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Direct Observation of Internalization and ROS Generation of Amyloid β-Peptide in Neuronal Cells at Subcellular Resolution

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The pathological role of the amyloid β-peptide (Aβ) in Alzheimer's disease (AD) is one of the central questions in current neurodegenerative research.[1,2] There is consensus that an aberrant accumulation of Aβ within affected areas of the brain contributes directly to the development of AD.[1,2] The progressive accumulation of Aβ in the extracellular space yields amyloid plaques which trigger a series of reactions leading to synaptic and neuronal dysfunction and degeneration in brain areas responsible for distinct memory functions.[3] Recently, increasing evidence indicates a dynamic exchange between the intra- and extracellular Aβ pools.[4] It has been proposed that extracellular Aβ deposits are dynamic structures, and that Aβ can be released from the amyloid plaques and interact directly with neurons, triggering pathological cascades.[5] A few lines of evidence suggest that Aβ peptides can insert into the cell membrane and form multimeric channels with a central pore-like structure (the "channel hypothesis"), increasing the permeability of the membrane and providing a direct pathway for unregulated calcium influx.[6] In contrast to the channel hypothesis, a few other studies suggested that extracellular Aβ could be internalized and accumulated in neurons,[7] either by a dynamin-dependent and RhoA-mediated endocytosis pathway[7b] or via an energy independent nonendocytotic pathway.[7c] Thus the mode of Aβ interacting with cells and the mechanism of associated neurotoxicity remain unclear.[7d]

We have been developing novel strategies for AD therapy involving an anti-Fenton mechanism[8a,b] as well as molecular imaging probes which allow the direct observation of certain relevant cellular species or events at subcellular resolution in real time.[8c,d] In this study, we have covalently labeled the Aβ peptide ($Aβ_{1-40}$, the major form of Aβ found in amyloid plaques) with a fluorophore and used it as an optical probe to directly observe its interactions with neuronal cells and its subcellular distribution under a confocal microscope. Moreover, by utilizing other organelle trackers, membrane integrity probes and reactive oxygen species (ROS) sensors, we obtained direct evidence of Aβ-induced ROS production at subcellular resolution in live human neuroblastoma SH-SY5Y cells, a cell line that has been generally used as a cellular model to study AD.

For labeling Aβ, we first synthesized the succinimidyl ester derivative of lissamine rhodamine B (LRB-X-SE, compound **5**, Scheme S1 in the Supporting Information), which is one of the most amine-selective and stable reactive reagents for labeling proteins. Lissamine rhodamine B sodium salt (**1**, Scheme S1) was chosen as the starting material, from which lissamine rhodamine B sulfonyl chloride (LRB-SC, **2**, Scheme S1) was synthesized in a low cost and scalable route.[9] Then, using an improved procedure,[10] in which we replaced 6 M HCl with trifluoroacetic acid, **3** was hydrolyzed with high efficiency (Scheme S1). Aβ was then fluorescently labeled using LRB-X-SE (the product named AβL) following a standard protocol and the unbound dye was removed by gel filtration (see the Experimental Section and the Supporting Information).

The interactions of AβL with live SH-SY5Y cells were recorded as a series of snapshots over 24 h as shown in Figure 1 and Figure S2. The whole process could be divided into three sequential stages: 1) AβL binding to the surface of cells: After incubating cells with AβL for 0.5 h, a few scattered red points indicated that extracellularly applied AβL was binding to the surface of cells (Figure S2 A, indicated by white arrows). 2) AβL

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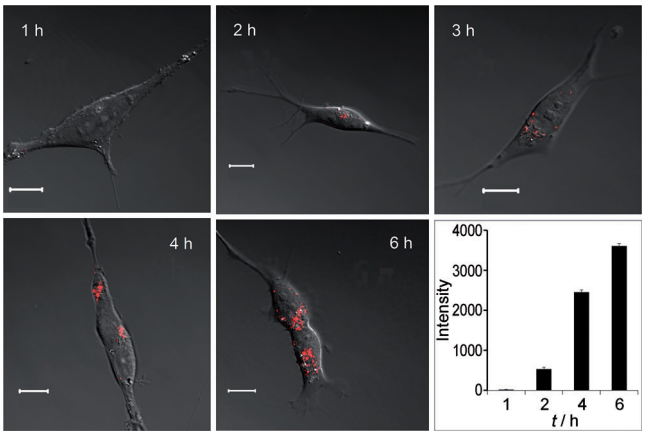

Figure 1. The time course of the internalization and accumulation of AβL in live SH-SY5Y cells. Confocal fluorescence microscopy of SH-SY5Y cells incubated with AβL (0.5 μM) was carried out at different time points (1–6 h) during the incubation process and the fluorescence intensity of each time point is shown in the bar graph. Scale bars: 10 μm.

being internalized into cells: After incubation for ~1–1.5 h, a small amount of AβL, appearing as weak scattered points, was being internalized into the intracellular space. Some of these points attached, or were close to, the inner side of the plasma membrane (Figures 1 and S2B). Interestingly, we were able to capture a few typical moments of AβL being internalized into the plasma membrane. Figures 2, S3 and S4 show an-

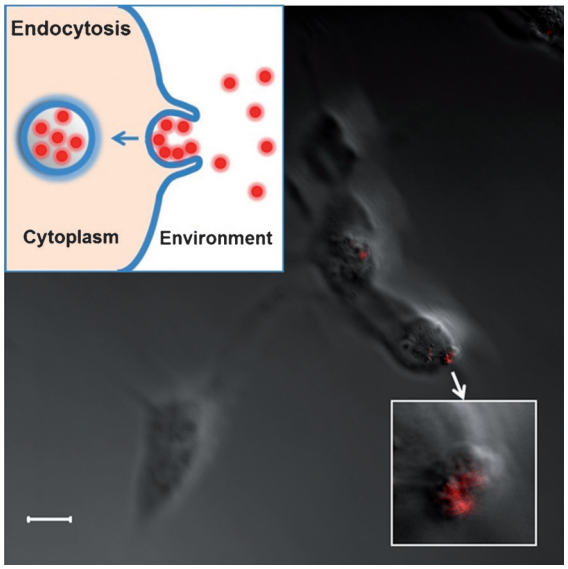

Figure 2. Typical internalization of AβL (0.5 μM) in the plasma membrane of a live SH-SY5Y cell viewed under a laser-scanning confocal microscope. Red fluorescence indicates the emission of AβL. Inset: a diagram of typical endocytosis. Scale bar: 10 μm.

nular-invagination structures (zoom-in images in insets) with about 3.5 μm diameter centered with AβL on the plasma membrane of the cells. It looked like a "mouth" of the cell was engulfing AβL, indicating a typical endocytosis process.[7] As shown in Figure S3, from A to D, the four continuous scanning layers of the sample from the outside to the inside of the cells gave the detailed structural information on the moment that internalization of AβL occurred. It should be noted that there were small amounts of AβL already observable inside the cell at this stage. 3) The accumulation of internalized AβL: Along with the increase in incubation time from 2 to 6 h, more AβL was internalized and accumulated inside the cells, appearing as isolated red spots inside the intracellular space (Figures 1 and S2C). After 6 h, the internalization and accumulation seemed to reach their peaks. Extending the incubation time (up to 24 h) did not result in more accumulation of AβL in cells (Figure 5B).

The scattered pattern of the internalized AβL suggested that it was localized in certain subcellular compartments. To investigate further their subcellular localization, colocalization experiments[8c] were performed using MitoTracker Green FM (a green-fluorescent dye which localizes to mitochondria in live cells regardless of mitochondrial membrane potential) and LysoTracker Blue DND-22 (a blue-fluorescent dye that stains acidic compartments such as endosomes and lysosomes in live cells). As shown in Figure 3 and Figure S5, it can be seen that

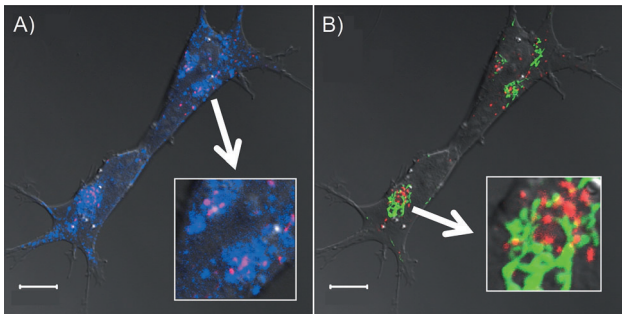

Figure 3. The colocalization of AβL (0.5 μM) with A) endosomes/lysosomes and B) mitochondria. The fluorescence emissions of AβL, LysoTracker and MitoTracker are indicated in red, blue and green, respectively. Integration of the signals revealed that ~88% of AβL signals colocalized with LysoTracker and ~12% colocalized with MitoTracker. Scale bars: 10 μm.

most of the AβL signals (red) colocalized with LysoTracker Blue (colocalized signals appeared in purple) with minor colocalizing with MitoTracker Green (colocalized signals appeared in yellow), suggesting that AβL is enriched in endosomes/lysosomes after internalization, with minor fractions (~12%) reaching mitochondria. Previous studies have suggested that Aβ species can accumulate in lysosomes[11] or mitochondria,[12] and agree with our data which has provided more direct and unambiguous evidence.

To investigate the mechanisms by which Aβ damages neurons, we first assessed the effect of AβL on the membrane integrity of SH-SY5Y cells using propidium iodide (PI), a fluorescent dye that cannot enter cells with intact plasma membranes.[13] Up to 24 h after AβL incubation, the cells remained PI-negative compared with the cells without AβL treatment (Figure S6), suggesting that the plasma membranes of AβL-treated cells remained intact under these conditions.

As several lines of evidence have implied that Aβ exerts its toxicity intracellularly through the production of ROS,[1,2,14] we then investigated the cellular ROS production using a general ROS probe carboxy-H_2DCFDA. As shown in Figure 4, marked increase in cellular ROS levels was observed in the AβL-treated cells compared to the control. However, detection of ROS in subcellular compartments was not possible with this probe due to its poor fluorescence properties.

As hydrocyanine dyes displayed much better sensitivity to ROS and excellent photostability,[15] we further applied hydro-Cy5, the reduced form of Cy5 that displays fluorescent emis-

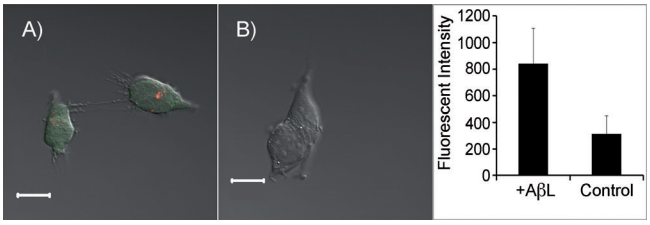

Figure 4. The ROS-inducing effect of AβL (0.5 μM) in SH-SY5Y cells (24 h) monitored by carboxy-H_2DCFDA (5 μM). A) ROS (green fluorescence) in AβL (red)-treated cells; B) ROS in control cells without AβL treatment. The bar graph shows the intensity of ROS under each condition ($n=6$). Scale bars: 10 μm.

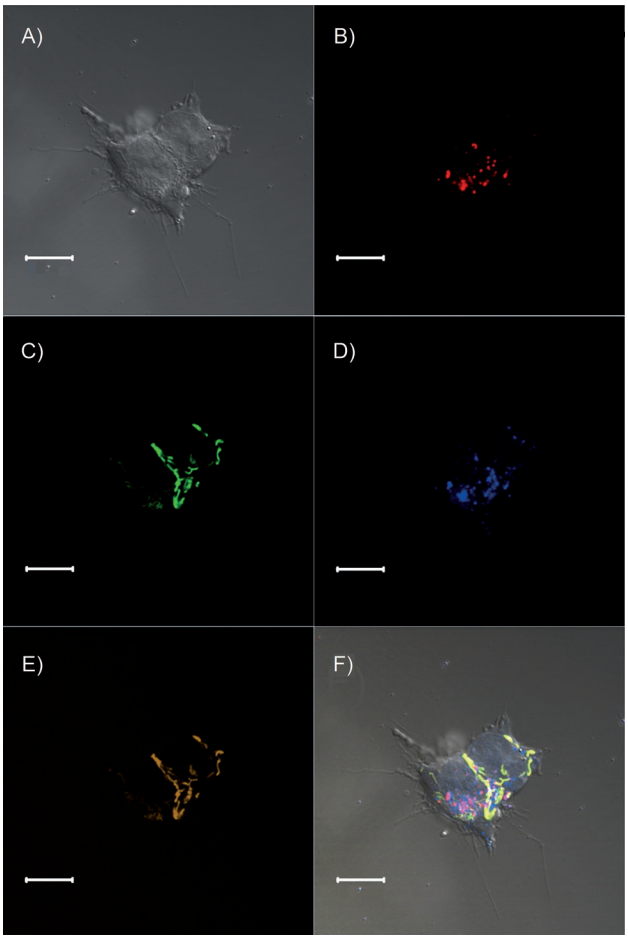

Figure 5. Representative confocal images of intracellular colocalization studies of hydro-Cy5 (10 μM) incubated with AβL-treated live human SH-SY5Y cells (24 h) colabeled with MitoTracker and LysoTracker. A) DIC image of cells. B) AβL fluorescence collected at 571–629 nm (red). C) MitoTracker fluorescence collected at 493–562 nm (green). D) LysoTracker fluorescence collected at 409–480 nm (blue). E) Cy5 fluorescence collected at 639–727 nm (gold). F) DIC image of (A) and fluorescence images of (A)–(D) were merged. The colocalization of ROS signals (gold, Cy5 channel) with MitoTracker Green signals (colocalized signal appeared in light yellow in panel (F) revealed that the ROS detected by hydro-Cy5 was localized in mitochondria. Scale bars: 10 μm.

sions in the near-infrared region, as a ROS sensor to monitor the ROS-inducing effect of AβL in SH-SY5Y cells. As shown in FigureS7 and Figure 5, AβL treatment caused significant ROS production in SH-SY5Y cells. The superior fluorescence properties of Cy5 provided us the opportunity to probe the subcellular localization of the ROS detectable by hydro-Cy5. Colocalization experiments using MitoTracker Green and LysoTracker Blue were performed with the cells incubated with AβL and hydro-Cy5. As shown in Figure 5, the colocalization of the ROS signals (Cy5, gold) with MitoTracker Green signals (colocalized signal appeared in light yellow in panel F) revealed that AβL-induced production of ROS in mitochondria of SH-SY5Y cells. Aβ is known to induce mitochondrial dysfunction and mitochondrial oxidative damage has been reported as an early event in AD progression.[16] However, the causal factors are still unclear.[7d] Overproduction of ROS in mitochondria causes apoptosis of neurons,[17] thus the AβL-induced production of ROS in mitochondria may directly linked to the neurotoxicity of Aβ.

In summary, our data have demonstrated that extracellularly applied AβL can be readily internalized into human SH-SY5Y cells by endocytosis. The internalized AβL colocalizes mainly in endosomes/lysosomes with a small portion reaching mitochondria. The internalized AβL does not damage the cell membrane directly but induces marked overproduction of ROS in SH-SY5Y cells as well as mitochondrial ROS production. The unambiguous evidence in this study paves the way for a clearer understanding of the cellular mechanisms of Aβ toxicity in AD.

Experimental Section

Chemicals: $A\beta_{1\text{-}40}$ (sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) was purchased from GL Biochem Ltd. (Shanghai, China), confirmed by MALDI-TOF MS (calculated 4329.9/observed 4330.0) and HPLC (>95%). Lissamine rhodamine B sodium salt was purchased from TCI, Inc. O-(N succinimidyl)-*N*,*N*,*N*,*N*-tetramethyluronium tetrafluoroborate, methyl 6-aminohexanoate hydrochloride, oxalyl chloride and PI were purchased from Sigma–Aldrich. Carboxy-H_2DCFDA, LysoTracker Blue DND-22 and MitoTracker Green FM were purchased from Life Technologies, Inc. (Eugene, OR). Hydro-Cy5 was synthesized following a protocol described previously.[15] Other chemicals and solvents were of analytically pure grade.

Instrumentation: NMR spectra were recorded on a Bruker DRX-300 spectrometer. ESI-MS analyses were performed using a PerkinElmer API 150EX mass spectrometer at 298 K. UV/Vis spectra were recorded on a PerkinElmer Lambda 25 spectrometer at 293 K. Fluorescence spectra were recorded on a PerkinElmer LS55 luminescence spectrometer at 293 K. The pH measurements were carried out using a Corning pH meter equipped with a Sigma–Aldrich micro combination electrode calibrated with standard buffer solutions. The fluorescence imaging of the fluorophore-labeled $A\beta_{1-40}$ in live cells under various conditions was investigated under a Zeiss LSM 710 laser-scanning confocal microscope. The REUSE function controlled by Zeiss software was applied to guarantee that the spectra were recorded under the same instrumental conditions if necessary.

Synthesis and characterization of LRB-X-SE: As shown in Scheme S1, the succinimidyl ester of the aminohexanoic acid sulfonamide of lissamine rhodamine B sulfonyl chloride (LRB-X-SE, **5**) was synthesized. We chose lissamine rhodamine B sodium salt (**1**) as the starting raw material, from which lissamine rhodamine B sulfonyl chloride (LRB-SC, **2**) can be synthesized in a low-cost and scalable route according to a reported procedure.[9] Then following an improved procedure,[10] in which we replaced 6 M HCl with trifluoroacetic acid resulting in a highly efficient hydrolysis of **3**, the target **5** was synthesized, and characterized by NMR and ESI-MS. The ^{1}H NMR spectrum of **5** is shown in Figure S1. ^{1}H NMR (300 MHz; $(CD_3)_2SO$; Me_4Si): $\delta = 8.41$ (s, 1H, ArH), 7.95–7.92 (m, 2H, 2ArH), 7.48–7.46 (d, 1H, ArH), 7.06–7.03 (m, 2H, 2ArH), 6.98 (d, 1H, ArH), 6.94 (s, 2H, 2ArH), 3.65–3.63 (q, 8H, 4CH_2), 2.89–2.85 (m, 2H, CH_2), 2.80 (s, 4H, 2CH_2), 2.68–2.64 (m, 2H, CH_2), 1.63–1.58 (m, 2H, CH_2), 1.46–1.40 (m, 4H, 2CH_2), 1.20 ppm (t, 12H, 4CH_3); ESI-MS (*m*/*z*): 769.3 $[M+H]^+$, 791.2 $[M+Na]^+$, $C_{37}H_{44}N_4O_{10}S_2$ requires 768.25.

Fluorescence labeling of $A\beta_{1-40}$ by LRB-X-SE: $A\beta_{1-40}$ was treated for 2 h with 100% trifluoroacetic acid (TFA) at RT and the excess TFA was purged under an argon stream until a clear film remained

in the test tube,[18] so as to obtain a chemically homogeneous sample. The pretreated $A\beta_{1-40}$ was then dissolved in DMSO (10 mg mL^{-1}, 3.08 mM) and stored at −20 °C. The exact concentration of the stock solution was determined by absorption spectroscopy of the tyrosine residue of $A\beta_{1-40}$ (275 nm) using an extinction coefficient of 1410 L mol^{-1} cm^{-1},[19] and the spectroscopic concentration, 3.09 mM, is consistent with the concentration by weight.

The fluorescence labeling of $A\beta_{1-40}$ at its free amine sites was performed using the LRB-X-SE reactive dye using a similar previously described procedure.[20] In short, the pH of a solution of $A\beta_{1-40}$ (100 μM, 300 μL) was adjusted to pH ~ 8.5 with of $NaHCO_3$ (1 M, 30 μL), followed by addition of the reactive dye in DMSO (3.0 mM, 50 μL). The reaction was stirred at RT for approximately 75 min. Then, the labeling reaction mixture was loaded onto a Sephadex G-25 column or a disposable PD-10 desalting column packed with Sephadex G-25 medium resin for removal of unreacted dye (Figure S8). The resulting eluant (10 μM, pH 7.4) was aliquoted and stored at −20 °C or used immediately for cellular uptake experiments. Successful labeling of the peptide was confirmed by UV/Vis spectroscopy.

Cell culture and confocal imaging: Human SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in a 1:1 mixture of Eagle's Minimal Essential medium (ATCC) and Ham's F12 medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS, ATCC) without antibiotics and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured by using 0.05% trypsin-EDTA solution (ATCC). The cells were seeded on 2-chamber slides for 48 h at 5×10^4 cells per chamber and grown until each chamber was 20–30% confluent. The fluorescently labeled $A\beta_{1-40}$ (AβL, 10 μM) was prediluted to a concentration of 0.5 μM in culture medium without FBS. Cell culture medium was removed from the chambers and replaced with fresh medium containing AβL (0.5 μM). Cells were imaged on a laser-scanning confocal microscope (Zeiss LSM 710). After being cultured for 24 h in the medium with or without AβL, cells were washed with PBS and plasma membrane damage was investigated using PI (10 μM, 10 min) as described previously.[21] ROS generation was imaged by using carboxy-H_2DCFDA or hydro-Cy5 as ROS probes. After treatment with or without AβL, cells were washed with PBS. Carboxy-H_2DCFDA (5 μM) or hydro-Cy5 (10 μM) was added to the cell culture and incubated for 15 min before being examined by laser-scanning confocal microscopy (Zeiss LSM 710). Colocalization experiments were performed similarly as described previously.[8d]

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