room (4°C) for incubation. The solutions were incubated without agitation for 24h or 72h in the cold room. At the end of the incubation period, the solution was centrifuged for 10 min at 14,000 x g and 4°C, and the supernatant used for further experiments. The final amount of A β in the supernatant was not quantified; instead, this solution was labeled as '100 μ M A β ', and all subsequent stated concentrations of A β follows from this solution. For example, '10 μ M A β ' represents a 10x dilution of this supernatant.

2.2. SDS-PAGE analysis of aggregated A β

All SDS-PAGE-related materials, reagents, staining kits and equipment used were purchased from ThermoFisher (Waltham, MA). To verify the presence of small A β aggregates were produced via the aggregation procedure, SDS-PAGE was conducted with the supernatant obtained. Solutions of A β , blanks and protein standard were prepared to run on a NuPAGE Novex 10-well, 4-12% bis-tris minigel. The NuPAGE MES SDS running buffer was used as the A β peptide was very small (MW = 4514). The Mark 12 unstained protein standard provided adequate range of marker proteins (2.5 – 200 kDa). We expected there to be several types of aggregates and wanted to detect all of them, therefore an unspecific detection method was preferable over Western blotting. We decided to utilize silver staining was chosen over Coomassie Blue because of the higher sensitivity (as low as 0.5 ng per the manufacturer's specifications). A suitable amount of A β , typically ~1 μ g total peptide, was mixed with pre-chilled LDS sample buffer. For heated samples, the solution was heated in Eppendorf tubes on a heating block for 10 min at 70°C and immediately placed back on ice. No reducing agent was used in the SDS sample buffer, since A β does not contain cysteine residues and therefore should not have disulphide cross linkages and so there should be no difference with or without reducing agents. Because the aggregation of A β is quick and spontaneous in buffers at or near physiological pH and ionic strength, the entire gel electrophoresis process was carried out in the cold room to minimize aggregation over the course of the experiment. The electrophoresis chamber and minigel were prepared and immersed in an ice-water bath and placed in the cold room prior to addition of the sample to the wells. 20 μ L of each sample was added to each well in the minigel and electrophoresis was conducted for 55 min at 200V constant voltage. Gels were removed from the chamber and washed twice

with MiliQ water for 5min on a rotating shaker at 60 rpm to remove residual SDS. Silver staining was done using a SilverExpress silver staining kit per the manufacturer suggested protocol. Stained gels were photographed using a BioRad Gel-Doc imaging device and manufacturer provided software.

2.3. Cell Culture and Immunocytochemistry

All cell culture reagents were purchased from ThermoFisher (Waltham, MA) unless otherwise stated. SH-SY5Y human neuroblastoma cells and Neuro-2a mouse neuroblastoma cells were obtained from the American Type Culture Collection (Manassas, VA). Newly obtained cells were designated “Passage 1” and cells were not used beyond Passage 15. U87 glioma cells were a kind gift from Dr. Harry Asada. Cells were maintained in filter-capped T-25 flasks in an humidified incubator at 37°C, 21% O₂ and 5% CO₂. Complete standard medium was prepared for each cell type as follows: *SH-SY5Y cells* - DMEM/F12 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep); *SH-SY5Y neuronal differentiation* – Neurobasal medium with 2% B27, 1% GlutaMAX and 1% pen-strep (designated ‘neuronal medium’ when fully supplemented); *Neuro-2a cells* – EMEM with non-essential amino acids (Sigma, St. Louis, MO) with 10% FBS, 1% pen-strep; *U87 cells* – DMEM high glucose with 10% FBS, 1% pen-strep. All medium was sterile filtered with a 0.2 µm filter after preparation. Cells were passaged per ATCC recommended protocol. All reagents were pre-warmed in a 37°C water bath before use with live cells. To passage cells, the old cell culture medium was first aspirated, and the cells washed twice with DPBS (Lonza, Basel, Switzerland). DPBS was only used to wash cells prior to passaging as it did not contain Ca²⁺ and Mg²⁺, which are important co-factors for cell adhesion proteins. For all other washing, or incubation periods requiring a buffer instead of complete medium, HBSS with Ca²⁺ and Mg²⁺ (Sigma, St. Louis, MO) was used to prevent undesired detachment of cells from the culture surface, because HBSS has glucose as an energy source and because it is a better buffer at atmospheric CO₂. After washing, cells were incubated with TrypLE cell dissociation reagent for 1 min in the incubator and dislodged from the cell culture flask by gentle tapping. The action of TrypLE was stopped via dilution by at least 2:1 with complete medium and the cells triturated by pipetting. 2 samples were taken for cell counting using a Bio-Rad cell counter and the counts averaged to get the cell density. Appropriate aliquots of the cell solution were made and the centrifuged at

room temperature for 5 min at 200 x g to pellet the cells. The supernatant was discarded and the cells were re-suspended in the appropriate complete medium for seeding. For experiments, cells were seeded at a density of $8.5 - 9.5 \times 10^3$ cells/cm² 2-3 days prior to the experiment to allow for proper attachment and acclimatization of the cells in the cell culture vessels.

Differentiation of SH-SY5Y cells. All-trans retinoic acid (RA) and poly-D-lysine (PDL, MW 70,000 – 150,000) were purchased from Sigma (St. Louis, MO). Cell culture vessels were first coated with 0.1 mg/ml PDL. The vessels were treated prior to PDL coating with plasma to render their surfaces hydrophilic. An adequate amount of PDL was then added to cover the entire culture surface and incubated for 5 min at room temperature. After coating, the solution was then aspirated and the surfaces washed twice with sterile ddH₂O and allowed to dry for at least 2h in an oven at 70°C before use. SH-SY5Y cells were seeded at a density of 6×10^3 cells/cm² in neuronal medium with 10 µM RA for 7 days. During differentiation, 50% of the medium was changed every 2-3 days. After 7 days, the medium was replaced by retinoic-acid free medium and the cells were used for experiments. Exposure to the atmosphere was minimized to avoid oxidative damage or drying out of the cells, and all pipetting action was done as gently as possible because the differentiated cells detach easily if mechanically agitated.

Immunocytochemistry of differentiated SH-SY5Y cells. To verify that differentiated SH-SY5Y cells have a more neuronal phenotype, immunocytochemistry was conducted to check whether the cells expressed the following neuron-specific proteins: the intermediate filament NF-H, dendritic marker MAP-2 and axonal marker GAP43. Primary antibodies (all rabbit IgG) were purchased from Abcam (Cambridge, UK). Alexa 488-conjugated mouse anti-rabbit IgG, rhodamine-phalloidin, Hoechst 33342 and Nunc Labtek 8-well chambered glass coverslips were purchased from ThermoFisher (Waltham, MA). Bovine serum albumin (BSA, Cohn's fraction V), sucrose, Triton X-100, paraformaldehyde (PFA) and HBSS were purchased from Sigma (St. Louis, MO). Fixing solution – PFA, sucrose, both 4% w/v, in HBSS. Permeabilization solution – Triton X-100 0.1% v/v in HBSS. Blocking and washing solutions – BSA in HBSS, 5% w/v and 0.5% w/v respectively. All antibodies were diluted in washing solution. Cell nuclei were stained with Hoechst 33342 and counterstained with RFP-phalloidin. The fixing solution was

stored at -20°C and all other solutions were stored at 4°C. Blocking and washing solutions were prepared fresh before each experiment by diluting a stock solution (10% BSA in HBSS).

The fixing and staining procedure is as follows: Cells were seeded and differentiated in LabTek chambered glass coverslips. HBSS and fixing solution were pre-warmed in a 37°C water bath. Differentiated cells were washed using HBSS with Ca^{2+} and Mg^{2+} and fixed at room temperature by incubating for 10 min the fixing solution. Cells were then washed once with HBSS and permeabilized by incubating for 10 min with 0.1% Triton X-100 (Sigma). After removal of the Triton X-100 solution, cells were washed once with HBSS and incubated for 3h at room temperature in blocking solution followed by washing once with washing solution. For each wash, cells were incubated in washing solution for 5 min at room temperature. Cells were incubated overnight at 4°C with primary antibodies, and washed three times with washing solution. Cells were then incubated with secondary bodies and RFP-phalloidin concurrently for 2h at room temperature and washed twice with washing solution. Finally, cells were incubated with a Hoechst solution (3 $\mu\text{g/mL}$) for 15 min at room temperature to stain the DNA. Cells were then washed one final time and imaged. Imaging was done using an Olympus FV1200 confocal microscope and images were analyzed using Imaris and ImageJ.

2.4. Cell viability and caspase activation assay

The ApoLive Glo assay (Promega, Madison, WI) was used to assess the cytotoxic activity of the aggregated A β . Cells were seeded in black-walled, clear-bottom 96-well tissue culture test plates. Cells were treated with either A β (1-42) or A β vehicle. Three concentrations of A β solutions were administered to the cells; 1, 10 and 20 μM total A β . The solutions were prepared as follows: first the the A β aggregation solution was centrifuged as detailed in section 2.1, and the supernatant retained. The following volumes stated are for a single well in a 96-well plate and scaled according to the number of wells treated: 1, 10 or 20 μL of A β supernatant (100 μM A β)was aliquoted into microcentrifuge tubes. The 1 and 10 μL aliquots, were topped up to 20 μL with vehicle. 80 μL of complete medium was then added to each tube for a final volume of 100 μL with final concentrations of 1, 10 and 20 μM A β . For the control tube, 20 μL vehicle was used instead of the A β solution. A β vehicle was

prepared using the identical procedure as the A β aggregation solution, except that no A β was present. A minimum of 3 wells (samples) per treatment were prepared for each experiment. All solutions were kept on ice during the preparation procedure, until the final 80 μ L of complete medium was added. The final solutions were mixed and added to cells as quickly as possible with gentle pipetting was maintained to avoid dislodging the cells, especially the differentiated SH-SY5Y cells. Negative controls were prepared by simply replacing the old culture medium with fresh complete medium. The plates were then returned to the incubator for the designated A β treatment time of 24, 48 or 72h. No medium change was done during the period of treatment. Assay controls were done by treating the cells with 0.5 μ M staurosporine for $\geq$ 6h. Blank controls for assay baseline readings were wells containing cell culture medium without any cells.

Cell viability and caspase activation was assessed using the ApoLive-Glo Multiplex assay. Cell viability is assessed using a cell permeant molecule, Gly-Phe-AFCoumarin. The molecule is cleaved by a protease in the cell to release fluorescent AFCoumarin. The protease is labile in cells with compromised cell membranes and will not cleave the substrate, therefore only cells with intact cell membranes will generate a positive fluorescent signal which can be read using a plate reader. In other words, the assay measures cell viability as a function of cell plasma membrane integrity. Caspase 3/7 activity is assessed by a luciferase-based method. The reagent contains aminoluciferin conjugated to the caspase cleavage sequence DEVD, and active caspases cleave the sequence to release luciferin, a substrate for the luciferase reaction by luciferase in the reagent, resulting in luminescence. The caspase reagent also contains a chemical that lyses the cells to release the caspase in the cell, therefore cells are not viable at the end of the assay. The higher the caspase 3/7 activity, the higher the generated luminescence will be. 20 μ L of the viability reagent was added to each well, the plate shaken gently to mix the contents and incubated for 30 min at 37°C, following which the fluorescence was read from the bottom of the plate. 100 μ L of the caspase reagent was then added to each of the wells and the plate shaken to mix the contents before incubating for another 30 min at 37°C, and the luminescence in each well read. Endpoint fluorescence and luminescence were read using a Tecan Infinite m200 microplate reader and its associated software, using manufacturer recommended settings for the assay kit. Data was represented as relative fluorescence units (RFU) and relative luminescence units (RLU) and

analyzed using Microsoft Excel and GraphPad Prism. First, average RFU and RLU values were obtained for vehicle control and blank control samples. The average blank control readings were subtracted from the average vehicle control readings. Typically, blank control readings do not exceed a few hundred RFU/RLU and do not significantly change the average vehicle control readings (typically 10^4 to 10^5) by an appreciable amount. All other readings (A β -treated, negative and positive controls) were normalized against the average vehicle control for each separate experiment. This was done to enable comparison of results across experiments conducted on different occasions. Finally, these ratios obtained were used in statistical analysis for evaluating cell viability and apoptosis.

2.5. A β uptake into cells

Wild type (wt-A β) and N-terminal tagged, fluorescent (fl-A β) A β (1-42) was prepared using the same method detailed in section 2.1. SH-SY5Y cells were seeded in 8-well chambered coverslips and differentiated as detailed in section 2.3. wt-A β and fl-A β were incubated in a 3:1 ratio for the aggregation phase. The A β mixture was diluted in complete medium to a concentration of 10 μ M and cells were incubated with this solution for 24h. Cell membranes were then stained with DiI with the A β -containing medium removed and set aside during the staining time (~20 min). The A β medium was returned to the cells after washing off excess DiI. Cells were left in the incubator for another 30 min to equilibrate, and then imaged using a confocal laser scanning microscope (Olympus FV1200). Images were analyzed and adjusted using Imaris and ImageJ.

2.6. Confocal Fluorescence Correlation Spectroscopy (confocal FCS)

We first began probing the cell membrane fluidity of A β -treated cells using confocal FCS. Cells were seeded in LabTek 8-well chambered coverslips per the procedures detailed in section 2.2. A β was prepared per the protocol detailed in section 2.1. Cells were treated with 1 μ M A β (1-42) or A β -vehicle for 24h. After 24h, the A β -containing medium was removed from the wells, set aside and the cell membrane was stained with Alexa 488-conjugated CtxB (Sigma, St. Louis, MO). Stock solutions of CtxB were prepared by dissolving dry, lyophilized CtxB in HBSS to obtain a 10 μ M solution. The solution was aliquoted and stored at -20°C to minimize

multiple freeze-thaw cycles. A staining solution was prepared by diluting the stock solution in HBSS to a final concentration of 10 nM. Cells were incubated with the staining solution for 20 min at 37°C, and washed twice with pre-warmed HBSS. The A β medium that had been set aside was then returned to the wells to ensure that A β was present in the environment for the whole experiment. FCS measurements were done using an Olympus FV300 laser scanning confocal microscope coupled to a hardware correlator. A water immersion objective (60x, 1.2 NA, Olympus) was used for imaging. A 488 nm argon laser (emission filter: FF01 512/17-25) was used for the excitation of fluorophores. The system was calibrated using Atto488 as the reference dye. Laser power was maintained at 15 μ W to reduce photobleaching and phototoxicity to the cells. The apical plasma membrane was located by adjusting the Z position of the stage while looking at the signal intensity trace in point scanning mode. Once the position with the highest signal intensity was located, the signal was collected and correlated by the hardware correlator. At least 4 consecutive measurements were made on each spot, with each measurement lasting 30 – 45s. The correlation curve is obtained live and can be used to determine if the observation volume was properly located on the plasma membrane. Should the location not be accurate, a bad auto-correlation function (ACF) will be obtained where the degree of correlation does not decay well with increasing time. Raw ACFs were then analyzed using the Igor Pro software and fitted with suitable diffusion models to obtain τ_D values of the probe. The 2-dimensional, 2-particle (2D_2p) model was used for particles diffusing on a membrane and the 3-dimensional, 1-particle, 1-triplet (3D_1p1t) model was used for particles diffusing in solution. Koppel's formula was used as the weighting method to fit the ACFs. ACFs which returned poor curve fitting results were excluded from the final analysis. Mean and standard deviations of the τ_D values for each sample were calculated using Microsoft Excel. All confocal FCS measurements were conducted on an open microscope stage at room temperature. The mean translational diffusion time, τ_D , was obtained for each sample in each experiment. Differences in the calibration of the equipment on different occasions directly affect τ_D values, therefore τ_D values from different experiments were not pooled even if the treatments were identical – for statistical analysis, only sets of data from experiment conducted in a single run, that is, data obtained using the same calibration of the equipment, were compared.

2.7. Imaging Total Internal Reflectance-FCS (ITIR-FCS)

To measure diffusion on the bilayers via ITIR-FCS, a TIRF microscope (Olympus IX-71, Olympus) and a high magnification, high NA oil immersion objective lens (PlanApo, 100x, NA 1.45) were used. Data is acquired in the form of images. An EMCCD camera (Andor iXON 860) the Andor Solis software was used for image acquisition. A 10-pixel by 10-pixel region of A 10 x 10-pixel region of interest (ROI) on the cell membrane was selected for imaging, which is typically the largest size that can be used without exceeding the area of the basal cell membrane of differentiated SH-SY5Y cells, which had the smallest cell body out of all the cell types we used. For consistency sake, the size of the ROI was set as 10 x 10 pixels for all ITIR-FCS experiments, except for a few earlier measurements with undifferentiated SH-SY5Y (12 x 12 pixels). Each pixel on the image has a side length corresponding to 240 nm in the sample at 100x magnification. 30,000 frames with 2 ms exposure time were recorded for the slower-diffusing probes, CtxB and GPI, and 50,000 frames with 1 ms exposure time was used for faster diffusing probe DiI as well as for Rho-PE in measurements on supported planar bilayers. Longer exposure times improve signal-to-noise ratios but shorter exposure times are necessary when using fast diffusing probes, as temporal resolution will be compromised if the exposure time is longer than typical transitional diffusion times for the observed probe. Less frames were recorded for CtxB to avoid photobleaching effects. This is undesirable as the gradual decrease in average fluorescence intensity over the time of a single measurement would pose difficulties when generating auto-correlation functions. ITIR-FCS images were analyzed with an IGOR Pro-based homebrew software, ImFCS, software using the two-dimensional 1-particle fitting model (diffusion on a membrane) (Sankaran et al., 2010). Statistical analysis was done by comparing sample means using GraphPad Prism.

ITIR-FCS on supported planar lipid bilayers. All lipids used were purchased from Avanti Polar Lipids (Alabaster, AL). The protocol for making supported planar bilayers (SPB) was adapted from Nirmalya Bag and Thorsten Wohland (Bag et al., 2013b). All glassware was cleaned with the following procedure to remove impurities that may hinder formation of the SPB: first, glassware was scrubbed with Hellmanex detergent (Sigma, St. Louis, MO), diluted 1:10 with distilled water, and washed with distilled water. The glassware was then filled with detergent and

sonicated for 30 minutes in a bath sonicator. After sonication, the glassware was rinsed 30 times with distilled water, filled with 2 M H₂SO₄ and sonicated again for 30 minutes. After that, glassware was rinsed 50 times with distilled water, filled up with distilled water and sonicated once more for 30 minutes. Finally, the glassware was rinsed once with 100% ethanol, and stored filled with ethanol until ready for use.

Stock solutions of lipids were prepared by dissolving the dry lipids in chloroform. GM1 was dissolved in a chloroform/methanol 1:1 mixture. Stock lipids were stored in glass round-bottomed flasks at -20°C. Stock lipids were warmed to room temperature and agitated briefly by pulsing with a vortex machine to ensure total solvation of the lipids before use. Referencing Kenji Sasahara and Shinya Kyoko (Sasahara et al., 2013), SPBs were prepared with a lipid composition of DOPC/SM/Chol (0.5/0.25/0.25). Appropriate amounts of each lipid was transferred to a round-bottomed flask using glass Hamilton syringes (Sigma, St. Louis, MO), for a total lipid concentration excluding cholesterol of 0.5 mM in the final target lipid solution volume (2 mL). GM1 and Rho-PE were also added to final concentrations of 0.0125 mM (2.5%) and 40 nM (0.008%) respectively, Rho-PE being the fluorescent membrane probe. The syringes were rinsed with three times each with absolute ethanol and chloroform before use. Between each transfer of lipids, the syringes were rinsed thrice with chloroform. The round-bottomed flask was then affixed to a rotating vacuum vaporizer and the solvent allowed to evaporate, leaving behind a thin film of lipid on the bottom of the flask. The lipid film was then dissolved in 2 mL of buffer, which we call “Buffer A” (100 mM HEPES, 150 mM NaCl, pH 7.4, all reagents from Sigma, St Louis, MO) to give a 0.5 mM lipid solution. Unused lipid solutions were stored in the fridge at 4°C. On the day of the experiment, lipid solutions were warmed to room temperature and pulsed on a vortex machine before use. First, the lipid solution was sonicated in a bath sonicator for 20 minutes to form lipid vesicles. Next, the vesicle/lipid mixture was added 1:1 to Buffer A in new, clean chambered coverslips, ensuring that the lipid solution spread throughout the well. The coverslips were then incubated in the oven at 65°C for 20 minutes followed by cooling at room temperature for another 20 minutes for fusion of liposomes to form a planar lipid bilayer on the surface of the coverslip. After that, half of the solution was removed followed by addition of an equal amount of Buffer A. This was repeated 50 times to wash off excess lipid vesicles. The resulting supported planar bilayer was then used for ITIR-FCS experiments.

Cholesterol depletion and A β experiments. 8-well chambered coverslips containing the SPBs in each well was mounted in an environmental chamber on the microscope stage. The settings for the environmental chamber was 37°C with humidified air and 5% CO₂ to maintain identical conditions that will be used for live cell experiments. A stock (20 mM) solution of m β CD (Sigma, St. Louis, MO) was prepared by dissolving dry m β CD in Buffer A. m β CD solution was added to each well for a final concentration of 10 mM. For vehicle controls, an equal amount of Buffer A alone was added. As each SPB is expected to be a homogenous lipid bilayer, measurements taken from different spots on a single membrane are not expected to show vast differences in diffusivity. For kinetic measurements, images were recorded on different spots but on the same SPB every 5 min over a total time of 30 min following addition of m β CD or Vehicle. Recordings were taken from 1 SPB each for both treatment and controls. For endpoint measurements, images were recorded from 5 different spots on a single SPB after incubation with the treatment solution for 30 min. Recordings were taken from 3 SPBs each for both treatment and controls. 50,000 frames with an exposure time of 1 ms each were recorded for each measurement on the SPB. SPBs were similarly prepared for A β experiments. All the buffer was removed from the bilayer and replaced with either ddH₂O or A β solution, as quickly as possible to prevent drying out of the membrane. A mixture of images at either 50,000 frames at 1 ms exposure or 30,000 at 2 ms exposure were taken. All ACFs were fitted with the ImFCS software using the 2-dimensional 1-particle model for diffusion. Further consolidation and analysis of the data was done using Microsoft Excel and GraphPad Prism. For live-cell experiments, similar recording parameters were employed, except that the membrane was stained with CtxB and m β CD was dissolved in HBSS instead of Buffer A. All cells were kept in HBSS for the duration of the experiment.

ITIR-FCS on living cell membranes. Cells were seeded in LabTek 8-well chambered coverslips per the procedures detailed in section 2.3. Cells were exposed to A β (1-42) or A β vehicle for varying amounts of time up to 48h. Stock CtxB solutions were prepared according to the procedure detailed in section 2.7. Stock DiI solutions were prepared as such: a small amount of C-18 DiI perchlorate salt (ThermoFisher, Waltham, MA) was picked using a micropipette tip, and dissolved in 1 mL of anhydrous DMSO in a microcentrifuge tube. The solution was vortexed for 30s to ensure that all the DiI was dissolved. The absorbance spectrum of the DiI solution

was obtained using a Cary UV-Vis spectrophotometer. Concentration of the DiI was determined using the Beer-Lambert law, given by $A = \epsilon cl$, where A is the peak absorbance, ϵ is the molar extinction coefficient, c is the molar concentration and l is the pathlength of the cuvette. Fresh staining solutions were prepared before each experiment by diluting the stock probe solutions in HBSS to obtain a final concentration of 50 nM and 100 nM for CtxB and DiI respectively. To stain the cells, the cell culture medium was first removed and set aside, and cells were incubated with staining solutions for 20 min at 37°C followed by washing twice with pre-warmed HBSS. It is crucial that the staining solution contains Ca^{2+} and Mg^{2+} , otherwise cells may detach during the staining procedure. The original cell culture medium was kept warm in the incubator and was returned to the respective cell culture wells after staining to ensure that A β was present in the medium during the entire FCS experiment. For GFP-GPI experiments, cells were transiently transfected with a GFP-GPI plasmid using the Lipofectamine 3000 transfection reagent (ThermoFisher, Waltham, MA) per manufacturer recommended protocol. No staining step was required for the GFP-GPI samples. In all ITIR-FCS experiments involving living cells, chambered coverslips containing cells were mounted on the microscope stage in an environmental chamber that provided humidified air and maintained a temperature of 37°C and 5% CO_2 throughout the experiment. The living cell membrane can be highly heterogeneous, especially for lipids such as GM1 that diffuse in and out of various types of membrane subdomains (Renner et al., 2009). Therefore, for membrane probes tracking these types of lipids, such as CtxB, some curves will be poorly fitted because they are outliers compared to the bulk of the observed probe. Such poorly-fitted curves that were not included in further analysis of the data. FCS diffusion law analysis was run using the same software to obtain τ_0 , the transit time of the fluorescent molecules at an effective observation area (A_{eff}) of 0, which is representative of the degree of heterogeneity of the membrane with respect to the molecules being observed. Further consolidation and analysis of the data was done using Microsoft Excel.

2.8. Data and Statistical Analysis

Data were plotted and analyzed using MS Office Excel and GraphPad Prism. All statistical tests were carried out as unpaired, two-tailed tests with 95% confidence interval (95% C.I.).

3. Results

3.1. Generation and characterization of small, soluble A β (1-42) aggregates

Small, soluble A β aggregates were reliably generated using the protocol that had been adapted from William Klein and Mary Jo LaDu (Klein, 2002; Stine et al., 2011). We first tried to replicate the results of previously released studies as well as optimize our protocol. The protocols we had adapted our protocol from called for the use of F12 medium as the main diluent for the generation of small, soluble toxic A β aggregates, typically ranging from dimers to tetramers. F12 medium was used as it is at a physiological pH and ionic strength, which are important factors influencing A β aggregation. In our study, DMEM/F12 instead. We started by incubating the peptide for 24h at three temperatures – at 4°C in the cold room, at room temperature on the lab bench and at 37°C in the cell culture incubator. The aggregates present comprise mainly trimers and tetramers with molecular weights approximating 13.5 kDa and 18 kDa respectively, some faint bands of what seem to be dimers at 9 kDa and a comparatively large amount of unaggregated monomers with MW of 4.5 kDa (Fig. 10). The aggregates appear to be moderately heat labile, as heating for 10 min at 70°C reduced amounts of trimers and tetramers, especially the tetramers. The quantity of trimers and tetramers in the samples incubated at room temperature and 37°C seem to be less than in the sample incubated at 4°C. A possible reason for this could be that in these samples, large, insoluble aggregates had formed that were either pelleted during the centrifuging step, or unable to enter the PAGE gel, which in our set up is effective for separating proteins of between 3.5 – 160 kD. The types of aggregates generated at all three incubation temperatures (Fig. 10) were comparable to data from several previous studies (Dahlgren et al., 2002; Davis et al., 2011; Klein, 2002; Stine et al., 2003), indicating that our modified protocol is effective for the generation of A β oligomers.

Because we wanted to work with differentiated SH-SY5Y cells, which are cultured with Neurobasal/B27 instead of DMEM/F12 as the base medium, we wanted to make the A β aggregates using Neurobasal medium as the base medium so that there would be minimal changes in the cell culture conditions for differentiated SH-SY5Y cells when treated with A β . This is because we expect the differentiated SH-SY5Y cells to be more sensitive due to increased neuronal character. Using supplement-free

Neurobasal medium as the diluent for aggregating A β allowed the generation of A β aggregates that were identical to that generated with DMEM/F12 as the diluent (Fig. 11a, lanes 1 and 2 vs 3 and 4), at both 4°C (Lanes 1 and 3) and room temperature. We attempted to increase the quantity of trimers and tetramers in the solution by incubating the peptide solution for a longer time. Incubating A β in Neurobasal medium at 4°C for 72h resulted in an increase in the quantity of trimers and tetramers generated (Fig. 11b). Interestingly, there were no ‘dimer’ bands visible in the gels in these and subsequent SDS-PAGE experiments, even at higher aggregate quantities (Fig. 11b, 12). With the evidence that Neurobasal medium allowed for similar A β aggregates to form as in DMEM/F12, we used Neurobasal medium as the diluent for all subsequent A β preparations. Apart from wild-type A β , we also used fluorophore-tagged amyloid-beta, HiLyte Fluor 488-A β (1-42) (referred to as “fl-A β ”) and scrambled A β (1-42) (referred to as “scr-A β ”) for our cell uptake and cytotoxicity studies respectively. Thus, we also examined the aggregation profile of these

Figure 10. SDS-PAGE analysis of A β aggregation

Samples containing a total A β concentration of 100 μ M were incubated for 24h at different temperatures to allow for generation of small, soluble A β aggregates. These aggregates were small enough not to be pelleted after centrifuging for 10 min at 14,000x g. Monomers, trimers and tetramers were generated at 4°C, room temperature and at 37°C, suggesting that formation of these types of aggregates are temperature-independent, probably being related more to the pH and ionic strength of the aqueous medium. Faint bands corresponding to what seems to be dimers are also observed. It is unclear whether these bands are truly indicative of dimers or simply an artefact. Heated samples were subject to 10 min of heating in Eppendorf tubes at 70°C on a heating block.

Figure 11. SDS-PAGE analysis of A β aggregate preparation using Neurobasal medium.

(a) 1 and 2 – A β incubated for 24h in supplement-free Neurobasal medium. 3 and 4 – A β incubated for 24h in supplement-free DMEM/F12. Lanes 1 and 3 were samples incubated at 4°C, Lanes 2 and 4 were samples incubated at room temperature. “Mo” – A β monomers, “Tri” – A β trimers, “Tet” – A β tetramers. (b) A β incubated for 72h at 4°C in Neurobasal medium. As can be observed, incubating A β for a longer time at 4°C increases the amount of small, soluble aggregates (trimers/tetramers)

Figure 12. SDS-PAGE analysis of variant A β peptides.

wt-A β - wild-type sequence A β (1-42); fl-A β – N-terminal HiLyte 488-tagged A β (1-42); scr-A β – scrambled sequence A β (1-42). ‘wt-fl’ – A β mixtures containing wt-A β and fl-A β at the stated ratios

peptides. Pure solutions of fl-A β formed similar types of aggregates as wild-type A β (1-42) (referred to as “wt-A β ”) (Fig. 12). When mixed together with wt-A β at wt-fl ratios of 3:1, 1:1 and 1:3, the resulting mixture also formed similar aggregates, although at varying quantities (Fig. 12). No aggregates were formed that were not also present in pure wt-A β preparations. and all species of aggregates formed in pure wt-A β preparations were also found in all fl-A β -containing preparations, suggesting that fl-A β aggregated in an identical way as wt-A β , albeit at a different rate. The scr-A β preparation had a very different aggregation profile compared to that of wt-A β – only dimers were present, as well as a large amount of unaggregated peptide (Fig. 12, rightmost lane).

3.2. Selection of cell types for experimentation with amyloid-beta

Several different types of cells have been used to conduct *in vitro* studies with A β . In our study, we decided to use SH-SY5Y human neuroblastoma cells as the primary cell type, along the U87 human glioma and Neuro-2a mouse neuroblastoma cells (Fig. 13). These cell types were chosen due to their origin from neuronal or brain tissue. According to ATCC documentation, SH-SY5Y cells originated from a metastatic bone tumor, U87 were obtained from malignant gliomas and Neuro-2a were obtained from a spontaneous tumor of an albino mouse. The SH-SY5Y and Neuro-2a cell lines are also commonly used in A β -related studies, and the SH-SY5Y cell line can be induced to differentiate by culturing in medium containing 10 μ M retinoic acid over a period of 7 days, after which they become more sensitive to A β -induced cytotoxicity (Lambert and Stevens, 1994). The cell types are referred to as the following from this point on: SH-ND or ND – non-differentiated SH-SY5Y cells; SH-RAI or RAI – retinoic acid-induced differentiated SH-SY5Y cells; U87 – U87 glioma; and N2a – Neuro-2a mouse neuroblastoma. We also conducted immunocytochemistry experiments on retinoic acid-differentiated SH-SY5Y cells (referred to as ‘SH-RAI’ for the rest of this report) to examine those cells for the neuronal markers MAP-2, GAP43 and NF-H (Supplementary Fig. S1).

Figure 13. Cell types used in the present study.

(a) SH-SY5Y human neuroblastoma cells. (b) retinoic acid-differentiated SH-SY5Y cells, (c) U87 human glioma cells and (d) Neuro-2a mouse neuroblastoma cells. Scale bar = 50 μm

3.3. A β is internalized by all selected cell types

Using a preparation of A β mixed at a wt-fl ratio of 3:1, we investigated whether cells would internalize soluble A β in the solution. The three cell types that we selected for experiments with A β all could internalize the peptide within 24h of exposure to 10 μM of A β . Internalized A β show up as punctate green areas within the cytosol, delineated by the cell plasma membrane, which is shown in red, stained with the lipophilic dye C18-DiI (Fig. 14). Different staining patterns of the cell plasma membrane were observed for each cell type, possibly due to differences in staining efficiency caused by variations in lipid content of the different plasma membranes. Cell nuclei were stained with Hoechst 33342 and are shown in blue.

Figure 14. Internalization of A β by SH-SY5Y, U87 and Neuro-2a cells

(g and h overleaf) (a) and (b) – non-differentiated SH-SY5Y human neuroblastoma cells; (c) and (d) – retinoic acid-induced differentiated SH-SY5Y cells; (e) and (f) – U87 glioma cells; (g) and (h) Neuro-2a mouse neuroblastoma cells. (a), (c), (e), (g) – Vehicle-treated controls, (b), (d), (f), (h) – cells incubated with 10 μ M of wt-fluorescent (3:1) A β mixture for 24h. Blue – cell nuclei (Hoechst), green – fluorescent (HiLyte-488) A β , red – cell plasma membrane (DiI). Scale bar – 20 μ m

3.4. A β induces time- and concentration-dependent apoptosis in selected cells

The effect of A β on the selected cell types were assessed via viability and caspase activation assays. A β does not cause significant reduction of cell viability or induction of apoptosis within 24h. A β treatment significantly reduced viability and induced apoptosis compared to vehicle-treated cells in a concentration-dependent manner in SH-SY5Y cells within 48 – 72h (Fig. 15). Following this, we assessed the activity of A β (1-42) against a scrambled A β peptide in the U87 and Neuro-2a cell lines (Tables 1a – 1c). We limited the test to 72h because in the earlier experiment with SH-SY5Y cells, significant effects had been observed within 72h of A β treatment. In addition to statistical significance of the wt-A β response over either vehicle or scrambled-A β response, the following conditions were deemed to be necessary for a exposure time/A β -concentration combination to be considered significant: 1. An observation at a certain concentration should also be observed at higher concentrations at the same time point, 2. Caspase 3/7 activation can occur before cell viability is reduced but not vice versa, 3. Reduction in cell viability should always be accompanied with significant caspase 3/7 activation at the same time point and 4. Scrambled A β should cause worse outcomes for cells than wild-type A β , in which case wt-A β would be deemed to have no significant cellular activity. A β was deemed to have

significant effects on a cell type if it both reduced cell viability *and* triggered apoptosis within 72h with a maximum of 20 μ M A β (Table 1c.). The results here indicate two things – firstly, that the effects observed are expressly due to wt-A β and secondly, different cell types have different sensitivity to A β , with differentiated SH-

SY5Y cells (human neuroblastoma) being the most sensitive, followed by undifferentiated SH-SY5Y cells and then equally by U87 (human glioma) and Neuro-2a (mouse neuroblastoma).

a) Viability of A β -treated SH-SY5Y cells

b) Caspase 3/7 Activation in A β -treated SH-SY5Y cells

Figure 15. Effect of A β on SH-SY5Y cells

(a) Assessment of cell viability and (b) assessment of caspase activation in A β -treated cells. Caspase 3/7 activation is an indicator of apoptosis. RAI – retinoic acid-induced differentiated SH-SY5Y cells. ND – non-differentiated SH-SY5Y cells. Veh – vehicle control-treated cells. Concentrations indicated are that of wt-A β in the cell culture medium. Statistical analysis: two-tailed two-way ANOVA with Tukey's post-hoc test, ** - $p < 0.01$, † - $p < 0.0001$, 95% C.I

Assessment of A β effects on cell viability: wild-type peptide vs. scrambled peptide						
Cell Type	Significant difference vs. scrambled?				Does the wt-treated have higher or lower viability?	Does A β change Cell Viability of these cells?
	Time	1 μ M	10 μ M	20 μ M		
SH-RAI	48h	N	N	Y	Lower	Yes
	72h	Y	Y	Y	Lower	
SH-ND	48h	N	N	Y	Lower	Yes
	72h	N	N	Y	Lower	
U87	48h	N	N	N	-	No
	72h	N	N	N	-	
N2a	48h	N	N	Y	Higher	No
	72h	N	N	N	-	

Table 1a. Assessment of wt-A β effect on cell viability within 72h of exposure

Assessment of A β effects on caspase 3/7 activation: wild-type peptide vs. scrambled peptide						
Cell Type	Significant difference vs. scrambled?				Are caspase 3/7 levels higher or lower in the wt-treated cells?	Does A β trigger apoptosis in these cells?
	Time	1 μ M	10 μ M	20 μ M		
SH-RAI	48h	N	N	Y	Higher	Yes
	72h	N	Y	Y	Higher	
SH-ND	48h	N	N	Y	Higher	Yes
	72h	N	Y	Y	Higher	
U87	48h	N	N	N	-	Yes
	72h	N	N	Y	Higher	
N2a	48h	N	N	Y	Higher	Yes
	72h	N	N	Y	Higher	

Table 1b. Assessment of wt-A β effect on caspase 3/7 activation within 72h of exposure

Cell response within 72h of exposure to A β (up to 20 μ M)			
Cell Type	A β Reduces Viability	A β Triggers Apoptosis	Does A β have a detrimental effect* on cell health within 72h?
SH-RAI	Yes	Yes	Yes
SH-ND	Yes	Yes	Yes
U87	No	Yes	No
N2a	No	Yes	No

Table 1c. Summary of the effect of wt-A β on selected cell types within 72h of exposure

SH-RAI - retinoic acid-induced differentiated SH-SY5Y cells. SH-ND – non-differentiated SH-SY5Y cells. U87 – U87 glioma cells. N2A – Neuro-2a mouse neuroblastoma cells. Statistical analysis: two-tailed unpaired two-way ANOVA with Tukey’s post-hoc test, 95% C.I, minimum acceptable p value - $p < 0.05$. Detailed statistical analysis and raw data available in Supplementary Tables S1a – d.

3.5. Validation of our confocal FCS system for studying membrane diffusion

Confocal FCS was sensitive enough to distinguish between a small molecule (Atto-488 dye), cholera-toxin B (Alexa 488-CtxB) in solution and membrane-bound CtxB (Fig. 16). Atto-488 is the reference dye used to calibrate the system before each

experiment. Calibration is done to ensure that the shape of the confocal detection volume is in an acceptable range. This is the “k-factor”, which is the ratio between the longitudinal length and the radius of the beam waist of the focused beam (z and ω respectively, refer to Fig. 4), with a ratio between 5 and 10 considered acceptable. This is important as the main readout obtained from confocal FCS experiments is the transitional diffusion time of the molecule, τ_D , which depends on the dimensions of the detection area/volume. In our results presented below, the calculated k-factor of the calibrations for all experiments ranged from 5.7 to 7.3. We chose CtxB as the membrane probe as it binds to GM1, and is a commonly used marker for the liquid-ordered phase or raft-type membranes. The instrument used in Hossain and Pramanik’s study on membrane-bound A β had a k-factor of 5 ($\omega = 0.25 \mu\text{m}$ and $z = 1.25 \mu\text{m}$) (Hossain et al., 2007). In their experiment, unbound Rho-Lys-A β (~5.1 kDa) had a τ_D of 100 μs . Nag and Maiti reported the τ_D of fluorescein (0.33 kDa) in

Figure 16. Confocal FCS is a sensitive tool for measuring diffusion of small particles.

Experimentally obtained auto-correlation functions (red, fluctuating lines) for the reference dye (Atto 488) and our membrane probe (CtxB) were fitted (solid lines) with appropriate diffusion models. Atto-488 is a water-soluble fluorescent dye with a molecular weight of about 0.8 kDa. It is commonly used as a calibration dye for confocal FCS because its diffusion characteristics is well known. Atto-488 has a typical τ_D of ~40 μs in water. The molecular weight of pentameric CtxB is ~60 kDa and has a measured τ_D of 215 μs in Neurobasal/B27 medium. In DMEM/F12 with 10% FBS, the τ_D of CtxB is 340 μs . τ_D of CtxB bound to the apical membrane of SH-SY5Y cells usually range between 20 – 80 ms. For the figure above, a representative curve for each molecule with similar τ_D as the mean value for that molecule is shown. The fitted curve for CtxB on the apical cell membrane (black line) is only fitted for times $> 10^{-4}$ s because the 2-dimensional, 2 particle model was used for fitting, which does not include the triplet state (typically not exceeding 10^{-5} s).

water to be 32 μs , although they did not state the s and u of their instrument (Nag et al., 2010). In our experiments, we measured the τ_D of Atto-488 (0.8 kDa) in water to be 40 μs and the τ_D of Alexa 488-CtxB (approx. 60 kDa) to be 215 μs and 340 μs in aqueous, serum-free and serum-containing medium respectively. Our measured τ_D of CtxB is only about 2-3 times that of Pramanik's reported τ_D for Rho-Lys-A β despite the MW of CtxB being about 12 times larger than A β . Due to the power term (1/3) in equation 1.7, in effect a 12-fold increase in MW would result in only a 2.2-fold increase in the translational diffusion time, τ_D . Taking into account the effect of serum components on viscosity and the possible binding of serum proteins to CtxB in serum-containing medium, our measured τ_D values of both standard dyes and our chosen membrane probe are within expectations and validates the calibration of our instrument for FCS.

3.6. Validation of ITIR-FCS as a suitable method of studying membrane fluidity

To validate the ability of ITIR-FCS to detect changes in membrane fluidity a cholesterol depletion experiment was set up using artificially created supported planar bilayers (SPB). Taking reference from Sasahara and Shinya (Sasahara et al., 2013), we used a SPB composed of similar lipids in similar proportions. Sasahara and Shinya made a DOPC/DOPS/SM/Chol (0.4/0.1/0.25/0.25) bilayer, which simulates raft-type membrane conditions, with a GM1 content ranging from 1.3 – 3.8% and 1% Texas Red-PE (TR-PE) as the fluorescent probe. We used a DOPC/SM/Chol (0.5/0.25/0.25) bilayer with GM1 content of 2.5% and 0.008% Rho-PE as the fluorescent probe. Sasahara and Shinya, using fluorescence recovery after photobleaching (FRAP), reported the diffusivity of TR-PE in resting membranes to be 1.10 $\mu\text{m}^2\text{s}^{-1}$. In our setup, the diffusivity of Rho-PE in resting membranes was $0.91 \pm 0.26 \mu\text{m}^2\text{s}^{-1}$ (Table 2) and agrees with previously reported values. We attempted to induce changes in the membrane fluidity of the SPB and see whether ITIR-FCS would detect the change. First, we measured the endpoint diffusion coefficient, of cholesterol-depleted membranes. SPBs were treated for 30 minutes with either m β CD or control vehicle, after which images were taken and analyzed. Diffusivity of the membrane is expected to increase upon cholesterol depletion, since cholesterol increases the degree of order in the membrane. As expected, D values of

Effects of cholesterol depletion on supported lipid bilayers (endpoint)

Figure 17. Endpoint observation of cholesterol depletion of supported planar bilayers

The diffusivity of Rho-PE in DOPC/SM/Chol (0.5/0.25/0.25) supported planar bilayers with an added 2.5% molar concentration GM1 content was measured using ITIR-FCS. Diffusion coefficients, D , are presented as mean $\pm$ S.D. Detailed D data in Table 2.

Cholesterol depletion of supported planar bilayers by mβCD (endpoint)				
State / Treatment of SPB	Mean D ($\mu\text{m}^2 \text{s}^{-1}$)	N (SPBs/Acquisitions)	ANOVA	p -value
Resting	0.9 ± 0.3	2 / 10	-	-
Vehicle	1.0 ± 0.2	3 / 15	N.S.	0.521
10 mM mβCD	1.3 ± 0.3	3 / 16	**	0.005

Table 2. Endpoint diffusion coefficients of supported planar bilayers treated with mβCD

Multiple images, typically 5, were acquired from different spots each SPB. Statistical analysis: two-tailed unpaired one-way ANOVA with post-hoc Dunnett's multiple comparisons test, 95% C.I.

the membrane increased after mβCD treatment, (mβCD-treated = $1.26 \pm 0.32 \mu\text{m}^2 \text{s}^{-1}$, vehicle-treated control = $1.02 \pm 0.16 \mu\text{m}^2 \text{s}^{-1}$; resting = $0.91 \pm 0.26 \mu\text{m}^2 \text{s}^{-1}$) (Table 2, Fig.18). S.D. values of the mean D for all 3 conditions were ~25%, which shows that the membrane diffusion coefficients measured by ITIR-FCS are consistent for a given condition and are reliable. Next, we did a kinetic measurement of the membrane. 5 images were taken on two separate SPBs and the average D taken to be the resting state diffusivity of the membrane. Next, either 10 mM mβCD or control vehicle was added to the membrane and 1 image was taken every 5 min for the next 30 min. Images were taken from different spots on the membrane and the D . The plot of the change in D shows that within 5 min of addition of mβCD, the membrane diffusivity had increased significantly and stayed at an elevated level for at least 30 min (Fig. 18). At every time point measured, D for the mβCD-treated membrane was higher than that of the resting state up to 30 min (Table 3).

Effects of cholesterol depletion on supported planar bilayers (kinetic)

Figure 18. Kinetic measurements of effects cholesterol-depletion on SPBs

The diffusivity of Rho-PE in DOPC/SM/Chol (0.5/0.25/0.25) supported planar bilayers (SPBs) with an added 2.5% molar concentration GM1 content was measured using ITIR-FCS. To obtain the average resting D of the SPB, Five images were taken from separate spots on the each SPB used. This D value was used for the D at time = 0. After addition of either 10 mM mβCD or vehicle, 1 image was taken every 5 min for a total of 30 min.

Cholesterol depletion of supported planar bilayers by mβCD (kinetic)						
Time after treatment (min)	D (Vehicle) ($\mu\text{m}^2 \text{s}^{-1}$)	ANOVA	p -value	D (mβCD) ($\mu\text{m}^2 \text{s}^{-1}$)	ANOVA	p -value
0	0.94 ± 0.4	-	-	0.89 ± 0.2	-	-
5	0.97 ± 0.3	N.S.	0.888	1.5 ± 0.4	***	< 0.001
10	1.0 ± 0.3	N.S.	0.154	1.7 ± 0.5	***	< 0.001
15	0.86 ± 0.2	N.S.	0.158	1.2 ± 0.3	***	< 0.001
20	0.83 ± 0.3	*	0.021	1.2 ± 0.4	***	< 0.001
25	1.0 ± 0.3	N.S.	0.065	1.4 ± 0.5	***	< 0.001
30	0.55 ± 0.2	***	< 0.001	1.4 ± 0.5	***	< 0.001

Table 3. Real-time ITIR-FCS measurements of diffusion coefficients of supported planar bilayers treated with mβCD

Diffusion coefficients, D , are represented as mean ± S.D. The D of an SPB at each time point was compared against the D for the same SPB at time = 0. Statistics: two-tailed unpaired one-way ANOVA with Dunnett's multiple comparisons test, 95% C.I.

3.7. Validating the utility of ITIR-FCS for studying membrane fluidity in living cells

To make sure that our set up was sensitive enough to capture changes in membrane fluidity, we measured the diffusion of the bulk membrane probe, C18-DiI and the

raft-type membrane probe, CtxB. Fluidity of the bulk plasma membrane was changed by simply changing the ambient temperature of the surroundings via means of an environmental chamber. Diffusion of cell plasma membrane-bound DiI was first measured at 25°C, followed by a ramping up of the temperature to 37°C and the diffusion of DiI measured again. CtxB binds GM1 in the raft-type domains of the cell plasma membrane. These membrane domains are cholesterol-dependent and thus we used mβCD to deplete membrane cholesterol levels (5 mM mβCD, 30 min) and change the diffusivity of CtxB. The results are plotted in Table 4. SH-SY5Y cells were the most sensitive to these treatments, with DiI and CtxB values changing significantly post-treatment. Neuro-2a cells were similarly sensitive with respect to DiI diffusivity, but less sensitive with respect to CtxB. For U87 cells, no significant changes in diffusivity were found for either DiI or CtxB post-treatment. This suggests that DiI and CtxB may not be optimal membrane probes for U87. It is notable however that mean *D* values for DiI in U87 cells do increase after the temperature was increased, although this increase was not statistically significant perhaps due to the small sample size (*N* = 4) and relatively higher S.D compared to SH-SY5Y and Neuro-2a cells. Detailed ITIR-FCS data for control experiments can be found in the Supplementary Data.

Measuring changes in bulk and raft-type membrane fluidity by ITIR-FCS				
	Mean <i>D</i> of DiI (μm ² s ⁻¹)		Mean <i>D</i> of CtxB (μm ² s ⁻¹)	
	25°C	37°C	Resting	5 mM mβCD
SH-ND	1.5 ± 0.14	2.2 ± 0.20	0.16 ± 0.05	0.08 ± 0.008
<i>N</i> (cells)	4	4	4	4
ANOVA <i>p</i> -value	0.028 *		< 0.001 ***	
U87	0.69 ± 0.44	1.3 ± 0.42	0.09 ± 0.04	0.11 ± 0.03
<i>N</i> (cells)	4	4	4	4
ANOVA <i>p</i> -value	0.1121 (N.S.)		0.4805 (N.S.)	
N2a	1.0 ± 0.14	1.4 ± 0.22	0.12 ± 0.03	0.08 ± 0.02
<i>N</i> (cells)	4	4	4	4
ANOVA <i>p</i> -value	0.021 *		0.0741 (N.S.)	

Table 4. Control experiments for selected membrane probes DiI and CtxB

DiI is a lipophilic dye that inserts into the cell membrane, and is a reporter of bulk membrane fluidity. The bulk membrane fluidity can be easily altered by changing the ambient temperature. CtxB binds to the ganglioside GM1, found in cholesterol and sphingolipid-dependent raft-type membrane domains. Fluidity of raft-type membrane domains can be altered by cholesterol depletion. Diffusion coefficients presented as mean ± S.D. Statistical analysis: two-tailed unpaired Student's *t*-test with Welch's correction, 95% C.I.

3.8. Sub-toxic concentrations of A β do not change fluidity of CtxB on live cell membranes

We began probing the effects of A β on cell membrane fluidity by using confocal FCS to measure the diffusion of CtxB on live SH-SY5Y cells. For this experiment, the cells were grown in Neurobasal/B27 for 5 days before exposure to 1 μ M of A β for 24h. Confocal FCS measurements of CtxB on the apical cell membrane of these cells show that there is no significant difference in the diffusion of membrane-bound CtxB between A β -treated and control vehicle-treated cells (Table 5). Auto-correlation functions were fitted with a 2-dimensional, 2-particle (2D_2p) diffusion model. The τ_D of freely diffusing CtxB was previously experimentally determined to be about 215 μ s in Neurobasal/B27 medium (Fig. 16, blue line) and this value was fixed as the τ_D of the first particle in fitting of the ACF, representing non-membrane bound CtxB. The τ_D of membrane-bound CtxB was calculated from the fit for each measurement in the sample, and the sample average τ_D is represented in Table 5 as mean $\pm$ S.D. The number N represents the number of ACFs (individual measurements) successfully fitted, with the number in parentheses representing the total number of cells measured. For example, in experiment 1, 4 measurements per cell were made on 10 cells in the control set, from which a total of 30 measurements

Confocal FCS analysis of CtxB diffusion on SH-SY5Y cells							
Experiment	Mean τ_D Vehicle Control (ms)	Mean τ_D A β -Treated (ms)	Measurement time (s)	N Control	N Treated	Did A β cause a statistically significant change in fluidity?	Increase or Decrease?
1	34.1 $\pm$ 11.7	26.7 $\pm$ 9.1	30	30 (10)	34 (11)	No	N/A
2	18.5 $\pm$ 5.7	18.7 $\pm$ 6.2	45	45 (15)	46 (20)	No	N/A
3	46.7 $\pm$ 16.7	58 $\pm$ 33.9	45	47 (12)	40 (12)	Yes ($p = 0.0046$)	Increase
4	63.9 $\pm$ 36	56.6 $\pm$ 19.9	45	52 (16)	72 (20)	Yes ($p = 0.0303$)	Decrease
5	32.7 $\pm$ 11.4	34.4 $\pm$ 9.8	45	38 (10)	33 (15)	No	N/A
6	35.2 $\pm$ 13.7	29.4 $\pm$ 10.9	30	59 (21)	62 (22)	No	N/A

Table 5. Confocal FCS measurements of membrane-bound Alexa 488-CtxB on SH-SY5Y cells

Translational diffusion times (τ_D) of membrane-bound CtxB were obtained from the apical membrane of SH-SY5Y cells cultured in neuronal medium and treated with 1 μ M A β for 24h. Each measurement run consists of 4-6 consecutive measurements of the fluorescence intensity on a single spot on the cell membrane of a single cell, each giving one auto-correlation function. The measurement time refers to the time per single measurement. Measurements were made on several separate cells in each sample. N here refers to the total number of recordings; the number in parentheses indicate the number of separate cells measured for each respective sample. Statistical analysis – two-tailed unpaired one-way ANOVA with Fisher's LSD for comparison of means within each group (experiment), 95% C.I.

were successfully fitted with the 2D_2p diffusion model. Some ACFs could not be properly fitted because of various reasons, including photobleaching effects, presence of large CtxB aggregates and movement of the apical cell membrane out of the detection volume during the acquisition process, all of which will negatively affect fitting of the ACFs. Only in 2 of the 6 experiments (Table 5, experiments no. 3 and 4) was there a significant difference between fluidity of membrane-bound CtxB on cells treated with 1 μ M A β versus vehicle-treated control cells. Additionally, the trend is not consistent – in experiment 3, A β -treated cells exhibited an increase in fluidity of CtxB but in experiment 4 the trend is the opposite. We therefore conclude that exposure of SH-SY5Y cells to 1 μ M A β for 24h does not result in any significant change to the fluidity of membrane-bound CtxB.

3.9. A β does not affect the bulk fluidity of live cell membranes

Detailed ITIR-FCS data for A β -treated cells can be found in the Supplementary Data.

We utilized ITIR-FCS to investigate the effect of A β (1-42) on the membrane fluidity of living cells. In accordance with the observation that 10 μ M of A β was sufficient to significantly trigger apoptosis in sensitive cell types, 10 μ M A β was selected to be the concentration to treat living cells with for membrane fluidity studies. Cells were treated with either 10 μ M A β or vehicle for 30 min, 24h or 48h. The cell membrane was then labeled with the fluorescent lipid dye, C18-DiI and FCS measurements made. A β was present in the cell culture medium always during the measurement, except during staining of the membrane (~20 min). For the 30-min group, A β was added to the cell culture medium after the membrane was stained. Membrane fluidity of the bulk phase of the cells' plasma membranes were obtained by measuring the diffusion rate of C18-DiI in the membrane. C18-DiI is a lipid dye – a fluorophore, the tetramethylindocarbocyanine group, linked to two 18-carbon acyl tails, enabling it to insert into the cell plasma membrane and diffuse as a lipid in the liquid disordered phase. The sensitivity of DiI to changes in the bulk membrane was demonstrated by changing the ambient temperature of the cells, where a decrease in temperature result in a decreased membrane diffusion rate and vice-versa (Section 3.7, Table 4). In SH-SY5Y cells, diffusivity of DiI was not changed by A β treatment, from the time immediately following exposure (30 min) up to 48h. *D* values for DiI on differentiated SH-SY5Y cells, the most sensitive cell

type, typically remained consistent up to 48h post A β -exposure, ranging between 2.31 to 2.58 $\mu\text{m}^2 \text{s}^{-1}$ for vehicle-treated controls and 2.38 to 2.76 $\mu\text{m}^2 \text{s}^{-1}$ for A β -treated cells (Table 6, “SH-RAI”). In undifferentiated SH-SY5Y cells, the diffusion of DiI was even more stable, holding steadily at $\sim 2.2 \mu\text{m}^2 \text{s}^{-1}$ regardless of treatment and time point measured (Table 6, “SH-ND”). Similar trends are observed in the other two cell types. DiI diffusion rates of A β -treated cells were not significantly different from that of vehicle-treated U87 cells (Vehicle-treated: 1.66 – 1.72 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 1.67 – 1.84 $\mu\text{m}^2 \text{s}^{-1}$) and Neuro-2a cells (Vehicle-treated: 1.28 – 1.48 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 1.48 – 1.67 $\mu\text{m}^2 \text{s}^{-1}$) (Table 6, “U87” and “N2a”). Altogether, these results indicate that A β does not alter bulk membrane fluidity in living SH-SY5Y, U87 and Neuro-2a cells, neither immediately after addition of A β to the medium nor at up to 48h of exposure.

<i>D</i> values of DiI on living cell plasma membranes after A β treatment ($\mu\text{m}^2 \text{s}^{-1}$)						
Time	30 min		24h		48h	
Treatment	Vehicle	10 μM A β	Vehicle	10 μM A β	Vehicle	10 μM A β
SH-RAI	2.6 $\pm$ 0.2	2.8 $\pm$ 0.5	2.3 $\pm$ 0.3	2.4 $\pm$ 0.4	2.4 $\pm$ 0.4	2.4 $\pm$ 0.2
<i>N</i> (cells)	6	6	29	29	10	10
ANOVA	N.S.		N.S.		N.S.	
SH-ND	2.3 $\pm$ 0.3	2.2 $\pm$ 0.4	2.2 $\pm$ 0.2	2.2 $\pm$ 0.1	-	-
<i>N</i> (cells)	11	12	8	8	-	-
ANOVA	N.S.		N.S.		-	
U87	1.7 $\pm$ 0.2	1.7 $\pm$ 0.4	1.7 $\pm$ 0.2	1.8 $\pm$ 0.1	-	-
<i>N</i> (cells)	6	7	7	8	-	-
ANOVA	N.S.		N.S.		-	
N2a	1.5 $\pm$ 0.4	1.7 $\pm$ 0.3	1.5 $\pm$ 0.3	1.6 $\pm$ 0.2	1.3 $\pm$ 0.2	1.5 $\pm$ 0.3
<i>N</i> (cells)	6	6	11	11	4	4
ANOVA	N.S.		N.S.		N.S.	

Table 6. Effect of A β on DiI diffusion on plasma membranes of living cells

SH-RAI – differentiated SH-SY5Y cells; SH-ND – non-differentiated SH-SY5Y cells; U87 – U87 glioma cells; N2a – Neuro-2a cells. Diffusion coefficients presented as mean $\pm$ S.D. Statistical analysis: two-tailed unpaired one-way ANOVA with Tukey’s post-hoc test for multiple comparisons, unpaired, 95% C.I.

3.10. A β does not affect raft-type membrane fluidity

In addition to CtxB being a putative marker for raft-type membrane domains, its ligand, the ganglioside GM1, is also believed to interact with A β or be affected by A β interactions with lipid membranes (Eckert et al., 2010; Matsuzaki et al., 2010; Sasahara et al., 2013). We thought it worthwhile, therefore, to examine the effect of A β (1-42) on the diffusion of membrane-bound CtxB using ITIR-FCS. Test

conditions were identical to that of the DiI tests in the preceding section, except that the cell plasma membrane was stained with CtxB instead of DiI. Diffusion of membrane-bound CtxB on differentiated SH-SY5Y cells were not significantly altered within 48h of exposure to A β (Vehicle-treated: 0.19 – 0.20 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 0.19 – 0.20 $\mu\text{m}^2 \text{s}^{-1}$) (Table 7, “SH-RAI”). Undifferentiated SH-SY5Y cells and the non-neuronal U87 cells were similarly unaffected by A β treatment (SH-SY5Y – Vehicle-treated: 0.16 – 0.17 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 0.16 – 0.18 $\mu\text{m}^2 \text{s}^{-1}$; U87 – Vehicle-treated: 0.11 – 0.12 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 0.12 $\mu\text{m}^2 \text{s}^{-1}$) (Table 7, “SH-ND” and “U87” respectively). Additionally, for CtxB, we measured its diffusivity at 6h post-exposure to A β and at concentrations of A β up to 20 μM (supplementary data, Fig. S2). In only one instance, at 20 μM A β at 24h post-exposure on differentiated SH-SY5Y cells, was the mean diffusion coefficient of CtxB significantly different compared to vehicle-treated controls (supplementary Fig. S2a). The trend was not observed at later time points and thus was regarded as an artefact. On Data for CtxB for certain treatment conditions and the Neuro-2a cell line are unavailable due to difficulties in staining the membrane with CtxB during those experiments. Nevertheless, our results are sufficient for to conclude that A β does not affect the fluidity of membrane-bound CtxB between the time that the cells start to respond to A β and the time they are killed by it

<i>D</i> values of CtxB on living cell plasma membranes after A β treatment ($\mu\text{m}^2 \text{s}^{-1}$)						
Time	30 min		24h		48h	
Treatment	Vehicle	10 μM A β	Vehicle	10 μM A β	Vehicle	10 μM A β
SH-RAI	-	-	0.20 $\pm$ 0.04	0.19 $\pm$ 0.04	0.19 $\pm$ 0.05	0.20 $\pm$ 0.05
<i>N</i> (cells)	-	-	26	33	22	21
ANOVA	-		N.S.		N.S.	
SH-ND	0.17 $\pm$ 0.05	0.16 $\pm$ 0.06	0.17 $\pm$ 0.04	0.18 $\pm$ 0.05	0.16 $\pm$ 0.04	0.18 $\pm$ 0.06
<i>N</i> (cells)	9	9	22	27	14	26
ANOVA	N.S.		N.S.		N.S.	
U87	0.11 $\pm$ 0.03	0.12 $\pm$ 0.02	0.12 $\pm$ 0.06	0.12 $\pm$ 0.05	-	-
<i>N</i> (cells)	3	4	12	12	-	-
ANOVA	N.S.		N.S.		-	

Table 7. Effect of A β on CtxB diffusion on plasma membranes of living cells

SH-RAI – differentiated SH-SY5Y cells; SH-ND – non-differentiated SH-SY5Y cells; U87 – U87 glioma cells; N2a – Neuro-2a cells. Diffusion coefficients presented as mean $\pm$ S.D. Statistical analysis: two-tailed one-way ANOVA with Tukey’s post-hoc test for multiple comparisons, unpaired, 95% confidence interval.

3.11. A β does not affect membrane fluidity of GPI-anchored GFP

CtxB is generally accepted as a marker of raft-type membrane domains via its binding to the ganglioside GM1. Because GM1 is only one of several lipid types that are commonly found in raft-type membrane domains, it is possible that A β can affect raft-type membrane domains and yet somehow not affect GM1 diffusion. To test whether this was the case, cells were transiently transfected with a plasmid expressing a GPI-linked GFP that would be anchored on the outer plasma membrane. GPI-linked proteins are regularly sequestered into transiently-formed raft-type membrane domains and can move in and out of these domains. In all tested cell types, membrane-diffusion rate of GFP-GPI was not affected by exposure to A β (Table 8). Data is not available for differentiated SH-SY5Y cells due to the low expression efficiency of the plasmid in this cell type in our experiments. The diffusion rate of GFP-GPI was not significantly changed within 48h A β treatment in all cell types. *D* of GFP-GPI in undifferentiated SH-SY5Y cells ranged between 0.54 – 0.67 for vehicle-treated cells and between 0.58 – 0.62 $\mu\text{m}^2 \text{s}^{-1}$. The corresponding values for U87 and Neuro-2a cells are as follows: U87 – vehicle-treated: 0.42 – 0.58 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 0.42 – 0.49 $\mu\text{m}^2 \text{s}^{-1}$; Neuro-2a – vehicle-treated: 0.64 – 0.67 $\mu\text{m}^2 \text{s}^{-1}$, A β -treated: 0.61 – 0.66 $\mu\text{m}^2 \text{s}^{-1}$. Thus, A β also did not affect the diffusion of GFP-GPI in all the cell types tested in our study.

<i>D</i> values of GFP-GPI on living cell plasma membranes after A β treatment ($\mu\text{m}^2 \text{s}^{-1}$)						
Time	30 min		24h		48h	
Treatment	Vehicle	10 μM A β	Vehicle	10 μM A β	Vehicle	10 μM A β
SH-ND	0.67 $\pm$ 0.1	0.58 $\pm$ 0.2	0.54 $\pm$ 0.1	0.58 $\pm$ 0.3	0.62 $\pm$ 0.1	0.62 $\pm$ 0.3
<i>N</i> (cells)	4	8	9	11	6	6
ANOVA	N.S.		N.S.		N.S.	
U87	0.58 $\pm$ 0.1	0.46 $\pm$ 0.2	0.42 $\pm$ 0.1	0.49 $\pm$ 0.1	0.51 $\pm$ 0.2	0.42 $\pm$ 0.1
<i>N</i> (cells)	4	5	6	9	3	4
ANOVA	N.S.		N.S.		N.S.	
N2a	-	-	0.64 $\pm$ 0.2	0.61 $\pm$ 0.2	0.67 $\pm$ 0.1	0.66 $\pm$ 0.3
<i>N</i> (cells)	-	-	9	16	10	12
ANOVA	-		N.S.		N.S.	

Table 8. Effect of A β on GPI-linked GFP diffusion on plasma membranes of living cells

SH-RAI – differentiated SH-SY5Y cells; SH-ND – non-differentiated SH-SY5Y cells; U87 – U87 glioma cells; N2a – Neuro-2a cells. Diffusion coefficients presented as mean $\pm$ S.D. Statistical analysis – unpaired one-way ANOVA with Tukey's post-hoc test for multiple comparisons, 95% C.I.

3.12. Interaction of A β with supported planar bilayers

Unexpectedly, pre-aggregated A β (1-42) peptide did not cause changes to both bulk and raft-type membrane fluidity of the cell types examined in our study, and this in spite of A β clearly having cellular activity. We thus decided to examine the effects of A β on supported planar lipid bilayers (SPBs) by replicating a recent study by Kenji Sasahara (Sasahara et al., 2013). This time, SPBs were prepared with the same composition as used in Sasahara's study – DOPC/DOPS/SM/Chol (0.4/0.1/0.25/0.25) with 2.5% GM1 and Rho-PE as the marker. The resting membrane diffusion values of Rho-PE in our SPB as measured by ITIR-FCS is $0.88 \pm 0.13 \mu\text{m}^2 \text{s}^{-1}$, a value closely matching that of Sasahara's SPB, which was measured by FRAP ($0.99 \pm 0.1 \mu\text{m}^2 \text{s}^{-1}$). We also prepared A β (1-42) peptide in the same way as described in Sasahara's study. Briefly, dried A β peptide was dissolved in anhydrous DMSO followed by dilution with pure water to 90.2 μM and kept cool on ice. Immediately before use, A β was further diluted with pure water to 10 μM . Additionally, we examined the response of our SPB to A β prepared per our standard protocol, with both wt-A β and scrambled-A β . We were unable to replicate the results of Sasahara et al. In their study, addition of A β to the bilayer immediately caused a drastic but reversible reduction in membrane fluidity. In our experiment, mean diffusion coefficients did not change significantly upon exposure of the SPB to A β , whether it was prepared per Sasahara's protocol or our protocol (Table 9). However, the standard deviation of the mean of the SPB increased quite a bit when treated with

Effect of A β on DOPC/DOPS/SM/Chol SPBs					
	Resting	ddH ₂ O	10 μM A β in ddH ₂ O	10 μM A β Oligomer	10 μM scrambled A β
<i>D</i> ($\mu\text{m}^2 \text{s}^{-1}$)	0.88 ± 0.1	0.91 ± 0.1	0.98 ± 0.1	0.89 ± 0.2	1.0 ± 0.5
ANOVA <i>p</i> (<i>D</i>)	-	N.S.	N.S.	N.S.	N.S.
FCS diffusion law y-intercept	-0.05 ± 0.1	-0.1 ± 0.1	-0.01 ± 0.1	-0.04 ± 0.1	0.07 ± 0.2
<i>N</i> (Acquisitions)	17	6	6	6	6
ANOVA <i>p</i> (diff-law)	-	N.S.	N.S.	N.S.	N.S.

Table 9. Effect of A β on supported planar bilayers

SH-RAI – differentiated SH-SY5Y cells; SH-ND – non-differentiated SH-SY5Y cells; U87 – U87 glioma cells; N2a – Neuro-2a cells. Diffusion coefficients presented as mean $\pm$ S.D. Statistical analysis – unpaired one-way ANOVA with Tukey's post-hoc test for multiple comparisons, 95% C.I.

A β from our preparation protocol, by ~66% and ~260% for wt-A β and scrambled-A β respectively. We therefore wondered if there were some changes perhaps in membrane *heterogeneity*, even though the *fluidity* of the membrane probe, Rho-PE, did not change. We thus conducted FCS diffusion laws analysis on the data obtained. FCS diffusion laws analysis gives an indication on the phase of the membrane, indicated by the y-intercept when A_{eff}/D is plotted against A_{eff} – negative values indicating restricted diffusion, a zero-value indicating perfectly free diffusion and increasingly positive values indicating increasing heterogeneity or presence of phase-separated domains. The y-intercept values calculated from our diffusion data suggested that the resting state of the SPB is largely free and homogenous, with a slight restricted diffusion character (Table 9). There was no statistically significant change in the diffusion law y-intercept, indicating that the character of the membrane did not change significantly. Thus, in summary, A β did not change the average fluidity of the SPB; it seemed like there were some perturbations in the membrane heterogeneity but we cannot be certain with the current data.

4. Discussion

4.1. Generation of A β aggregates

Because the 42-amino acid variant of A β has been shown to be more fibrillogenic and cytotoxic to cells, we decided to use this variant in our studies. It had been previously reported that small diffusible A β aggregates caused cytotoxicity and could be the more relevant species of A β in Alzheimer's disease pathology (Benilova et al., 2012). We have therefore opted to adapt well-established protocols to generate small, soluble A β aggregates for our experiments. It is understood that A β aggregation is dependent mainly on ionic strength and pH of the environment, with a low pH favoring fibril formation and physiological pH favoring formation of small, soluble aggregates (Dahlgren et al., 2002). Published protocols that we referenced used F12 medium as the incubation medium for generating A β aggregates (Dahlgren et al., 2002; Klein, 2002; Stine et al., 2011). We were unable to obtain F12 medium and thus decided to use DMEM/F12 as a substitute. Incubating 100 μ M of A β (1-42) in DMEM/F12 allowed us to generate trimers and tetramers, with a very low amount of dimers and a major proportion of monomers (Fig. 10). This is a similar result to the reference protocols, such as that of Mary Jo-LaDu's group (Dahlgren et al., 2002).

In our case, incubation at different temperatures (4°C, room temperature and 37°C) did not affect the *type* of aggregates formed, but rather the *quantity* of aggregates and monomers detectable in the sample after incubation (Fig. 10). Interestingly, the quantity of monomers and aggregates detected decreased as the incubation temperature increased, even though the starting amount of A β was the same in all the samples. Additionally, in the sample incubated at room temperature, there is a faint smear present between 50 – 200 kDa, which is not present at other temperatures. Because room temperature is an intermediate between 4°C and 37°C, this may indicate that higher temperatures promote formation of larger, non-soluble aggregates, that are either pelleted during the centrifuging step or too large to enter the SDS-PAGE gel. Supporting this hypothesis is the observation that amount of A β monomers are greatly reduced as the temperature is increased to 37°C – quite possibly, the monomers have been sequestered into larger aggregates that were not detectable using this protocol.

It is puzzling why dimers are the least abundant aggregate type in the samples, since a reasonable assumption would be that dimers are kinetically the first species formed in the process of A β aggregation, followed by larger aggregates such as trimers and tetramers. The first deduction from this observation would be that dimers very short lived and quickly undergo either further aggregation into trimers/tetramers or dissociate into monomers again, thus the much lower levels of dimers in the solution. However, trimers and tetramers seem to be different in nature from dimers. This is evident in the fact that trimers/tetramers are moderately heat-labile; the quantity of tri-/tetramers was reduced by heating for 10 min at 70°C. In contrast, the quantity of dimers seems to remain approximately the same before and after heat treatment (Fig.10, “boiled” samples). This exceptional heat stability of dimers could mean either that dimers are structurally distinct from tri-/tetramers, or perhaps dimers are simply an artefact of the SDS-PAGE process, forming quickly and spontaneously in the presence of some component in the SDS-PAGE reagents. The latter conclusion is possible, as there have been some reports that SDS can induce A β aggregation. Supporting this conclusion is the observation that the quantity of dimers does seem to neither decrease nor increase after heat treatment. Possibly, during the incubation period in the aggregation protocol, monomers aggregate directly into tri-/tetramers and this type of aggregates in turn dissociate directly into monomers, bypassing the dimeric stage, hence the unchanging quantity of dimers despite the breaking down

of tri-/tetramers by heating. The dimers detected here could have arisen via an aggregation pathway of A β monomers that takes place only in the presence of SDS reagents. In opposition to this theory, however, is the observation that as with the trimers and tetramers, the quantity of dimers detected was negatively correlated with the incubation temperature, suggesting that dimers arose from the aggregation process and not the SDS-PAGE process. Curiously, dimers were not detected in subsequent PAGE experiments, whether with DMEM/F12 or Neurobasal/B27 as the aggregation medium (Fig. 11). This absence of dimers in a pre-aggregated A β solution is similar to previously published research using similar aggregation protocols (Dahlgren et al., 2002; Klein, 2002; Stine et al., 2011). The most plausible explanation for the dimers detected in the first experiment then, may be that they were simply a one-off artefact introduced in the handling of the procedures then.

After verifying that we could generate the desired small, soluble A β aggregates with our protocol, we next substituted serum-free Neurobasal medium for the DMEM/F12 medium because we intended to differentiate and culture SH-SY5Y cells in Neurobasal medium, which is a standard medium used in neuronal culture. Even though SH-SY5Y cells are not true neurons, we decided that Neurobasal medium is best base medium for culturing differentiated SH-SY5Y cells as the composition of Neurobasal medium is specifically suited for neuronal cell culture. Incubating A β in supplement-free Neurobasal medium at 4°C and room temperature for 24h resulted in formation of aggregates identical to that of A β incubated in DMEM/F12 (Fig. 11a). There seemed to be slightly less aggregated A β in the Neurobasal-incubated A β (Fig. 11a, lanes 1 and 2) than the DMEM/F12-incubated A β (Fig. 11a, lanes 3 and 4), therefore the incubation time was increased to increase the quantity of trimers and tetramers generated. Incubating the A β at 4°C for 72h successfully resulted in an increase in the quantity of trimers and tetramers present in the sample (Fig. 11b). As we expected differentiated SH-SY5Y cells to be the most sensitive cell type to variations in the cell culture medium, we decided to standardize our A β aggregation protocol to incubate 100 μ M of A β in Neurobasal medium for 72h at 4°C. It must be kept in mind that once aggregated, the *actual concentration of each A β species is not known*; A β concentrations described in subsequent experiments refer to the total monomeric A β present at the very beginning of the aggregation protocol (refer to section 2.1 for details).

Variant A β (1-42) peptides aggregated with varying similarity to that of wild-type A β . HiLyte 488-A β (1-42) (fl-A β) had an aggregation profile identical to that of wt-A β , but oligomers were less abundant (Fig. 12, “fl-A β ”). When mixed with wt-A β , the aggregation profile did not change but the abundance of oligomers varied according to the wt-A β :fl-A β ratio. At a wt-to-fl ratio of 1:1, the quantity of tri-/tetramers is much less than that of pure wt-A β and somewhat equal to that of pure fl-A β . At a wt-to-fl ratio of 1:3, the quantity of tri-/tetramers is also much less than that of pure wt-A β and seems to be less than even pure fl-A β . At a wt-to-fl ratio of 3:1, the quantity of oligomers is conversely relatively high, seemingly even more than that of wt-A β . Given that the initial total concentration of A β is 100 μ M in all samples, this suggests that the addition of the fluorophore hinders the rate of aggregate formation, but the presence of wt-A β can modulate the aggregation rate depending on its relative abundance compared to fl-A β . Thus, fl-A β may limit aggregation rate but is still able to form “normal” aggregates, and possibly hybridizes with wt-A β to form aggregates at different rates depending on the wt-A β to fl-A β ratio. As the type of aggregates are not affected by fl-A β , we decided that fl-A β was an acceptable A β variant for the visualization of A β via fluorescence microscopy.

While N-terminal HiLyte 488-tagged fluorescent A β (1-42) could form the same type of aggregates as wt-A β , The scrambled peptide A β variant (scr-A β) did not. Scrambled A β (1-42) had an aggregation profile that only showed dimerization and nothing more. A majority of the peptide remained as monomers, just as the case is with wt-A β . Thus, scrambling of the A β peptide sequence does not change amino acid content, it completely alters the aggregating ability of the peptide. Because the toxicity of A β is thought to be associated with its aggregation, and since aggregation is related to peptide sequence, this makes the scrambled A β peptide a suitable control peptide for studies attempting to correlate exposure to A β with cellular response or activity. All in all, the experiments and results discussed in this section provide ample support for our A β aggregation protocol, and for the use of fl-A β and scr-A β as method of visualizing A β and as a control peptide for wt-A β respectively.

4.2. Cells internalize A β

To determine whether A β interacted with our selected cell types by examining whether they internalized the peptide. Because the A β mixture with a wt-to-fl ratio

of 3:1 looks most like wt-A β in terms of aggregate profile and abundance of aggregates, we exposed cells to A β of this mixture. A β was added to cell culture medium to a final concentration of 10 μ M for 24h, following which the cell membrane and cell nuclei were stained with DiI and Hoechst 33342 respectively and visualized using confocal fluorescence microscopy. All the cell types tested – both differentiated and undifferentiated SH-SY5Y cells, U87 cells and Neuro-2a cells – internalized a enough A β within 24h to be visualized. In the SH-SY5Y and Neuro-2a cell types, internalized A β showed up as small punctate areas, an observation that agrees with reports that internalized A β is located mainly in the lysosomes and multi-vesicular bodies (LaFerla et al., 2007). In U87 cells, however, A β does not seem to be distributed in the same way as the former two cell types. Instead, there seems to be a much more internalized A β , in larger sized vesicles. This may be due to the difference in the tissue of origin – both SH-SY5Y are neuroblastomas (neuronal in origin) while U87 is a glioma (glial in origin). Regardless of the difference in distribution and amount of internalized A β , we can say that A β is internalized by all tested cell types within 24h of exposure at a A β concentration of 10 μ M. Interestingly, there does not seem to be any fluorescence from A β at the cell plasma membrane of all tested cell types, which could mean either that A β does not remain at the plasma membrane, or that A β is present at the membrane in concentrations too low to be visualized by confocal fluorescence microscopy. These observations, taken together with the finding from the FCS experiments that A β does not change membrane fluidity of these cells, suggest that *A β may interact with the cell membrane in some way such as to be internalized, yet not in a way such as to perturb normal cell membrane organization and function.*

4.3. A β triggers apoptosis and lowers cell viability

We first decided to use the SH-SY5Y neuroblastoma cell line as a model for studying A β effects on cell membrane because these cells are human and neuronal in origin. These cells express neuron-specific proteins and a ganglioside expression profile that changes upon differentiation in a way that is like that of typical neuronal development (Encinas et al., 2000; Rebhan et al., 1994). Previous research had shown that SH-SY5Y cells are more sensitive to A β cytotoxicity after they are differentiated (Lambert and Stevens, 1994), therefore we decided to include differentiated neuroblastoma cells in our study. Additionally, we used the U87

glioma cell line, which are human but glial instead of neuronal in origin, and the Neuro-2a cell line, which is neuronal but mouse instead of human in origin. We decided to use cell lines instead of primary cells in our study because cell lines are easily obtained, easily replenished and are less heterogeneous than primary tissue samples, which should reduce confounding factors arising from sample heterogeneity.

Cytotoxicity studies with the SH-SY5Y showed that the A β could induce apoptosis and reduce cell viability of these cells in both the undifferentiated and differentiated state, and that differentiated cells were more sensitive to A β -induced cytotoxicity (Tables 1a – 1c). In our experiments, the minimum required concentrations required to induce apoptosis *and* reduce cell viability within 72h were 20 μ M for undifferentiated cells and 10 μ M for differentiated cells. The cytotoxic effect of A β on SH-SY5Y cells was concentration-dependent; for example, 1 μ M A β could not induce apoptosis nor reduce cell viability in differentiated cells within 48h but 10 μ M A β could (Fig. 15). Similarly, for the undifferentiated cells, even though 10 μ M of A β could not reduce cell viability by 72h (Fig. 15a), 20 μ M of A β both induced apoptosis and reduced cell viability by then (Fig. 15, a and b). Cytotoxicity also seemed to be time-dependent; for example, in differentiated SH-SY5Y cells, 10 μ M of A β induced apoptosis and reduced cell viability after at least 24h. Similarly, for undifferentiated SH-SY5Y cells, 20 μ M of A β induced apoptosis only after at least 24h. While both undifferentiated and differentiated cells are sensitive to A β -induced cytotoxicity, differentiated cells are more sensitive, as effects set in at both a lower concentration of A β (Fig. 15 a and b, “RAI 10 μ M” vs. “ND 10 μ M”) *and* at an earlier time point (Fig. 15a, “RAI 20 μ M” vs. “ND 20 μ M”)

In the experiments described in the preceding paragraph, the effects of A β on cells was compared to that of a vehicle control. To determine whether the observed effects were specifically caused by A β (1-42), we conducted another set of experiments comparing the effects of wild-type A β (1-42) to a scrambled A β (1-42) peptide. Although there are some variations in the effects observed, for example, in differentiated cells, 10 μ M of A β could only trigger apoptosis and reduce cell viability between 48 – 72h compared to between 24 – 48h in the previous experiment (Tables 1a and 1b, “SH-RAI” and “SH-ND”), a similar trend is observed: both undifferentiated and differentiated SH-SY5Y cells are sensitive to A β -induced

cytotoxicity, and that differentiated SH-SY5Y cells are more sensitive to A β . We also included the U87 and Neuro-2a cell lines in these experiments to see whether the observed effects on cells are of a general or specific nature with respect to cell type. A β had a less robust effect on U87 and Neuro-2a cell types. In both these cell types, A β did not reduce viability within 72h of exposure, and apoptosis was induced only at the highest concentration of A β tested, 20 μ M, and after at least 48h of A β exposure. These results are presented summarily in Tables 1a – 1c. From this experiment, two things are clear: first, the effects of A β presented and described in Fig. 15 are specific to A β (1-42) as the scrambled peptide does not elicit similar effects; and second, A β does trigger apoptosis in all four tested cell types (SH-RAI, SH-ND, U87 and N2a), but the degree to which the cells are detrimentally affected by A β depends on the cell type. Clearly, something about the SH-SY5Y cell line predisposes it to A β -induced cytotoxicity as compared to U87 and N2a cells, and between the undifferentiated and differentiated states, the differentiated state is more sensitive. Indeed, although all tested cell types originate from brain tissue, they are not similar in nature. U87 cells are of a glial type, which is not known to be the tissue type primarily affected in AD, and Neuro-2a cells are murine in origin – the mouse being an organism that does not generate A β endogenously and indeed does not even express the amyloid-precursor protein. All in all, our cytotoxicity studies suggest that A β prepared in the method describe in our study can cause cytotoxicity via induction of apoptosis, and different cell type and states are differentially sensitive to A β .

4.4. Resolving issues pertaining to the preparation of A β and its effects on cells

Up to this point in this study, it has been assumed that the SDS-PAGE data of the A β solutions produced by the A β aggregation protocol detailed here definitely produces A β aggregates, at least of the trimer and tetramer species. However, two potentially contentious issues need to be considered. First, because there have been some claims that SDS can trigger A β aggregation, could it be that what we take to be tri-/tetramers in our A β preparation are not actually that? In other words, are we really adding A β trimers and tetramers, the assumed toxic A β species, to cells, or are there some other forms of A β that we do not know about present in our A β prep? Second, the response of Neuro-2a cells to A β in this study seem not to concur with previously published studies. Dahlgren et al. showed that oligomeric A β was toxic to these cells at as little as 10 nM. Unaggregated A β peptide was not toxic at

nanomolar concentrations, but at high ($> 1 \mu\text{M}$) concentrations, it had similar cytotoxic effect as oligomeric A β (Dahlgren et al., 2002). Because our membrane fluidity studies hinge on the A β functioning as it should, it is important that we address these issues before moving on to the actual membrane fluidity studies.

R. W. Hepler and associates showed in a study published in 2006 that the SDS-PAGE analysis of the “standard” A β oligomerization protocol presented by William Klein (Klein, 2002) and adapted for use in our present study, may be inaccurate (Hepler et al., 2006). In summary, Hepler et al. presented evidence that the staining pattern of A β in SDS-PAGE gels were similar regardless of whether the A β was subject to the oligomerization procedure or not. The same A β solutions analyzed by size exclusion chromatography (SEC) and atomic force microscopy (AFM) did show differences. For example, AFM images of oligomerized peptide showed a higher quantity of small globular structures on a backdrop of A β monomers, while images of unaggregated peptide showed a more-or-less homogenous field of A β monomers with a few larger structures. SEC similarly could differentiate between unaggregated and oligomerized A β solutions, high- and low-mass peak eluting at different times, with the MW of the low-mass peak corresponding to that of monomeric A β . However, SDS-PAGE gels of unaggregated, oligomerized and fibrillar solutions of A β all showed similar staining patterns when run on a 10 – 20% tricine gel and stained with SYPRO-ruby. This staining pattern is practically identical to that presented in other studies, such as Dahlgren et al. (Dahlgren et al., 2002), and in our study (Figs. 10 and 11). Additionally, we do have data, although only from a single experiment, that unaggregated peptide does show a similar SDS-PAGE staining pattern as peptide that had been subject to an oligomerization step (Supplementary Fig. S3). One curious observation is that the tri-/tetramer bands in SDS-PAGE are heat labile, and did not re-form in the time taken to run the SDS-PAGE experiment after being removed from heat. This observation holds for both pre-aggregated peptide (Fig. 10, “24h incubation, boiled”) and un-aggregated peptide (Supplementary Fig. S3a, lane 4, Supplementary Fig. S3b, lane 2), and is counter-intuitive to a conclusion that SDS (or in our case, LDS) is the agent that causes the tri-/tetramer bands to form in the SDS-PAGE experiments, for even the sample prepared and immediately used for SDS-PAGE has detectable tri-/tetramer bands (Supplementary Fig. S3b, lane 1). Regardless, the state of available SDS-PAGE data in this study, coupled with observations by others such as Hepler et al., indicate that

SDS-PAGE data may not accurately represent the aggregation present in each A β solution and the aggregates observed via SDS-PAGE may simply be an artifact of the SDS-PAGE procedure. This experiment with the un-aggregated peptide was conducted rather much earlier during my study, and during the time it was conducted, I, the author of this study, was not aware of the findings of Hepler et al. Since at there had been prior observations, for example by Konrad Beyreuther (Brody et al., 2011) and Jarrett et al. (Jarrett et al., 1993) that A β (1-42) aggregates almost instantaneously in aqueous solution, I took the phenomenon that un-aggregated peptide also produced tri-/tetramer bands in SDS-PAGE to mean that sufficient A β oligomers had formed during the sample handling time to be detectable, and thus did not think to continue using un-aggregated A β for any subsequent cell-based experiments. It must also be noted that while Hepler et al., showed that *SDS-PAGE data does not correlate* with presence of oligomeric or any higher-level A β aggregates, they *do not disprove that A β aggregates are formed* using the “standard Klein protocol”, on which the protocol in my study is based. In fact, the AFM data provided by Hepler et al. agrees with AFM data provided by others such as Klein (Klein, 2002) and Dahlgren et al. (Dahlgren et al., 2002; Stine et al., 2003), and verifies that the aggregation protocol *does* allow for generation of a variety of A β species in solution, from the seemingly ever-present monomers to various small and higher-order aggregates. This finding is corroborated with the SEC data also provided by Hepler et al. One conclusion from Hepler’s 2006 study is that solution-based analysis of A β solutions are a better method of telling what A β types are present. Because there was no solution-based analysis of the A β solutions used in this study, I cannot claim here to have definitively produced A β aggregates, and any interpretation of subsequent data can only be taken as “effects of A β subjected to a pre-aggregation protocol believed to generate small, soluble oligomers” – which, for brevity, I shall refer to as “A β ” in this study.

With the above factors considered, the ensuing issue is this: since the cytotoxicity studies presented in this study showed that only at high concentrations of A β (≥ 10 μ M) was apoptosis induced in all three tested cell types (SH-SY5Y, Neuro-2a and U87), could it be that the A β solution prepared here did not in fact contain the supposedly toxic A β oligomers, but rather only monomers? This possibility is enhanced by the fact that Dahlgren et al. in their 2002 paper showed that unaggregated A β (1-42) could not reduce cell viability of Neuro-2a cells at low (sub-

micromolar) concentrations but at high ($\geq 1 \mu\text{M}$), could reduce cell viability, even to the same degree as that of pre-aggregated A β at $10 \mu\text{M}$ (Dahlgren et al., 2002). Additionally, Sarkar et al. showed in their 2013 paper that monomeric A β (1-42) binds to cell membranes at much lower affinities than pre-aggregated, oligomeric A β (1-42) (Sarkar et al., 2013). In that study, binding of A β to the cell membrane was visually determined via confocal imaging of a fluorescent, rhodamine-labeled A β and differences in membrane localization between monomeric and oligomeric A β was apparent within 30 min of addition of A β to the cells. In my present study, after incubation of cells for 24h with fluorescent HyLite-488 A β mixed with wt-A β , no fluorescent signal from A β was detected at the membrane even though intracellular puncta were observed (Fig. 14, b, d, f and h). Thus, the results presented in this study would seem to imply that the A β solution used to treat the cells with are predominantly monomeric rather than oligomeric. Nevertheless, it must also be noted that several other studies utilizing the same SH-SY5Y cell line (Campioni et al., 2010; Lambert and Stevens, 1994; Lee et al., 2015; Qu et al., 2011), and even human cortical neurons (Deshpande et al., 2006) used A β concentrations similar to that shown in this study to be able to trigger apoptosis (10^{-6} to 10^{-5} M) Deshpande and Busciglio reported in their study using the HCN-1 human cortical neuron cell line, that $5 \mu\text{M}$ of A β -derived diffusible ligands caused cytotoxicity only after 5-7 days (Deshpande et al., 2006). In our study, $10 \mu\text{M}$ of A β triggered apoptosis in differentiated SH-SY5Y cells within 48h. Thus, the concentration of A β ($1 - 2 \times 10^{-5}$ M) used in our study that triggers cytotoxicity does not disagree totally with the earlier studies.

While there is a discrepancy between the results presented in my study and that presented in Dahlgren et al., there are also other studies which present similar findings as is presented here in this study. The difference between my results and Dahlgren et al. (or indeed any other study) could be due to variances in cell culture conditions, sample handling and/or the cell passage number and other unknown factors, and it is not implausible that a micromolar range of A β is required to trigger detrimental effects to cells under *in vitro* cell culture conditions. Nevertheless, because no solution-based analysis was made of the A β preparations in our current study, based on SDS-PAGE data alone, we cannot be certain that A β oligomers were indeed present in the solutions that were finally used to treat the cells. Thus, we must allow for the possibility that the A β solutions used in this study contained only

monomers or at least predominantly contained monomers. Supporting the notion that primarily monomers are present in the A β solution in this study, is the observation that a high concentration of A β (~10 μ M) is required to trigger apoptosis and that no fluorescent A β is found to be localized at the cell membrane. Supporting the notion that A β oligomers could be present in the A β solution is the fact that several other researchers have shown using solution-based assays that the aggregation method used here does produce small globular A β oligomers. Without further information, we must accept the uncertainty that the cytotoxic effect of A β on cells presented in this study could be entirely due to A β monomers *or* it could be due to aggregates present in the solution. Possibly, and in any related future studies, solution-based determination of A β aggregates should be included, in order that the researcher may be certain of what A β species are actually present – as has been proposed by Benilova et al. in a more recent review of the field (Benilova et al., 2012)

Previous research has suggested that A β can depolarize the mitochondrial membrane, causing it to become leaky (Demuro et al., 2005; Deshpande et al., 2006). The mitochondria and endoplasmic reticulum are intracellular stores of calcium. In addition to calcium release, mitochondria with compromised membrane integrity also release cytochrome c, a component of the electron transport chain in the mitochondrial inner membrane. Once in the cytosol, cytochrome c binds to the apoptotic protease-activating factor (Apaf-1), triggering the proteolytic cleavage of procaspase-9 to release caspase 9, further triggering the activation of caspases 3 and 7, resulting in an apoptotic caspase cascade. A second possible calcium-related apoptotic mechanism involves the cytochrome c-mediated release of calcium from the endoplasmic reticulum, which leads to further release of cytochrome c from the mitochondria, forming a positive feedback loop leading to an apoptotic cascade. A recent study has also shown that apoptosis in undifferentiated SH-SY5Y cells is accompanied by cytochrome c release (Lee et al., 2015). In our study, reduction of cell viability is preceded by caspase activation, implying that this is an apoptotic process. We show that A β does have cytotoxic effects on the SH-SY5Y cell line, which is human and neuronal in origin, and we also show that different cell states, for example, differentiated vs. undifferentiated SH-SY5Y cells, and different cell types originating from brain tissue, for example, SH-SY5Y vs. Neuro-2a vs. U87 cells, are affected to different degree by A β exposure (Tables 1a and 1b). This agrees with the observation that in clinical cases of AD, within the brain, not all cell types

are affected to a similar degree by A β . We also show that scrambled A β (1-42) does not damage cells compared to wild-type A β (1-42) and that scrambled A β does not aggregate in a similar fashion as wild-type A β as analyzed by SDS-PAGE (Fig. 12, “scr-A β ” vs. “wt-A β ”). Thus, the effects of the A β solution prepared using the protocol detailed in Section 2.1 are both cell-specific and peptide-specific – not all cells respond in the same way to this A β and not any peptide with the same amino acid composition as wt-A β can kill cells like wt-A β does.

4.5. Confocal FCS studies suggest that sub-toxic concentrations of A β do not change membrane fluidity of living cells

Although there have been several previous studies that tried to establish the effect of A β on the fluidity of cell membranes, there has not been a conclusive answer. As discussed on before, two of the major possible reasons why there has not been a consensus could be firstly, because the probes used introduced biases in the results obtained, and secondly, there may be disadvantages in the membrane model used due to the complexities in the relationship between membrane lipids and membrane fluidity. Because of these reasons, we have elected to use the plasma membrane of living cells as the model membrane to investigate A β effects on membrane fluidity. FCS is an ideal technique for this setup, because of the ability to make measurements in a real-time fashion and its single-molecule sensitivity, which allows one to use a very low concentration of fluorescent probe to label the cell. This is important, since the probes themselves have potential to perturb membrane fluidity, especially if they can trigger phase separation in the membrane. To our knowledge, our study is the first to utilize FCS to observe membrane fluidity in live cells after A β treatment.

As this is the first time we are attempting to observe A β -induced changes on membrane fluidity in a live cell set up, we were not certain what to expect – would there be any change at all, and if so, would the magnitude of the changes be large enough for us to detect? Therefore, before we first sought to validate our system’s accuracy and reliability for studying diffusion. We chose CtxB as the membrane probe because it binds to the ganglioside GM1, which along with being a typical raft-type membrane marker, is also thought to interact with A β . Thus, the diffusivity of GM1 should be affected if A β does interact with it. Also, since liquid-ordered, raft-type membrane domains are heavily dependent on cholesterol, A β interacts with

membrane cholesterol, as suggested by several previous studies, the diffusivity of GM1 should also be affected. τ_D , or translational diffusion time, is one of the basic information obtained about the probe particle from a confocal FCS experiment. τ_D is dependent on the area or volume observed in the sample, as opposed to diffusion coefficient, D , which is in units of area per unit time and is independent of the observed area. Because of this, it is important to calibrate the instrument before each experiment depending on the lasers (wavelength used), lenses and optical filters used, to obtain a suitable detection volume. The k-factor is ratio between the longitudinal width and the beam width (radius) of the detection volume and describes the shape of the detection volume and a k-factor between 5 and 10 is considered acceptable. Calibration is done using well-characterized reference dye, such as rhodamine-6-G or fluorescein, where one knows the expected τ_D if the detection volume is in an acceptable range. In our experiments, we use Atto-488, which is expected to have a τ_D of about 40 μ s, as the calibration dye for Alexa-488 CtxB, and the k-factor obtained in all experiments ranged between 5 to 8. Our confocal FCS measurements of Atto-488 in water, CtxB in medium and membrane-bound CtxB yielded τ_D values that agreed well with previously published studies (Fig. 16, Section 3.5) (Hossain et al., 2007; Nag et al., 2010). We therefore were confident that confocal FCS could reveal changes in membrane fluidity if A β did indeed cause any.

We took care in the analysis and treatment of our data to avoid type I (false positive) errors. Importantly, we needed to select the appropriate fitting model for our FCS data, one that properly accounts for the shapes of the auto-correlation functions generated. Because the confocal detection volume is typically a couple of microns in length parallel to the light beam, and the cell membrane is no more than a few nanometers in thickness, most the detection volume will consist of space not on the cell membrane, including the cytosol and the extracellular medium. Therefore, one must anticipate the presence of probe molecules that are not membrane bound. The source of these non-membrane bound probe species are usually internalized probe (cytosolic), either taken in by the cell from the medium or as part of the membrane during endocytic processes, or excess unbound probe in the medium after staining (extracellular). In an FCS experiment, these populations of probe will be treated as a diffusing species, along with the membrane bound probe, and contribute to the fluorescent intensity signal, and thus the auto-correlation function. One can minimize the effects of these undesired species by first, adequate washing of the cells

after staining, and second, making measurements as soon as possible after staining before significant internalization of the probe occurs. Nevertheless, because we expect that there will still be some non-membrane-bound probe present, we experimentally determined the τ_D of Alexa 488-CtxB in Neurobasal/B27 medium (Fig. 16). Molecules diffusing in solution are expected to have τ_D values in the 10^{-5} to low 10^{-4} s (microsecond) range. Membrane bound particles are expected to be in the 10^{-3} s (millisecond) range, which is relatively slow compared to the typical diffusion times of particles in solution and the timescale of the fluorophore triplet state dynamics, which are in the order of 10^{-6} s, and which also is a component of the ACF as it is a fluorescent effect that can be detected. Although several theoretical models of diffusion exist that try to account for several components contributed by various fluorescent phenomena to an ACF obtained in an FCS experiment, it is easier to obtain a good fit to the ACF if there are fewer components to account for. We therefore decided to truncate the ACF to exclude the shorter timescales (10^{-6} s) and use a two-dimensional two-particle diffusion model to fit the data, so as to exclude the triplet-state component of the ACF and account only for the CtxB probe, both in-solution and membrane-bound. The τ_D of Alexa 488-CtxB in solution was determined to be 215 μ s, and was fixed as the τ_D of the first particle, τ_{D1} , when fitting ACFs of membrane-bound CtxB. The calculated τ_D of the second particle, τ_{D2} , would then be the τ_D of the membrane-bound CtxB. As expected, the proportion of particles with τ_{D2} in the fitted data was the majority species, typically 75% or more of the entire observed population of the fluorescent CtxB probe (data not presented), and the τ_{D2} values were mostly in the low – mid 10^{-3} s range. This confirms that we were indeed measuring membrane-bound CtxB.

Because concentrations of A β on the order of 10^{-6} M had previously been shown to be toxic to cells cultured *in vitro*, we decided to start investigating the effects of A β on cell membrane fluidity by treating cells with 1 μ M of A β (1-42) for 24h. The range of measured translational diffusion times, τ_D , of CtxB on the cell membrane of SH-SY5Y cells that had been cultured in Neurobasal/B27 medium was quite wide, between 18 – 70 ms, in spite of the small k-factor range of 5.7 – 7.3 for the six experiments conducted. This could be due to inherent heterogeneity in the cell populations examined or due to variation in the detection volume. Nevertheless, it is possible to compare τ_D values within each experiment as the measurements were made on the instrument using the same calibration. The mean τ_D values of all samples

had a standard deviation of about 30%, indicating that the diffusivity of CtxB is quite consistent and reproducible within each sample. Of the six experiments conducted, mean τ_D values of A β -treated samples either remained unchanged or decreased compared to vehicle-treated samples. However, only in two experiments was there a statistically significant difference between the mean τ_D of the vehicle treated samples versus A β -treated samples when analyzed using Student's T-test (Table 5, experiments 1 and 6, statistical testing method detailed in Section 2.2.9). Coincidentally, an acquisition time of 30s was used in those two experiments versus 45s in the other experiments. A longer acquisition time is generally preferred as the auto-correlation function generated will be better. This can be understood as having more time points to correlate the fluorescence signal intensity with, resulting in better noise reduction. There is no arbitrary condition for deciding on the acquisition time, but typically longer times are better for molecules that diffuse slower, such as membrane-bound particles. A balance must be struck between longer acquisition times and avoiding photobleaching of the fluorophore, which greatly affects the ACF if it occurs during the acquisition time, because it is effectively a reduction in the concentration of the probe molecule. Even though a shorter acquisition time of 30s was used for experiments 1 and 6, the mean τ_D values obtained does not seem to be biased, as they are not unlike τ_D values obtained in the other experiments. Since in only 2 out of the 6 experiments were the τ_D of A β -treated cells significantly different from control cells, we concluded from the confocal FCS experiments that sub-toxic (1 μ M), short-term (24h) treatment of cells with A β (1-42) does not change the membrane fluidity of CtxB.

4.6. ITIR-FCS is a suitable method to measure membrane fluidity in living cells

We then decided to switch from using confocal FCS to using imaging total-internal reflectance FCS (ITIR-FCS), as its technical features are better suited for studying the fluidity of the cell membrane. The greatest advantage ITIR-FCS offers over conventional confocal FCS is the ability to observe a large, two-dimensional area in a single measurement, greatly increasing throughput in terms of area of membrane measured. Additionally, because the set up relies on the correlation of pixel brightness from an image taken by a fixed camera, the image pixel size is always the same for a given set of objective lens and there is no need to calibrate the detection area each time the system is used as is required in confocal FCS. One limitation is

that this technique can only be used to observe the basal membrane of the cell, since TIRF illumination can only be achieved at the interface between the coverglass and the sample (see Fig. 7 for illustration). The region of interest selected for each cell was 10 pixels by 10 pixels, corresponding to a square with a side of 2.4 μm in the image plane, generating 100 ACFs per measurement. FCS data was correlated with a homebrew software, ImFCS, using the two-dimensional, one particle model of diffusion (Sankaran et al., 2010). In the analysis stage, ACFs that were obviously bad, such as curves that were not characteristic of membrane-bound diffusion, or that had calculated D values that were far outliers, for example, a value several times higher than the median D of the cell were excluded from the final summary. If ACFs from any cell contained too many undesirable ACFs, data from that cell was discarded. The mean diffusion coefficient, D , for each cell was calculated, and all the mean D for a certain set of conditions, for example, “Vehicle-treated supported planar bilayers, resting state” (Table 3) was averaged to obtain the sample mean D for that set of conditions.

As with the confocal FCS experiments, we sought to validate the reliability of our ITIR-FCS system as a tool to study membrane fluidity of living cells. This was done via a series of control experiments on both artificial lipid membranes and on live cells. The first of these were cholesterol depletion experiments on supported planar bilayers, an artificial membrane system. Cholesterol is an essential component of the cell plasma membrane and its main role is in regulating the order and thus fluidity of the membrane via interaction with the lipid acyl tails in the hydrophobic core of the membrane bilayer. In membranes composed of unsaturated fatty acids, cholesterol increases order by interacting with and limiting the free movement of the acyl tails. In membranes composed more of saturated fatty acids, cholesterol decreases order by disrupting the close packing of the long, saturated lipid acyl tails. Therefore another way to describe the role of cholesterol in the membrane is that it increases fluidity in the membrane below the gel to liquid-crystalline phase-transition temperature and decreases fluidity at temperatures above it (Diaz et al., 2012). This phase transition temperature, in turn, depends on the types of lipids making up the membrane. Thus, cholesterol modulates the fluidity of the cell membrane, preventing it from either becoming too fluid or too rigid, either of which case would disrupt normal cell membrane function. One key role of cholesterol is its involvement in the formation of lipid “rafts” – transient membrane microdomains

particularly enriched in sphingomyelin and phosphatidylcholine, along with other long-chain, saturated lipids such as the ganglioside GM1. Methyl- β -cyclodextrin (m β CD) is a cyclic oligosaccharide consisting of seven glucopyranose molecules, with the 2, 3 and 6 –OH sites variably substituted with methyl groups to increase its solubility in water. m β CD has a hydrophobic core which allows it to interact with and sequester cholesterol.

Taking reference from Sasahara et al., bilayer composed of DOPC/SM/Chol (0.5/0.25/0.25) was prepared (Sasahara et al., 2013). 2.5% GM1 was added and 0.008% Rho-PE was used as the fluorescent marker to measure diffusion. We did not use DOPS in our bilayer; instead we made up for it by using more DOPC, as both DOPC and DOPS are structurally similar. Additionally, they used 1% TR-PE as the fluorescent probe, which is very much higher than in our membrane. The high concentration is necessary for their experiment since they used FRAP, but for our ITIR-FCS, only a concentration of 40 nM is needed. ITIR-FCS measurements of the resting bilayer returned a D value of $0.91 - 0.94 \text{ } \mu\text{m}^2 \text{ s}^{-1}$ (Table 2, “resting” and Table 3, time = 0), which agrees with Sasahara’s reported resting D of $0.99 \text{ } \mu\text{m}^2 \text{ s}^{-1}$ for their membrane (Sasahara et al., 2013). Our kinetic and endpoint measurements with m β CD-treated SPBs also showed expected increases in the fluidity of the membranes. In the kinetic measurements, we observed that the membrane quickly (< 5 min) became more fluid upon addition of 10 mM m β CD and remained at the higher levels than the resting state throughout the course of the experiment. This shows that the ITIR-FCS system can detect quick changes of membrane fluidity when it is perturbed. In the endpoint measurements, we again observed that the cholesterol-depleted SPBs were more fluid than the resting state, while control-treated SPBs remained unchanged. Five images were taken at different spots per SPB on a total of 3 SPBs for both control and m β CD-treated samples. With a 10-pixel by 10-pixel region of interest, this yielded 1,500 separate ACFs per condition. The standard deviations of the mean D for each condition were no higher than 25.3% for these measurements (Table 3), demonstrating that ITIR-FCS is a reliable and accurate method for measuring membrane diffusion.

We next moved on to apply the ITIR-FCS technique to living cells to see whether we could achieve the same level of sensitivity and accuracy as we did with the artificial lipid membranes, starting with the bulk membrane marker, the

carbocyanine dye C18-DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine). C18-DiI is a lipophilic dye that comprises a fluorescent head group coupled with two acyl tails, enabling it to be incorporated into the plasma membranes of living cells and has long been used to study membrane diffusion (Klausner and Wolf, 1980; Wolf et al., 1981). C18-DiI has also been used a marker of the liquid disordered or bulk phase in studying the membrane fluidity of living cells (Bag et al., 2013b). In our study, fluidity of the bulk phase of the cell plasma membrane was altered by simply changing the ambient temperature. By increasing ambient temperature from 25°C to 37°C, diffusion of DiI was increased by ~40% in both SH-SY5Y and Neuro-2a plasma membranes (SH-SY5Y: 25°C = $1.510 \pm 0.142 \mu\text{m}^2 \text{s}^{-1}$, 37°C = $2.148 \pm 0.202 \mu\text{m}^2 \text{s}^{-1}$; Neuro-2a: 25°C = $1.010 \pm 0.135 \mu\text{m}^2 \text{s}^{-1}$, 37°C = $1.430 \pm 0.216 \mu\text{m}^2 \text{s}^{-1}$) (Table 4, DiI). The mean diffusion coefficient of DiI in U87 cell plasma membranes also increased by nearly 100% (25°C = $0.686 \pm 0.440 \mu\text{m}^2 \text{s}^{-1}$, 37°C = $1.253 \pm 0.421 \mu\text{m}^2 \text{s}^{-1}$), but this was not statistically significant, possibly due to the small sample size ($N = 4$) and relatively high standard deviation of the mean. All in all, these experiments proved the ability of our ITIR-FCS system in detecting changes in bulk membrane fluidity.

We next attempted to measure any change in the fluidity of the raft-type membrane marker, CtxB, in response to changes in membrane lipid composition. Since CtxB binds to the raft-associated ganglioside GM1 in the plasma membrane, there should be an observable change in the diffusion of membrane-bound CtxB upon cholesterol depletion of the membrane. We measured the diffusion coefficient of CtxB diffusion on SH-SY5Y, U87 and Neuro-2a plasma membrane before and after treatment with 5 mM of m β CD. Mean D values of membrane-bound CtxB was reduced by almost 50% in SH-SY5Y cells (resting = $0.157 \pm 0.056 \mu\text{m}^2 \text{s}^{-1}$, treated = $0.081 \pm 0.008 \mu\text{m}^2 \text{s}^{-1}$) (Table 4). The mean diffusion coefficient of CtxB on Neuro-2a cells was also reduced by ~35% (resting = $0.124 \pm 0.034 \mu\text{m}^2 \text{s}^{-1}$, treated = $0.080 \pm 0.016 \mu\text{m}^2 \text{s}^{-1}$) (Table 4). This reduction was not statistically significant, however, when analyzed with a two-tailed, one-way ANOVA with a 95% confidence interval, possibly due to the small sample size. Diffusion coefficient of CtxB on U87 cells were not affected by m β CD treatment. Because cholesterol depletion is expected to decrease membrane order and *increase* fluidity, our observation that the fluidity of membrane-bound CtxB was *reduced* after m β CD may initially seem counterintuitive. There is nonetheless a plausible reason for such a phenomenon. Possibly, with reduced

cholesterol content, the degree of packing of GM1-containing raft-type domains increased because less cholesterol is available to limit the degree to which the acyl tails of the lipids associate with each other in raft-type membrane microdomains. Additionally, CtxB exists in solution and binds to GM1 as a pentamer, which inherently increases the order of packing of GM1 in the membrane. Therefore, reduction of membrane cholesterol may have allowed GM1 to associate even more closely with each other and with other raft-associated lipids, thus reducing its diffusion rate. Regardless of the reason behind this phenomenon, it is evident that changes in diffusion coefficient of membrane-bound CtxB could be detected by our ITIR-FCS system.

FCS measurements of two markers, CtxB and DiI, show that it is possible to differentiate between different membrane phases. CtxB exists as a pentamer and binds to the ganglioside GM1 in cell membranes (Aman et al., 2001; Merritt et al., 1994). This property of CtxB has led it to be used as a marker to study membrane diffusion as a raft or liquid-ordered phase marker (Day and Kenworthy, 2009), such as in synthetic membranes (Sasahara et al., 2013), liposomes (Sezgin et al., 2012) and even in studying synaptic membranes in living neurons (Renner et al., 2009). In our experiments, ITIR-FCS measurements of CtxB diffusion on undifferentiated SH-SY5Y cells at rest returned a mean D of $0.157 \mu\text{m}^2 \text{s}^{-1}$ (Table 4), similar to previously reported values for CtxB in primary rat hippocampal neuron membranes using single-particle tracking of quantum dot-labeled CtxB (Renner et al., 2009). ITIR-FCS measurements of DiI diffusion in SH-SY5Y cells at rest at 37°C returned mean D of $2.15 \mu\text{m}^2 \text{s}^{-1}$ (Table 4), similar to previously reported values of DiI in SH-SY5Y cells (Bag et al., 2013b). Additionally, mean D values obtained for both probes in A β vehicle-treated cells are alike regardless of the duration of treatment and in samples measured in separate experiments (Tables 7 and 8) demonstrating the consistency of ITIR-FCS for measuring membrane diffusion. The standard deviation of the mean D of each sample was generally no more than about 30%, meaning that the D values obtained in our measurements are consistent and reproducible for any given sample condition. This is also in contrast with confocal FCS experiments, where the τ_D could vary rather widely for similarly treated cells between different experiments, even though the instrument was calibrated with similar κ values each time. Therefore, we believe ITIR-FCS is an ideal method for probing the effects of A β on membrane fluidity.

4.7. A β (1-42) does not alter the plasma membrane fluidity of living cells

Confocal FCS experiments indicated that at the sub-toxic A β concentration of 1 μ M did not perturb the fluidity of membrane-bound CtxB on live SH-SY5Y cells. We thus decided to treat the cells with a higher concentration of A β – 10 μ M – because this concentration had been experimentally determined to be cytotoxic to both differentiated and undifferentiated SH-SY5Y cells. Although 10 μ M A β *did not* trigger apoptosis in U87 and Neuro-2a cells within 72h, we still decided to use this concentration of A β to treat these cells. Firstly, we had seen from confocal microscopy that U87 and Neuro-2a cells do internalize A β at this concentration. Secondly, absence of apoptosis does not mean absence of other cellular response. Thirdly, our goal was not to kill the cells outright but to examine whether there were any changes in the membrane fluidity that was caused by A β , and 10 μ M A β had already been shown to be potent enough to kill the more sensitive SH-SY5Y cells within 48-72h, thus proving its functionality. Fourth and finally, keeping a consistent A β concentration for experimenting with all cell types will provide greater insight on the activity of A β with respect to different cell types. We next determined three time points at which we would measure the cells' membrane fluidity after treating them with A β – 30 min, 24h, 48h. Examining the cells at 30 min should reveal any effects resulting from immediate addition of A β to the cell culture medium. The later time points of 24h and 48h were chosen because a) A β has certainly already been internalized by 24h and b) apoptosis had begun between 24 - 48h for the most sensitive SH-SY5Y cells, and between 48 – 72h for the U87 and Neuro-2a cells albeit at 20 μ M A β instead of 10 μ M. Apoptosis and reduced cell viability are not necessarily correlated with A β -related *membrane changes*, but these give an indication of A β -related *cell activity/response*. Any change in membrane fluidity should also occur before cell death, therefore we believed that 24h and 48h are suitable time points at which to examine the cells for changes in membrane fluidity. To study the effect of A β on the cell plasma membrane fluidity, we compared the diffusion behavior of three probes – C18-DiI, CtxB and GFP-GPI – after cells were exposed to either A β or vehicle. C18-DiI was selected as it inserts into and diffuses within the plasma membrane, reporting on the bulk membrane behavior. CtxB was chosen because of its status as a putative “raft”-marker, and because it binds to the GM1 ganglioside which is itself thought to have certain interactions with A β . A third probe, GFP-GPI, was chosen as a second marker of raft-type membrane fluidity, in

case CtxB/GM1 diffusion was not affected by A β . Thus, by reporting the diffusion behavior of the bulk membrane, of raft-type membrane microdomains and of GM1-specific/GM1-containing raft-type membrane domains, we believe that these three probes are sufficient to give an overview of the response of the cell plasma membrane to A β .

ITIR-FCS analysis indicated that A β did not have any effect on the fluidity of DiI, CtxB and GFP-GPI in the most sensitive SH-SY5Y cell type, neither at the early time point immediately following addition of A β to the culture medium (30 min) nor at the later time points (24 – 48h) when apoptosis had already begun. Similar observations were made for the U87 and Neuro-2a cell types. Against the backdrop of prior studies that had shown either an increase or decrease in the fluidity of biological membranes attributable to A β , this result does not immediately strike us as likely. We thus attempted to replicate the results of a recent, earlier study which showed activity of A β with artificial lipid membranes (Sasahara et al., 2013), with the intention of verifying the efficacy of our A β . However, we were unable to replicate those results, even though we used identical SPB lipid content and A β preparation methods (Table 9). We also probed the effects of both wild-type and scrambled A β prepared with our protocol, however those too did not perturb the fluidity of the SPB, save that the S.D. of the mean were increased versus the resting state (in Buffer A) and SPBs treated with water without any peptide. FCS diffusion law did not reveal any significant changes in membrane heterogeneity or diffusion behavior upon A β treatment of the membranes. Thus, we have a conundrum – since A β does have expected cytotoxic activity, how does it not also affect plasma membrane fluidity, given that several studies have previously demonstrated such effects?

4.8. A β does not alter cell membrane fluidity – potential confounding factors

That A β did not change membrane fluidity whatsoever in the cells examined in our study does not come as expected. There are indeed several potential confounders in the experimental set up used in this study, which will now be discussed.

Firstly, as discussed in Section 4.4, the A β solutions prepared in this study could possibly consist entirely or predominantly of A β monomers, which may not be

biologically active within the conditions tested in this study. However, we have also discussed and shown that firstly, the A β preparation method in question has been shown by several other researchers to reliably generate A β oligomers. In this study, solution-based methods were not used to analyze my A β solutions and thus we cannot be certain that said A β solutions do contain oligomeric A β . An attempt was made to replicate the experiment of Sasahara et al. with artificial lipid bilayers (Sasahara et al., 2013) to verify that A β did have certain effects on lipid membranes (Section 3.12, Table 9). However, ITIR-FCS measurements failed to detect any change in membrane fluidity of the artificial bilayer used, whether treated with wt-A β /scrambled-A β prepared per my protocol or prepared according to that of Sasahara et al. (Table 9). The reason for this observation is currently unclear. Nevertheless, the A β solutions prepared using the protocol listed in this study clearly do trigger apoptosis in the tested cell types within a reasonable time frame (48 – 72h) when compared to data from other *in-vitro* cell-based studies (as discussed in greater detail in Section 4.4). Therefore, it must be noted that in this study, the specific type of A β species present in the solution is unknown, the method of A β preparation produced an A β solution that is biologically active to the cells used, and the results obtained from cell-based membrane fluidity assays in this study are attributable to this form of A β , whatever its exact composition may be.

Secondly, could A β have induced either subtle or complex changes in membrane fluidity that were somehow not detected by ITIR-FCS? This is certainly possible. Bailey et al., in studying the effects of apoptosis on plasma membrane biophysics, observed that the typical spectroscopic measurements of laurdan general polarization in apoptotic cells did not show changes in membrane fluidity, but two-photon microscopy of the same dye in the same cells *did* show some changes (Bailey et al., 2009). To this end, I shall discuss a study published in 2014 by Guo et al, which also uses ITIR-FCS as a means of assaying membrane fluidity of living cells (Guo et al., 2014). To describe briefly, Guo and his colleagues applied Bayesian analysis to the standard ITIR-FCS data to obtain greater spatial contrast for the images, allowing for the determination of diffusion coefficients that were initially not resolvable spatially. Thus, they could discern different membrane phases on images of cell membranes which were not apparent before applying Bayesian analysis, based solely by the calculated diffusion coefficients of each pixel in the image. Applying this technique to SH-SY5Y cells treated with another amyloidogenic peptide, the human

islet amyloid polypeptide (hIAPP), and using a two-component model, they showed that there was a fast component and slow component in the same observed area of membrane and that the slow component gradually increased in size (or proportion) over a period of 60 min. This two-component Bayesian analysis showed an initial increase in diffusion coefficients (increase in membrane fluidity) of both the fast and slow diffusing membrane components in the first 10 min, which remained unchanged for the next 50 min. Standard ITIR-FCS analysis of the plasma membrane instead showed an initial increase in diffusion coefficients in the first 10 min, followed by a gradual decrease over the next 50 min. Taken together, this indicates that standard ITIR-FCS can reveal changes in membrane fluidity if these changes are of sufficient contrast to the resting state, but more complex or detailed membrane dynamics could be missed if no further analysis is conducted. Notably, these changes happened quickly, on the order of minutes, with a peptide concentration of 1 μ M. In my current study with A β , the time points (30 min, 6h, 24h and 48h) at which membrane fluidity was assayed after addition of A β to the medium should sufficiently cover both the early and late stages of peptide interaction with the cells. Since only standard ITIR-FCS was used, the absence of changes in membrane fluidity of cells exposed to A β could either be because A β indeed did not cause any change to plasma membrane fluidity, or that A β did cause some changes that are too subtle or of too low magnitude to be observed by standard ITIR-FCS – a factor that must be kept in mind when interpreting the finality or impact of the results presented in this study.

Thirdly, because apoptosis was triggered in the A β -treated cells and apoptosis itself can potentially cause changes in membrane fluidity, could it be that the effects of apoptosis masked any effect A β had on membrane fluidity? There has indeed been some information that apoptosis itself may have effects on cell membrane organization and concomitantly, membrane fluidity. Rachel Bailey and associates, in studying the phenomenon of lipid hydrolysis by secreted phospholipase A₂ (sPLA₂), reported that firstly, general polarization measurements of the membrane dye, laurdan, showed no change in membrane fluidity when apoptosis was induced in S49 lymphoma cells by the glucocorticoid dexamethasone (Bailey et al., 2009). While not definitive, their study suggested that upon glucocorticoid-induced apoptosis, the membrane initially becomes less ordered (more fluid) and then gradually more ordered (less fluid) as apoptosis progresses. A later, related study by

Elizabeth Gibbons and associates went into deeper analysis of this phenomenon and showed that membrane fluidity indeed first increased and then decreased in SH-SY5Y cells treated with the apoptosis-inducer, thapsigargin (Gibbons et al., 2013). General polarization measurements of Patman and anisotropy measurements of TMA-DPH (both being membrane dyes) showed that membrane fluidity began increasing immediately upon treatment of cells with thapsigargin, reaching a maximum increase somewhere between 3 – 5h and then decreased again from around 6h onwards. Both Bailey and Gibbons show that towards the late stages of apoptosis, the cell membrane becomes leaky and the dye starts to permeate into the cell. Thus, it is quite certain that apoptosis itself has effects on membrane biophysics, including fluctuations in membrane fluidity. Could such effects indeed be masking any effects of A β on the cell membrane, thus explaining the reason no net change of membrane fluidity in our present study? A simple answer would be “yes”, but there is reason to believe otherwise. First, we must consider that the apoptosis-related changes in membrane fluidity as reported by Bailey and Gibbons occur quickly and concurrently with the onset of apoptosis. Specifically, thapsigargin, an apoptosis-inducer that works by increasing cytosolic Ca²⁺ concentration, caused maximum caspase activation and susceptibility to sPLA₂ hydrolysis by ~3h post-treatment (Gibbons et al., 2013). In our study, we showed that the earliest onset of A β -induced apoptosis, as evidenced by caspase activation, was 48h, and that only in the most sensitive differentiated SH-SY5Y cells (Fig. 15, Table 1b). In undifferentiated SH-SY5Y cells (Fig. 15, Table 1b), Neuro-2a cells and U87 cells (Table 1b), apoptosis only set in between 48 – 72h. The caspase assay showed no caspase activation within 24h for *all* tested cell types. Treating cells with a 0.5 mM staurosporine, a pan-apoptosis inducer, triggered intense caspase activation and massive cell death within 6h (assay control, data not shown), assuring us that the tested cells do have intact apoptosis mechanisms. Apart from A β , no other reagent that could possibly have caused apoptosis was present in the cell culture medium. Identical amounts of DMSO was present in both A β -treated and vehicle control-treated samples and did not trigger apoptosis in vehicle control-treated samples, and scrambled A β peptide also did not trigger apoptosis (compared to vehicle-treated controls). Thus, we can be quite certain that apoptosis observed in the A β -treated cells is due to the presence of A β alone. Cell viability of differentiated SH-SY5Y cells drastically decreased by 72h (Fig. 15a). Assuming A β -induced apoptosis was triggered and reached a maximum between 24 – 48h for differentiated SH-SY5Y cells and at $\geq$ 48h for all other cell types, the accompanying apoptosis-

related changes in membrane fluidity should also occur and be most pronounced between 24 – 72h for differentiated SH-SY5Y cells and at ≥ 48 h for the other cell types. In this study, ITIR-FCS assaying of membrane fluidity was conducted at 30 min, 6h, 24h and 48h after addition of A β to the cell culture medium (Tables 5, 6, 7, 8, Supplementary Fig S2). Thus, even if all data obtained after 24h, where effects of apoptosis could possibly mask effects of A β on membrane fluidity, were excluded from the analysis, the remaining data (30 min, 6h and 24h) still indicates no change in membrane fluidity upon exposure of cells to cytotoxic concentrations of A β . It is believed that one key pathway by which A β triggers apoptosis in cells is that it causes abnormal increases in cytosolic Ca²⁺ concentration, either by making the plasma membrane leaky (Arispe and Doh, 2002; Campioni et al., 2010; Evangelisti et al., 2012) or by compromising mitochondrial membrane integrity (Demuro et al., 2005; Deshpande et al., 2006). This is a similar net effect as that caused by thapsigargin, which inhibits Ca²⁺ uptake by the endoplasmic reticulum, thus increasing cytosolic Ca²⁺ concentration, and which could cause apoptosis-related changes in membrane fluidity (Gibbons et al., 2013). It could well be possible that A β *does* cause apoptosis-related changes to membrane fluidity, and these effects may not have been detected by the standard ITIR-FCS method. However, the goal of this study is to examine the effects of A β exposure itself, and not A β -induced apoptosis on cell membrane fluidity. Thus, the current methodology of assaying membrane fluidity at times earlier than when significant increases in cellular caspase levels occurs should suffice to answer the research question of whether A β does perturb cell membrane fluidity, with the caveat that there could be changes happening that were not apparent under standard ITIR-FCS.

Fourth, I shall discuss the cell types used. It can be argued that *in vitro* cell culture may not be representative of the situation in real, living tissue or organisms and thus A β does not affect these cells in ways that we would expect it to. In our study, the 3 cell types chosen all originated from brain tissue. SH-SY5Y cells are both human and neuronal in origin, while U87 cells are human but non-neuronal and Neuro-2a cells are neuronal but non-human. We found that all three cell types can internalize A β (Section 3.3) and have varying sensitivities to A β exposure, with differentiated SH-SY5Y cells being the most sensitive, followed by non-differentiated SH-SY5Y cells and finally U87 and Neuro-2a cells (Section 3.4). The sensitivity of these cell types may be correlated with their phenotype, which would make sense given our

understanding that cortical and hippocampal neurons are the most affected cell types in Alzheimer's disease, and that A β production or AD does not occur naturally in mice. Further, this also shows that A β is not simply some kind of "pan-cytotoxic" molecule, but it has different potencies in different cell types depending on the nature of the cells. Thus, the cell types chosen are sufficiently sensitive to A β to be relevant, even if they are not the same as primary cortical or hippocampal neurons or as brain tissue. It must, however, be taken into account that the methodology presented in this study is confined to the *in-vitro*, and while useful as a tool to glean information on basic cellular mechanisms, the findings may be very different from the situation *in-vivo*, where effects of organism homeostasis are in play.

Fifth, I shall discuss the choice of the membrane probes. Are DiI, CtxB and GFP-GPI suitable probes for assaying potential membrane fluidity changes caused by A β ? DiI is a lipid analogue and is used as a general membrane stain. Several variants of DiI exist and can be purchased from commercial vendors, including short chain (e.g. C12) or mono-unsaturated variants. The choice of C18-DiI was made in this study based on related studies that have reliably established DiI as a liquid-disordered phase marker of cell plasma membranes (Bag and Wohland, 2014; Bag et al., 2013a; Bag et al., 2013b; Guo et al., 2014; Sezgin et al., 2012). GFP-GPI and indeed other GPI-anchored proteins in living cells have also previously been shown to be amenable to changes in ambient temperature and cholesterol depletion, and useful for tracking membrane fluidity dynamics of liquid-ordered membrane phases (Bag et al., 2013a; Day and Kenworthy, 2009; Renner et al., 2009; Saha et al., 2015). CtxB specifically binds GM1 on the cell membrane and like GFP-GPI, has been used as a marker of raft or the liquid-ordered membrane phase (Bacia et al., 2004; Renner et al., 2009; Sezgin et al., 2012; Zhang et al., 2009). In my study, control experiments done with DiI (temperature-dependent behavior, Section 3.7, Table 4) and CtxB (cholesterol-dependent behavior, Section 3.7, Table 4) showed that the ITIR-FCS system used is sensitive enough to detect pan-membrane changes in either the bulk membrane or in cholesterol-dependent membrane domains. As reviewed earlier (Sections 1.5 – 1.7), cholesterol is also thought to be a major factor in A β interaction with cell membranes, with various theories being proposed for its role, such as modulating A β insertion into the membrane. Should A β have indeed perturbed the availability or distribution of membrane cholesterol, we believe our system would have detected it, as evidenced by clear changes in membrane fluidity of CtxB upon

cholesterol depletion. One caveat to consider is that the ganglioside GM1 is believed to interact directly with A β , perhaps acting as a sort of seed molecule for membrane-based aggregation of A β (Wakabayashi and Matsuzaki, 2007; Wakabayashi et al., 2005). It is unknown whether A β could trigger effects on the membrane diffusion of CtxB that are a specific result of this type of A β interaction with GM1. However, two liquid-ordered phase markers were used (CtxB and GFP-GPI) in our study, which should allow for sufficient coverage of the liquid-ordered phase. All in all, we showed reproducible behavior of our chosen probes, DiI, CtxB and GFP-GPI, under well understood and tested conditions. Certainly, these three probes are by no means exhaustive or even highly comprehensive, and this should be considered when pondering the results of this study.

While there are perhaps other potentially confounding factors in the interpretation of the results obtained in this present study regarding the effects of A β on cell plasma membrane fluidity, I have discussed in this section five of the more critical ones. Overall, it can be taken that the results of this study show what happens to the cell plasma membrane when exposed to A β prepared via the oligomer-aggregation method, using three membrane probes that report on both the liquid-disordered bulk membrane and the liquid-ordered or raft-type membrane. The specific type of A β species in the solution is unknown, and the probes are by no means comprehensive. These factors must be considered when considering the results presented in this study. Bearing this in mind, I continue with further discussion of my findings and their implications.

4.9. Can A β perturb cell plasma membrane fluidity?

Our observation that A β does not change fluidity of CtxB and DiI seems to conflict with data reported by some earlier studies, which report either an increase or a decrease in membrane fluidity, but not no change. There are two big questions that need to be answered: Firstly, why does A β not perturb the fluidity of the plasma membrane in our study, although it has been previously shown to do so in several membrane models? Secondly, does this mean that the cell plasma membrane is totally unaffected by A β ?

First, let us consider this question: should we expect A β to affect membrane fluidity, and if so, what are the underlying mechanisms? Even though several studies, such as those of the Wood and Muller groups (Avdulov et al., 1997; Chochina et al., 2001; Eckert et al., 2010; Muller et al., 1998; Müller et al., 1995) are in conflict with regard to the effects A β have on membrane studies, there have been other precedents to suggest that binding of A β to a lipid membrane can perturb membrane fluidity. Two recent studies, both published in 2013, give us some insight to the possible biophysical process related to beta-amyloid's ability to affect cell membrane fluidity. Filippov and Antzutkin found, using NMR spectroscopy of two-phase DOPC/SM bilayers with varying amounts of cholesterol, that the presence of A β (1-40) resulted in different changes to the lateral diffusion (D_L) of liquid ordered (lo) and liquid disordered (ld) phases. At low (20%) membrane cholesterol, D_L of the lo phase decreased while D_L of the ld phase increased. They suggested that this was due to cholesterol being transferred from the ld phase to the lo phase. At high (33%) membrane cholesterol, the D_L of *both* ld and lo phases decreased. They posited that only the removal of cholesterol from both phases can cause such a result – that is the interaction of A β (1-40) with the membrane somehow led to a sequestration of cholesterol into a third – possibly a solid – phase (Filippov and Antzutkin, 2013). They also suggested that, based on their observation that the solubility of cholesterol in bilayers comprising only of one type of lipid is dependent on the polarity of the lipid head groups, A β probably caused the sequestration (lower solubility) of cholesterol by modifying the interaction of the lipid head groups either with each other or with cholesterol. The second study is that by Kenji Sasahara and associates, who found that adding A β (1-42) to DOPC/DOPS/SM/Chol bilayers caused a sharp decrease in lateral diffusion of the membrane (Sasahara et al., 2013). This effect was more pronounced when GM1 was added to the membrane. Sasahara et al. suggested that the latter effect was due to A β interacting with GM1 in the membrane, leading to phase separation of the A β -GM1 complexes into raft-type domains or possibly even sequestration of these peptide-lipid complexes. They did not, however, explain or suggest the reason for reduction in lateral diffusion even in the absence of GM1. Finally, Guo et al., studying the effects of hIAPP, also an amyloidogenic peptide, on live cell membranes of SH-SY5Y cells, found that while the mean diffusivities of the fast- and slow-diffusing membrane fraction does not change much over time after hIAPP was added to the cell culture, the size of the slow-diffusing fraction increased while the size of the fast-diffusing fraction decreased leading to an overall decrease

in the mean diffusivity of the membrane (Guo et al., 2014). They suggested a ‘carpet model’ of peptide aggregation on the membrane, whereby the peptide first bound to the membrane, then extracted lipids from the membrane, triggering a phase separation of the membrane with an increasing slow-fraction size over time. In my study, control experiments were performed where methyl- β -cyclodextrin (m β CD) was used to deplete membrane cholesterol (Section 3.7). In the SH-SY5Y cells, diffusion of raft-type marker CtxB was markedly altered when cells were treated with m β CD (Table 4, “CtxB” column). While the experiments described here differ in the set up and reagents used, one thing is clear – diffusivity of a membrane can be altered if there are changes in the membrane lipid composition. This would likely be the way in which A β would cause changes in membrane fluidity in the cell membrane, if it indeed could.

We currently understand, to a certain extent, the interplay of factors contributing to the decrease in membrane fluidity of neuronal membranes in old age and in neurodegenerative diseases such as Alzheimer’s disease. One of the key factors seems to be the balance in the levels of cholesterol, poly-unsaturated fatty acids (PUFA) and long-chain poly-unsaturated fatty acids (LC-PUFA) in the aging neuronal membrane (Yehuda et al., 2002). Raquel Marin and associates have also shown that in transgenic mouse models of Alzheimer’s disease, the lipid rafts isolated from the mouse brain were less fluid even though cholesterol levels in the rafts of aged transgenic mice were 12% lower than that of wild-type mice of the same age, which theoretically should increase fluidity of the membranes (Diaz et al., 2012). They attributed this to the greater reduction (30%) in the level of LC-PUFAs in the rafts of transgenic mice and concluded that while membrane fluidity is controlled by the presence of lipids that promote greater hydrophobic packing, unsaturated fatty acids are ultimately key in modulating the overall membrane fluidity. From this perspective, it seems that the control of membrane fluidity in biological membranes is intimately linked to the *lipid composition* and by association, the *organization in the lipids* in the membrane. Possibly, exposure of cells to A β in the setting of our study does not alter the lipid composition of the cell membrane by an appreciable degree in living cells. Although there have been several previous studies showing a link between A β and membrane fluidity, to our knowledge, this is the first study where cell membrane fluidity was observed live upon the treatment of cells with A β . There may be factors influencing membrane fluidity in living cells that are not

apparent in cell-free systems, such as the role of lipid metabolism and homeostasis in maintaining membrane composition, and other structural factors such as the sub-membrane actin meshwork or other non-lipid components of the membrane that ‘buffer’ the cell membrane from any acute changes in fluidity. In several A β -related studies on the membrane, A β is assumed to attach to and insert into the membrane bilayer, resulting in either changes in membrane fluidity or membrane permeabilization. Notably, in Kenji Sasahara’s study, even though micromolar concentrations of A β dramatically reduced membrane fluidity of a liquid-ordered type of supported planar bilayer (A β was assumed to be monomeric at the time of addition to the membrane), membrane fluidity *recovered once A β was desorbed from the membrane* by flushing with buffer (Sasahara et al., 2013). This has implications for the association of membrane fluidity effects with A β . Firstly, if changes in membrane fluidity caused simply by binding of A β are non-permanent, this type of interaction alone may not be able to cause long-lasting changes membrane fluidity. Secondly, the change in fluidity observed in a cell-free system such as this may purely be an *in vitro* biophysical phenomenon, due, for example, to changes in the membrane phase when proteins bind to the it (Forstner et al., 2006), and is not necessarily replicable in living organisms or cells. The cells in our experiments were always exposed to A β , except for the brief periods during which the cell membrane was stained with the fluorescent probes, yet there were no marked, permanent changes in membrane fluidity. Additionally, we probed the membrane fluidity at time points whereby cell viability was already reduced and apoptosis had been triggered, which means that A β had started to affect the cells detrimentally. Despite these conditions, we did not observe changes in both the bulk membrane (measured by DiI) nor in ordered, raft-type membrane subdomains (measured by CtxB and GFP-GPI). Although another amyloidogenic peptide, the hIAPP, could trigger phase separation in living cell membranes (Guo et al., 2014). In Guo’s study, it was suggested that hIAPP ‘carpets’ the cell membrane and forms lipid-peptide aggregates on it, triggering phase separation and thus changes in membrane fluidity. This mechanism of interaction may not hold true for A β , because while both can form amyloid fibrils, they are nevertheless unrelated peptides. It is currently unclear how A β interacts with the cell membrane in the set up used in our study – whether it forms clusters, inserts into or ‘carpets’ the membrane, or some other interaction. Bearing this in mind, our conclusion would be that while the nature of the A β -membrane interaction in living cells is unclear, we know that presence of the A β

peptide alone does not alter membrane fluidity despite being in sufficient amounts to trigger programmed cell death, and that this is probably because the A β peptide does not trigger membrane phase separation or changes in lipid composition when applied to cells in the manner described in this study.

4.10. The role of the plasma membrane in a cell's interaction with A β

If A β indeed does not perturb plasma membrane fluidity in the short-term, how does it exert detrimental effects on the cell? One possibility is to consider the effect that A β has on cellular homeostasis. One hypothesis of A β -mediated cytotoxicity posits that the detrimental effects A β has on cells is due firstly to dysregulation of cholesterol metabolism, leading to greater production and aggregation of A β , altered gene regulation and increased oxidative stress, and secondly, dysregulation of sphingolipid and ceramide metabolism, resulting in increased A β production, decrease levels of sphingomyelin and increased levels of ceramide, which also increases oxidative stress and triggers apoptosis-related cell signaling (Cutler et al., 2004; Grosgen et al., 2010). It is believed that A β causes these changes via its interaction with HMG-CoA reductase and sphingomyelinases. From this perspective, A β causes *changes in cellular metabolism*, leading to *apoptosis-mediated cytotoxicity*. Another hypothesis for A β -induced cellular toxicity, the calcium dysregulation hypothesis, suggests that A β increases permeability of the cell membrane, either directly via the insertion of A β into the cell membrane to form cation channels, or otherwise via some hitherto unknown mechanism (Benilova et al., 2012). An alternative mechanism might be that internalized A β somehow triggers release of intracellular calcium stores (LaFerla et al., 2007). The calcium dysregulation hypothesis postulates that neuronal excitotoxicity, inhibition of long-term potentiation, augmented long-term depression, or a combination of these effects are a result of sustained increase in intracellular calcium levels. These processes are indeed neuron-specific and are closely related to calcium homeostasis, and their disruption may result in cytotoxic effects. However, abnormal increases of intracellular calcium are also detrimental for non-neuronal cells, triggering cell death via the cytochrome c-mediated apoptosis pathway.

In our study, we established that A β does have cellular activity to the effect of inducing apoptosis. Is it possible that A β can exert its effects on cells *without*

perturbing the cell plasma membrane? This scenario does seem quite improbable – given the role of the plasma membrane as the primary barrier between the interior and exterior of a cell, any exogenous factor, protein or otherwise, must somehow interact with the plasma membrane as the first step of its encounter with a cell. What role, then, does the plasma membrane play in mediating the effects of A β on the cell? Whether altered lipid metabolism or calcium dysregulation, for A β to cause apoptosis, it must either trigger a signaling event at the plasma membrane, which when transduced to the cell cytoplasm eventually results in apoptosis, or A β itself, when internalized, triggers apoptosis via interacting with related biochemical pathways within the cytosol. One interesting point to consider is that A β is thought to bind to the ganglioside GM1 on the cell membrane, which is the very same receptor that the cholera toxin utilizes to gain entry to the cell via endocytosis. In the case of cholera toxin, the pentameric B-subunit causes clustering of GM1, which presumably triggers endocytosis of the toxic A-subunit into the cell. Mutant cholera toxin, which has fewer GM1 binding sites on the B subunit, has reduced ability to cluster GM1 resulting in reduced endocytic uptake of the toxin and reduced toxicity (Wolf et al., 2008). In our study, we treated cells with A β solutions comprised initially of monomers, trimers and tetramers. It is currently unclear whether any one specific type of aggregate, part or perhaps even all of them, can cluster GM1 in a way that triggers their cellular uptake. Research exists indicating that A β is indeed internalized by neurons as well as differentiated SH-SY5Y cells and is linked to a plethora of detrimental effects (LaFerla et al., 2007; Saavedra et al., 2007; Zheng et al., 2012). If the internalization of A β leads to cellular toxicity, the role of plasma membrane would be in regulating the entry of A β into the cell. Possibly, differences in the plasma membrane of different cells play a part in this regulation. Strong evidence has been produced by Massimo Stefani to support this hypothesis (Cecchi et al., 2005; Evangelisti et al., 2012). In the first study, Cecchi et al. showed that sensitivity of different cell types to the bacterial self-aggregating peptide, HypF-N, is related to membrane cholesterol content. Cell lines with lower membrane cholesterol were more susceptible to HypF-N toxicity than those with higher membrane cholesterol. In the latter study, Evangelisti et al. showed using SH-SY5Y cells, that in addition to cholesterol content, membrane GM1 content was also related to the sensitivity of cells to toxic peptide aggregates. Cells with more GM1 in their membranes were more sensitive to HypF-N induced cytotoxicity. There are three points of particular note in Evangelisti's study: first, in addition to pre-fibrillar HypF-

N aggregates, the cytotoxicity of oligomeric A β (1-42) was also examined and shown to be similar to HypF-N. Second, the researchers showed a link between membrane lipid composition and permeability of the membrane to the toxic peptide aggregates – that is, low cholesterol and high GM1 results in increased membrane permeability to HypF-N and A β (1-42). Third, they showed that internalization of the toxic aggregates resulted in increased cytosolic Ca²⁺ and caspase 3 activation. There has been some studies done on the membrane lipid content of native SH-SY5Y cells (Reynolds et al., 2001), but to date there has not been a comprehensive survey of the lipid contents of differentiated SH-SY5Y cells. Nevertheless, Rebhan et al. showed that the ganglioside content of SH-SY5Y cells change upon differentiation (Rebhan et al., 1994). Markedly, the GM1 content was almost 8-fold higher in differentiated cells than in undifferentiated cells, along with increases in other gangliosides such as GD1a, GT1a, GD1b and GT1b (Rebhan et al., 1994). The U87 cells type, which were used in our study, have been shown to lack GM1 in their plasma membranes, at least not at levels detectable by TLC (Ecsedy et al., 1999). In our study, we showed that differentiated SH-SY5Y cells are more sensitive to A β -mediated cytotoxicity than undifferentiated SH-SY5Y cells, and SH-SY5Y cells are on the whole more sensitive than either U87 or Neuro-2a cells. These observations suggest that the *cell plasma membrane could indeed be involved in A β effects on cells but the plasma membrane may not necessarily be perturbed by A β in a way such as to change its fluidity, whether via changes in lipid composition or otherwise.*

4.11. Proviso – A β does not perturb membrane fluidity under acute cytotoxic conditions attained *in vitro*

In the two preceding sections, we explored the different interactions A β is known to have with the cell plasma membrane and stated that A β might conceivably not perturb plasma membrane fluidity. We must qualify however, that our study involves cells grown *in vitro*, exposed to A β -in a very unnatural manner that triggers acute cytotoxicity – something that probably does not happen clinically. First, in our study, the concentrations of A β that the cells are exposed to are much higher than what occurs in nature. For membrane fluidity experiments, cells were exposed to between 1 - 10 μ M of A β , whereas soluble A β is typically detected at only picomolar to nanomolar levels in the human brain (Johnson et al., 2011). Secondly, in our study, the cells experience the detrimental effects of A β treatment in a very short time frame

– apoptosis begins within as little as 48h in differentiated SH-SY5Y cells. We discussed in Section 4.9 that changes in cell membrane fluidity in the brain is known to be related changes in lipid composition because of aging or disease. This is a process that happens over extended periods of time, and it could very well be the reason why we do not pick up changes in the membrane fluidity of A β -treated cells in our study. Indeed, when we used m β CD to deplete cholesterol, a membrane lipid, from the cells, we were immediately able to detect a decrease in membrane fluidity of CtxB. Instead, it seems that in our study, the cells experience something akin to an ‘acute’ condition or disease, where a high concentration of A β quickly triggers apoptotic cell death, likely related to A β -induced signaling events. There is some experimental basis to this statement. Previously, Atul Deshpande and his colleagues showed that micromolar concentrations of A β prepared in a similar manner as in our study triggered cell death in human cortical neurons via a pathway involving the release of cytochrome c and apoptosis-inducing factor (AIF) following membrane damage in mitochondria. They state that mitochondrial damage happens immediately following treatment with A β , and neurotoxicity sets in within 5 – 7 days of treatment with 5 μ M of ADDLs. This timescale is not dissimilar to what we have observed in our study. More recently, Lee et al. reported similar findings using similar A β preparations and SH-SY5Y cells, although they did not differentiate the cells in their study (Lee et al., 2015). All these factors taken into consideration, we may conclude that in our study, cells were subjected to an ‘acute’ exposure to A β , which led to the triggering of cell death via apoptosis. Possibly, the fluidity of the cell plasma membrane is not affected in this scenario. This is more in line with the perspective presented in section 4.10, where the role of the plasma membrane is to prevent toxic exogenous molecules from entering the cell, failing which the cell suffers detrimental effects leading to programmed cell death. Thus, *membrane fluidity may be affected by A β exposure but not in a way that is apparent or that occurs via the scenario explored in our study.*

4.12. A β and plasma membrane fluidity – a ‘chicken and egg’ question?

How does the cell plasma membrane and its functions relate to the pathology and symptoms of Alzheimer’s disease? Membrane fluidity plays an important role in neuronal membrane and cell function, therefore changes in the fluidity of the membrane may indeed cause the symptoms of Alzheimer’s disease, such as the loss

of synapses, inability to form new memories due to impaired long-term potentiation and augmented long-term depression, and/or excitotoxicity resulting from undesirable influx of Ca^{2+} ions across leaky cell membranes. On the other hand, as we have observed in our experiments and as other studies have indicated, $\text{A}\beta$ can cause very quick cell death (within days) due to induction of apoptosis. Significantly, in our experiments with differentiated and undifferentiated SH-SY5Y cells, which are human, cerebral and neuronal in origin, $\text{A}\beta$ caused similar effects despite the more neuronal-phenotype of differentiated cells, albeit differentiated cells were more sensitized to $\text{A}\beta$ -induced apoptosis. The typical symptoms of Alzheimer's disease happen over a period of years, even decades, and may not be directly correlated with gross cell death. We also know that cell membrane fluidity is tightly linked to lipid composition, which is controlled by lipid metabolism and homeostasis, which changes with age. Additionally, the cell membrane is responsible for permitting or denying entry of exogenous material to the cell, including $\text{A}\beta$. This then begs the proverbial 'chicken-and-egg' question – when it comes to $\text{A}\beta$ -related cellular damage, which is the primary factor? Which comes first, the $\text{A}\beta$ or the plasma membrane? Is it the accumulation and aggregation of $\text{A}\beta$ in the brain which harms the neurons, resulting in cell death and the onset of pathological symptoms of AD, or is it changes in lipid metabolism and thus membrane composition, that sensitizes the cell to $\text{A}\beta$ -induced abnormalities, such as calcium dysregulation, structural damage to cellular components such as the microtubules, and apoptosis? A complicating factor is the fact that $\text{A}\beta$ itself potentially affects lipid metabolism, especially for cholesterol and sphingolipids (Cutler et al., 2004; Grosvenor et al., 2010), and that APP processing and $\text{A}\beta$ generation seem to be related to membrane function (Schneider et al., 2008), since the β - and γ -secretases and APP are themselves integral or membrane-associated proteins. We observe $\text{A}\beta$ -related effects in cells often at high (micromolar) concentrations; in the human body, $\text{A}\beta$ is usually present at much lower (pico – nanomolar) concentrations, which is sometimes not only non-toxic, but may even be trophic (Dahlgren et al., 2002). Possibly, low-yet-abnormal concentrations of $\text{A}\beta$ can cause changes in lipid metabolism and/or APP processing that eventually lead to the more acute, more toxic effects of $\text{A}\beta$ to neurons in the brain. These aspects most likely can only be properly examined via studies in animal models, where diet and organism-level effects are in play. To this end, research like that done by Raquel Marin has thus far shown through experiments with transgenic, APP-expressing mice, that even though $\text{A}\beta$ does correlate with decreased raft-type

membrane fluidity at the organism level, the decrease is also correlated with changes in the composition of more than one type of membrane lipid, including cholesterol and fatty acids (Diaz et al., 2012). Crucially, the group has recently reported similar findings from analysis of the brains of actual AD patients (Diaz et al., 2015). Therefore, the situation *in vivo* is likely to be not as straightforward as simply '*A β causes such-and-such of an effect to the cell plasma membrane*', but a more adequate description would be, '*exposure to amyloid-beta, with factors such as age, diet, metabolism and other underlying conditions may lead to changes in the lipid composition, and therefore membrane fluidity, of plasma membranes of cells in the brain*'.

5. Conclusion

Because of aging populations in much of the developed world, age-related neurodegenerative diseases, such as dementia, will continue to grow in prevalence and in significance in the next few decades (Abbott, 2011). Alzheimer's disease is one of these diseases, and there is currently a knowledge gap with regard to disease progression, which hampers us from developing effective strategies for treatment and therapies for affected or at-risk individuals. In nature, Alzheimer's disease progression does not occur quickly; there may be decades between the onset of mild cognitive impairment and full-blown Alzheimer's disease (Gandy, 2011; Williams, 2011). The amyloid-beta peptide is implicated at both early and late stages of the disease, and is therefore a 'main character' to be studied if we are to properly understand the disease. Because reduced membrane fluidity in the brain is thought to be disease-causing in aged individuals, one of the areas of interest in A β research has been to investigate its effects on membrane fluidity. To that end, several studies have been conducted, yet reports have been at times conflicting and there has not been a conclusive answer to the question. This could be due to the heterogeneity in the design of the various studies, where the species of A β , type of model membrane, type of probe and even the measurement technique may introduce biases into the results obtained. In our study, we first tried to optimize the experimental setup by using relevant A β species and living cell membrane as the model, coupled with a sensitive and reliable technique of measuring membrane fluidity, in the hope that this will help us to conclusively answer the question of what, if any, effects does A β have on cell membrane fluidity? Our results do appear to be in conflict with

previously reported results, because we detected neither an increase nor decrease in cell membrane fluidity of both liquid ordered and liquid disordered phases in the membranes of A β -treated cells, even though the treatment was sufficient to induce cytotoxicity. Looking deeper however, our results do not actually preclude that A β may cause changes in cell membrane fluidity. This may be because membrane fluidity is largely controlled by the lipid content of the membrane and lipid content does not change in the short term following A β treatment. It is possible that at a lower, non-cytotoxic concentrations of A β , and with a longer period of exposure, A β -mediated changes in the membrane lipid composition and membrane fluidity will become apparent. Raquel Marin has previously shown, by measuring fluorescence anisotropy of DPH and TMA-DPH in purified brain membranes from mice, that fluidity of rafts in the brain is affected by A β and that poly-unsaturated fatty acids play a key role in this change (Diaz et al., 2012). The same group has followed up on that finding with another study, this time with membranes from human brain tissue, and report a similar finding (Diaz et al., 2015). The methods used in those studies are similar to that of some of the earlier studies where measurements may have been biased due to the methodology; nevertheless these are important findings that lead us a step further in our understanding of A β and Alzheimer's disease pathology. To properly answer the question of how A β affects membrane fluidity, it will also be important to carefully choose the experimental methodology, so as to avoid the pitfalls that led to conflicting results from some of the earlier studies done in this area. Ideally, the experiments should be conducted with 'chronic' conditions of A β -treatment, with probes that introduce minimal bias into the measurements and with membranes that are as close as possible to the native state in living animals or tissues. Indeed, even the use of cell lines must be carefully evaluated, as it has been shown that the lipid content in cell lines can vary greatly from that of cells from their native tissue (Diaz et al., 2012; Martin et al., 2006; Reynolds et al., 2001).

Even though several earlier studies have reported that A β can cause changes in the fluidity of lipid membranes with similar concentrations and types of A β as we used in our study, to our best knowledge, our study is the first to observe the cell membrane fluidity of *living cell membranes in response to A β exposure*. In our study, we treated live, differentiated SH-SY5Y neuroblastoma cells with both toxic (10 μ M) and non-toxic (1 μ M) concentrations of A β . The exact composition of the A β solutions used is unknown, but we do know that it is biologically active, being able

to trigger apoptosis in at least three cell types – SH-SY5Y, Neuro-2a and U87 cells. We showed that this preparation of A β does not affect cell membrane fluidity of living cells exposed to high concentrations of A β that is sufficient to cause apoptosis within a few days. We posit that the reason is because there are mechanisms in living cells that prevent A β from causing significant changes to membrane lipid content and membrane fluidity. Supporting this conclusion is our observation that even though A β is internalized by cells, indicating some form of interaction between A β and the cell membrane, it did not change both the bulk and raft-type membrane fluidity in living cells. In this study, only three membrane probes – C18-DiI, CtxB and GFP-GPI were used. It could be possible that the fluidity of some other membrane probe that report on different sub-membrane domains may be affected. Also, the method of A β exposure is more similar to an acute, high dose of toxic compound, rather than chronic low dosage exposure – the latter of which is probably more representative of what happens *in-vivo*. Therefore, the results of this study must be accepted with the following caveats: 1) the plasma membrane fluidity of some types of lipid or membrane sub-domains may be affected, and can only be verified with the use of other membrane probes and 2) perhaps fluidity of the probes used in this study, as well as other aspects of membrane function such as membrane permeability are affected in cases of chronic A β exposure. Within such a context, future work may include examining whether the cell membrane had been compromised in any way at all, for example by measuring intracellular calcium levels; determining whether a solution enriched for A β oligomers *can* trigger membrane fluidity changes; and finding out the differences between the cell lines that make each cell differentially sensitive to A β , for example, SH-SY5Y cells versus U87 or Neuro-2a cells. In order to conclusively determine the effects of A β on membrane fluidity, more research will have to be done. Future researchers should consider using methodologies that allow for examination of the cell membrane in as unobtrusive a manner as possible, and where possible, utilize cell and tissue samples from either animal models or actual human AD patients that have undergone natural AD progression.

References:

- Abbott, A., 2011. Dementia: a problem for our age. *Nature* 475, S2-4.
- Alzheimer, A., Stelzmann, R.A., Schnitzlein, H.N., Murtagh, F.R., 1995. An English translation of Alzheimer's 1907 paper, "Über eine eigenartige Erkrankung der Hirnrinde". *Clin Anat* 8, 429-431.
- Aman, A.T., Fraser, S., Merritt, E.A., Rodighiero, C., Kenny, M., Ahn, M., Hol, W.G., Williams, N.A., Lencer, W.I., Hirst, T.R., 2001. A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity. *Proc Natl Acad Sci U S A* 98, 8536-8541.
- Ariga, T., Wakade, C., Yu, R.K., 2011. The pathological roles of ganglioside metabolism in Alzheimer's disease: effects of gangliosides on neurogenesis. *International journal of Alzheimer's disease* 2011, 193618-193618.
- Arispe, N., Doh, M., 2002. Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease A β P (1-40) and (1-42) peptides. *FASEB J* 16, 1526-1536.
- Arispe, N., Rojas, E., Pollard, H.B., 1993a. Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc Natl Acad Sci U S A* 90, 567-571.
- Arispe, N., Pollard, H.B., Rojas, E., 1993b. Giant multilevel cation channels formed by Alzheimer disease amyloid β -protein [A β P-(1-40)] in bilayer membranes. *Proceedings of the National Academy of Sciences USA* 90, 10573-10577.
- Avdulov, N.A., Chochina, S.V., Igbavboa, U., O'Hare, E.O., Schroeder, F., Cleary, J.P., Wood, W.G., 1997. Amyloid beta-peptides increase annular and bulk fluidity and induce lipid peroxidation in brain synaptic plasma membranes. *J Neurochem* 68, 2086-2091.
- Bacia, K., Scherfeld, D., Kahya, N., Schwille, P., 2004. Fluorescence correlation spectroscopy relates rafts in model and native membranes. *Biophysical journal* 87, 1034-1043.
- Bag, N., Wohland, T., 2014. Imaging fluorescence fluctuation spectroscopy: new tools for quantitative bioimaging. *Annual review of physical chemistry* 65, 225-248.
- Bag, N., Yap, D.H., Wohland, T., 2013a. Temperature dependence of diffusion in model and live cell membranes characterized by imaging fluorescence correlation spectroscopy. *Biochim Biophys Acta*.
- Bag, N., Ali, A., Chauhan, V.S., Wohland, T., Mishra, A., 2013b. Membrane destabilization by monomeric hIAPP observed by imaging fluorescence correlation spectroscopy. *Chem Commun (Camb)* 49, 9155-9157.
- Bailey, R.W., Nguyen, T., Robertson, L., Gibbons, E., Nelson, J., Christensen, R.E., Bell, J.P., Judd, A.M., Bell, J.D., 2009. Sequence of physical changes to the cell membrane during glucocorticoid-induced apoptosis in S49 lymphoma cells. *Biophys J* 96, 2709-2718.

- Benilova, I., Karran, E., De Strooper, B., 2012. The toxic A β oligomer and Alzheimer's disease: an emperor in need of clothes. *Nature neuroscience* 15, 349-357.
- Brody, H., Grayson, M., Apoorva, M., Scully, T., Nick, H., 2011. Alzheimer's Disease. *Nature Outlook* 475, S1-S42.
- Busciglio, J., Gabuzda, D.H., Matsudaira, P., Yankner, B.A., 1993. Generation of beta-amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proc Natl Acad Sci U S A* 90, 2092-2096.
- Campioni, S., Mannini, B., Zampagni, M., Pensalfini, A., Parrini, C., Evangelisti, E., Relini, A., Stefani, M., Dobson, C.M., Cecchi, C., Chiti, F., 2010. A causative link between the structure of aberrant protein oligomers and their toxicity. *Nat Chem Biol* 6, 140-147.
- Cecchi, C., Baglioni, S., Fiorillo, C., Pensalfini, A., Liguri, G., Nosi, D., Rigacci, S., Bucciantini, M., Stefani, M., 2005. Insights into the molecular basis of the differing susceptibility of varying cell types to the toxicity of amyloid aggregates. *J Cell Sci* 118, 3459-3470.
- Cecchi, C., Rosati, F., Pensalfini, a., Formigli, L., Nosi, D., Liguri, G., Dichiarà, F., Morello, M., Danza, G., Pieraccini, G., Peri, a., Serio, M., Stefani, M., 2008. Seladin-1/DHCR24 protects neuroblastoma cells against A β toxicity by increasing membrane cholesterol content. *Journal of cellular and molecular medicine* 12, 1990-2002.
- Chochina, S.V., Avdulov, N.A., Igbavboa, U., Cleary, J.P., O'Hare, E.O., Wood, W.G., 2001. Amyloid beta-peptide 1-40 increases neuronal membrane fluidity: role of cholesterol and brain region. *J Lipid Res* 42, 1292-1297.
- Cribbs, D.H., Pike, C.J., Weinstein, S.L., Velazquez, P., Cotman, C.W., 1997. All-D-enantiomers of beta-amyloid exhibit similar biological properties to all-L-beta-amyloids. *J Biol Chem* 272, 7431-7436.
- Cutler, R.G., Kelly, J., Storie, K., Pedersen, W.A., Tammara, A., Hatanpaa, K., Troncoso, J.C., Mattson, M.P., 2004. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci U S A* 101, 2070-2075.
- Dahlgren, K.N., Manelli, A.M., Stine, W.B., Jr., Baker, L.K., Krafft, G.A., LaDu, M.J., 2002. Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem* 277, 32046-32053.
- Davis, R.C., Marsden, I.T., Maloney, M.T., Minamide, L.S., Podlisny, M., Selkoe, D.J., Bamberg, J.R., 2011. Amyloid beta dimers/trimers potently induce cofilin-actin rods that are inhibited by maintaining cofilin-phosphorylation. *Mol Neurodegener* 6, 10.
- Day, C.a., Kenworthy, A.K., 2009. Tracking microdomain dynamics in cell membranes. *Biochimica et biophysica acta* 1788, 245-253.

- Demuro, A., Mina, E., Kayed, R., Milton, S.C., Parker, I., Glabe, C.G., 2005. Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. *The Journal of biological chemistry* 280, 17294-17300.
- Deshpande, A., Mina, E., Glabe, C., Busciglio, J., 2006. Different Conformations of Amyloid β Induce Neurotoxicity by Distinct Mechanisms in Human Cortical Neurons. *The Journal of Neuroscience* 26, 6011-6018.
- Di Paolo, G., Kim, T.-W., 2011. Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature reviews. Neuroscience* 12, 284-296.
- Diaz, M., Fabelo, N., Martin, V., Ferrer, I., Gomez, T., Marin, R., 2015. Biophysical alterations in lipid rafts from human cerebral cortex associate with increased BACE1/AbetaPP interaction in early stages of Alzheimer's disease. *J Alzheimers Dis* 43, 1185-1198.
- Diaz, M.L., Fabelo, N., Marín, R., 2012. Genotype-induced changes in biophysical properties of frontal cortex lipid raft from APP/PS1 transgenic mice. *Frontiers in physiology* 3, 454-454.
- Dickson, D.W., Yen, S.H., 1989. Beta-amyloid deposition and paired helical filament formation: which histopathological feature is more significant in Alzheimer's disease? *Neurobiol Aging* 10, 402-404; discussion 412-404.
- Eckert, G.P., Wood, W.G., Muller, W.E., 2001. Effects of aging and beta-amyloid on the properties of brain synaptic and mitochondrial membranes. *J Neural Transm* 108, 1051-1064.
- Eckert, G.P., Wood, W.G., Muller, W.E., 2010. Lipid membranes and β -amyloid: a harmful connection. *Current Protein and Peptide ...*, 319-325.
- Ecsedy, J.A., Holthaus, K.A., Yohe, H.C., Seyfried, T.N., 1999. Expression of mouse sialic acid on gangliosides of a human glioma grown as a xenograft in SCID mice. *J Neurochem* 73, 254-259.
- Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., Comella, J.X., 2000. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *Journal of Neurochemistry* 75, 991-1003.
- Estus, S., Tucker, H.M., van Rooyen, C., Wright, S., Brigham, E.F., Wogulis, M., Rydel, R.E., 1997. Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant "apoptotic" pattern of gene induction. *J Neurosci* 17, 7736-7745.
- Evangelisti, E., Cecchi, C., Cascella, R., Sgromo, C., Becatti, M., Dobson, C.M., Chiti, F., Stefani, M., 2012. Membrane lipid composition and its physicochemical properties define cell vulnerability to aberrant protein oligomers. *J Cell Sci* 125, 2416-2427.

- Filippov, A., Antzutkin, O.N., 2013. Influence of Alzheimer's β -amyloid peptide on the lateral diffusion of lipids in raft-forming bilayers. *Mendeleev Commun* 23, 316-318.
- Forstner, M.B., Yee, C.K., Parikh, A.N., Groves, J.T., 2006. Lipid lateral mobility and membrane phase structure modulation by protein binding. *J Am Chem Soc* 128, 15221-15227.
- Gandy, S., 2011. Perspective: prevention is better than cure. *Nature* 475, S15.
- Gibbons, E., Pickett, K.R., Streeter, M.C., Warcup, A.O., Nelson, J., Judd, A.M., Bell, J.D., 2013. Molecular details of membrane fluidity changes during apoptosis and relationship to phospholipase A(2) activity. *Biochim Biophys Acta* 1828, 887-895.
- Glabe, C.G., Kaye, R., 2006. Common structure and toxic function of amyloid oligomers implies a common mechanism of pathogenesis. *Neurology* 66, S74-78.
- Good, T.A., Murphy, R.M., 1995. Aggregation state-dependent binding of beta-amyloid peptide to protein and lipid components of rat cortical homogenates. *Biochem Biophys Res Commun* 207, 209-215.
- Grosen, S., Grimm, M.O., Friess, P., Hartmann, T., 2010. Role of amyloid beta in lipid homeostasis. *Biochim Biophys Acta* 1801, 966-974.
- Guo, L., Har, J.Y., Sankaran, J., Hong, Y., Kannan, B., Wohland, T., 2008. Molecular diffusion measurement in lipid bilayers over wide concentration ranges: a comparative study. *Chemphyschem* 9, 721-728.
- Guo, S.M., Bag, N., Mishra, A., Wohland, T., Bathe, M., 2014. Bayesian total internal reflection fluorescence correlation spectroscopy reveals hIAPP-induced plasma membrane domain organization in live cells. *Biophys J* 106, 190-200.
- Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., et al., 1992. Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature* 359, 322-325.
- Hepler, R.W., Grimm, K.M., Nahas, D.D., Breese, R., Dodson, E.C., Acton, P., Keller, P.M., Yeager, M., Wang, H., Shughrue, P., Kinney, G., Joyce, J.G., 2006. Solution state characterization of amyloid beta-derived diffusible ligands. *Biochemistry* 45, 15157-15167.
- Hossain, S., Grande, M., Ahmadkhanov, G., Pramanik, A., 2007. Binding of the Alzheimer amyloid beta-peptide to neuronal cell membranes by fluorescence correlation spectroscopy. *Exp Mol Pathol* 82, 169-174.
- Howland, D.S., Trusko, S.P., Savage, M.J., Reaume, A.G., Lang, D.M., Hirsch, J.D., Maeda, N., Siman, R., Greenberg, B.D., Scott, R.W., Flood, D.G., 1998. Modulation of secreted beta-amyloid precursor protein and amyloid beta-peptide in brain by cholesterol. *J Biol Chem* 273, 16576-16582.

- Ittner, L.M., Götz, J., 2011. Amyloid- β and tau--a toxic pas de deux in Alzheimer's disease. *Nature reviews. Neuroscience* 12, 65-72.
- Jarrett, J.T., Berger, E.P., Lansbury, P.T., 1993. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 32, 4693-4697.
- Johnson, R.D., Schauerte, J.A., Wisser, K.C., Gafni, A., Steel, D.G., 2011. Direct observation of single amyloid-beta(1-40) oligomers on live cells: binding and growth at physiological concentrations. *PLoS One* 6, e23970.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., Muller-Hill, B., 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325, 733-736.
- Kannan, B., Guo, L., Sudhakaran, T., Ahmed, S., Maruyama, I., Wohland, T., 2007. Spatially resolved total internal reflection fluorescence correlation microscopy using an electron multiplying charge-coupled device camera. *Anal Chem* 79, 4463-4470.
- Klausner, R.D., Wolf, D.E., 1980. Selectivity of fluorescent lipid analogues for lipid domains. *Biochemistry* 19, 6199-6203.
- Klein, W.L., 2002. A β toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochemistry International* 41, 345-352.
- Klein, W.L., Krafft, G.A., Finch, C.E., 2001. Targeting small A β oligomers: the solution to an Alzheimer's disease conundrum? *Trends in neurosciences* 24, 219-224.
- Kremer, J.J., Pallitto, M.M., Sklansky, D.J., Murphy, R.M., 2000. Correlation of beta-amyloid aggregate size and hydrophobicity with decreased bilayer fluidity of model membranes. *Biochemistry* 39, 10309-10318.
- Lackowicz, J.R., 2006. *Principles of Fluorescence Spectroscopy*. New York, N.Y. : Springer.
- LaFerla, F.M., Green, K.N., Oddo, S., 2007. Intracellular amyloid-beta in Alzheimer's disease. *Nature reviews. Neuroscience* 8, 499-509.
- Lambert, M.P., Stevens, G., 1994. β /A4-evoked degeneration of differentiated SH-SY5Y human neuroblastoma cells. *Journal of Neuroscience Research* 38, 377-385.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., Klein, W.L., 1998. Diffusible, nonfibrillar ligands derived from A β (1-42) are potent central nervous system neurotoxins. *Proceedings of the National Academy of Sciences of the United States of America* 95, 6448-6453.
- Lee, G., Thangavel, R., Sharma, V.M., Litersky, J.M., Bhaskar, K., Fang, S.M., Do, L.H., Andreadis, A., Van Hoesen, G., Ksiazek-Reding, H., 2004. Phosphorylation of tau by fyn: implications for Alzheimer's disease. *J Neurosci* 24, 2304-2312.

- Lee, J.Y., Park, Y., Pun, S., Lee, S.S., Lo, J.F., Lee, L.P., 2015. Real-time investigation of cytochrome c release profiles in living neuronal cells undergoing amyloid beta oligomer-induced apoptosis. *Nanoscale* 7, 10340-10343.
- Lenaz, G., 1987. Lipid fluidity and membrane protein dynamics. *Biosci Rep* 7, 823-837.
- Lesne, S.E., 2014. Toxic oligomer species of amyloid- β in Alzheimer's disease, a timing issue. *Swiss medical weekly* 144, w14021-w14021.
- Lin, M.S., Chen, X.B., Wang, S.S., Chang, Y., Chen, W.Y., 2009. Dynamic fluorescence imaging analysis to investigate the cholesterol recruitment in lipid monolayer during the interaction between beta-amyloid (1-40) and lipid monolayers. *Colloids Surf B Biointerfaces* 74, 59-66.
- Liour, S.S., Kapitonov, D., Yu, R.K., 2000. Expression of gangliosides in neuronal development of P19 embryonal carcinoma stem cells. *J Neurosci Res* 62, 363-373.
- Malchiodi-Albedi, F., Paradisi, S., Matteucci, A., Frank, C., Diociaiuti, M., 2011. Amyloid oligomer neurotoxicity, calcium dysregulation, and lipid rafts. *International journal of Alzheimer's disease* 2011, 906964-906964.
- Mann, D.M., Iwatsubo, T., Ihara, Y., Cairns, N.J., Lantos, P.L., Bogdanovic, N., Lannfelt, L., Winblad, B., Maat-Schieman, M.L., Rossor, M.N., 1996. Predominant deposition of amyloid-beta 42(43) in plaques in cases of Alzheimer's disease and hereditary cerebral hemorrhage associated with mutations in the amyloid precursor protein gene. *Am J Pathol* 148, 1257-1266.
- Martin, V., Almansa, E., Fabelo, N., Diaz, M., 2006. Selective polyunsaturated fatty acids enrichment in phospholipids from neuronal-derived cell lines. *J Neurosci Methods* 153, 230-238.
- Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., Beyreuther, K., 1985a. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A* 82, 4245-4249.
- Masters, C.L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R.N., Beyreuther, K., 1985b. Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *EMBO J* 4, 2757-2763.
- Matsuzaki, K., Kato, K., Yanagisawa, K., 2010. Abeta polymerization through interaction with membrane gangliosides. *Biochimica et biophysica acta* 1801, 868-877.
- Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., Rydel, R.E., 1992. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J Neurosci* 12, 376-389.
- Merritt, E.A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J.A., Hol, W.G., 1994. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci* 3, 166-175.

- Morris, R., Cox, H., Mombelli, E., Quinn, P.J., 2004. Rafts, little caves and large potholes: how lipid structure interacts with membrane proteins to create functionally diverse membrane environments. *Subcell Biochem* 37, 35-118.
- Muller, W.E., Eckert, G.P., Scheuer, K., Cairns, N.J., Maras, A., Gattaz, W.F., 1998. Effects of beta-amyloid peptides on the fluidity of membranes from frontal and parietal lobes of human brain. High potencies of A beta 1-42 and A beta 1-43. *Amyloid* 5, 10-15.
- Müller, W.E., Koch, S., Eckert, A., Hartmann, H., Scheuer, K., 1995. β -Amyloid peptide decreases membrane fluidity. *Brain research* 674, 133-136.
- Nag, S., Chen, J., Irudayaraj, J., Maiti, S., 2010. Measurement of the attachment and assembly of small amyloid-beta oligomers on live cell membranes at physiological concentrations using single-molecule tools. *Biophys J* 99, 1969-1975.
- Näslund, J., Schierhorn, a., Hellman, U., Lannfelt, L., Roses, a.D., Tjernberg, L.O., Silberring, J., Gandy, S.E., Winblad, B., Greengard, P., 1994. Relative abundance of Alzheimer A beta amyloid peptide variants in Alzheimer disease and normal aging. *Proceedings of the National Academy of Sciences of the United States of America* 91, 8378-8382.
- Oda, T., Wals, P., Osterburg, H.H., Johnson, S.A., Pasinetti, G.M., Morgan, T.E., Rozovsky, I., Stine, W.B., Snyder, S.W., Holzman, T.F., et al., 1995. Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress. *Exp Neurol* 136, 22-31.
- Peters, I., Igbavboa, U., Schütt, T., Haidari, S., Hartig, U., Rosello, X., Böttner, S., Copanaki, E., Deller, T., Kögel, D., Wood, W.G., Müller, W.E., Eckert, G.P., 2009. The interaction of beta-amyloid protein with cellular membranes stimulates its own production. *Biochimica et biophysica acta* 1788, 964-972.
- Pike, C.J., Walencewicz, A.J., Glabe, C.G., Cotman, C.W., 1991. In vitro aging of beta-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Res* 563, 311-314.
- Qu, M., Zhou, Z., Xu, S., Chen, C., Yu, Z., Wang, D., 2011. Mortalin overexpression attenuates beta-amyloid-induced neurotoxicity in SH-SY5Y cells. *Brain Res* 1368, 336-345.
- Rebhan, M., Vacun, G., Bayreuther, K., Rösner, H., 1994. Altered ganglioside expression by SH-SY5Y cells upon retinoic acid-induced neuronal differentiation. *Neuroreport* 5, 941-944.
- Renner, M., Choquet, D., Triller, A., 2009. Control of the postsynaptic membrane viscosity. *J Neurosci* 29, 2926-2937.
- Reynolds, L.M., Dalton, C.F., Reynolds, G.P., 2001. Phospholipid fatty acids and neurotoxicity in human neuroblastoma SH-SY5Y cells. *Neurosci Lett* 309, 193-196.
- Saavedra, L., Mohamed, A., Ma, V., Kar, S., de Chaves, E.P., 2007. Internalization of beta-amyloid peptide by primary neurons in the absence of apolipoprotein E. *J Biol Chem* 282, 35722-35732.

- Saha, S., Lee, I.H., Polley, A., Groves, J.T., Rao, M., Mayor, S., 2015. Diffusion of GPI-anchored proteins is influenced by the activity of dynamic cortical actin. *Mol Biol Cell* 26, 4033-4045.
- Sakono, M., Zako, T., 2010. Amyloid oligomers: formation and toxicity of Abeta oligomers. *The FEBS journal* 277, 1348-1358.
- Sankaran, J., Manna, M., Guo, L., Kraut, R., Wohland, T., 2009. Diffusion, transport, and cell membrane organization investigated by imaging fluorescence cross-correlation spectroscopy. *Biophys J* 97, 2630-2639.
- Sankaran, J., Shi, X., Ho, L.Y., Stelzer, E.H., Wohland, T., 2010. ImFCS: a software for imaging FCS data analysis and visualization. *Opt Express* 18, 25468-25481.
- Sarkar, B., Das, A.K., Maiti, S., 2013. Thermodynamically stable amyloid-beta monomers have much lower membrane affinity than the small oligomers. *Front Physiol* 4, 84.
- Sasahara, K., Morigaki, K., Shinya, K., 2013. Effects of membrane interaction and aggregation of amyloid β -peptide on lipid mobility and membrane domain structure. *Physical chemistry chemical physics : PCCP* 15, 8929-8939.
- Schnabel, J., 2011. Amyloid: little proteins, big clues. *Nature* 475, S12-14.
- Schneider, A., Rajendran, L., Honsho, M., Gralle, M., Donnert, G., Wouters, F., Hell, S.W., Simons, M., 2008. Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. *J Neurosci* 28, 2874-2882.
- Sengupta, P., Balaji, J., Maiti, S., 2002. Measuring diffusion in cell membranes by fluorescence correlation spectroscopy. *Methods* 27, 374-387.
- Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., et al., 1992. Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. *Nature* 359, 325-327.
- Sezgin, E., Levental, I., Grzybek, M., Schwarzmann, G., Mueller, V., Honigsmann, A., Belov, V.N., Eggeling, C., Coskun, U., Simons, K., Schwille, P., 2012. Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim Biophys Acta* 1818, 1777-1784.
- Shi, X., Wohland, T., 2010. Fluorescence Correlation Spectroscopy. In: Diaspro, A. (Ed.), *Nanoscopy and Multidimensional Optical Fluorescence Microscopy*. Chapman and Hall/CRC, pp. 6-1 - 6-34.
- Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.D., McKay, D.M., Tintner, R., Frangione, B., et al., 1992. Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science* 258, 126-129.
- Simons, M., Keller, P., Dichgans, J., Schulz, J.B., 2001. Cholesterol and Alzheimer's disease: is there a link? *Neurology* 57, 1089-1093.

- Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C.G., Simons, K., 1998. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. *Proc Natl Acad Sci U S A* 95, 6460-6464.
- Sponne, I., Fifre, A., Koziel, V., Oster, T., Olivier, J.-L., Pillot, T., 2004. Membrane cholesterol interferes with neuronal apoptosis induced by soluble oligomers but not fibrils of amyloid-beta peptide. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18, 836-838.
- Stillwell, W., 2013. An introduction to biological membranes; from bilayers to rafts. 367-367 pp.
- Stine, W.B., Jungbauer, L., Yu, C., LaDu, M.J., 2011. Preparing synthetic A β in different aggregation states. *Methods in molecular biology (Clifton, N.J.)* 670, 13-32.
- Stine, W.B., Jr., Dahlgren, K.N., Krafft, G.A., LaDu, M.J., 2003. In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J Biol Chem* 278, 11612-11622.
- Tashima, Y., Oe, R., Lee, S., Sugihara, G., Chambers, E.J., Takahashi, M., Yamada, T., 2004. The effect of cholesterol and monosialoganglioside (GM1) on the release and aggregation of amyloid beta-peptide from liposomes prepared from brain membrane-like lipids. *J Biol Chem* 279, 17587-17595.
- Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., Hansen, L.A., Katzman, R., 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol* 30, 572-580.
- Terzi, E., Hölzemann, G., Seelig, J., 1997. Interaction of Alzheimer beta-amyloid peptide(1-40) with lipid membranes. *Biochemistry* 36, 14845-14852.
- Tucker, H.M., Rydel, R.E., Wright, S., Estus, S., 1998. Human amylin induces "apoptotic" pattern of gene expression concomitant with cortical neuronal apoptosis. *J Neurochem* 71, 506-516.
- Vereb, G., Szollosi, J., Matko, J., Nagy, P., Farkas, T., Vigh, L., Matyus, L., Waldmann, T.A., Damjanovich, S., 2003. Dynamic, yet structured: The cell membrane three decades after the Singer-Nicolson model. *Proc Natl Acad Sci U S A* 100, 8053-8058.
- Wakabayashi, M., Matsuzaki, K., 2007. Formation of amyloids by Abeta-(1-42) on NGF-differentiated PC12 cells: roles of gangliosides and cholesterol. *Journal of molecular biology* 371, 924-933.
- Wakabayashi, M., Okada, T., Kozutsumi, Y., Matsuzaki, K., 2005. GM1 ganglioside-mediated accumulation of amyloid beta-protein on cell membranes. *Biochemical and biophysical research communications* 328, 1019-1023.
- Wawrezinieck, L., Rigneault, H., Marguet, D., Lenne, P.-F., 2005. Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization. *Biophysical journal* 89, 4029-4042.

- Williams, R., 2011. Biomarkers: warning signs. *Nature* 475, S5-7.
- Wolf, A.a., Jobling, M.G., Saslowsky, D.E., Kern, E., Drake, K.R., Kenworthy, A.K., Holmes, R.K., Lencer, W.I., 2008. Attenuated endocytosis and toxicity of a mutant cholera toxin with decreased ability to cluster ganglioside GM1 molecules. *Infection and immunity* 76, 1476-1484.
- Wolf, D.E., Kinsey, W., Lennarz, W., Edidin, M., 1981. Changes in the organization of the sea urchin egg plasma membrane upon fertilization: indications from the lateral diffusion rates of lipid-soluble fluorescent dyes. *Dev Biol* 81, 133-138.
- Wong, P.T., Schauerte, J.A., Wisser, K.C., Ding, H., Lee, E.L., Steel, D.G., Gafni, A., 2009. Amyloid-beta membrane binding and permeabilization are distinct processes influenced separately by membrane charge and fluidity. *J Mol Biol* 386, 81-96.
- Yang, X., Sun, G.Y., Eckert, G.P., Lee, J.C., 2014. Cellular membrane fluidity in amyloid precursor protein processing. *Mol Neurobiol* 50, 119-129.
- Yankner, B.A., Duffy, L.K., Kirschner, D.A., 1990. Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* 250, 279-282.
- Yehuda, S., Rabinovitz, S., Carasso, R.L., Mostofsky, D.I., 2002. The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. *Neurobiol Aging* 23, 843-853.
- Yu, R.K., Tsai, Y.T., Ariga, T., Yanagisawa, M., 2011. Structures, biosynthesis, and functions of gangliosides--an overview. *J Oleo Sci* 60, 537-544.
- Zhang, C., Qiu, H.E., Krafft, G.A., Klein, W.L., 1996. A beta peptide enhances focal adhesion kinase/Fyn association in a rat CNS nerve cell line. *Neurosci Lett* 211, 187-190.
- Zhang, D., Manna, M., Wohland, T., Kraut, R., 2009. Alternate raft pathways cooperate to mediate slow diffusion and efficient uptake of a sphingolipid tracer to degradative and recycling compartments. *Journal of cell science* 122, 3715-3728.
- Zheng, L., Cedazo-Minguez, A., Hallbeck, M., Jerhammar, F., Marcusson, J., Terman, A., 2012. Intracellular distribution of amyloid beta peptide and its relationship to the lysosomal system. *Transl Neurodegener* 1, 19.

Supplementary Data

THE EFFECT OF THE AMYLOID-BETA PEPTIDE ON THE FLUIDITY OF CELL MEMBRANES: A STUDY BY FLUORESCENCE CORRELATION SPECTROSCOPY

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**The Effect of the Amyloid-Beta Peptide on Cell Membrane Fluidity -
Supplementary Data**

Immunocytochemistry of SH-SY5Y cells

Figure S1. Immunocytochemistry of SH-SY5Y cells

(a), (c), (e) and (g) – undifferentiated SH-SY5Y human neuroblastoma cells. (b), (d), (f), (h) retinoic acid-differentiated SH-SY5Y cells. (a) and (b) – blank controls (no primary antibody added). Green stain: (c) and (d) – GAP43; (e) and (f) – MAP-2; (g) and (h) – NF-H. Red – rhodamine-phalloidin counterstain, blue – Hoechst 33342 (DNA). All primary antibodies were rabbit IgG. Secondary antibody – Alexa 488-conjugated mouse anti-rabbit IgG. Both undifferentiated and differentiated SH-SY5Y cells express GAP-43 (axonal marker), MAP-2 (dendritic marker) and NF-H (neuronal intermediate filament). Distribution of GAP-43 and MAP-2 are markedly different for differentiated cells versus undifferentiated cells. GAP-43 in undifferentiated cells seem to be exclusively perinuclear while in differentiated cells they are less associated with the cell nucleus and distributed more along long neuritic processes. MAP-2 in undifferentiated cells seem to be less abundant and generally distributed throughout the cytosol. In differentiated cells, MAP-2 is more concentrated (brighter) and distributed along long neuritic processes. NF-H distribution is somewhat like MAP-2 for both undifferentiated and differentiated cells, albeit the differences in staining pattern are less pronounced. Images were recorded in a single sitting and laser power, HV and gain settings were kept constant throughout. Offset was

Statistical and raw data for cell viability and caspase activation assays

Assessment of A β effects on cell viability: wild-type peptide vs. scrambled peptide				
Cell Type	ANOVA <i>p</i> -value			
	Time	1 μ M	10 μ M	20 μ M
SH-RAI	48h	0.9985	> 0.9999	0.0004
	72h	0.0222	0.0044	< 0.0001
SH-ND	48h	0.9649	0.9649	0.0366
	72h	0.1192	0.4913	< 0.0001
U87	48h	> 0.9999	0.6602	0.9428
	72h	0.8414	0.9795	0.1915
N2a	48h	0.8305	0.9902	0.0056
	72h	0.4913	0.9795	0.9313

Table S1a. Statistical data for cell viability assay

Mean normalized Relative Fluorescence Units (Viability): wild-type peptide vs. scrambled peptide					
Cells	Time	Peptide	1 μ M	10 μ M	20 μ M
SH-RAI	48h	wt-A β	0.96	1.00	0.87
		Scrambled A β	0.97	1.00	1.02
	72h	wt-A β	1.02	0.87	0.66
		Scrambled A β	0.92	0.98	1.04
SH-ND	48h	wt-A β	1.02	0.92	0.88
		Scrambled A β	0.99	0.95	0.98
	72h	wt-A β	0.95	0.80	0.65
		Scrambled A β	0.87	0.84	0.89
U87	48h	wt-A β	0.96	0.95	1.02
		Scrambled A β	0.95	1.00	0.99
	72h	wt-A β	0.95	0.95	1.02
		Scrambled A β	0.92	0.94	0.95
N2a	48h	wt-A β	0.98	1.02	1.03
		Scrambled A β	1.02	1.04	0.90
	72h	wt-A β	1.04	1.07	1.07
		Scrambled A β	0.99	1.05	1.04

Table S1b. Raw data for cell viability assay

Assessment of A β effects on caspase 3/7 activation: wild-type peptide vs. scrambled peptide				
Cell Type	ANOVA <i>p</i> -value			
	Time	1 μ M	10 μ M	20 μ M
SH-RAI	48h	0.9289	0.3061	< 0.0001
	72h	0.6464	< 0.0001	< 0.0001
SH-ND	48h	0.9718	0.4865	< 0.0001
	72h	0.5745	0.0001	< 0.0001
U87	48h	0.9955	0.9807	0.1526
	72h	0.9080	0.9423	< 0.0001
N2a	48h	0.9874	0.7966	0.0067
	72h	0.9759	0.8872	< 0.0001

Table S1c. Statistical data for caspase 3/7 activation assay.

Mean normalized Relative Luminescence Units (Caspase): wild-type peptide vs. scrambled peptide					
Cells	Time	Peptide	1 μ M	10 μ M	20 μ M
SH-RAI	48h	wt-A β	0.86	0.96	1.45
		Scrambled A β	0.81	0.84	0.87
	72h	wt-A β	0.83	1.15	2.01
		Scrambled A β	0.74	0.72	0.86
SH-ND	48h	wt-A β	0.96	0.99	1.82
		Scrambled A β	0.92	0.89	0.86
	72h	wt-A β	0.87	1.07	2.83
		Scrambled A β	0.78	0.78	0.84
U87	48h	wt-A β	0.96	0.92	1.11
		Scrambled A β	0.93	0.88	0.99
	72h	wt-A β	0.92	0.91	1.65
		Scrambled A β	0.86	0.86	0.92
N2a	48h	wt-A β	0.85	0.83	0.97
		Scrambled A β	0.89	0.90	0.78
	72h	wt-A β	0.94	0.80	1.02
		Scrambled A β	0.90	0.86	0.70

Table S1d. Raw data for caspase 3/7 activation assay

Tables 1a and 1c: Statistical analysis for cell viability and caspase activation assays – two-tailed unpaired two-way ANOVA, Tukey's post-hoc test for multiple comparisons of mean RFU (viability)/RLU (caspase) values. Only wt-A β vs. scrambled-A β comparisons were tabled, although comparisons were made between all possible pairings within a group (cell type + time point). Statistically significant *p*-values at a 95% confidence interval are indicated in red. Tables 1b and 1d: raw data for cell viability and caspase activation assays – assay was conducted in triplicate wells on separate 96-well test plates for each time point, 48h and 72h. The experiment was run at least twice but the data presented here are from a single experiment run due to complications in other experiments that rendered part of the data inadmissible. Vehicle controls were run in triplicate on all plates and data was normalized by averaging individual readings from each peptide-treated well against the average reading from 3 wells from the vehicle control sample set. The normalized reading per well (3 per peptide treatment for each time point) were then averaged to get the mean normalized RFU (viability) or RLU (caspase activation) for that peptide treatment condition. For statistical analysis, all peptide-treatment RFU/RLU values per time point (3 readings each for 2 peptides, 3 concentrations and 4 cell types, total 72 individual readings per

a) Effect of A β on CtxB diffusion on SH-RAI cells

b) Effect of A β on CtxB diffusion on SH-ND cells

Figure S2. Effect of A β on CtxB diffusion on SH-SY5Y cells

(a) retinoic acid-differentiated SH-SY5Y cells (SH-RAI), (b) undifferentiated SH-SY5Y cells (SH-ND). Statistical analysis: two-tailed unpaired two-way ANOVA, Tukey's post-hoc test for multiple comparisons of sample means. * - $p < 0.05$, 95% confidence interval

SDS-PAGE of aggregated vs. unaggregated A β peptide

Figure S3. SDS-PAGE analysis of pre-aggregated vs. unaggregated A β peptide

a) Lanes 1 and 2 – 100 μ M A β pre-aggregated in Neurobasal medium for 24h in the cold room; Lanes 3 and 4 – 100 μ M A β prepared and left on ice for 1h just before the SDS-PAGE experiment. Lanes 1 and 3 – non-heat treated peptide samples, Lanes 2 and 4 – samples that had been heated for 10 min at 70°C prior to gel loading. 1.35 μ g total protein per well. b) 100 μ M A β prepared and immediately used for SDS-PAGE. Lane 1 – non-heat treated peptide, Lane 2 – peptide heated for 10 min at 70°C prior to gel loading. 450 ng total protein per well. A β was prepared as detailed in Section 2.1, SDS-PAGE and silver staining was performed as detailed in Section 2.2, except that Novex Sharp Pre-Stained protein standard (ThermoFisher, Waltham, MA) was used instead of Mark12 unstained protein standard.

ITIR-FCS *D* Values of DiI on Aβ-treated Cells

<i>D</i> Values (μm ² s ⁻¹) of DiI on Differentiated SH-SY5Y cells					
30 min		24h		48h	
Vehicle	Aβ	Vehicle	Aβ	Vehicle	Aβ
2.405	1.947	1.359	1.612	1.890	2.046
2.442	2.419	1.763	1.666	2.057	2.237
2.464	2.824	1.913	1.948	2.146	2.296
2.679	2.834	2.028	1.962	2.217	2.300
2.718	3.036	2.048	1.987	2.459	2.330
2.754	3.481	2.049	2.057	2.555	2.428
		2.076	2.084	2.579	2.456
		2.084	2.113	2.606	2.475
		2.142	2.191	2.768	2.486
		2.143	2.250	3.077	2.695
		2.221	2.268		
		2.252	2.282		
		2.307	2.354		
		2.348	2.359		
		2.367	2.379		
		2.376	2.405		
		2.384	2.449		
		2.396	2.453		
		2.408	2.506		
		2.436	2.540		
		2.501	2.556		
		2.515	2.562		
		2.532	2.621		
		2.539	2.697		
		2.626	2.716		
		2.660	2.758		
		2.679	2.882		
		2.707	2.973		
		3.244	3.250		

<i>D</i> Values (μm ² s ⁻¹) of DiI on U87 cells			
30 min		24h	
Vehicle	Aβ	Vehicle	Aβ
1.374	1.061	1.241	1.499
1.641	1.061	1.669	1.827
1.725	1.777	1.670	1.847
1.797	1.832	1.697	1.870
1.853	1.891	1.716	1.890
2.010	2.075	1.766	1.910
	2.106	1.846	1.911
			1.939

<i>D</i> Values (μm ² s ⁻¹) of DiI on Undifferentiated SH-SY5Y cells			
30 min		24h	
Vehicle	Aβ	Vehicle	Aβ
1.754	1.683	1.809	1.970
1.887	1.835	1.925	2.113
2.036	1.928	2.141	2.182
2.269	1.966	2.244	2.191
2.275	1.970	2.283	2.233
2.279	2.021	2.365	2.343
2.293	2.229	2.394	2.352
2.407	2.319	2.473	2.411
2.433	2.436		
2.481	2.520		
2.631	2.583		
	3.090		

<i>D</i> Values (μm ² s ⁻¹) of DiI on Neuro-2a cells					
30 min		24h		48h	
Vehicle	Aβ	Vehicle	Aβ	Vehicle	Aβ
0.860	1.386	0.784	1.270	1.065	1.176
1.307	1.480	1.104	1.459	1.175	1.231
1.455	1.482	1.255	1.480	1.360	1.645
1.606	1.595	1.365	1.581	1.500	1.861
1.668	2.006	1.440	1.646		
1.992	2.067	1.519	1.655		
		1.538	1.662		
		1.581	1.673		
		1.588	1.722		
		1.842	1.806		
		2.006	2.022		

Table S2: *D* values of DiI on Cells

Each value represents the mean *D* of an area of membrane, at least 240 x 240 μm in size (100 pixels or data points), from an individual cell. Values are arranged in ascending order. Aβ-treated cells were exposed to 10 μM of Aβ. Data was collected over several separate experiments.

Plot of ITIR-FCS Data of A β -treated cells: DiI

Figure S4: Plot of D values of DiI on A β -treated Cells

Bars – sample mean

Sample ITIR-FCS data: DiI

Figure S5: Example ITIR-FCS data of DiI on A β -treated cells

The data shown here is of single measurements (100 pixels, 240 x 240 μm) of an area of membrane from undifferentiated SH-SY5Y cells. Each set of data – the auto-correlation functions (ACF), diffusion coefficient (D) histogram/heat map, particle number (N) histogram/heat map – is of an individual cell in the sample. ACF graphs allow us to determine whether a good curve fitting was achieved. The heat maps allow us to see at-a-glance the state of the area of membrane being examined. The mean D , shown above the D histogram, is the value of interest that is retained for comparison of sample mean D between different treatments.

ITIR-FCS *D* Values of CtxB on A β -treated Cells

<i>D</i> Values ($\mu\text{m}^2\text{s}^{-1}$) of CtxB on Differentiated SH-SY5Y cells			
24h		48h	
Vehicle	A β	Vehicle	A β
0.162	0.082	0.110	0.098
0.170	0.125	0.124	0.128
0.171	0.137	0.146	0.132
0.171	0.139	0.147	0.142
0.177	0.139	0.148	0.158
0.181	0.155	0.159	0.170
0.181	0.158	0.169	0.174
0.182	0.163	0.169	0.177
0.187	0.163	0.174	0.208
0.188	0.164	0.176	0.210
0.193	0.164	0.178	0.212
0.196	0.177	0.178	0.214
0.196	0.181	0.185	0.217
0.196	0.187	0.192	0.223
0.200	0.192	0.199	0.223
0.201	0.194	0.202	0.228
0.201	0.198	0.204	0.232
0.208	0.200	0.210	0.235
0.217	0.200	0.216	0.252
0.221	0.207	0.231	0.281
0.232	0.209	0.301	0.311
0.233	0.211	0.327	
0.237	0.212		
0.238	0.215		
0.248	0.225		
0.328	0.233		
	0.235		
	0.238		
	0.239		
	0.239		
	0.248		
	0.250		
	0.288		

<i>D</i> Values ($\mu\text{m}^2\text{s}^{-1}$) of CtxB on Unifferentiated SH-SY5Y cells					
30 min		24h		48h	
Vehicle	A β	Vehicle	A β	Vehicle	A β
0.108	0.105	0.094	0.087	0.104	0.099
0.126	0.116	0.121	0.123	0.112	0.108
0.138	0.118	0.135	0.127	0.135	0.111
0.149	0.120	0.135	0.133	0.146	0.111
0.149	0.126	0.138	0.134	0.150	0.112
0.157	0.137	0.147	0.137	0.154	0.128
0.175	0.144	0.148	0.143	0.155	0.133
0.176	0.173	0.149	0.143	0.157	0.142
0.185	0.180	0.154	0.148	0.159	0.150
0.192	0.183	0.156	0.156	0.162	0.153
0.232	0.194	0.158	0.156	0.186	0.155
0.283	0.199	0.161	0.172	0.206	0.166
	0.209	0.163	0.176	0.226	0.168
	0.302	0.168	0.181	0.245	0.175
		0.179	0.181		0.176
		0.181	0.183		0.181
		0.198	0.195		0.187
		0.222	0.197		0.189
		0.223	0.197		0.190
		0.224	0.210		0.198
		0.233	0.213		0.210
		0.274	0.226		0.232
			0.234		0.237
			0.242		0.259
			0.270		0.298
			0.277		0.335
			0.304		

<i>D</i> Values ($\mu\text{m}^2\text{s}^{-1}$) of CtxB on U87 cells			
30 min		24h	
Vehicle	A β	Vehicle	A β
0.081	0.085	0.031	0.072
0.105	0.115	0.052	0.079
0.142	0.125	0.062	0.082
	0.138	0.083	0.088
		0.098	0.093
		0.108	0.096
		0.113	0.119
		0.140	0.130
		0.148	0.151
		0.159	0.172
		0.160	0.189
		0.233	0.218

Table S3: *D* values of CtxB on A β -treated Cells

Each value represents the mean *D* of an area of membrane, at least 240 x 240 μm in size (100 pixels or data points), from an individual cell. Values are arranged in ascending order. A β -treated cells were exposed to 10 μM of A β . Data was collected over several separate experiments.

Plot of ITIR-FCS Data of Aβ-treated cells: CtxB

Figure S6: Plot of D values of CtxB on Cells

Bars – sample mean

Sample ITIR-FCS data: CtxB

Figure S7: Example ITIR-FCS data of CtxB on cells

The data shown here is of single measurements (100 pixels, 240 x 240 μm) of an area of membrane from undifferentiated SH-SY5Y cells. Each set of data – the auto-correlation functions (ACF), diffusion coefficient (D) histogram/heat map, particle number (N) histogram/heat map – is of an individual cell in the sample. ACF graphs allow us to determine whether a good curve fitting was achieved. The heat maps allow us to see at-a-glance the state of the area of membrane being examined. The mean D , shown above the D histogram, is the value of interest that is retained for comparison of sample mean D between different treatments.

ITIR-FCS *D* Values of GFP-GPI on Aβ-treated Cells

<i>D</i> Values (μm ² s ⁻¹) of GFP-GPI on Unifferentiated SH-SY5Y cells					
30 min		24h		48h	
Vehicle	Aβ	Vehicle	Aβ	Vehicle	Aβ
0.530	0.220	0.375	0.189	0.507	0.341
0.608	0.464	0.379	0.194	0.615	0.353
0.699	0.468	0.447	0.402	0.532	0.457
0.839	0.581	0.521	0.444	0.769	0.706
	0.591	0.540	0.470	0.741	0.768
	0.640	0.577	0.562	0.547	1.102
	0.649	0.593	0.570		
	1.047	0.602	0.709		
		0.827	0.848		
			0.940		
			1.000		

<i>D</i> Values (μm ² s ⁻¹) of GFP-GPI on N2A cells			
30 min		24h	
Vehicle	Aβ	Vehicle	Aβ
0.224	0.400	0.523	0.155
0.592	0.429	0.584	0.369
0.601	0.430	0.589	0.533
0.613	0.448	0.658	0.538
0.629	0.473	0.663	0.546
0.713	0.553	0.689	0.649
0.724	0.564	0.689	0.654
0.805	0.575	0.729	0.736
0.841	0.613	0.755	0.740
	0.639	0.844	0.786
	0.688		0.826
	0.697		1.329
	0.792		
	0.800		
	0.819		
	0.856		

<i>D</i> Values (μm ² s ⁻¹) of GFP-GPI on U87 cells					
30 min		24h		48h	
Vehicle	Aβ	Vehicle	Aβ	Vehicle	Aβ
0.464	0.412	0.282	0.345	0.304	0.360
0.635	0.746	0.370	0.351	0.492	0.408
0.462	0.512	0.399	0.366	0.739	0.428
0.737	0.357	0.462	0.440		0.498
	0.285	0.473	0.454		
		0.556	0.575		
			0.608		
			0.644		
			0.665		

Table S4: *D* values of GFP-GPI on Cells

Each value represents the mean *D* of an area of membrane, at least 240 x 240 μm in size (100 pixels or data points), from an individual cell. Values are arranged in ascending order. Aβ-treated cells were exposed to 10 μM of Aβ. Data was collected over several separate experiments.

Plot of ITIR-FCS Data of Aβ-treated cells: GFP-GPI

Figure S8: Plot of D values of GFP-GPI on Cells

Bars – sample mean

Sample ITIR-FCS data: GFP-GPI

Figure S9: Example ITIR-FCS data of GFP-GPI on cells

The data shown here is of single measurements (100 pixels, 240 x 240 μm) of an area of membrane from undifferentiated SH-SY5Y cells. Each set of data – the auto-correlation functions (ACF), diffusion coefficient (D) histogram/heat map, particle number (N) histogram/heat map – is of an individual cell in the sample. ACF graphs allow us to determine whether a good curve fitting was achieved. The heat maps allow us to see at-a-glance the state of the area of membrane being examined. The mean D , shown above the D histogram, is the value of interest that is retained for comparison of sample mean D between different treatments.

Control Experiment: DiI diffusion on cell membranes

Temperature-dependent diffusion of DiI on membranes <i>D</i> Values ($\mu\text{m}^2\text{s}^{-1}$)		
Cell Type	25°C	37°C
SH-ND	1.382	1.912
	1.394	2.054
	1.608	2.270
	1.654	2.354
Neuro-2a	0.824	1.161
	1.034	1.430
	1.034	1.437
	1.147	1.690
U87	0.195	0.730
	0.456	1.238
	0.927	1.281
	1.164	1.761

Table S5: ITIR-FCS control experiments with DiI

Figure S10: Plot of D from ITIR-FCS control experiments with DiI

Bars – sample mean

Sample ITIR-FCS data: DiI controls

Figure S11: Example ITIR-FCS data of control experiments of DiI on cell membrane

The data shown here is of single measurements of an area of membrane from undifferentiated SH-SY5Y cells. DiI exhibits a temperature-dependent shift in fluidity which is very evident when measured by ITIR-FCS. The ambient temperature was controlled using a stage-mounted environmental chamber, with humidified air and 5% CO₂.

Control Experiment: CtxB diffusion on cell membranes

Cholesterol-dependent diffusion of CtxB on membranes <i>D</i> Values ($\mu\text{m}^2\text{s}^{-1}$)		
Cell Type	Vehicle	5 mM m β CD
SH-ND	0.114	0.072
	0.130	0.077
	0.146	0.088
	0.238	0.088
Neuro-2a	0.077	0.061
	0.121	0.071
	0.145	0.092
	0.151	0.095
U87	0.029	0.087
	0.100	0.092
	0.109	0.109
	0.119	0.142

Table S6: ITIR-FCS Control Experiments with DiI

Figure S12: Plot of D from ITIR-FCS control experiments with CtxB

Bars – sample mean

Sample ITIR-FCS data: CtxB controls

Figure S13: Example ITIR-FCS data of control experiments of CtxB on cell membrane

The data shown here is of single measurements of an area of membrane from undifferentiated SH-SY5Y cells. DiI exhibits a cholesterol-dependent shift in fluidity which is very evident when measured by ITIR-FCS. Cholesterol was depleted from the cell membrane by treatment with 5 mM methyl-β-cyclodextrin for 30 min.