의학박사 학위논문

A53T 파킨슨 병 랫드 모델에서 광유전 기술을 이용한 반응성 성상세포의 억제가 GABA 분비 및 파킨슨 증상에 미치는 효과

(Effect of optogenetic inhibition of reactive astrocytes on the GABA secretion and recovery of parkinsonism in A53T Parkinson's disease rat model)

울 산 대 학 교 대 학 원

의 학 과

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이 논문을 의학박사 학위 논문으로 제출함

2022 년 5 월

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국문 요약

배경 및 목적

파킨슨병 (Parkinson's disease)에서 알파-시누클레인(α-synuclein, α-Syn) 단백질 응집에 따른 도파민 (Dopamine, DA) 신경 세포 (neuron)의 퇴행성 변화는 비가역적인 것으로 인식되었으나 최근 한 연구에서 tyrosine hydroxylase (TH)가 발현되지 않지만 회복 가능한 휴면기 상태의 DA neuron 의 존재를 보고하였다. 즉, 파킨슨병에서 나타나는 반응성 성상세포 (reactive astrocytes)는 비정상적으로 강직성 r-aminobutyric acid (GABA)를 분비하여 DA neurons 을 억제하는데, monoamine oxidase B (MAO-B)의 억제제를 투여하였을 GABA 분비가 감소하며 DA neurons 의 활성과 TH 발현이 회복되고 파킨슨 운동 증상이 개선되는 것으로 나타났다. 그러나 MAO-B 는 GABA 생성에 관여하는 것 외에도 DA 를 분해하고 산화 스트레스를 증가시켜 신경 퇴행을 유발하는 등 다양한 기전을 통해 파킨슨 증상 발현에 기여하며, 파킨슨 증상 개선에 대한 MAO-B 억제제의 효과 역시 복합적이다. 따라서 본 연구에서는 구체적으로 reactive astrocyte 의 억제 효과를 알아보기 위해 세포 특이적으로 이온 수용체를 발현하여 활성도를 조절할 수 있는 광유전 기술을 이용하였으며, 파킨슨병 동물 모델에서 reactive astrocyte 의 억제가 GABA 생성 또는 분비 및 파킨슨 운동 증상 개선에 미치는 영향을 검증하고자 하였다.

연구 방법

Wistar rat 16 마리의 흑색질 밀부 (substantia nigra pars compacta, SNpc) 등쪽 경계에 AAV2-CMV-*α-synuclein (A53T)*-EGFP 를 주입하여 α-Syn 과발현 파킨슨 동물을 만들었으며, 2 주 후 실험군 (n = 8)에는 AAV_{DJ}-GFAP104-e*NpHR3.0*-mCherry 을 주입하여 할로로돕신 (halorhodopsin, NpHR) 광유전자를 reactive astrocytes 에 형질 도입 시켰고, 대조군 (n = 8)에는 phosphate-buffered saline 를 주입하였다. 2 주 후 모든 동물에서 광섬유를 삽입하였으며, 1 주 후 590 nm 파장의 빛을 50 Hz, 10 ms pulse duration 으로 1 시간 동안 조사하였다. 빛 자극 전후 stepping test 를 통한 파킨슨 운동 증상 변화, TH 발현, glial fibrillary acidic protein (GFAP), GABA, 그리고 α-Syn 양을 실험군과 대조군 간에 비교하였다.

연구 결과

실험군에서 NpHR 은 GFAP (+) 세포의 30.1±19.5 (0.9–68.2)%에서 발현되었으며, 두 그룹 간에 GFAP 발현에 통계적 차이는 없었으나, 광 자극 후 GABA 양이 대조군에 비해 실험군에서 72.6% 로 상당히 감소하였다 ($p = 0.0486$). 또한 실험군에서 α-Syn 응집체 양이 대조군에 비해 67.5% ($p < 0.0001$)로 상당히 감소하였다. 한편, 실험군에서 TH (+) 세포의 30.3±11.6 (11.1–49.6) % 에서 NpHR 이 발현되었으며, TH (+) 세포 수가 대조군과 비교하여 27% ($p = 0.0132$) 감소하였다. 행동 검사에서 광 자극 후 실험군에서는 자극 전과 비교하여 stepping 횟수가 81.4±7.2 (71.6–90.9) % 크게 개선된 반면 (p = 0.0002), 대조군에서는 49.5±23.2 (9.3–85.1) % 악화되었다 ($p = 0.0003$).

결론

본 연구는 α-Syn 과발현 파킨슨 동물 모델에서 reactive astrocytes 에 NpHR 광유전자를 형질 도입 후 광 자극을 함으로써, reactive astrocytes 를 기능적으로 억제하면 GABA 생성이 감소하고 α-Syn 응집체 감소가 일어나 파킨슨 운동 증상이 개선됨을 증명하였다. 이는 reactive astrocytes 의 활성 조절이 파킨슨병 치료의 새로운 치료 전략이 될 수 있음을 시사하는 소견으로, 향후 광유전자를 통해 reactive astrocyte 만을 특이적이고 선택적으로 조절하는 방법에 대한 연구 및 reactive astrocytes 억제에 의한 α -Syn 응집체 감소 기전, 그리고 나아가 파킨슨병에서 reactive astrocyte 발생과 α -Syn 응집체 형성의 선후 관계 및 병인의 중요도를 규명하는 연구가 필요하다.

중심어: 알파-시누클레인, 가바, 할로로돕신, 광유전학, 파킨슨병, 반응성 성상세포

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Figure 5. Halorhodopsin (NpHR) was transduced also in TH (+) cells, in addition to reactive astrocytes. After illumination, TH (+)- cell number, -total area, and signal intensity significantly decreased in NpHR group compared to control group; However, as α-Syn aggregates significantly decreased, the TH (+) cells co-localized with α-Syn also decreased in NpHR group, and there was no difference in net-TH (+) cells without α-Syn aggregates, which are functioning, between experimental and control groups.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, in which misfolded α-synuclein (α-Syn) aggregates themselves and accumulates, forming intraneuronal inclusion bodies called Lewy bodies. [1-4] α-Syn aggregates are toxic and make injury to several micro-organelles, causing synaptic dysfunction and, ultimately, progressive neuronal cell loss, particularly in dopamine-producing neurons. [5] Therefore, it has been long believed that the accumulation of pathological α-Syn is the origin of PD, and several strategies to decrease the α-Syn aggregates via antagonization, reducing the formation, or enhancing their clearance have been considered first-line. However, in recent years, reactive astrocytes abundantly present in neurodegenerative regions in PD have received particular attention as they were revealed to upregulate r-aminobutyric acid (GABA) synthesis and aberrantly release tonic GABA, suppressing dopamine (DA) neurons. [6-8] A recent study revealed the presence of dormant DA neurons that are functionally inactive, with tyrosine hydroxylase (TH) being negative but alive with still expressing dopa decarboxylase (DDC). [9] Given that astrocytic GABA synthesis occurs by monoamine oxidase B, the study treated animals with MAO-B inhibitors such as selegiline and safinamide in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine) PD rat models, and reported significantly decreased GABA intensity in astrocytes, normalized $GABA_A$ receptor-mediated tonic GABA current in SNpc DA neurons, and increased TH expression in TH-/DDC+ DA neurons. This reversibility of TH expression implied the possibility of the rescue of functionally inactive DA neurons, and disease-modifying therapies targeting the reactive astrocyte has been proposed. However, in addition to being involved in GABA production, as MAO-B contributes to PD through various mechanisms, such as degrading DA and increasing oxidative stress to induce neurodegeneration, the effects of MAO-B inhibitors are also complex. Therefore, this study aimed to specifically inhibit reactive astrocytes by transducing halorhodopsin (NpHR) to verify the impact of inhibition of reactive astrocytes on GABA amount and TH expression, α-Syn aggregates, and alleviation of parkinsonian motor symptoms in α-Syn overexpression PD rat model.

Methods and Materials

Experimental Animals

Sixteen male Wistar rats (Orient Bio Inc., Seongnam, South Korea) weighing 250–300 g at the beginning of the experiment were housed in a room with a 12/12 h light/dark cycle and had free access to food and water. All procedures were conducted following the Institutional Animal Care and Use Committee of the

Asan Institute for Life Sciences guidelines. The Ethics Committee for Animal Experiments of the Asan Institute for Life Sciences approved the experimental protocol (Seoul, South Korea).

Study design

To investigate the effect of inhibition of reactive astrocytes on the recovery of parkinsonism and elucidate its underlying mechanisms, we designed transducing the halorhodopsin gene into astrocytes in animal PD models using the adeno-associated virus engineered to have the glial fibrillary acid protein (GFAP) gene as a promoter in the experimental group. The adequacy of PD model formation and improvement of parkinsonism were evaluated through a stepping test. The timelines of the experiments are summarized in Figure 1.

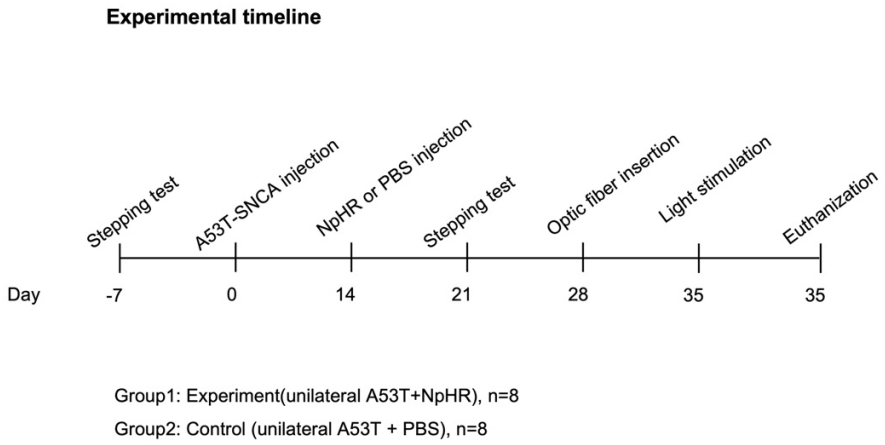


Figure 1. Time schedule of the experiment

A53T α-synuclein overexpression model

To establish the animal PD model, viral vectors AAV2-CMV- *α-synuclein (A53T)*-EGFP, overexpressing human mutant-A53T-α-synuclein, were used (purchased from the Korea Institute of Science and Technology virus facility (Seoul, South Korea); viral titer 1.16×10^{13}GC/mL). The animals were generally anesthetized by intraperitoneal injection of 35 mg/kg of Zoletil and 5 mg/kg of Rompun and placed in a stereotactic frame. The AAV vector plasmid in a volume of 2μL was unilaterally injected into the dorsal border of the right substantia nigra pars compacta (SNpc) with the coordinates AP −5.4 mm, ML +2.0 mm relative to bregma, and DV −7.5 mm from the dura. The virus was delivered at a 0.3 μL/min rate using a

33-gauge Hamilton syringe and an automated microsyringe pump (Harvard Apparatus, Holliston, MA, USA). After injection, the needle was kept in place for 5 min to prevent the solution from flowing backward.

Halorhodopsin transduction

Viral vectors AAV_{DJ}-GFAP104-e*NpHR3.0*-mCherry (viral titer 1.54× 10^{13}GC/ml) were manufactured at the Korea Institute of Science and Technology virus facility (Seoul, Korea), aiming for the selective transduction of the halorhodopsin gene in astrocytes. Two weeks after injection of AAV2-CMV-A53T-*SNCA*-EGFP, 2μL of AAV_{DJ}-GFAP-*NpHR*-mCherry was stereotactically injected in the experiment group (n = 8) under general anesthesia as the same method as mentioned above at the same target. In the control group (n = 8), 2μL of phosphate-buffered saline (PBS) was injected instead of AAV_{DJ}-GFAP-*NpHR*-mCherry.

Optical fiber insertion and light stimulation

Two weeks after halorhodopsin transduction, all animals underwent general anesthesia and were placed in a stereotactic frame. After a scalp incision, four burr holes were made using electric drilling system, and screws were embedded in the skull. The optical fiber (core 200 μm, outer diameter 245 μm, numerical aperture 0.53, RM3 type, flat tip; Doric Lenses, Québec, QC, Canada) was inserted using a stereotactic cannula holder into the right side SNpc with the same target as the viral injection. Then, it was firmly secured in the skull via screws and dental cement (Vertex, Zeist, The Netherlands) (Fig. 2). Illumination with 590 nm light was performed in all animals with 50 Hz and 10 ms pulse duration for one hour under the control of a pulse generator (Berkeley Nucleonics Corp., San Rafael, CA, USA) a week later. During optic inhibition, animals freely moved in glass cylinders (diameter 20 cm, height 40 cm), with the optic fiber being connected to a LED fiber-optic light source (Doric lenses) through the fiber-optic rotatory joint.

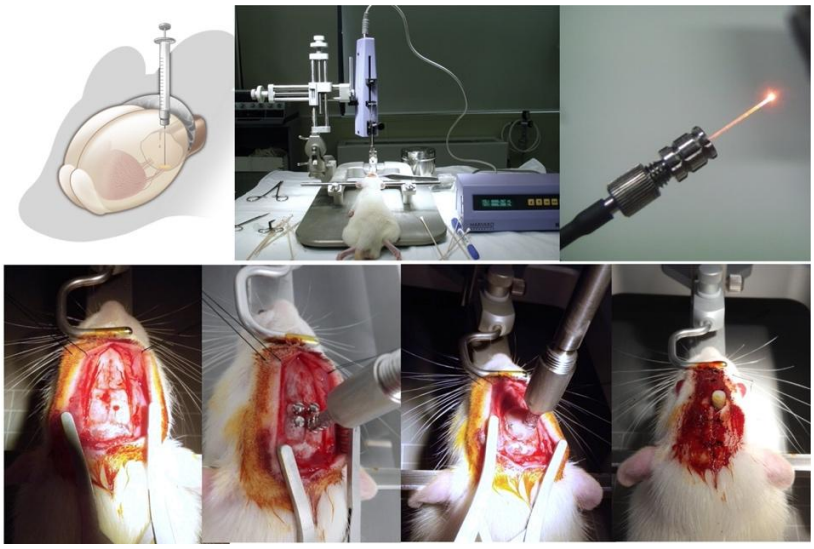


Figure 2.
Stereotactic surgical procedures of optical fiber implantation

Stepping Test

All animals were subjected to stepping tests 1 week before viral injection of AAV2-CMV- *α-synuclein (A53T)*-EGFP and 3 weeks and 5 weeks (immediate pre- and post-illumination) after. The test was performed on a treadmill (Jeung Do Bio & Plant Co., Seoul, South Korea), moving at 1.8 m/10s. One forelimb of the animal was allowed to spontaneously touch the moving treadmill track, with both hindlimbs and another forelimb being held to immobilize by the examiner. The tests were performed for both contralateral and ipsilateral forelimbs and were video recorded to allow counting of the number of adjusted steps for one minute by a forelimb of interest. All animals repeated the stepping test twice in each session, and the number of steps in two trials was averaged.

Tissue Processing

Immediately after the stepping test performed after one-hour illumination, the animals were euthanized under general anesthesia via intraperitoneal injection of 35 mg/kg of Zoletil and 5 mg/kg of Rompun.
They underwent transcardiac perfusion with 0.9% saline mixed with 10,000 IU heparin (Hanlim Pharm, Seoul, South Korea), followed by 4% paraformaldehyde in PBS. After decapitation, optical fibers were carefully removed, and brains were extracted and post-fixed in 4% paraformaldehyde for 12 hours, followed by dehydration in 30% sucrose solution until they sank. The brains were cut in a coronal plane with a 40 μm thickness from AP -4.8 to -6.0 mm using a cryotome (Thermo Scientific, Waltham, MA, USA). The brain sections were preserved under the free-floating condition in 0.08% sodium azide (Sigma) in PBS at 4℃.

Immunohistochemical Staining

Serial coronal sections of the SN were subjected to immunohistochemical staining separately for TH, GFAP, and GABA. The procedures were performed as described below. The brain sections were washed in 0.5% bovine serum albumin (BSA; Bioworld, Dublin, OH, USA) in PBS (pH 7.4) and incubated with a blocking solution containing BSA, Triton X-100 (Sigma), and sodium azide (Sigma) in PBS. The sections were incubated overnight with each primary antibody (mouse anti-TH antibody, 1:2,000, Sigma; rabbit anti-TH antibody, 1:1,000, Abcam, Cambridge, UK; rabbit anti-GFAP antibody, 1:100, Sigma; and guinea pig anti-GABA antibody, 1:500, Sigma) in 0.5% BSA in PBS (pH 7.4), and subsequently incubated for 2 h with Alexa Fluor 647 antibody (1:1,000; Invitrogen, Carlsbad, CA, USA). Fluorescent-labeled tissues were cover-slipped with a fluorescent mounting medium (DAKO, Glostrup, Denmark).

Imaging and stereological assessment

Fluorescent images were obtained using a confocal microscope (Carl Zeiss, Oberkochen, Germany) with ZEN microscope software (Carl Zeiss), with scanning in three channels: excitation λ = 633 nm (TH, GFAP, and GABA), 561 nm (NpHR), and 488 nm (α-Syn). Three coronal sections containing the SNpc were chosen for each TH, GFAP, and GABA immunohistochemical staining for quantitative analysis. The MetaMorph® software (BioVision Technologies, Pennsylvania, USA) that offers image analysis tools with a semiautomated quantification algorithm was used to compare the amounts of pathological human α-Syn, as well as the NpHR, TH, GFAP, and GABA expression between the experimental and control groups. SNpc was outlined manually on the MetaMorph® software, and α-Syn /NpHR/TH, α-Syn /NpHR/GFAP, and α-Syn/NpHR/GABA positive-cell number, -total area (µm2), -average area (µm2), and -signal intensity were computed.

Statistical Analysis

All data are presented as mean ± standard error. Statistical analyses were performed using Prism Software (GraphPad, La Jolla, CA, USA). To analyze the differences in time-dependent patterns of stepping tests, we performed two-way repeated-measures ANOVA tests using the Bonferroni post-test. Mann-Whitney U test was performed to compare the results of image quantification for α-Syn, NpHR, TH, GFAP, and GABA between the experimental and control groups, with a p-value < 0.05 being considered significant.

Results

Impact of optogenetic inhibition of the substantia nigra on mitigation of parkinsonism

All animals receiving AAV2-CMV- *α-synuclein (A53T)*-EGFP showed contralateral forelimb akinesia 3 weeks after viral injection. In the stepping test performed before optical illumination, The contralateral forelimb's adjusting steps significantly decreased to 16.5±6.6 (9.1–26.8) % and 17.8±3.9 (12.9–24.6) % of the ipsilateral forelimb in experimental (p = 0.0002) and control groups (p = 0.0002), respectively. After 1-hour optical illumination, the adjusting steps of the contralateral forelimb significantly improved, with the number of adjusting steps of the contralateral forelimb being increased to 89.2±11.3 (65.6–100) % of those of the ipsilateral forelimb in the experimental group (p = 0.0002); improving by 81.4±7.2 (71.6–90.9) % compared to the pre-illumination state. In contrast, in the control group receiving PBS instead of AAV_{DJ}-

GFAP-*NpHR*-mCherry, the contralateral forelimb akinesia was deteriorated after illumination, with the adjusting steps being decreased to 8.5±3.3 (3.1–13.2) % of the ipsilateral forelimb, worsening by 49.5±23.2 (9.3–85.1) % (p = 0.0003) compared to the pre-illumination state (Fig. 3).

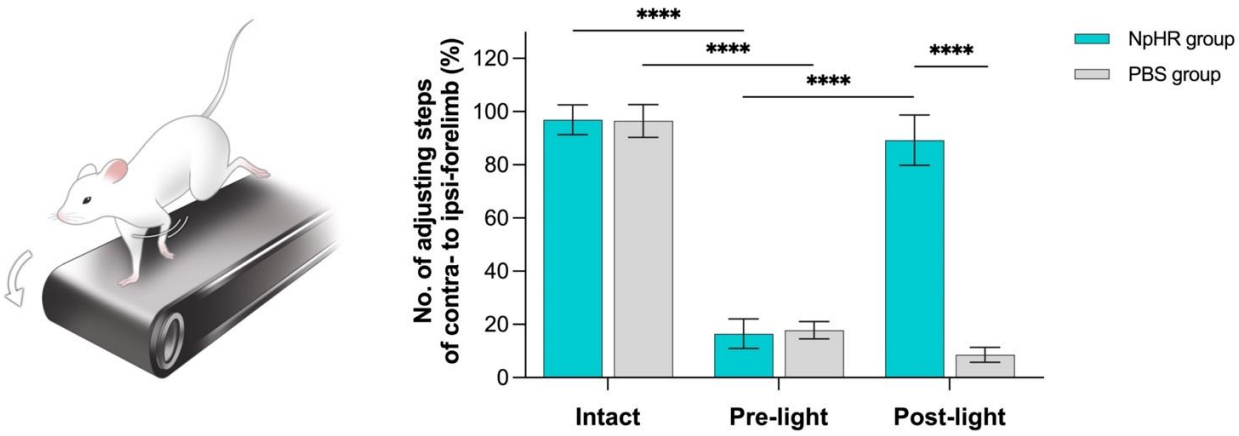


Figure 3. Comparisons the contralateral forelimb akinesia between pre- and post- illumination in experimental and control groups. Akinesia was assessed by the proportion of the adjusting step number of contralateral forelimb to that of ipsilateral forelimb.

Characteristics of pathological α-Syn transfection and halorhodopsin transduction in the substantia nigra pars compacta

The human pathological α-Syn was expressed in both TH (+) cells and GFAP (+) cells. In general, the signals of TH and GFAP were dimmed where the signal of the α-Syn aggregates was strong. The co-localization areas that α-Syn overlapped with TH and GFAP were in 26.5±8.0 (10.6–40.0) % and 8.3±5.3 (2.0–19.1) % of the α-Syn aggregates (+) area in the SNpc of the control group. Meanwhile, NpHR was transduced in 30.1±19.5 (0.9–68.2) % of GFAP (+) cells and 30.3±11.6 (11.1–49.6) % of TH (+) cells in the SNpc of the experimental group. It was also expressed in α-Syn (+) cells: The distribution of NpHR expression showed a relatively exclusive pattern to the α-Syn in the SNpc, with the co-localization area being only 14.4±11.0 (0.3–41.5) % of the α-Syn (+) area.

Optical inhibition reduced the amount of α-Syn in A53T-α-Syn overexpression PD model

The number of α-Syn (+) cells was comparable between the experimental and control groups with 52.9±37.2 (6–189) versus 56.4±18.8 (7–106), respectively (p = 0.2376). However, the total area, average

area per cell, and average signal intensity of the α-Syn aggregates were significantly reduced in the experimental group by 67.5% ($p < 0.0001$), 69.8% ($p < 0.0001$), and 0.7% ($p = 0.0152$), respectively, compared to the control group (Fig 4.).

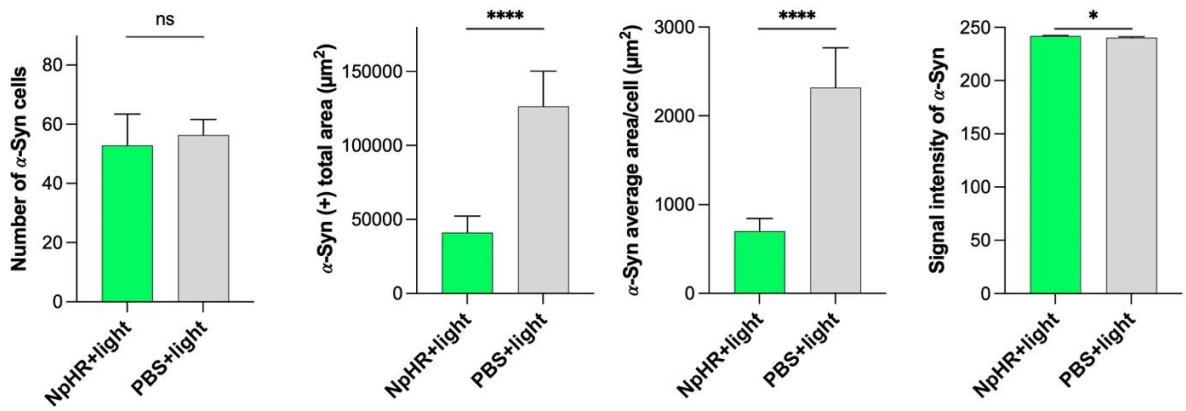


Figure 4. The amount of α-Syn aggregates significantly decreased in optogenetic stimulated halorhodopsin (NpHR) group comprared to control group

Effect of optical inhibition on TH expression

TH expression was significantly decreased in the experimental group compared to the control group in the TH (+)-cell count, -total area, and-signal intensity by 27% ($p = 0.0132$), 32.2% ($p = 0.0151$), and 6.4% ($p = 0.0425$), respectively. The TH (+)-average area was decreased in the experimental group by 13.1% compared to the control group, but the difference was not significant ($p = 0.0814$). However, the percentage of colocalization area where TH overlapped with the α-Syn aggregates was also significantly reduced in the experimental group compared to the control group with 15.2±10.8 (1.3–39.8) % versus 37.7±16.1 (9.7–74.0) %, yielding the reduction rate of 59.8% ($p < 0.0001$). When excluding the TH (+) cell in which the pathological α-Syn aggregates are accumulated, the TH (+)-cell count and -total area were comparable between the experimental and control groups, with the mean value of 78.5 versus 78.9 and 55062 μm^2 versus 59526 μm^2, respectively.

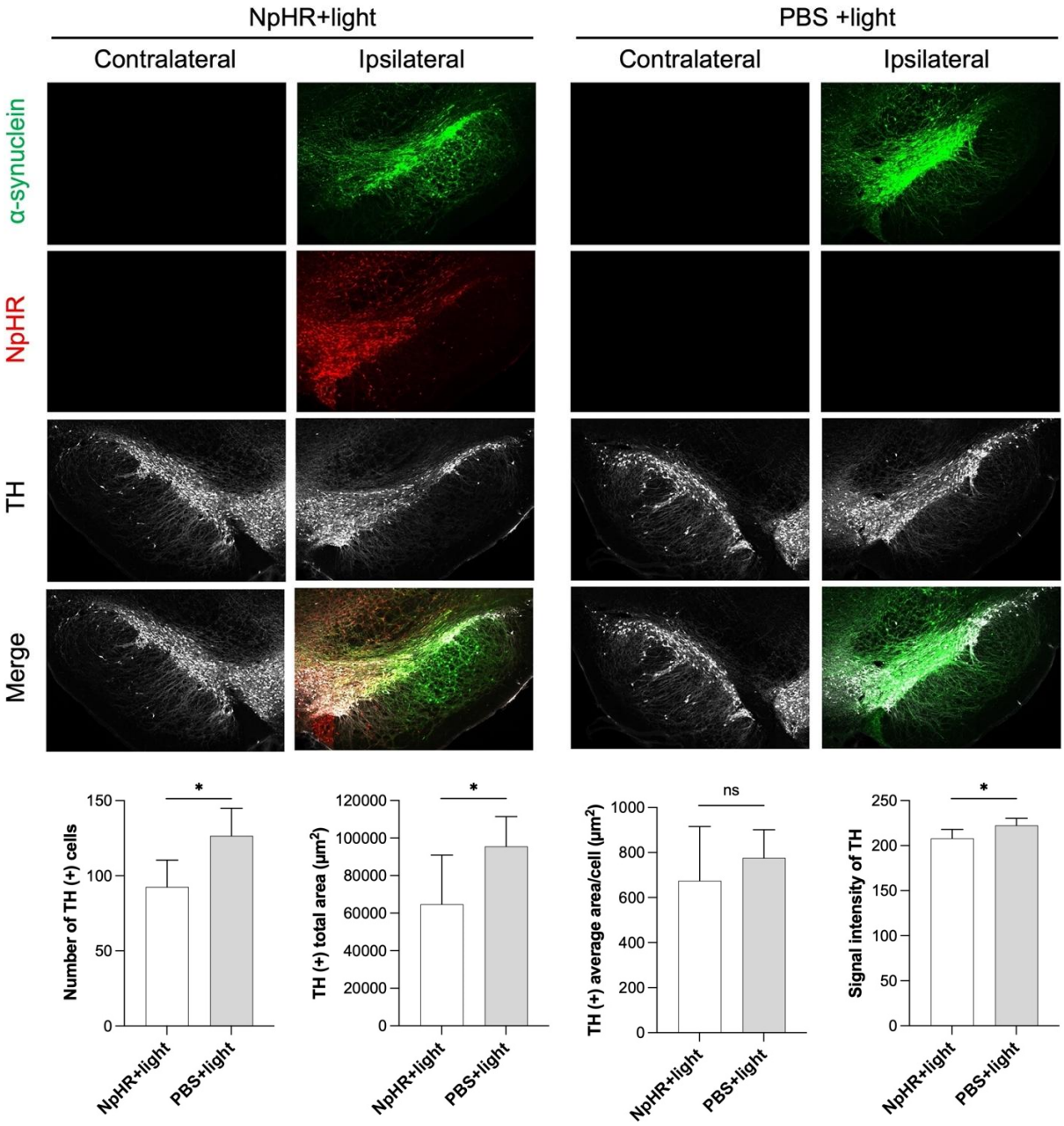


Figure 5. Halorhodopsin (NpHR) was transduced also in TH (+) cells, in addition to reactive astrocytes. After illumination, TH (+)- cell number, -total area, and signal intensity significantly decreased in NpHR group compared to control group; However, as α-Syn aggregates significantly decreased, the TH (+) cells co-localized with α-Syn also decreased in NpHR group, and there was no difference in net-TH (+) cells without α-Syn aggregates, which are functioning, between experimental and control groups.

Effect of optical inhibition on GABA secretion

The differences in GFAP (+)-particle number (p = 0.1014), -total area (p = 0.0734), -average area (p = 0.8357), and -signal intensity (p = 0.6014) between the experimental and control groups were not statistically significant. Meanwhile, the percentage of area where GFAP overlapped with the α-Syn aggregates in the GFAP (+) cells was significantly reduced in the experimental group compared to the control group with 5.6±4.9 (0.3–16.6) % versus 14.2±9.3 (3.1–34.0) %, decreasing by 60.6% (p = 0.0017).

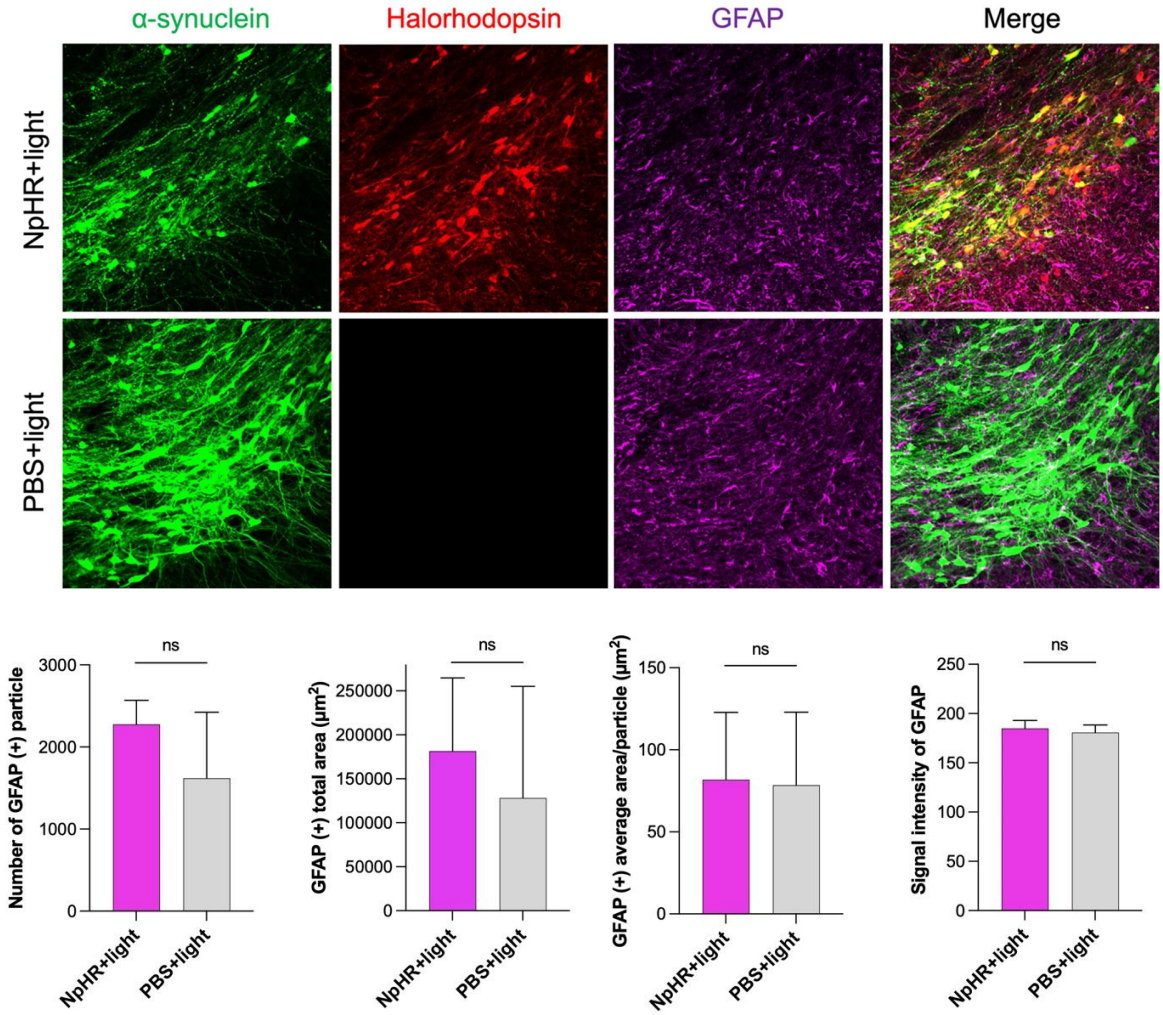


Figure 6. GFAP expression was not significantly different between the experimental and control groups

GABA (+)-cell count and -total area were significantly decreased in the experimental group compared to the control group by 52.0 % (p = 0.0387) and 72.6% (p = 0.0486). The percentage of co-localization area overlapping GABA with the α-Syn aggregates in the GABA (+) area was significantly decreased from

31.3±20.4 (1.3–63.2) % in the control group to 9.0±13.7 (0–46.5) % in the experimental group, eliciting the reduction rate of 71.2 % (p = 0.0002).

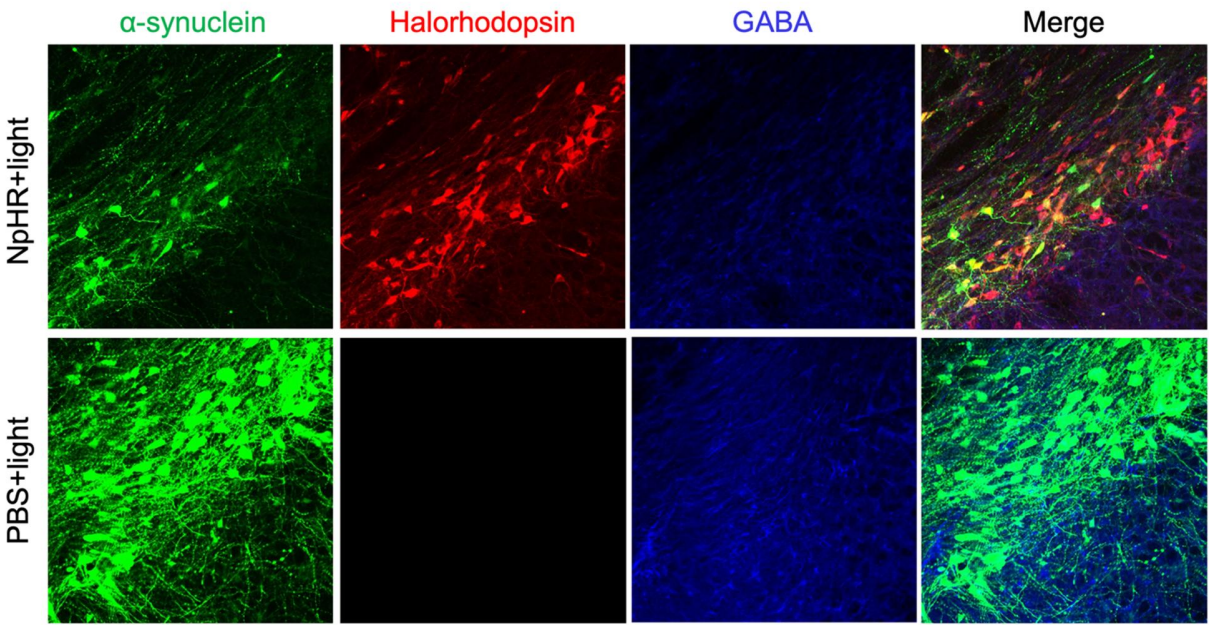
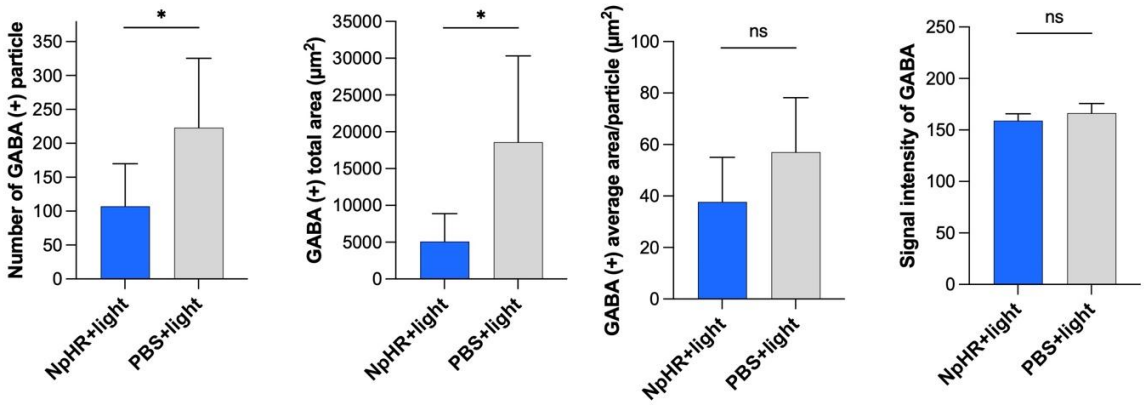


Figure 7. After illumination, GABA amount in the SNpc significantly decreased in the halorhodopsin



(NpHR) transduced group compared to the control group

Discussion

Pathological hallmarks of the PD are the widespread accumulation of intraneuronal α-Syn aggregates and progressive neuronal cell loss. DA neurons of the SNpc have been well known to be most vulnerable to the α-Syn pathologies.[3-5] α-Syn is considered a natively unfolded monomer and implicated in the modulation of synaptic activity by regulating the synaptic vesicle release, although its physiological function remains

to be fully elucidated. However, when a-syn is misfolded by disease-related mutations, it is prone to self-aggregate, and these synuclein aggregates are toxic and impair the functions of mitochondria, lysosomes, and endoplasmic reticulum, and interferes with microtubular transport, resulting in neurodegeneration. [10, 11] Also, the clearance of intraneuronal α-Syn aggregates in PD is impaired due to defects in proteosome and lysosome, facilitating the further accumulation of α-Syn protein and neurodegeneration. [12-14] It has been believed that dysfunction of the DA neurons in PD and consequential dopamine depletion is irreversible. However, a recent study demonstrated that 24-hour optogenetic stimulation of DA neurons transduced with Channelrhodopsin-2 in the SNpc of the A53T rat model recovered TH expression from TH [15] dormant DA neurons and alleviated parkinsonism. [9] Indeed, the expression of TH, the rate-limiting enzyme of dopamine biosynthesis, depends on neuronal activity and can be regulated by increasing or decreasing the firing of the action potential. [9, 16, 17] The reversibility of TH expression and dopamine production in the dormant DA neurons implicated the potential for a novel strategy of disease-modifying therapies which rescues the inactive DA neurons by restoring the neuronal activity in PD.

Astrocytes are a key player in response to CNS injury such as neuroinflammation. They undergo morphological and functional changes in the process of reactive astrogliosis. Reactive astrocytes are present in brain regions implicated in neurodegeneration in a wide of chronic neurodegenerative disease, including Alzheimer's disease and Parkinson's disease. [18] Although reactive astrocytes have long been considered a byproduct of the neuroinflammation process occurring secondary to neurodegeneration, recent studies suggested that they may participate in worsening of neuronal dysfunction and disease progression by augmenting the synthesis and release of the major inhibitory neurotransmitter GABA in the pathological conditions. [7, 8, 19-21] A recent study verified that the DA neuronal firing rate was significantly suppressed by approximately 60–80% in PD animal models. However, it recovered considerably after treatment with monoamine oxidase-B (MAO-B) inhibitors that block GABA production, such as selegiline and safinamide. [9] Recent study reported that the MAO-B inhibitor also significantly restored the TH level in the SNpc that was decreased depending on suppressed DA neuronal firing by aberrant tonic astrocytic GABA.

Based on these preceding findings of the literature, this study hypothesized that inhibition of neuroinflammatory reactive astrocytes would revitalize the DA neurons in the dormant or dysfunctional state, improving parkinsonian motor symptoms. Aiming to inhibit reactive astrocytes spatiotemporally selective, AAV containing NpHR under the promoter of GFAP was unilaterally introduced on the SNpc in the A53T PD rat model. NpHR gene encodes the chloride ion channel, which opens responsive to yellow light with a length of 590 nm, inducing hyperpolarization of membrane potential. [22, 23] Therefore, we

speculated that optogenetic inhibition of reactive astrocytes would disinhibit the suppressed DA neurons by blocking the astrocytic aberrant tonic GABA synthesis and secretion, resulting in ameliorating parkinsonism. This study verified that NpHR activation in the astrocytes markedly improved parkinsonism in the α-Syn overexpression animal PD model established using the human A53T mutant gene. Although it may be logical that GFAP expression would decrease after illumination in the experimental group expressing NpHR, there was a trend that the amount of GFAP expression was higher in the experimental group than in the control group. GFAP is a routine identifier of astrocytes, and increased GFAP in astrocytes has been used as a standard marker of astrocyte activity. [24, 25] Astrocytes undergo the process of reactive astrogliosis to exist in at least two distinct reactive states of being neurotoxic or neuroprotective, termed "A1" and "A2", respectively, depending on the type of stress. [18, 25] A1 neuroinflammatory reactive astrocytes (A1s) release many classical complements cascade components gene causing synaptic degeneration as well as neurotoxin. In contrast, A2 reactive astrocytes are known to upregulate many neurotrophic factors responsible for promoting the neuronal regeneration and synapse repair. [26-28] Unfortunately, GFAP expression is elevated in both types of reactive astrocytes, and an advanced study is needed to distinguish between different activation states of reactive astrocytes. [25] Apart from the upregulated GFAP levels, in this study, GABA in the SNpc was significantly decreased by NpHR activation in astrocytes by 590 nm light in the experimental group compared to the control group. Activated NpHR drives the influx of Cl^- ions into the cell, causing hyperpolarization. [29] Photostimulated Channelrhodopsin 2 in astrocytes leads to an influx of cations, namely, H^+, Na^+, and Ca^{2+}, and influences ion homeostasis in the synapses. A consequent increase in intracellular Ca^{2+} has been reported to cause the release of gliostransmitters, such as glutamate and ATP, enhancing both excitatory and inhibitory synaptic transmission. [29-32] Contrary to Channelrhodopsin 2, however, the impact of NpHR activation in astrocytes has not been evaluated on the regulation of flux of ions/neurotransmitters across the cell membrane, intracellular signaling, and synaptic transmission. Transmembrane Cl^- gradients play prominent roles in signaling in astrocyte physiology. [33] Also, GABA uptake and release via the GABA transporter pathway in the astrocytes occurs coupled with the flux of $2Na^+$ and Cl^- in the same directions. [34] Hence, it is plausible that the manipulation of Cl^- levels in astrocytes via optogenetic stimulation may induce signaling pathways involved in the downregulation of GABA synthesis or GABA release. The direct effect of increased Cl^- by NpHR activation on GABA production and secretion dynamics in astrocytes needs to be elucidated. We could not confirm the recovery of DA neuronal firing and increased TH expression following illumination in this experiment. Given that NpHR was partially transduced in the DA neurons in addition to the astrocytes, the absolute values of TH (+)-cell counts, -total area, and -signal intensity might be inevitably

decreased in the experimental group after illumination. However, the TH (+) cells in which the pathological α-Syn aggregates were accumulated were also decreased in the experimental group, and therefore, there was no significant difference in TH expression between the two groups when only DA neurons without the α-Syn overexpression were counted. More than that, the animals were euthanized immediately after 1-hour illumination, and thus, we consider that the TH level might not have been recovered in the experimental group due to the latent time for TH to be expressed. Notably, the amount of α-Syn aggregates was significantly reduced in the experimental group after illumination compared to the control group, which implies that clearance of the pathological α-Syn aggregates was increased. The clearance systems for intraneural inclusion bodies depend on neuronal activity and are suppressed in chronic neurodegenerative diseases such as PD[13, 14]; However, their functions can be recovered as the neuronal activity is restored. Indeed, a recent study on Alzheimer's disease and frontotemporal dementia animal models reported that synaptic activation by chronic DBS promotes autophagic-lysosomal degradation, thereby enhancing the clearance of tau oligomers.[35] We speculate that the immune systems, such as the autophagic-lysosomal system and the ubiquitin-proteosome system, were reactivated in the revived DA neurons in the SNpc, degrading the α-Syn aggregates[36-39]; and that restored synaptic activities of the neurons with decreased α-Syn aggregates may be the fundamental mechanism associated with improvement of contralateral forelimb akinesia.

Conclusion

Optogenetic stimulation of NpHR in reactive astrocytes of SNpc in the A53T PD model significantly decreased GABA which functionally suppressing DA neurons and the pathological α-Syn aggregates, resulting in restoration of parkinsonian motor symptoms. These findings suggest that regulating reactive astrocyte activity can be a novel therapeutic strategy for PD. A substantial amount of research is still needed to find the methods to precisely control optogenetic expression in the reactive astrocytes and to investigate the mechanism of reducing α-Syn aggregates by astrocyte inhibition. In addition, the antecedent relationship between the emergence of reactive astrocytes and α-Syn aggregates and which of them has a more significant impact on the pathogenesis of PD should also be elucidated.

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Abstract

Background and objectives

It has been long considered that degenerative changes in dopamine (DA) neurons by α-synuclein (α-Syn) aggregates are irreversible in Parkinson's disease (PD); however, a recent study revealed the presence of DA neurons in a dormant state that doesn't express the tyrosine hydroxylase (TH) and is functionally inactive but rescuable. That is, the administration of monoamine oxidase B (MAO-B) inhibitor, a r-aminobutyric acid (GABA) synthase in the astrocytes, decreased the aberrant tonic GABA secretion by reactive astrocytes and restored the DA neuronal activity, improving parkinsonian motor symptoms. However, in addition to being involved in GABA production, as MAO-B contributes to PD through various mechanisms, such as degrading DA and increasing oxidative stress to induce neurodegeneration, the effects of MAO-B inhibitors are also complex. Therefore, this study aimed to verify the impact of reactive astrocyte inhibition on GABA production/secretion and parkinsonian motor symptoms in the PD animal model through optogenetic technology that introduces ion channels into specific cells and regulates their activity.

Methods and materials

AAV2-CMV-α-synuclein (A53T)-EGFP was injected into the dorsal border of substantia nigra pars compacta (SNpc) of 16 Wistar rats to establish an α-Syn overexpressing PD model. After 2 weeks, the experimental group (n = 8) was injected with AAV_{DJ}-GFAP104-eNpHR3.0-mCherry to transduce a halorhodopsin (NpHR) optogene into reactive astrocytes, and phosphate-buffered saline was injected into the control group (n = 8). After 2 weeks, optical fibers were inserted in all animals, and after 1 week, light with a wavelength of 590 nm was applied for 1 hour at 50 Hz and 10 ms pulse duration. Changes in contralateral forelimb akinesia before and after light stimulation, TH expression, glial fibrillary acidic protein (GFAP), GABA, and α-Syn levels were compared between the experimental group and the control group.

Results

In the experimental group, NpHR was expressed in 30.1±19.5 (0.9–68.2) % of GFAP (+) cells. There was no statistical difference in GFAP expression between the two groups. However, the amount of GABA was significantly reduced in the experimental group by 72.6% (p = 0.0486) after illumination. Also, the amount of α-Syn aggregates in SNpc in the experimental group was significantly reduced by 67.5% (p <

0.0001) compared to the control group. Meanwhile, NpHR was also expressed in 30.3±11.6 (11.1–49.6) % of TH (+) cells in the experimental group, and thus, the number of TH (+) cells was reduced by 27% (p = 0.0132) compared to the control group. In the behavior test, contralateral forelimb akinesia in the experimental group was significantly improved by 81.4±7.2 (71.6–90.9) % after illumination compared to the pre-light stimulation state (p = 0.0002), while motor symptom was worsened by 49.5±23.2 (9.3–85.1) % (p = 0.0003) in the control group.

Conclusions

Functional inhibition of reactive astrocytes by optogenetic stimulation of NpHR in α-Syn overexpression PD animal model decreased GABA production and α-Syn aggregates, improving parkinsonian motor symptoms. This finding suggests that regulating reactive astrocyte activity can be a novel therapeutic strategy for PD. Further studies are needed to find the methods to precisely control optogenetic expression in the reactive astrocytes and to investigate the mechanism of reducing α-Syn aggregates by astrocyte inhibition. In addition, the antecedent relationship between the emergence of reactive astrocytes and α-Syn aggregates and which of them has a more significant impact on the pathogenesis of PD should also be elucidated.

Keywords: alpha-synuclein, GABA, Halorhodopsin, optogenetics, Parkinson's disease, reactive astrocyte