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Restoration of Adult Neurogenesis by Intranasal Administration of Gangliosides GD3 and GM1 in The Olfactory Bulb of A53T Alpha-Synuclein-Expressing Parkinson's-Disease Model Mice

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting the body and mind of millions of people in the world. As PD progresses, bradykinesia, rigidity, and tremor worsen. These motor symptoms are associated with the neurodegeneration of dopaminergic neurons in the substantia nigra. PD is also associated with non-motor symptoms, including loss of smell (hyposmia), sleep disturbances, depression, anxiety, and cognitive impairment. This broad spectrum of non-motor symptoms is in part due to olfactory and hippocampal dysfunctions. These non-motor functions are suggested to be linked with adult neurogenesis. We have reported that ganglioside GD3 is required to maintain the neural stem cell (NSC) pool in the subventricular zone (SVZ) of the lateral ventricles and the subgranular layer of the dentate gyrus (DG) in the hippocampus. In this study, we used nasal infusion of GD3 to restore impaired neurogenesis in A53T alpha-synuclein-expressing mice (A53T mice). Intriguingly, intranasal GD3 administration rescued the number of bromodeoxyuridine + (BrdU +)/Sox2 + NSCs in the SVZ. Furthermore, the administration of gangliosides GD3 and GM1 increases doublecortin (DCX)-expressing immature neurons in the olfactory bulb, and nasal ganglioside administration recovered the neuronal populations in the periglomerular layer of A53T mice. Given the relevance of decreased

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Competing interests The authors have no relevant financial or non-financial interests to disclose.

Declarations

Ethics approval All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Augusta University (AU) according to the National Institutes of Health (NIH) guidelines and performed with the approved animal protocols (references AUP 2009–0240 and 2014–0694).

Consent to participate N/A.

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The gangliosides nomenclature are based on Svennerholm [1] and IUPAC–IUB [2].

ganglioside on olfactory impairment, we discovered that GD3 has an essential role in olfactory functions. Our results demonstrated that intranasal GD3 infusion restored the self-renewal ability of the NSCs, and intranasal GM1 infusion promoted neurogenesis in the adult brain. Using a combination of GD3 and GM1 has the potential to slow down disease progression and rescue dysfunctional neurons in neurodegenerative brains.

Keywords

Ganglioside GD3; Ganglioside GM1; A53T alpha-synuclein; Parkinson's disease; Neural stem cell; Neurogenesis

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the progressive loss of motor functions, leading to bradykinesia, rigidity, and tremor. Etiological analysis identified that the degeneration of dopaminergic neurons at the substantia nigra pars compacta, which projects into the striatum, is the main cause of the disease. Neurodegeneration in the midbrain is induced by the deposition of alpha-synuclein (aSyn), which forms cytotoxic oligomers and aggregates that build inclusion bodies, referred to as Lewy bodies [3].

Gangliosides, sialic acid-containing glycosphingolipids, are particularly abundant in the nervous system, and their metabolism is closely associated with brain development. We have identified that GD3 is the predominant ganglioside species in neural stem cells (NSCs) [4] and that GD3 modulates NSC self-renewal by interacting with epidermal growth factor receptor (EGFR) signaling [5]. In postnatal brains, GD3 is crucial for the long-term maintenance of NSCs [6–8]. Deficiency in GD3 leads to developmental and behavior deficits such as depression and impairment in hippocampus-dependent memory function. The synthesis of GD3 is switched to the synthesis of complex, brain-type gangliosides including GM1, resulting in terminal differentiation and loss of stemness of NSCs. We have reported that histone acetylation status of glycosyltransferase genes directly links the developmental profiles of ganglioside composition, and nuclear gangliosides contribute to neuronal cell fate specification by an epigenetic regulatory mechanism in mouse brains [9–13]. GM1 has been reported to be the key molecule for the pathogenesis of PD since GM1 binds to aSyn with high affinity to stabilize the alpha-helical state and inhibit oligomerization [14]. Further, GM1-deficient mice exhibit PD-like movement disorders, elevated aSyn expression and reduced expression of tyrosine hydroxylase (TH), which is the enzyme responsible for the primary step of dopamine synthesis in the substantia nigra pars compacta [15–20]. In addition, the depletion of GM1 is reported to result in most of PD's symptoms, including motor impairment, striatal dopamine depletion, selective loss of TH-expressing neurons, gastrointestinal dysfunction, cardiac pathology, and cognitive impairment, suggesting that the deficiency of GM1 neuropathologically correlates with motor- and non-motor symptoms of PD in mice and humans. Significantly, B3galt4 (GM1 synthase) gene expression which catalyzes a conversion of ganglioside GM2 to GM1 was reduced in the substantia nigra of PD patients [21]. Further, total ganglioside levels

including GM1 and GD3 were reduced in the substantia nigra and occipital cortex and of PD patients compared with non-PD age-matched controls [17, 22, 23].

Interestingly, intraperitoneal injection of GM1 or the GM1 analog LIGA20 reduced the deposition of aSyn and ameliorated the motor deficit symptoms in PD patients and GM1-deficient rodents, despite the remaining unsolved problems regarding the difficulty of GM1 passing through the brain-blood barrier and the possible cytotoxicity of LIGA20 [22, 24–30]. In recent years, intranasal administration has been attracting wide attention as a promising route to deliver drugs, nutritional factors, and stem cells to the brain [31–34]. In fact, intranasally administered gangliosides were delivered into the brain of A53T aSyn-expressing PD-model mice (A53T mice), which develop intracellular aggregation of aSyn like PD patients [35], and intranasal infused GM1 or GD3 removed the toxic aSyn accumulation from the brain of A53T mice. Further, intranasal infused GM1 restored TH expression via recruiting the transcriptional factor Nurr1 into the nuclei of dopaminergic neurons [10]. Therefore, intranasal administration is a promising ganglioside delivery route, bypassing the blood–brain barrier and raising a potential strategy for the treatment of PD and other neurodegenerative diseases. Taken together, reduction of GM1 tends to increase aSyn, and further aSyn upregulation might consume endogenous GM1 to reduce aSyn toxicity. Therefore, intranasal infusion of GM1/GD3 could supplement the shortfall in the endogenous gangliosides to prevent PD pathogenesis. PD patients and model mice also reveal non-motor symptoms including loss of smell (hyposmia), sleep disturbances, depression, anxiety, and cognitive function disorder in the premotor phase impacting daily life [36–38]. It is suggested that smell impairment is partially related to the NSC dysfunctions and neurogenic deficits of the olfactory bulb (OB). It is desirable to elucidate the relationship between non-motor symptoms and adult neurogenesis for a better understanding of the disease mechanism and a therapeutic strategy.

Although the biological significance of adult hippocampal neurogenesis in humans is still under debate [39–44], the detection of adult neurogenesis has brought about the hypothesis that the adult brain exhibits more plasticity than previously thought, and that this contributes to memory and the pathogenesis of neurodegenerative diseases [40, 42, 44–47]. The clinical significance of altered neurogenesis in the neurodegenerative disease pathogenic mechanism is not settled. Although it is reported that the proliferation of NSCs at the subventricular zone (SVZ) was not changed and that dopaminergic neurons in the OB of postmortem PD brains were increased [48–50], other studies reported decreased proliferating cells in the SVZ of human postmortem PD brains [51] and in human A30P aSyn-expressing PD-model mice [52, 53]. Further, newborn neurons at the OB were decreased in human aSyn- or A30P aSyn-overexpressing mice [52, 54]. It is conceivable that there is an active level of neurogenesis in the early/moderate onset of the disease, but neurogenesis then becomes sluggish in the late/severe neurodegenerative disease brains [40, 42, 44, 55–57]. Altered NSC activities in the distinct disease phases (early or late stage) are a partial mechanism for this controversial issue in abnormal neurogenesis during neurodegenerative diseases. Diminished neurogenesis occurs in normal aging, and it is hypothesized that an accelerated loss of the NSC pool is one mechanism for the transition from having a healthy brain to neurodegenerative disease [42, 44, 45]. It has been reported that A53T mice developed

olfactory dysfunction and impaired neurogenesis in the SVZ and dentate gyrus (DG) [58–64].

Many fundamental cellular processes in NSC fate-determination and self-renewal are heavily influenced by glycoconjugates, including gangliosides [65, 66]. We showed that the intracranial administration of GD3 into the lateral ventricle of GD3-deficient adult mice resulted in an increase of the NSC population in the SVZ and DG, which might be evoked by enhanced EGF signaling through the interaction of EGFR with GD3 on the microdomain of NSCs [5–7]. On the other hand, GM1 has a specific role in adult neurogenesis to promote neuronal gene expression for neuronal differentiation [12, 67, 68], and in the sustenance of neuronal gene expression for mature neuronal functions [10]. Intracerebroventricularly (icv) administered GD3 augments self-renewal, and cells expressing the multipotent marker, sex determining region Y (SRY)-box 2 (Sox2), and GM1 increases newly generated immature bromodeoxyuridine + (BrdU +) / doublecortin + (DCX +) neurons in five familial Alzheimer disease (5XFAD) mouse brains [7]. These studies suggest that intranasal delivery of GD3 and GM1 into the brain might increase the NSC self-renewal-replication and their progeny in the SVZ and OB of mice.

Here we show the reduced NSC self-renewal capacity with elevated cyclin-dependent kinase (CDK) inhibitor p21 (also known as p21(WAF1/Cip1)) expression at the SVZ of A53T mice, whereas intranasal infusion of GD3 reversed this cell cycle arrest in NSCs, and the DCX + immature neurons were increased at the OB by administration of GM1/GD3. Further, GM1 treatment restored olfactory neurons, including tyrosine hydroxylase (TH)- and calretinin (CR)-expressing cells at the periglomerular layer (PGL) in the OB of A53T mice. These results suggest that olfactory dysfunction, a non-motor pathology of PD, could be recovered via restoration of adult neurogenesis to sustain olfactory neurons by combinatorial intranasal administration of GD3 and GM1.

Materials and methods

Antibodies

For this study, the following antibodies were purchased: mouse anti-BrdU (RRID:AB_10015222; BD Biosciences, San Jose, CA, USA, #555,627), rabbit anti-Sox2 (RRID:AB_823640; Cell Signaling Technology, Danvers, MA, USA, #2748S), rabbit anti-p21 (RRID:AB_823586; Cell Signaling Technology, #2947S), mouse anti-Nestin (RRID:AB_396354; BD Biosciences, #556,309), rabbit anti-GFAP antibody (RRID:AB_10013382; Agilent Dako, Santa Clara, CA, USA, #z-0334), mouse anti-DCX (RRID:AB_10610966; Santa Cruz Biotechnology, CA, USA, #sc271390), rabbit anti-TH (RRID:AB_390204; Millipore, St. Louis, MO, USA, #AB152), rabbit anti-CR (Synaptic Systems GmbH, Göttingen, Germany, #214 102), rabbit anti-actin (RRID:AB_476693; Sigma, St. Louis, MO, USA, #A2066), Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) (RRID: AB_2536161; Invitrogen, #A28175), Alexa Fluor 488-conjugated goat anti-rabbit IgG (RRID:AB_143165; Invitrogen, #A11008), Alexa Fluor 568-conjugated goat anti-rabbit IgG (RRID:AB_143157; Invitrogen, #A11011), Alexa Fluor 647-conjugated goat anti-rabbit IgG (RRID:AB_2536101; Invitrogen, #A27040), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (RRID:AB_2556546; Invitrogen, #R37118),

Alexa Fluor 488-conjugated donkey anti-mouse IgG (RRID: AB_2556542; Invitrogen, #R37114) antibodies, and Alexa Fluor 594-conjugated cholera toxin subunit B (CtxB; Invitrogen, #C34777).

Experimental mouse models

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Augusta University (AU) according to the National Institutes of Health (NIH) guidelines and were performed with approved animal protocols (references AUP 2009-0240 and 2014-0694). For the PD model, mice expressing A53T mutant human aSyn under the murine prion promoter (B6.Cg-2310039L15RikTg (Prnp-SNCA*A53T) 23Mkle/J) (RRID:IMSR_JAX:006,823) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA, stock no 006823) and are referred to as the A53T mice. C57B6L/6 J mice were also obtained from The Jackson Laboratory (stock no. 000664). The original GD3-synthase (GD3S, sialyltransferase-II; ST-II, or St8Sia1)-knockout (KO) mice (RRID:MMRRC_000037-MU) and their wild-type (WT) mates were kindly provided by Dr. Richard Proia (NIDDK, NIH, Bethesda, MD) and were crossed to generate heterozygous mice with C57BL/6 J. The heterozygous male and female mice were mated and subjected to PCR screening for genotyping. Littermate WT mice were used as controls. All mice were housed in standard conditions with food and water provided ad libitum and maintained on a 12-h dark/12-h light cycle. Male mice were used in all experiments. No animals were excluded in this study.

Intranasal ganglioside administration

The acquisition, care, and experimental procedure for animals used in this study were in compliance with the NIH guidelines as published in the Guide for the Care and Use of Laboratory Animals. Currently, icv administration is the most reliable method to deliver gangliosides into the brain. We successfully developed a more convenient, non-invasive delivery procedure by intranasal infusion of gangliosides. GM1 was infused into 8-month-old GM2 synthase (GM2S, N-acetylgalactosamine transferase (GalNAc-T); B4galnt1)-KO mice (RRID:MMRRC_000036-MU) [10]. Since these mice do not have GM1, exogenous GM1 can be detected. Immunohistochemical analyses revealed that intranasally administered GM1 was distributed to brain tissues [10], including cortex, olfactory bulb (OB), subventricular zone (SVZ), hippocampus, midbrain, and cerebellum. Intranasal infused GM1 was visualized with Alexa Fluor 594-conjugated cholera toxin subunit B (CtxB, 1:5,000: CtxB has been frequently used as a probe of GM1) (Supplementary Figure. S1). Partial specific volumes of GM1 and GD3 were similar (estimated 0.781 mL/g and 0.772 mL/g, respectively) [69]. The critical micelle concentrations of monosialogangliosides and disialogangliosides are reported as $8.5 \times 1\ 0^{-5}$ M and $9.5 \times 1\ 0^{-5}$ M, correspondingly [70]. Those chemical characteristics of GD3 predict that GD3 is easy to intranasally enter to the brain, similar to GM1. According to our previous experiments [10] and that neurogenesis could be detected with 28 days [6], we chose 5 mg/kg/day for 28 days as intranasal ganglioside treatment without any anesthetic treatment. Gangliosides (GD3 or GM1; 5 mg/kg/day) were intranasally administered [71] into 8-month-old WT (C57B6/J), A53T mice. Also, 5 mg/kg/day of GD3 was intranasally infused into GD3S-KO mice at 5–7-month-old (male) for 28 days using capillary tips (Bio-Rad, Hercules, CA, #2,239,915)

with 6 μL into the right and the left nares twice daily (6 μL × 2 nares = total 24 μL per day for 28 days), then the animals were housed for 28 days without treatment, and their olfaction was analyzed at 7–9-month-old. For intranasal administration, the mouse was held on the back of the neck using the thumb and pointer finger, and then inverted with the ventral side facing up towards the ceiling. Six μl of ganglioside solution or saline was intranasally infused into the nare of the mouse. The infused animal was held this position for 30 s. The procedure was repeated for 4 times (total 24 μl infusion). The GD3 and GM1 used in this study were isolated from either bovine buttermilk or brains in our laboratory by established procedures [72–74]. GD3 and GM1, being amphipathic, were easily dissolved in saline. Animals were divided into five groups: (1) WT and (2) A53T mice with saline infusion as placebo groups; (3) A53T with GD3 (5 mg/kg/day) infusion group; (4) A53T with GM1 (5 mg/kg/day) infusion group; and (5) GD3 infusion plus GM1 infusion group (GD3 at 5 mg/kg/day for 14 days, then GM1 at 5 mg/kg/day for the other 14 days). Each group consisted of n = 3–4 animals. BrdU (Sigma; 50 mg/kg body weight) was administered intraperitoneally during the final week. Also, 5 mg/kg/day of GD3 was intranasally infused into GD3S-KO mice (5–7-month-old, male) for 28 days.

Immunohistochemistry

Mice were anesthetized with isoflurane using an open-drop method and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde (PFA). The brains were collected and post-fixed with 4% PFA overnight, followed by cryoprotection with 30% sucrose in PBS at 4 °C overnight; this was repeated more than three times with fresh sucrose-PBS. After embedding in the Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA), the brains were quickly frozen in liquid nitrogen. Cryosectioning was performed to obtain 20 μm thick coronal sections using a cryostat (Leica, Wetzlar, Germany). For staining of DCX, CR, or co-staining of Sox2 and BrdU, or Nestin and GFAP, sections were treated in a microwave for 5 min in pre-boiled 10 mM citrate buffer (pH 6.0), followed by permeabilization with PBS containing 0.5% Triton X-100 for 5 min and blocked with PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature, and then incubated with anti-DCX antibody (1:50, Santa Cruz, #sc271390), or anti-CR antibody (1:100, Synaptic Systems GmbH, #214 102), rabbit anti-Sox2 antibody (1:100, Cell Signaling Technology, #27,485) and mouse anti-BrdU antibody (1:500, BD Biosciences, #555,627) or mouse anti-Nestin antibody (1:100, BD Biosciences, #556,309) and rabbit anti-GFAP antibody (1:100, Agilent Dako, #z-0334) at 4 °C overnight. For immunostaining p21, antigen retrieval was performed by autoclave treatment in 10 mM citrate buffer (pH 6.0) at 105 °C for 10 min, followed by permeabilization and blocking as described above. Then, sections were subjected to reaction with rabbit anti-p21 antibody (1:100, Cell Signaling Technology, #2947S) followed by incubation with Alexa Fluor-conjugated secondary antibody for 2 h at room temperature. Nuclei counterstaining was performed with 1 μg/mL 40,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, #D1306) for 30 min. After every incubation with antibodies or chemicals, sections were washed three times with PBS. Specimens were mounted with VectaMount (Vector Laboratories, Burlingame, CA, USA).

Microscopy and image processing

Images of labeled sections were acquired by a Zeiss LSM700 (Carl Zeiss, Land Baden-Württemberg, Germany) with a 63x (1.4 NA oil immersion, Plan Apochromat) objective as 10 μm of z-stacks of optical slices or a Nikon A1R MP + multiphoton/confocal microscope (Nikon, Tokyo, Japan) with an ApoLWD 25 × (1.10 W DIC, N2) objective. The microscope settings were kept constant for each staining. Serial z-images were stacked using Fiji (NIH, Bethesda, MD, USA) and merged using Photoshop (Adobe, San Jose, CA, USA). For quantification, Nestin and GFAP, Sox2 and BrdU, and p21 in the SVZ were counted at the DAPI-stained germinal zone. TH and CR-labeled cells were counted in the periglomerular layer of the OB for each saline or ganglioside-treated group ($n = 3 – 4$ mice, 5 sections per each mouse; $n = 15 – 20$ sections per group). Immunofluorescence higher than background and specific staining pattern/localization were considered to determine positive/negative for each staining when cells were counted using Fiji image analysis. To identify activated NSCs, Sox2 and BrdU double-positive cells were counted when their staining was overlapped with DAPI. BrdU-positive cells within the SVZ were counted 5 Sects. (200 μm apart) per animal. To calculate the total number of marker-double-positive cells, at least 1,000 Sox2-positive cells per group were analyzed. For counting DCX +, TH +, or CR + cells, 5 Sects. (200 μm apart) per animal and at least 1,000 DAPI-positive cells in each region of interest per group were analyzed. The blinding procedures and randomized field approach for images were performed to acquire unbiased results. To generate robust and unbiased results, the recommended conventions for double-blinding in mouse experiments were followed.

Buried pellet test

The WT and GD3S-KO mice (4–6-month-old and 7–9-month-old GD3 infused mice; male) fasted for 24 h before testing in a home cage with a water bottle. On the day of the experiment, the mice were habituated in a testing cage with 3 cm depth bedding for 10 min and placed back in the home cage. Then a food pellet (1 cm^3) was placed under the bedding in the testing cage. The mouse was placed in the center of the testing cage and the latency (seconds) to uncover the food pellet was measured. If the mouse did not find the pellet within 5 min, the trial was finished and a score of 300 s was noted for the mouse. The time until mice uncover the food pellet in bedding was measured as an average of 4 trials. A pellet was embedded beneath the bedding at a different position in each trial. After all mice were tested, they were fed. Behavioral experiments were performed between 8 and 11 am.

Statistical analysis

All statistical procedures were performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). There was no test for outliers and no pre-determined exclusion criteria. We determined our sample sizes according to our previous study [10]. To estimate the biological variability, ensure the quality and consistency between biological samples, and meanwhile maximize the cost-effectiveness, we chose to use 3–5 animals per condition for ganglioside infusion experiments according to our published study. Group data were analyzed using the Shapiro–Wilk test and Brown-Forsythe test to examine their normality and the homogeneity of variances of datasets, respectively. When the data set passed both, one-way ANOVA with a Dunnett's multiple comparison test was performed. Kruskal Wallis test followed by

Dunn's multiple comparison test was performed when the data sets failed to exhibit a normal distribution nor homogeneity. An unpaired two-tailed Student's *t*-test was performed if the Shapiro–Wilk test and F-test reported a normal distribution and homogeneity. If not, the Mann Whitney test was performed. In all cases, p values < 0.05 were regarded as significant, as shown in the figure legends. The current research was performed after the previous study [10], so we used almost the same scale of mice. In addition, post-hoc power $(1 - \beta)$ was calculated using G*Power 3.1.9.7 (Heinrich-Heine-Universität, Düsseldorf, Germany). The comparison between WT ($n = 8$ mice) vs. GD3S-KO ($n = 9$ mice) for olfactory functional test (Fig. 7A) was Power $(1 - \beta) = 0.881$. And the comparison GD3S-KO ($n = 3$ mice) with GD3S-KO + GD3 ($n = 5$ mice) in Fig. 7C was Power $(1 - \beta) = 0.806$. Our experiments (minimum $n = 3$ mice) were above the desired Power level (0.8). To estimate the biological variability, ensure the quality and consistency between biological samples and meanwhile maximize the cost-effectiveness, we chose to use 3–5 animals per condition for ganglioside infusion experiments according to our published study [10]. All data distribution was depicted by a box and whisker plot (Figs. 2, 3, 4, 5, 6, and 7). A box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values outside of the upper and lower quartiles. The median values are represented by bars in the boxes.

Results

Intranasal GD3 infusion restored the self-renewal ability of NSCs in the SVZ of A53T mice

The molecular structures of GD3 and GM1 (Fig. 1) indicate that both molecules are amphiphilic due to the lipid moiety of ceramide and the hydrophilic carbohydrate headgroup with N-acetylneuraminic acid [75]. It was reported that neuronal precursor cells reached the OB from the SVZ taking 21 days [76]. We have confirmed that icv injection of GD3 for 7 days promoted the self-renewal of Sox2 + cells in the SVZ [7]. Further, we have reported that GM1 reached the brain by intranasal infusion for 7 days [10]. Based on these studies, we assumed that it might take 7 days for activation of NSCs and a further 21 days for migration of progenitor cells to the OB. Thus, we chose 28 days of infusion for cumulative effect of intranasal delivered gangliosides. Our previous study demonstrated that intranasal infusion of GD3 or GM1 significantly reduces intracellular aSyn levels in the brain of A53T mice [10]. Additionally, icv administered GD3 and GM1 have neurogenic effects in adult mice [7]. Therefore, we attempted intranasal ganglioside infusion to modulate adult neurogenesis as a novel treatment strategy for non-motor symptoms of patients with PD. To investigate the self-renewal ability of NSCs in the SVZ after 4 weeks (28 days) of intranasal saline or ganglioside infusion, we intraperitoneally administered BrdU into A53T mice for 5 days prior to sacrifice (Fig. 2A). BrdU was incorporated in 37.94 ± 5.46% (Mean ± SEM) of Sox2-expressing cells ($n = 1680$) in the SVZ of WT animals (Fig. 2B–C). In contrast, only 16.83 ± 1.80% of Sox2 + cells ($n = 1467$) were labeled with BrdU in the SVZ of A53T mice (A53T + saline), showing a greater than 50% decrease in BrdU incorporation in the SVZ compared with WT. Meanwhile, GD3-infused A53T mice showed a partial but significant restoration of self-renewal ability of Sox2 + cells to 29.65 ± 2.15% ($n = 1326$). GM1 or GD3/GM1 treatments increased the percentage of BrdUlabeled cells in Sox2-expressing

cells (GM1: 23.91 ± 3.0% (n = 1200), GD3/GM1: 25.79 ± 1.92% (n = 1014), although there was no statistical significance.

GD3 suppressed the expression of the cell cycle inhibitor, p21, in the SVZ of A53T mice

It is hypothesized that neurogenesis can be modulated by cell cycle kinetics, such as the cell cycle length, and that the CDK inhibitor p21 can bind proliferating nuclear antigen (PCNA) and suppress PCNA-dependent DNA synthesis [77, 78]. To clarify the underlying mechanism of the results shown in Fig. 2, we examined the expression of p21 in A53T mice with and without intranasal ganglioside treatments (Fig. 3A). Only a very small fraction of cells (1.08 ± 0.18%) expressed p21 in the SVZ of WT mice at 9 months of age (Fig. 3B–C). Notably, 15-fold higher elevated expression (16.41 ± 5.60%) of p21 in the SVZ of A53T mice was observed. Importantly, overexpression of p21 was overridden by intranasal infusion of GD3 to only 1.51 ± 0.90% of cells expressing p21. To investigate the population of adult NSCs, we counted the Nestin and GFAP double-positive Type B cells in the adult brain (Fig. 3B). Although Nestin +/GFAP + radial glia-like NSCs tended to be reduced in the SVZ of A53T mice (WT; 58.40 ± 9.22% vs. A53T; 43.57 ± 10.62%), there is no significant difference between WT and A53T mice (Fig. 3D–3E). These results demonstrated that aSyn and GD3 act in an opposing manner for NSC self-replication through regulating p21 expression in the SVZ of A53T mice. aSyn suppresses NSC self-renewal, which results in a decline of adult neurogenesis in A53T mice, while GD3 promotes NSC activities to sustain the NSC pool in the adult brain.

Intranasal administration of gangliosides recovered DCX expression in the OB

Intranasal treatment with GD3 restored the self-renewal ability of NSCs in the SVZ of A53T mice (Fig. 2 and 3), consistent with our previous reports [6, 7, 68]. Further, increasing evidence has shown that GM1 is involved in neuronal maturation [12, 13, 77–82]. For neurogenesis in the OB, the immature neurons migrate through the rostral migratory stream (RMS) after being produced in the SVZ [83–85]. To address the question if the administration of gangliosides affects this process, we examined the effect of intranasal ganglioside infusion on neuroblast lineages in the OB by the expression of DCX, a marker for immature neuronal cells (Fig. 4A–C). The expression of DCX appeared to have a tendency to be downregulated in the OB of A53T mice, possibly due to reduced neurogenesis in this mouse line (Fig. 4B). DCX + cells in GD3-treated mice tended to be increased whereas the intranasal administration of GM1 and GD3/GM1 restored DCX + cells in A53T PD brain compared with A53T mice (WT + saline, 549.72 ± 95.36 cells/mm^2; A53T + saline, 377.83 ± 140.93 cells/mm^2; A53T + GD3, 978.47 ± 169.64 cells/mm^2; A53T + GM1, 995.28 ± 308.05 cells/mm^2; A53T + GD3/GM1, 1055.90 ± 613.94 cells/mm^2; Fig. 4C). These results demonstrate that while the intranasal administration of GD3 restored NSC self-renewal in the SVZ, intranasally infused GM1 plays a more critical role in neuronal maturation and migration in A53T mice.

Neuronal cells in the periglomerular layer in the OB were restored by intranasal administration of gangliosides

Sensory activities of perception and discrimination of odors are important parts of our daily life. Olfactory dysfunctions are one of the major non-motor symptoms in PD. The OB is the

first brain region where odors are processed. TH + neurons are involved in the processing of odors and the adaptation of the bulbar network functions from external conditions, while the function of CR + neurons is still difficult to identify [86]. Neurogenesis continues in the SVZ throughout life, and NSCs from SVZ contribute to those OB neurons that are integrated in the existing neuronal circuit [87]. An estimation suggested that10,000 new neurons are generated in the SVZ of adult mice daily and are incorporated into the OB via the RMS. For example, CR-positive granule neurons are mostly produced from the RMS and the wall of the anterior SVZ, and dopaminergic progenitor cells are produced in the dorsolateral region of the SVZ. Next, we investigated the population of TH-expressing dopaminergic neurons and CR-expressing neurons in the periglomerular layer (PGL), where old neurons are thought to be replaced by newly generated ones [86, 88], after the ganglioside treatment for 4 weeks (Fig. 5A). In the PGL of WT animals, 11.07 ± 0.74% cells were TH + neurons (Fig. 5B–C), and 11.66 ± 0.71% cells were CR + neurons (Fig. 6B–C); these quantities are comparable with those reported elsewhere [89]. While the TH + neurons were reduced (4.40 ± 1.35%) by more than 60% in the PGL of A53T mice compared to WT, intranasal GM1 infusion significantly restored the number of TH + cells to 8.73 ± 1.51% in A53T PD brain. CR + neurons were also reduced (6.00 ± 0.70%) to about half in the PGL of A53T mice compared to WT. We further demonstrated that the population of CR + neurons was also significantly restored (19.44 ± 2.86%) after GM1 administration (Fig. 6). Interestingly, the effect of combination treatment (GD3/GM1) differed between TH + dopaminergic and CR + neurons (TH + : 5.79 ± 0.29%; CR + 19.91 ± 1.51%). GD3 treatments did not increase the TH + cell population in A53T mice; however, CR + neurons are upregulated to the WT level by GD3 infusion (11.43 ± 1.46%). These results reflect a lower turnover rate of CR + neurons than the dopaminergic neurons after the administration of gangliosides in A53T mice, as reported previously [90].

GD3 plays a vital role in olfaction

We have reported that GD3S-KO mice exhibit a decreased neurogenesis in the OB [6]. Since the old neurons need to be replaced by newborn neurons in the OB to maintain olfaction [91–93], we hypothesized that GD3 is essential to the sense of smell and that GD3S-KO mice have olfactory impairment. The buried pellet test, which relies on the animal's natural tendency to use olfactory cues for foraging, is used to confirm the ability to smell. To evaluate the importance of GD3 in olfaction of adult mice (4–6-monthold), we performed the buried pellet test after 24 h of fasting [94, 95]. The time that it takes for mice to uncover the food pellet in bedding was measured. As expected, GD3Sdeficient mice needed more than twice the time period to find the buried food pellet beneath the bedding (WT vs. GD3S-KO; 45.43 ± 7.26 s vs. 103.4 ± 17.21 s) (Fig. 7A). We then investigated whether intranasal administration of GD3 rescues olfaction via activation of NSC self-renewal and neuronal maturation. Since we used 8–9-month-old of A53T mice for all experiments, we chose 7–9-month of age to assess olfactory functions of GD3S-KO mice. GD3 was intranasally infused into 5–7-month-old of GD3S-KO mice for 4 weeks (28 days). Mice were housed without any extra treatment for another 4 weeks for migration through the RMS and neuronal maturation of NSCs (Fig. 7B). GD3-infused GD3S-KO mice (7–9-month-old) exhibited partially, but significantly, improved olfaction than the saline-treated counterpart (GD3-KO vs. GD3S-KO + GD3; 280.8 ± 19.24 s vs. 214.7 ± 10.11 s) (Fig. 7C). These

results demonstrated that GD3 has an important role in olfactory function and that intranasal infusion of GD3 helped mice recover from olfactory dysfunction, suggesting an effective therapy for neurodegenerative diseases with olfactory disorders.

Discussion

In this study, we demonstrated that intranasally administered gangliosides recovered the NSC pool in the SVZ and neuronal population in the OB of A53T mice. Further, we discovered that GD3 is necessary for the proper function of olfactory sensation. Our previous report demonstrated that intranasal infusion of GM1 protects dopaminergic neurons at the substantia nigra pars compacta from cytotoxic aSyn deposit, which might restore dopaminergic innervation in the striatum of A53T mice [10]. Recently, Yoon et al. demonstrated that aSyn regulates the expression of p21 to promote cell cycle arrest. Highly upregulated p21 was observed in aSyn-expressing SH-SY5Y cells and the cortex and the hippocampus of aSyn transgenic (Tg) mice with DNA damage and senescence [96]. A significant increase in the expression of p21 in the midbrain of PD patient postmortem brain compared with age-matched controls has been reported [97, 98]. Those data suggested that a p21-induced, senescencelike phenotype may contribute to pathogenesis in PD patient brains. In this study, we focused on the neurogenic defects found in A53T mice and demonstrated that the decreased cellular proliferation accompanied the upregulation of p21 in the SVZ of A53T mice. Intranasal GD3 infusion likely stimulates EGF signaling, suppresses p21 expression, and eliminates aSyn in the SVZ [6, 10]. Therefore, intranasal infusion of GD3 is a possible strategy for resilience to impaired neurogenesis-related symptoms through the inhibition of p21-induced cell cycle arrest.

Although we observed a neurogenic effect of gangliosides, further investigation is needed to determine the proper amount and administration period of ganglioside required for effective neurogenesis. This would include determining the efficacy of delivery from nasal passage to the central nervous system as well as the pharmacokinetics and pharmacodynamics of intranasal gangliosides. Combinatorial intranasal infusion of GD3 and GM1 is expected to be more effective for neurogenesis, although it may require some methodological optimization. Combination therapies can include administration of the active agents together in the same admixture or in separate admixtures. In early-to-moderate stages of neurodegenerative diseases, GM1 alone might be sufficient to maintain neuronal functions. On the other hand, at more severe stages of disease, GD3 would be needed at first to amplify NSCs and then GM1 could be given to support neuronal differentiation of NSCs. Since the aggregation of aSyn in the OB induces hyposmia [99], intranasal infusion of gangliosides not only removes aSyn but also promotes the survival of olfactory neurons and maintains functional neurons. In this research, we administered GD3 for the first 2 weeks and GM1 for the next 2 weeks to promote NSC self-renewal and maturation of neurons, respectively. This combination therapy was effective for CR + cells but not TH + cells, possibly due to the maturation rate. Kohwi et al. demonstrated that 23.0 ± 5.0% of BrdU + cells expressed CR whereas only 4.1 ± 2.8% of BrdU + cells expressed TH, as determined 15 days after BrdU injection [90]. CR-positive cells are immature and therefore are supposed to contribute to a reserve pool of interneurons that could be recruited according to need [86]. These immature neurons can be upregulated by GD3. Longer terms of GM1 treatment (e.g., 4 weeks) after

GD3 infusion might be needed to increase the number of TH + neurons in the PGL. GD3 maintains stemness and inhibits further neuronal differentiation, on the other hand GM1 promotes neuronal differentiation. Distinctive biological characteristics of both gangliosides can be utilized for a particular process for neurogenesis.

We previously demonstrated that GM1 recruited the dopaminergic neuron-associated transcription factor, Nurr1, to the TH promoter region to activate TH gene expression [10]. In addition, GM1 also recruited paired-like homeodomain transcription factor 3 (Pitx3), a critical transcription factor for the survival of midbrain dopaminergic neurons. Meanwhile, we showed that icv administration of GD3 clearly increased the expression of the NSC-associated transcription factor SOX2 in cells at the SVZ and DG in the adult mouse brain [7]. In this study, we showed that GD3 downregulates p21, a major inhibitor of cell proliferation, in nuclei of NSCs; thus, GD3 likely upregulates its self-renewal capability. In this manner, it is likely that GD3 sustains stemness and inhibits further neuronal differentiation such as upregulation of TH expression for TH + neuronal differentiation. We are currently investigating how GD3 modulates expression of certain molecules to maintain stem cell activities and regulate NSC fate determination during disease progression and resilience. Although previous research indicated that there are relatively small population of neural progenitors in the RMS of adult human brain, it has been reported that NSCs exist in the adult rodent and human OB contributing new granular and periglomerular neurons in the adult human olfactory system [100–104]. Understanding the functional roles of GD3 and GM1 using human NSCs derived from induced pluripotent stem cells of PD patients to validate the effectiveness of human neurogenesis would be desirable.

We demonstrated that GD3 deficiency leads to impaired olfaction and that intranasal GD3 revived the sense of smell in GD3S-KO mice. Intriguingly, A53T aSyn-BAC Tg mice exhibit hyposmia in the olfactory avoidance test [105], and A53T mice have been reported to develop olfactory dysfunction and impaired neurogenesis [58–64]. Hyposmia is commonly observed prior to motor deficits especially in PD patients carrying a pathogenic A53T aSyn mutation, which might cause aSyn deposition in the OB [106–109]. We previously discovered that intranasal GD3 and GM1 significantly reduce toxic aSyn levels in the mouse brain [10, 110]. In this study, we showed that intranasal GD3 sustains NSC activities in SVZ-OB and GM1 promotes neuronal differentiation in OB. In such a way, gangliosides have multifunctions to eliminate toxic proteins, such as aSyn and to restore postnatal neurogenesis for improvement of non-motor functions including olfaction. It is desired to investigate whether ganglioside treatment ameliorates PD symptoms including motor and non-motor dysfunctions. Reduction of TH + and CR + neurons at the PGL in mouse prion promoter-driven A53T mice implicates hyposmia in these mice. This study focused on the neurogenic process at the SVZ and RMS and the neuronal population in OB as a mechanism of olfactory dysfunction associated with PD pathology. The mechanisms of the other non-motor symptoms, including cognitive dysfunction, depression, and anxiety that are associated with neurogenesis at the DG in the hippocampus, need to be investigated in the future research. Further investigation will clarify the functional relationship between neurogenesis and olfactory sensation regulated by gangliosides in PD pathology. According to our analyses, upregulation of cellular proliferation at the SVZ by GD3 intranasal infusion and sequential administration of GM1 might increase the number of newborn neurons,

suggesting that curative gangliosides improve the impaired olfaction of PD. Our findings in restoring the sense of smell of GD3S-KO hyposmia mice after GD3 intranasal infusion imply the probable restoration of NSC self-renewal in the SVZ and subsequent neurogenesis in the OB. To demonstrate the possibility of this strategy for PD, further validation such as the buried pellet test and olfactory avoidance test will be needed after intranasal ganglioside treatment using PD-model mice.

As > 95% of patients with PD present with significant olfactory loss [107], our research emphasizes the potential translational benefit of our ganglioside therapy. However, there are several challenges for a clinical trial to overcome. The difference in size of olfactory bulbs in mouse and primate (including human) and limitation of neuronal migration along the rostral migratory stream in humans, which may affect dosing parameters for clinical trials, it is necessary to address how this will affect intranasal drug delivery. In a next step, it is desirable to determine pharmacokinetics and pharmacodynamics of intranasal ganglioside in the monkey brain. In non-human primate experiments, we will choose the suitable intranasal delivery device and ganglioside nasal formulation for aerosol distribution. The monkey study will be a bridge between mouse and human to establish efficient nasal ganglioside therapy for PD patients.

Further, risks for side effects including tumorigenesis should be considered. Tumorigenesis is a potential concern when utilizing a factor that can stimulate cell proliferation. NSCs and cancer cells share certain common biomarker molecules, such as Nestin, Sox2, EGFR, Prominin (CD133), and GD3. This does not mean that endogenous NSC biomarkers would necessarily induce tumorigenesis. Indeed, NSCs in the brain are not cancer cells. In our experiments, no detectable adverse effects occurred during or after intranasal administration of gangliosides. Tissue sections were analyzed with hematoxylin and eosin (HE) staining, and no abnormal structures were found in the olfactory epithelium, brain, eye, spinal cord, tongue, thymus, lung, liver, spleen, kidney, stomach, intestine, colon, bone, or bone marrow after intranasal GD3 or GM1 infusion. Neither histological damage nor tumor formation was observed. Our study demonstrated that intranasally administered gangliosides are safe and show no toxicity. However, investigators need to pay close attention to the possibility of tumorigenesis with NSC marker molecules.

In summary, we demonstrated that GD3 activated NSC self-renewal via suppression of p21 overexpression induced by aSyn in the SVZ, and that GM1 and GD3/GM1 sustained neuronal populations in the OB of A53T mice. Further, GD3 is critical for the sense of smell, and olfactory dysfunction by GD3 deficiency was significantly restored by intranasal GD3 infusion. Taken together, our study highlights a conceptual foundation for a potential therapeutic strategy using gangliosides, with advances in its easy and noninvasive delivery against non-motor PD symptoms related to adult neurogenesis as well as movement symptoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The authors agree to share information regarding the conduct of this research and the results obtained from this investigation upon written requests from interested investigators. The authors agree to share any animal models, antibodies, and reagents that are employed in this study upon written requests.

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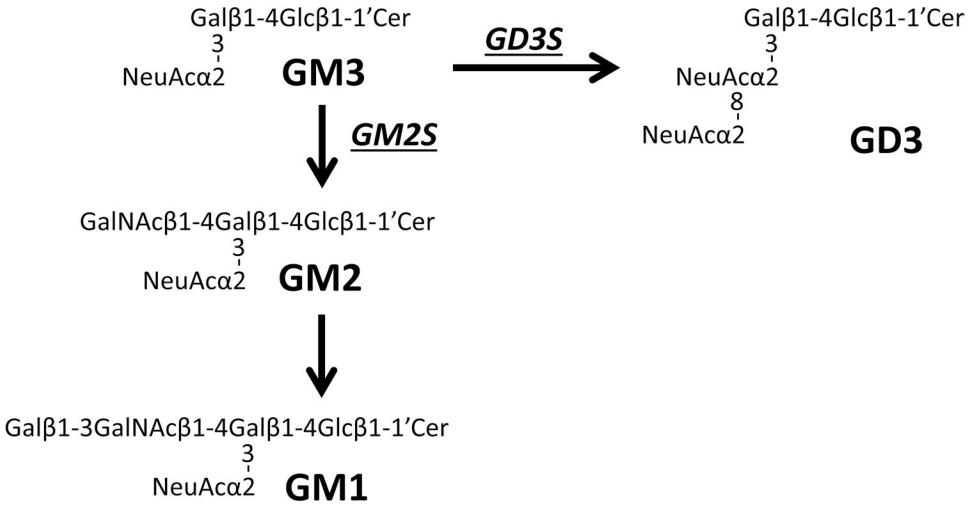
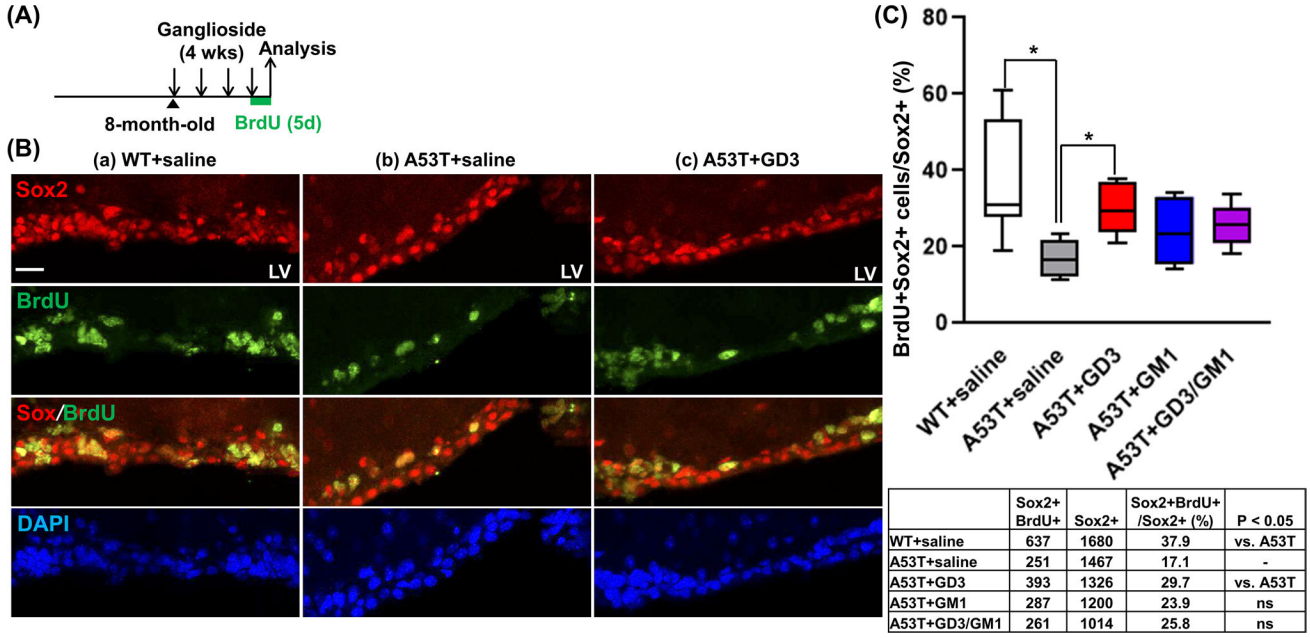


Fig. 1.

Molecular structures of gangliosides GD3 and GM1. The nomenclature for gangliosides and their components are based on that of Svennerholm (1963) and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature (1977). Glycosyltransferases (underlined) catalyze the biosynthesis of gangliosides. Cer: ceramide, Gal: galactose, GalNAc: N-acetylgalactosamine, GD3S: GD3 synthase, St8Sia1, Glc: glucose, GM2S: GM2 synthase, B4galnt1, NeuAc: N-acetylneuraminic acid



	Sox2+ BrdU+	Sox2+	Sox2+BrdU+ /Sox2+ (%)	P < 0.05
WT+saline	637	1680	37.9	vs. A53T
A53T+saline	251	1467	17.1	-
A53T+GD3	393	1326	29.7	vs. A53T
A53T+GM1	287	1200	23.9	ns
A53T+GD3/GM1	261	1014	25.8	ns

Fig. 2.

Intranasally administered GD3 restored BrdU incorporation in Sox2-positive cells at the subventricular zone (SVZ) in A53T nice. **A** Time-line diagram of the experiment. Gangliosides (5 mg/kg/day for 28 days) were intranasally infused into A53T mice. **B** Immunohistochemistry for Sox2 (red) and BrdU (green) for WT + saline, A53T + saline, and A53T + GD3 mice. LV: lateral ventricle. **C** Quantification of BrdU and Sox2 double-positive cells in Sox2 + cells from imaging data of (B). Counted numbers of cells in each group are shown in the table (ns; not significant). Brown-Forsythe test failed to detect a homogeneity ($p = 0.041$). Then Kruskal–Wallis test followed by Dunn's multiple comparison test was performed. WT + saline vs. A53T + saline: $p = 0.0012$, A53T + saline vs. A53T + GD3: $p = 0.0057$, $^{*}p < 0.05$. $n = 3$–4 mice/group; WT + saline: 4mice, A53T + saline: 3 mice, A53T + GD3: 3 mice, A53T + GM1: 3 mice, A53T + GD3/GM1: 3 mice. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes. Scale bar, 20 μm

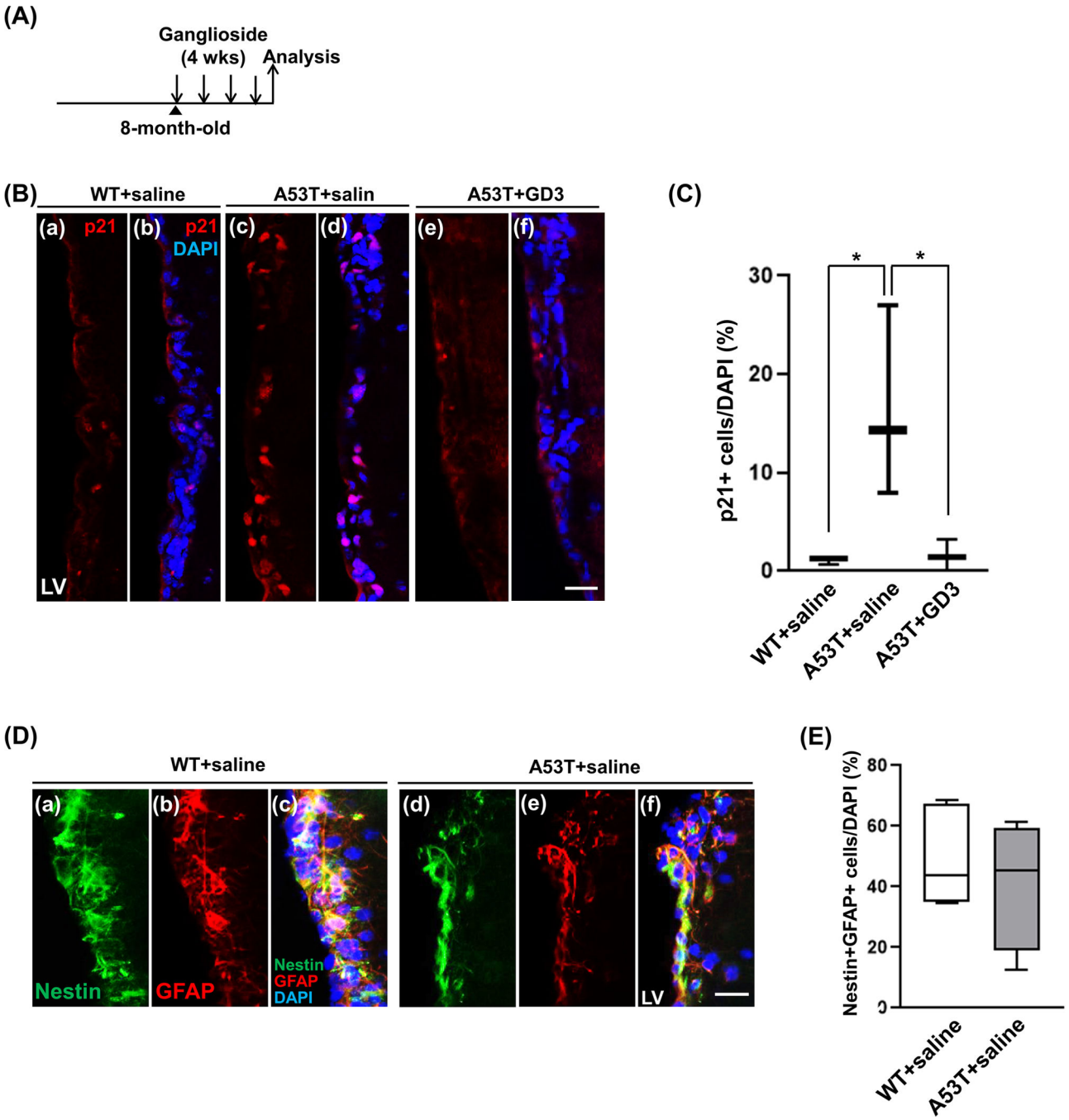


Fig. 3.

Intranasally administered GD3 suppressed the expression of cyclin-dependent kinase (CDK) inhibitor p21 in the SVZ of A53T mice. **A** Time-line diagram of the experiment. GD3 (5 mg/kg/day for 28 days) was intranasally infused into A53T mice. **B** Immunohistochemistry for p21 in the SVZ. Red p21; blue, nuclear DAPI. LV: lateral ventricle. **C** Quantification of p21-expressing cells from imaging data of (B). WT + saline vs. A53T + sline: $p = 0.029$, A53T + saline vs. A53T + GD3: $p = 0.032$, $*p < 0.05$. One-way ANOVA with Dunnett's multiple comparison test. **D** Immunohistochemistry for Nestin and GFAP in the SVZ. Green,

Nestin; red GFAP; blue, nuclear DAPI. **E** Percentage of Nestin and GFAP double-positive Type B NSCs was quantified imaging data from (D). WT + saline vs. A53T + saline: $p = 0.325$. $n = 3–4$ mice/group; WT + saline: 4mice, A53T + saline: 3 mice, A53T + GD3: 3 mice, A53T + GM1: 3 mice, A53T + GD3/GM1: 3 mice. Student's unpaired t test was performed. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes. Scale bars, 20 μm

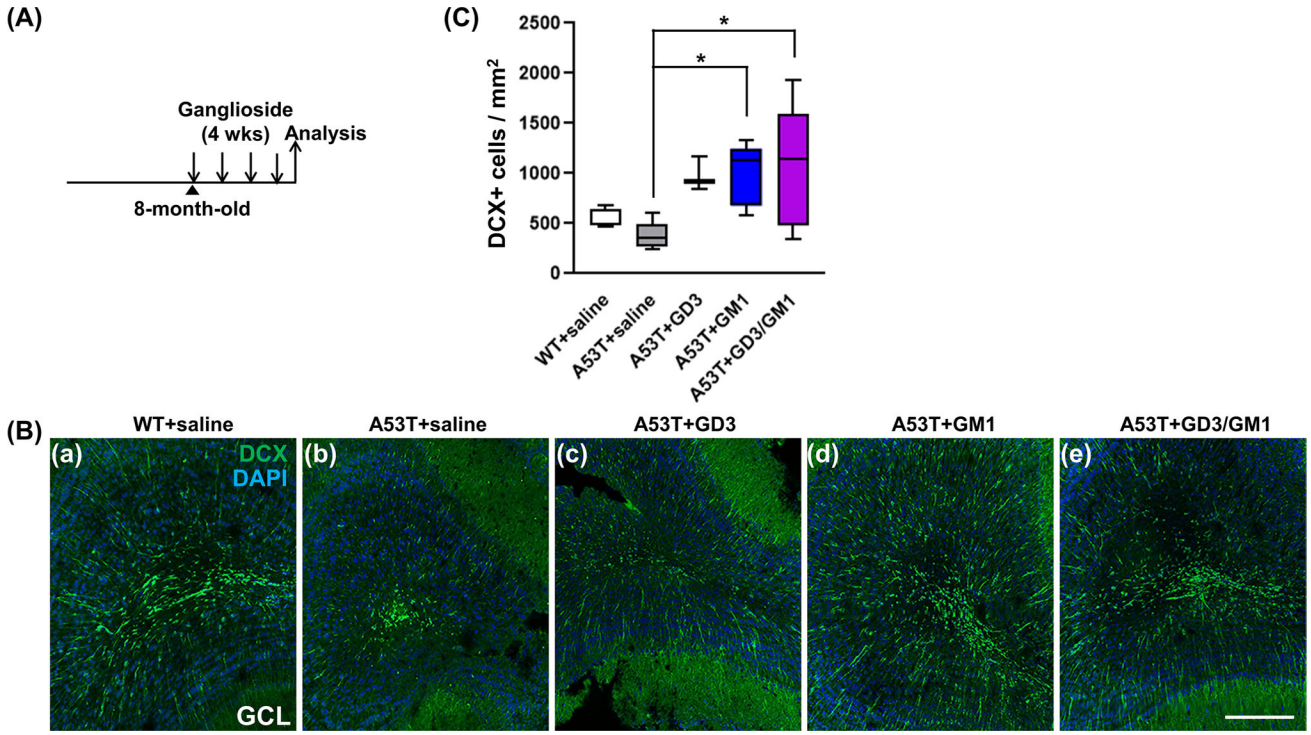


Fig. 4.

Intranasal administration of GM1 restored DCX expression in the OB of A53T mice. **A** Time-line diagram of experiment. Gangliosides (5 mg/kg/day for 28 days) were intranasally infused into A53T mice. **B** Immunohistochemistry for Doublecortin (DCX) in the olfactory bulb (OB). Green, DCX; blue, nuclear DAPI. (C) DCX + cells in the granule cell layer of the OB. A53T + saline vs. A53T + GM1: $p = 0.033$, A53T + saline vs. A53T + GD3/GM1: $p = 0.018$, $^{*}p < 0.05$. $n = 3$–4 mice/group; WT + saline: 4mice, A53T + saline: 3 mice, A53T + GD3: 3 mice, A53T + GM1: 3 mice, A53T + GD3/GM1: 3 mice. One-way ANOVA with Dunnett's multiple comparison test. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes. Intranasal infusion of GM1 and GD3/GM1 combination restored the immature DCX + cells, in OB. Scale bar, 200 μm

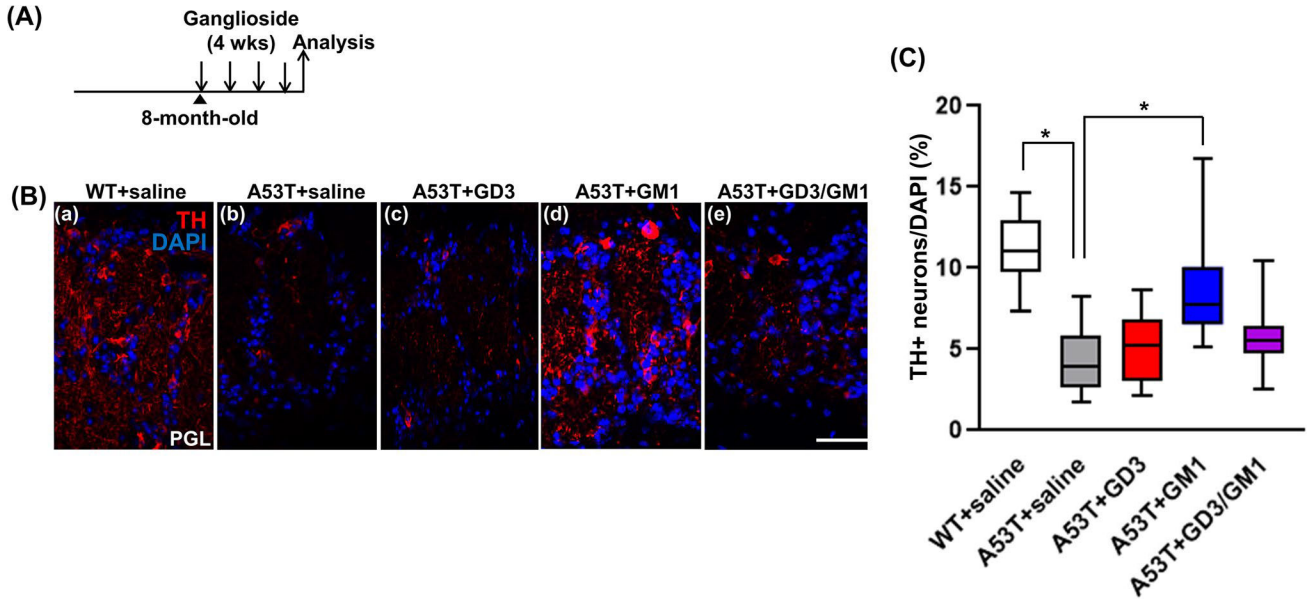


Fig. 5.

Restoration of the population of TH + neurons by intranasally administered GM1 in the periglomerular layer (PGL) of A53T mice. **A** Time-line diagram of experiment. Gangliosides (5 mg/kg/day for 28 days) were intranasally infused into A53T mice. **B** Immunohistochemistry for tyrosine hydroxylase (TH) in the OB. Red, TH; blue, nuclear DAPI. **C** Quantification of TH + neurons from imaging data of (B). WT + saline vs. A53T + saline: $p < 0.0001$, A53T + saline vs. A53T + GM1: $p = 0.0023$, * $p < 0.05$. $n = 3–4$ mice/group; WT + saline: 4mice, A53T + saline: 3 mice, A53T + GD3: 3 mice, A53T + GM1: 3 mice, A53T + GD3/GM1: 3 mice. Shapio-Wilk test failed to detect a normal distribution ($p = 0.036$). Then Kruskal–Wallis test followed by Dunn's multiple comparison test was performed. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes. Scale bar, 50 μm

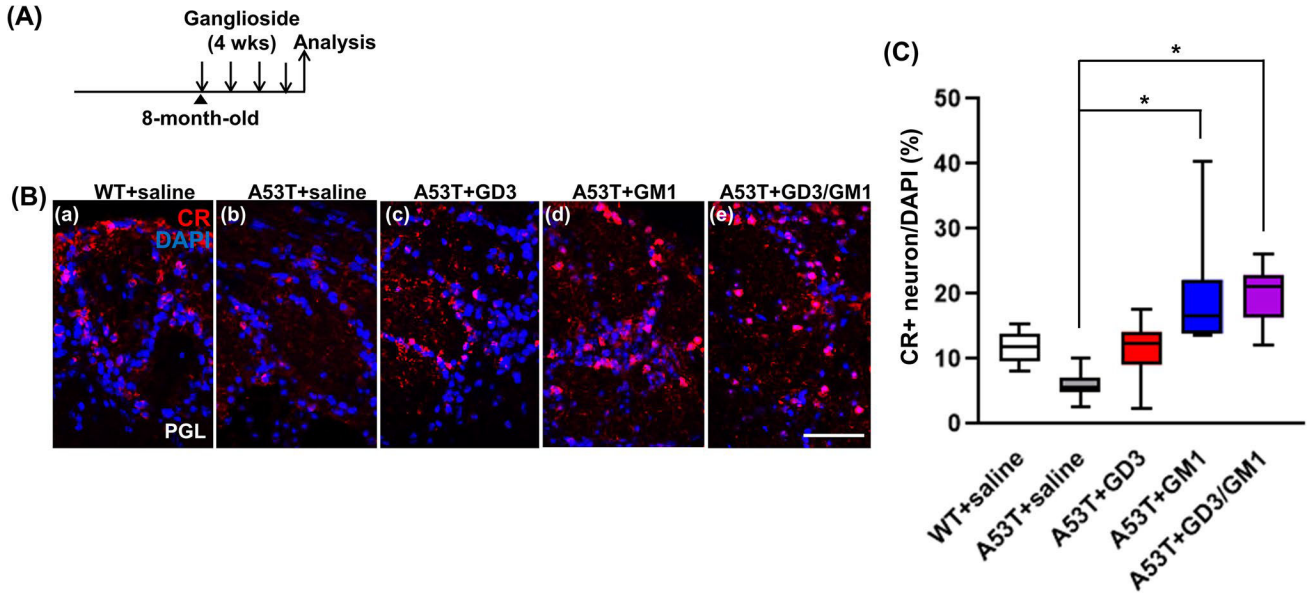


Fig. 6.

Restoration of the population of CR + neurons by intranasally administered gangliosides in the periglomerular layer (PGL) of A53T mice. **A** Time-line diagram of the experiments. Gangliosides (5 mg/kg/day for 28 days) were intranasally infused into A53T mice. **B** Immunohistochemistry for calretinin (CR) in the OB. Red, CR; blue, nuclear DAPI. **C** Quantification of CR + from imaging data of (B). WT + saline vs. A53T + saline: $p = 0.038$, A53T + saline vs. A53T + GM1: $p < 0.0001$, A53T + saline vs. A53T + GD3/GM1: $p < 0.0001$, * $p < 0.05$. $n = 3–4$ mice/group; WT + saline: 4mice, A53T + saline: 3 mice, A53T + GD3: 3 mice, A53T + GM1: 3 mice, A53T + GD3/GM1: 3 mice. Shapio-Wilk test failed to detect a normal distribution ($p = 0.002$). Then Kruskal–Wallis test followed by Dunn's multiple comparison test. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes. Scale bar, 50 μm

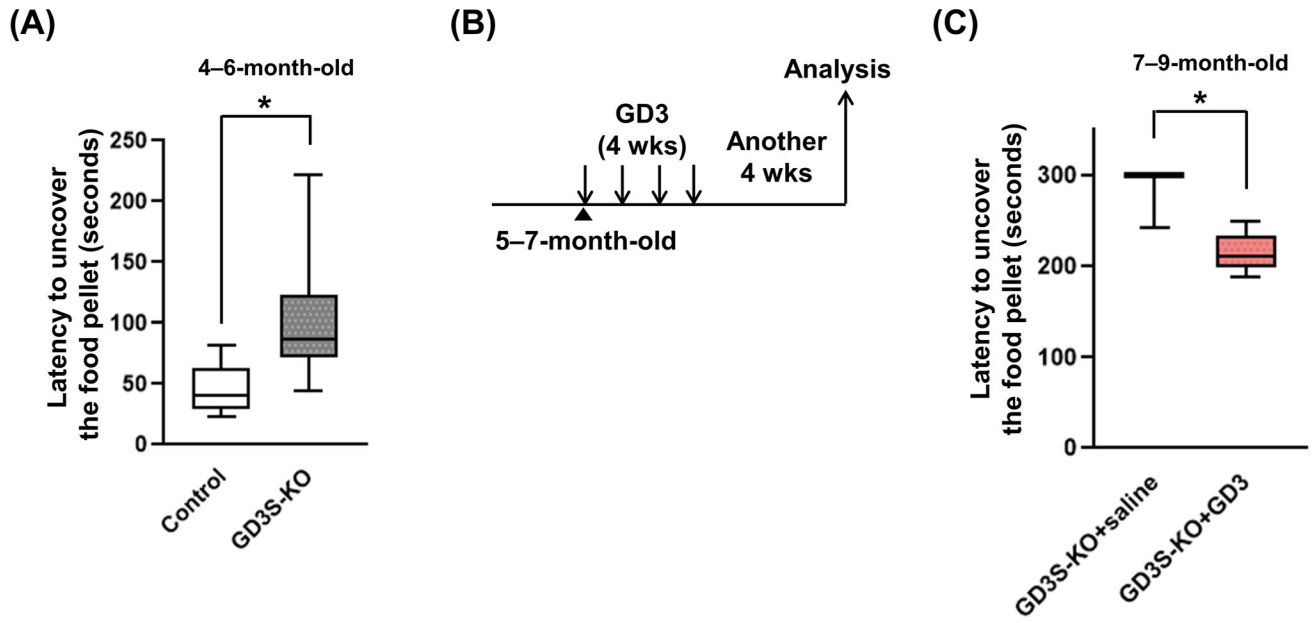


Fig. 7.

Depletion of GD3 impairs olfactory sensation, and intranasal GD3 infusion restored olfaction of GD3S-KO mice. The buried pellet test, which relies on the animal's natural tendency to use olfactory cues for foraging, is used to confirm the ability to smell. The time until mice uncovered the food pellet in bedding was measured as an average of 4 trials. **A** Latency to uncover the food pellet beneath the bedding and statistical analysis of the experiments. The line on the dot blots indicate means. Littermates WT ($n = 8$) vs. GD3S-KO ($n = 9$): $p = 0.0025$. F-test failed to detect a normal distribution ($p = 0.025$). Then Mann Whitney test was performed. **B** Time-line diagram of the experiment. Ganglioside GD3 (5 mg/kg/day for 28 days) was intranasally infused into A53T mice, and the buried pellet test was performed four weeks after treatment. **C** Latency to uncover the food pellet beneath the bedding and statistical analysis of the tests. GD3S-KO + saline ($n = 3$ mice) vs. GD3S-KO + GD3 ($n = 5$ mice): $p = 0.0147$, * $p < 0.05$. Student's unpaired t test was performed. The box range represents upper and lower quartiles, and the end of whiskers represent the minimum and maximum values. The median values are represented by bars in the boxes