CNTNAP4 partial deficiency exacerbates α -synuclein pathology through astrocyte–microglia interplay

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Research Article

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Abstract

Background

Parkinson's disease (PD) is a common progressive neurodegenerative movement disorder, which is characterized by dopaminergic (DA) neuron death and the aggregation of neurotoxic α -synuclein. *CNTNAP4*, a risk gene of autism, has been implicated to participate in DA neuron degeneration in PD pathogenesis. However, the role of *CNTNAP4* in α -synuclein pathology remains unclear.

Methods

Heterozygous *CNTNAP4* null mice injected with adeno-associated viral vector (AAV)-mediated human α -synuclein (AAV-*h* α -Syn) were used to evaluate the impact of partial *CNTNAP4* loss on α -synuclein pathology. The resulting physiological/behavioral effects were assessed using behavioral tests, western blotting, immunohistochemistry, and transmission electron microscopy. The underlying mechanism was studied using RNA-sequencing. Cytokine expression and secretion were examined using quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Magnetic Luminex assay kit.

Results

Here we showed *CNTNAP4* partial deficiency exacerbates α -synuclein pathology, nigrostriatal DA neuron degeneration and motor impairment, induced by AAV-*h* α -Syn overexpression. Mechanistically, aggravated α -synuclein pathology stimulates complement C3 release by astrocytes, activating microglial C3a receptor, which in turn triggers microglia to secrete complement C1q and pro-inflammatory cytokines. The astrocyte–microglia crosstalk further drives DA neuron death and motor dysfunction in PD. Furthermore, we showed that *in vivo* depletion of microglia rescues the aggravated α -synuclein pathology resulting from partial loss of *CNTNAP4*.

Conclusions

Our results indicate that *CNTNAP4* plays a key role in α -synuclein pathogenesis by regulating glial crosstalk and may be a potential target for PD treatment.

Background

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by abnormal movements, such as tremors and bradykinesia. Dopaminergic (DA) neuron degeneration and the presence of proteinaceous inclusions termed Lewy bodies (LBs) are the hallmarks of PD[1, 2]. Pathologically, LBs consist primarily of α -synuclein. In addition, α -synucleinopathies are important biomarkers for other diseases, such as dementia with LBs and multiple system atrophy[2–4]. The α -synuclein (encoded by *SNCA*) is a 14-kDa intracellular protein enriched in the presynaptic terminals, where it binds to lipids and modulates the release of synaptic vesicles. Genetically, duplications, triplications,

and N-terminal point mutations of *SNCA* (A30P, E46K, H50Q, G51D, A53E, and A53T) cause autosomal dominant forms of familial PD[5–11].

The molecular mechanisms underlying neuron death in PD due to α -synuclein involve multiple pathways, such as dysfunctional synaptic-vesicle trafficking, mitochondrial dysfunction, oxidative stress, altered calcium homeostasis, defective autophagic degradation, impaired organelle dynamics, and neuroinflammation[12–16]. Over the last few decades, increasing evidence has suggested that astrocytes and microglia contribute to the α -synuclein pathology in PD[17]. Although most cytoplasmic α -synuclein inclusions are found in neurons, immunoreactive α -synuclein is also found in a subset of glia (astrocytes and oligodendrocytes) in the midbrain and basal ganglia of PD patients[18]. Mechanistically, loss of function of lysosomal *ATP13A2* in astrocytes and impaired tunneling nanotubes (TNTs) between astrocytes contribute to α -synuclein accumulation and propagation in PD[19]. Astrocytic dynamin-dependent endocytosis promotes the efficient uptake of α -synuclein fibrils[20]. Emerging evidence indicates that microglia also play a critical role in α -synuclein pathology in PD. Active microglia are closely associated with α -synuclein-positive deposits in the olfactory bulb, substantia nigra (SN), and pons of PD patients[21, 22]. Microglia-expressed lymphocyte-activation gene 3 (LAG3) is genetically linked with PD and is an identified receptor for misfolded α -synuclein fibrils. Besides, LAG3 participates in the α -synuclein spread between cells[23–25]. Recently, microglia were found to remove neuron-released α -synuclein via TLR4-NF- κ B-p62-mediated selective autophagy[26]. In addition, microglia cooperatively degrade α -synuclein fibrils via TNTs[27]. Both astrocytes and microglia participate in α -synuclein pathology; however, communication between these glial cells remains poorly understood.

Previously, we reported that loss of function of contactin-associated protein-like 4 (CNTNAP4) induces parkinsonian phenotypes, such as DA neuron death and movement disorders, through regulating mitophagy[28]. Herein, we report that partial loss of *CNTNAP4* accelerates α -synuclein pathology, nigrostriatal neuron degeneration, and motor disorders induced by the injection of adeno-associated viral vector (AAV)-mediating human α -synuclein overexpression (AAV-*h* α -Syn). Mechanistically, we found that partial loss of *CNTNAP4* exacerbates α -synuclein pathology and CNTNAP4 is involved in modulating the interplay between astrocytes and microglia through the Complement 3 (C3)-C3a receptor (C3aR) signaling pathway. *In vivo* depletion of microglia by PLX-3397 reduces *CNTNAP4* deficiency-aggravated α -synuclein pathology. Hence, our study reveals a novel role of *CNTNAP4* deficiency in PD pathogenesis through astrocyte–microglia crosstalk.

Materials And Methods

Reagents

Anti-CNTNAP4 (bs-11076R-2) and anti-CNTNAP4 (orb544737) antibodies were purchased from Bioss (Beijing, China) and Biorbyt LLC (San Francisco, CA, USA), respectively. Anti-human α -synuclein (ab138501), phospho- α -synuclein (Ser129) (ab51253), CD68 (ab53444), C1q (ab71940), and GABA_A α 2 (ab193311) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-mouse α -synuclein

(610786) antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-glial fibrillary acidic protein (GFAP, MAB360) antibody was purchased from Millipore (Billerica, MA, USA). Anti-ionized calcium binding adapter molecule (Iba1) (019–19741) antibody was purchased from FUJIFILM Wako (Osaka, Japan). Anti-synapsin β (#5297), anti-syntaxin (#18572), anti-SNAP25 (#5308), and anti-PSD-95 (#3450) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-synapsin β (106 303) antibody was purchased from Synaptic Systems (Goettingen, Germany). Anti-tyrosine hydroxylase (TH) (F-11, sc-25269), anti-C3 (sc-28294), anti-C3aR (sc-74522), anti-GABA_A α 5 (sc393921), and anti-GABA_A β 3 (sc-376252) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-GAPDH (60004–1) antibody was purchased from Proteintech Group (Rosemont, IL, USA). DyLight 488 goat anti-mouse IgG (H + L) (70- GAM4882) and DyLight 594 goat anti-rabbit IgG (H + L) (70- GAR5942) antibodies were purchased from Multi Sciences (Hangzhou, China). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and HRP-labeled goat anti-mouse IgG were purchased from Beyotime Biotechnology (Shanghai, China).

Animals

Heterozygous male CNTNAP4 null (CNTNAP4^{+/-}) mice (12–14 weeks) were used to evaluate the α -synuclein pathology in this study. Generally, CNTNAP4^{+/-} mice were obtained by mating CNTNAP4 knockout mice with wild-type (WT) C57BL/6J mice. CNTNAP4 knockout mice with C57BL/6J genetic background have been reported in our previous studies[28] and were generated by Shanghai Model Organisms Center, Inc. (Shanghai, China). Age- and sex-matched littermates were used as controls. Animals were housed in a 12-h dark–light cycle and had free access to water and food.

AAV-*h* α -Syn generation and stereotaxic injection

AAV-*h* α -Syn virus used in this study has been described in previous studies[29, 30]. Generally, AAV9 virus encoding overexpression of either human wild-type α -synuclein or green fluorescent protein (AAV-GFP) was driven by the Syn I promoter and enhanced using the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). WT or CNTNAP4^{+/-} mice were stereotactically injected into the bilateral substantia nigra pars compacta (SNpc) with either AAV-GFP or AAV-*h* α -Syn-GFP, as stated previously[29]. Briefly, mice were anesthetized and fixed on a stereotactic frame (RWD Life Sciences Corp., China). AAV-GFP (1.14×10^{12} vg/mL) or AAV-*h* α -Syn-GFP (7.38×10^{12} vg/mL) (packaged by Sunbio Medical Biotechnology, Shanghai, China) in 0.5- μ L volume were injected into the bilateral SNpc at the target site, as reported previously (Bregma AP, -3.0 mm, ML, $\pm$ 1.3 mm, DV, -4.7 mm)[29]. The syringe was left in place for 5 min before being slowly withdrawn. Eight weeks after the stereotaxic injections, behavioral tests were performed.

Microglial depletion

Similar to a previous study[31], the brain microglia were eliminated using intragastric administration of PLX3397, an inhibitor of the receptor for colony-stimulating factor 1 (CSF1R). PLX3397 (Selleck, Houston,

TX, USA) was dissolved in DMSO at 200 mg/ml and diluted to the working concentration. Eight weeks after AAV-*hα-Syn* injection, mice received intragastric feeding with 40 mg/kg of PLX3397 on 30 consecutive days. The control group received similar intragastric feeding with vehicle.

Behavioral tests

Open field test (OFT)

The procedure for OFT has been described previously[32]. Mice were placed in the center of a rectangular plastic box (40 × 40 × 40 cm). The movement of mice was recorded using a video tracking system (EthoVisione XT software, Beijing, China) for 15 min. The total distance, movement speed, and time spent by mice in the central zone were analyzed.

Rotarod test

Before the rotarod test, mice were placed on the Rotarod (Ugo Basile SRL, Gemonio, VA, Italy) at a speed of 10 rpm for training. Three days later, mice were placed on the rotarod cylinder that was accelerated from 4 to 40 rpm within 5 min. The latency time to falling of the animals was recorded.

Grasping test

Mice were suspended on a horizontal metal wire of 1 mm diameter, placed 30 cm above the ground for 10 s using the two front paws. The grasping score was recorded as 3, 2, 1, and 0 if mice grasped the wire with two hind paws, mice grasped the wire with one hind paw, mice failed to grasp the wire, and mice fell, respectively.

Y maze test

Mice were placed within the center zone with three equal angles between the arms, with walls that were 30 cm long, 10 cm wide, and 20 cm high, for 8 min. The alternation score (%) and number of arm entries for each mouse was recorded. Non-overlapping entrance sequences were defined as spontaneous alternations.

Elevated plus maze (EPM)

EPM consisted of two open arms (30 × 5 cm), two closed arms (30 × 5 × 15 cm), and a central zone (5 × 5 cm). Mice were placed in the central intersection and the total distance travelled, movement speed, open arm entries, and time spent in the open arm (%) were recorded using the video tracking system.

RNA-sequencing (RNA-seq) and bioinformatics analysis

RNA-seq and bioinformatics analysis were performed similar to our previous study[29]. Briefly, RNA was isolated using Trizol (Invitrogen, San Diego, CA, USA), and cDNA libraries were prepared using the TruSeq Stranded mRNA LT Prep Kit (Illumina). Libraries were sequenced on a HiSeq 2500 instrument (Illumina) at

the MGH Next Generation Sequencing Core Facility, using paired-end 50-bp sequencing. Sequencing reads were mapped by Novogene (Beijing, China). Read counts over transcripts were calculated using HTseq, followed by differential expression analysis using EdgeR. Genes were classified as differentially expressed based on the cutoffs of fold change (FC) > 1.6, false discovery rate (FDR) < 0.1, and $p < 0.005$. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes (DEGs) were performed using the R package (v 3.5.1).

Immunohistochemistry and immunofluorescence analyses

Mice brains were collected, post-fixed in 4% paraformaldehyde, and dehydrated in 20–30% sucrose solution. Brains were embedded in optimal cutting temperature (OCT) compound and cut into 15- μ m serial coronal sections using a freezing microtome (Leica). Free-floating sections were blocked with 5% BSA and incubated with the primary antibody. In the immunohistochemistry assay, sections were incubated with biotin-conjugated antibody, followed by DAB staining using the UltraSensitive SP IHC Kit (MXB biotechnologies, China). Images were scanned under a microscope (Leica CS2, Hamburg, Germany). In the immunofluorescent assay, sections were incubated with fluorescent-labeled secondary antibody and images were acquired using a confocal microscope (SP8, Leica). Quantitative analysis was performed using the Image-Pro Plus 6.0 photogram analysis system (IPP 6.0, Media Cybernetics, Bethesda, MD, USA). The interaction of indicators, such as Iba1 and GFAP, was evaluated by quantifying the fluorescence signal intensity.

Immunoblot analysis

Protein expression levels were examined using western blotting. Briefly, tissues were homogenized in RIPA buffer (Beyotime, Shanghai, China) and the supernatants were collected. Protein concentrations were quantified using the BCA Kit (Beyotime, Shanghai, China), and electrophoresis was performed using SDS-PAGE gels. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% BSA and incubated at 4°C overnight with primary antibodies, followed by HRP-conjugated secondary antibodies. The bands were visualized using enhanced chemiluminescence (ECL, Beyotime, Shanghai, China). Images were captured using the GeneGnome XRQ Chemiluminescence imaging system (Gene Company, Hong Kong, China). Image J software was used to analyze the optical density of bands.

Measurement of cytokine secretion

Serum cytokine levels were examined using the customized mouse Magnetic Luminex assay kit (Bio-Techne, Minneapolis, MN, USA) according to the manufacturer's instructions. The measurements were performed using a Bio-Rad BioPlex 200 system (Bio-Rad Laboratories, Basel, Switzerland) and data were analyzed using the Bio-Plex software 6.0 (version 6.0, Bio-Rad Laboratories).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, San Diego, CA, USA), and RNA quantity was assessed using Nanodrop (Agilent Technologies, California, USA). Then, cDNA was generated from 1 µg of total RNA per sample using the cDNA Reverse Transcription Kit (QIAGEN, Waltham, MA, USA). Quantitative PCR was performed using the primers listed in **Table 1**. Each sample was compared with GAPDH as the internal control. Data are from three separate experiments, and each performed in triplicate.

Transmission electron microscopy (TEM)

The ultrastructural morphologies of nigral synaptic vesicles, mitochondria, and autolysosome were analyzed using TEM, similar to our previous study[32]. After fixing and dehydration with different concentrations of ethanol and acetone, SN tissues were embedded with 812 embedding agents (SPI-Pon 812 Epoxy Resin Monomer, SPI, Shanxi, China). Following polymerization, sections were cut using a Leica EM UC7 (Leica Microsystems, Germany) and placed on copper grids. The grids were post-stained with uranyl acetate and bismuth subnitrate. The sections were observed using TEM (HT7700, Hitachi, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using two-way ANOVA followed by Bonferroni's multiple comparisons test or one-way ANOVA followed by Tukey's *post hoc* test, as appropriate. Differences with a *p*-value < 0.05 were considered statistically significant. The statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). *P*-values are represented as **p* < 0.05 and ***p* < 0.01.

Results

CNTNAP4 partial deficiency exacerbates α-synuclein pathology and nigral DA neuron death

Previously, we established a line of *CNTNAP4* knockout mice, which exhibits aggravated PD phenotype[28]. To evaluate the impact of *CNTNAP4* loss on α-synuclein pathology, we used heterozygous *CNTNAP4* null mice. The AAV-mediated overexpression of α-synuclein in the nigrostriatal system of mice has been found to successfully induce parkinsonian changes, such as the formation of cytoplasmic α-synuclein inclusions, progressive nigrostriatal DA neuron death, and movement disorders[30]. In this study, WT and *CNTNAP4*^{+/-} mice received bilateral stereotaxic injections of either AAV-*hα*-Syn or AAV-GFP in the SNpc for 8 weeks (Fig. 1a). We found that human α-synuclein was immunoreactive in the TH-positive neurons of mice injected with AAV-*hα*-Syn, and the number of α-synuclein immunoreactive cells was increased in *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice (Figs. 1b, c and e). We also found that α-synuclein spread to the striatum through the nigrostriatal pathway (Figs. 1c and d). Moreover, AAV-*hα*-Syn increased the protein expression level of human α-synuclein and phosphorylation of serine 129 of α-synuclein, which was the dominant pathological modification of α-synuclein[33], in the SN rather than striatum of *CNTNAP4*^{+/-} mice (Figs. 1f–j, Supplementary Figs. 1a and b). Furthermore, AAV-*hα*-Syn induced

nigrostriatal DA neuron death, which was much more apparent in the SNpc of *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice (Figs. 1k and l, Supplementary Figs. 1c and d, and Supplementary Fig. 2). AAV-*hα*-Syn decreased synapsin I in the SN of *CNTNAP4*^{+/-} mice, and both AAV-*hα*-Syn and *CNTNAP4*^{+/-} reduced nigral PSD-95 expression, suggesting that these two factors may affect synaptic plasticity (Supplementary Figs. 3a–c).

Thus, we successfully established an exogenous α -synuclein mouse model, in which *CNTNAP4* partial deficiency aggravates α -synuclein pathology and nigral DA neuron death.

Partial absence of *CNTNAP4* accelerates DA terminal damages and motor dysfunction after α -synuclein overexpression

Because we observed DA neuron death and alterations in synaptic protein expression, we investigated the ultrastructural changes after AAV-*hα*-Syn injection in *CNTNAP4*^{+/-} mice. Similar to reduced TH-positive neurons, the number of synaptic vesicles were significantly reduced in the DA terminal of AAV-*hα*-Syn, *CNTNAP4*^{+/-}, and *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice (Figs. 2a and b). Interestingly, we found that AAV-*hα*-Syn injection decreased the number of mitochondria and increased autolysosomes in heterozygous *CNTNAP4* null mice compared to AAV-*hα*-Syn or *CNTNAP4*^{+/-} mice (Figs. 2a, c, and d). Significant mitochondrial morphological changes (mitochondrial swelling and disappearance of cristae) were found in the nigral neurons of AAV-*hα*-Syn and *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice, which was much more severe in latter group (indicated by red arrows in Fig. 2a). These observations suggest that dysfunctional mitochondrial metabolism induces more severe nigral DA neuron death in the *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice.

We further evaluated the effect of α -synuclein overexpression and *CNTNAP4* partial deficiency on the behavioral parameters. *CNTNAP4* partial deficiency decreased the total travelled distance, number of entries to the center zone, and movement speed in OFT (Figs. 3a–c), and the performance of mice in the rotarod and grasping tests (Figs. 3e and f), which were consistent with our previous report that *CNTNAP4* null impaired the motor function[28]. Rotarod and grasping tests also showed that AAV-*hα*-Syn induces motor function (Figs. 3e and f). In particular, motor function was further decreased in *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice compared to AAV-*hα*-Syn or *CNTNAP4*^{+/-} mice (Figs. 3a–c, and 3e). Additionally, both AAV-*hα*-Syn and *CNTNAP4*^{+/-} reduced the spontaneous alterations in Y maze, suggesting they may partially affect cognitive function (Supplementary Figs. 4a and b). Although AAV-*hα*-Syn slightly reduced the open arm entries in the control mice in EPM test, AAV-*hα*-Syn or *CNTNAP4*^{+/-} showed no obvious effects on the time spent in the center zone of OFT and other parameters in the EPM test (Fig. 3d, Supplementary Figs. 4c–f). These data further support that AAV-*hα*-Syn aggravates DA neuron damages in *CNTNAP4* partial deficiency mice, which is responsible for the impaired motor function.

Transcriptomic analysis of α -synuclein burden in *CNTNAP4* partial deficiency mice

We then used RNA-seq to investigate the molecular mechanisms underlying DA neuron death and motor impairment in *CNTNAP4*^{+/-} + AAV-*hα*-Syn mice. PCA score plots revealed a distinct separation of

components in the WT, AAV-*hα*-Syn, CNTNAP4^{+/-}, and CNTNAP4^{+/-} + AAV-*hα*-Syn groups (Fig. 4a). The volcano plot showing DEGs between CNTNAP4^{+/-} + AAV-*hα*-Syn and CNTNAP4^{+/-} or AAV-*hα*-Syn group, and gene expression distribution of four groups are presented in Figs. 4b and c, and Supplementary Fig. 5a. Herein, we listed the representative DEGs enriched in the KEGG pathways between CNTNAP4^{+/-} + AAV-*hα*-Syn and CNTNAP4^{+/-} or AAV-*hα*-Syn group (Figs. 4d–g). AAV-*hα*-Syn significantly increased the DEGs (such as *Il1b*, *Il1m*, *Cxcl2*, *Ccl12*, *Ccl28*, *Csf1*, *Csf1r*, *Tnfsf8*, and *Tnfrsf1b*) enriched in the “cytokine-cytokine receptor interaction” pathway in the absence of CNTNAP4 (Figs. 4d and h), suggesting that AAV-*hα*-Syn induce the pro-inflammatory effects in CNTNAP4^{+/-} mice. Notably, the upregulated and downregulated DEGs between CNTNAP4^{+/-} + AAV-*hα*-Syn and CNTNAP4^{+/-}/AAV-*hα*-Syn groups were involved in “complement and coagulation cascades” and “dopaminergic synapse” pathways (Figs. 4d–g). These data indicate that AAV-*hα*-Syn may exacerbate DA neuron death through pro-inflammatory effects in CNTNAP4^{+/-} mice. The representative upregulated DEGs between CNTNAP4^{+/-} + AAV-*hα*-Syn and CNTNAP4^{+/-} mice are shown in a heatmap (Fig. 4h). The upregulated DEGs (*C1qb*, *C1qc*, *C1s1*, *C3*, *C4b*, *Vwf*, *Cfh*, and *A2m*) were enriched in the “complement and coagulation cascades” pathway (Fig. 4i). We also verified the RNA-seq data in these four groups by qRT-PCR and found that AAV-*hα*-Syn increased the expression levels of *C1qa*, *C1qc*, *C3* and *C4b* in CNTNAP4^{+/-} mice compared to CNTNAP4^{+/-} or AAV-*hα*-Syn mice (Figs. 4j–o).

The upregulated and downregulated DEGs between CNTNAP4^{+/-} + AAV-*hα*-Syn and CNTNAP4^{+/-} groups were enriched in KEGG pathways and the downregulated DEGs were enriched in the “dopaminergic synapse” and “synaptic vesicle cycle” pathways (Supplementary Figs. 5b, 6a, and b). Downregulation of the “synaptic vesicle cycle” pathway indicated that AAV-*hα*-Syn damaged synapse assembly in CNTNAP4^{+/-} mice, which was consistent with our ultrastructural analysis. Additionally, RNA-seq data suggested that CNTNAP4^{+/-} + AAV-*hα*-Syn had downregulated expression levels of genes involved in GABAergic synapse compared to AAV-*hα*-Syn. However, AAV-*hα*-Syn increased the protein expression levels of GABA_A receptors in CNTNAP4^{+/-} mice (Supplementary Figs. 7a and b). Thus, based on the RNA-seq data, pro-inflammatory activation may aggravate the parkinsonian phenotype and α-synuclein burden in CNTNAP4^{+/-} mice.

Overexpression of α-synuclein activates a pro-inflammatory response in CNTNAP4 partial deficient mice via astrocyte–microglia communication

As stated previously, our RNA-seq data suggest that AAV-*hα*-Syn activates the complement pathway (C1q, C3, and C4) in CNTNAP4^{+/-} mice. Previously, a striking physical interaction between astrocytes and microglia mediated by C3-C3aR signaling was reported in murine models of neuromyelitis optica and epilepsy[34, 35]. Therefore, we explored whether AAV-*hα*-Syn also used this pathway to activate astrocyte–microglial communications in the partial absence of *CNTNAP4*. First, we examined astrocyte and microglia numbers by staining the SNpc with their markers GFAP and Iba1. Intriguingly, the expression level of GFAP, a marker of astrocytes, was significantly increased in the CNTNAP4^{+/-} + AAV-

hα-Syn group (Figs. 5a and c), suggesting that astrocytes were activated. Additionally, the soma volume of astrocytes in the SNpc of CNTNAP4^{+/-} + AAV-*hα-Syn* mice was greater and their processes were thicker than astrocytes from other groups (Figs. 5a and d). The immunofluorescence staining demonstrated an increased microglial volume and a reduction in microglial process complexity in the SNpc of CNTNAP4^{+/-} + AAV-*hα-Syn* mice compared to the other groups (Figs. 5b, e and f), indicating that the microglia change from a “resting” ramified phenotype to an “activated” bushy phenotype. We proved that AAV-*hα-Syn* activated microglia in CNTNAP4^{+/-} mice by co-staining Iba1 and CD68 (a microglial activation marker), and Iba1 and TH (Figs. 5g and i, Supplementary Fig. 8). Surprisingly, in contrast to the other groups, astrocytes merged with microglia in the SNpc of CNTNAP4^{+/-} + AAV-*hα-Syn* group (Figs. 5h and j).

Being the main source of C3 in the brain, AAV-*hα-Syn* significantly induced activated astrocytes to produce extracellular C3 in CNTNAP4^{+/-} mice (Figs. 6a and c). Astrocytic C3 can bind with the C3a receptor to induce an inflammatory reaction[34, 36]. In the present study, we found that the microglia in the CNTNAP4^{+/-} + AAV-*hα-Syn* mice exhibited striking C3aR upregulation (Figs. 6b and d). In line with the RNA-seq data, we also found that C1q immunoreactivity, another microglial activation marker, was increased in the CNTNAP4^{+/-} + AAV-*hα-Syn* group (Figs. 6e and g). Microglia are the principal source of C1q in the central nervous system and *hα-Syn* was co-localized with C1q (Figs. 6f and h), suggesting that C1q may drive microglia to engulf exogenous α-synuclein in the CNTNAP4^{+/-} + AAV-*hα-Syn* group.

To investigate whether activated astrocyte–microglial crosstalk induces an immune response, we then evaluated the cytokine mRNA and protein expression levels in SN and serum. AAV-*hα-Syn* increased the mRNA expression of pro-inflammatory cytokines (*IL-1β* and *IFN-γ*) and microglial homeostatic markers (*CSF1R*, *CX3CR1*, *TMEM119*, and *P2RY12*) (Figs. 7a, d, f–i), which was consistent with the view that pathological α-synuclein is a trigger for excessive inflammatory responses in PD[26, 37]. We also found increased *IL-1β* and *TNF-α* mRNA expression levels in CNTNAP4^{+/-} mice (Figs. 7a and c). Compared to AAV-*hα-Syn*, CNTNAP4^{+/-} + AAV-*hα-Syn* increased *IL-1β* and *TNF-α* mRNA expression levels, and serum IL-6 and granulocyte colony-stimulating factor (G-CSF) protein expression levels (Figs. 7a, c, j, and l). CNTNAP4^{+/-} + AAV-*hα-Syn* increased the *IL-1β*, *IL-6*, *IFN-γ*, *CSF1R*, *CX3CR1*, *TMEM119*, and *P2RY12* mRNA expression levels compared to the CNTNAP4^{+/-} mice (Figs. 7a, b, d, f–i). AAV-*hα-Syn* reduced systematic IL-3 expression in WT and CNTNAP4^{+/-} mice (Fig. 7k), while AAV-*hα-Syn* or CNTNAP4^{+/-} showed no obvious effects on other serum cytokines, such as IL-2, IL-10, and IL-17 (Supplementary Figs. 9a–i and Supplementary Fig. 10a–j). These data reveal that AAV-*hα-Syn* induced a pro-inflammatory response in CNTNAP4^{+/-} mice.

These observations demonstrate that AAV-*hα-Syn* promotes astrocyte–microglia interplay through C3-C3aR pathway to induce an inflammatory response in CNTNAP4^{+/-} mice.

Eliminating microglia ameliorates AAV-*hα-Syn*-induced parkinsonian lesions in the partial absence of CNTNAP4 by interrupting astrocyte–microglia interplay

To investigate whether the interruption of the C3-C3aR pathway could rescue AAV-*h*α-Syn-induced parkinsonian damages in *CNTNAP4*^{+/-} mice, we eliminated microglia using PLX3397 (Fig. 8a), an inhibitor of the CSF1 receptor[38]. Notably, the elimination of microglia attenuates the movement disorder in the *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice in the OFT and grasping test (Figs. 8b–e). Furthermore, elimination of the microglia improved nigrostriatal DA neuron death in the *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 8f–h) and slightly decreased endogenous mouse α-synuclein level (Fig. 8i). However, it had no apparent effects on the expression of exogenous and mouse phosphorylated α-synuclein in the *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 8i–k).

Histopathological staining with Iba1 showed that PLX3397 significantly eliminated the resident microglia in the SNpc of *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 9a and b). Additionally, elimination of microglia significantly reduced the mRNA expression levels of pro-inflammatory cytokines (*IL-1β*, *IL-6*, *TNF-α*, and *IFN-γ*) and microglial homeostatic markers (*CSF1R*, *CX3CR1*, *TMEM119*, and *P2RY12*) in the *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 9c–k). These data suggest that the elimination of microglia attenuates the immune response caused by AAV-*h*α-Syn in *CNTNAP4*^{+/-} mice.

Our results proved that the astrocyte–microglial C3-C3aR signaling pathway plays a vital role in AAV-*h*α-Syn-induced inflammatory response in *CNTNAP4*^{+/-} mice. Next, we examined the effects of microglial elimination on this pathway. Elimination of microglia decreased the expression of two microglial activation markers, CD68 and C1q, in the SNpc of *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 10a–d). Moreover, microglial depletion interrupted the interaction between astrocytes and microglia, and reduced the astrocytic production of C3 in the SNpc of *CNTNAP4*^{+/-} + AAV-*h*α-Syn mice (Figs. 10e–h). Therefore, our results suggest that pharmacological inhibition of microglia attenuates AAV-*h*α-Syn-induced motor impairment, DA neuron death, and immune reaction in *CNTNAP4*^{+/-} mice through astrocyte–microglia interaction.

Discussion

PD is the most common neurodegenerative movement disorder. Numerous studies have shown that α-synuclein pathology not only is a hallmark of PD but can also cause neuronal death and motor dysfunction[2, 37, 39]. In the present study, we found that *CNTNAP4* partial deficiency exacerbated α-synuclein pathology, nigral neuron degeneration, and the onset of motor dysfunction in an AAV-*h*α-Syn mouse model of PD. The underlying mechanism involves astrocyte–microglia communication due to the α-synuclein burden in the partial absence of *CNTNAP4*.

CNTNAP4 is highly enriched in cortical interneurons and nigral DA neurons. It is genetically linked with autism and functionally associated with GABAergic transmission[40, 41]. Previously, we and other groups revealed the crucial role of *CNTNAP4* in PD and other aging-related diseases[28, 42]. Mechanistically, we found that *CNTNAP4* deficiency induced nigral DA neuron death, α-synuclein pathology, and motor dysfunction through damaging the mitochondrial function[28]. In the present study, we found that AAV-

hα-Syn induces a more severe parkinsonian phenotype in *CNTNAP4*^{+/-} mice compared to AAV-*hα-Syn* or *CNTNAP4*^{+/-} mice. Because *CNTNAP4* is not expressed in astrocytes or microglia[40, 41], we conclude that these defects may be derived from *CNTNAP4* partial deficiency-induced diseased DA neurons that are susceptible to the exogenous α-synuclein deposition. These duplicated effects mediate DA neuron death and α-synuclein release. Then, the released α-synuclein induces astrocyte–microglia crosstalk and inflammatory reaction. *CNTNAP4* partial loss damages DA neuron partially due to mitochondrial dysfunction, as evidenced by the distinct mitochondrial morphological changes and induction of autolysosomes in *CNTNAP4*^{+/-} + AAV-*hα-Syn* mice, which were consistent with our previous observations[28]. Furthermore, single injection of AAV-*hα-Syn* induces lower DA neuron death compared to a previous study[43], possibly because our AAV-*hα-Syn* system did not contain the cytomegalovirus immediate-early (CMVie)-enhancer, which increases the expression of transduced proteins and related neurodegeneration. Therefore, we did not observe the astrocyte–microglia crosstalk in AAV-*hα-Syn* injected mice. Our results support the view that *CNTNAP4* deficiency participates in PD pathogenesis.

In the brain, astrocytes are located adjacent to the synapses and blood vessels to form a complex network, whereas microglia constantly travel around. Astrocytes communicate with microglia through signaling factors, such as neurotransmitters, cytokines, chemokines, and extracellular vesicles[44–46], which contributes to synapse development, pruning, and maintenance of the local environment[47, 48]. However, it may also be harmful in some conditions. Over the last few decades, astrocyte–microglia crosstalk has been widely studied in neurological diseases[49]. Microglia augment astrocyte-mediated inflammatory activation in manganese exposure, traumatic brain injury, and stroke[44, 45, 50]. Previously, microglia activation was found to convert astrocytes into a neurotoxic A1 phenotype in PD[51]. However, the mechanism underlying the contribution of their interplay to α-synuclein pathology remains poorly understood. In this study, we detected that, in the partial absence of *CNTNAP4*, α-synuclein burden induced the interaction between astrocytes and microglia. Furthermore, this crosstalk may be involved in AAV-*hα-Syn*-induced nigral DA neuron death and motor impairment in *CNTNAP4*^{+/-} mice because microglial depletion interrupts this crosstalk and attenuates AAV-*hα-Syn* pathology in *CNTNAP4*^{+/-} mice.

In the present study, we report that astrocytes communicate with microglia through the C3-C3aR signaling pathway. C3 complement is a crucial component of the innate immune system and, together with other complement proteins, forms a major host mechanism for the detection and clearance of potential pathogens. C3 is closely associated with PD pathogenesis, and high C3 levels in the serum and cerebrospinal fluid correlate with worse quality of life and memory ability in PD patients[52–54]. Astrocytes are the main source of C3 in the central nervous system, and astroglial Kir6.1/K-ATP channel deficiency was previously reported to contribute to PD pathogenesis by inducing astrocyte–neuronal interaction through C3-C3aR signaling[55]. Using α-synuclein preformed fibril-injected mice and human A53T α-synuclein transgenic mice, Ma et al. identified that the complement and coagulation cascade pathways are the significant differential pathways compared to the controls[56]. Moreover, they found that C3-positive astrocytes were increased in the ventral midbrain of PD mice and that astrocyte-secreted C3 could induce DA neuron degeneration[56]. Intriguingly, similar to these observations, our results reveal

that the complement and coagulation cascade pathways are the common pathway between *CNTNAP4*^{+/-} + AAV-*hα-Syn* and AAV-*hα-Syn* or *CNTNAP4*^{+/-} mice. AAV-*hα-Syn* promotes C3 secretion from astrocytes in *CNTNAP4*^{+/-} mice, which correlates with induced nigral DA neuron death and motor dysfunction. Since C3aR is mainly expressed in microglia, we proved that astrocytic C3 induced microglial C3aR expression and its downstream inflammatory pathway. Thus, our study provides evidence that astrocyte–microglial C3-C3aR signaling is required for the AAV-*hα-Syn*-mediated inflammatory response in *CNTNAP4*^{+/-} mice.

Our results indicate that astrocyte–microglial C3-C3aR signaling pathway activates microglia. Recently, targeting microglia and its bidirectional signals with other neuronal cells have been reported to be attractive therapeutic options for PD and other neurodegenerative diseases, for which several strategies have been developed[57–61]: (1) Strategies to inhibit inflammation or pro-inflammatory cytokines, such as minocycline and NOD-like receptor protein 3 (NLRP3) inhibitor, effectively suppress microglial inflammation, nigral DA neuron death, and α -synuclein pathology in PD[60, 62, 63]. (2) Active or passive immunization to produce antibodies against α -synuclein and passive immunotherapy targeting α -synuclein have shown beneficial results in preclinical studies through augmenting α -synuclein clearance by microglia. Several immunotherapeutic strategies for PD are being evaluated in phase 2 clinical trials[59, 64, 65]. (3) Targeting bidirectional signals between astrocytes and microglia, such as the glucagon-like peptide 1 receptor agonist NLY01, was revealed to protect DA neurons against inflammatory responses elicited by astrocytes and microglia[51]. In the present study, we eliminated microglia using a CSF1R antagonist, PLX3397, and found that microglial depletion reduces astrocyte–microglia interaction, inflammatory response, and DA neuron loss. The development of an inhibitor or antagonist of C3aR in microglia would be beneficial in targeting the α -synuclein pathology in PD in the future.

Conclusions

We provide evidence that *CNTNAP4* partial deficiency accelerated α -synuclein pathology, nigrostriatal neuron degeneration, and motor disorders in an AAV-*hα-Syn* mouse model of PD. Additionally, astrocyte–microglial C3-C3aR signaling pathway was required for *CNTNAP4* partial deficiency-exacerbated α -synuclein pathology. Microglia elimination attenuated these effects. Therefore, *CNTNAP4* deficiency is critical to PD pathogenesis and *CNTNAP4* merits further research as a therapeutic target for PD (Fig. 11).

Abbreviations

AAV

adeno-associated viral vector

C3

Complement 3

C3aR

Complement 3a receptor

CMVie

cytomegalovirus immediate-early

CNTNAP4

contactin-associated protein-like 4

CSF1R

colony-stimulating factor 1

DA

Dopaminergic

DEGs

differentially expressed genes

EPM

elevated plus maze

G-CSF

granulocyte colony-stimulating factor

GFAP

glial fibrillary acidic protein

Iba1

ionized calcium binding adapter molecule

KEGG

Kyoto Encyclopedia of Genes and Genomes

LAG3

lymphocyte-activation gene 3

LBs

Lewy bodies

NLRP3

NOD-like receptor protein 3

OFT

open field test

PD

Parkinson's disease

qRT-PCR

quantitative reverse transcription polymerase chain reaction

RNA-seq

RNA-sequencing

SN

substantia nigra

SNpc

substantia nigra pars compacta

TEM

transmission electron microscopy

TH

tyrosine hydroxylase

TNTs

impaired tunneling nanotubes

WPRE

woodchuck hepatitis virus posttranscriptional regulatory element.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were performed according to the guidelines of Institutional Animal Care and Use Committee of Guangzhou Medical University and National Institute of Health guidelines on the care and use of animals (NIH Publications No. 8023, revised 1978).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YLZ designed the experiments, guided and supervised the project. YLZ and HXX discussed and wrote the manuscript. PYX edited the manuscript. WLZ, LYD, JWG, and MRZ performed the experiments and

analyzed the data. RFM, SHZ and ZLZ helped with animal surgery and behavioral experiments as well as data analysis. All authors read and approved the final manuscript.

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References

1. Kalia LV, Lang AE. Parkinson's disease. *Lancet*. 2015;386:896-912.
2. Koga S, Sekiya H, Kondru N, Ross OA, Dickson DW. Neuropathology and molecular diagnosis of Synucleinopathies. *Mol Neurodegener*. 2021;16:83.
3. Shahnawaz M, Mukherjee A, Pritzkow S, Mendez N, Rabadia P, Liu X, et al. Discriminating alpha-synuclein strains in Parkinson's disease and multiple system atrophy. *Nature*. 2020;578:273-277.
4. Alegre-Abarrategui J, Brimblecombe KR, Roberts RF, Velentza-Almpani E, Tilley BS, Bengoa-Vergniory N, et al. Selective vulnerability in alpha-synucleinopathies. *Acta Neuropathol*. 2019;138:681-704.
5. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 1997;276:2045-2047.
6. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet*. 1998;18:106-108.
7. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I, et al. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol*. 2004;55:164-173.
8. Appel-Cresswell S, Vilarino-Guell C, Encarnacion M, Sherman H, Yu I, Shah B, et al. Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. *Mov Disord*. 2013;28:811-813.
9. Lesage S, Anheim M, Letournel F, Bousset L, Honore A, Rozas N, et al. G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. *Ann Neurol*. 2013;73:459-471.
10. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. alpha-Synuclein locus triplication causes Parkinson's disease. *Science*. 2003;302:841.
11. Chartier-Harlin MC, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, et al. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet*. 2004;364:1167-1169.
12. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkman J, et al. Parkinson disease. *Nat Rev Dis Primers*. 2017;3:17013.
13. Rocha EM, De Miranda B, Sanders LH. Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease. *Neurobiol Dis*. 2018;109:249-257.
14. Nguyen M, Wong YC, Ysselstein D, Severino A, Krainc D. Synaptic, Mitochondrial, and Lysosomal Dysfunction in Parkinson's Disease. *Trends Neurosci*. 2019;42:140-149.
15. Ge P, Dawson VL, Dawson TM. PINK1 and Parkin mitochondrial quality control: a source of regional vulnerability in Parkinson's disease. *Mol Neurodegener*. 2020;15:20.

16. Park JH, Burgess JD, Faroqi AH, DeMeo NN, Fiesel FC, Springer W, et al. Alpha-synuclein-induced mitochondrial dysfunction is mediated via a sirtuin 3-dependent pathway. *Mol Neurodegener.* 2020;15:5.
17. Bartels T, De Schepper S, Hong S. Microglia modulate neurodegeneration in Alzheimer's and Parkinson's diseases. *Science.* 2020;370:66-69.
18. Wakabayashi K, Hayashi S, Yoshimoto M, Kudo H, Takahashi H. NACP/alpha-synuclein-positive filamentous inclusions in astrocytes and oligodendrocytes of Parkinson's disease brains. *Acta Neuropathol.* 2000;99:14-20.
19. Tsunemi T, Ishiguro Y, Yoroisaka A, Valdez C, Miyamoto K, Ishikawa K, et al. Astrocytes Protect Human Dopaminergic Neurons from alpha-Synuclein Accumulation and Propagation. *J Neurosci.* 2020;40:8618-8628.
20. Filippini A, Mutti V, Faustini G, Longhena F, Ramazzina I, Rizzi F, et al. Extracellular clusterin limits the uptake of alpha-synuclein fibrils by murine and human astrocytes. *Glia.* 2021;69:681-696.
21. Doorn KJ, Goudriaan A, Blits-Huizinga C, Bol JG, Rozemuller AJ, Hoogland PV, et al. Increased amoeboid microglial density in the olfactory bulb of Parkinson's and Alzheimer's patients. *Brain Pathol.* 2014;24:152-165.
22. Imamura K, Hishikawa N, Sawada M, Nagatsu T, Yoshida M, Hashizume Y. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. *Acta Neuropathol.* 2003;106:518-526.
23. Mao X, Ou MT, Karuppagounder SS, Kam TI, Yin X, Xiong Y, et al. Pathological alpha-synuclein transmission initiated by binding lymphocyte-activation gene 3. *Science.* 2016;353.
24. Cui SS, Du JJ, Liu SH, Meng J, Lin YQ, Li G, et al. Serum soluble lymphocyte activation gene-3 as a diagnostic biomarker in Parkinson's disease: A pilot multicenter study. *Mov Disord.* 2019;34:138-141.
25. Guo W, Zhou M, Qiu J, Lin Y, Chen X, Huang S, et al. Association of LAG3 genetic variation with an increased risk of PD in Chinese female population. *J Neuroinflammation.* 2019;16:270.
26. Choi I, Zhang Y, Seegobin SP, Pruvost M, Wang Q, Purtell K, et al. Microglia clear neuron-released alpha-synuclein via selective autophagy and prevent neurodegeneration. *Nat Commun.* 2020;11:1386.
27. Scheiblich H, Dansokho C, Mercan D, Schmidt SV, Bousset L, Wischhof L, et al. Microglia jointly degrade fibrillar alpha-synuclein cargo by distribution through tunneling nanotubes. *Cell.* 2021;184:5089-5106 e5021.
28. Zhang W, Zhou M, Lu W, Gong J, Gao F, Li Y, et al. CNTNAP4 deficiency in dopaminergic neurons initiates parkinsonian phenotypes. *Theranostics.* 2020;10:3000-3021.
29. Gong J, Zhang W, Ding L, Zhang M, Zheng S, Ma R, et al. 4,4'-Dimethoxychalcone regulates redox homeostasis by targeting riboflavin metabolism in Parkinson's disease therapy. *Free Radic Biol Med.* 2021;174:40-56.
30. Faustini G, Longhena F, Varanita T, Bubacco L, Pizzi M, Missale C, et al. Synapsin III deficiency hampers alpha-synuclein aggregation, striatal synaptic damage and nigral cell loss in an AAV-based

- mouse model of Parkinson's disease. *Acta Neuropathol.* 2018;136:621-639.
31. Wang J, Lai S, Li G, Zhou T, Wang B, Cao F, et al. Microglial activation contributes to depressive-like behavior in dopamine D3 receptor knockout mice. *Brain Behav Immun.* 2020;83:226-238.
 32. Zhang W, Chen H, Ding L, Gong J, Zhang M, Guo W, et al. Trojan Horse Delivery of 4,4'-Dimethoxychalcone for Parkinsonian Neuroprotection. *Adv Sci (Weinh).* 2021;8:2004555.
 33. Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, et al. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem.* 2006;281:29739-29752.
 34. Chen T, Lennon VA, Liu YU, Bosco DB, Li Y, Yi MH, et al. Astrocyte-microglia interaction drives evolving neuromyelitis optica lesion. *J Clin Invest.* 2020;130:4025-4038.
 35. Wei Y, Chen T, Bosco DB, Xie M, Zheng J, Dheer A, et al. The complement C3-C3aR pathway mediates microglia-astrocyte interaction following status epilepticus. *Glia.* 2021;69:1155-1169.
 36. Lian H, Litvinchuk A, Chiang AC, Aithmitti N, Jankowsky JL, Zheng H. Astrocyte-Microglia Cross Talk through Complement Activation Modulates Amyloid Pathology in Mouse Models of Alzheimer's Disease. *J Neurosci.* 2016;36:577-589.
 37. Grozdanov V, Bousset L, Hoffmeister M, Bliederhaeuser C, Meier C, Madiona K, et al. Increased Immune Activation by Pathologic alpha-Synuclein in Parkinson's Disease. *Ann Neurol.* 2019;86:593-606.
 38. Elmore MR, Najafi AR, Koike MA, Dagher NN, Spangenberg EE, Rice RA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron.* 2014;82:380-397.
 39. Dehay B, Bourdenx M, Gorry P, Przedborski S, Vila M, Hunot S, et al. Targeting alpha-synuclein for treatment of Parkinson's disease: mechanistic and therapeutic considerations. *Lancet Neurol.* 2015;14:855-866.
 40. Karayannis T, Au E, Patel JC, Kruglikov I, Markx S, Delorme R, et al. Cntnap4 differentially contributes to GABAergic and dopaminergic synaptic transmission. *Nature.* 2014;511:236-240.
 41. Shangguan Y, Xu X, Ganbat B, Li Y, Wang W, Yang Y, et al. CNTNAP4 Impacts Epilepsy Through GABAA Receptors Regulation: Evidence From Temporal Lobe Epilepsy Patients and Mouse Models. *Cereb Cortex.* 2018;28:3491-3504.
 42. Iakoubov L, Mossakowska M, Szwed M, Duan Z, Sesti F, Puzianowska-Kuznicka M. A common copy number variation (CNV) polymorphism in the CNTNAP4 gene: association with aging in females. *PLoS One.* 2013;8:e79790.
 43. Oliveras-Salva M, Van der Perren A, Casadei N, Stroobants S, Nuber S, D'Hooge R, et al. rAAV2/7 vector-mediated overexpression of alpha-synuclein in mouse substantia nigra induces protein aggregation and progressive dose-dependent neurodegeneration. *Mol Neurodegener.* 2013;8:44.
 44. Shi SX, Li YJ, Shi K, Wood K, Ducruet AF, Liu Q. IL (Interleukin)-15 Bridges Astrocyte-Microglia Crosstalk and Exacerbates Brain Injury Following Intracerebral Hemorrhage. *Stroke.* 2020;51:967-974.

45. Kirkley KS, Popichak KA, Afzali MF, Legare ME, Tjalkens RB. Microglia amplify inflammatory activation of astrocytes in manganese neurotoxicity. *J Neuroinflammation*. 2017;14:99.
46. Liao K, Niu F, Hu G, Yang L, Dallon B, Villarreal D, et al. Morphine-mediated release of miR-138 in astrocyte-derived extracellular vesicles promotes microglial activation. *J Extracell Vesicles*. 2020;10:e12027.
47. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, et al. Synaptic pruning by microglia is necessary for normal brain development. *Science*. 2011;333:1456-1458.
48. Vainchtein ID, Chin G, Cho FS, Kelley KW, Miller JG, Chien EC, et al. Astrocyte-derived interleukin-33 promotes microglial synapse engulfment and neural circuit development. *Science*. 2018;359:1269-1273.
49. Vainchtein ID, Molofsky AV. Astrocytes and Microglia: In Sickness and in Health. *Trends Neurosci*. 2020;43:144-154.
50. Clark DPQ, Perreau VM, Shultz SR, Brady RD, Lei E, Dixit S, et al. Inflammation in Traumatic Brain Injury: Roles for Toxic A1 Astrocytes and Microglial-Astrocytic Crosstalk. *Neurochem Res*. 2019;44:1410-1424.
51. Yun SP, Kam TI, Panicker N, Kim S, Oh Y, Park JS, et al. Block of A1 astrocyte conversion by microglia is neuroprotective in models of Parkinson's disease. *Nat Med*. 2018;24:931-938.
52. Vesely B, Dufek M, Thon V, Brozman M, Kiralova S, Halaszova T, et al. Interleukin 6 and complement serum level study in Parkinson's disease. *J Neural Transm (Vienna)*. 2018;125:875-881.
53. Sun C, Yu W, Zhao Z, Song C, Liu Y, Jia G, et al. Peripheral Humoral Immune Response Is Associated With the Non-motor Symptoms of Parkinson's Disease. *Front Neurosci*. 2019;13:1057.
54. Wang Y, Hancock AM, Bradner J, Chung KA, Quinn JF, Peskind ER, et al. Complement 3 and factor h in human cerebrospinal fluid in Parkinson's disease, Alzheimer's disease, and multiple-system atrophy. *Am J Pathol*. 2011;178:1509-1516.
55. Chen MM, Hu ZL, Ding JH, Du RH, Hu G. Astrocytic Kir6.1 deletion aggravates neurodegeneration in the lipopolysaccharide-induced mouse model of Parkinson's disease via astrocyte-neuron cross talk through complement C3-C3R signaling. *Brain Behav Immun*. 2021;95:310-320.
56. Ma SX, Seo BA, Kim D, Xiong Y, Kwon SH, Brahmachari S, et al. Complement and Coagulation Cascades are Potentially Involved in Dopaminergic Neurodegeneration in alpha-Synuclein-Based Mouse Models of Parkinson's Disease. *J Proteome Res*. 2021;20:3428-3443.
57. Lewcock JW, Schlepckow K, Di Paolo G, Tahirovic S, Monroe KM, Haass C. Emerging Microglia Biology Defines Novel Therapeutic Approaches for Alzheimer's Disease. *Neuron*. 2020;108:801-821.
58. Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, El Fatimy R, et al. The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity*. 2017;47:566-581 e569.
59. Mandler M, Valera E, Rockenstein E, Weninger H, Patrick C, Adame A, et al. Next-generation active immunization approach for synucleinopathies: implications for Parkinson's disease clinical trials. *Acta Neuropathol*. 2014;127:861-879.

60. Gordon R, Albornoz EA, Christie DC, Langley MR, Kumar V, Mantovani S, et al. Inflammasome inhibition prevents alpha-synuclein pathology and dopaminergic neurodegeneration in mice. *Sci Transl Med.* 2018,10.

61. McFarthing K, Buff S, Rafaloff G, Dominey T, Wyse RK, Stott SRW. Parkinson's Disease Drug Therapies in the Clinical Trial Pipeline: 2020. *J Parkinsons Dis.* 2020,10:757-774.

62. Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, et al. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci U S A.* 2001,98:14669-14674.

63. Investigators NN-P. A randomized, double-blind, futility clinical trial of creatine and minocycline in early Parkinson disease. *Neurology.* 2006,66:664-671.

64. Bae EJ, Lee HJ, Rockenstein E, Ho DH, Park EB, Yang NY, et al. Antibody-aided clearance of extracellular alpha-synuclein prevents cell-to-cell aggregate transmission. *J Neurosci.* 2012,32:13454-13469.

65. Masliah E, Rockenstein E, Mante M, Crews L, Spencer B, Adame A, et al. Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease. *PLoS One.* 2011,6:e19338.

Tables

Table 1. Primer sequences used for qRT-PCR.

Mice genes	Primer sequence (5'-3')
<i>C1qa</i>	F: GGA CTGGTATCCGAGGTTTAA R: GATATTGCCTGGATTGCCTTTC
<i>C1qb</i>	F: GCTAGAGCAAGAGGAGGTTGTTAC R: TCAGGGAAAAGCAGAAAGCCAGTG
<i>C1qc</i>	F: TACTTCGTCTACTACACATCGC R: GGAAACAGTAGGAAACCAGAGA
<i>C1s1</i>	F: GCATTGTCTTTCAA ACTGACCT R: ACACAGGTTATCTTCACCACAT
<i>C3</i>	F: GTTTCTGAACACAGCCAAAGAT R: GACATCTGTTTGATATTGGGCC
<i>C4b</i>	F: GAGTCCAGAGTTCAGTACACTG R: CAGTCTCAAAGTGACTCACGTA
<i>IL-1β</i>	F: AATGCCACCTTTTGACAGTGAT R: TGCTGCGAGATTTGAAGCTG
<i>IL-6</i>	F: AGGATACCACTCCCAACAGACC R: AAGTGCATCATCGTTCATACA
<i>TNF-α</i>	F: CACGTCGTAGCAAACCACC R: TGAGATCCATGCCGTTGGC
<i>IFN-γ</i>	F: TGGCAGGAGATGTCTACACT R: GAAGCACCAGGTGTCAAGTC
<i>TGF-β</i>	F: ATTCCTGGCGTTACCTTGG R: AGCCCTGTATTCCGTCTCCT
<i>CSF1R</i>	F: CCTCAAACGTGGAGACACCAA R: CGTGTGCCAACATCATTGCT
<i>CX3CR1</i>	F: CAACCCCTTTATCTACGCCTT R: GACCCATCTCCCTCGCTTG
<i>TMEM119</i>	F: CTGACATTCTGGCTGCTACC R: CACCCTTCACAGGCTTTGCTC
<i>P2RY12</i>	F: TTTGCTGGGCTCATCACGAAC

	R: ACTGAAGTAACTTGGCACACC
<i>GAPDH</i>	F: ACGGGAAGCTCACTGGCATGGCCTT
	R: CATGAGGTCCACCACCCTGTTGCTG

The primer sequences of *C1qa*, *C1qb*, *C1qc*, *C1s1*, *C3*, *C4b*, *IL-1 β* , *IL-6*, *TNF- α* , *IFN- γ* , *TGF- β* , *CSF1R*, *CX3CR1*, *TMEM119*, *P2RY12*, *GAPDH*.

Figures

Figure 1

***CNTNAP4* partial deficiency exacerbates α -synuclein pathology and nigral DA neuron death.** (a) Mice were stereotactically injected with AAV-GFP or AAV-*ha-Syn* virus for 8 weeks, after which behavioral tests were performed and mice were sacrificed. (b) Immunofluorescence staining of TH and human α -synuclein (*ha-Syn*) in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. Scale bars, 40 μ m. Magnified co-staining of TH and *ha-Syn*-positive cells in the SNpc are shown in the right

column of panel b. Scale bars, 8 μ m. (c) Immunohistochemical staining of *ha*-Syn-positive cells in the striatum and SNpc of WT and CNTNAP4^{+/-} mice injected with either AAV-GFP or AAV-*ha*-Syn. Scale bars, 1 mm in the striatum and 100 μ m in the SNpc. Magnified images of *ha*-Syn-positive cells in the SNpc are shown in the right column of panel c. Scale bars, 50 μ m. (d and e) Quantification of *ha*-Syn density and positive cells in the striatum and SNpc. N = 4–6. (f–j) The protein expression levels of CNTNAP4, *ha*-Syn, phosphorylation of α -synuclein at serine 129, and mouse α -synuclein were determined by Western blotting. N = 3 per group. (k) Immunohistochemical staining of TH-positive cells in the SNpc of WT and CNTNAP4^{+/-} mice injected with either AAV-GFP or AAV-*ha*-Syn. (l) Quantification of TH-positive cells in the SNpc. Scale bars, 100 μ m. Magnified images of TH-positive cells in the SNpc are shown in the right column of panel k. N = 6–10. Scale bars, 50 μ m. Results are expressed as mean $\pm$ SEM. ** p < 0.01, * p < 0.05 vs. WT, ## p < 0.01, # p < 0.05 vs. AAV-*ha*-Syn, && p < 0.01, & p < 0.05 vs. CNTNAP4^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 2

Overexpression of α -synuclein hampers synaptic plasticity and mitochondrial homeostasis in

CNTNAP4^{+/-} mice. (a) Ultrastructural analysis of synaptic vesicles, mitochondria, and autolysosomes in the SNpc of WT and CNTNAP4^{+/-} mice injected with either AAV-GFP or AAV-*ha*-Syn. Scale bars, 500 nm for detecting synaptic vesicles, 2 μ m for detecting mitochondria, and 500 nm for magnified images, 5 μ m for detecting autolysosome and 500 nm for magnified images. Red arrows show mitochondrial swelling and mitochondrial cristae disappearance. (b–d) Quantification of the numbers of synaptic vesicles, mitochondria, and autolysosomes in the SNpc. N = 10 per group. Results are expressed as mean $\pm$ SEM.

****** $p < 0.01$ vs. WT, **##** $p < 0.01$, **#** $p < 0.05$ vs. AAV-*hα*-Syn, **&&** $p < 0.01$ vs. CNTNAP4^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 3

CNTNAP4 partial deficiency accelerates motor dysfunction in AAV-*hα*-Syn mice. (a–d) Total distance travelled, number of entries to the center zone, movement speed, and time spent in the center of the open-field in WT and CNTNAP4^{+/-} mice injected with either AAV-GFP or AAV-*hα*-Syn. (e) The rotarod test was used to examine the motor coordination of mice. (f) The grasping test was used to examine the grip strength of mice. N = 11, 11, 10, and 7 in the WT + AAV-GFP, WT + AAV-*hα*-Syn, CNTNAP4^{+/-} + AAV-GFP, and CNTNAP4^{+/-} + AAV-*hα*-Syn groups, respectively. Results are expressed as mean ± SEM. ****** $p < 0.01$, ***** $p < 0.05$ vs. WT, **##** $p < 0.01$, **#** $p < 0.05$ vs. AAV-*hα*-Syn, **&&** $p < 0.01$, **&** $p < 0.05$ vs. CNTNAP4^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 4

Transcriptomic profiles of α -synuclein burden in *CNTNAP4* partial deficiency mice. (a) PCA score plots revealed a distinct separation of components in WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. N = 2 per group. (b and c) DEGs between *CNTNAP4*^{+/-} + AAV-*ha-Syn* and *CNTNAP4*^{+/-} or AAV-*ha-Syn* groups were shown in a volcano plot. (d and e) The representative KEGG pathways enriched by up- and down-regulated DEGs between *CNTNAP4*^{+/-} + AAV-*ha-Syn* and *CNTNAP4*^{+/-} group are shown. (f and g) The representative KEGG pathways enriched by up- and down-regulated DEGs between *CNTNAP4*^{+/-} + AAV-*ha-Syn* and AAV-*ha-Syn* group are shown. (h) Upregulated DEGs between *CNTNAP4*^{+/-} + AAV-*ha-Syn* and *CNTNAP4*^{+/-} mice are shown in a heatmap. Note that the genes (*C1qb*, *C1qc*, *C1s1*, *C3*, *C4b*, *Vwf*, *Cfh*, and *A2m*) were enriched in “complement and coagulation cascades” pathway, (*Il1b*, *Il1m*, *Cxcl2*, *Ccl12*, *Ccl28*, *Csf1*, *Csf1r*, *Tnfsf8*, and *Tnfrsf1b*) were enriched in “Cytokine-cytokine receptor interaction” pathway. (i) RNA-seq data revealed the expression levels of *C1qb*, *C1qc*, *C1s1*, *C3*, *C4b*, *Vwf*, *Cfh*, and *A2m* in the four groups. (j–o) The mRNA expression levels of *C1qa*, *C1qb*, *C1qc*, *C1s1*, *C3*, and *C4b* were determined by qRT-PCR. N = 3 per group. Results are expressed as mean $\pm$ SEM. ***p* < 0.01, **p* < 0.05.

0.05 vs. WT, $^{##}p < 0.01$ vs. AAV-*ha-Syn*, $^{&&}p < 0.01$, $^{&}p < 0.05$ vs. *CNTNAP4*^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 5

Exogenous α -synuclein burden induces interaction between astrocytes and microglia in *CNTNAP4* partial deficient mice. (a) Immunofluorescence staining of GFAP-positive cells in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. Scale bars, 20 μm. Magnified images of GFAP-positive cells in the SNpc are shown in the right column of panel a. Scale bars, 5 μm. (b) Immunofluorescence staining of Iba1-positive cells in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. Scale bars, 20 μm. Magnified Iba1-positive skeletal images in the SNpc are shown in the right column of panel b. Scale bars, 5 μm. (c and d) Quantification of the number and volume of GFAP-positive cells in Fig. a. (e and f) Quantification of the volume and branch length of Iba1-positive cells in Fig. b. (g) Immunofluorescence staining of Iba1-merging CD68 cells in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. Scale bars, 40 μm. Magnified images are shown in the right column of panel g. Scale bars, 8 μm. (h) Immunofluorescence staining of GFAP-merging Iba1 cells in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*ha-Syn*. Scale bars, 40 μm. Magnified images are shown in the right column of panel h. Scale bars, 8 μm. (i and j)

Quantification of the interaction area of Figs. g and h. N = 5–10. Results are expressed as mean $\pm$ SEM. $**p < 0.01$, $*p < 0.05$ vs. WT, $##p < 0.01$, $#p < 0.05$ vs. AAV-*h* α -Syn, $\&\&p < 0.01$ vs. *CNTNAP4*^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 6

Astrocyte–microglial C3-C3aR signaling is activated by α -synuclein in *CNTNAP4* partial deficient mice. Immunofluorescence staining and quantification of GFAP-merging C3 cells (**a** and **c**), Iba1-merging C3aR cells (**b** and **d**), Iba1-merging C1q cells (**e** and **g**), and *h* α -Syn-merging C1q cells (**f** and **h**) in the SNpc of WT and *CNTNAP4*^{+/-} mice injected with either AAV-GFP or AAV-*h* α -Syn. Scale bars, 40 μ m. Magnified images are shown in the right column of panel a, b, e, and f. Scale bars, 8 μ m. N = 5–8. Results are expressed as mean $\pm$ SEM. $**p < 0.01$ vs. WT, $##p < 0.01$, $#p < 0.05$ vs. AAV-*h* α -Syn, $\&\&p < 0.01$, $\&p < 0.05$ vs. *CNTNAP4*^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 7

CNTNAP4 partial loss increased pro-inflammatory cytokine expression levels in AAV-ha-Syn mice. (a–i) The mRNA expression levels of *IL-1 β* , *IL-6*, *TNF- α* , *IFN- γ* , *TGF- β* , *CSF1R*, *CX3CR1*, *TMEM119*, and *P2RY12* in the SN of WT and CNTNAP4^{+/-} mice injected with either AAV-GFP or AAV-ha-Syn. N = 3 per group. (j–l) Serum expression levels of IL-6, IL-3, and G-CSF were determined using Mouse Magnetic Luminex Assay. N = 8, 8, 8, and 6 in the WT + AAV-GFP, WT + AAV-ha-Syn, CNTNAP4^{+/-} + AAV-GFP, and CNTNAP4^{+/-} + AAV-

ha-Syn groups, respectively. Results are expressed as mean $\pm$ SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. WT, $^{##}p < 0.01$, $^{\#}p < 0.05$ vs. AAV-*ha*-Syn, $^{\&}p < 0.01$, $^{\&}p < 0.05$ vs. CNTNAP4^{+/-}. Statistical significance was determined using two-way ANOVA + Bonferroni's multiple comparisons test.

Figure 8

Depletion of microglia reduces motor dysfunction and DA neuron death in CNTNAP4^{+/-} mice injected with AAV-*ha*-Syn. (a) During the administration of AAV-GFP or AAV-*ha*-Syn virus, as shown in Fig. 1a, mice were intragastrically fed with PLX3397 for 4 weeks. Following behavioral tests, mice were sacrificed. (b–d) Total distance travelled, movement speed, and number of entries to the center zone in WT, CNTNAP4^{+/-} + AAV-*ha*-Syn, and CNTNAP4^{+/-} + AAV-*ha*-Syn + PLX3397 groups. (e) The grasping test was used to examine the grip strength of mice. N = 10, 12, and 8 in the WT, CNTNAP4^{+/-} + AAV-*ha*-Syn, and CNTNAP4^{+/-} + AAV-*ha*-Syn + PLX3397 groups, respectively. (f) Immunohistochemical staining of TH-positive cells in WT, CNTNAP4^{+/-} + AAV-*ha*-Syn, and CNTNAP4^{+/-} + AAV-*ha*-Syn + PLX3397 groups. Scale bars, 1 mm for striatum, 100 μ m for SNpc. Magnified images of TH-positive cells in the SNpc are shown in the right column of panel f. Scale bars, 50 μ m. (g and h) Quantification of TH-positive cells in the striatum and SNpc. N = 6–11. (i–k) Protein expression levels of CNTNAP4, *ha*-Syn, phosphorylation of α -synuclein at serine 129, and mouse α -synuclein were determined using Western blotting. N = 3 per group. Results are expressed as mean $\pm$ SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. WT, $^{##}p < 0.01$, $^{\#}p < 0.05$ vs. CNTNAP4^{+/-} + AAV-*ha*-Syn. Statistical significance was determined using one-way ANOVA and Tukey's tests for *post hoc* comparisons.

Figure 9

Microglial depletion reduces the inflammatory response in CNTNAP4^{+/-} mice injected with AAV-ha-Syn.

(a) Immunohistochemical staining of Iba1-positive cells in WT, CNTNAP4^{+/-} + AAV-ha-Syn, and CNTNAP4^{+/-} + AAV-ha-Syn + PLX3397 groups. Scale bars, 50 μ m. (b) Quantification of Iba1-positive cells in the SNpc. (c-k) The mRNA expression levels of *IL-1 β* , *IL-6*, *TNF- α* , *IFN- γ* , *TGF- β* , *CSF1R*, *CX3CR1*,

TMEM119, and *P2RY12* in the SN of WT, CNTNAP4^{+/-} + AAV-*ha-Syn*, and CNTNAP4^{+/-} + AAV-*ha-Syn* + PLX3397 groups, respectively. N = 3 per group. Results are expressed as mean ± SEM. ***p* < 0.01, **p* < 0.05 vs. WT, ##*p* < 0.01 vs. CNTNAP4^{+/-} + AAV-*ha-Syn*. Statistical significance was determined using one-way ANOVA and Tukey's tests for *post hoc* comparisons.

Figure 10

PLX3397 interrupts astrocyte–microglial C3-C3aR signaling in CNTNAP4^{+/-} mice injected AAV-*ha-Syn*. Immunofluorescent staining and quantification of Iba1-merging CD68 cells (**a** and **c**), Iba1-merging C1q cells (**b** and **d**), GFAP-merging Iba1 cells (**e** and **g**), and GFAP-merging C3 cells (**f** and **h**) in the SNpc of WT, CNTNAP4^{+/-} + AAV-*ha-Syn*, and CNTNAP4^{+/-} + AAV-*ha-Syn* + PLX3397 groups. Scale bars, 40 μm. Magnified images are shown in the right column of panel a, b, e, and f. Scale bars, 8 μm. N = 7–9. Results

are expressed as mean $\pm$ SEM. $^{**}p < 0.01$ vs. WT, $^{##}p < 0.01$ vs. CNTNAP4^{+/-} + AAV-*h* α -Syn. Statistical significance was determined using one-way ANOVA and Tukey's tests for *post hoc* comparisons.

Figure 11

Schematic model of this study. *CNTNAP4* partial deficiency augments DA neuron death mediated by α -synuclein burden. Damaged DA neuron then releases α -synuclein and induces the astrocyte–microglia crosstalk which leads to astrocytic release of complement C3. Upon activation by C3a receptor, microglia secretion of complement C1q and pro-inflammatory cytokines further drives DA neuron death and aggravate motor dysfunction in PD.

Supplementary Files

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- [SupplementaryMaterials.pdf](#)