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Article

Live Cell FRET Imaging Reveals Amyloid β-Peptide Oligomerization in Hippocampal Neurons

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Abstract: Amyloid β-peptide (Aβ) oligomerization is believed to contribute to the neuronal dysfunction in Alzheimer disease (AD). Despite decades of research, many details of Aβ oligomerization in neurons still need to be revealed. Förster Resonance Energy Transfer (FRET) is a simple but effective way to study molecular interactions. Here we use a confocal microscope with a sensitive Airyscan detector for FRET detection. By live cell FRET imaging, we detect Aβ42 oligomerization in primary neurons. The neurons were incubated with fluorescently labelled Aβ42 in the cell culture medium for 24 hours. Aβ42 were internalized and oligomerized into the lysosomes/late endosomes in a concentration-dependent manner. Both the cellular uptake and intracellular oligomerization of Aβ42 were significantly higher than for Aβ40. These findings provide a better understanding of Aβ42 oligomerization in neurons.

Keywords: amyloid β-peptide; oligomerization; aggregation; FRET

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1. Introduction

Alzheimer disease is the most common cause of dementia, and affects mainly older people [1]. It is estimated to affect over 100 million people worldwide in 2050, and its pathogenesis remains unclear [2]. The aggregation of the amyloid β-peptide (Aβ) is believed to play a crucial role in the pathogenesis of Alzheimer disease [3].

Aβ is a 38–43 amino acids peptide cleaved from a transmembrane protein, the Aβ precursor protein (APP) [4]. It is generated intracellularly and secreted to the extracellular space [5,6]. Among the various Aβ isoforms, Aβ42 is more toxic than other species because it is more prone to aggregate [7]. Aβ oligomers – both intracellular and extracellular - are suggested to impair synaptic function and contribute to cognitive decline during the early stages of AD [8]. Increased intracellular Aβ42 has been shown to correlate with AD neuropathology [9,10]. However, more studies on intracellular Aβ oligomers are needed to understand the pathogenesis in early AD.

Oligomeric Aβ exist in a dynamic equilibrium, which is sensitive and influenced by the environment and the biophysical analysis can affect the oligomers [11][12]. Several techniques have been used for studying Aβ oligomerization, such as Thioflavin T assays, SDS polyacrylamide gel electrophoresis (PAGE), and Fluorescence microscopy [13]. However, only a few of them are suitable to use in living cells under physiological conditions.

Förster Resonance Energy Transfer (FRET) is widely used to measure protein interactions in molecular biology. Due to its strong dependence on the distance between the donor and acceptor [14], it has been used for studying cellular Aβ oligomerization and showed unique capability [15].

Several studies have shown that extracellular Aβ could be internalized and form aggregates in cells [15,16]. However, some key questions regarding the fate of internalized

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Aβ still need to be addressed, such as under what conditions, in which subcellular compartments and to what extent Aβ could oligomerize in neurons.

In this study, we performed live cell FRET imaging to detect Aβ42 oligomerization in primary neurons, by using a high-resolution confocal microscope with a sensitive Airyscan detector. We found that monomeric Aβ could be taken up into primary neurons and oligomerize mainly in the lysosomes and/or late endosomes in a concentration-dependent manner.

2. Results

2.1. Internalized Aβ oligomerizes in lysosomes/late endosomes

Fluorescence Correlation Spectroscopy (FCS), Fluorescence cross-correlation spectroscopy (FCCS), and the combination of FRET and FCS (FRET-FCS) were used to investigate the oligomerization state of Aβ in the cell culture medium. These techniques have been shown capable of detecting Aβ oligomers in solution and FRET-FCS can even detect FRET-active oligomers at fractions below 1% compared to monomers [17]. We prepared 500 nM HiLyte™ Fluor 488 (HF488) Aβ42 and 500 nM HiLyte™ Fluor 647 (HF647) Aβ42 in the cell culture medium. The FCCS analysis showed that Aβ aggregation was low in the medium after 24 hours (Figure 1, Figure S1-S3).

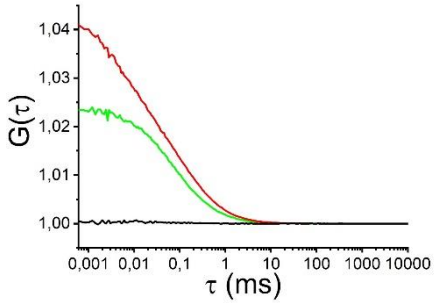

Figure 1. FCCS measurements on a sample consisting of 500 nM HF488 Aβ42 (green) + 500 nM HF647 Aβ42 (red). Auto- (green and red) and cross-correlation (black) curves were collected in 10 minutes measurement after 24 hours incubation. Laser power: 0.4 % 488 nm and 1.2 % 633 nm. The lack of cross-correlation indicates that a very low number of oligomers, if any, are present. If there were, the black cross-correlation curves would have an amplitude rising above the 1.0-line, like the green and red auto-correlation curve.

Sensitized emission FRET was used to detect Aβ oligomerization in primary neurons (Figure 2a). In this approach, we excited the donor HF488 Aβ42, which transfers energy directly to the acceptor HF647 Aβ42 if it is within 10 nm distance, resulting in emission from the acceptor. The emitted light is then detected by a confocal microscope using emission filter for the acceptor. Theoretically, it can be excluded that FRET arises due to the low mean monomer-to-monomer distance of Aβ even at high concentration. Thus, FRET signal in this study indicates Aβ oligomerization.

It should be noted that fluorescence crosstalk from donor, cross excited acceptor, and noises like autofluorescence may interfere with the analysis. To acquire the corrected FRET from crosstalk, spectral bleed-through parameters of the donor and acceptor were determined with cells treated with only HF488 Aβ42 or only HF647 Aβ42, respectively. The cell autofluorescence was measured in a non-treated control group. The image analysis was processed using the ImageJ plug-in PixFRET.

Primary hippocampal neurons (21 days in vitro) were incubated in the presence of 500 nM HF488 Aβ42 and/or 500 nM HF647 Aβ42 for 24 hours and imaged by a confocal microscope equipped with an Airyscan detector. Both HF488 Aβ42 and HF647 Aβ42 were accumulated in the vesicles of the soma (Figure 2b-c). After correction, FRET signal was found in most of the Aβ-containing vesicles (Figure 2d-e).

The corrected FRET can still be affected by the concentration of fluorophores. To get normalized FRET, the corrected FRET value was divided by the square root of the product

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of donor and acceptor intensities in each pixel. Figure 2f shows the normalized FRET of the vesicles in the soma of neuron.

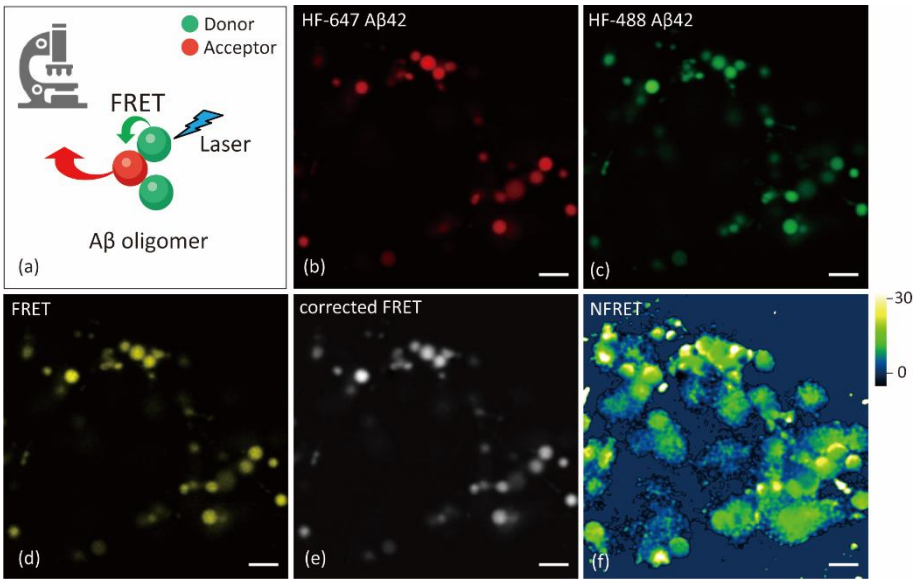

Figure 2. FRET analysis to detect aggregated Aβ42 in primary neurons. (**a**) Principle of FRET. Equimolar Aβ42 monomers labeled with HF488 and HF647 are used for oligomerization detection. If the emission of excited donor overlaps with the absorption of the acceptor, energy transfer from donor to ground state acceptor when their distance is 10 nm or less; (**b-d**) Acceptor channel (640 nm excitation, 650-700 nm emission), donor channel (488 nm excitation, 480-520 nm emission) and FRET channel (488 nm excitation, 650-700 nm emission) of representative FRET image from confocal microscope; (**e-f**) the corrected FRET and normalized FRET (NFRET) image from ImageJ PixFRET plug-in. Scale bar: 2 μm.

Next, to identify the subcellular location where Aβ is accumulated, we used the lysosomal marker SiR-lysosome in neurons that were treated with 1000 nM Aβ42 for 24h. Most of Aβ-containing vesicles were colocalized with the lysosomal marker (Figure 3). This suggests Aβ42 is internalized and oligomerized into lysosomes and late endosomes.

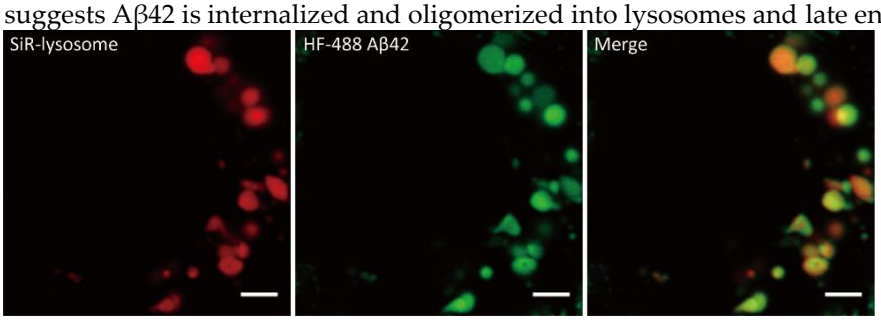

Figure 3. Internalized Aβ42 in primary neurons. DIV 21 neurons were treated with 1000 nM HF488 Aβ42 (green) for 24h. SiR-lysosome (red) was used to label lysosome and late endosomes. Scale bar: 2 μm.

2.2. Aβ42 oligomerization occurs at both surface and vesicles of neurons

A comparison of Aβ oligomerization in vesicles distributed in soma with vesicles in neurites showed no significant differences ($P > 0.05$, Fig. 4). The weak Aβ signal on the cell surface was difficult to distinguish from the background, while imaging the vesicles with high intensities in soma without over-saturation. Thus, higher laser power and detector gain were applied to measure the Aβ aggregation on the cell surface. The normalized FRET value of Aβ42 on the neuron surface or nearabout could be several times higher than in vesicles of soma and neurite. This is consistent in two groups treated with different Aβ concentrations (Figure 5). Similar strong oligomerization was also observed along the neurites although we did not identify if it came from the surface of neurites .

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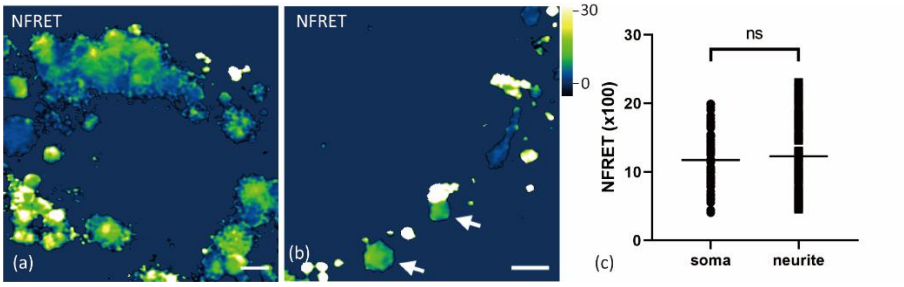

Figure 4. Comparison of FRET between Aβ vesicles in soma and neurites. Primary neurons were treated with 500 nM HF488 Aβ42 and 500 nM HF647 Aβ42 for 24h. (**a**) normalized FRET (NFRET) image of Aβ vesicles in soma; (**b**) NFRET image of Aβ vesicles (white arrow) in neurites; (**c**) NFRET value of 63 vesicles in soma and 64 vesicles in neurite were compared using Mann-Whitney test ($P > 0.05$). Scale bar: 2 μm.

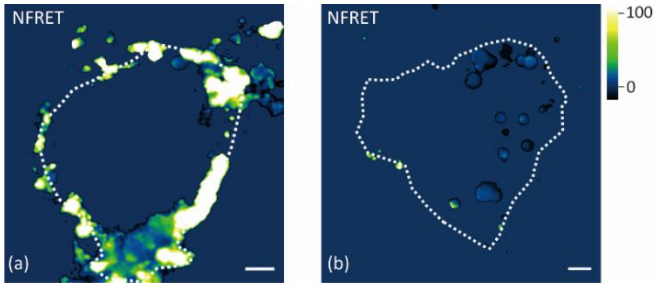

Figure 5. Normalized FRET (NFRET) images of Aβ on the cell surface. Primary neurons were treated with 500 nM HF488 Aβ42 + 500 nM HF647 Aβ42 (**a**) or 125 nM HF488 Aβ42 + 125 nM HF647 Aβ42 (**b**) for 24h. The cell bodies of neurons are outlined with the white dashed lines. Scale bar: 2 μm.

2.3. Internalized Aβ42 oligomerization in soma is concentration-dependent

To further confirm the association between Aβ42 oligomerization and concentration in soma, primary neurons were treated with three different Aβ concentrations (HF488 Aβ42 and HF647 Aβ42 at 1:1 ratio; 250, 500 and 1000 nM Aβ in total) for 24h. As shown in Figure 6, Aβ oligomerization occurred in all the three groups, and increased with extracellular Aβ42 concentration. We also found a negative correlation between the diameter of the Aβ vesicles and the normalized FRET level in two cell groups treated with higher Aβ concentration. The smaller vesicles tend to present higher oligomerization level in cells treated with 1000 nM Aβ42 ($r = -0.46$, $P < 0.05$) and 500 nM Aβ42 ($r = -0.28$, $P < 0.05$). There was no significant correlation in 250 nM group ($r = 0.23$, $P > 0.05$).

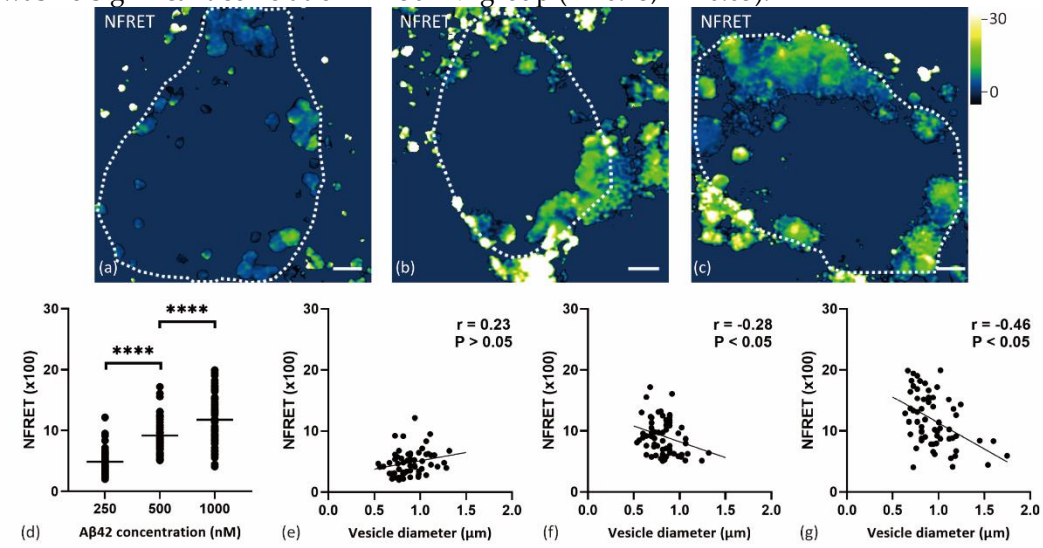

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Figure 6. Internalized Aβ42 oligomerization in neuronal soma. (**a-c**) normalized FRET (NFRET) image of vesicles in soma. Primary neurons were treated with 250 nM Aβ42 (**a**) or 500 nM Aβ42 (**b**) or 1000 nM Aβ42 (**c**) for 24h; (**d**) NFRET values of 63 vesicles from each group were calculated and compared using ANOVA test ($P < 0.0001$); (**e-g**) Pearson correlation of Aβ vesicle size and NFRET values in neurons treated with 250 nM Aβ42 (**e**) or 500 nM Aβ42 (**f**) or 1000 nM Aβ42 (**g**) for 24h.

2.4. Internalized Aβ42 oligomerizes in neurons with higher efficenciy than Aβ40

Finally, we treated primary neurons with fluorescently labeled Aβ40, and compared the polymerization of internalized Aβ40 and Aβ42. Confocal image analysis showed that more Aβ42 were taken up into neurons than Aβ40 using the same concentrations for 24 hours, and the Aβ42 oligomerization level was significantly higher as well (Figure S4).

Because the difference of oligomerization could be due to different Aβ concentrations in vesicles, we then compared vesicles with similar intensity of Aβ42 and Aβ40. Aβ concentration in vesicles of cells treated with 1000 nM Aβ40 for 24 hours was comparable to some of those treated with 250 nM Aβ42 (Figure 7 a-b). Therefore, we selected vesicles with similar Aβ concentration from these two groups and compared their normalized FRET level. Aβ42 treatment was found to induce more oligomerization than Aβ40, under similar Aβ concentrations in vesicles (Figure 7 c-f).

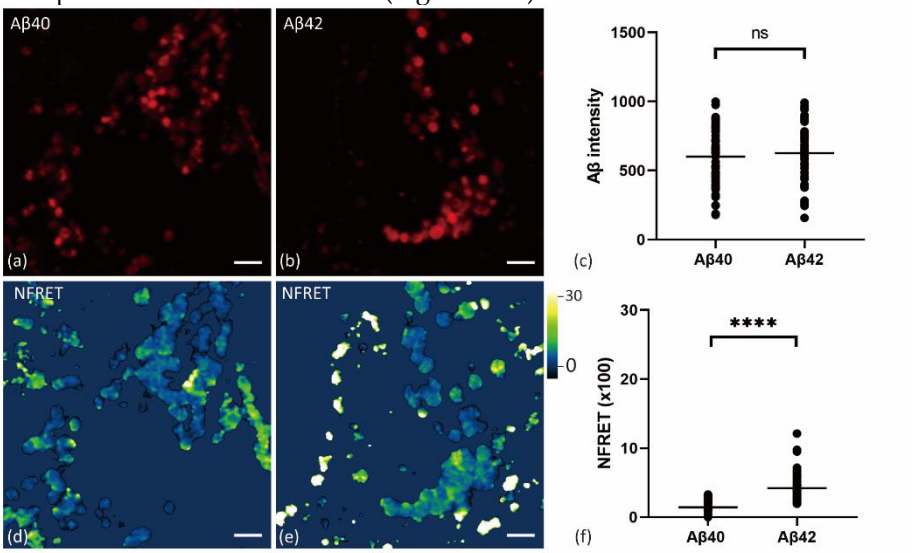

Figure 7. FRET images of neurons treated with Aβ40 or Aβ42. Primary neurons were treated with 1:1 mixed 1000 nM Aβ40 (**a, d**) or 250 nM Aβ42 (**b, e**) for 24h. (**a-b**) Confocal images of Aβ vesicles (HF647) in primary neurons, the same acquisition and display settings were used for both groups; (**c**) Aβ intensity (HF647) of selected vesicles in neurons treated with 1000 nM Aβ40 (N=65) and 250 nM Aβ42 (N=54) were compared using Mann-Whitney test ($P > 0.05$); (**d-e**) normalized FRET (NFRET) images of Aβ vesicles in primary neurons; (**f**) NFRET values of selected Aβ vesicles in neuron treated with Aβ40 and Aβ42 were compared using Mann-Whitney test ($P < 0.0001$).

Figure 8 shows the scatter plot of normalized FRET values and Aβ concentration in the vesicles of cells treated with different Aβ concentrations. The aggregation of both Aβ42 and Aβ40 showed strong trends to increase with their concentrations in vesicles. Despite lower external concentration, Aβ42 still aggregated to a higher extent than Aβ40.

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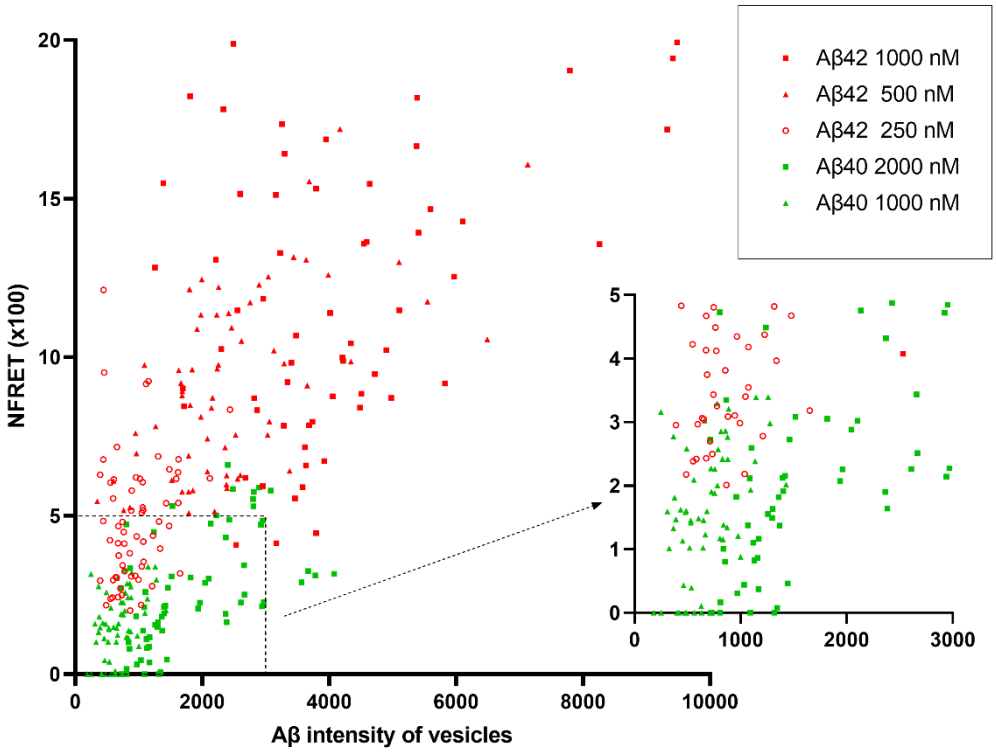

Figure 8. Scatter plot of normalized FRET (NFRET) and Aβ intensity (HF647) in vesicles of neuronal soma. Primary neurons were treated with different concentrations of Aβ40 (green) or Aβ42 (red) for 24 hours. The right graph shows enlarged details in the dashed box.

3. Discussion

Aβ aggregation is thought to have a crucial role in the progression of AD. However, the detection of Aβ oligomers is challenging due to lacking stability and being easily influenced during analysis [18]. FRET has been used to measure molecular distances at the nanoscale for a long time. Here by measuring FRET with a confocal microscope with an Airyscan unit, we confirm that extracellular monomeric Aβ could be internalized into the murine hippocampal neurons to form oligomers in lysosomes and/or late endosomes.

It has been suggested that intraneuronal Aβ accumulation could be an early event in the pathogenesis of AD [4]. In this study, extracellular Aβ is taken up by neurons and oligomerize intracellularly, making it a possible source of intraneuronal Aβ aggregation. We observed strong Aβ oligomerization occurred in primary neurons treated with only 250 nM Aβ42 for 24 hours. Previously, Hu et al. did not detect Aβ aggregation in SHSY5Y cells treated with 500 nM Aβ42 or below for 5 days, using agarose gel electrophoresis-SDS [16]. This might be due to the cellular uptake difference of Aβ, or because Aβ oligomers are SDS-instable species and this analysis could result in disassembly of oligomers [13].

In our study, Aβ in neurons aggregates in a concentration-dependent manner. This is consistent with previous study in SHSY5Y cells and in buffers [16,19]. Besides, the size of Aβ vesicles also showed a significant negative relation with aggregation in cells with 24 hours treatment of higher Aβ concentration, but not in lower concentration. We suggest that cells with lower Aβ concentration treatment could be at an earlier stage of uptake at 24 hours, compared with those with high concentrations.

The Aβ oligomer distribution in neuron is also interesting. It is reported that more lysosomes located in soma than in neurites [20]. However, the oligomerization level in lysosomes/late endosomes of soma and neurites seems similar. We also identified the strongest normalized FRET signal on the neuron surface based on the following consideration. First, the fluorescence on the cell surface lacks movement during imaging, different from those in the cellular organelles. Secondly, such distinctly high normalized FRET was

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observed only on the cell border and along neurites, but not in soma. Besides, it has been reported that Aβ form oligomers on lipid rafts in the cell membrane [21].

Regarding intraneuronal Aβ42 aggregation, another question is where this progress begins. First, we detected only little Aβ42 oligomerization at a 1000 nM concentration in the cell culture medium. Secondly, we noticed high Aβ oligomerization on the surface of neuron. The values of normalized FRET are several times higher than in the soma. The normalized FRET can be affected by the distance-dependent FRET efficiency and the ratio of "FRET" donor-acceptors to all the donors and acceptors [22]. In this study, such a large difference in the normalized FRET between soma and membrane cannot be explained by the FRET efficiency. Thus, we infer that lysosomes/late endosomes in soma contain non-FRET monomeric Aβ42. The concentration of Aβ42 in those vesicles is much higher than in extracellular space [16]. Low pH in the lysosomes/late endosomes might promote Aβ aggregation [19,23]. Considering the high concentration and low pH, Aβ monomers in these vesicles are more likely to be taken up extracellularly rather than depolymerized from oligomers. We suggest that at least part of Aβ is internalized in the monomeric form, then aggregates in the late endosomes/lysosomes. However, it is possible that part of Aβ oligomerize at the cell surface before entering late endosomes/lysosomes. It is also possible that oligomers that formed in neurons are released and then re-internalized to the endo-lysosomal system.

Compared with Aβ42, Aβ40 is less prone to form fibrils [24]. In the present study, Aβ42 aggregation dominance in neurons is partly owing to the cellular uptake preference, which results in high Aβ42 concentration in vesicles. Under similar concentrations in vesicles, Aβ42 still oligomerizes more than Aβ40, however the difference is less than we expected. Although normally Aβ42 is present at lower concentrations than Aβ40 [25], it can induce more oligomers due to the enhanced cellular uptake and its strong aggregation tendency. This novel finding is in line with our previous finding showing elevated levels of Aβ42 in neurons at risk in AD brain [9]. Thus, we hypothesize that Aβ42 accumulation in neurons by enhanced uptake is an early pathogenic event in AD, and that inhibition of this process could be a pharmacological strategy for treating AD.

In this study, we chose sensitized emission FRET for several reasons. It is simple, nondestructive, suitable for live imaging, and our study also fit the stoichiometry of 1:1 donor and acceptor. The fast and sensitive Airyscan unit allowed us to detect FRET signal in moving vesicles, but it also detected unwanted autofluorescence. To minimize the caused false positive FRET, autofluorescence was measured from the non-treated vesicles that present the fluorescence. However, detected autofluorescence existed only in part of vesicles, so background subtraction from the whole image might underestimate the FRET of those compartments with less autofluorescence, or even result in false negative values. In addition, although the bleed-through parameters were determined carefully, it was still difficult to measure accurate FRET data quantitatively with sensitized emission FRET, especially when the FRET signal was weak. This limits the use of sensitized emission FRET in some applications, like to determine the lowest Aβ concentration or the earliest timepoint to aggregate. Nevertheless, live cell FRET imaging can provide in situ detection with almost no intervention, making it a robust and unique technique for oligomerization measurement, when designed properly and conducted under controlled conditions.

In summary, our study suggests that Aβ42 monomer can be internalized in neurons and oligomerize in the late endosome/lysosomes with high efficiency. In such a way, Aβ42 oligomers might accumulate gradually in neurons in decades of lifetime, and cause the neuronal dysfunction.

4. Materials and Methods

4.1. Synthetic human Aβ Peptide and Preparation

Aβ (1–40) and Aβ (1–42) peptides, labeled with HiLyte™ Fluor 488 or HiLyte™ Fluor 647 at the N-termini, were purchased from AnaSpec (CA, USA). Aβ peptides were

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reconstituted by adding 40-50 µl 1% NH4OH to 0.1 mg dry peptide and diluted to 1 mg/ml with PBS, then aliquoted and stored at -20°C.

4.2. Primary neuron culture

Hippocampal neurons were isolated from the hippocampi of E16.5 C57/BL6 mice mouse embryo brain, seeded on the inner 10-mm microwell of poly-d-lysine-coated glass-bottom of P35G-1.5-10-C culture dishes (MatTek Corporation), as described previously [26]. Cortical neurons were seeded at the pre-coated edges of the plate as a support layer. Both hippocampal and cortical neurons were grown in selective Neurobasal medium containing 2% B27 (Invitrogen) and 1% L-glutamine (Invitrogen) at 37 °C in a cell incubator (humidified, 5% CO_2), and cultured for 21 days in vitro (DIV).

4.3. Lysosomes/late endosomes labelling in live cell imaging

Lysosomes/late endosomes were labelled using SiR-Lysosome (Spirochrome AG). Primary neurons were treated with 1000 nM SiR-Lysosome at 37°C, 5% CO2, 0.5 hour before imaging.

4.4. Confocal microscope and FRET measurements

Confocal and FRET images were acquired by Zeiss LSM 800 confocal microscope with Airyscan detector, using a Plan-Apochromat 63x/1.4 oil-immersion objective and the 488 and 640 nm laser lines as excitation source. For FRET measurements, the laser power and detector gain for all channels were fixed through the whole experiment. Each channel was subsequently scanned by a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT) Airyscan detector with a scan speed of 2.2 µs/pixel. 5–10 images were captured per cell culture dish.

The cells were incubated with either HF488 Aβ or HF647 Aβ, or an equimolar mixture of them. The donor control group were imaged sequentially in donor emission (488 nm excitation, 480-520 nm emission) and donor bleed-through (488 nm excitation, 650-700 nm emission). The acceptor control group included acceptor emission (640 nm excitation, 650-700 nm emission) and acceptor crosstalk (488 nm excitation, 650-700 nm emission). The FRET group contained three image sets: donor emission, acceptor emission, and FRET channel (488 nm excitation, 650-700 nm emission). The non-treated neurons group that used to detect the cell autofluorescence and background shared the same setting with FRET group.

FRET analysis was performed using ImageJ plug-in PixFRET as previously described [27]. Concisely, spectral bleed-through were first determined in each image acquired from donor control group and acceptor control group, by using a constant model in PixFRET. A Gaussian blur value of 2.0 was applied during the determination. By applying PixFRET computation, the original FRET images were then corrected from the spectral bleed-through and generated the FRET and normalized FRET image. “FRET/sqrt (Donor*Acceptor)” was selected as output of normalized FRET, in which values for each pixel were divided by the square root of donor and acceptor intensities from same pixels. Gaussian blur (2.0) and threshold (1.0) values were set for all the images. To clearly distinguish the low and high values, normalized FRET images were displayed in a lookup table “Green Fire Blue”. For comparison, the negative normalized FRET values will be set to 0. The quantification of normalized FRET values and Aβ intensity in vesicles were measured with ImageJ. Aβ intensity is calculated with HF647 Aβ because acceptor emission is not affected by FRET.

FCS, FCCS and FCS-FRET measurements were using a Zeiss 780 confocal laser scanning microscope equipped for FCS, with a Zeiss water immersion objective, C-Apochromat 40×/1.2 NA. The measurements have been described previously [17]. In this study, FRET-FCS was performed on the cell-free neuronal culture medium with 24 hours treatment of 500 nM HF488 Aβ42 and 500 nM HF647 Aβ42. The diffusion time (τ) refers to the mean time of fluorescent particles passing through the detection focus.

4.5. Statistics

Statistical tests were using the GraphPad Prism 8 software. Normality was assessed using D'Agostino & Pearson test. Non-parametric Mann–Whitney test was used for the data were not normally distributed. One-way ANOVA was used for comparison of three groups. Correlation analysis were tested by Pearson method. When the p value was less than 0.0001, it is stated as "****", and "*" for $p < 0.05$. If the p was greater than 0.05, it is stated as $p > 0.05$ or "ns".

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, Y.G., S.S-W., and L.T.; methodology, Y.G., S.W., S.S-W., and L.T.; formal analysis, Y.G. and S.W.; writing—original draft preparation, Y.G., and S.W.; writing—review and editing, Y.G., S.W., S.S-W., and L.T.; visualization, Y.G. and S.W.; supervision, B.W., S.S-W., and L.T.; funding acquisition, S.S-W., B.W., and L.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The ethical permit is approved by Linköping ethical committee (2018/01/25). No experiments are performed on live animals.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. 1. Ballard, C.; Gauthier, S.; Corbett, A.; Brayne, C.; Aarsland, D.; Jones, E. Alzheimer's Disease. *Lancet Lond. Engl.* 2011, 377, 1019–1031, doi:10.1016/S0140-6736(10)61349-9.
2. 2. 2020 Alzheimer's Disease Facts and Figures. *Alzheimers Dement.* 2020, 16, 391–460, doi:https://doi.org/10.1002/alz.12068.
3. 3. Viola, K.L.; Klein, W.L. Amyloid β Oligomers in Alzheimer's Disease Pathogenesis, Treatment, and Diagnosis. *Acta Neuropathol. (Berl.)* 2015, 129, 183–206, doi:10.1007/s00401-015-1386-3.
4. 4. LaFerla, F.M.; Green, K.N.; Oddo, S. Intracellular Amyloid-β in Alzheimer's Disease. *Nat. Rev. Neurosci.* 2007, 8, 499–509, doi:10.1038/nrn2168.
5. 5. Turner, R.S.; Suzuki, N.; Chyung, A.S.; Younkin, S.G.; Lee, V.M. Amyloids Beta40 and Beta42 Are Generated Intracellularly in Cultured Human Neurons and Their Secretion Increases with Maturation. *J. Biol. Chem.* 1996, 271, 8966–8970, doi:10.1074/jbc.271.15.8966.
6. 6. Tienari, P.J.; Ida, N.; Ikonen, E.; Simons, M.; Weidemann, A.; Multhaup, G.; Masters, C.L.; Dotti, C.G.; Beyreuther, K. Intracellular and Secreted Alzheimer Beta-Amyloid Species Are Generated by Distinct Mechanisms in Cultured Hippocampal Neurons. *Proc. Natl. Acad. Sci. U. S. A.* 1997, 94, 4125–4130, doi:10.1073/pnas.94.8.4125.
7. 7. Walsh, D.M.; Selkoe, D.J. Aβ Oligomers – a Decade of Discovery. *J. Neurochem.* 2007, 101, 1172–1184, doi:https://doi.org/10.1111/j.1471-4159.2006.04426.x.
8. 8. Karisetty, B.C.; Bhatnagar, A.; Armour, E.M.; Beaver, M.; Zhang, H.; Elefant, F. Amyloid-β Peptide Impact on Synaptic Function and Neuroepigenetic Gene Control Reveal New Therapeutic Strategies for Alzheimer's Disease. *Front. Mol. Neurosci.* 2020, 13, doi:10.3389/fnmol.2020.577622.
9. 9. Hashimoto, M.; Bogdanovic, N.; Volkmann, I.; Aoki, M.; Winblad, B.; Tjernberg, L.O. Analysis of Microdissected Human Neurons by a Sensitive ELISA Reveals a Correlation between Elevated Intracellular Concentrations of Abeta42 and Alzheimer's Disease Neuropathology. *Acta Neuropathol. (Berl.)* 2010, 119, 543–554, doi:10.1007/s00401-010-0661-6.
10. 10. Aoki, M.; Volkmann, I.; Tjernberg, L.O.; Winblad, B.; Bogdanovic, N. Amyloid β-Peptide Levels in Laser Capture Microdissected Cornu Ammonis 1 Pyramidal Neurons of Alzheimer's Brain. *NeuroReport* 2008, 19, 1085–1089, doi:10.1097/WNR.0b013e328302c858.
11. 11. Nag, S.; Sarkar, B.; Bandyopadhyay, A.; Sahoo, B.; Sreenivasan, V.K.A.; Kombrabail, M.; Muralidharan, C.; Maiti, S. Nature of the Amyloid-β Monomer and the Monomer-Oligomer Equilibrium. *J. Biol. Chem.* 2011, 286, 13827–13833, doi:10.1074/jbc.M110.199885.

12. 12. Hayden, E.Y.; Teplow, D.B. Amyloid β-Protein Oligomers and Alzheimer's Disease. *Alzheimers Res. Ther.* 2013, 5, 60, doi:10.1186/alzrt226.
13. 13. Bruggink, K.A.; Müller, M.; Kuiperij, H.B.; Verbeek, M.M. Methods for Analysis of Amyloid-β Aggregates. *J. Alzheimers Dis.* 2012, 28, 735–758, doi:10.3233/JAD-2011-111421.
14. 14. Algar, W.R.; Hildebrandt, N.; Vogel, S.S.; Medintz, I.L. FRET as a Biomolecular Research Tool — Understanding Its Potential While Avoiding Pitfalls. *Nat. Methods* 2019, 16, 815–829, doi:10.1038/s41592-019-0530-8.
15. 15. Wesén, E.; Gallud, A.; Paul, A.; Lindberg, D.J.; Malmberg, P.; Esbjörner, E.K. Cell Surface Proteoglycan-Mediated Uptake and Accumulation of the Alzheimer's Disease Peptide Aβ(1–42). *Biochim. Biophys. Acta BBA - Biomembr.* 2018, 1860, 2204–2214, doi:10.1016/j.bbamem.2018.08.010.
16. 16. Hu, X.; Crick, S.L.; Bu, G.; Frieden, C.; Pappu, R.V.; Lee, J.-M. Amyloid Seeds Formed by Cellular Uptake, Concentration, and Aggregation of the Amyloid-Beta Peptide. *Proc. Natl. Acad. Sci.* 2009, 106, 20324–20329, doi:10.1073/pnas.0911281106.
17. 17. Wennmalm, S.; Chmyrov, V.; Widengren, J.; Tjernberg, L. Highly Sensitive FRET-FCS Detects Amyloid β-Peptide Oligomers in Solution at Physiological Concentrations. *Anal. Chem.* 2015, 87, 11700–11705, doi:10.1021/acs.analchem.5b02630.
18. 18. Mroczko, B.; Groblewska, M.; Litman-Zawadzka, A.; Kornhuber, J.; Lewczuk, P. Amyloid β Oligomers (AβOs) in Alzheimer's Disease. *J. Neural Transm.* 2018, 125, 177–191, doi:10.1007/s00702-017-1820-x.
19. 19. Burdick, D.; Soreghan, B.; Kwon, M.; Kosmoski, J.; Knauer, M.; Henschen, A.; Yates, J.; Cotman, C.; Glabe, C. Assembly and Aggregation Properties of Synthetic Alzheimer's A4/Beta Amyloid Peptide Analogs. *J. Biol. Chem.* 1992, 267, 546–554, doi:10.1016/S0021-9258(18)48529-8.
20. 20. Kulkarni, V.V.; Maday, S. Neuronal Endosomes to Lysosomes: A Journey to the Soma. *J. Cell Biol.* 2018, 217, 2977–2979, doi:10.1083/jcb.201806139.
21. 21. Kim, S.-I.; Yi, J.-S.; Ko, Y.-G. Amyloid β Oligomerization Is Induced by Brain Lipid Rafts. *J. Cell. Biochem.* 2006, 99, 878–889, doi:https://doi.org/10.1002/jcb.20978.
22. 22. Xia, Z.; Liu, Y. Reliable and Global Measurement of Fluorescence Resonance Energy Transfer Using Fluorescence Microscopes. *Biophys.* J. 2001, 81, 2395–2402.
23. 23. Su, Y.; Chang, P.-T. Acidic PH Promotes the Formation of Toxic Fibrils from β-Amyloid Peptide. *Brain Res.* 2001, 893, 287–291, doi:10.1016/S0006-8993(00)03322-9.
24. 24. Jarrett, J.T.; Berger, E.P.; Lansbury, P.T. 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* 1993, 32, 4693–4697, doi:10.1021/bi00069a001.
25. 25. Asami-Odaka, A.; Ishibashi, Y.; Kikuchi, T.; Kitada, C.; Suzuki, N. Long Amyloid Beta-Protein Secreted from Wild-Type Human Neuroblastoma IMR-32 Cells. *Biochemistry* 1995, 34, 10272–10278, doi:10.1021/bi00032a022.
26. 26. Yu, Y.; Jans, D.C.; Winblad, B.; Tjernberg, L.O.; Schedin-Weiss, S. Neuronal Aβ42 Is Enriched in Small Vesicles at the Presynaptic Side of Synapses. *Life Sci. Alliance* 2018, 1, doi:10.26508/lsa.201800028.
27. 27. Feige, J.N.; Sage, D.; Wahli, W.; Desvergne, B.; Gelman, L. PixFRET, an ImageJ Plug-in for FRET Calculation That Can Accommodate Variations in Spectral Bleed-Throughs. *Microsc. Res. Tech.* 2005, 68, 51–58, doi:https://doi.org/10.1002/jemt.20215.