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Excessive microglial activation aggravates olfactory dysfunction by impeding the survival of newborn neurons in the olfactory bulb of Niemann–Pick disease type C1 mice

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ABSTRACT

Progressive olfactory impairment is one of the earliest markers of neurodegeneration. However, the underlying mechanism for this dysfunction remains unclear. The present study investigated the possible role of microgliosis in olfactory deficits using a mouse model of Niemann–Pick disease type C1 (NPC1), which is an incurable neurodegenerative disorder with disrupted lipid trafficking. At 7 weeks of age, NPC1 mutants showed a distinct olfactory impairment in an olfactory test compared with age-matched wild-type controls (WT). The marked loss of olfactory sensory neurons within the NPC1 affected olfactory bulb (NPC1-OB) suggests that NPC1 dysfunction impairs olfactory structure. Furthermore, the pool of neuroblasts in the OB was diminished in NPC1 mice despite the intact proliferative capacity of neural stem/progenitor cells in the subventricular zone. Instead, pro-inflammatory proliferating microglia accumulated extensively in the NPC1-OB as the disease progressed. To evaluate the impact of abnormal microglial activation on olfaction in NPC1 mice, a microglial inhibition study was performed using the anti-inflammatory agent Cyclosporin A (CsA). Importantly, long-term CsA treatment in NPC1 mice reduced reactive microgliosis, restored the survival of newly generated neurons in the OB and improved overall performance on the olfactory test. Therefore, our study highlights the possible role of microglia in the regulation of neuronal turnover in the OB and provides insight into the possible therapeutic applications of microglial inhibition in the attenuation or reversal of olfactory impairment.

1. Introduction

The olfactory system is an essential sensory element for survival, and it is primarily based on well-organized neuronal communications between the olfactory epithelium (OE) and the olfactory bulb (OB). Every process associated with proliferation, maturation, integration and apoptosis of OB neurons must be fine-tuned to maintain olfaction functional integrity [1,2]. Interestingly, the progressive loss of olfaction is often observed in the initial stages of neurological disorders, including Alzheimer's disease (AD) [3], Parkinson's disease (PD) [4], dementia and Down's syndrome [5,6], which suggests that olfactory dysfunction is an early sign of neurodegeneration [7]. The presence of amyloid-β (Aβ) or α-synuclein pathology has been linked to this phenomenon [8,9]. However, the pathological basis of olfactory dysfunction in the neurodegenerative process has not been thoroughly explained.

Niemann–Pick disease type C1 (NPC1) is a fatal metabolic disorder caused by a mutation in the NPC1 gene, which leads to the disruption

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid precursor protein; Arg1, arginase 1; BrdU, 5-bromo-2′-deoxyuridine; CBD, calbindin; CsA, cyclosporin A; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DCX, doublecortin; GAD65, glutamate decarboxylase 65; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gap43, growth associated protein 43; GC, granule cell; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; GL, glomerular layer; IL-1β, interleukin-1β; LP, laminar propria; LV, lateral ventricle; NeuN, neuronal nuclei; NPC1, Niemann–Pick disease type C1; NSC, neural stem cell; OB, olfactory bulb; OE, olfactory epithelium; OMP, olfactory marker protein; OSN, olfactory sensory neuron; PD, Parkinson's disease; PGC, periglomerular cell; qRT-PCR, quantitative real time-PCR; Str, striatum; SVZ, subventricular zone; TNF-α, tumor necrosis factor-α; TH, tyrosine hydroxylase; WT, wild type

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of the lipid trafficking system and lipid sequestration within the lysosomal and late endosomal compartments [10]. Considering that the presence of neurological complications is significantly correlated with disease severity [11], elucidating the neurodegenerative process is an important step to establish therapeutic strategies for NPC1. A patterned neuropathy has been observed in NPC1-affected humans and mouse models with distinctive signs of inflammation usually triggered by activation of microglia and/or astroglia [11,12]. Because a neural-specific loss of the NPC1 gene is sufficient to reproduce the NPC1 phenotype [13], neuronal damage might precede reactive microgliosis and astrocytosis in NPC1. However, several studies have also shown that a neural pathology could develop in a non-autonomous manner in NPC1 [14]. Indeed, intact NPC1 function in astrocytes seems to be important in supporting neurons in vitro [15], and NPC1 gene-null oligodendrocytes fail to maintain proper CNS myelination patterns, which results in Purkinje cell loss in the cerebellum that resembles NPC1 pathology [16]. Furthermore, TNF-α, a pro-inflammatory cytokine, is involved in NPC1-associated liver damage [17], and anti-inflammatory agents relieve symptoms and increase the life span of NPC1 mutant mice [18]. Therefore, the role of abnormal microgliosis and inflammation in NPC1 progression still needs to be determined.

Herein, we assessed the pathological changes in olfaction using an NPC1 mouse model. Our results demonstrated that NPC1 mice showed distinct signs of olfactory dysfunction in a behavior test compared with WT controls. Our results also revealed a drastic loss in olfactory sensory neurons (OSNs) and neuroblasts with excessive microglial activation in the NPC1-OB. Notably, the administration of the anti-inflammatory drug CsA considerably improved olfactory function and increased neural survival in NPC1 mice through microglial inhibition. These data indicate that the regulation of microglial activation might be important for the treatment of olfactory dysfunction in neurological disorders.

2. Materials and methods

2.1. Animal model

Breeding pairs of heterozygous NPC1 null mice (Balb/c NPC1^{NIH}; NPC1) were purchased from Jackson Laboratories (Bar Harbor, MA). The genotyping was performed as previously described [19], and homozygous WT and NPC1 mice were used in this study. All animals were handled in accordance with the regulations of the Institute of Laboratory Animal Resources (SNU-110517-3, Seoul National University, Korea). No specific gender-based differences were observed in each experiment. The number of animals used is indicated in the Results section and figure legends.

2.2. Buried food finding test

To evaluate olfaction in mice, the buried food test was used as previously described [20] with some modifications. Briefly, 7-week-old mice from each experimental group were fasted for 24 h before the test. Next, the mice were individually habituated for 10 min in a new cage with fresh bedding. During the habituation step, a piece of standard chow (1 × 1 cm) was buried under the bedding in the middle of a new cage (test cage) to a depth of 0.5 cm. The subject was then placed in the left corner of the test cage and allowed to move freely to seek the hidden food for 3 min. During this time, all activities, including sniffling around or digging in the bedding, were recorded until the mouse discovered the food and began to eat (latency). If the mouse was unable to locate the food within 3 min, the result was categorized as a 'failure' (Figs. 1B and 6B) and was also excluded from the mean latency calculations (Figs. 1C and 6C). Directly after the test, each mouse underwent another trial with the food placed on top of the bedding (exposed food test) to ensure that the buried food test was based on olfaction and not on visual ability.

2.3. Cyclosporin A (CsA) administration

Chronic microglial inhibition was conducted by CsA treatment (Chong Kun Dang, South Korea), a broad anti-inflammatory drug. Four-week old NPC1 mice were injected with CsA (5 mg/kg, prepared in normal saline) every second day for 4 weeks and the control group received the same volume of normal saline without CsA.

2.4. 5-bromo-2′-deoxyuridine (BrdU) administration

To trace the fate of proliferating cells in the OB, 4-week-old mice received a single injection of BrdU (Sigma–Aldrich, St. Louis, MO) (50 mg/kg, dissolved in 0.9% NaCl) intraperitoneally (i.p.) and sacrificed after one month. Meanwhile, the short-term characterization of proliferating cells was achieved by a BrdU injection (100 mg/kg, i.p) into 4- or 8-week-old mice 4 or 24 h before euthanasia. The control subjects received the same volume of normal saline.

2.5. Tissue processing

Tissue preparation was performed as previously described [19]. For RNA and protein extraction, mice were sacrificed by cervical dislocation, and whole brains were immediately collected. Then, OBs were collected for homogenization with Trizol (Invitrogen, Carlsbad, CA) (for RNA) or lysis buffer (Pro-prep; Intron Biotechnology, Korea) (for protein) using a TissueLyser II (Qiagen, Valencia, CA). For immunohistochemistry, mice were perfused with normal saline and then with 4% paraformaldehyde for 20 min each. The isolated whole brains containing OBs were post-fixed in 4% paraformaldehyde for another 24 h and transferred to a 30% sucrose solution for 3–4 days until they sank. In some experiments, the OE was also isolated after perfusion and immersed in a 0.5 M EDTA/10% sucrose solution for decalcification. Tissues were then placed into a mold filled with infiltration mixture (OCT compound) (Sakura Finetek, Japan) and stored at −80 °C until processing for cryosections.

2.6. Quantitative real time-PCR (qRT-PCR) based gene expression analysis

RNA quantification and reverse transcription PCR were performed as previously described [19]. qRT-PCR was performed by mixing cDNA with primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using an ABI 7500 Realtime-PCR System with the supplied software (Applied Biosystems) according to the manufacturer's instruction. The primer sequences used in this study were as follows: Tumor necrosis factor-α (TNF-α), forward 5′-AGGCTGTGCATTGCACCT CA-3′ and reverse 5′-GGGACAGTGACCTGGACTGT-3′; interleukin-1β (IL-1β), Forward 5′-GATCCACACTCTCCAGCTGCA-3′ and reverse 5′-CAACCAACAAGTGATATTCTCCAT-3′; IL-6, forward 5′-AAGTGCAT CATCGTTGTTCATACA-3′ and reverse 5′-GAGGATACCACTCCCAACAG ACC-3′; and arginase 1 (Arg1), forward 5′-GAACACGGCAGTGGCTTT AAC-3′ and reverse 5′-TGCTTAGCTCTGTCTGCTTTGC-3′. Each relative mRNA level was calculated using the comparative Ct method and then normalized to Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA levels.

2.7. Western blot analysis

Protein quantification and Western blot analysis were performed as previously described [19]. Equal amounts of each protein sample were loaded onto 8–15% SDS-PAGE gels and then transferred to a nitrocellulose membrane for standard blocking with bovine albumin serum followed by incubating with primary antibodies. The following primary antibodies were used: Olfactory marker protein (OMP) (1:500; Osenses, Australia), Glutamate decarboxylase 65 (GAD65) (1:1000; Abcam, Cambridge, MA), Tyrosine hydroxylase (TH) (1:1000; Millipore, Billerica, MA), Calbindin (CBD) (1:1000; Millipore), Doublecortin (DCX)