Alpha-synuclein oligomerization increases its effect on promoting NMDA receptor internalization

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Abstract: The internalization of NMDA receptors (NMDARs) is promoted by monomeric α-synuclein (α-syn). Because of the pathogenic role of oligomeric α-syn, the effect of aggregated α-syn on this regulation deserves investigation. Several α-syn oligomers were prepared by incubating recombinant human α-syn in phosphate-buffered saline (PBS), plasma of normal controls (NC) and patients with Parkinson’s disease (PD). The α-syn oligomers formed in PBS are not phosphorylated and are different from the α-syn oligomers formed in the plasma of NC and PD that are moderately and highly phosphorylated at serine 129, which is a key phosphorylation site of the α-syn molecule in PD patients. After being added into the culture medium, the α-syn monomers and its oligomers formed in different methods and rapidly entered into MES23.5 dopaminergic cells and induced an increase in the expression of Rab5B, an endocytic protein that has been shown to regulate clathrin-mediated endocytosis of NMDARs. The levels of surface GluN1, a subunit obligatory for the assembly of functional NMDARs, were reduced, but the total GluN1 changes didn’t show a parallel reduction of the surface of GluN1, indicating the internalization of GluN1. Compared with the monomers, the oligomers, especially those formed in PD plasma, were more potent in promoting GluN1 internalization, and were abolished by clathrin inhibitor pitstop2. The above results suggest that α-syn oligomers, especially those formed in PD plasma, increase the effect of α-syn in promoting the internalization of NMDAR GluN1 subunits, possibly through a clathrin-mediated endocytic mechanism.

Keywords: α-synuclein, NMDA receptor, endocytosis, parkinson’s disease, plasma

Introduction

The N-methyl-D-aspartate receptor (NMDAR) is a subtype of the ionotropic glutamate receptor that plays critical roles in regulating synaptic plasticity and cognitive and motor functions [1-3]. The NMDAR complex constitutes heterotetrameric transmembrane proteins, two GluN1 and two GluN2 or GluN3 subunits, and after it is activated, it allows the positively charged ions to flow through the cell membrane. A number of studies have demonstrated that the binding densities of the NMDAR subunits tend to decrease in the brains of patients with neurodegenerative disorders such as Parkinson’s disease (PD) and Alzheimer’s disease (AD) [4, 5], leading to the hypofunction of NMDARs. A progressive increase in the severity of NMDAR hypofunction within the brain induces a range of clinically relevant effects on brain function, involving local and distributed circuitry. This may in turn underlie the observed cognitive and behavioral disturbances in some of the neurodegenerative diseases such as PD and AD [1, 6, 7].

Alpha-synuclein (α-syn) is a 140-amino acid protein enriched in the presynaptic terminals of neurons [8] that plays a role in synaptic plasticity and neurotransmission [9]. Aberrant expression and aggregation of α-syn are thought to cause neurodegeneration of the brain [10-14]. Increased α-syn expression and aggregation are associated with cognitive and behavioral deficiencies [11, 15, 16]. The fact that NMDARs and α-syn are implicated in the cognitive and behavioral deficiencies in the neurodegenera-
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tive brain suggests that there may be a potential link between the two proteins.

The substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) are the two brain areas that are involved in the pathogenesis of PD [17, 18]. Dopaminergic (DA) neurons from these two areas of the brain have projections to the striatum, nucleus accumbens (ventral striatum), limbic systems, hippocampus and prefrontal cortex. These in turn regulate voluntary movement control and cognitive functions such as emotions, motivation, rewards, and addictive behaviors [19-23]. In PD patients, both SNpc and VTA displayed lesions by α-syn-containing Lewy pathology [24]. It has been previously reported that the intracellular accumulation of α-syn monomers, either due to intracellular translocation of extracellular α-syn protein or the intracellular overexpression of the α-syn gene, promote the internalization of GluN1 subunits on the cell surface through a clathrin-mediated endocytic mechanism by the participation of the endocytic protein Rab5B [25, 26]. Due to the pathogenic role of α-syn oligomers [27-30], the effect of oligomerized α-syn on the expression of surface GluN1 in DA neurons deserves further investigation.

We have previously shown that recombinant human α-syn incubated in plasma from PD patients can aggregate into oligomers that display increased cytotoxicity compared with those formed in the PBS and plasma of normal health controls (NC) [31]. In the present study, α-syn oligomers were prepared by incubating recombinant human α-syn in either PBS or plasma from PD patients and NC, and sublethal concentrations of the oligomers were used to treat cultured dopaminergic cells to compare their effects on the expressions of Rab5B and surface GluN1. The potential mechanism and pathological relevance for the regulation of these were discussed.

Materials and methods

Plasma samples

Plasma samples were obtained from 20 clinically diagnosed idiopathic PD patients and NC. The PD patients were enrolled at the Department of Neurology, Xuanwu Hospital of Capital Medical University, and diagnosed by a consultant neurologist based on the UK Parkinson’s Disease Society Brain Bank criteria for idiopathic PD [32]. The subjects in the NC group were recruited from the Physical Examination Center of the hospital and were matched in age and gender with those of the PD patients. All participants provided informed consent, and underwent an evaluation that consisted of medical history, physical and neurological examinations, laboratory tests, and neuropsychological assessments. The protocol was approved by the Ethics Committee of the Xuanwu Hospital. Blood was collected in EDTA-coated vacuum tubes, and the plasma was separated by centrifugation at 3,000×g for 20 min. The plasma samples were aliquoted and stored at -80°C until use.

Cell culture

The MES23.5 dopaminergic cells were obtained as a generous gift from Dr Wei-Dong Le. The cells were cultured and expanded as described previously [33] in a DMEM/F12 medium (Gibco, NY, USA) and supplemented with 5% fetal bovine serum (Gibco, NY, USA), 100 U/100 ml penicillin/streptomycin, and Sato’s ingredients. All flasks were pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA).

Preparation of recombinant human α-syn

Recombinant human α-syn was prepared by the transformation of the plasmid pET-15b-NACP into Escherichia coli BL21 cells and then purified by sequential ion exchange chromatography, hydrophobic chromatography, and reverse phase chromatography [34]. The α-syn proteins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their identity was confirmed by western blotting using an anti-α-syn antibody. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Preparation of α-syn oligomers

Individual PD or NC plasma was blended, and then removed the endogenous α-syn and potential hemoglobin by affinity purification using an overdose of antibodies against α-syn and hemoglobin. The blended PD or NC plasma was then diluted to 1/3 with PBS (pH 7.4). To prepare the α-syn oligomers, 100 μM of recombinant human α-syn was either dissolved in PBS or in diluted PD or NC plasma, and then incubated at 37°C for 48 h with continuous
shaking (650 rpm) on an Eppendorf Thermomixer Comfort (Eppendorf AG 22331, Hamburg, Germany).

To obtain purified α-syn oligomers, the α-syn molecules of various sizes were first isolated from the plasma according to the method that was described previously [35]. Briefly, the plasma containing α-syn molecules was allowed to pass through the CNBr-activated Sepharose 4B column (GE healthcare, Uppsala, Sweden) coupled with anti-α-syn antibody. Then, the α-syn molecules captured in the column were specifically eluted by a glycine buffer (0.1 M, pH 2.5) followed by immediate neutralization with a Tris-HCl buffer (1 M, pH 9.0). The α-syn oligomers in the eluates were separated by SDS-PAGE from the monomers and dimers and then were recovered using a Micro Protein Recovery Kit (Sangon, Biotech, Shanghai, China) [36]. The α-syn oligomers formed in the PBS were directly separated by SDS-PAGE and then recovered using the Micro Protein Recovery Kit. Protein concentrations were determined using the BCA Protein Assay Kit as described above.

Detection of oligomeric and phosphorylated α-syn

α-Syn oligomers were measured using an enzyme-linked immunosorbent assay (ELISA) as described initially by El-Agnaf and his colleagues [37]. Briefly, the non-biotinylated and biotinylated 3D5 anti-α-syn monoclonal antibodies were used for capturing and detection, respectively. After completion of the immunoreaction, the contents of each well of the ELISA plate were incubated with ExtrAvidin alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) followed by a reaction with enzyme substrate p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was read at 405 nm using a microplate reader (Multiskan MK3, Thermo Scientific, UT, USA).

To detect phosphorylated α-syn, an anti-pS129-α-syn polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to capture the antibody. The remaining steps were the same as those for the detection of α-syn oligomers.

Transmission electron microscopy

Purified oligomeric α-syn samples were placed on the copper grids coated with Formvar. The samples on the grids were washed thrice with distilled water and stained with 2% uranyl acetate. Excess staining was removed by blotting and air drying. The samples were then visualized under a JEM-2100 (Japan) transmission electron microscope [38].

Preparation of protein extracts

The cells were washed thrice with ice-cold PBS and lysed using a lysis buffer containing Tris-Cl (50 mM, pH 7.5), NaCl (150 mM), EGTA (5 mM), EDTA (5 mM), SDS (2% w/v), and a protease inhibitor cocktail. The lysates were centrifuged at 12,000×g for 30 min at 4°C, and the supernatants were used as whole cell lysates [39]. The cell surface proteins were isolated according to the method described before. In brief, the cells were washed with ice-cold (PBS), and the cell surface proteins were biotinylated with 0.5 mg/mL EZ-Link-sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL, USA) in PBS for 30 min at 4°C. Then, the biotinylation reaction was terminated by incubating the cells with 20 mmol/L glycine. After being washed with ice cold PBS, the cells were lysed using a RIPA buffer, followed by centrifugation at 5000×g for 5 min at 4°C. After that the supernatant was collected and incubated with avidin-conjugated agarose beads for 2 h at 4°C. The cell surface proteins captured by avidin-coupled beads were analyzed by western blotting [40].

Western blot analysis

Western blot analysis was performed as described before [25]. Samples (20 μg proteins/lane) were separated by 10% SDS-PAGE, transferred onto a polyvinyldene difluoride (Millipore, Bedford, MA, USA) membrane, and incubated at 4°C overnight with each primary antibody against the following proteins: GluN1 (1:1000; BD PharmingenTM, Franklin Lakes, NJ, USA), Rab5B (1:10000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-syn [1:5000 for 3D5 monoclonal antibody; 1:1000 for anti-pan-α-α-syn antibody (Abcam, Cambridge, UK); 1:1000 for anti-pS129-α-syn polyclonal antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA)]; β-tubulin (1:10000; Abcam, Cambridge, UK) and calnexin (1:10000; Abcam, Cambridge, UK), followed by 1 h reaction at room temperature using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5000; Vector Laboratories, Inc., CA, USA).
Figure 1. Oligomerization and phosphorylation of α-syn in different incubating conditions. 100 μM of recombinant human α-syn was incubated in PBS, NC, and PD plasma, respectively. After incubation, the levels of oligomeric (A) and phosphorylated (B) α-syn in the samples were measured by ELISA. The α-syn proteins in the incubating solutions were purified using immunoaffinity chromatography, which were then examined by Coomassie brilliant blue (CBB) staining (C) and western blot analysis using 3D5 anti-α-syn antibody (D) and anti-pS129 α-syn antibody (E). Lanes 1-4 show the samples before affinity purification (1), the non-specific eluates (2, 3), and the specific α-syn eluates (4). (F-H) The α-syn oligomers formed in PBS (lane 1) and purified from NC (lane 2) and PD (lane 3) plasma.
The immunoreactivity was visualized by an enhanced chemiluminescence reagent (Promega, Madison, WI, USA).

**Immunofluorescence staining**

Cells were cultured for 24 h before treatment with α-syn for 60 min. The cells were then fixed at 4°C for 30 min with 4% (w/v) paraformaldehyde to reduce cellular autofluorescence [41], and then blocked for 30 min with 3% (w/v) bovine serum albumin. To observe the intracellular translocation of α-syn and total GluN1 expression, the cells were initially permeibilized with 0.3% (v/v) Triton X-100 for 30 min at room temperature, incubated overnight at 4°C with 3D5 anti-α-syn antibody (1:1000) or mouse monoclonal anti-GluN1 antibody (1:1000), followed by 2 h reaction at room temperature with Alexa Fluor 594 goat anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA, USA). For detection of the GluN1 (1:1000) cell surface, the cells were directly incubated overnight at 4°C with anti-GluN1 antibody without permeabilization with Triton X-100, followed by 2 h reaction with Alexa Fluor 594 goat anti-mouse IgG. The cells were then counterstained with DAPI before being observed under a confocal laser microscope (Leica TCS-SP8, Heidelberg, Germany).

**Statistical analysis**

Data are expressed as the means ± standard deviation (SD). Statistical analyses were performed using SPSS 22.0. A one-way ANOVA followed by Tukey's multiple comparison test were performed to evaluate the differences between the groups. P < 0.05 was considered to be statistically significant.

**Results**

**Purification and characterization of α-syn oligomers**

After incubation, the α-syn proteins in the PBS and NC/PD plasma were analyzed with ELISA that was specific for oligomeric and phosphorylated α-syn. The high and moderate levels of the α-syn oligomers were quantified in the plasma of both PD and NC, respectively, which were highly and moderately phosphorylated. No phosphorylated α-syn was detected in PBS, but the levels of the α-syn oligomers were lower than those in the plasma of PD and NC (Figure 1A, 1B).

To obtain pure α-syn oligomers, the α-syn proteins in the NC and PD plasma were first isolated from the plasma using immunoaffinity chromatography and then analyzed by Coomassie brilliant blue (CBB) staining (Figure 1C) and western blot using antibodies against non-phosphorylated (Figure 1D) and phosphorylated α-syn (Figure 1E). In the specific eluates, the monomers, dimers, trimers, and large-sized polymers of α-syn were detected by the anti-non-phosphorylated α-syn antibody (lane 4, Figure 1D). However, only the trimers and large-sized polymers of α-syn were revealed by the anti-pS129 α-syn antibody (lane 4, Figure 1E). This indicated that the phosphorylated α-syn was more prone to aggregate into oligomers. The α-syn oligomers in the specific eluates and in PBS were separated by SDS-PAGE and recovered using a Micro Protein Recovery Kit to purify the oligomers. After purification, only the oligomers were detected in the recovered solutions (Figure 1G), which was in contrast to the non-purified samples (Figure 1F). In addition, the α-syn oligomers purified from the PD and NC plasma were differentially phosphorylated, but were absent from those oligomers purified from PBS (Figure 1H).

The α-syn aggregates were further examined under a transmission electron microscope (TEM). Under TEM, the α-syn aggregates appeared granular in shape and touched each other. The α-syn granules formed in the NC/PD plasma appeared bigger than those formed in PBS. In addition, the assemblies in the PD plasma were bigger than those in the NC plasma gathered from (Figure 1I).
Intracellular translocation and accumulation of extracellularly added α-syn oligomers

According to Ahn et al., the extracellular α-syn can rapidly enter into living cells in a non-endocytic manner [42] without being degraded by the cellular proteolytic systems [43]. The ability of monomeric α-syn to penetrate the cell membrane has been confirmed by several studies [25, 26, 44-47]. To establish a cell model with an intracellular accumulation of α-syn oligomers, the α-syn oligomers that were initially formed in the PBS were added to the culture medium of MES23.5 cells and then underwent
Intracellular translocation of α-syn oligomers formed in different conditions. The α-syn monomers (0.4 μM) and oligomers (0.4 μM) formed in PBS, NC and PD plasma were added to the culture medium of MES23.5 dopaminergic cells. After 1 h incubation, the levels of α-syn monomers and oligomers in the whole cell lysates were measured and quantified by western blotting using 3D5 anti-human α-syn antibody (A). In cells treated with α-syn monomers, only monomeric α-syn was detected. In cells treated with different α-syn oligomers, only oligomeric α-syn was identified. The cells were also labeled immunofluorescently for α-syn (red) and counterstained for nuclei (blue) using DAPI (B). In the untreated cells, no α-syn-positive signals were detected by the 3D5 anti-human α-syn antibody. However, in the α-syn-treated cells, positive α-syn signals were detected by the anti-human α-syn antibody, presenting in an either diffused (in the monomers-treated cells) or in granular (in the oligomers-treated cells) appearance. Con: PBS control; PBS-mAS: α-syn monomers in PBS; PBS-oAS: α-syn oligomers formed in PBS; NC-oAS: α-syn oligomers formed in NC plasma; PD-oAS: α-syn oligomers formed in PD plasma. Bar = 10 μm.

examination of their intracellular translocation after 1 h of incubation. Western blot analysis using an anti-pan-α-syn showed that only low levels of endogenous α-syn were detected in the untreated control cells, which was not recognized by 3D5 anti-human α-syn (Figure 2A). In contrast, in the cells treated with α-syn monomers or oligomers, the 3D5 anti-human α-syn revealed a single band at 18 kD or 54 kD that was similar to the molecular size of α-syn monomers or trimers, respectively (Figure 2B). In addition, augmenting concentrations of extracellular α-syn oligomers led to the increase of α-syn trimers in the cells (Figure 2C). In agreement with the western blot results, immunofluorescence staining using 3D5 anti-human α-syn revealed granular α-syn-positive structures in the oligomer-treated cells, which were in contrast to the diffused staining of α-syn in the monomer-treated cells. In the untreated
cells, the endogenous α-syn could be detected using the anti-pan-α-syn, but not by 3D5 anti-human α-syn (Figure 2D). Therefore, in the following experiments, we used the 3D5 anti-human antibody to detect the exogenous recombinant human α-syn. The above results indicated that α-syn oligomers could enter the MES23.5 cells as its monomers without any obvious degradation.

We next compared the intracellular translocations of α-syn oligomers formed in different conditions. For this purpose, the α-syn oligomers formed in PBS and NC/PD plasma were added to the culture medium. After 1 h of incubation, the cells were lysed for western blot analysis or fixed for immunofluorescence staining. The western blot results showed that the oligomeric α-syn was detected in the cells treated with different α-syn oligomers (Figure 3A), indicating that the α-syn oligomers entered into the cells. Compared with cells treated with α-syn oligomers formed in PBS, wherein only α-syn trimers at 54 kD were detected, the cells treated with α-syn oligomers formed in NC/PD plasma showed two bands, where one at 54 kD corresponded to the α-syn trimers, and another at 108 kD was identical to the α-syn hexamers (Figure 3A). Again, there was only a single band at 18 kD to be detected in the cells treated with α-syn monomers, which was absent in the untreated cells if 3D5 anti-human α-syn was used (Figure 3A). As illustrated by immunofluorescence staining, the cells treated with different α-syn oligomers presented granular α-syn-positive structures in the cells. The cells treated with α-syn monomers exhibited the diffused staining of α-syn (Figure 3B).

**Effects of different α-syn oligomers on surface GluN1 internalization**

As shown in the above results, the extracellular α-syn oligomers could enter into the MES23.5 dopaminergic cells, leading to the accumulation of α-syn oligomers in the cells. After that, α-syn monomers and different α-syn oligomers were added to the culture medium of the MES23.5 cells, we observed their effects on GluN1 expression. After 1 h of incubation, the cells were either lysed for western blot analysis or fixed for immunofluorescent staining. The most significant reduction in the levels of surface GluN1 was observed in cells treated with α-syn oligomers formed in PD plasma. Also, cells treated with α-syn oligomers formed in NC plasma showed a moderate reduction of surface GluN1 expression (Figure 4A-C). The α-syn oligomers formed in PBS and α-syn monomers induced only a mild reduction of surface GluN1 expression. In all α-syn-treated cells, the levels of total GluN1 were stable, indicating an internalization of GluN1 from the cell surface.

The α-syn-induced internalization of surface GluN1 could be observed by immunofluorescence staining. In cells not permeabilized with Triton X-100, the anti-GluN1 antibody could only bind to the surface GluN1. The results of immunofluorescent staining revealed that the most intensive GluN1 signals were found on the surfaces of untreated cells, diminishing successively in cells treated with α-syn monomers, α-syn oligomers formed in PBS, and α-syn oligomers formed in NC and PD plasma (Figure 4B). In contrast, in cells permeabilized with Triton X-100, such as the anti-GluN1 antibody, could also bind to the intracellular GluN1, and both surface and cytoplasmic GluN1 could be stained and demonstrated similar GluN1 signal intensities among various α-syn-treated cells (Figure 4C).

**Effects of α-syn oligomers on Rab5B expression**

Rab5B is a small GTPase that is involved in clathrin-mediated endocytosis [48] that has been shown to participate in α-syn-induced NMDAR internalization [25, 26]. The differential effects of various α-syn oligomers on surface GluN1 expression suggested that they may affect the expression of Rab5B differently. Indeed, when α-syn monomers and different oligomers were used to treat the cells, they induced different levels of increase in Rab5B expression. The strongest effect was observed for the oligomers formed in PD plasma, followed by the oligomers formed in the NC plasma and PBS (Figure 5).

**Inhibition of α-syn-induced GluN1 internalization by pitstop2**

Rab5B has been reported to participate in the regulation of the abundance of cell surface NMDA receptors through clathrin-mediated endocytosis [49-51]. If α-syn-induced GluN1 internalization was also mediated by clathrin-mediated endocytosis, then the clathrin inhibitor could block this process. To test this possibility, some of the cells were exposed to...
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A

\[ \begin{array}{c|ccccc}
\alpha\text{-syn concentration} & 0 & 0.4 & 0.4 & 0.4 & 0.4
\hline
\text{Surface GluN1} & & & & & \\
\text{Total GluN1} & & & & &
\end{array} \]

102 kDa

Surface GluN1/Total GluN1 (%)

B

\[ \begin{array}{cccccc}
\text{\textit{α}-syn concentration (0.4 μM)} & \text{Con} & \text{PBS-mAS} & \text{PBS-oAS} & \text{NC-oAS} & \text{PD-oAS}
\hline
\text{GluN1} & & & & & \\
\text{DAPI} & & & & & \\
\text{Merge} & & & & &
\end{array} \]

C

\[ \begin{array}{cccccc}
\text{\textit{α}-syn concentration (0.4 μM)} & \text{Con} & \text{PBS-mAS} & \text{PBS-oAS} & \text{NC-oAS} & \text{PD-oAS}
\hline
\text{GluN1} & & & & & \\
\text{DAPI} & & & & & \\
\text{Merge} & & & & &
\end{array} \]
pitstop2, a selective inhibitor of clathrin-mediated endocytosis [52], before incubation with α-syn monomers or various oligomers. In the absence of pitstop2, α-syn monomers and various oligomers induced different levels of reductions in the expression of surface GluN1. In the presence of pitstop2, the reductions of surface GluN1 expression were abolished (Figure 6). This suggested the possibility that GluN1 internalization, induced by either α-syn monomers or different oligomers, involved a clathrin-mediated endocytic mechanism.

Discussion

In the present study, we prepared different α-syn oligomers by incubating recombinant α-syn in PBS, NC, or PD plasma. We found that the α-syn oligomers formed in the NC and PD plasma were phosphorylated at serine 129. This phosphorylation was much greater in the PD plasma compared to the NC plasma. The discrepancy for the α-syn aggregates formed in different conditions was also reflected in the images of TEM. The granular α-syn aggregates formed in NC/PD plasma were bigger than those in PBS. In addition, they touched each other to form irregular, large assemblies, which were bigger in PD plasma than in NC plasma. The above results indicated that the α-syn oligomers formed under different conditions have different conformations. This discrepancy may be due to their differential phosphorylation and other potential factors present in the plasma.

As mentioned before, extracellular α-syn rapidly enters into the living cells in a non-endocytic manner without being degraded by cellular proteolytic systems [42, 43]. We have previously demonstrated that the extracellular addition of monomeric α-syn can enter into MES23.5 dopaminergic cells and lead to the intracellular accumulation of α-syn monomers [25, 44], inducing surface GluN1 internalization [25]. The effect of extracellular α-syn on surface GluN1 was similar to that of intracellular overexpression of α-syn [25], indicating that the extracellular addition of α-syn exerted this effect after entering into the cells. To investigate the effects of α-syn oligomers on surface GluN1,
we added various purified α-syn oligomers to the culture medium of MES23.5 dopaminergic cells and then observed their intracellular translocation. As demonstrated by western blotting and immunofluorescent staining, all kinds of α-syn oligomers as well as α-syn monomers entered into the cells. Simultaneously, different levels of the reduction of surface GluN1 were detected. As the levels of total GluN1 were unchanged and the surface GluN1 was reduced, this indicated an internalization of GluN1 on the cell surface. This effect was more prominent for the oligomers than the monomers. In addition, the oligomers formed in PD plasma were more potent than those formed in NC plasma/PBS in promoting GluN1 internalization.

The underlying mechanism regarding the effect of α-syn oligomers on GluN1 internalization remains to be clarified, and the available data strongly suggest that Rab5B may play a role in this regulation. Rab5B is a small ATPase molecule that functions as a regulator in the early endocytic pathway [53]. It has been suggested that Rab5B participates in the regulation of cell surface NMDA receptors through clathrin-mediated endocytosis [49-51]. Our previous studies showed that the GluN1 internalization induced by the intracellular accumulation of α-syn monomers was accompanied by the upregulation of Rab5B expression, which was abolished by silencing Rab5B expression [25]. In the present study, we found that the intracellular accumulation of α-syn oligomers was also accompanied by an increase in the expression of Rab5B. Besides, the levels of Rab5B were closely related to the surface expressions of GluN1 in the cells treated with different α-syn oligomers. For example, the most prominent increase in the levels of Rab5B was observed in the cells treated with α-syn oligomers formed in PD plasma, and was associated with the most significant reduction of surface GluN1 expression in the same cells. Accordingly, mild and moderate upregulations of Rab5B in the cells treated with α-syn oligomers formed in NC plasma and PBS were associated with mild and moderate downregulations of surface GluN1 in the corresponding cells. Taken together, the above results suggest that the differential regulations of surface GluN1 expression by different α-syn oligomers depend on their different regulations of Rab5B. Since Rab5B participates in the regulation of cell surface NMDA receptors through clathrin-mediated endocytosis [49-51], we speculated that the α-syn oligomers-induced GluN1 internalization, which was accompanied by increased Rab5B expression, might be also mediated by clathrin. To demonstrate the role of clathrin in this regulation, we applied clathrin specific inhibitor pistop2 before the addition of α-syn oligomers to the culture medium. We

Figure 6. Effect of the clathrin inhibitor on α-syn-induced surface GluN1 reduction. A portion of the cells were pre-incubated with pitstop2 (15 μM) for 10 min before treatment with α-syn monomers and purified oligomers formed in various incubating conditions. One hour later, the levels of surface and total GluN1 were measured by western blotting. Data are expressed as the means ± SD; n = 4. *P < 0.05 vs. control, †P < 0.05 vs. α-syn monomers, ‡P < 0.05 vs. α-syn oligomers formed in PBS, §P < 0.05 vs. α-syn oligomers formed in NC plasma. Con: PBS control; P2: Pitstop2; PBS-mAS: α-syn monomers in PBS; PBS-oAS: α-syn oligomers formed in PBS; NC-oAS: α-syn oligomers formed in NC plasma; PD-oAS: α-syn oligomers formed in PD plasma.
observed a complete inhibition of GluN1 internalization. Our previous study suggested that α-syn monomers-induced GluN1 internalization was mediated by clathrin-mediated endocytosis [25, 26]. This result indicates that the clathrin-mediated endocytosis may also participate in the α-syn oligomers-induced GluN1 internalization.

The present study provided evidence for the first time that α-syn oligomers can promote NMDAR GluN1 subunit internalization. In particular, the present study demonstrated that the α-syn oligomers formed in PD plasma, are highly phosphorylated, and exhibit a more potent effect on GluN1 internalization. Because GluN1 is an obligatory subunit that is essential for the assembly of functional NMDAR [54], its reduction on the cell surface indicated the impairment of NMDAR function. Most studies support that α-syn oligomerization is toxic to neurons [10, 27, 55, 56]. The present study suggests that one of the potential mechanisms for the neurotoxicity of α-syn oligomer is its detrimental effect of NMDARs.

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Disclosure of conflict of interest

None.

Abbreviations

PD, Parkinson’s disease; AD, Alzheimer’s disease; NMDAR, N-methyl-D-aspartate receptor; α-syn, Alpha-synuclein; PBS, Phosphate-buffered saline; SDS, Sodium dodecyl sulfate; ELISA, Enzyme-linked immunosorbent assay; EDTA, Ethylene diamine tetraacetic Acid; EGTA, Ethylene glycol tetraacetic acid; KF, potassium fluoride; DTT, dithiothreitol.

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