Role of Membrane Tension Sensitive Endocytosis and Rho GTPases in the Uptake of the Alzheimer’s Disease Peptide Aβ(1-42)

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ABSTRACT: Intraneuronal accumulation of amyloid-β (Aβ) is an early pathological signum of Alzheimer’s disease, and compartments of the endolysosomal system have been implicated in both seeding and cell–cell propagation of Aβ aggregation. We have studied how clathrin-independent mechanisms contribute to Aβ endocytosis, exploring pathways that are sensitive to changes in membrane tension and the regulation of Rho GTPases. Using live cell confocal microscopy and flow cytometry, we show the uptake of monomeric Aβ(1-42) into endocytic vesicles and vacuole-like dilations, following relaxation of osmotic pressure-induced cell membrane tension. This indicates Aβ(1-42) uptake via clathrin independent carriers (CLICs), although overexpression of the bar-domain protein GRAF1, a key regulator of CLICs, had no apparent effect. We furthermore report reduced Aβ(1-42) uptake following overexpression of constitutively active forms of the Rho GTPases Cdc42 and RhoA, whereas modulation of Rac1, which is linked to macropinosome formation, had no effect. Our results confirm that uptake of Aβ(1-42) is clathrin- and dynamin-independent and point to the involvement of a new and distinct clathrin-independent endocytic mechanism which is similar to uptake via CLICs or macropinocytosis but that also appear to involve yet uncharacterized molecular players.

KEYWORDS: Amyloid-β, Aβ(1-42), Alzheimer’s disease, cellular uptake, clathrin-independent endocytosis (CIE), uptake mechanism

INTRODUCTION

Alzheimer’s disease (AD) is characterized by the aggregation of amyloid-β (Aβ) peptides resulting in the formation of extracellular plaque deposits in the brain, alongside the formation of intracellular neurofibrillary tangle structures. Aβ has been suggested as a causative agent of AD pathology; many familial forms of AD are associated with mutations that alter its production, processing, and clearance. It is formed by proteolytic cleavage of the amyloid precursor protein (APP), which is linked to macropinosome formation, had no effect. Our results confirm that uptake of Aβ(1-42) is clathrin- and dynamin-independent and point to the involvement of a new and distinct clathrin-independent endocytic mechanism which is similar to uptake via CLICs or macropinocytosis but that also appear to involve yet uncharacterized molecular players.

Keywords: Amyloid-β, Aβ(1-42), Alzheimer’s disease, cellular uptake, clathrin-independent endocytosis (CIE), uptake mechanism
While the mechanisms and functions of clathrin-mediated endocytosis (CME) have been well studied in many physiological contexts, CIE mechanisms remain much less well-defined due to their diversity and complexity. It is not yet understood how many distinct CIE mechanisms a cell actually has nor is it clear how extensively these pathways are used in different cell types. Reports range from almost exclusive use of CME, to situations where the majority of the cellular endocytic volume is internalized via CIE. CIE is, importantly, active in neurons, contributing to cargo uptake and fast regulation of membrane turnover at synapses where Aββ peptides are also present. In addition to the implicated role in capture and local membrane bending assisted by the well-recognized Aββ/β endophilin-mediated endocytosis (FEME), CIE also contributes to the neuronal internalization of the APP-processing enzyme BACE1, the amyloidogenic human PrP protein, as well as several types of amyloid oligomers and fibrils.

The molecular and mechanistic classification of CIE has been complicated by the apparent lack of specific cargoes and exclusive regulators. Nonetheless, at least three distinct pathways exist, all regulated by activating/deactivating cycling of specific small signaling G-proteins of the Rho GTPase family (Figure 1). Macropinocytosis is initiated by large (micrometer size-range), actin-driven membrane protrusions and is activated by specific signals, such as growth factors, chemokines, cationic peptides, and amyloid assemblies. Rac1 activation is highly coupled to macropinocytosis. Fast endophilin-mediated endocytosis (FEME) results from cargo capture and local membrane bending assisted by the endophilin BAR-domain and other cytosolic proteins. FEME is important in assisting fast membrane recycling at the synapse and occurs under the regulatory control of dynamin as well as the Rho GTPases Rac1 and RhoA. It is further activated upon inhibition of Cdc42. The dynamin independence of Aββ endocytosis suggests, however, that FEME is not involved; notably this was further reinforced by our study. Finally, CIE uptake can also occur via clathrin-independent carriers (CLICs) into glycosylphosphatidylinositol (GPI)-anchored protein enriched endocytic compartments (GEECs; CLIC/GEEC). CLIC/GEEC is a constitutive and cargo clustering driven pathway important for the uptake of GPI-anchored proteins, glycosylated cargoes, certain toxins, and glycosphingolipids. CLICs are further involved in the endocytosis of the brain abundant lipid GM1, which is a well-recognized Aββ binding partner. CLIC formation is regulated by Cdc42, and the formation of at least one subpopulation of CLICs is also dependent on the GTase-regulatory and BAR-domain containing protein GRAF1.

Endocytosis via CLIC/GEEC, but also via macropinocytosis, is modulated by changes in plasma membrane tension, and the mechanisms are upregulated under conditions where cells need to rapidly internalize excess plasma membrane. Their involvement in cargo uptake can therefore be explored by altering the toxicity of the cell culture medium as explored here.

The small Rho GTPases in this study are not only important regulators of CIE, they are also involved in both neuronal development and neurodegeneration, and their activity (specifically that of Rac1 and RhoA) has been observed to decrease in the brains of patients with AD. Furthermore, Rac1 activation increases the production of Aβ from APP and results in hyperphosphorylation of tau, thereby providing a possible link between these two AD-relevant pathological hallmarks. It has also been suggested that soluble Aβ(1-40) peptides can reduce neurite length in a neuroblastoma model by inducing RhoA activity, whereas application of fibrillar Aβ(1-42) to neurons has been reported to result in dysregulated actin polymerization through altered activity of Rac1 and Cdc42. Furthermore, Aβ(1-42) oligomers have been observed to exert cellular toxicity in a RhoA-dependent manner, following internalization. By being key regulators of several AD-related signals, Rho GTPases have also been suggested as possible therapeutic targets. Thus, effects of Aβ internalization on Rho GTPase regulation have been established in several AD-relevant contexts, but it has not yet been explored how modulation of various Rho GTPases per se affect the clathrin-independent endocytic uptake of Aββ itself. Lastly, several reports link AD to dysregulated cholesterol metabolism, and it has been shown in a variety of in vitro biophysical contexts that amyloid assemblies, including Aββ, are in themselves potent modulators of membrane organization, integrity, and bending, suggesting they might have direct, cargo-mediated effects on various types of CIE.

This study extends our previous work on Aββ endocytosis by focusing specifically on the role of CIE in the uptake of Aββ(1-42) monomers. We probe CIE mechanisms by modulating cell membrane tension, and the activity of the CLIC/GEEC specific GTase activating protein GRAF1. We show that the uptake of Aββ(1-42), supplied to human neuroblastoma SH-SYSY cells, is catalyzed upon relief of hypotonic media-induced plasma membrane tension via a mechanism that is under regulatory control of the small GTPases Cdc42 and RhoA but not Rac1. These findings suggest that actin polymerization is highly important but that internalization is not via macropinocytosis in its most classical description. Furthermore, Aββ(1-42), despite being previously shown to occur at the leading edge of cells and in areas of high membrane ruffling, was found to be independent of GRAF1, suggesting that its CIE uptake involves other, yet unidentified, endocytic membrane sculpting proteins. Altogether, this work has revealed new insights into how components of CIE are related to the endocytosis of Aββ(1-42) and, significantly, pointed out that AD-relevant dysregulations of Rho GTPase activities could importantly influence the extent of intraneuronal Aββ accumulation.

Figure 1. Clathrin-independent endocytosis (CIE). Uptake via the CIE paths macropinocytosis, FEME, and CLIC/GEEC, highlighting the respective involvement of the Rho GTPases Cdc42, Rac1 and RhoA, as well as that of GRAF1 and sensitivity to changes in membrane tension.
RESULTS AND DISCUSSION

Cellular Uptake of Monomeric Aβ(1-42) Is Increased by the Alleviation of Hypotonic-Media Induced Membrane Tension. Uptake into endolysosomal compartments and subsequent intraneuronal accumulation have been implicated in both seeding and cell–cell propagation of Aβ(1-42). Previous work has shown that uptake of monomeric Aβ(1-42) is clathrin-independent, and in order to further map out the involved endocytic paths and regulators, we here focus on clathrin-independent endocytosis (CIE).

First, we explored how modulation of the plasma membrane tension, achieved by altering the tonicity of the incubation medium, influenced the cellular uptake of Aβ(1-42), applied in highly monomeric form, to cultured SH-SYSY neuroblastoma cells. Such acute changes in membrane tension have previously been described in studies on CLIC/GEEC in molluscan neurons exposed to hypotonic medium (50% MQ). In our experiments, we observe little or no influence on the cellular uptake of Aβ(1-42) concentration (2 μM); also the uptake of dextran, which is a nonspecific fluid phase endocytosis marker, is low. During exposure to hypotonic conditions, the cells expanded and rolled up due to an osmotic pressure-induced increase in cell volume. Previous studies have shown that this is accompanied by an increase in plasma membrane tension, which was increased by a factor of 3 (from 0.04 to 0.12 mN/m) in molluscan neurons exposed to hypotonic medium (50% MQ). In our experiments, we observe little or no internalization of Aβ(1-42) and dextran (Supplementary Movie 2 and Figure 2A, middle row). During re-exposure to isotonic conditions (recovery), the cells readopted normal morphology and resumed to internalize Aβ(1-42) and dextran, respectively. Inserts display zoomed areas. (B) Zoomed images of one representative cell with AF647-labeled dextran 10 kDa-filled VLDs (internalized during exposure to 250 μg/mL dextran for 10 min at recovery conditions). The time-lapse was started after the 10 min recovery period and a 1× wash, and the cells were imaged by confocal microscopy. The scale bar is 5 μm. The time lapse movie is displayed as Supplementary Movie 4.
the hypotonic treatment (Figure 2A, bottom row). Contrasting to Aβ(1-42), this fluid-phase marker only appeared to internalize into VLDs (Supplementary Movie 3), indicating a behavioral difference and a higher degree of specificity of Aβ(1-42) towards small vesicles. The small Aβ(1-42) containing endosomes could either originate from a specific upregulation of a distinct endocytic pathway or from a specific type of uptake from the VLDs as such. We therefore monitored the fate of the dextran-containing VLDs in the SH-SYSY cells by time lapse microscopy (Figure 2B, Supplementary Movie 4; recordings started when the cells had been kept for 10 min in the recovery phase), observing both tubulation and concurrent fission of the tubules. This confirms that the VLDs are internal and dynamic structures that are being degraded and eventually eliminated by the cell. An interesting observation in this regard is that the VLDs we observe in SH-SYSY cells persist longer (in general >10 min), compared to VLDs in HeLa or mouse embryonic fibroblasts (where they disappear within minutes). The number of VLDs per SH-SYSY cell also appears to be high. It is possible that these differences stem from the fact that SH-SYSY cells, like all neuroblastoma cells, lack caveolin-172 and therefore cannot regulate their cell surface in response to membrane tension reduction by caveolae formation.33,41 We also characterized the response of SH-SYSY cells to the acute changes in membrane tension applied in our experiments by imaging the actin cytoskeleton in cells transfected with CellLight Actin-GFP (Supplementary Movie 5 and Figure S2). While actin filaments were clearly visible in cells before the hypotonic treatment, these structures rapidly disassembled following addition of MQ in agreement with previous reports. Furthermore, within the timespan of the recovery experiment, the actin filaments did not completely re-establish; instead more punctate filaments were formed.

To quantify how changes in membrane tension affected cell uptake of Aβ(1-42), Trf, and dextran, we used flow cytometry. Figure 3A shows mean fluorescence intensities (normalized relative to uptake in isotonic media), supporting the imaging data (Figure 2A and Supplementary Figure S1). Interestingly, the extent of Aβ(1-42) uptake during the recovery phase merely doubles, whereas the uptake of dextran is increased ~8 times, suggesting a difference in uptake mechanism and membrane tension dependence of the two macromolecules. This was further substantiated by the observation that the uptake of Aβ(1-42) and dextran during recovery has different linear concentration dependence (Figure 3B, Supplementary Figure S3); the concentration dependence for Aβ(1-42) has a slope of 4.3 ± 0.7 compared to 13.5 ± 2.1 for dextran.

We next explored how the uptake of Aβ(1-42) and dextran during recovery depended on the magnitude of the hypotonic shock, probing also smaller changes in membrane tension (Figure 3C,D). Aβ(1-42) and dextran have very similar trends, and clear effects are only observed above 50% MQ, which has also been applied in other studies. Exposure of the cells to hypertonic media (addition of 100 mM NaCl to the culture medium) to reduce membrane tension did not increase Aβ(1-42) or dextran uptake (Supplementary Figure S4), suggesting that uptake during membrane tension reduction is directly related to the cell’s need to reduce its cell membrane surface area.

Cellular Uptake of Aβ(1-42) Is Independent of GRAF1. Building on the results above, demonstrating a membrane tension-sensitive endocytosis of Aβ(1-42), we next explored if the uptake was mediated via GRAF1-dependent CLIC/GEEC. We used an engineered HeLa Flp-In T-REx cell line with an inducible expression of GFP-GRAF1, but it has also been shown that GRAF1 is constitutively expressed and thus relevant for CIE in SH-SYSY cells. The HeLa cell line was transfected with mCherry-tagged Cdc42 (WT and dominant active (DA) Q61L mutant). Upon doxycycline-induced low level expression of GFP-GRAF1, punctuate and tubular GRAF1-positive structures appeared in cells expressing DA Cdc42 Q61L but not Cdc42 WT, as reported by Vidal-Quadras et al. CLIC/GEEC ligands are expected to be trapped in these GRAF1-structures but unable to transit further due to the DA Cdc42 Q61L overexpression, thus enabling visualization of GRAF1-mediated uptake via CLIC/GEEC, which is otherwise a very fast event. Cells were incubated with 1 μM Aβ(1-42) for 40 min, resulting in the formation of Aβ(1-42)-containing endosomal vesicles that did not colocalize with, or appear to display any similar intracellular distribution pattern as, GRAF1 in cells transfected with Cdc42 Q61L (Figure 4A). Since it is possible that the lack of colocalization between Aβ(1-42) and GRAF1 is due to the fairly long incubation time (40 min; needed to achieve high signal-to-noise images of intracellular Aβ(1-42) in this case), it cannot be excluded that Aβ(1-42) has been trafficked from CLICs to downstream endosomal organelles by the time of analysis. Therefore, we also exposed cells to a shorter incubation pulse (15 min) at a higher peptide concentration.
modulates uptake. Since Cdc42 (and other Rho GTPases) regulates the actin cytoskeleton,74 this would be consistent with our previously published result that inhibitors of actin polymerization reduced Aβ(1-40) and Aβ(1-42) but not Trf internalization in SH-SY5Y cells.13 To follow up on this finding, we explored systematically how Aβ(1-42) uptake depends on overexpression of WT, DN, and DA forms of Cdc42 as well as Rac1 and RhoA, which are two additional well-described Rho GTPases that furthermore have been implicated in AD pathology75 and to some extent appear to be regulated by the presence of Aβ peptides.57,58

First, cells were transfected with EGFP-Cdc42 variants (WT, DN, DA), followed by 1 h treatment with 1 μM Aβ(1-42) (Figure 5A) or 5 min with 5 μg/mL Trf (Supplementary Figure S6). The EGFP-Cdc42 expressing cells have altered morphologies, with increased filopodia formation, consistent with the effect of Cdc42 on the actin skeleton.74 Furthermore, the cell-to-cell variation in EGFP intensity shows that transfected cells expressed different amounts of the EGFP-Cdc42 variants, as confirmed by flow cytometry (Figure 5B). We took advantage of this when quantifying Aβ(1-42) and Trf uptake, by gating cells as nontransfected (overlapping with the intensity of mock cells transfected with MQ water, see Supplementary Figure S7) and low, medium, and high transfected (Figure 5B) based on their EGFP intensity, as previously described.13 Within each gate, we determined the mean cellular fluorescence intensity of HF647-labeled Aβ(1-42) or AF647-labeled Trf (Figure 5C). We found that both Aβ(1-42) and Trf uptake is reduced by overexpression of DA EGFP-Cdc42 Q61L, suggesting that Cdc42 modulation can effect on the intrinsic endocytic capacity of the cells. Overexpression of WT or DN EGFP-Cdc42 had no effect on Aβ(1-42) and Trf uptake (Figure 5C).

We repeated this set of experiments, exploring also the Rho GTPases Rac1 and RhoA. Overexpression of EGFP-tagged Rac1 (DA Q61L, WT, and DN T17N) induced lamellipodia-like morphologies in transfected cells as expected from the literature.76 None of the Rac1 variants had any effect on Aβ(1-42) uptake (Figure 6A,B), not even at the highest levels of overexpression (see Supplementary Figure S10 for depiction of the flow cytometry gates), although a reduction of Trf uptake...
in the cells with the highest concentration of WT and DN Rac1 was observed. The latter result is contrasting observations by Lamaze et al.,75 investigating Trf uptake in HeLa cells, highlighting how highly variable endocytic responses can be among cell types. We also observe that cells with a “medium” expression level of DA Rac1 appear to internalize slightly more Trf than control, suggesting that endocytic pathways may be sensitively fine-tuned by the transient concentration of adaptors. Modulation of Rac1 did not alter the endocytic capacity of cells, as measured by the uptake of dextran (Supplementary Figure S8). Considering the involvement of Cdc42 and Rac1 in macropinocytosis,30 which we have previously inhibited by IPA-3 and wortmannin showing reductions in the uptake of Aβ(1-40) and Aβ(1-42),15 it is noteworthy that we do not observe any effects on Aβ(1-42) uptake with Rac1. This is furthermore interesting in relation to a study showing that the uptake of fibrillar Aβ(1-42) by microglia depends on Rac1.76 This points to differences in the uptake mechanisms of soluble and fibrillar Aβ forms. We have recently observed similar, seemingly mechanistic differences in the uptake of preformed fibril fragments and monomers of the Parkinson’s disease related protein α-synuclein.40

Lastly, cells were transfected with EGFP-tagged RhoA (DA Q63L, WT, and DN T19N) and exposed to Aβ(1-42) and Trf, followed by analysis by confocal microscopy (Aβ(1-42) in Figure 7A, Trf in Supplementary Figure S11) and flow cytometry (Figure 7B, gates in Supplementary Figure S12). This showed that the uptake of both Aβ(1-42) and Trf is reduced following overexpression of all variants of RhoA. The most extensive concentration-dependent effect is seen with DA Q63L, but statistically significant reductions in uptake were also observed following overexpression of WT and T19N (T19N; high transfection gate not included due to too few cells). The uptake reduction in cells expressing DA RhoA could, at least partially, be explained by an overall decrease in endocytic activity as the uptake of dextran 10 kDa is reduced by up to 50% (Supplementary Figure S8). However, reports by Yu et al.59 demonstrate involvement of RhoA in endocytosis of Aβ(1-42) oligomers, in-line with our results. Also, the results do not point toward any importance of fast endophilin-

Figure 5. Cellular uptake of Aβ(1-42) is reduced by Cdc42 Q61L overexpression. (A) Confocal microscopy images of SH-SY5Y cells transfected with EGFP-tagged Cdc42 (DA Q61L, WT, or DN T17N) following incubation with 1 μM HF647-labeled Aβ(1-42) for 1 h. The scale bar is 20 μm and representative for all images. The asterisks (*) marks the positions of cells that are not expressing Cdc42. (B) Representative flow cytometry histograms (cell count vs intensity) of cells analyzed 24 h post transfection with EGFP-labeled Cdc42. For further analysis, the cells were gated for peptide uptake based on transfection efficiency and the extent of EGFP-Cdc42 overexpression (e.g., green fluorescence; none, low, medium, and high) as indicated in the figures. (C) Quantification of Aβ(1-42) and Trf uptake in cells transfected with Cdc42. The cells were incubated with either 1 μM HF647-labeled Aβ(1-42) for 1 h or 5 μg/mL AF647-labeled Trf for 5 min, washed, and analyzed for intracellular peptide signal by flow cytometry. Uptake is reported as relative mean cellular fluorescence in relation to uptake in nontransfected cells (N = 3, n = 4). Asterisk (*) marks uptake levels that are significantly different from uptake in nontransfected cells (adjusted p-value <0.05) by one-way ANOVA with matched data followed by multiple comparisons with Bonferroni posthoc test (adjusted p-values were Q61L, Aβ(1-42) none vs high 0.0001 and Trf none vs high <0.0001).
mediated endocytosis (FEME)\textsuperscript{45} in Aβ(1-42) internalization, as the uptake levels after perturbation of Rho GTPase activity are different compared to what would be expected if FEME (which is sensitive to perturbation of Rac1 and RhoA) is highly consistent with the findings here on the important regulatory roles of key CIE regulatory Rho GTPases. In previous work, we also observed reductions in Aβ(1-42) uptake upon pharmacological macropinocytosis inhibition.\textsuperscript{13} Our observations of sensitivity to Cdc42 activity reinforce the putative importance of this path, although the lack of sensitivity to Rac1, an activator of the formation of the large membrane protrusions that drive the formation of macropinosomes,\textsuperscript{30} suggests that macropinocytotic Aβ(1-42) uptake may be of a nonclassical type. The sensitivity to reduction of membrane tension could support macropinocytosis\textsuperscript{53} but is mainly consistent with uptake via constitutively active CLIC/GEEC.\textsuperscript{52} Importantly, we have previously observed polarized internalization of Aβ(1-42) in CHO cells, resulting from lamellipodia,\textsuperscript{20} which are CLIC-enriched areas.\textsuperscript{35} Furthermore, CLICs are important regulators of the cell uptake of the brain-abundant and AD-relevant glycosphingolipid GM1, which could be a putative receptor due to its reported tight binding to Aβ\textsubscript{peptides}\textsuperscript{41,49}, influencing also their aggregation\textsuperscript{77} and toxicity.\textsuperscript{78} Notably, we have observed that NIH-3T3 fibroblasts internalize more Aβ(1-42) than CHO and SH-SYSY, consistent with their inherent endocytic capacities.\textsuperscript{13} We here note that fibroblasts, in particular, have an exceptionally high constitutive activation of CLICs.\textsuperscript{35} Interestingly, in this regard, we find that Aβ(1-42) uptake is independent of the BAR domain containing and membrane sculpting protein GRAF1, which has been put forward as a key regulator of CLIC formation.\textsuperscript{30,51}

Altogether, we report that Aβ(1-42) internalizes into cells via a specific and distinctive CIE mechanism that is highly sensitive to changes in membrane tension and the regulatory

\textbf{CONCLUSION}

In this study, we have used live cell confocal fluorescence microscopy and flow cytometry to study how perturbation of endocytic mechanisms that are sensitive to changes in membrane tension and the regulatory control of Rho GTPases influence the cellular uptake of monomeric Aβ(1-42). This extends previous work by us and others in this area and provides additional insight into cell biological mechanisms and pathways that contribute to the Alzheimer’s disease relevant endolysosomal accumulations of Aβ(1-42).

We report that Aβ(1-42) uptake into SH-SYSY cells is sensitive to osmotic pressure-induced alterations in membrane tension, which points to the involvement of CLICs or macropinocytosis. Importantly, Aβ(1-42) behaves distinctively different from the CME-cargo Trf, but also dextran, which is an unspecific fluid-phase endocytosis marker. This suggests that Aβ(1-42) uptake occurs by a specific and differently regulated uptake path. We have previously demonstrated that Aβ(1-42) uptake is sensitive to perturbations of actin,\textsuperscript{53} which appears highly consistent with the findings here on the important

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\caption{Cellular uptake of Aβ(1-42) is not sensitive to changes in Rac1 expression. (A) SH-SYSY cells transfected with EGFP-tagged Rac1 (DA Q61L, WT, or DN T17N) and incubated with 1 μM HF647-labeled Aβ(1-42) for 1 h imaged by confocal microscopy. The scale bar is 20 μm. The asterisks (*) marks an example of a cell that is not expressing Rac1. (B) Quantification of Aβ(1-42) and Trf uptake in cells transfected with Rac1. The cells were incubated with either 1 μM HF647-labeled Aβ(1-42) or 5 μg/mL AF488-labeled Trf for 1 h or 5 min, respectively, washed and analyzed for intracellular peptide signal by flow cytometry. Uptake is reported as relative mean cellular fluorescence and based on level of Rac1-expression (none, low, medium, and high) in relation to uptake in nontransfected cells (adjusted p-values were Q61L, Trf none vs medium 0.0355; WT, Trf none vs high 0.0001; T17N, Trf none vs high <0.0001).}
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control of small Rho GTPases. The uptake mechanism appears similar to uptake via CLICs or possibly macropinocytosis, but the independence of Rac1 and GRAF1 suggests that it also involves yet uncharacterized membrane-sculpting and vesicle coating proteins of these incompletely characterized clathrin-independent endocytic paths. Better insights into this molecular machinery, guided by the results presented here, will undoubtedly provide a clearer cellular and molecular understanding of how uptake relates to endolysosomal accumulation. Putatively, this could also have important implications toward the development of future Aβ-clearing therapies, possibly targeting the endocytic path.

**METHODS**

**Reagents.** Synthetic Aβ(1-42) peptide, conjugated to the HiLyte Fluor HF488 or HF647 at the N-terminus, were from Anaspec Inc. (Fremont). The peptide purity was >95% as determined by Anaspec Inc. by MS and RP-HPLC. AlexaFluor488 (AF488)- and AlexaFluor647 (AF647)-labeled Transferrin (Trf) and AF647-labeled dextran 10 kDa were from Molecular Probes and purchased via ThermoFisher Scientific. Synthetic Aβ(1-42) peptide powders were dissolved in hexafluoro-2-propanol to disrupt any aggregates and monomerize the peptide. The solutions were vortexed briefly and aliquoted at 4 °C until further use. We have demonstrated that this protocol results in highly monomeric samples. For concentration determinations, the peptide films were snap frozen in liquid nitrogen and kept at ~80 °C until use. We have demonstrated that this protocol results in highly monomeric samples. For concentration determinations, the peptide film was dissolved in 1% ammonium hydroxide (v/v), and the absorption of the dye label was measured on a Cary 4000 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, CA). Extinction coefficients of 70 000 M⁻¹ cm⁻¹ at 504 nm and 250 000 M⁻¹ cm⁻¹ at 649 nm was used for the HF488 and HF647 dye labels, respectively, according to the information provided by the manufacturer. Prior to each experiment, one peptide film was dissolved in a small volume 1% ammonium hydroxide (v/v) and diluted with cell culture medium supplemented with 2% B-27 and 30 mM HEPES. The concentration of ammonium hydroxide was kept below 0.01% and was matched in controls to ensure identical treatment of all samples. Unused samples were discarded in order to avoid Aβ(1-42) aggregation induced by freeze–thawing.

**Figure 7.** Cellular uptake of Aβ(1-42) is reduced by RhoA overexpression. (A) SH-SY5Y cells transfected with EGFP-tagged RhoA (DA Q63L, WT, or DN T19N) and incubated with 1 μM HF647-labeled Aβ(1-42) for 1 h imaged by confocal microscopy. The scale bar is 20 μm. The asterisks (*) mark an example of a cell that is not expressing RhoA. (B) Quantification of Aβ(1-42) and Trf uptake in cells transfected with RhoA. The cells were incubated with either 1 μM HF647-labeled Aβ(1-42) or 5 μg/mL AF647-labeled Trf for 1 h or 5 min, respectively, washed and analyzed for intracellular peptide signal by flow cytometry. Uptake is reported as relative mean cellular fluorescence and based on the level of RhoA expression (none, low, medium, and high) in relation to uptake in nontransfected cells (N = 4, n = 4). T19N-transfected cells displayed few cells in the high transfected-gate, and this data was thus not included. The gates applied are displayed in Supplementary Figure S12. Asterisk (*) marks uptake levels that are significantly different from uptake in nontransfected cells (adjusted p-value <0.05) by one-way ANOVA with matched data followed by multiple comparisons with Bonferroni post hoc test (adjusted p-values were Q63L, Aβ(1-42) none vs medium 0.0013, none vs high <0.0001, Trf none vs medium <0.0001, none vs high <0.0001; WT, Aβ(1-42) none vs high 0.0224, Trf none vs medium 0.0059, none vs high <0.0001; T19N, Aβ(1-42) none vs medium 0.0098, Trf none vs medium 0.0003).
Cell Culture and Sample Preparation. Cell Maintenance and Seeding. SH-SY5Y cells were grown in a 1:1 mixture of minimal essential medium (MEM) and nutrient mixture F-12 Ham supplemented with 10% heat-inactivated fetal bovine serum, 1% MEM nonessential amino acids, and 2 mM l-glutamine. The cells were detached (trypsin-EDTA 0.25%, 3 min) and passaged twice a week. Cells were plated 1 day prior to experiments in either flat-bottomed 96 well plates (Nunc or VWR; 50 000 cells/well) for flow cytometry or in glass-bottomed culture dishes (MatTek; 25 000 cells (untreated cells) or 100 000 (transfected cells)/14 mm dish) for microscopy. Fp-In T-Rex HeLa cells with inducible expression of GFP-GRAF1 were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2% l-glutamine, and with the addition of 100 μg/mL hygromycin B and 5 μg/mL blasticidin S HCl. The cells were detached (trypsin-EDTA 0.25%, 3 min) and passaged twice a week. Transfected cells were plated 1 day prior to experiments in glass-bottomed culture dishes (MatTek; 50 000 cells/14 mm dish) for microscopy.

Alterations in Membrane Tension. SH-SY5Y cells were incubated with 0.1 M glycine-HCl buffer pH 2.5 with 150 mM NaCl; Trf) and harvested for analysis by confocal microscopy and flow cytometry. For uptake at isotonic conditions, the cells were washed 1X with serum-free medium, incubated with either 2 μM AF647-labeled dextran 10 kDa in serum-free medium with the addition of 2% B-27 for 10 min, washed 1X with serum-free medium for microscopy, or 3X/2 min on ice with either ice-cold serum-free medium (Af-1-42 and dextran) or acidic buffer (0.1 M glycine-HCl buffer pH 2.5 with 150 mM NaCl; Trf) and harvested for analysis by flow cytometry. For uptake at hypotonic conditions, the cells were first pretreated for 10 min in isotonic medium where after they were incubated with the peptides and dextran for 10 min as outlined above but diluted in hypotonic medium (25–75% MQ water added to the isotonic medium). Lastly, cells analyzed for uptake at recovery conditions were first pretreated with isotonic medium for 10 min followed by treatment in hypotonic medium for 10 min, whereupon they were exposed to the peptides and dextran in isotonic medium for 10 min.

Transfection of Rho GTPases and Induction of GRAF1 Expression. SH-SY5Y cells were passaged 2 days prior to transfection, grown to ∼70% confluency, and transfected with plasmids encoding for Rho GTPases (EGFP-tagged DA, WT, and DN variants of Cdc42, Rac1, and RhoA; see the above section Reagents) by electroporation using a Neon Transfection System (Invitrogen, Carlsbad, CA), following the protocol provided by the manufacturer and applying a single pulse of 1 100 V with a pulse width of 50 ms. The cells were transfected using 1 μg of plasmid DNA/100 000 cells in a 10 μL Neon Tip or 10 μg of plasmid DNA/106 cells in a 100 μL Neon Tip and plated immediately after. Based on the initial experiments, the analysis was set to ~24 h, ~27 h, and ~30 h post-transfection for Cdc42, Rac1, and RhoA, respectively, by evaluating the time it took until the cells expressed the proteins and adapted the expected morphologies. The Fp-In T-Rex HeLa cells with inducible expression of GFP-GRAF1 were seeded in 6 well plates (250 000 cells/well) 24 h prior to induction. GFP-GRAF1 expression was induced by addition of 1 ng/mL doxycycline hyclate, and 3 h postinduction the cells were transfected with mCherry-tagged Cdc42 WT or DA Q61L by Lipofectamine 2000 (0.5 μg of DNA and 1 μL of Lipofectamine/well) following the protocol provided by the manufacturer. At 5 h post-transfection, the cells were harvested and reseeded in glass-bottomed dishes followed by Af expression and imaging 24 h post-reseeding.

Confocal Microscopy. Confocal images were acquired on a Nikon C2+ confocal microscope equipped with a C2-DUVB GaAsP Detector Unit and using an oil-immersion 60x 1.4 Nikon APO objective (Nikon Instruments, Amsterdam, The Netherlands). The sample was excited and detected with appropriate excitation laser lines and emission filters, sequentially for samples including fluorophores excited at 488 nm, 561 nm, and 640 nm, or simultaneously using a filter cube to split the channels when imaging only fluorophores excited at 488 and 640 nm.

Flow Cytometry. Flow cytometry was used for quantification of cellular uptake. Prior to analysis, the cells were washed 3X/2 min in ice-cold serum free medium (Af-(1-42) and dextran 10 kDa) or acidic buffer (0.1 M glycine-HCl buffer pH 2.5 with 150 mM NaCl; Trf) and detached by trypsin-EDTA 0.25% for 7 min followed by addition of ice-cold FBS-supplemented cell culture medium to inhibit further proteolytic degradation of the cells. All samples were kept on ice until they were analyzed on a Guava EasyCyte BHT (Millipore, Darmstadt, Germany) that automatically retrieves samples from a 96-well plate. In order to exclude effects due to difference in delay time, we used mixed order of analysis, loading only a few samples at a time with the remaining samples kept on ice. Only the central cell cluster on the forward/side scatter (FSC/SSC) dot plot was analyzed, and for each sample 5 000 cells from within the gate were counted. The EGF, HF-488, and AF-488 fluorophores were excited by a 488 nm laser, and fluorescence was detected through a 525/30 nm filter. HF647 and AF647 was excited with a 635 nm laser and detected through a 661/19 nm filter. The mean cellular uptake or level of Rho GTPase expression was estimated as the average fluorescence intensity of all cells within the gate. The mean cellular uptake was baseline corrected by subtracting the signal recorded for untreated cells. Each cell treatment was performed in three or four technical replicates (n = 3–4) and repeated on at least two separate occasions (N ≥ 2). All flow cytometry data was analyzed in InCyte software (Millipore, Darmstadt, Germany) and displayed using Origin software (Origin-Lab, Northampton, MA).

Statistics. Statistical analysis was performed by matched sample ANOVA using GraphPad Prism software (GraphPad, San Diego, CA) on data that had been normalized so that the uptake in nontransfected cells was 100% for the individual experiments. Matched sample ANOVA was followed by multiple comparisons with the Bonferroni posthoc test to test for differences in mean peptide uptake between nontransfected cells and uptake at the different transfection efficiencies. This means that the reported individual p-values have been adjusted for the number of comparisons that were relevant to the experiment.

Associated Content

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscchemneuro.0c00053.

Supplementary Movie 1, cellular exposure to Af-(1-42) and dextran 10 kDa during isotonic conditions (AVI)

Supplementary Movie 2, cellular exposure to Af-(1-42) and dextran 10 kDa during hypotonic conditions (AVI)

Supplementary Movie 3, cellular exposure to Af-(1-42) and dextran 10 kDa during recovery conditions (AVI)

Supplementary Movie 4, tubulation and fission of dextran 10 kDa-filled VLDs (AVI)

Supplementary Movie 5, cellLight Actin-GFP staining of cells exposed to changes in membrane tension (AVI)

Supplementary Movie 6, Af-(1-42) and GRAF1 in cells expressing Cdc42 Q61L (AVI)

Associated confocal microscopy movie descriptions and supporting confocal microscopy and flow cytometry data (PDF)

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