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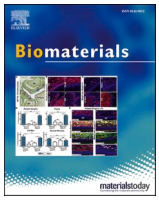

Direct evidence for the involvement of intestinal reactive oxygen species in the progress of depression via the gut-brain axis

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ABSTRACT

Depression is a serious global social problem. Various therapeutic drugs have been developed based on the monoamine hypothesis; however, treatment-resistant depression is a common clinical issue. Recently, the gut-brain axis, which is associated with the hypothesis that the intestinal environment affects the brain, has garnered significant interest, and several studies have attempted to treat brain disorders based on this axis. These attempts include fecal transplantation, probiotics and prebiotics. In this study, we focused on intestinal reactive oxygen species (ROS) because excessive ROS levels disturb the intestinal environment. To elucidate the impact of scavenging intestinal ROS on depression treatment via the gut-brain axis, a novel polymer-based antioxidant (siSMAPo^{TN}), which was distributed only in the intestine and did not diffuse into the whole body after oral administration, was used. siSMAPo^{TN} selectively scavenged intestinal ROS and protected the intestinal environment from damage caused by chronic restraint stress (CRS). In addition, siSMAPo^{TN} suppressed physiological and behavioral depression-related symptoms in the CRS mouse model.

1. Introduction

Depression is one of the most common brain dysfunctions and has become a social issue on a global scale [1]. Symptoms vary among patients, and the detailed mechanism is still not well understood. Various antidepressants have been developed based on the "monoamine hypothesis," which suggests that depression is caused by a deficiency or imbalance in monoamine neurotransmitters [2]. However, serious limitations are associated with the monoamine theory, and treatment-resistant depression is a common clinical problem [3]. Hence, an alternative strategy that targets outside the monoamine systems also should be considered. Recently, a communication system between the gut and brain, called the "gut-brain axis" has been garnering significant interest [4]. The gut-brain axis refers to the crosstalk between biological signals in the brain and the gut. This signaling pathway comprises various mediators that interact with the brain. Several important gut-brain axis components, such as proinflammatory cytokines, interleukins, stress response hormones, and metabolites of intestinal bacteria, have been identified as products or triggers of brain disorders [4]. However, the network between the gut and brain is muti-channel and complicated, and thus, the detailed mechanism underlying the brain dysfunction mediated by disturbances in the gut environment is still unclear. Methods for the treatment by which therapeutic molecules act only in the gut and work on brain disorders based on the gut-brain axis are limited to probiotics, prebiotics, and fecal transplantation [5].

Several factors can disrupt the gut environment. Oxidative stress caused by reactive oxygen species (ROS) is a well-known cause of inflammation [6]. ROS are highly oxidative substances derived from oxygen and are among the most important factors affecting the intestinal environment [7]. They are produced in vivo by the respiratory mechanism of mitochondria and phagocytosis of immune cells and are essential for biological activities. ROS levels are appropriately controlled by endogenous enzymes for maintaining their function as signaling molecules. However, under conditions involving uncontrolled inflammation and chronic stress, ROS are overproduced and lead to unexpected events, such as excessive inflammation, immune dysfunction, and inflammatory bowel disease (IBD) [8]. We have been studying nanoparticle-type antioxidants for more than ten years. During our investigation, we confirmed that the orally administered nanoparticle-based antioxidant clearly eliminated the overproduced

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intestinal ROS in an ulcerative colitis mouse model and suppressed inflammation significantly [9]. Since antioxidative nanoparticles possess poly (ethylene glycol) (PEG) on the outermost layer, they show high dispersion stability, biocompatibility, and sustained accumulation in the intestine. Given the importance of ROS, removal of overproduced ROS in the intestine is expected to maintain intestinal homeostasis and regulate systemic inflammation and the immune system, which are remarkably influenced by the intestinal environment. Another important feature of nanoparticle-type antioxidants is their extremely few adverse effects since several tens of nanometer size decrease its internalization in healthy cells and avoid dysfunction of intracellular redox balance including an electron transport chain. It would also be interesting to investigate how intestinal ROS scavengers influence the progression of depression. Although several attempts and clinical trials using antioxidants for the treatment of depression have been reported, it is essential to develop antioxidants that are present only in the intestine and are not distributed systemically to confirm the effect of intestinal ROS on brain function via the gut-brain axis. In this study, a novel antioxidative nanoparticle (siSMAPo^{TN}) with a PEG shell and a cross-linked core with a size of several tens of nanometers was designed. High colloidal stability of siSMAPo^{TN} due to PEG at the outer surface is expected to be effective to accumulate in the intestine [10]. The developed antioxidant nanoparticles can scavenge ROS only in the intestine and can be used to investigate the intrinsic relationship between intestinal ROS and brain function via the gut-brain axis (Fig. 1). This study clearly showed that scavenging intestinal ROS inhibits the progression of stress-dependent development of depression.

2. Materials and methods

2.1. Materials

poly (styrene/maleic anhydride) copolymer (PSMA) [67:33] (MW = 7500) was purchased from Polysciences, Inc. (USA). PEG monomethyl ether (MeO-PEG-OH, MW = 5000) and chloramine-T were purchased from Sigma-Aldrich Inc. (USA). Diethyl ether, phosphate-buffered saline (PBS) powder, trichloroacetic acid, ethanol, ethyl acetate, guanidine hydrochloride, formaldehyde solution, and nitric acid were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Japan). PBS powder was dissolved in distilled and deionized water (dd water) and autoclaved for sterilization before use. Superdehydrated tetrahydrofuran (THF) was purchased from Kanto Chemical Co., Inc. (Japan). Superdehydrated solvents were further purified using a solvent purification system (GlassContour, Nikko-Hansen, Japan). Butyllithium (BuLi) (1.6 M in hexane) was purchased from ACROS Organics (USA). 3-Aminopropyltrimethoxysilane (APTMS), 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl free radical (4-NH_2-TEMPO), and 2,4-dinitrophenylhydrazone (DNPH) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). $Na^{125}I$ was purchased from PerkinElmer (Waltham, MA). Heparin was purchased from Mochida Pharmaceutical Inc. (Japan).

2.2. Preparation of siSMAPo^{TN}

A total of 25.0 g of MeO-PEG-OH (5 kDa) was vacuum dried at 110 °C overnight and then mixed with dehydrated THF (100 mL) under a nitrogen atmosphere, followed by the addition of 3.4 mL of BuLi (1.6 M in hexane solvent). The resulting solution was mixed with a solution of PSMA (7.5 kDa) (37.5 g) in THF (100 mL) and stirred for 90 min. The reaction mixture was poured into 2 L of diethyl ether, and the resulting precipitate was separated by decantation. The precipitate was washed several times with diethyl ether and vacuum-dried overnight using a desiccator, leading to the formation of PSMA-*g*-PEG (56 g). The successful synthesis was confirmed by ^{1}H NMR and gel permeation chromatography (GPC). PSMA-*g*-PEG (18 g) was dissolved in THF (200 mL), and APTMS (5.8 mmol) and amino-TEMPO (29 mmol) were added and stirred for 3 h. The resulting solution was dialyzed against water using a semipermeable membrane (MWCO 3500 Da; Spectrum, Japan). The dialysate was exchanged every 12 h. After two days of dialysis, nanoparticles denoted as siSMAPo^{TN} were obtained, and the concentration was adjusted. The introduction efficiencies of TEMPO and 3-trimethoxysilylpropyl groups were confirmed by electron spin resonance spectroscopy (ESR) and inductively coupled plasma atomic emission spectroscopy (ICP-AES), respectively. The size of the nanoparticles was confirmed by dynamic light scattering (DLS).

2.3. Animals

All animal experiments were performed using eight-week-old male ICR and c57BL/6J mice purchased from Charles River, Japan. Inc. (Kanagawa, Japan). Mice were housed at the University of Tsukuba's Animal Testing Facility, and the study was conducted in accordance with the University of Tsukuba's Guide for the Care and Use of Laboratory Animals (registration number:21–067, 21–068). Mice were maintained at a temperature of 23.5 ± 2.5 °C, humidity of 52.5 ± 12.5%, and a light/dark cycle of 14 h:10 h.

2.4. Pharmacokinetics of siSMAPo^{TN}

The pharmacokinetics of siSMAPo^{TN} were analyzed using

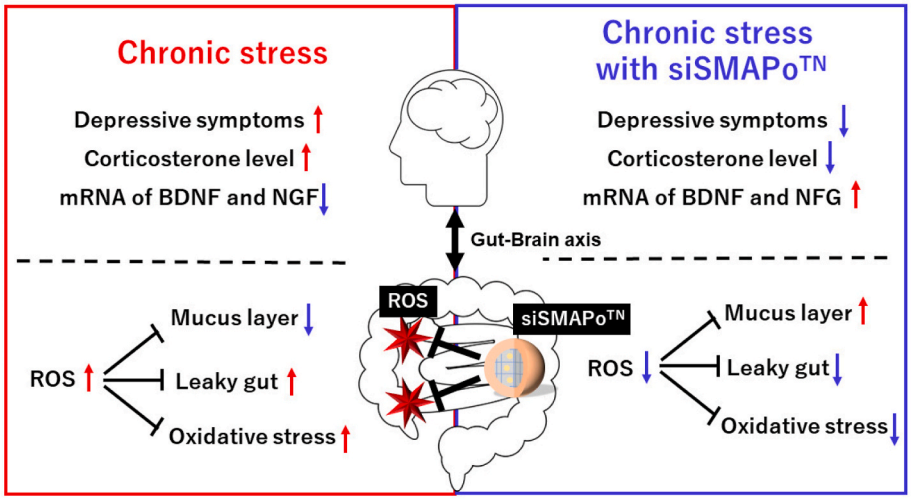


Fig. 1. Schematic illustration of the effect of overproduced ROS and siSMAPo^{TN} nanoparticles in the intestines.

radioisotope iodine (^{125}I) labeling. ^{125}I-labeled nanoparticles were obtained by the reaction of Na^{125}I and siSMAPo^{TN} using the chloramine-T method. Briefly, 74 μL of Na^{125}I solution (0.1 M), 200 μL of chloramine T (300 mM), and 1 mL of siSMAPo^{TN} solution (10 mg/mL) were mixed at room temperature. After 20 min, gel filtration was conducted using a PD-10 column (GE Healthcare, Piscataway, NJ, USA) to eliminate unreacted ^{125}I. Gel filtration of the solution was repeated several times using a PD-10 column to completely separate the free I^{125}.

The concentration of labeled nanoparticles was adjusted with dd water to 125,000 Bq/mL. Mice were fasted for 12 h, and then 0.2 mL (25,000 Bq/mouse) of the labeled nanoparticle solution was administered by oral gavage. Each mouse was euthanized using anesthesia after 0.5, 2, 5, and 24 h, and blood and intestines were collected. The radioactivity in each organ was measured using a γ-counter (ARC-380; Aloka, Japan).

2.5. Depression model

In this study, the chronic restraint stress (CRS) model was adopted based on previously published studies [11]. Briefly, mice were restrained in a 50 mL plastic tube with air holes for 6 h daily. Under conditions involving CRS, c57BL/6J mice in the healthy group were left in cages without food or water. Mice were randomly divided into three groups (healthy group, n = 8; CRS group, n = 8; CRS + siSMAPo^{TN} group; n = 8), and each gauge had free access to water and food, except during the 6 h of stress restraint. The siSMAPo^{TN} group was provided ad libitum access to a nanoparticle solution (650 mg-polymer/kg; 5 mmol TEMPO/L) instead of water. During experiments, there was no difference in food and water or sample intake between all groups. CRS was continued for three weeks. At the end of the experiment, all mice were fasted for 12 h and anesthetized using isoflurane. Blood, colon, and brain samples were collected during dissection. Plasma with heparin was immediately isolated by centrifugation and placed on ice. For tissue staining, the colon was soaked in formalin solution for 2 h and then transferred into 70% ethanol. The colons and brains used for RT-qPCR were soaked in an RNA-later solution. All other samples were placed in liquid nitrogen and immediately frozen.

2.6. Forced swimming test (FST)

Mice were then placed in a 2 L beaker filled with 1500 mL of water (25 °C), and the immobility time, defined as the time when animals were immobilized except for demonstrating movements required to keep their noses/heads above the water, was measured. The measurements were performed in the last 4.5 min of the test duration of 6 min. After the test, mice were carefully dried and returned to their home cage. Mice were randomized, and measurements were recorded by video and evaluated by an analyst who was blinded to the animal information.

2.7. Tail suspension test (TST)

Mice were suspended from a rod with the tip of their tails fixed 50 cm from the tip of the rod, and the immobility time was recorded during the last 4 min of the test duration of 6 min. The time when the suspended mouse was completely immobilized was considered immobility time. Mice were randomized, and measurements were recorded by video and measured by an analyst who was blinded to the animal information.

2.8. Measurement of lipid oxidation

The collected colonic tissue was homogenized in RIPA buffer and centrifuged (11,000×*g*, 10 min). The supernatant (200 μL) of the sample was mixed with 20% trichloroacetic acid and 50% thiobarbituric acid (50 μL) and incubated at 95 °C for 1 h. The samples were mixed with 300 μL of a butanol/pyridine mixture (15:1) and centrifuged (11,000×*g*, 10 min) at room temperature. Finally, 200 μL of the supernatant was transferred to a 96-well plate, and the absorbance was measured at 532 nm.

2.9. Protein carbonyl assay

The protein carbonyl level in the colonic tissue was measured according to a previous study [12]. Briefly, 200 mL of colonic tissue homogenized in radioimmunoprecipitation (RIPA) buffer was mixed with 800 μL of 2.5 M DNPH and HCl (for blank). The solution was vortexed and incubated on a plate shaker at room temperature in the dark. After 1 h, 250 μL of 50% trichloroacetic acid was added to the incubated solution, followed by vortexing and centrifugation (20,600 *g* for 5 min). After discarding the supernatant, the precipitate was washed with 1 mL of ethanol/ethyl acetate mixture (1:1) and centrifuged (20,600 *g*, 5 min) again. The wash-centrifugation process was repeated thrice to remove unreacted DNPH. The precipitate was then dissolved in 1 mL of 6 mM guanidine chloride. The solution (200 μL) was then transferred to a 96-well plate, and the absorbance was measured at 370 nm using a spectrophotometer. The protein carbonyl content was determined using a molar absorption coefficient of 22,000 cm^{-1}. The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., MA, USA) was used to measure protein concentration, and the protein carbonyl value was measured as nmol per mg of protein.

2.10. Immunostaining of mucin and goblet cells

The samples were stained according to the protocol published in a previous study [11]. Briefly, the colon containing feces was placed in a formalin fixative immediately after collection. The tissue was embedded in paraffin and cut into 5 μm-thick sections. The tissue sections were dewaxed, hydrated, and stained with alcian blue. Measurements were taken for one section of each mouse and in at least ten different sectional regions.

2.11. Intestinal permeability experiment

After three weeks of CRS, 0.15 mL of FITC-labeled dextran (Sigma-Aldrich Inc., USA, 4 kDa) was orally administered to mice under fasting conditions. Two hours later, blood was collected from the heart, and plasma was obtained by centrifugation with heparin. The fluorescence intensity of each sample was measured using a plate reader (Thermo Fisher Scientific, MA, USA).

2.12. Measurement of mRNA expression level by RT-qPCR

Tissue samples were homogenized in 1 mL tubes with TRIZOL reagent (0.5 mL). Chloroform (0.1 mL) was added followed by centrifugation (12,000 *g*, 15 min) after vortexing. The aqueous layer was removed, and centrifugation was performed again (12,000 g, 10 min) with isopropanol (IPA) (0.2 mL). The removed precipitates were washed with 75% ethanol (1 mL), and the total mRNA was centrifuged (7500×*g*, 5 min). RT-qPCR was performed using the One-Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time). The expression data were normalized to the mRNA expression levels of GAPDH. The standard curve method of RT-qPCR was used to determine the relative quantity of the target gene. The gene-specific primers used for qPCR were as follows.

GAPDF Fw:5′-GCCATTTGCAGTGGCAAAGTGG-3’.
GAPDH Rev:5′-GATGGGCTTCCCGTTGATGACAA-3’.
BDNF Fw:5′-TGGCTGACACTTTTGAGCACGTC-3’.
BDNF Rev: 5′-GCTCCAAAGGCACTTGACTGCTG-3’.
IL-6 Fw:5′– CCAAGAGGTGAGTGCTTCCC-3‘
IL-6 Rev:5’- CTGTTGTTCAGACTCTCTCCCT-3’.
ZO-1 Fw:5′- GCCGCTAAGAGCACAGCAA-3’.
ZO-1 Rev:5′- TCCCCACTCTGAAAATGAGGA-3’.

2.13. Measurement of plasma corticosterone and IL-6

The corticosterone concentration in the collected plasma was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Arbor assays, USA). The assay was performed according to the manufacturer's instructions. The absorbance was measured using a plate reader (Thermo Fisher Scientific Inc., USA) at a wavelength of 412 nm to determine corticosterone concentration. Separately, plasma corticosterone levels were evaluated using an LC-MS/MS system (HPLC: LaChrom Ultra, Hitachi, Japan; column: TSKgel ODS-100Z 3 μm, TOSOH, JAPAN, MS:API 2000, AB SCIEX, Canada). Plasma samples (50 μL) were mixed with 800 μL of ethyl acetate and allowed to stand for a while for precipitation of plasma proteins. The supernatant (600 μL) was dried and redissolved with methanol (500 μL) and applied for the analysis using methanol as an eluent at a flow rate of 0.2 mL/min. Plasma IL-6 levels were measured by ELISA using mouse IL-6 ELISA kit (Sigma-Aldrich Inc., USA).

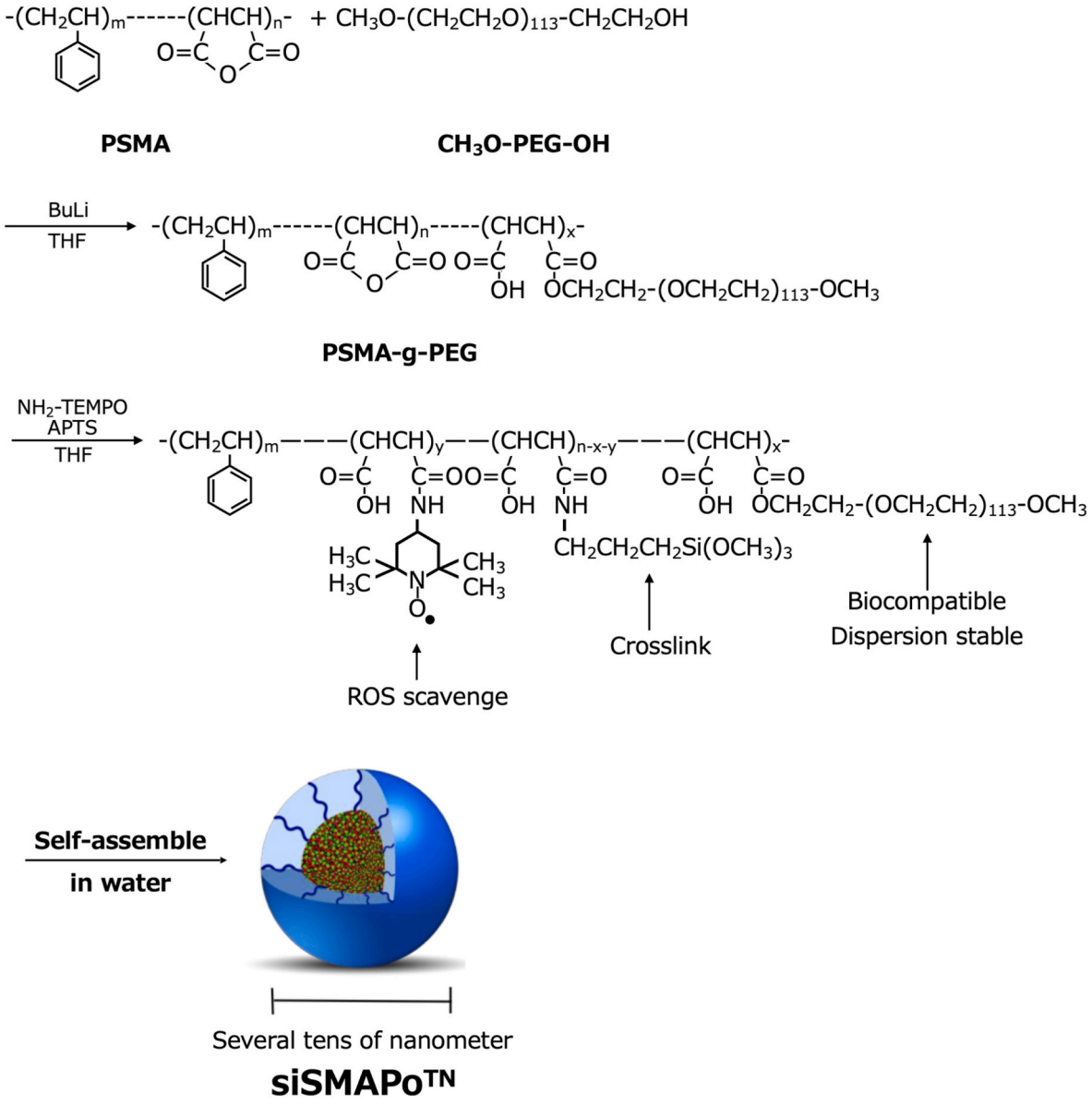
2.14. Statistical analysis

For statistical analysis, one-way analysis of variance and multiple comparison tests were performed using the software GraphPad Prism 8.3.0 (GraphPad Software, USA). Data are presented as the mean ± SEM. The statistical differences were determined using one-way ANOVA followed by Turkey's multiple comparison test and set at $p \leq 0.05$ (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

3. Results

3.1. Synthesis and characterization of siSMAPo^{TN} nanoparticles

In order to investigate the effect of intestinal ROS scavenging on brain function, the design of antioxidative nanoparticles, which are distributed only in the intestine and do not spread into the whole body, is important. In this study, chemically crosslinked antioxidative nanoparticles (siSMAPo^{TN}) based on PSMA were prepared (Scheme 1). Since PSMA possesses maleic anhydride repeating units in its main chain, it is convenient to introduce versatile nucleophiles as side chains. PEG is a



Scheme 1. The synthesis scheme of PSMA-*g*-PEG and siSMAPo^{TN} polymer and its self-assembling in water.

unique water-soluble polymer that has high biocompatibility and improves the dispersion stability of nanoparticles. Since the size and dispersion stability of nanoparticles affect their retention in the intestinal mucosa [10], PEG was introduced as a graft chain of PSMA. GPC of PSMA-*g*-PEG showed a shift to a higher molecular weight than that of PSMA, indicating the successful synthesis of PSMA-*g*-PEG (Fig. 2). The number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (Mw/Mn) of PSMA-*g*-PEG were 5,100, 13,900, and 2.47, respectively. The ^{1}H NMR spectra (Fig. S1) also supported the successful synthesis of PSMA-*g*-PEG, in which PEG was introduced into the maleic anhydride moiety of PSMA.

PSMA-*g*-PEG was further modified by amino-TEMPO (as an antioxidant moiety) and aminopropyl (trimethoxy)silane (APTMS), which were introduced to form chemically stable crosslinking particles (Fig. S2). The modification efficiencies of the TEMPO and silica moieties were determined using ESR and ICP-AES, respectively. The polymer was confirmed to possess 11 μg/mg of silica and 55 μg/mg of amino-TEMPO, which corresponded to four units of APTMS and 20 units of TEMPO per chain, respectively. After dialysis of the resulting amphiphilic polymer from the THF solution against water, nanoparticles with a size of approximately 60 nm, as determined by DLS, were successfully prepared (Fig. S3). The surface charge of siSMAPo^{TN} was −30 mV, which was attributed to the carboxylic acid groups acting as the side chains of the polymer.

3.2. Pharmacokinetic study of siSMAPo^{TN}

Selective localization of siSMAPo^{TN} in the intestine is important to understand the effect of intestinal ROS scavenging on brain function. The pharmacokinetics of siSMAPo^{TN} were investigated after radiolabeling with ^{125}I. When free ^{125}I was administered orally, it was distributed not only into the GI tract but also into the blood. On the contrary, the radiation was only observed in the small and large intestines and not in the blood after the oral administration of ^{125}I-labeled siSMAPo^{TN} (Fig. 3A and B). It is interesting to note that radiation emissions continued for an extended period. For example, more than 30% of the administered siSMAPo^{TN} was observed in the intestine even 24 h after oral administration.

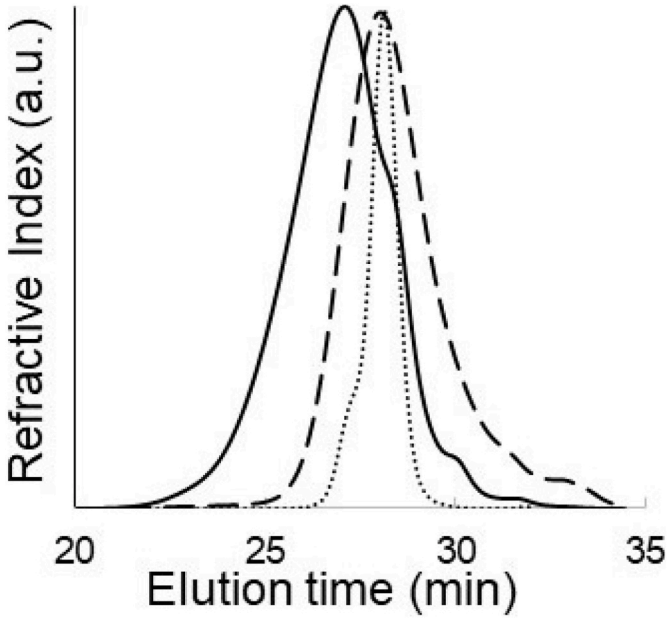


Fig. 2. Gel permeation chromatograms (GPCs) of PEG, PSMA and synthesized polymers (PSMA-*g*-PEG). Dotted line: PEG, dashed line: PSMA, solid line: PSMA-*g*-PEG.

3.3. Oral administration of siSMAPo^{TN} improved CRS-induced depression-like behavior

CRS has been widely used to induce depression-like symptoms, such as behavioral changes and inflammation [11]. First, we aimed to examine whether oral administration of siSMAPo^{TN} influenced depression-like syndromes. To investigate the protective effect of depression, effects on depression-like behavior were investigated using FST and TST, which are typical depressive behavioral tests. In both tests, mice subjected to CRS showed increased immobility time, indicating clear depression-like behavior, as shown in Fig. 4A and B. On the contrary, the siSMAPo^{TN} group inhibited the increase in their immobility times. The results described here indicate that the intestinal ROS scavenger siSMAPo^{TN} also suppresses the stress-dependent development of depression-like behavior.

3.4. siSMAPo^{TN} reduced intestinal oxidative damage caused by CRS

The induction of oxidative damage in the colon has also been reported in CRS mice [13]. To investigate the antioxidative properties of siSMAPo^{TN}, the intestinal levels of malondialdehyde (MDA) and protein carbonyl which are representative biomarkers of oxidative stress were evaluated (Fig. 5). The intestinal MDA level was increased under conditions involving CRS, consistent with a previous report [14]. siSMAPo^{TN} significantly inhibited the increase in MDA levels at the jejunum (Fig. 5A), ileum (Fig. 5D) and colon (Fig. 5G), indicating the antioxidative properties of siSMAPo^{TN} in the intestine. Reduced oxidative damage was also confirmed by protein carbonylation levels (Fig. 5B, E and 5H). Interestingly, the intestinal interleukin 6 (IL-6) level, which was increased by CRS, was also suppressed by siSMAPo^{TN} (Fig. 5C, F and 5I). The levels of IL-6, a proinflammatory cytokine, also increase along with ROS levels in inflamed areas [15]. Described data suggests siSMAPo^{TN} reduced oxidative damage attributed to overproduced ROS during CRS and suppressed inflammation by reducing the levels of proinflammatory cytokines.

To further investigate the effects of siSMAPo^{TN} in the intestinal environment under CRS, Alcian blue stain was used, which can stain the goblet cells and mucin layer in the colon. As shown in Fig. 6A and B, the CRS group showed a significant decrease in the colonic mucosal area and goblet cells filled with mucin, which is consistent with previous studies [11], indicating a reduction in mucus barrier function. It is interesting to note that the siSMAPo^{TN}-treated group showed mitigation of the reduction in the number of goblet cells and the destruction of the intestinal environment, suggesting a protective effect of siSMAPo^{TN} in the intestinal mucosa.

3.5. siSMAPo^{TN} prevented CRS-induced leaky gut syndrome

Intestinal inflammation increases intestinal paracellular permeability, leading to a condition called “leaky gut” [16]. The leaky gut syndrome is a condition in which the barrier function of the intestines is destroyed, allowing the leakage of toxic substances, such as lipopolysaccharide (LPS) and intestinal bacteria that do not normally leak into the bloodstream to spread throughout the body [16]. Since siSMAPo^{TN} significantly suppressed the intestinal inflammation caused by CRS, we aimed to investigate how the ROS-scavenging property of siSMAPo^{TN} affected the leaky gut. Mice were administered FITC-labeled dextran (4 kDa) via oral gavage, and plasma samples were collected 2 h after administration. The fluorescence intensity of the plasma was analyzed to confirm changes in intestinal permeability. Although no significant difference was observed between the control and the stressed mice groups, there was a tendency of increased plasma FITC intensity ($p = 0.16$) (Fig. 7A). Importantly, the plasma FITC level of the stressed mice treated with siSMAPo^{TN} group was significantly reduced compared to the stressed mice group, indicating the suppression of the CRS-induced intestinal permeability by siSMAPo^{TN} treatment. To further evaluate

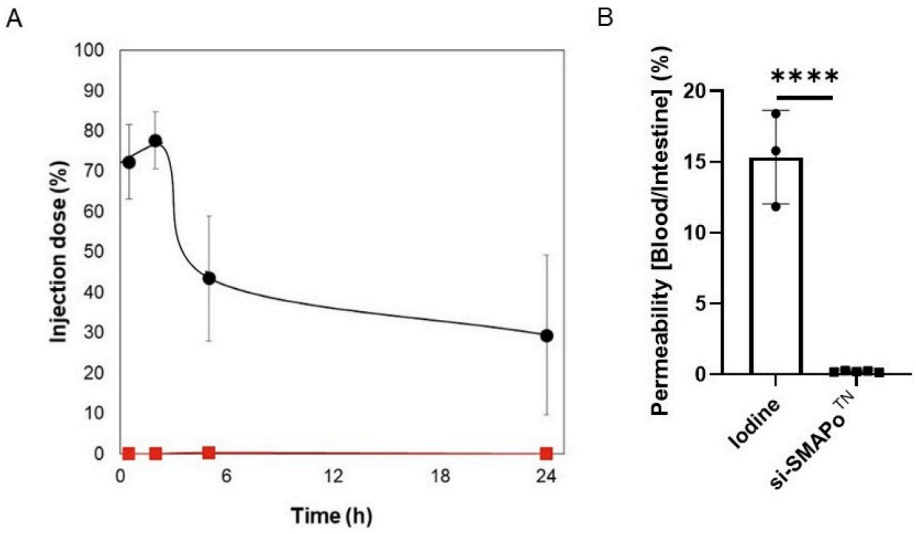


Fig. 3. Biodistribution of siSMAPo^{TN} nanoparticles administered orally. Distribution was determined using a gamma counter. a) The radioactivity after oral administration of the nanoparticles in vivo. Circle: intestines (small and large intestine), square: blood. b) The permeability ratio of blood against intestines. Data are expressed as mean ± SEM, n = 3–5, ****p < 0.001.

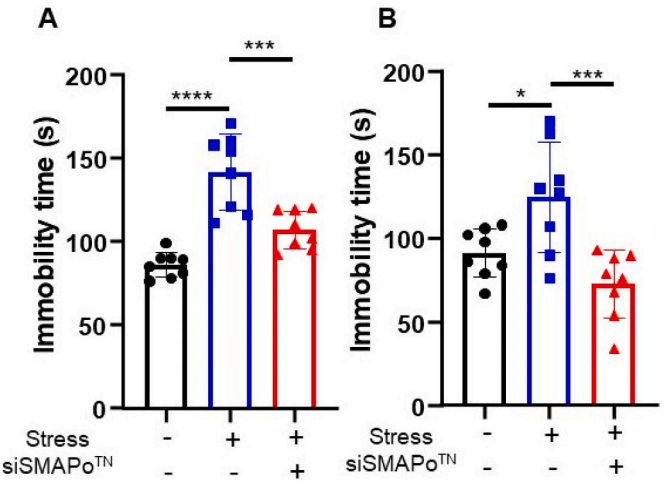


Fig. 4. Antidepressant-like effects of oral administration of siSMAPo^{TN}. The immobility time of the forced swimming test (A) and tail suspension test (B) were increased by CRS, whereas the tendencies were suppressed by the administration of siSMAPo^{TN}. Data are expressed as mean ± SEM, n = 8. *p < 0.05, ***p < 0.005, ****p < 0.001.

the colon condition, mRNA levels of the tight junction protein ZO-1 in the colon tissue were measured by RT-qPCR. The oral administration of siSMAPo^{TN} improved the tendency of reduction (Fig. 7B) induced by CRS. These results indicated that siSMAPo^{TN} prevents stress-induced leaky gut syndrome.

3.6. siSMAPo^{TN} suppressed changes in depression-related biomarkers in the plasma and brain

Taken together, the aforementioned data indicate that scavenging the overproduced ROS prevented the disruption of the intestinal environment and behavioral signs of depression caused by CRS. To obtain further evidence concerning the relationship between intestinal ROS and CRS-induced depression-like symptoms, as well as to evaluate the protective effect of intestinal ROS scavengers on the progression of depression, biological markers of depression in the blood and brain were investigated. Blood corticosteroid levels are one of the most representative markers of depression [11]. Since corticosterone is an adrenocortical hormone that participates in maintenance of the homeostatic balance in the body and is secreted in response to stress, chronic elevation of corticosterone levels is observed in mice subjected to CRS [11]. In this study, corticosterone levels in mouse plasma were measured using ELISA and LC-MS/MS (Fig. 8A and B, respectively). The CRS group showed a significant increase in corticosterone levels. In contrast, in the group administered siSMAPo^{TN}, no increase in the corticosterone levels was observed.

Meta-analysis showed elevated IL-6 levels in patients with major depressive disorder [17]. Increased plasma IL-6 levels were also observed in our CRS mice model. Interestingly, the stressed mice treated with siSMAPo^{TN} group did not show an increase in the IL-6 levels in plasma (Fig. 8C), indicating that siSMAPo^{TN}, which was located only in the intestine, alleviated the inflammation in the body caused by the CRS.

In addition to the blood hormone and inflammatory cytokine levels, the expression of mRNA of a typical neurotrophic factor in the hippocampus was measured by RT-qPCR. The levels of brain-derived neurotrophic factor (BNDF) and nerve growth factor (NGF) are known to decrease in CRS-based models of depression [18]. The mRNA levels of these factors decreased in the CRS group without treatment with siSMAPo^{TN}, whereas those in the CRS group treated with siSMAPo^{TN} did not decrease, as shown in Fig. 8D and E. These data indicate that orally administered siSMAPo^{TN} not only protected the intestinal environment from oxidative damage and excessive inflammation but also helped to maintain plasma corticosteroid homeostasis and neurotrophic factors in the hippocampus.

4. Discussion

A significant relationship between the gut environment and various organs has been demonstrated, and its disruption induces the development of various disorders in the body [19]. Among them, the gut-brain axis, which involves bidirectional communication between the gut and brain, has garnered significant interest in recent years [4]. However, few studies have investigated the effects of specific molecules in the gut environment on brain function. As a result, attempts to treat brain dysfunction via the gut-brain axis have been limited to probiotics, prebiotics, and fecal transplantation [5]. In this study, we focused on the intestinal oxidative damage caused by ROS. Although intestinal ROS are important signaling molecules for the maintenance of intestinal homeostasis, excessive levels of ROS disrupt the intestinal environment and lead to disorders, inflammation, and dysfunction of the intestinal

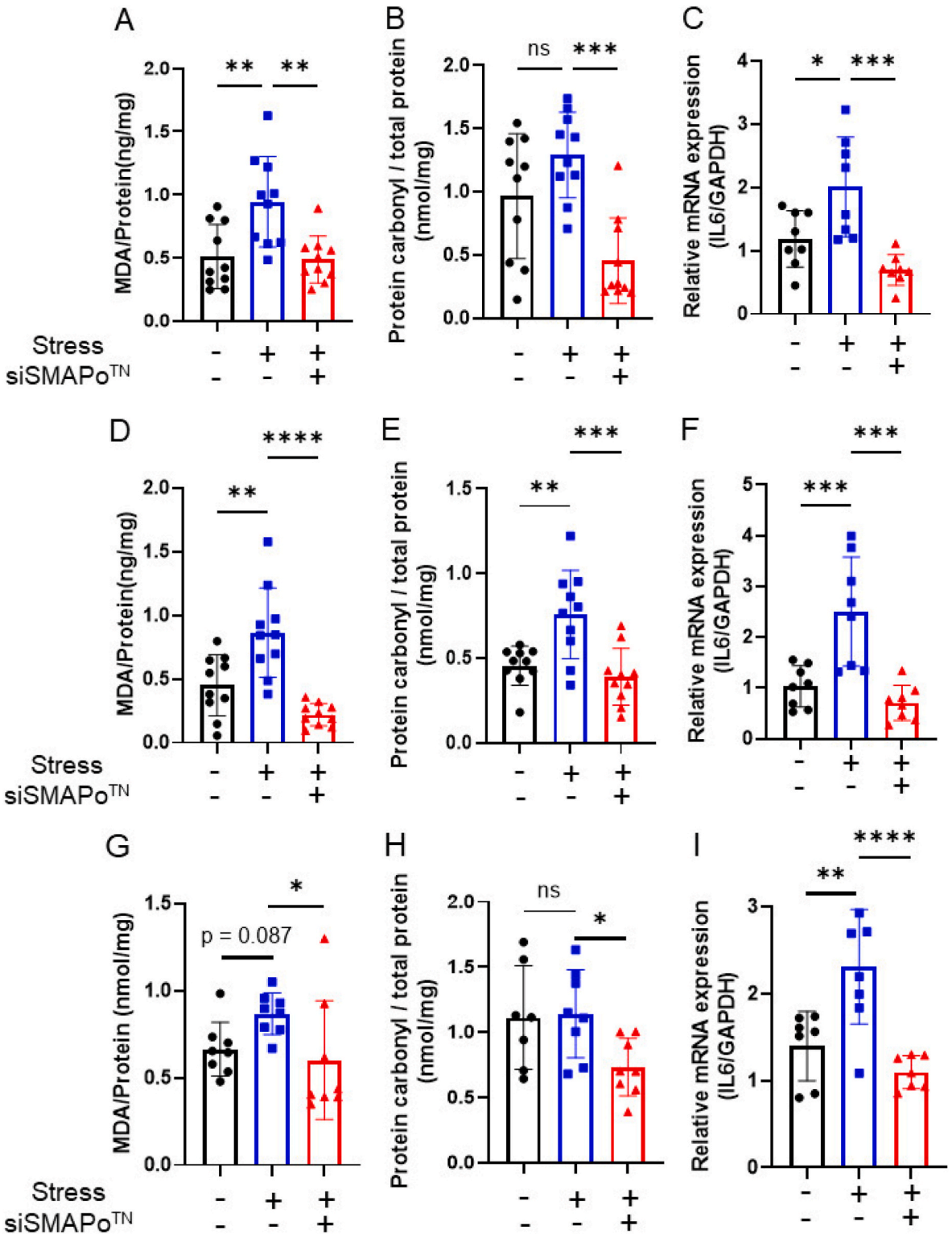


Fig. 5. Measurement of oxidative stress in the intestine. MDA, protein carbonylation and relative mRNA levels of inflammatory cytokines (IL-6) were evaluated in the jejunum (A, B and C), ileum (D, E and F) and colon (G. H and I). Data are expressed as mean ± SEM, n = 7–10, *p < 0.05, **p < 0.01, ****p < 0.001.

immune system [7,8]. We hypothesized that excessive generation of ROS in the intestine affects brain function via the gut-brain axis, and scavenging intestinal ROS may be a novel strategy for depression treatment.

We have previously reported antioxidative nanoparticles that can scavenge excessively generated ROS in vivo [9]. For instance, Long et al. reported self-assembling polymer nanoparticles for the treatment of inflammatory bowel disease (IBD) [10]. Polymer-based antioxidants drastically alter the pharmacokinetics and biodistribution of low-molecular-weight (LMW) antioxidants. In this study, we further optimized the material design, in which PEG, TEMPO, and silanol were covalently conjugated to PSMA which were confirmed by ^{1}H NMR, ESR and ICP-AES. The resulting nanoparticle, siSMAPo^{TN}, exhibited high dispersion stability, biocompatibility, and antioxidant capacity. We

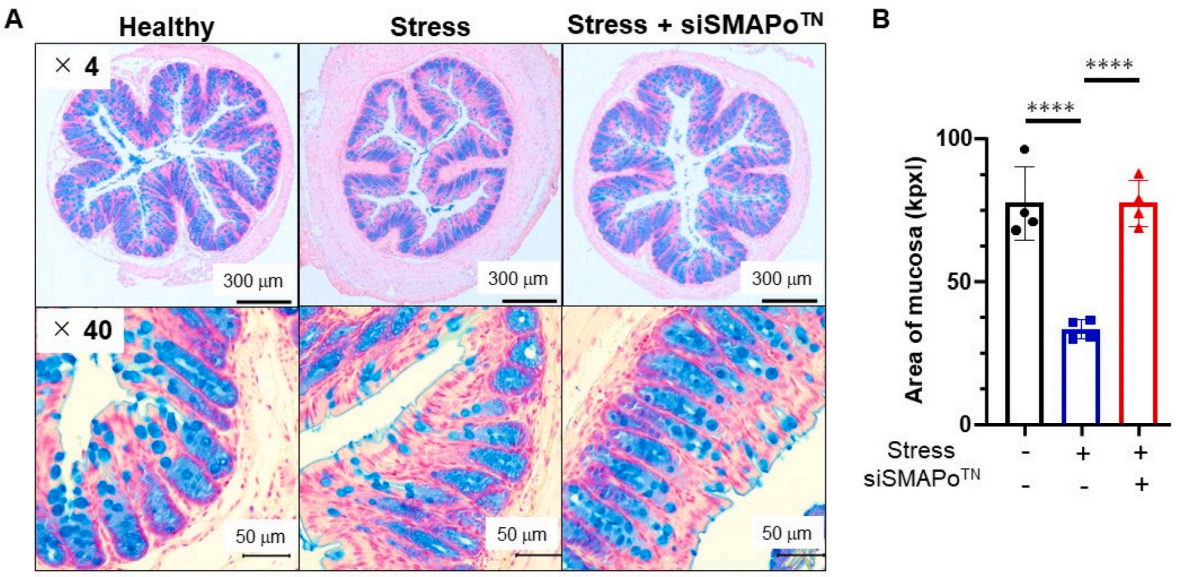


Fig. 6. CRS reduced the colonic mucus barrier. A) Representative Images of the colonic mucosa layer stained by Alcian blue which stains mucus layer and goblet cells filled with mucin. B) Quantification of the mucosal area by Image-J. Data are expressed as mean ± SEM, n = 4, ****p < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

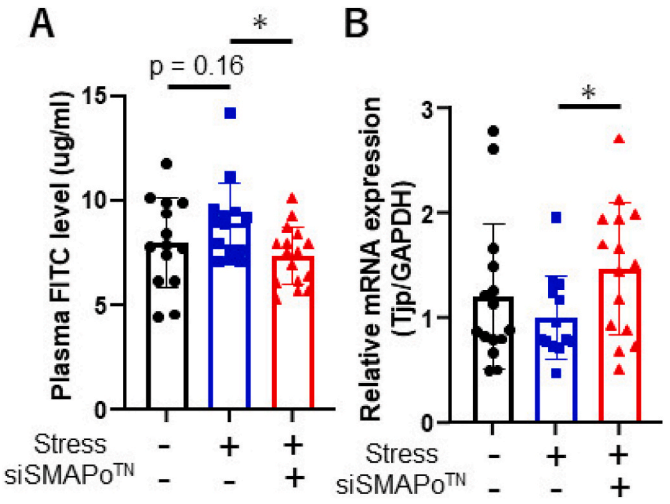


Fig. 7. The oral administration of siSMAPo^{TN} improved leaky gut syndrome. A) FITC-dextran (4 kDa) levels in the plasma, B) Relative mRNA levels of the tight junction protein (Tjp) in the colonic tissue. Data are expressed as mean ± SEM, n = 14 or 16, *p < 0.05.

have previously confirmed that SMAPo^{TN}, which possesses no silanol moiety in the polymer backbone of PSMA, was internalized in the bloodstream after oral administration [20]. In contrast, the chemically cross-linked structure of siSMAPo^{TN} inhibits the disintegration of self-assembled micelles in vivo and prevents their spread via leakage into the bloodstream. This characteristic is in sharp contrast to that of LMW antioxidants, which are distributed into the bloodstream after oral administration and rapidly diffuse throughout the body. siSMAPo^{TN} can remain only in the intestine and exert a ROS-scavenging effect without leaking into the bloodstream. Importantly, siSMAPo^{TN} can exist in the intestine for more than 24 h because of its high dispersion stability and biocompatibility. Thus, siSMAPo^{TN} is a suitable material for examining the relationship between intestinal ROS and depression via the gut-brain axis.

CRS, which is often used to establish a simple rodent model of depressive disorder, causes various changes in the entire body. For instance, it leads to the disruption of the colonic mucosal layer [11], increases intestinal ROS and inflammatory cytokine levels [13–15], and causes leaky gut syndrome [21]. These symptoms were also confirmed in this study. CRS increased the colonic MDA levels, which are a representative marker of oxidative damage in the cellular membrane. We found that continuous administration of siSMAPo^{TN} reduced colonic oxidative stress and protected the mucosal layer and intestinal barrier function. These findings indicate that the removal of excess ROS can ameliorate the damage caused by CRS in the intestinal environment. We further confirmed that selective scavenging of intestinal ROS prevented intestinal damage including leaky gut syndrome, which is consistent with the previous studies [22].

The leaky gut syndrome is often accompanied by an imbalance in hormones and chronic inflammation of the body [23]. In this study, suppression of leaky gut syndrome by siSMAPo^{TN} inhibited the increase in plasma corticosterone levels. Corticosterone is a typical stress biomarker whose levels are elevated during systemic inflammation. Increased corticosterone levels have been reported in various depression models, and elevated human cortisol levels, equivalent to corticosterone levels, have also been reported in patients with depression [24]. Although the role of corticosterone in depression has not been fully investigated, the relationship between corticosterone and BDNF, which promotes neuronal maturation and whose levels are reduced in patients with depression, has been reported [25]. Agasse et al. reported that corticosterone reduced BDNF levels and hippocampal neurogenesis via CDK5 phosphorylation of huntingtin [26]. The findings of the present study are consistent with this result; siSMAPo^{TN} prevented the increase in corticosterone levels and the decrease in BDNF levels. These data suggest the importance of controlling corticosterone levels in the blood, and the data described here clearly indicate that scavenging intestinal ROS prevents unexpected systemic inflammation caused by excessive corticosterone levels.

Current antidepressants, which are commonly used to treat depression, modulate neurotransmitter systems and induce neurogenesis in the brain [27]. These antidepressant drugs must cross the blood-brain barrier (BBB). The BBB limits the internalization of drugs into the brain, preventing an increase in therapeutic efficiency [28]. Another approach for depression treatment targets the gut microbiome and improves brain function via the gut-brain axis. These approaches include fecal transplantation, probiotics, and prebiotics that target the intestinal

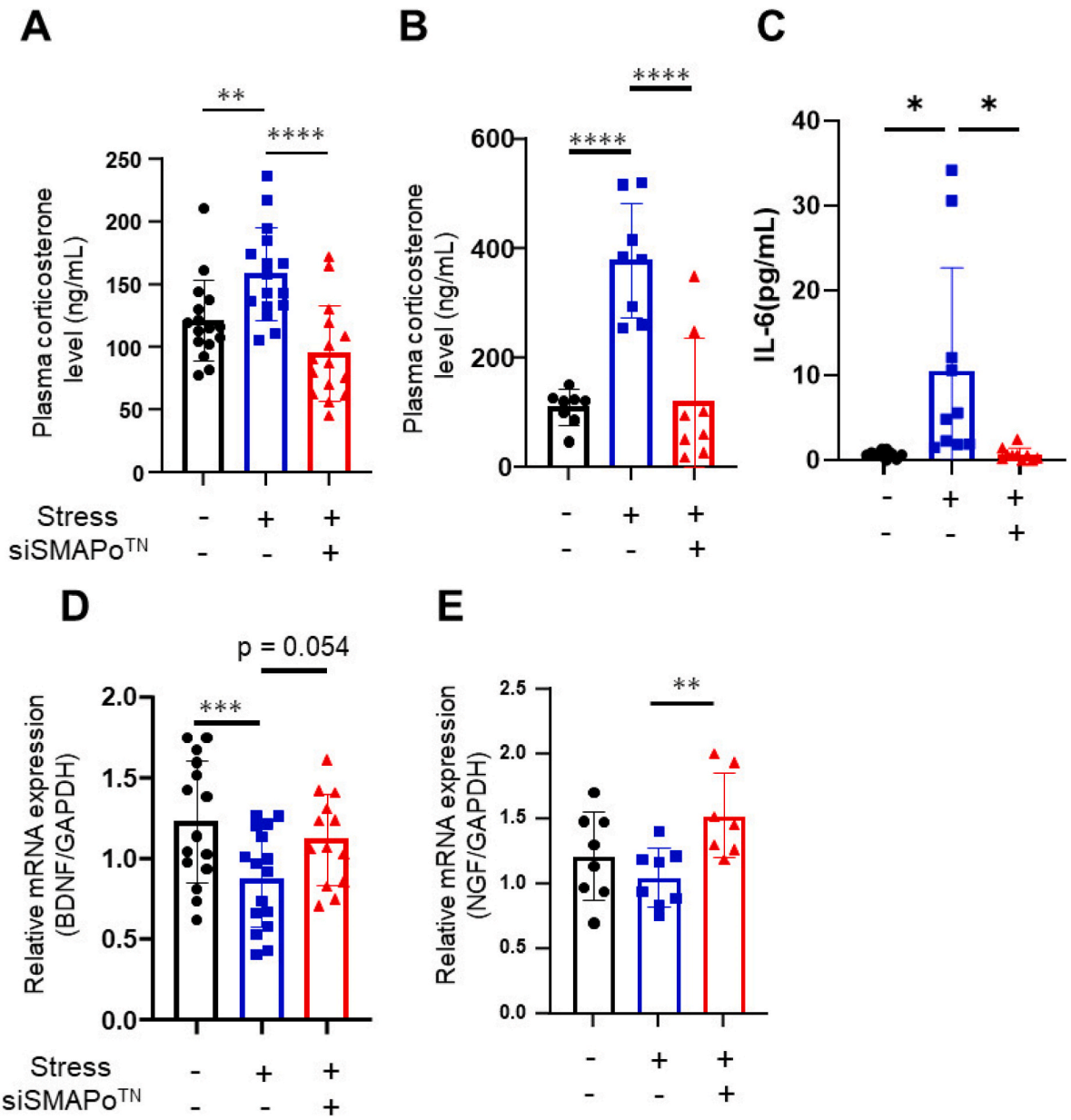


Fig. 8. Biomarkers related to depression were analyzed after euthanasia. The plasma corticosterone level was measured by ELISA (A) and LC-MS/MS (B), and plasma IL-6 level (C) measured by ELISA. Relative mRNA levels of BDNF (D) and NGF (E) in the hippocampus. Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01, ****p < 0.001.

environment. However, the clinical effects of probiotics and prebiotics are limited [29], and issues associated with infection have been observed with fecal transplantation [30]. Accordingly, another strategy to treat depression via the gut-brain axis should be considered. In this study, we focused on intestinal ROS as target molecules. Orally administered siSMAPo^{TN} protected the intestine from oxidative damage and maintained homeostasis. Surprisingly, siSMAPo^{TN} not only protected the intestine from oxidative damage but also alleviated the inflammation in the body and behavioral depression symptoms caused by CRS, despite its antioxidant effect being limited to the intestine and not observed in the blood or other organs, such as the brain. As far as we know, this is the first report that clearly demonstrates the prevention of depressive symptoms induced by CRS via the elimination of intestinal ROS. The proposed intestinal ROS scavenger is a novel antidepressant candidate that can be used to treat depression via the gut-brain axis.

Credit author statement

Yutaka Ikeda: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft.; Naoki Saigo: Methodology; Investigation; Formal analysis; Validation; Roles/Writing -review & editing.; Yukio Nagasaki: Project administration; Supervision; Conceptualization; Funding acquisition; Investigation; Validation; Roles/Writing - review & editing.; All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The University of Tsukuba possesses a patent for this material, which is licensed to CrestecBio, Inc. YN is the advisor and shareholder of CrestecBio, Inc., which holds registered or applying for patents on redox

nanoparticles. The other authors have no conflict of interest to declare.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2023.122053.

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