Neuroprotectin D1 Protects Against Postoperative Delirium-like Behaviors in Aged Mice by Regulating Neuroinflammation Induced by Surgical Trauma

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Research

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Abstract

Background

Postoperative delirium (POD) is the most common postoperative complication affected elderly patients, yet the underlying mechanism is elusive and without effective therapy. The neuroinflammation hypothesis has been emerging as the pathogenesis of POD. Recently, accumulative evidence has supported the role of specialized proresolving lipid mediators (SPMs) in regulating inflammation. NPD1, a novel member of SPMs, is identified with potent immune-resolvent and neuroprotective effect. We aimed to investigate the role of NPD1 in neuroinflammation and postoperative cognitive function with a mice model of POD.

Methods

Aged male C57BL/6 mice were subjected to laparotomy under isoflurane anesthesia with NPD1 prophylaxis. The general and memory behaviors were assessed by buried food test, open field test and Y maze test at 6, 9, and 24 hours after the surgery. Expression level of inflammatory cytokines were analyzed by ELISA. The permeability of blood-brain barrier (BBB) was detected by spectrophotometric quantification of extravascular dextran tracer from brain tissue extracts, and the expression of tight junction (TJ) associated proteins by Western blotting. The reactive states of astrocytes and microglia were examined by immunofluorescence. The protective effects of NPD1 was also evaluated through macrophage polarization by flow cytometry, using the bone marrow-derived macrophages cultures.

Results

NPD1 prophylaxis decreased the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines in peripheral blood, hippocampus and prefrontal cortex, and limited the leakage of BBB by increasing the expression of TJ associated proteins. Besides, NPD1 prevented the activation of microglia and astrocytes both in the hippocampus and prefrontal cortex, which resulted in improved general and memory function of mice. Furthermore, NPD1 treatment modulated the inflammatory cytokine profile and promoted the macrophage polarization toward M2 in LPS-stimulated macrophages.

Conclusions

These findings verify the anti-inflammatory and proresolving activities of NPD1 in the inflammatory milieu both *in vivo* and *in vitro*, and provide better insight into the pathophysiology and potential therapy for POD.

Background

Postoperative delirium (POD), defined as delirium occurring mainly within 1 week after surgery, is a common neuropsychiatric complication characterized by fluctuating and concurrent disturbances of attention, cognition, psychomotor behavior, emotion and sleep-wake rhythm. POD has been linked to higher mortality, prolonged hospitalization, increased risk of long term cognitive impairment [1, 2], and presents with extra medical burden on governments and society [1, 3]. The incidence of POD is ranged from 14% in the general medical units to 82% in the intensive care unit, particularly with higher tendency in elderly patients [4–6]. As aging has become a common issue in global, the number of elderly who need surgery/anesthesia treatment has been increasing, as well as the prevalence of POD. However, there are no effective therapies for this complication for the undefined underlying pathophysiology.

Recent studies highlight the importance of neuroinflammation in the development of POD [7, 8]. Surgical trauma activates the innate immune system, leading to the systemic release of cytokines [7]. The humoral pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6), have been reported to be associated with the leakage of the blood-brain barrier (BBB), which leads to the entrance of pro-inflammatory cytokines and monocyte-derived macrophages, resulting in the activation of glia, including microglia and astrocytes [9, 10]. This process is mainly affected by the bone marrow-derived macrophages (BMDMs), which react dually to the microenvironmental cues to initiate the neuroinflammation [11–13]. Interaction between peripheral immune and brain amplifies the inflammation in the central nervous system (CNS) [14, 15], and the cascade of neuroinflammation induces the synaptic dysfunction and neuronal apoptosis, ultimately impairs the cognitive function [16, 17]. On this basis, treatments targeting at reversing neuroinflammation show great potential to be candidate therapies for POD.

Along with passive termination of inflammation, resolution actively participates in the restoration of acute inflammation as a coordinated program, which is regulated by specialized proresolving lipid mediators (SPMs) [18]. SPMs are endogenous biosynthesis from essential fatty acids with potent properties of anti-inflammation and immunoregulation [19, 20]. Protectin D (PD) family, specially termed as neuroprotectin D1 (NPD1) when synthesized in the neural system, is one of the SPMs derived from omega-3-polyunsaturated fatty acid docosahexaenoic acid (DHA), sharing similar biological activities with other lipid mediators such as resolvins and maresins, including accelerating nonphlogistic macrophage phagocytosis, inhibiting neutrophil infiltration and regulating the production of cytokines and chemokines [20–22]. Additionally, NPD1 has been demonstrated to be neuroprotective in the preclinical models of Alzheimer's disease, which shares some characteristics with POD such as memory impairment [23, 24]. However, there are not any reports about the role of NPD1 in the POD.

Based on these discoveries, we proposed the hypothesis that prophylaxis with NPD1 could improve the POD-like behavior of aged mice through its proresolving effect on the inflammation induced by surgical trauma. To validate this hypothesis, we assessed the effects of NPD1 on the postoperative behavior of aged mice, and inflammation events both in the periphery and in CNS. Furthermore, we aimed to

determine that NPD1 exerts anti-inflammatory and proresolving properties by promoting macrophage polarization, which is pivotal in promoting the restorative process in the acute inflammation [25].

Methods

Animals

The experimental protocol was approved by the Animal Ethics Committee of Zhongnan Hospital of Wuhan University, and all experiments were performed following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Female C57BL/6 mice (Changsha Tianqin Biotechnology CO., LTD., Changsha, China), 18 months old, and weighing 30–40 g were group-housed 4–5 per cage on a 12-hour light/dark cycle in a temperature-controlled (25 ± 2˚C) room with free access to standard rodent water and food.

Experimental Protocol

The mice were randomly divided into the control group, surgery group, NPD1 group, or NPD1 + surgery group. NPD1 (Cayman Chemical, Ann Arbor, MI, USA) was given at 2 μg/ml in saline with 1.4% ethanol, i.p. 600 ng (300 μl) per mouse in NPD1 group and NPD1 + surgery group, while the equal volume of 1.4% ethanol in saline was given in control group and surgery group. One hour after administration of NPD1 or vehicle, mice in the surgery group and NPD1 + surgery group were subjected to a simple laparotomy under isoflurane anesthesia, while the mice in the control group and NPD1 group were placed in their home cages with 100% oxygen for two hours without surgery treatments. The mice had multiple behavioral tests at 24 hours before the surgery (baseline), and at 6, 9, and 24 hours after the surgery/anesthesia. Within each group, separate cohorts were subjected to assessment at each time point (n = 8–10 per cohort). To measure BBB permeability by immunohistochemistry and spectrophotometric quantification, mice were given with 10-kDa dextran i.v. at 6 hours after surgery/anesthesia, and decapitated 15 min later to harvest the brain (n = 5 per cohort). To detect inflammatory cytokine, mice were sacrificed at 6, 9, and 24 hours after the surgery/anesthesia to collect blood, hippocampus, and prefrontal cortex for Western blotting and ELISA (n = 5 per cohort). At 24 hours postoperatively, mice were anesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Then, hippocampal and prefrontal cortex tissues were collected for immunostaining (n = 3–5 per cohort).

Surgical Model

A simple laparotomy was performed under isoflurane anesthesia using the methods as described in our previous studies [26, 27]. Specifically, each mouse was induced with 1.4% isoflurane in 100% oxygen in a transparent acrylic chamber. Fifteen minutes after induction, the mouse was moved out of the chamber and placed on a heating pad to maintain body temperature between 36 ℃ to 37 ℃ during the surgery. Isoflurane anesthesia was maintained via a cone device with a 16-gauge needle sensor monitoring the

concentration of isoflurane. A longitudinal midline incision was made from the xiphoid to the 0.5 cm proximal pubic symphysis on the skin, abdominal muscles and peritoneum. Abdominal organs were partially exposed for 2 min, then the incision was sutured layer by layer with 5 – 0 Vicryl thread. The procedure for each mouse lasted about 10 minutes, then the mouse was put back into the anesthesia chamber for up to 2 hours to receive the rest of the anesthesia. Blood pressure was monitored with the mouse-tail blood pressure cuff (Softron BP-2010A, Softron Beijing Biotechnology Co., Ltd., Beijing, China), and blood gas and blood glucose levels were tested by a blood gas analyzer (i-STAT, Abbott Point of Care Inc., Princeton, NJ, USA). Analgesia was given 5 min before skin incision, at the end of the procedure and every 8 hours for one day postoperatively with EMLA cream (2.5% lidocaine and 2.5% prilocaine).

Behavioral Tests

The behavioral changes were detected by multiple behavioral tests in the order of buried food test, open field test and Y maze test at 24 hours before the surgery, and at 6, 9, and 24 hours after the surgery as described in our previous studies [26]. In all the tests each apparatus was cleaned with 75% ethanol after each mouse to remove odors.

Firstly, for buried food test, each mouse was given 2 pieces of sweetened cereal two days before the test in their home cage. Mice were habituated for one hour before the test by placing the home cage with mice in the testing room. The test cage was contained 3 cm deep of clean bedding and a piece of sweetened cereal pellet buried underneath 0.5 cm below the surface. The location of the food pellet was randomly changed every time. The mouse was placed in the center of the test cage for 5 minutes, and the latency to eat the food was measured as the time interval from the mouse placed in the test cage to when it uncovered the food pellet and grasped it in the forepaws and/or teeth. If the mouse failed to find the pellet within 5 minutes, the latency was defined as 300 seconds.

Secondly, for open field test, mouse was placed in the center of an open field chamber (40 × 40 × 40 centimeters) in a quiet and illuminated room and allowed to freely explore for 5 minutes. The movement parameters of the mouse were monitored and analyzed via a video camera connected to the Any-Maze animal tracking system software (Xinruan Information Technology Co. Ltd., Shanghai, China). Parameters of total distance moved, freezing time and time spent in the center were recorded and analyzed.

At last, the Y maze test was executed in a two trials task in a quiet and illuminated room, with the apparatus consists of three arms (width 8 cm × length 30 cm × height 15 cm) at 120° angles extending from a central space, and each wall of the arms were pasted with cardboards in different patterns as spatial cues. Three arms of Y maze were randomly distributed into the novel arm, which is blocked at the first trial but opened at the second trial; start arm, in which the mouse starts to explore; and another arm, which is always open. The first trial is the training trial which allowed the mouse to explore the start arm and the other arm for 10 minutes, with the novel arm being blocked. After 2 h (for the tests of 6 and 24 hours after surgery) or 4 hours (for the tests of 9 hours after surgery) time interval, the second trial as the

retention trial was conducted. The mouse was placed back in the maze in the same start arm with free access to all the 3 arms for 5 minutes. A video camera, which was linked to the Any-Maze animal tracking system software, was installed 200 centimeters above the chamber to monitor and analyze the number of entries and the time spent in each arm.

Bbb Permeability Assay

The method is based on the established BBB dye-injection assay with slight modification [28, 29]. Specifically, 6 h after surgery, each mouse was injected intravenously through tail veins with 100 μl 10-kDa dextran Texas Red (Invitrogen, D1863). Fifty minutes after injection, each mouse was anesthetized and decapitated. The brains were harvested and fixed by immersion in 4% paraformaldehyde overnight at 4 ℃, then cryopreserved in 30% sucrose and frozen in TissueTek OCT (Sakura). Frozen sections of 20 μm were collected and post-fixed in 4% PFA at room temperature (20–25 ℃) for 15 min, washed in PBS and were blocked with 10% goat serum (Boster Biologic Technology, China) for 2 hours, permeabilized with 0.5% Triton X-100, then incubated with isolectin B4 (20 μg/ml, I21411, Molecular Probes, San Francisco, CA, USA) for immunostaining to visualize blood vessels. The fluorescence images of the injected tracer and isolectin were detected under 40· objective lens.

Spectrophotometric quantification of extravascular 10-kDa dextran Texas Red was carried out with extracts of the hippocampus and prefrontal cortex at 6 hours after surgery. Specifically, anesthetized animals were perfused transcardially for 5 min with 50 ml PBS, then the brains were removed and homogenized in 1% Triton X-100 in PBS (100 μl/100 mg brain tissue). Brain lysates were centrifuged at 16,000 r.p.m. for 20 min and the relative fluorescence of the supernatant was measured on a fluorometer POLARstar Omega (BMG Labtech) (ex/em 595/615 nm).

Western Blot Analysis

The total protein samples from hippocampal and prefrontal tissues were homogenized using RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris, 1% Triton, 0.1% sodium dodecyl sulfate, and 0.5% deoxycholate) containing protease and phosphatase inhibitors. The lysate was centrifuged at 12,000 rpm for 5 minutes at 4 ℃ to remove the sediment. The supernatants were collected, and the protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit (Aspen, Wuhan, China). After the determination of the contents, the proteins were separated by SDS-PAGE (8–12%) and then transferred to PVDF membranes (Aspen, Wuhan, China). After being blocked with 5% skim milk for 1 hour at room temperature, the membranes were incubated overnight at 4 ℃ with the following primary antibodies: anti-ZO-1 (1:500, Abcam, ab96587), anti-occludin (1:2000, Abcam, ab167161), anti-claudin-5 (1:500, Biorbyt, orb214680), anti-β-actin (1:10,000, TDY Biotech, ab37168). The membranes were washed three times with TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Aspen, AS1107) for 30 minutes at room temperature. Specific immunoreactivity was detected using enhanced

chemiluminescence (Aspen, Wuhan, China). The bands were measured using image analysis software (AlphaEaseFC software).

Enzyme-linked Immune-sorbent Assay (elisa)

The concentrations of TNF-α, IL-6, IL-10, IL-12 in the plasma and brain tissue of mice or the primary bone marrow-derived macrophages for *in vitro* experiments were determined using ELISA kits (eBioscience) according to the manufacturer's instructions.

Immunofluorescence

At 24 hours after surgery, mice were deeply anesthesia with isoflurane and perfused transcardially with ice-cold 0.1 M PBS followed by 4% PFA in 0.1 M PBS at pH 7.4. Brains were harvested and post-fixed in 4% PFA in 0.1M PBS at 4 ℃, and cryoprotected in 30% sucrose for 72 hours. Brains were freeze-mounted in TissueTek OCT (Sakura), and were cut sequentially to 20 μm. After washed in PBS and permeabilized in 0.5% Triton X-100, the coronal sections were blocked with 10% goat serum in PBS for 2 hours at room temperature to block non-specific binding, then the following primary antibodies were used: mouse anti-glial fibrillary acidic protein (GFAP) (1:500, Abcam, I21411), rabbit anti-Iba-1 (1:200, Abcam, ab178847) at 4 ℃ overnight. For secondary detection, (goat anti-mouse, goat anti-rabbit) conjugated with Alexa Fluor dyes (405 and 488) from Invitrogen (1:500) were used. Immunolabelled sections were coverslipped with 40,6-diamidino-2-phenylindole (DAPI; Invitrogen) and visualized by microscopy (Olympus, Tokyo, Japan). Five high magnifications were chosen in three non-overlapping fields randomly acquired in hippocampal subregions using a counting frame size of 0.4 mm^2. Images were processed and the area of the astrocytes and microglia quantified using ImageJ software (NIH). The area of the selected cells was converted into a binary image using the dilation method and the cell outline measured. Total immunoreactivity was calculated as percentage area density defined as the number of pixels (positively stained areas) divided by the total number of pixels (sum of positively and negatively stained area) in the imaged field.

Primary Cell Culture And Grouping

Bone marrow-derived macrophages (BMDMs) were purchased from iCell Bioscience Inc (MIC-iCELL-i017, Shanghai, China), and were cultured in DMEM/F-12 containing 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin and supplementary factor (iCell Bioscience Inc, PriMed-iCell- 011) 37˚C under 5% CO2 and 95% air. Cells were then stimulated with 10 ng/ml lipopolysaccharide (LPS, Sigma, L2880), with or without NPD1 (80 ng/ml) for 24 h, respectively. The same batches of BMDMs were untreated as control.

Flow Cytometry Analysis

For cell staining, anti-mouse CD 68-PE, CD16/CD32-PE-Cy7, CD206-PE (Invitrogen) were used. The cells were detached and suspended in flow cytometry staining buffer and treated with Fc-receptor blocker antibody for 20 min at 4 °C to eliminate nonspecific binding. Then the cells were stained by these antibodies for 30 min at 4 ℃ in dark. After washed twice in flow cytometry staining buffer and resuspended in the staining buffer, samples were detected using a BD FACSCalibur system was used for analysis.

Statistical analysis

The statistical analyses were performed with SPSS 19.0 (IBM, New York, USA) or GraphPad Prism 6 (GraphPad, New York, USA). Quantitative data are expressed as the means ± standard error of the mean (SEM). Statistical significance was determined using 1-way or 2-way analysis of variance, followed by the Bonferroni post hoc test. A *p* value less than 0.05 was considered statistically significant.

Results

NPD1 prophylaxis ameliorates POD-like behaviors induced by surgery/anesthesia in aged mice

To determine whether surgery/anesthesia affects general and cognitive behavior of aged mice, we performed a battery of behavioral tests with food buried test, open field test and Y maze test at 24 hours before surgery and 6, 9, 24 hours after surgery in the present study as we previously reported [26, 27].

We first executed the buried food test to explore whether surgery/aesthesia affected the mice's ability to associate odorant with food reward [30]. The latency to eat food was markedly increased in the Surgery group compared to the Control group at 6 hours after surgery ($P < 0.01$, Fig. 1A), while pretreatment with NPD1 (600 ng/mouse) improved the impaired ability of finding and eating food induced by surgery/anesthesia ($P < 0.05$, Fig. 1A). No significant changes were observed between the NPD1 group and Control group.

Then we executed the open field test to examine the locomotor ability and exploratory behavior of mice with surgery/anesthesia or NPD1 treatment [31]. There were no significant differences in total distance traveled by mice between four groups at any time points, indicating that surgery/anesthesia did not affect the motor function of aged mice (Fig. 1B). Surgery/anesthesia significantly decreased the time spent in the center at 6 and 9 hours after surgery ($P < 0.05$), and preemptive administration of NPD1 ameliorated this phenomenon at 9 hours after surgery ($P < 0.05$, Fig. 1C). Besides, surgery/anesthesia significantly decreased the freezing time at 6, 9, 24 hours after surgery ($P < 0.05$, Fig. 1D), while preoperative treatment with NPD1 increased the freezing time at 9 and 24 hours after surgery ($P < 0.05$,

Fig. 1D). NPD1 administration alone did not change these parameters compared to the control condition (Fig. 1B-D).

At last we conducted Y maze for assessing the hippocampus-dependent spatial memory in aged mice as previously validated [32]. Surgery/anesthesia did not alter the number of arm visits among four groups (Fig. 1E). However, surgery/anesthesia significantly reduced the number of entries in the novel arm at 6 hours after surgery ($P < 0.05$, Fig. 1F) and the duration in the novel arm at 6 and 9 hours after surgery ($P < 0.05$, Fig. 1G), as compared to the control condition. Pretreatment with NPD1 increased the number of entries in the novel arm and duration in the novel arm at 6 hours after surgery ($P < 0.05$, Fig. 1F-G). NPD1 administration *per se* did not affect the performance of aged mice in the Y maze test at any tome point.

In conclusion, prophylaxis with NPD1 attenuated the impairment of general behaviors (buried food test and open field test) and learned behaviors (Y maze test) induced by surgery/anesthesia of aged mice in a fluctuating way.

NPD1 modulates the expression of inflammatory cytokines after surgery both in periphery and in CNS

To assess the effects of NPD1 on the systemic inflammation and neuroinflammation, we firstly measured the changes of TNF-α, IL-6 and IL-10 in blood plasma after surgery. Surgery/anesthesia significantly increased the level of TNF-α and IL-6 at 6 and 9 hours after surgery ($P < 0.05$, Fig. 2A-B) but did not change the expression of IL-10 (Fig. 2C). Though a single dose of NPD1 did not completely reverse the increase of proinflammatory cytokines to the control condition, it markedly reduced the levels of TNF-α and IL-6 at 6 hours after surgery ($P < 0.05$, Fig. 2A-B). Besides, pretreatment of NPD1 increased the expression of IL-10, a crucial cytokine during the resolution phase of inflammation, at 6 hours after surgery ($P < 0.05$, Fig. 2C). Secondly, we measured these cytokines in the hippocampus and prefrontal cortex, two key brain regions related to memory network [33, 34]. Surgery/anesthesia induced a marked increase in the expression of TNF-α and IL-6 at 6 and 9 hours after surgery both in the hippocampus and prefrontal cortex compared to the control condition ($P < 0.05$, Fig. 2D-E, G-H). Prophylaxis NPD1 significantly decreased the expression of TNF-α and IL-6 at 6 and 9 h compared to the Surgery group in these brain regions ($P < 0.05$, Fig. 2D-E, G-H). Notably, pretreatment with NPD1 increased the expression of IL-10 not only in the hippocampus at 6 and 9 hours after surgery ($P < 0.05$, Fig. 2F), but also in the prefrontal cortex at 6 h after surgery ($P < 0.05$, Fig. 2L). No effects on these cytokines were reported when treated with NPD1 alone.

NPD1 prophylaxis alleviates the leakage of BBB induced by surgery/anesthesia

The breakdown of blood-brain barrier (BBB) has been reported to be associated with delirium and perioperative neurocognitive disorders [35, 36], herein we employed a well-established dye injection assay

to investigate the integrity of BBB [28, 29] under the treatment of surgery/anesthesia with or without NPD1.

The immunofluorescence images revealed that 10-kDa dextran was primarily confined to vessels in the Control group, NPD1 group and NPD1 + Surgery group. By contrast, the signal of dextran were detected in the brain parenchyma around vessels in the Surgery group (Fig. 3A). To quantitate the extravascular dextran, spectrophotometric quantification of 10-kDa dextran-Texas Red from brain tissue extracts was performed. Both in the hippocampus and prefrontal cortex, we found that surgery/anesthesia increased the level of extravascular 10-kDa dextran as compared to the control condition, while NPD1 prophylaxis decreased the leakage of dextran induced by surgery/anesthesia ($P < 0.05$, Fig. 3B-C).

We next examined the effects of NPD1 on the expression of occludin, claudin-5 and ZO-1 after surgery, which are the tight junction (TJ) associated proteins to maintain the integrity of BBB [37, 38]. By quantitative western blot we found that there was a marked decrease in the expression of ZO-1, claudin-5 and occludin both in the hippocampus and prefrontal cortex at 24 hours after surgery, while pretreatment with NPD1 significantly attenuated the reduction of these proteins ($P < 0.05$, Fig. 4C-H). Preemptive administration of NPD1 alone did not change the homeostasis of BBB.

NPD1 reverses the reactive states of astrocytes and microglia in the hippocampus and prefrontal cortex

We measured the changes of immunoreactivity of GFAP and Iba-1 in the hippocampus and prefrontal cortex to assess the reactive states of microglia and astrocyte, which represent the major pathological manifestation of neuroinflammation [39–42]. Astrocytes in the hippocampus and prefrontal cortex showed significant morphological changes with shorter and de-ramified processes, atrophic cell soma and reduced GFAP immunoreactive area after surgery compared to the control condition ($P < 0.05$, Fig. 5A, B, D). By contrast, mice underwent surgery but pretreated with NPD1 retained the stellate shape of classical astrocytes, with longer processes and similar immunoreactive area to the Control group ($P < 0.05$, Fig. 5A, B, D).

NPD1 also attenuated microglial activation as measured by changes in the expression of Iba-1. Surgery induced the amoeba-like morphology of microglia and increased Iba-1 immunoreactive area in the hippocampus and prefrontal cortex compared with the control condition ($P < 0.05$, Fig. 5A, C, E), while preemptive administration of NPD1 significantly restored the ramified shape of microglia and reduced cellular area ($P < 0.05$, Fig. 5A, C, E). There were neither significant changes in GFAP nor Iba-1 were observed in the NPD1 group.

NPD1 alleviates the expression level of pro-inflammatory cytokine and promotes the macrophage polarization toward

M2 in the LPS-stimulated BMDMs

NPD1 has been reported to exert proresolving effect by immunoregulation, including blocking neutrophil infiltration and promoting phagocytosis *in vivo* [19, 43], which process is related with the reaction of polarized macrophages [44, 45]. Herein we assessed the effect of NPD1 on LPS-stimulated BMDMs by testing the specific cell markers of polarized macrophages, and the expression of inflamatory cytokines. After identified the particular macrophage marker CD68 in primary BMDMs (Fig. 6A), we stimulated BMDMs with LPS, with or without NPD1 for 24 hours. The polarization of BMDMs were analyzed through the expression of M1 marker CD16/CD32 and M2 marker CD206 (Fig. 6B-C). Quantitative analysis of flow cytometry showed that the population of M1 was significantly increased in the LPS group compared to the Control group ($P < 0.05$, Fig. 6D). NPD1 co-incubation tend to downregulate the macrophage polarization to M1, and markedly increased the population of M2 as compared to incubated with LPS alone ($P < 0.01$, Fig. 6D-E). The levels of TNF-α and IL-12 was significantly increased by LPS stimulation ($P < 0.05$, Fig. 6F, H), while co-incubation with NPD1 decreased the expression of these two cytokines ($P < 0.05$, Fig. 6F, H). Incubation with LPS alone increased the level of IL-10 compared to control condition ($P < 0.05$, Fig. 6G), suggesting the there were spontaneous initiation of resolution in the inflammation, but co-cultured with NPD1 elevated the expression of IL-10 in further ($P < 0.05$, Fig. 6G).

Discussion

In the present study, we demonstrate that NPD1, a novel lipid derived mediator of SPMs, contributes to the postoperative recovery of POD-like behavior in aged mice by its anti-inflammatory and proresolving effect. Our results indicate that prophylaxis with NPD1 at peripheral injured site alleviates systemic inflammatory response and protects BBB integrity after laparotomy. What's more, it limits neuroinflammation both in the hippocampus and prefrontal cortex, according to the expression of inflammatory cytokines and reactive states of microglia and astroglia in these brain regions. These protective actions against inflammation displayed by NPD1 may relate to macrophage polarization toward M2, as we showed in the *in vitro* experiment. To the best of our knowledge, this is the first report of the effects of NPD1 in a rodent model of POD.

Accumulative evidence has identified the pivotal role of neuroinflammation in the occurrence of POD, while peripheral inflammation is considered to be the initiation of neuroinflammation [36, 46, 47]. In the aseptic surgery setting, injured cells activate BMDMs by releasing damage-associated molecular patterns (DAMPs) that bind to Toll-like receptors (TLRs) of BMDMs, accordingly upregulating the expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [48–50]. These cytokines can cause further activation of DAMPs in positive feedback, eventually leads to the augment of inflammation [47, 51]. Our results demonstrate that NPD1 attenuates the systemic release of TNF-α and IL-6 after surgery, which are the pivotal cytokines to appear after trauma [47]. These findings are consistent with the potent anti-inflammatory activity of NPD1 in many other disease models that associated with inflammation, for instance, peritonitis [52], corneal damage [53], asthma [54], and inflammatory pain [55]. In a peritonitis model of murine, PD1/NPD1 has been proven to effectively attenuate polymorphonuclear neutrophil

infiltration and the expression of pro-inflammatory cytokines even at a very small dose (1 ng/mouse) [19, 52, 56]. Furthermore, preemptive administration with NPD1 increases the systemic expression of IL-10, one of the most important mediators in the inflammatory resolution as a potent suppressor to classical macrophage activation [57]. It has been demonstrated that inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and IL-12, are strictly relevant to M1-like macrophages, while IL-10 is primarily secreted by M2-like macrophages [25, 44]. The changes of cytokine profile in the periphery suggest that the macrophage polarization toward M2 may be linked with NPD1.

The breakdown of BBB is seen as a hallmark of neuroinflammation, because this disruption facilitates the infiltration of peripheral immunocompetent cells and cytokines to the immunologically privileged brain [58–60]. The barrier function of BBB is mainly created by the tight junctions of brain microvascular endothelial cells [61]. TNF-α and IL-6 have been reported to disturb the integrity of BBB by reducing the expression of TJ associated proteins between neurovascular endothelium [62, 63]. Notably, TNF-α can upregulate cyclooxygenase 2 isozyme (COX2) in brain microvascular endothelium, thereby increase the local generation of prostaglandins, which has a potent ability to increase vascular permeability [64, 65]. NPD1, as well as its precursor DHA, has shown protective activity for BBB and neurocognitive behavior after experimental ischemic stroke [66, 67], which was also noted in our model. The restoration of impaired BBB by preemptive NPD1 administration may be indirect, attributing to the modulated profile of cytokines in circulation we discussed above, as the same mechanism of how NPD1 alleviated leakage in the laser induced choroidal neovascularization [68]. Interestingly, synthesis of PDs is an enzymatic process via lipoxygenase (LOX) mechanism, while the transcription of LOX is launched by the same signaling pathways for producing prostaglandin E2 and D2 [65, 69]. The production of IL-10 also requires the participation of prostaglandins [57]. This kind of temporal-spatial interaction between inflammatory mediators can explain, at least in part, why there is no valid evidence for inflammatory inhibitors to treat POD or other neurocognitive disorders, for they may hinder the resolution phase of inflammation. Therefore, NPD1, as well as other SPMs, may be desirable therapies for the inflammation-driven diseases.

In addition to mitigate peripheral inflammatory response, NPD1 subsides the activation of glia cells and the expression of inflammatory cytokines in the hippocampus and prefrontal cortex. As resident macrophages in CNS, microglia act as immune surveillance and respond to different kinds of pathological stimuli [70]. Once activated, microglia rapidly switch to a pro-inflammatory phenotype with stout morphology, and enhance the production of pro-inflammatory molecules such as IL-1α, TNF-α and complement component 1q (C1q) [71, 72]. These specific cytokines along with cell debris released by classically activated microglia can trigger the transformation of astroglia to A1, the detrimental reactive phenotype of astrocytes [39, 40, 72, 73]. A1 astrocytes lose the supportive abilities in CNS, *i.e.* maintaining synaptic functions and phagocytic capacity, and secretes neurotoxin to induce neuronal death at meanwhile [72, 74]. In our model of POD, NPD1 reverts the morphological changes of microglia and astrocytes both in the hippocampus and prefrontal cortex to their original forms, representing the restorative transformation from the inflamed phenotype to their resting states, and thus modified the pro-inflammatory milieu by modulating the secretion of inflammatory cytokines. It is thus not surprising that NPD1 pretreatment facilitates the recovery of POD-like behavior in aged mice, for these two beneficiary

brain regions act in concert to shape emotion, learn and organize memory, and transform information [75, 76]. Though microglia share similar properties with peripheral macrophages, it is may not be the target cell that affected by NPD1. Recently, parkin-associated endothelin-like receptor (Pael-R), also known as GPR37, has been identified to be the particular receptor for NPD1 [55]. GPR37 is enriched in oligodendrocytes and astrocytes, but not microglia [55, 77]. In this context, the anti-inflammatory and proresolving effects of NPD1 in CNS may harness through different cell types, and the underlying mechanism needs further investigation.

BMDMs have been shown to be the bridge to link the peripheral and central immune systems since they can infiltrate into the brain in conditions characterized by neuroinflammation [78, 79]. Their function can be deleterious or favorable, depending on their polarization states to extracellular milieu [25, 80]. Other members of SPMs that derived from the same precursor with NPD1 have been shown to induce the M2 polarization [81–83], which suggest the similar property maybe exist in NPD1. The phenotypic skewing of inflammatory mediators *in vitro* has been also promoted by NPD1, that is, attenuating the M1 macrophage markers (TNF-α and IL-12) and elevating the M2 macrophage marker (IL-10). Also, the shift of specific cell receptors on LPS-stimulated BMDMs from M1 to M2 has verified the M2 polarization is induced by NPD1 in further. These findings, along with those of *in vivo* in the present research, suggest that the proresolving effect of NPD1 may be linked to transformation in macrophage polarization to the M2 phenotype. However, three subsets of M2 phenotype, named as M2a, M2b, and M2c, each of which has different protective properties, have been identified among the M2 phenotype [84]. It is thus essential to explore the exhaustive effect of NPD1 on macrophages and inflammation in further.

There are several limitations to our research. Firstly, we only assessed the cellular constituents in CNS and morphological changes of glia cells, but not the influence of the interventions for neurons. The glia-neuron crosstalk, especially in the hippocampus, is highly involved in the normal function of neurons to form memory and consciousness [42, 85, 86]. Besides, a recent study has described the analgesic effect of NPD1 [55]. Though we used local analgesics to prevent postoperative pain, it is difficult to determine whether NPD1 enhanced the postoperative recovery of mice by acting in an additive fashion with local analgesics to relieve the pain. Secondly, we only focused on the impact of NPD1 in regulating inflammation in our study, but not the signaling pathways leading to the phenomenon. It has been documented that the neuroprotection of NPD1 is elicited through NF-κB or PI3K/Akt phosphorylation signaling [22, 87–89], which gives the reference to illuminate the particular pathways of NPD1 in the perioperative setting.

Conclusion

The present study identifies the novel role of NPD1 in regulating postoperative inflammation not only in periphery but also in the hippocampus and prefrontal cortex, which results in relieving the ensuing POD-like behavior of mice. Theses protective effects of NPD1 is related to its modulation of macrophage polarization. Collectively, these findings indicate the potential of NPD1 to be a novel therapy for neuroinflammation and POD.

Abbreviations

POD: Postoperative delirium; SPMs:Specialized proresolving lipid mediators; TJ:Tight junction; BMDMs:Bone marrow-derived macrophages; TNF-α:Tumor necrosis factor-α; IL-6:Interleukin-6; BBB:Blood-brain barrier; CNS:Central nervous system; PD:Protectin D; NPD1:Neuroprotectin D1; DHA:Omega-3-polyunsaturated fatty acid docosahexaenoic acid; PBS:phosphate-buffered saline; ; GFAP:Glial fibrillary acidic protein; IBA-1:Ionized calcium-binding adaptor molecule 1; SEM:Standard error of the mean

Declarations

Availability of data and materials

All data will be made available from the corresponding author, with a reasonable request.

Ethics approval

The experimental protocol was approved by the Animal Ethics Committee of Zhongnan Hospital of Wuhan University, Hubei, China, and all experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Author contributions

YZ designed and performed the experiment, collected and analyzed the data, and prepared the manuscript. JW and XL were involved in preparing the animal models and participated in interpreting the

results. KL contributed to behavioral testing. LC was involved in biochemical analysis. YZ and JW participated in the statistical analysis. MP contributed to the study concept and design, secured funding for the project, and prepared and critically revised the manuscript. All authors reviewed the manuscript.

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Figures

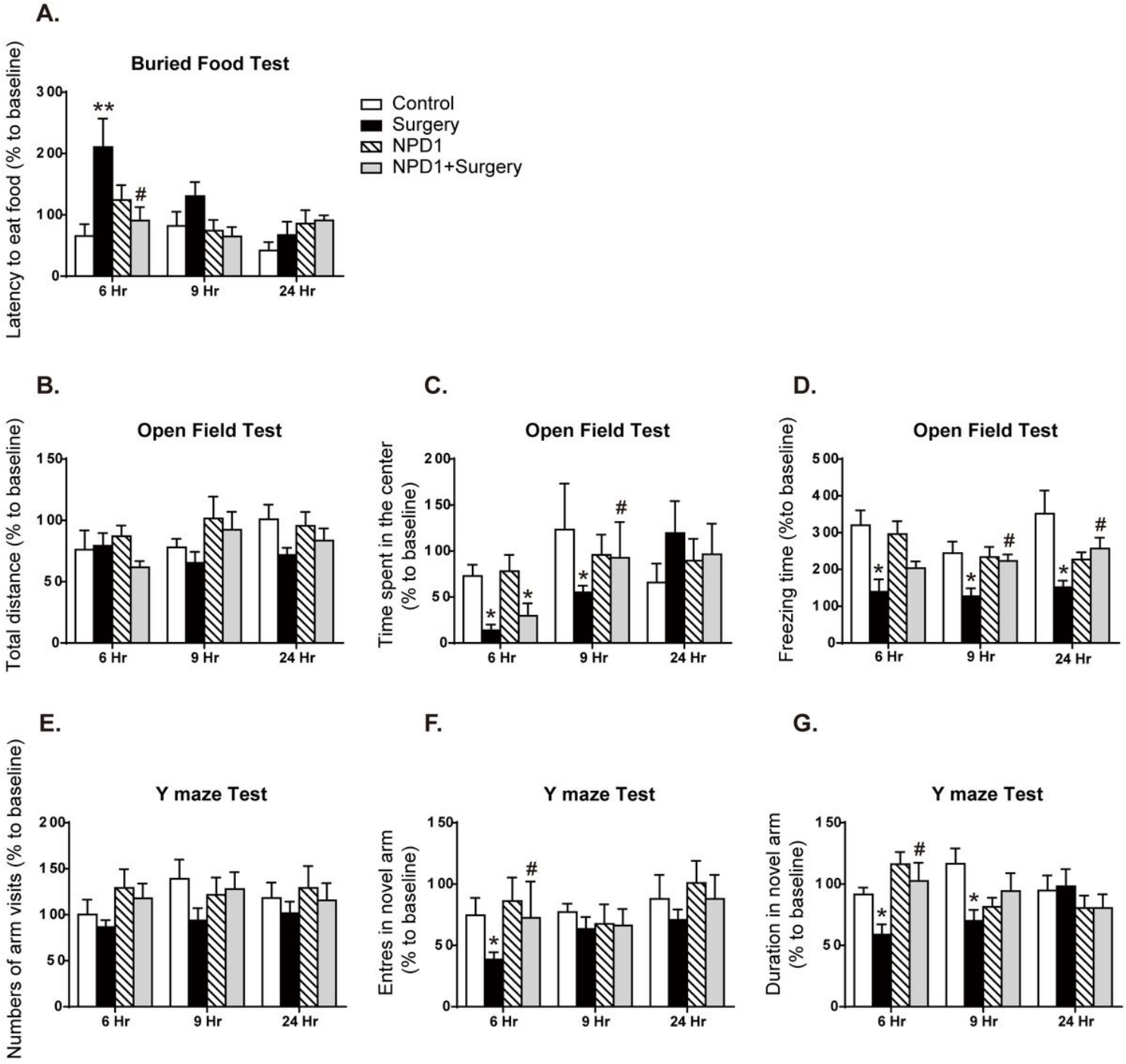

Figure 1

Surgery/Anesthesia induces POD-like behavior of aged mice, which can be ameliorated by the preemptive administration of NPD1. At 6, 9, 24 hours after surgery/anesthesia, the buried food test (A), open field test (B-D) and Y maze test (E-G) were executed ordinally. Data are presented as means ± SEM of the mean for each group (n = 8~10 per group). *P < 0.05 versus the Control group, **P < 0.01 versus the Control group, #P < 0.05 versus the Surgery group.

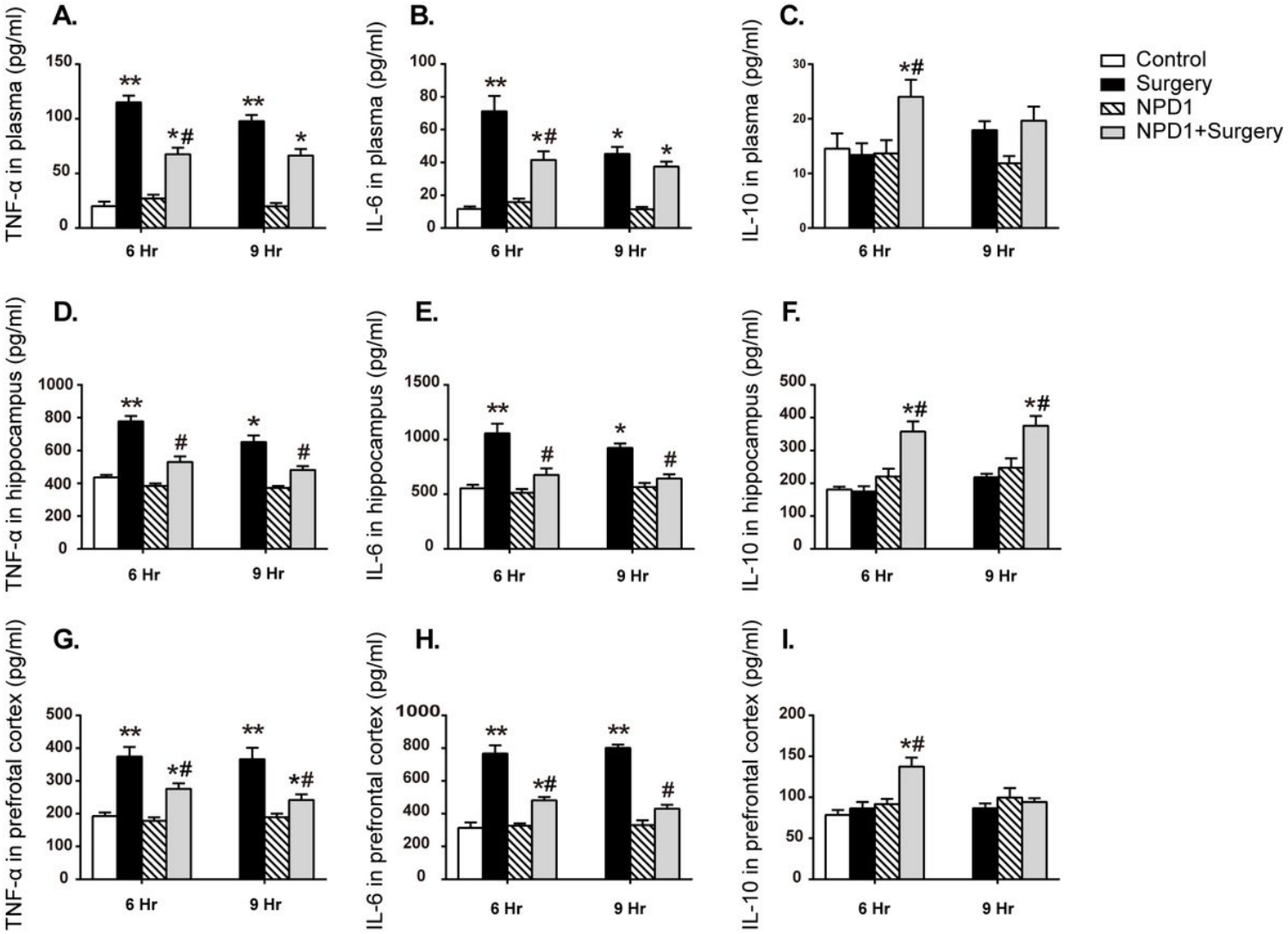

Figure 2

Effects of NPD1 on the expression of inflammatory factors in vivo. NPD1 pretreatment alleviated the surgery-induced upregulation of proinflammatory factors and promoted the expression of anti-inflammation factors both in the periphery blood and the central locations such as the hippocampus and prefrontal cortex (B)-(J). NPD1 and these cytokines were measured by ELASA. Data are presented as means ± SEM of the mean for each group (n = 4~5 per group). *P < 0.05 versus the Control group, **P < 0.01 versus the Control group, #P < 0.05 versus the Surgery group.

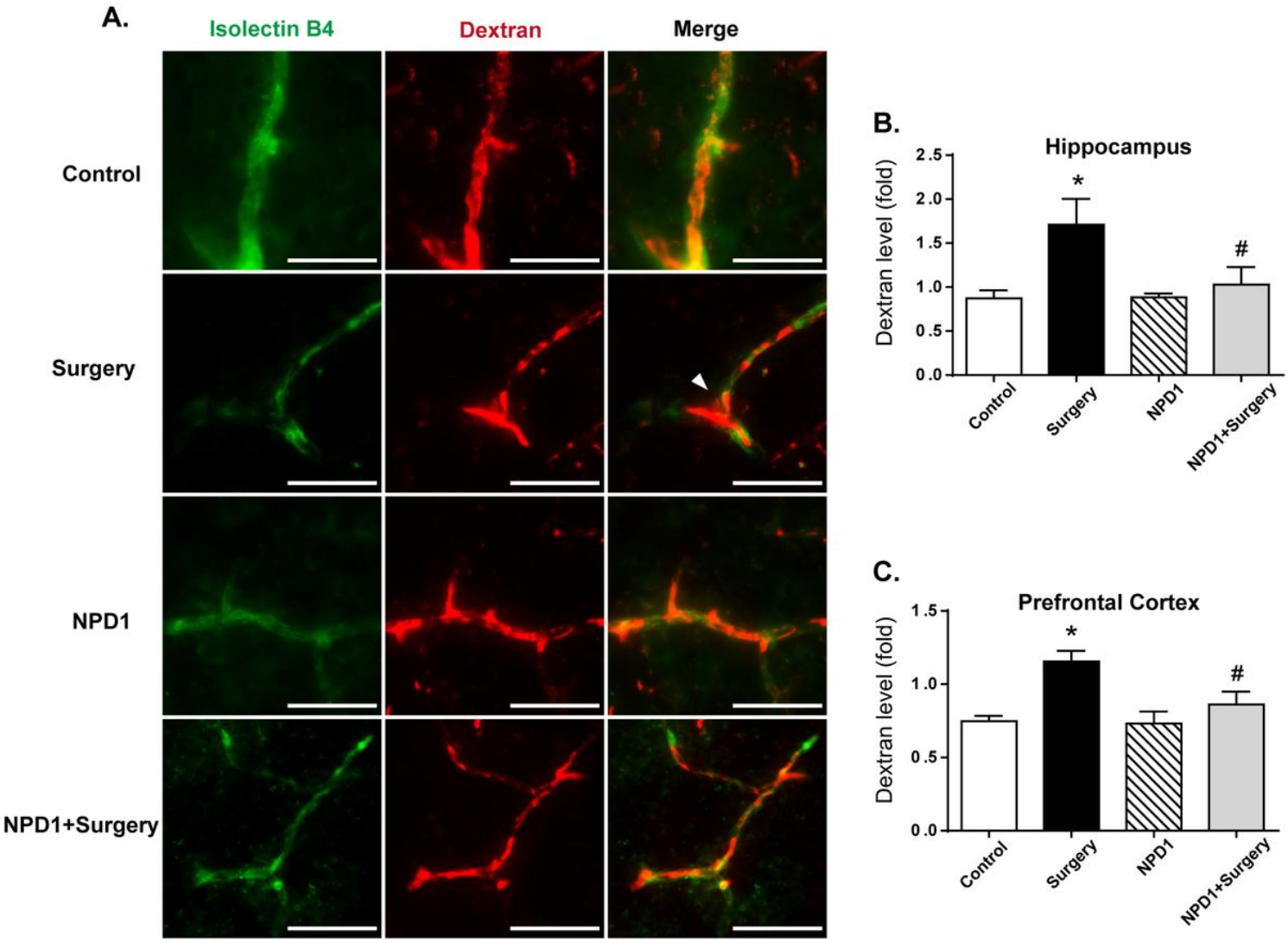

Figure 3

NPD1 protects against the leakage of BBB induced by surgery/anesthesia in the hippocampus and prefrontal cortex. Immunostaining of blood vessels (Isolectin B4, green) and intravenous injected dextran (10-kDa, red) in the brain section of the hippocampus at 6 hours after surgery (A). Arrowhead marked area indicated the dextran was extravascular. The spectrophotometric quantification of extravascular dextran (10-kDa) level in the extraction of the hippocampus and prefrontal cortex showed that the surgery/anesthesia increased the permeability of BBB as compared to control, and pretreatment with NPD1 attenuated this phenomenon (B-C). Data are presented as means ± SEM of the mean for each group (n = 4~5 per group). *P < 0.05 versus the Control group, #P < 0.05 versus the Surgery group. Scale bars represent 50 μm in (A).

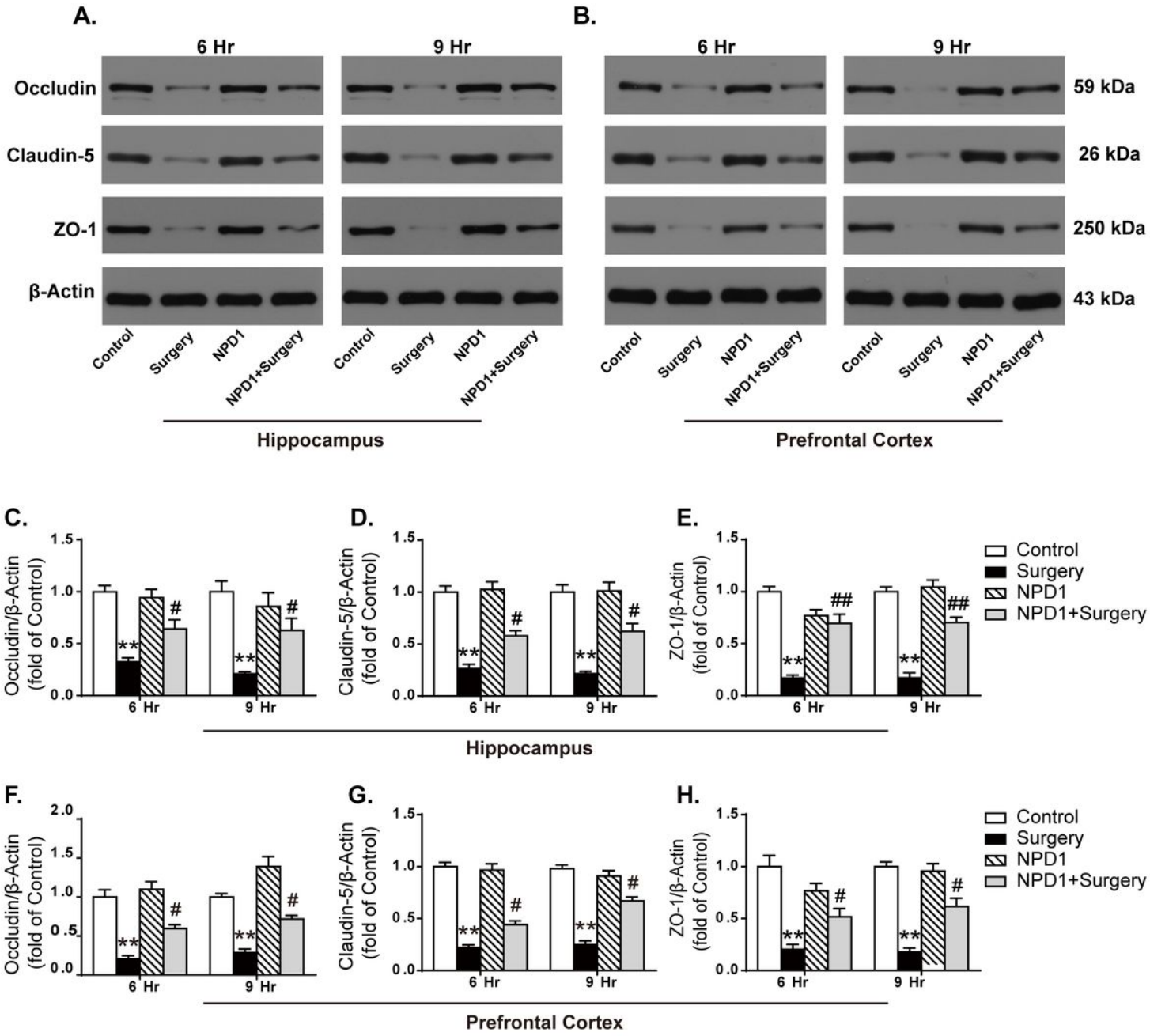

Figure 4

NPD1 modulates the expression of TJ associated proteins in the hippocampus and prefrontal cortex after surgery. Representative Western blotting bands of the expression of occludin, claudin-5 and ZO-1 in the hippocampus and prefrontal cortex at 6 and 9 hours after surgery (A, C). Quantification analyses of the expression of occludin, claudin-5 and ZO-1 were normalized to that of β-actin as internal control, and changes in protein levels were presented as folds of those in the Control group (B, D). Data are presented as means ± SEM of the mean for each group (n = 4~5 per group). **P < 0.01 versus the Control group, #P < 0.05 versus the Surgery group, ##P < 0.01 versus the Surgery group.

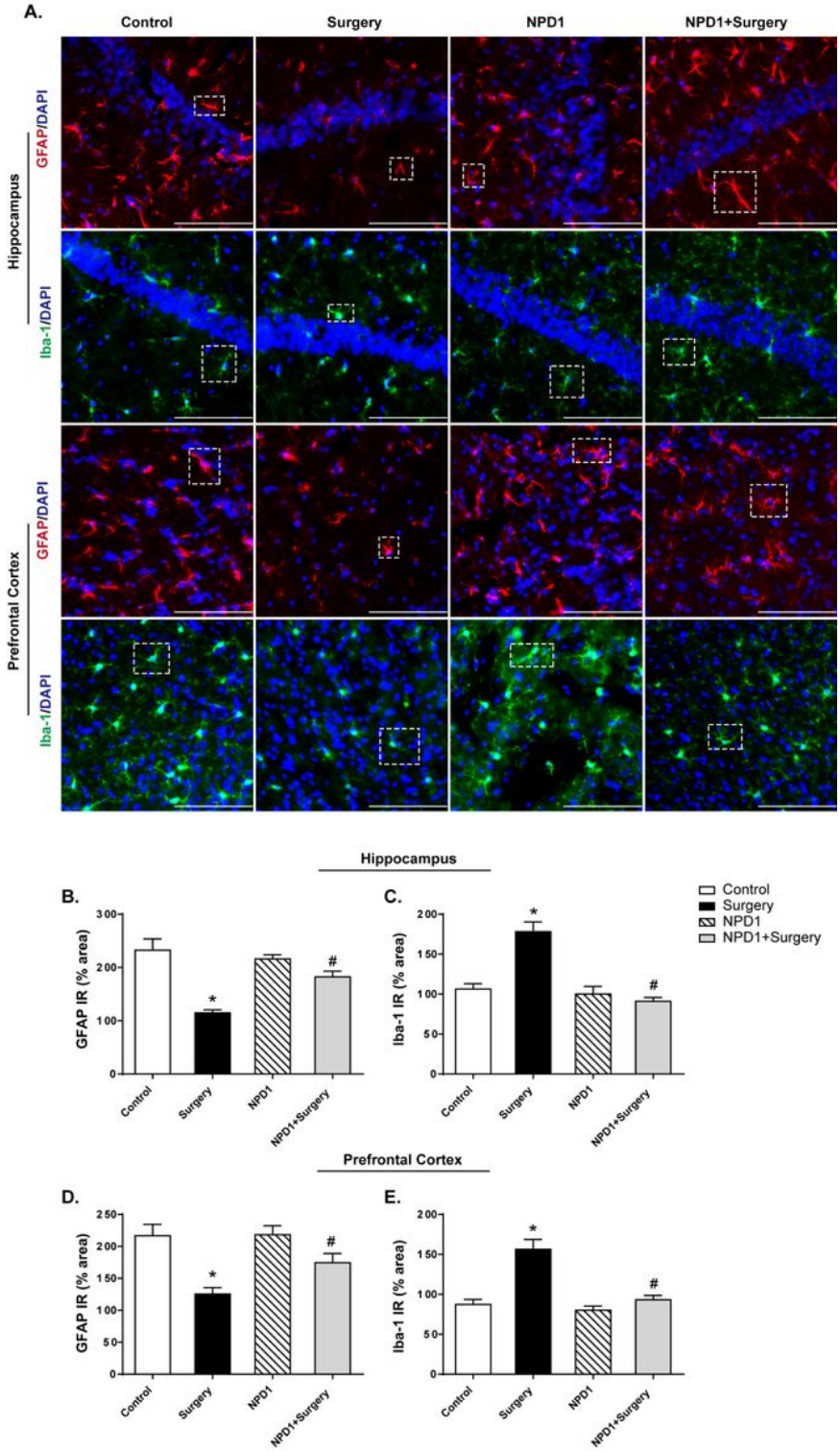

Figure 5

NPD1 pretreatment subsides the activation of astrocytes and microglia in the hippocampus and prefrontal cortex that elicited by surgery/anesthesia. Representative images of immunofluorescence showed the expression of GFAP and Iba-1 in the hippocampus and prefrontal cortex at 24 h after surgery (A-B, E-F). Surgery/anesthesia caused distinct changes in the morphology of glial cells with shorter processes and reduced cell soma for astrocytes, and amoeba-like morphology for microglial. Preemptive

administration of NPD1 significantly restored the classic stellate shape of astrocytes and the ramified shape of microglial. Quantification results of the immunostaining at 24 h after surgery showed in (C-D, G-H). Data are presented as means ± SEM of the mean for each group (n = 4~5 per group).*P < 0.05 versus the Control group, #P < 0.05 versus the Surgery group. Scale bars represent 100 μm in (A-B and E-F).

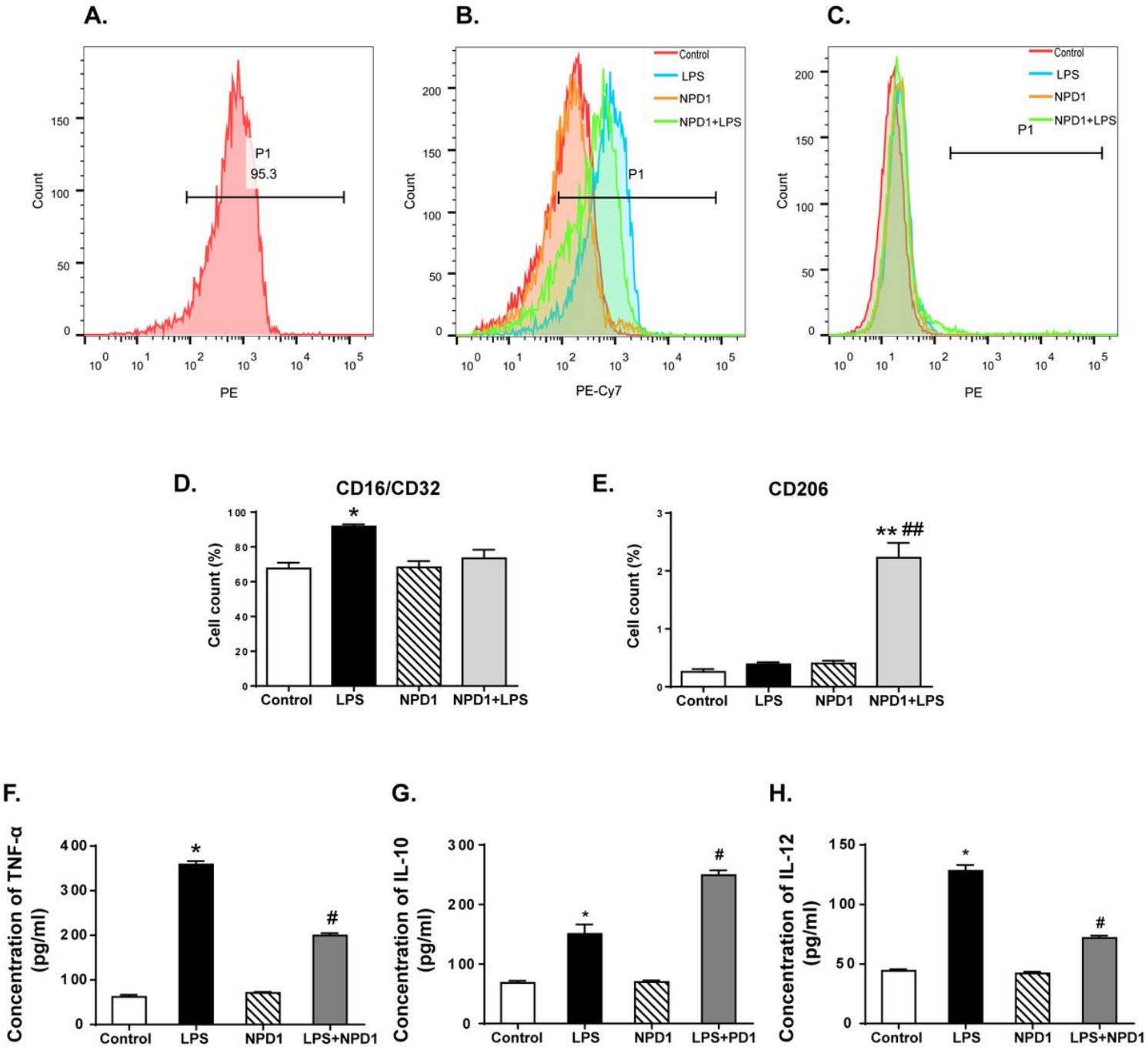

Figure 6

NPD1 promotes the macrophage polarization to M2 phenotype after proinflammatory stimulation on BMDMs, and modulates the expression of inflammatory cytokine profile. The expression of macrophage marker CD68 was showed in (A). NPD1 promoted the transformation of BMDMs cell markers from M1 to M2 phenotype, as measured by flow cytometry (B-C). The ratio of M1 and M2 phenotype in different

groups was calculated (D-E). The concentration of tumor necrosis factor-α (TNF-α), IL-12 and IL-10 were tested by ELASA (F-H). Data are presented as means ± SEM of the mean for each group (n = 5 per group). *P < 0.05 versus the Control group, **P < 0.01 versus the Control group, #P < 0.05 versus the Surgery group.