A-synuclein Induces Microglial Cell Migration through Stimulating HIF-1α Accumulation

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Microglial cell migration and infiltration plays a critical role in spinal cord injury after thoracoabdominal aortic surgery. In our previous study, α-synuclein, a presynaptic protein was shown to be released from injured neurons and cause microglial cell activation. Here, we aimed to explore the effect of α-synuclein on microglial cell migration. Primary microglial cells were isolated from Sprague–Dawley rats and then exposed different doses (0.2, 0.4, and 0.6 μM) of α-synuclein oligomers. The mRNA and protein levels of HIF-1α were then analyzed by qRT-PCR and Western blot. Cell migration was examined by a 96-well Boyden chamber. Moreover, toll-like receptor (TLR) 2-expression as well as TLR7/8-expression was inhibited by specific siRNA transfection. HIF-1α was overexpressed by Ad-HIF-1α transfection. In the results, α-synuclein was found to stimulate HIF-1α accumulation in microglial cells in a dose-dependent manner. Silencing HIF-1α expression dampened α-synuclein induced microglial cell migration. Furthermore, blockade of TLR7/8 expression but not TLR2 expression reduced HIF-1α accumulation in microglial cells. In addition, overexpressed HIF-1α, along with Src, prompted caveolin-1 expression and phosphorylation, as well as migration in microglial cells. A-synuclein acts via TLR7/8 and enhances HIF-1α expression, which might play a regulatory role in microglial cell migration.

Key words: microglial cell; spinal cord injury; HIF-1α; toll-like receptor 7/8; caveolin-1

INTRODUCTION

Numerous articles have reported that thoracoabdominal aortic surgery might result in spinal cord injury (SCI), the incidence of which reaches an average of 4.5% in 7309 patients (Uchida, 2014). Besides direct damage caused by this surgery, insidious and deleterious secondary injury occurs immediately, which includes disruption to the vascular supply of the spinal cord and inflammatory changes with consequent ischemia, cellular necrosis and apoptosis (Darian-Smith, 2009). Innate immune response has been involved in the SCI process. Microglial cells, the innate immune cells of the central nervous system (CNS), are quickly activated and take on an amoeboid-like phenotype for migration, resembling the infiltrating monocytes/macrophages (David and Kroner, 2011; Ghoshal et al., 2007). Microglial cells were observed in the lesion and mount to a peak by 3-7 days throughout the gray and white matter in rat spinal cord after injury (Sroga et al., 2003). Microglial cells can use invadosomes in peripheral tissues to adhere to and degrade the extracellular matrix (ECM) for efficient migration (Vincent et al., 2012). However, the mechanism responsible for microglial cell migration is still not completely understood. Notably, microglial cell exerts both negative and positive impacts on neuron function. In one respect, microglial cells can influence neuronal function and

SIGNIFICANCE

Spinal cord injury (SCI) may occur after thoracoabdominal aortic surgery and seriously affect numerous numbers of people. Microglial cell activation and migration have been involved in SCI. α-synuclein is a neuron-specific protein previously demonstrated to be released from injured neurons and cause microglial cells activation. The present study revealed the effect of α-synuclein on microglial cell migration depending on HIF-1α accumulation by stimulating TLR7/8. Accumulated HIF-1α, cooperating with c-Src, enhanced caveolin-1 expression and phosphorylation, thus leading to microglial cell migration.

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promote neurotoxicity through the release of several pro-inflammatory molecules, such as IL-1β, TNF-α, proteases, and reactive oxygen species (ROS) (Block et al., 2007; Skaper et al., 2012). In another respect, microglial cells can also be protective through glutamate uptake, cell debris clearance, and neurotrophic factors release, for instance insulin-like growth factor-1 (IGF-1), glial cell-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) (Lee et al., 2004; Nakajima et al., 2008; Parikhurst et al., 2013; Stoll and Jander 1999; Thored et al., 2009).

Alpha-synuclein was identified as a 140 amino acid protein and a neuron-specific protein distributed in the nucleus and presynaptic terminals (Cox et al., 2014); α-synuclein can be released from neurons (Qiao et al., 2015). The content of secreted extracellular α-synuclein could reach 2–12 nM from stable Neuroblastoma cells (SH-SY5Y cell lines) inducibly expressing wild-type α-synuclein (Emmanouilidou et al., 2010). When α-synuclein exists in its native state, it is an unstructured monomeric protein (Cox et al., 2014). Under conditions of physiological pH and temperature in vitro, α-synuclein is assembled into highly-ordered fibrils, the structure of which resembles that found in diseased brains (Cox et al., 2014). Moreover, α-synuclein was identified as a 140 amino acid protein and a neuron-specific protein distributed in the nucleus and presynaptic terminals (Cox et al., 2014); α-synuclein can be released from neurons (Qiao et al., 2015). The content of secreted extracellular α-synuclein could reach 2–12 nM from stable Neuroblastoma cells (SH-SY5Y cell lines) inducibly expressing wild-type α-synuclein (Emmanouilidou et al., 2010). When α-synuclein exists in its native state, it is an unstructured monomeric protein (Cox et al., 2014). Under conditions of physiological pH and temperature in vitro, α-synuclein is assembled into highly-ordered fibrils, the structure of which resembles that found in diseased brains (Cox et al., 2014). Generally, α-synuclein has been implicated in synaptic plasticity, neurotransmitter secretion, and synaptic vesicle pool maintenance (Burre et al., 2010; Mou et al., 2013; Scott and Roy 2012; Watson et al., 2009). This protein is also implicated in neuronal lesions. Intramuscular (IM) injection of fibrillar α-synuclein in mice can result in the rapid and synchronized development of hind limb motor weakness and robust lesion in CNS (Sacino et al., 2014).

Our previous study has demonstrated that ischemia/reperfusion-injured neurons could express and release increased levels of α-synuclein and cause microglial cells activation through TLR2 in vitro (Qiao et al., 2015). Other studies have reported that the extent of microglial activation in the SNpc correlates with α-synuclein accumulation (More et al., 2013). Moreover, microglial cells were demonstrated to co-localize with aggregated α-synuclein in the SNpc. Recently, Wang et al., 2015 reported that α-synuclein binds to membrane CD11b in microglial cells, leading to cell migration via H2O2 production by NADPH oxidase. However, it is not clear whether there are any other signals implicated in α-synuclein-induced microglial cell migration. In the current study, we sought to explore the effect of α-synuclein oligomers on rat primary microglial cell migration in vitro.

MATERIALS AND METHODS

Preparation and Culturing of Primary Rat Microglial Cell

Primary microglial cultures from 12 male Sprague–Dawley rats of 2 day old (Laboratory Animal Center, Xi’an Jiaotong University, Xi’an, China) were prepared as reported previously (Siddiqui et al., 2012). Microglia were harvested, centrifuged (1000 rpm for 10 min), and seeded onto 12-well plates at a density of 5 × 10^4 cells/well (purity ≥ 98%), being cultured by MEM supplemented with 2% fetal bovine serum. All experimental procedures involving animals were performed in accordance with the National Institutes of Health Guide for Care and Use of Animals and approved by the ethics committee of Xi’an Jiaotong University.

Recombinant α-synuclein (amino acid: 1-140) was provided by Prospec (catalog PRO-393). The protein was stored at -20°C after dissolving in sterile water at a concentration of 346 μM in aliquots. The aliquots were further diluted into 59 μM that was maintained at 4°C before the oligomerization. In the oligomerization process, α-synuclein was stirred vertically at 800 rpm at 37°C for 24 h in a Ther-mix (Integrated Technologies Ltd). For α-synuclein exposure, microglial cells were plated into 12-well plates at a density of 5 × 10^5 cells/well and then exposed to 0.2, 0.4, and 0.6 μM α-synuclein oligomers for 8 h before other evaluations. For MCP-1 and CCL3 treatment, microglial cells (5 × 10^5 cells/well) were challenged with 50 ng/mL MCP-1 or 50 ng/mL CCL3 for 8 h. In the blockade of CCR2 and CCR5 in microglia, 10 μM of CCR2 antagonist RS602868 and 10 μM of CCR5 antagonist maraviroc were added to the microglia culture and treated for 1 h. To activate TLR7/8, 0.1 μg/mL R848, a TLR7/8 agonist, was added to the microglia culture and incubated for 30 min. For the inhibition of c-Src, its specific inhibitor PP2 (10 μM) was added and incubated for 30 min. The concentration of PP2 was used according to previous reports (Callera et al., 2005; Yang et al., 2014a).

Microglial Cell Migration Assays

Microglial cell migration was assayed using a 96-well Boyden chamber. Each bottom well was filled with 200 μL of serum-free media with 0.2, 0.4, and 0.6 μM oligomeric α-synuclein. Each insert, with 5-μm pores in its filter membrane, was loaded with 1.0 × 10^6 microglia before the insert frame was placed back on the plate and incubated at 37°C overnight. The number of transmigrated cells was measured using a CytoQuant Kit (Life Technologies) according to the manufacturer’s protocol.

**SiRNA transfection.** HIF-1α siRNA, TLR2 siRNA, TLR7 siRNA or TLR8 siRNA, and scrambled siRNA (Santa Cruz Biotech) were transfected into microglial cells (1 × 10^5/well) with Lipofectamine 2000 (Invitrogen),
according to the manufacturer’s instructions. After the transfection of siRNA, Western Blot was employed to examine the transfection efficiency.

Adenovirus construction. Briefly, HIF-1α was amplified and sub-cloned into pAdTrack-CMV, an adenoviral shuttle plasmid, whereas GFP was used as a non-specific

Fig. 1. α-synuclein affects cell viability, HIF-1α expression and cell migration of microglial cells. Following the treatment of 0.2, 0.4, and 0.6 μM α-synuclein for 8 h, cell viability (A), HIF-1α mRNA (B) and protein (C) expression and migration (E) of microglia were evaluated respectively. $P < 0.05$ vs control, $P < 0.05$ vs 0.2 μM, $P < 0.05$ vs 0.6 μM. [Color figure can be viewed at wileyonlinelibrary.com]
control. Then, the recombinant shuttle plasmids pAdTrack-CMV and pAdEasy-1 were homologously recombined in the Escherichia coli strain BJ5183. The recombinant plasmids obtained were transfected into 293 cells to generate recombinant adenovirus. The virus was amplified and purified, and titers were determined by the p24 ELISA kit (Cell Biolabs, Inc.), before being stored at -80°C for subsequent use.

Measurement of cytokine expression. Culture supernatants of microglial cells were harvested at 8h following treatment with 0.6 μM α-synuclein. The secretion of TNF-α and IL-6 were determined using a commercially available ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Assessment of H2O2 production. The production of H2O2 in microglial cells was determined by a fluorometric assay with Amplex red reagent (Molecular Probes) according to manufacturer’s instructions. The absorbance at 560 nm was measured with a Microplate reader (Bio-Rad).

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted
using the TRizol RNA-extraction reagent (Bioteke), according to the manufacturer’s instructions. About 5 μg total RNA from each sample was reverse-transcribed into first strand cDNA for qRT-PCR analysis. The qRT-PCR was performed in a final volume of 10 μL, which contained 5 μL of TaqMan® Fast Advanced Master Mix (ThermoFisher), 1 μL of cDNA (1:50 dilution), and 2 μL each of the forward and reverse primers (1 mM). The following primers were used for amplification of HIF-1α (NC_000014.9), forwards: 5'-AGT GTA CCC TAA CTA GGC G-3', reverse: 5'-CAC AAA TCA GCA CCA AGC-3'. The steps in the qRT-PCR were as follows: 94°C for 2 min for initial denaturation; 94°C for 20 s, 58°C for 15 s, and 72°C for 15 s, with 2 s for plate reading for 40 cycles; and a melt curve from 65 to 95°C. B-actin was used as a quantitative and qualitative control to normalize the gene expression. The primers for β-actin amplification were, forwards: 5'-CCC ATC TAT GAG GGT TAC GC-3', reverse: 5'-TTT AAT CAC GAC CAC CTC-3'. Two-way ANOVA was employed in comparing α-synuclein expression (F = 8.215, n = 6, P < 0.001, one-way ANOVA; Fig. 1C, 1D). Therefore, these results indicate the induction of HIF-1α expression by α-synuclein oligomers. 

**Western blot analysis.** Microglial cells were homogenized and lysed with RIPA lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% TritonX-100, 1 mM EDTA, 10 mM b-glycerophosphate, 2 mM sodium vanadate, and a protease inhibitor) after respective treatment. Protein concentration was assayed using a micro-BCA protein kit (Pierce). Forty micrograms of protein per lane was separated by 12% SDS–PAGE and electroblotted onto PVDF membrane (Bio-Rad). Then, non-specific binding was blocked by incubating with 5% nonfat milk in TBST buffer at room temperature for 1 h. Primary antibodies including anti-HIF-1α (raised species: Halphex 67, antigen: amino acid 432–528 catalog no. sc-53546, 1:800 [Santa Cruz Biotechn]), anti-TLR2 (raised species: H-175, antigen: amino acid 180–354 catalog no. sc-10739, 1:1000, [Santa Cruz Biotechn]), anti-TLR7 (raised species: H-114, antigen: amino acid 27–140 catalog no.sc-30004, 1:900 [Santa Cruz Biotechn]), anti-β-actin were used as a quantitative control to normalize the gene expression. The primers for β-actin amplification were, forwards: 5'-CCC ATC TAT GAG GGT TAC GC-3', reverse: 5'-TTT AAT CAC GAC CAC CTC-3'. Data were analyzed using the formula: R = [Act sample-Act control].

**HIF-1α is Involved in A-synuclein Induced Microglia Migration.**

Previously, α-synuclein oligomers have been reported to be a chemorepellent and drive microglial migration via CD11b binding and H2O2 generation (Wang et al., 2015). To confirm the effect of α-synuclein on microglia migration, we used a 48-well chamber to examine cell migration. Compared with the addition of saline, α-synuclein oligomers significantly augmented microglia migration (F = 7.036, n = 6, P = 0.028, one-way ANOVA; Fig. 1E). MCP-1/CCR2 and CCL3/CCR5 have been demonstrated to drive microglia migration (Bianchi et al., 2011; Schuette-Nuetgen et al., 2012). To examine whether α-synuclein-induced microglia chemotaxis is mediated by MCP-1/CCR2 and CCL3/CCR5 signaling, CCR2 and CCR5 in microglia were blocked by OX40L antagonist RS504393 (10 μM, 1h) and CCR5 antagonist maraviroc (10 μM, 1h), respectively. CCR2 or CCR5 blockade significantly dampened MCP-1- or CCL3-induced microglia migration (antibody: F = 16.032, n = 8, P < 0.001, exposure: F = 19.225, n = 8, P < 0.001, two-way ANOVA; Fig. 2A). In contrast, blockade of CCR2 or CCR5 did not markedly influence α-synuclein-induced migration, which indicated that α-synuclein-induced migration was independent of MCP-1 and CCL3 expression (Fig. 2A). Notably, when HIF-1α expression was inhibited by HIF-1α siRNA transfection, the expression of CCR2 and CCR5 was not affected in microglia (for HIF-1α: F = 13.221, n = 6, P = 0.007, one-way ANOVA; for CCR2: F = 0.831, n = 6, P = 0.389, one-way ANOVA; for CCR5: F = 1.027, n = 6, P = 0.253, one-way ANOVA; Fig. 2B). Notably, microglia migration induced by α-synuclein was significantly impeded (F = 18.033, n = 8, P < 0.001, one-way ANOVA; Fig. 2C). These results feature a critical role of HIF-1α in α-synuclein-induced migration.
TLR7/8 but not TLR2 Mediates HIF-1α Expression Induced by A-Synuclein

Our previous study has found that α-synuclein oligomers bind to TLR2 and may activate microglia (Qiao et al., 2015). Hence, we next investigated whether HIF-1α accumulation induced by α-synuclein was mediated by TLR2. TLR2-specific siRNA was employed to knockdown TLR2 expression in microglial cells before α-synuclein exposure (0.6 μM). Compared with scramble siRNA transfection, TLR2 siRNA caused a significant reduction (decreased by 36%) of TLR2 expression (F = 10.09, n = 6, P = 0.011, one-way ANOVA; Fig. 3A, 3B). Consequently, no significant variation was found in HIF-1α protein expression after knockdown of TLR2 expression under α-synuclein exposure (F = 0.941, n = 6, P = 0.387, one-way ANOVA; Fig. 3A, 3B). However, inflammatory factors including TNF-α expression (F = 93.021, n = 8, P < 0.001, one-way ANOVA; Fig. 3C) and IL-6 expression (F = 89.986, n = 8, P < 0.001, one-way ANOVA; Fig. 3D) as well as H2O2 production (F = 22.322, n = 8, P < 0.001, one-way ANOVA; Fig. 3E) were downregulated by TLR2 knockdown. Toll-like receptor (TLR) 7 and 8 are crucial in immune response against single-stranded RNA viruses (Nicholas and Sumbayev, 2009). The activation of TLR7/8 leads to the accumulation of HIF-1α (Nicholas and Sumbayev, 2009). We speculated that TLR7/8 were involved in induction of HIF-1α by α-synuclein. Silencing TLR7 or TLR8 expression in microglial cells was performed using TLR7 siRNA or TLR8 siRNA, respectively (for TLR7: F = 19.227, n = 6, P = 0.002, one-way ANOVA; for TLR8: F = 18.089, n = 6, P = 0.002, one-way ANOVA; Fig. 3F, 3G, 3H, 3I). We found that knockdown of TLR7 or TLR8 expression led to a remarkable decrease in HIF-1α expression (F = 19.227, n = 6, P = 0.002, one-way ANOVA; Fig. 3F, 3G, 3H, 3I).
of HIF-1α accumulation (decreased by 43% and 49%, respectively) (TLR7 knockdown: F = 17.022, n = 6, P = 0.003, one-way ANOVA; TLR8 knockdown: F = 19.645, n = 6, P = 0.002, one-way ANOVA; Fig. 3F, 3G, 3H, 3I). In addition, R848, a specific TLR7/8 ligand was used as an agonist. Compared with the control (α-synuclein alone), R848 (0.1 μg/mL, 30 min) conditioning markedly raised HIF-1α accumulation in microglial cells (t = 11.023, n = 6, P < 0.001, paired-samples t-test; Fig. 3J, 3K). Overall, our results suggest that TLR7/8 that mediates HIF-1α accumulation induced by α-synuclein.

**HIF-1α Interacts with C-Src to Prompt Caveolin-1 Phosphorylation in Microglia**

Caveolin-1 is a major structural protein of caveolae, and can be phosphorylated by Src-family kinases, including c-Src, Lyn, and Hck (Vincent et al., 2012). In the lamellae of microglia, p-Tyr14Cav1 was enriched in the podonut and within individual podosomes it was in a ring-like pattern, closely associated with talin (Vincent et al., 2012). To further delineate the role of HIF-1α in microglia migration, HIF-1α was overexpressed in microglia by infection with recombinant adenovirus. As shown in the results, Ad-HIF-1α transfection boosted HIF-1α expression as well as caveolin-1 and p-Tyr14 caveolin-1 expression in microglia (for HIF-1α, transfection: F = 28.014, n = 6, P < 0.001, exposure: F = 24.091, n = 6, P < 0.001, two-way ANOVA; for caveolin-1, transfection: F = 21.031, n = 6, P < 0.001, exposure: F = 20.339, n = 6, P < 0.001, two-way ANOVA; for p-Tyr14 caveolin-1, transfection: F = 25.013, n = 6, P < 0.001, exposure: F = 23.983, n = 6, P < 0.001, two-way ANOVA; Fig. 4A, 4B). C-Src is a critical member of the tyrosine kinase family. In our results, this kinase was activated by α-synuclein exposure as well as by HIF-1α overexpression (for c-Src, transfection: F = 50.811, n = 6, P = 0.404, exposure: F = 0.663, n = 6, P = 0.522, two-way ANOVA; for p-cSrc, transfection: F = 23.672, n = 6, P < 0.001, exposure: F = 21.025, n = 6, P < 0.001, two-way ANOVA; Fig. 4A, 4B). Meanwhile, the inhibition of c-Src by its specific inhibitor PP2 (10 μM, 30 min) markedly reduced p-Tyr14 caveolin-1 expression levels but not caveolin-1 expression induced by HIF-1α overexpression.
(Fig. 4A, 4B). Furthermore, HIF-1α overexpression accelerated cell migration towards α-synuclein oligomers (transfection: $F = 33.895$, $n = 8$, $P < 0.001$, exposure: $F = 27.662$, $n = 8$, $P < 0.001$, two-way ANOVA; Fig. 4C, 4D).

**DISCUSSION**

After the occurrence of SCI, the innate immune response is rapidly triggered, with activated resident microglia, monocytes/macrophages as well as antigen-presenting cells, like dendritic cells (Popovich and Jones, 2003; Trivedi et al., 2006). In this process, the release of danger signals and pro-inflammatory mediators contributes largely to microglial cell activation and migration (Bowes and Yip, 2014). The present study investigated the effect of a presynaptic protein, α-synuclein, on microglial cell migration in vitro. Primary rat microglial cells were prepared and exposed to different doses of α-synuclein oligomers. Under physiological condition, plasma α-synuclein levels differed in several studies, varied from 3.6 ng/mL to 44.5 ng/mL (Koehler et al., 2015; Pchelina et al., 2014). However, as the activity of recombinant α-synuclein may be lower than that of α-synuclein in vivo. Our study, as well as other studies, used a high dose of α-synuclein (600 ng/mL) to treat microglial cells (Pacheco et al., 2015). This exposure led to an increased level of amoeboid microglial cells as well as elevated expression of a critical factor in response to hypoxia, HIF-1α, in these cells. HIF-1α has been reported to modulate microglial cell function. The increased release of pro-inflammatory cytokines was induced by hypoxia in microglia cultures through the HIF-1α-dependent pathway (Yang et al., 2014b). HIF-1α also mediated the regulatory role of adenosine in microglial cell function following injury (Merighi et al., 2015). Thus, we further delineated whether HIF-1α was involved in microglial cell migration.

Previously, Wang et al., 2015 characterized the role of α-synuclein, which acts as a chemoattractant in microglial cell migration. In line with previous studies, the present study found that α-synuclein oligomers might act as an attractant inducing microglial cell chemotaxis. Our results also examined and excluded the role of MCP-1/CCR2 and CCL3/CCR5 signaling in α-synuclein-induced migration. Notably, we found that HIF-1α was involved in α-synuclein-induced migration.

We have suggested that TLR2 might mediate microglial cell activation and migration induced by α-synuclein oligomers (Qiao et al., 2015). Nevertheless, we found that the knockdown of TLR2 expression did not alter HIF-1α accumulation in microglial cells, but resulted in a reduction of TNF-α and IL-6 release as well as H2O2 production. These results revealed that TLR2 might be dispensable for α-synuclein-induced HIF-1α expression but was necessary for the activation of microglial cells. Intriguingly, TLR7/8 were found to mediate HIF-1α expression by α-synuclein stimulation, as knockdown of their expression remarkably decreased HIF-1α expression, while TLR7/8 activation by agonists increased HIF-1α expression. TLR7 and TLR8 are phylogenetically and structurally related members of the TLR family. Activation of TLR7 was demonstrated to induce a neuroinflammatory response, leading to the production of multiple proinflammatory cytokines and chemokines, as well as the activation of astrocytes (Butchi et al., 2008). After treatment with α-synuclein, microglia can affect the immune response mediated by TLR2/1 and TLR7, increasing the secretion of the pro-inflammatory cytokines IL-6 and TNFα (Roodveldt et al., 2013).

In migrating cells (endothelial cells), a polarized distribution of caveolin-1 and caveolae is observed, which facilitates cell locomotion (Grande-Garcia and del Pozo, 2008). After SCI, higher expression of phosphorylated caveolin-1 (p-caveolin-1) was found in the macrophages/activated microglia (Shin, 2007). In this study, we overexpressed HIF-1α in microglial cells by recombinant virus. HIF-1α overexpression, along with c-Src, prompted caveolin-1 and p-Tyr14 Caveolin-1 expression in the transfected cells. In parallel, microglial cell migration was promoted by HIF-1α overexpression.

Collectively, the study reported here featured a central role of HIF-1α in α-synuclein-induced microglial cell migration after spinal cord injury and might provide a novel target for the prevention of spinal cord injury. Although directly dampening α-synuclein production and aggregation in vivo may be difficult at the present time, we could select other targets to reduce the effect of α-synuclein oligomers in spinal cord injury or other disorders with neuroinflammation.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare that there are no conflicts of interest.

**ROLE OF AUTHORS**

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HQ. Acquisition of data: HQ, XH, QZ, and NZ. Analysis and interpretation of data: NZ, LL, YH, WL, DW, and ZW. Drafting of the manuscript: HQ and XH. Statistical analysis: QZ and NZ. Obtained funding: HQ. Study supervision: HQ.

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