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ORIGINAL RESEARCH

A Dopamine D1-Like Receptor Agonist Ameliorates Traumatic Brain Injury Through its Immunosuppressive Effects

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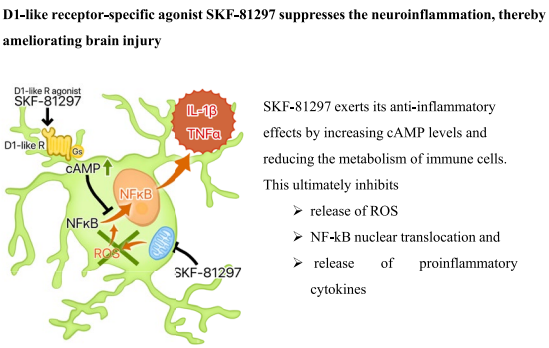
Abstract Traumatic brain injury (TBI) causes progressive nervous tissue degeneration long after the initial injury due to secondary neuroinflammatory reactions. G protein-coupled dopamine D1-like receptors, which elevate intracellular cAMP levels, have been shown to mediate the suppressive effects on lipopolysaccharide (LPS)-induced proinflammatory activation of microglia and macrophages. The present study investigated whether or not the D1-like receptor-specific agonist SKF-81297 (SKF) administered intraperitoneally once daily for 3days starting 1 h after TBI could ameliorate TBI in a rat model of stab wounds in the forebrain. SKF reduced the volume of TBI-induced brain tissue loss, increased mobile activity, and ameliorated cognitive dysfunction two months after TBI. A single dose of SKF suppressed the expression of IL-1β and TNFα in brain tissue by reducing oxidative injury at 24 h post-TBI. SKF decreased the energy metabolism of microglia, macrophages, and neutrophils in TBI brain. SKF prevented LPS-induced translocation of NFκB into the nuclei of primary cultured microglia. The agonist clenbuterol (CLB) for adrenergic β2 receptor, another Gs-linked GPCR, exerted comparable ameliorative effects in TBI model rats by suppressing neuroinflammation. In summary, SKF may exert anti-inflammatory effects by suppressing the NFkB pathway through increasing cAMP similarly to CLB and also by decreasing energy metabolism of inflammatory cells, leading to amelioration of TBI-induced brain degeneration.

Graphical Abstract



Keywords Traumatic brain injury · Microglia · Macrophage · SKF-81297 · LPS · NFκB · ROS

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Extended author information available on the last page of the article

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Introduction

Traumatic brain injury (TBI) is a leading cause of severe brain dysfunction, including motor, sensory, mental, and cognitive deficits, as well as acute mortality (Corps et al. 2015; Gyoneva and Ransohoff 2015; Maas et al. 2017, 2022). Even more worrying is the increasing number of stab wound-induced TBI caused by knives and firearms, which directly damage blood vessels and brain tissue, causing intracerebral hemorrhages (Ajayi et al. 2021; Lilford et al. 2024). Numerous studies have demonstrated that chronic brain dysfunction resulting from TBI is closely associated with neuroinflammation, secondary to TBI-induced primary neural cell death. In this secondary neuroinflammation, resident microglia and infiltrating leukocytes (monocytes, granulocytes, and lymphocytes) play crucial roles in exacerbating pathophysiological processes in and around the injured tissues (Corps et al. 2015; Gyoneva and Ransohoff 2015; Abe et al. 2018, 2020). These immune cells release various proinflammatory mediators, including cytokines, reactive oxygen species (ROS), and nitric oxide. Monocyte-derived macrophages and activated microglia also accelerate neural cell death through phagocytic activity that is a process known as phagoptosis (Neher et al. 2014; Butler et al. 2021).

In addition to their detrimental roles, microglia and circulating monocyte-derived macrophages may contribute to preventing degeneration and promoting the regeneration of injured tissues by eliminating degenerated materials and releasing anti-inflammatory cytokines and neuroprotective growth factors (Abe et al. 2020; Matsumoto et al. 2008; Smirkin et al. 2010; Islam et al. 2018). Furthermore, these cells have the potential to scavenge ROS while suppressing ROS-induced cell death, including ferroptosis (Wang et al. 2024; Toku et al. 1998; Tanaka et al. 1999). Single-cell RNA-sequencing data from a recent report using stab wound brain injury showed striking and distinct transcriptional changes in glial cell populations (Koupourtidou et al. 2024). Therefore, interventions that suppress the detrimental nature and promote the beneficial functions of these immune cells accumulating in the injured tissues are of great interest.

To date, various pharmacological interventions for TBI have been explored to suppress the detrimental effects and promote the beneficial functions of immune cells, particularly microglia and macrophages (Abe et al. 2018; Takeda et al. 2021, 2023a; Simon et al. 2017; Moppett 2007). Methylprednisolone, a corticosteroid studied for its potential to reduce inflammation of TBI, has been administered to patients with TBI (Simon et al. 2017; Moppett 2007). N-acetylcysteine, an antioxidant that may help reduce oxidative stress, has been investigated for its ameliorative effects in both laboratory (Chen et al. 2008; Pandya et al. 2014) and clinical settings (Conti et al. 2024; Khasnavis et al. 2024). In our previous study, classical hypnotic bromvalerylurea (BU), which has anti-inflammatory effects on microglia and macrophages in addition to enhancing GABAA receptor-mediated neurotransmission, markedly improved outcomes in rats with TBI (Abe et al. 2018; Takeda et al. 2023a, 2023b). BU exerts anti-inflammatory effects by activating Nrf2 (Takeda et al. 2023a) and by inhibiting JAK1(Kawasaki et al. 2017).

Agents that increase intracellular cAMP levels have been reported to suppress the proinflammatory activation of microglia (Mori et al. 2002; Zhang et al. 2002; Nishikawa et al. 2023). In particular, β2 adrenergic receptor (AR) agonists have strong immunosuppressive effects on lipopolysaccharide (LPS)-induced proinflammatory activation of microglia (Mori et al. 2002; Ishii et al. 2015). Microglia also express another G protein-coupled receptor (GPCR), the dopamine D1-like receptor, which is linked to Gs proteins (Nishikawa et al. 2023; Tanaka et al. 2024; Beaulieu and Gainetdinov 2011). Dopamine and the D1-like receptor-specific agonist SKF-81297 (SKF) suppress LPS-induced IL-1β and TNFα expression while increasing cAMP levels in primary rat microglia (Nishikawa et al. 2023; Tanaka et al. 2024; Miyaue et al. 2025).

Given the strong immunosuppressive effects of SKF, we investigated whether or not SKF ameliorates TBI in a rat model by suppressing the proinflammatory activities of microglia and infiltrating leukocytes.

Materials and Methods

Animals

All animal experimental protocols were carefully developed to minimize their suffering of animals and were conducted in accordance with the guidelines established by Ethics Committee for Animal Experimentation of Ehime University, Japan (approval number #05U50-2). Home-bred 10- to 12-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were used in this study. The TBI model was prepared by inducing stab wounds in the rat brain as described elsewhere (Abe et al. 2018). In brief, under deep anesthesia with isoflurane, the rat's head was secured in a stereotaxic apparatus (Narishige, Tokyo, Japan), and a longitudinal incision of approximately 15 mm was made in the skin to expose the skull. Two holes were drilled through the skull over the right hemisphere at approximately 2.5 and 4 mm right of the midline and 1 mm posterior to the bregma, respectively. A 26-gauge needle was inserted through each hole to a depth of approximately 7 mm from the surface of the

skull and moved in a fan-like manner from anterior to posterior, parallel to the midline. The needle was then withdrawn, and the skin incision was closed with quick-drying glue (Aron-Alpha; Toagosei, Tokyo, Japan). Soldem 3A solution (10 ml/rat/day; Terumo Corporation, Tokyo, Japan) was administered for postoperative care.

Primary Microglia, Macrophage and Neutrophils Cell Cultures

Rat primary microglial cell cultures were prepared as previously described (Tanaka et al. 1998), where cortices from newborn rat pups were mechanically dissociated into individual cells. Dissociated cells were cultured as mixed glial cultures in 75 cm^2 flasks with 10% fetal calf serum (FCS)-supplemented Dulbecco's Modified Eagle's medium (DMEM; Fujifilm Wako, Osaka, Japan). After 11 days, microglial cells were obtained by agitating the flasks at 200 rpm for 1 h at 37 °C. The pure microglial cells were purified with non-adhesive polystyrene dishes and were plated in poly L-lysine-coated 6-well culture plates. Rat primary macrophages were harvested from the peritoneal lavage of adult rats as described elsewhere (Kikuchi et al. 2015) and cultured in DMEM supplemented with 3% FCS-supplemented DMEM (Fujifilm Wako). The purity of the primary macrophages and microglia was confirmed using flow cytometry, where 98% of the cells were $CD11b^+$ cells. Rat primary cultured neutrophils were purchased from Cell Biologics Inc (Chicago, Illinois, USA). These neutrophils were then cultured according to the manufacturer's instructions using neutrophil medium (Cell Biologics Inc). For pharmacological studies, the culture medium was removed, and the cells were incubated for 6 h in serum-free E2 medium (DMEM containing 10 mM HEPES, pH 7.3; Gibco, Grand Island, NY, USA; 4.5 mg/mL glucose, Gibco; 5 µg/mL insulin, 5 nM sodium selenite, 5 µg/mL transferrin, Gibco; and 0.2 mg/mL bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA). Inflammatory cell culture models were prepared by exposing the cells to E2 medium containing 1 µg/mL LPS (from *Escherichia coli*, serotype 055:B5; Sigma-Aldrich) for 6 h or overnight, as required by each assay.

Knockdown Experiment

Protein knockdown was performed using RNA interference, where pre-validated sequences for rat (either control or D1r-specific) were purchased from ThermoFisher Scientific (Kopec et al. 2018). The scRNA, Silencer Select negative control (catalog #4,390,843), and siRNA, ID s127671 (catalog #4,390,771), were labeled with Cy3 (Mirus Bio, Madison, WI, USA). The labeled siRNA duplexes were exposed to 60 mm plated semiconfluent microglial cells for transfection using SilenceMag siRNA delivery reagents (OZ Bioscience, San Diego, USA) and placed on top of the magnetic plate (OZ Bioscience) for 15 min (Islam et al. 2018). The cells were incubated for 48 h for further studies.

Pharmacological Interventions

In Vivo Studies: The D1-like receptor-selective agonist SKF-81297 hydrobromide (SKF) from Tocris Bioscience (Bristol, UK) was dissolved in DMSO and diluted with normal saline to a concentration of 2.5%. Normal saline containing 2.5% DMSO was used as the Vcl as described in our previous study (Tanaka et al. 2024). SKF was administered intraperitoneally to TBI rats at a dose of 10 mg/kg, 1 h after TBI. In the long-term experiments, SKF was administered once per day for 3 days at the same dose. Clenbuterol hydrochloride (CLB; Sigma-Aldrich), dissolved in the same manner as SKF, was administered subcutaneously at a dose of 0.1 mg/kg body weight, following the same administration schedule as SKF. In Vitro Studies: LPS (1 µg/ml)-treated and untreated cells were exposed to 10 µM SKF as described in our previous study (Nishikawa et al. 2023).

Flow Cytometry (FCM) and Fluorescence-Activated Cell Sorting (FACS)

Brain tissues from the lesion area (approximately 100 mg) of TBI and normal rats were dissected and dissociated into single cells using a gentleMACS dissociator with 37C_ABDK (Miltenyi Biotec, Tokyo, Japan) and an adult brain dissociation kit (Miltenyi Biotec), as previously described (Abe et al. 2018). The prepared cell suspensions were subjected to FCM analyses and FACS. For multicolor immunofluorescence labeling, the cells were blocked with mouse anti-rat CD32 antibody (Rat BD Fc Block; BD Biosciences, Franklin Lakes, NJ, USA), followed by labeling with fluorescent antibodies against cell surface antigens by incubating the cells for 30 min at 4 °C using the antibodies listed in Supplementary Table 1. Cells labeled with antibodies for flow cytometry were analyzed using CytoFLEX S (Beckman Coulter, Tokyo, Japan). Live cells were gated using Zombie Green (BioLegend, San Diego, CA, USA). Data were analyzed using the FlowJo software program (version 10.9; Treestar, Ashland, OR, USA). For FACS, stained cells were stored overnight at 4 °C with a cell cover (Anacyte Laboratories, Hamburg, Germany). $CD11b^+/CD45^{low}$ cells were considered microglia, and $CD11b^+/CD45^{high}$ cells were considered macrophages. These cells were sorted in ice-cold phosphate-buffered saline (PBS) using a FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA) with

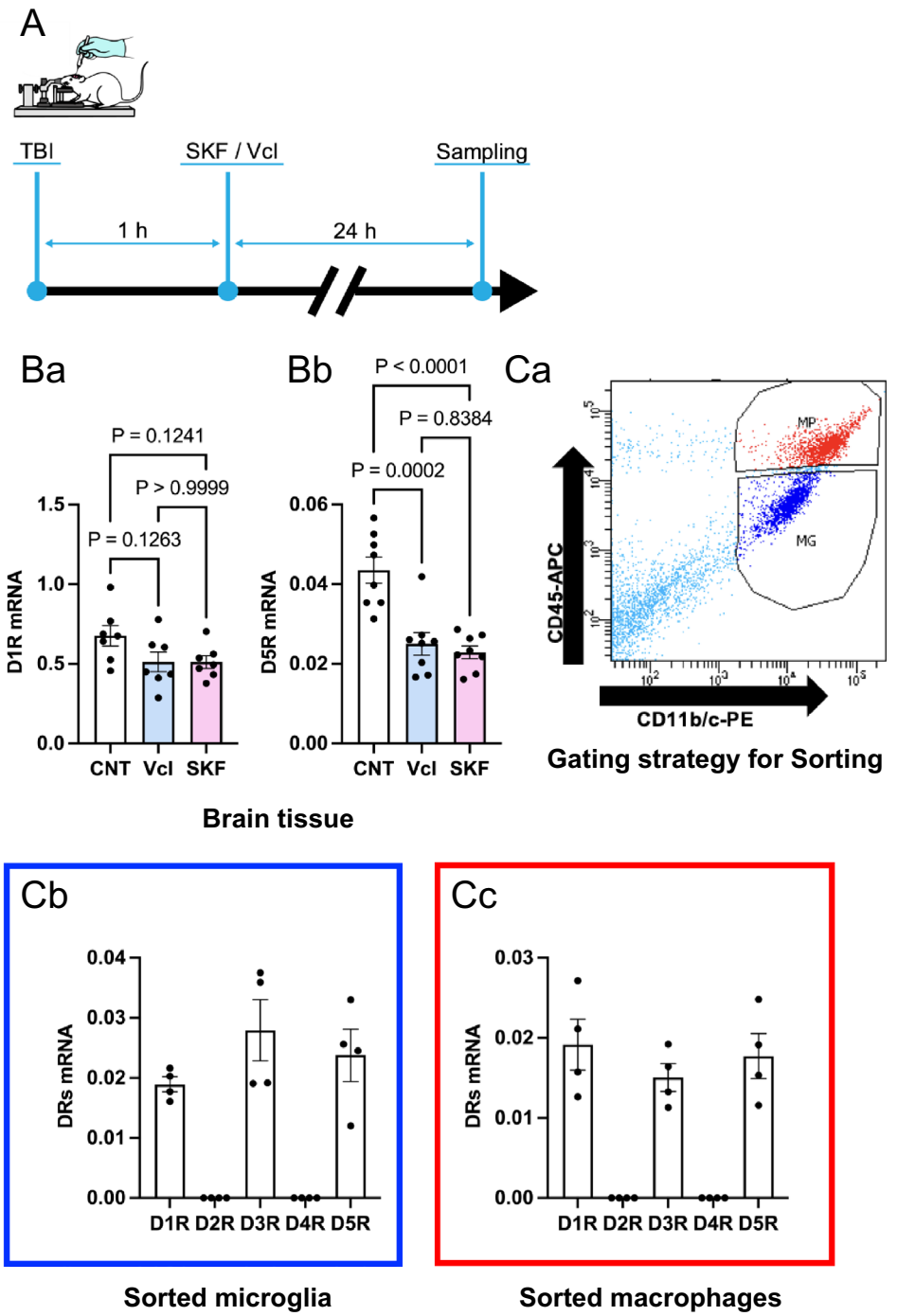


Fig. 1 Expression of DA receptors in the brain and microglia/macrophages. **A** Experimental timeline. **B** Expression of mRNA encoding D1-like receptors, D1R and D5R in normal healthy control (CNT) rat brains, vehicle (Vcl)- and SKF-treated TBI rat brains. A one-way ANOVA and Tukey's multiple comparison test. **C** By flow cytometry analysis, microglia (MG) and macrophages (MP) isolated from the injured brain tissues were distinguished by CD45 expression level. Expression of DA receptors by microglia (**Cb**) and macrophages (**Cc**). Expression of mRNA encoding D1R, D3R, and D5R was detected in both types of cells. $n=4$. The injured core and its surrounding tissue were enzymatically dispersed into single cells, which were subjected to an FCM analysis using antibodies against CD11b and CD45 (Fig. **Ca**). Infiltrated macrophages were identified as $CD11b^{+}/CD45^{high}$ cells and resident microglia as $CD11b^{+}/CD45^{low}$ cells. Both cell lines were sorted to prepare the total RNA for qPCR. The expression of mRNA encoding DR1, DR3, and DR5 was detected in both sorted microglia and macrophages (Fig. **Cb-c**)

an 85-µm nozzle and the BD FACSDIVA software program (BD Biosciences).

Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

Total RNA from brain tissues, and cells was isolated using the Maxwell RSC simplyRNA Tissue/Cells Kit (Promega, Madison, WI, USA). RNA was extracted from sorted microglia and macrophages using an RNeasy Micro Kit (QIAGEN, Valencia, CA, USA). cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix with a gDNA remover kit (Toyobo, Osaka, Japan). qPCR was conducted in triplicate using an MJ Mini instrument (Bio-Rad, Hercules, CA, USA) with THUNDERBIRD™ Next SYBR® qPCR Mix (Toyobo). All PCR primer sequences

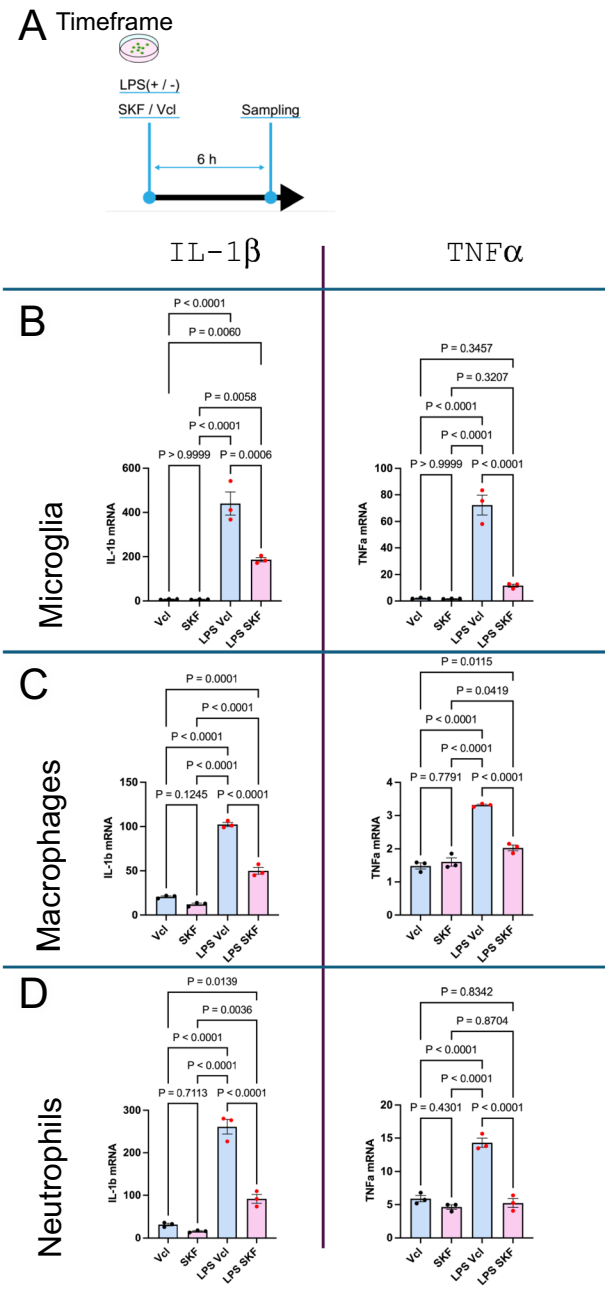


Fig. 2 Suppressive effects of SKF on proinflammatory responses of primary cultured cells. **A** Experimental timeline for cell culture studies. **B** Microglial cells were incubated with either none, LPS (1 mg/ml), or SKF (10 ng/ml) for 6 h, followed by the preparation of cDNA for qPCR. SKF significantly reduced the LPS induced mRNA expressions of both TNFα and IL-1β. n = 3. **C**, **D** SKF also exhibited a comparable effect on LPS-treated primary cultured macrophages and neutrophils. n = 3

are listed in Supplementary Table 2 (Hokkaido System Science Co. LTD, Sapporo, Japan). The qPCR data are presented as the percentage of GAPDH mRNA expression levels, calculated as $100 \times 1/2^{(\text{Ct of target gene} - \text{Ct of GAPDH gene})}$ (Islam et al. 2018).

RNA Sequencing (RNAseq) and Analyses

Total RNA from the brain tissue at the lesion area was isolated as described above. RNA integrity score (RIN) was calculated using the TapeStation system (Agilent Technologies, Santa Clara, CA, USA). Samples with an RIN value above 9.7 were used for further analyses. mRNA purification was performed using the NEBNext Poly(A) mRNA Magnetic Isolation Module. Library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit. Sequencing was carried out on a NovaSeq 6000 (Illumina, San Diego, CA, USA) in paired-end 2 × 150-bp cycle mode, generating a total of 26.7 million reads per sample. Data analyses were performed using the CLC Genomics Workbench Premium (QIAGEN) with default settings. In brief, fastq files were trimmed to remove adapter sequences and low-quality bases, followed by mapping and detection of differentially expressed genes (DEGs). Finally, an ingenuity pathway analysis (IPA) (QIAGEN) was used to identify the signaling pathways underlying the transcript sets that significantly predicted the effect of SKF.

Detection of Superoxide Anions Using Dihydroethidium (DHE)

DHE (10 mg/kg) was administered intraperitoneally to TBI model rats at 1 (day post-injury) dpi (Abe et al. 2018). Thirty min later, the animals were euthanized and their brains dissected. For FCM analyses, the tissue from the lesion area was processed into single cells and subjected to magnetic-activated cell sorting using CD11b microbeads to isolate myeloid cells, including microglia and macrophages. After sorting, the cells were analyzed using a violet laser and the V450 channel of CytoFLEX S, and the data were analyzed using the FlowJo software program.

Evaluation of Oxidative Stress Using 8-Hydroxy-2′-Deoxyguanosine (8-OHdG)

Brain tissue from the lesion area was isolated at 1 dpi, and DNA was extracted from the tissue using a DNA extraction TIS kit (Wako) and prepared for an enzyme-linked immunosorbent assay (ELISA) using the 8-OHdG Assay Preparation Reagent Set (Wako) (Abe et al. 2018). The levels of 8-OHdG were quantified using a high-sensitivity ELISA kit (Nikken SEIL, Shizuoka, Japan).

ELISA Details

Brain tissue from the injury site was harvested and homogenized in RIPA buffer (Wako). TNFα and IL-1β levels were measured using ELISA kits (Elabscience, Houston, TX,

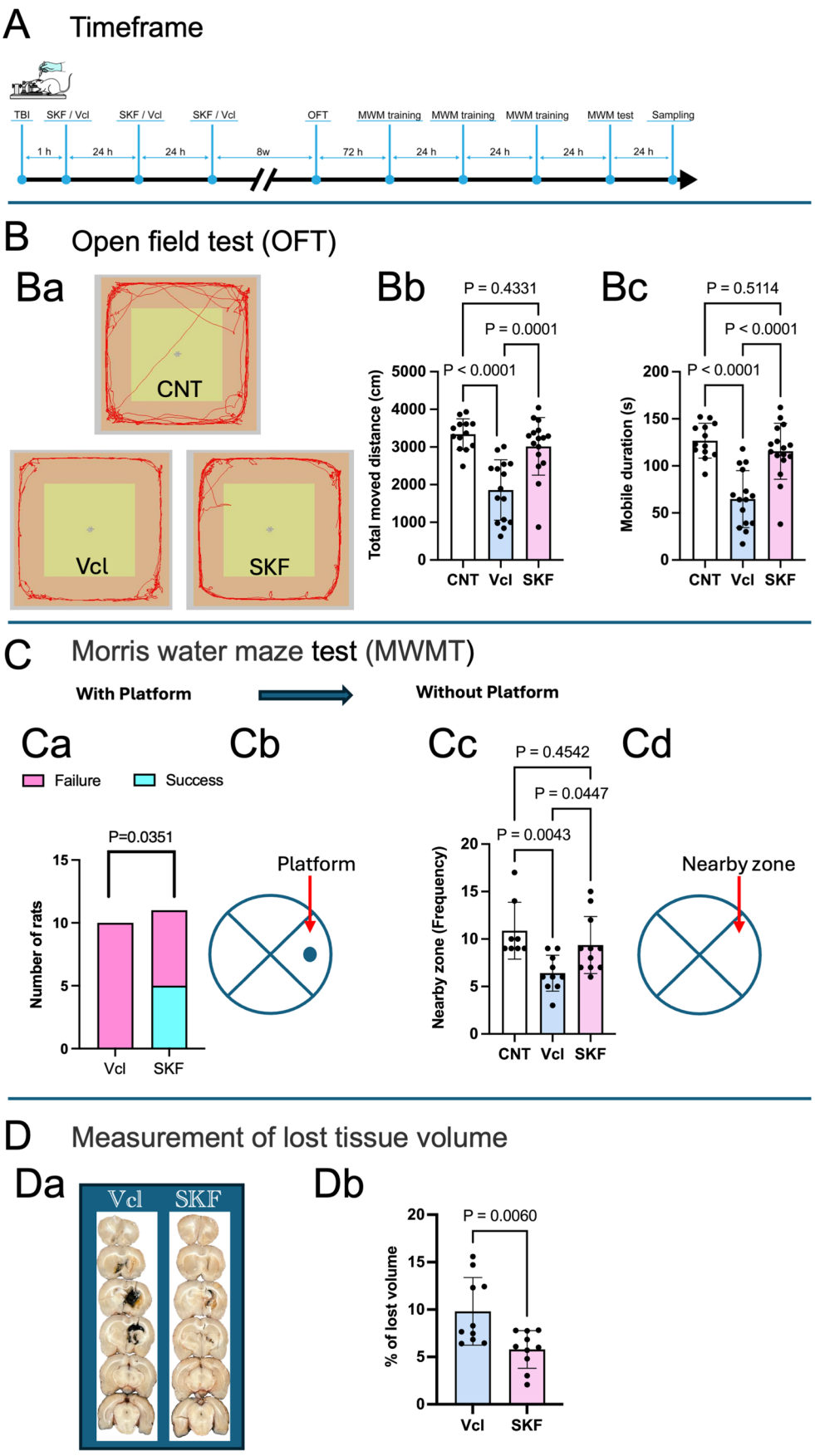


Fig. 3 Effects of SKF on the outcome of TBI. A) Experimental timeline. B) Open-field test.(Ba) Representative images of track visualization. (Bb) Total moved distance. (Bc) Mobileduration in 5 min in OFT. n = 13 (CNT), 15 (Vcl), and 16 (SKF). C) Morris water maze (MWM)test; Please refer to representative videos as Supplementary video 5(CNT), video 6(Vcl), video 7(SKF). (Ca) The ratio of success and failure of a Vcl and a SKF-treated rat; Please refer torepresentative videos as Supplementary video 8(CNT), video 9(Vcl), video 10 (SKF). $\chi 2$ testwith Fisher's exact test (Cb) The position where the platform was placed during the test wasindicated by an arrow. (Cc) The frequency of entering the nearby zone. n = 8 (CNT), 10 (Vcl),and 11 (SKF). See representative videos as Supplementary video 11 (CNT), video 12 (Vcl),video 13 (SKF). (Cd) An arrow denotes the target quadrant or the nearby zone. A one-wayANOVA and Tukey's multiple comparison test. Da) Representative brain slices of Vcl- andSKF-treated rats prepared two monthsafter TBI. SKF reduced TBI-caused brain tissue loss. (Db)Lost brain tissue volumes were statistically analyzed by comparing the ratio (%) of the right(ipsilateral side) to left (contralateral side) areas. n = 10. Unpaired two-tailed t test

USA) and assayed according to the manufacturer's instructions. The protein content of the brain tissue was quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) (Tanaka et al. 2024).

Immunoblotting

For the in vitro study, nuclear fractions of primary microglial cells were collected using a nuclear extraction kit (Active Motif, Tokyo, Japan) (Islam et al. 2018). The nuclear fractions were lysed with RIPA buffer, the amount of protein in each sample was quantified with a Pierce BCA Protein Assay Kit, and equal amounts of total protein were prepared with Laemmli sample buffer. For the in vivo study, brain tissue at the lesion area was homogenized in sample buffer at same concentration. The prepared samples were subjected to immunoblotting using the antibodies listed in Supplementary Table 3.

Immunohistochemistry

Under deep anesthesia, TBI rats at 1 dpi were subjected to perfusion fixation using 4% paraformaldehyde (PFA; Wako, Osaka, Japan), as described elsewhere (Abe et al. 2018). The brain region containing the injury was coronally cryosectioned at a thickness of 16 µm and subjected to immunohistochemical staining using the antibodies listed in Supplementary Table 4. Hoechst 33,342 (Sigma-Aldrich) was used for nuclear staining. Following immunohistochemical staining with antibodies, the specimens were observed under a FLUOVIEW FV4000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a 40 × objective lens. For DHE staining, the dissected brains were immersed in 4% PFA for 30 min and 15% sucrose for 2 h, and then 10-µm-thick frozen sections were prepared and immunostained.

Measurement of Cellular Bioenergetics

Quantification of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in rat primary cultured cells was performed using an XFp Extracellular Flux Analyzer (Agilent Seahorse Bioscience, Santa Clara, CA). After overnight incubation of cells with or without SKF, the plates were placed in the XFp analyzer, which was operated according to the manufacturer's instructions using oligomycin A (1 µM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 1 µM), rotenone (0.5 µM), and antimycin A (0.5 M), which were automatically and sequentially added to cells to determine mitochondrial and glycolytic activities (Abe et al. 2018).

Behavioral Assessments

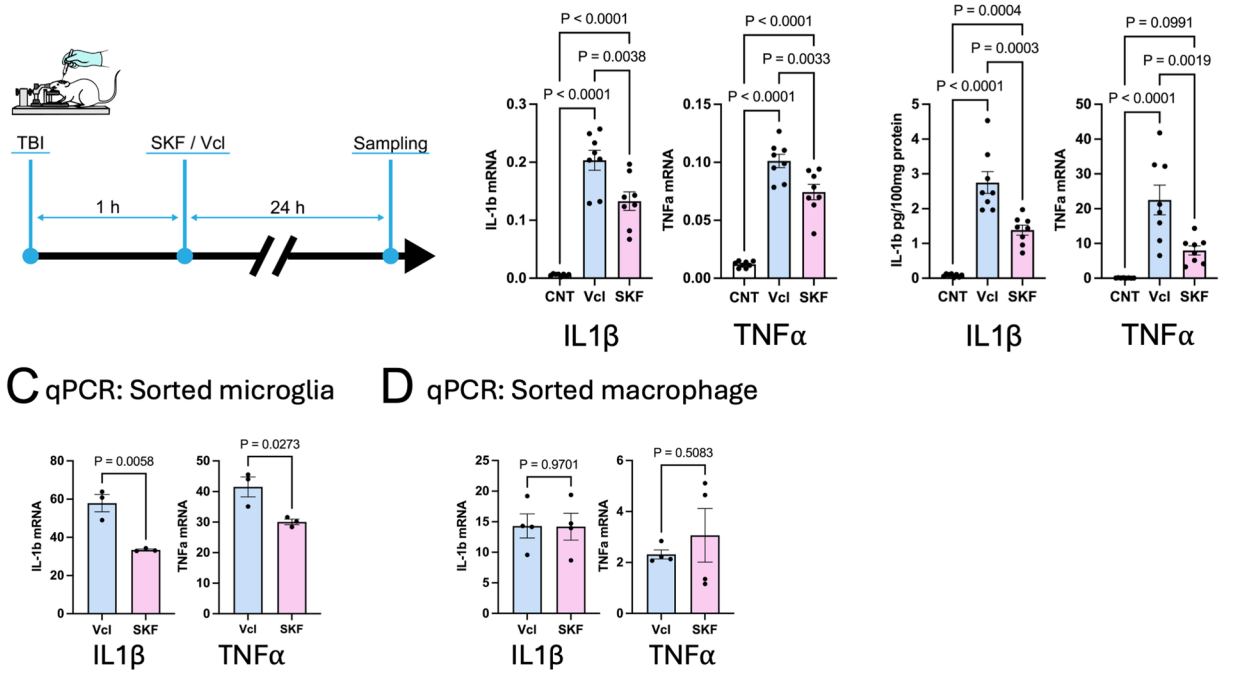
The behavioral tests were performed at 8 weeks following TBI. Using a video camera and tracking system (Ethovision XT 14; Noldus Info. Tech., Wageningen, Netherlands), the behaviors of TBI and normal control rats were evaluated. In one series of rats, motor incoordination was evaluated with a 5-min open-field test (OFT) using a square box (100 × 100 cm) with 50-cm-high walls (Miyanishi et al. 2019). From the day after the OFT, the Morris water maze (MWM) test was used to evaluate cognitive dysfunction using a 150-cm-diameter × 45-cm-tall circular pool. In MWM, the rats were trained for three consecutive days. They had three training sessions where they were forced to swim freely for 90 s from far, left and right points of the pool to find the platform with the flag. To memorize the location of the platform, rats were made to stay on it for 10 s. On the fourth day, two tests were performed: one with and another one without the platform, as described in our previous study (Abe et al. 2018).

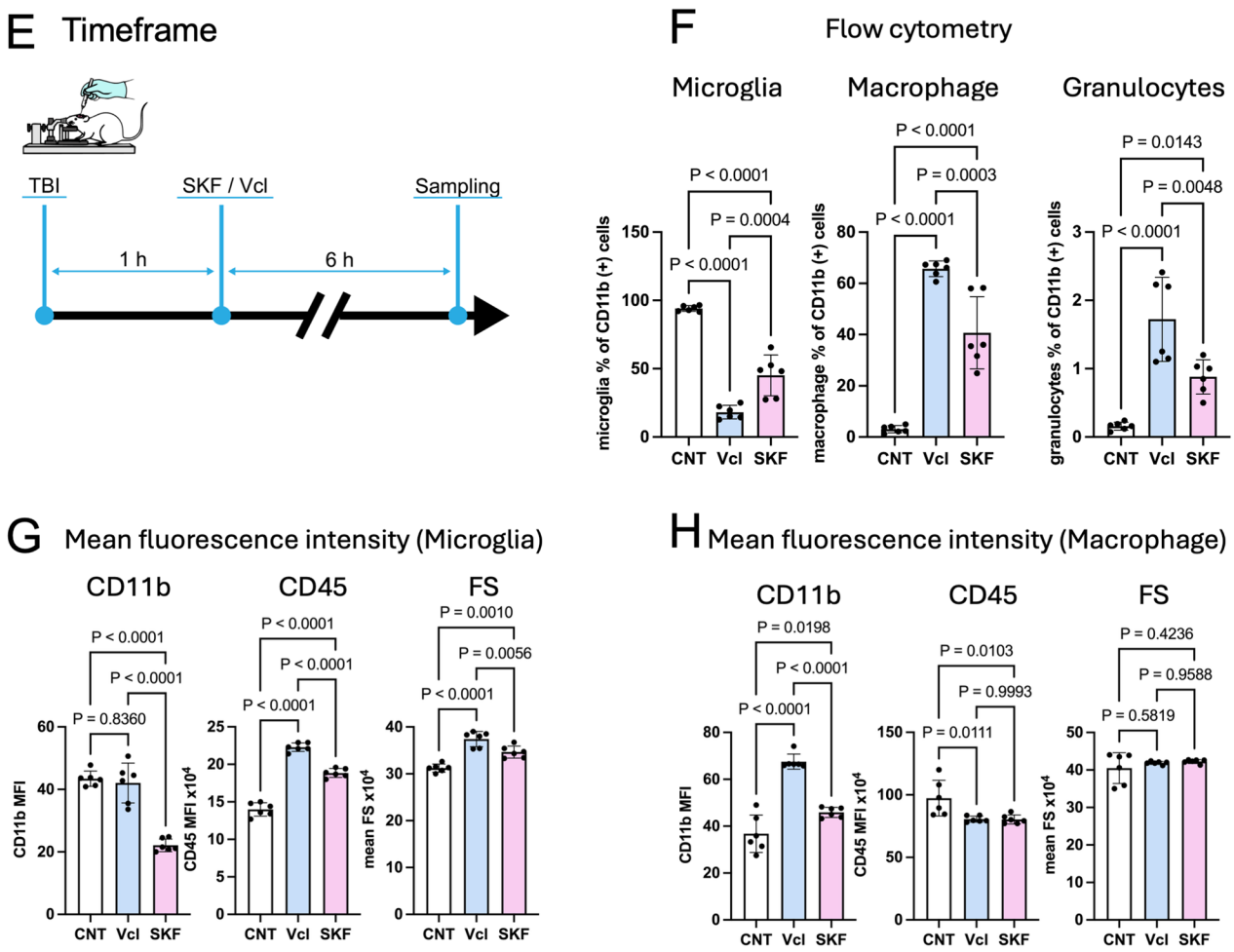
Measurements of Lost Brain Tissue Volume

Injured rat brains were dissected the day after behavioral tests and immersed in 4% PFA (Wako) for 1 week, and then six 2-mm-thick slices were prepared from the cerebrum to the olfactory bulb and placed on a black-colored slice board for photography. Using Adobe Photoshop CS5 Extended (Adobe Systems, San Jose, CA, USA), the photographed image was bitonalized, and the areas where the brain tissue was missing were processed as black, and the remaining tissue was as white. The percentage of lost volume was calculated as the remaining tissue of the left hemisphere minus that of the right hemisphere, which were further divided again by the left hemisphere as described in our previous studies (Nishihara et al. 2011; Abe et al. 2018).

Statistical Analyses

Data are expressed as the mean ± standard deviation (SD). Experimental data were analyzed using a two-tailed unpaired t-test, χ^2 test with Fisher's exact test, Welch's t-test, or one-way analysis of variance (ANOVA) with Tukey's post hoc test. The employed method is described in the figure legends. F-values, degrees of freedom, and the omnibus p-values for ANOVA analyses are listed in Supplementary Table 5. All analyses were performed using the Prism 9 software program (GraphPad Software, La Jolla, CA, USA). A p-value less than 0.05 was considered significant for all tests.


A Timeframe
TBI
SKF / Vcl
Sampling
1 h
24 h
Ba qPCR: tissue
IL-1b mRNA
TNFa mRNA
P < 0.0001
P = 0.0038
P < 0.0001
P < 0.0001
P = 0.0033
P < 0.0001
CNT Vcl SKF
IL1β
TNFα
Bb ELISA: tissue
IL-1b pg/100mg protein
TNFa mRNA
P = 0.0004
P = 0.0003
P < 0.0001
P = 0.0991
P = 0.0019
P < 0.0001
C qPCR: Sorted microglia
P = 0.0058
P = 0.0273
D qPCR: Sorted macrophage
P = 0.9701
P = 0.5083


E Timeframe
TBI
SKF / Vcl
Sampling
1 h
6 h
F Flow cytometry
Microglia
Macrophage
Granulocytes
microglia % of CD11b (+) cells
macrophage % of CD11b (+) cells
granulocytes % of CD11b (+) cells
P < 0.0001
P = 0.0004
P < 0.0001
P < 0.0001
P = 0.0003
P < 0.0001
P = 0.0143
P = 0.0048
P < 0.0001
CNT Vcl SKF
G Mean fluorescence intensity (Microglia)
CD11b
CD45
FS
CD11b MFI
CD45 MFI x10⁴
mean FS x10⁴
P < 0.0001
P < 0.0001
P = 0.8360
P < 0.0001
P < 0.0001
P < 0.0001
P = 0.0010
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P < 0.0001
H Mean fluorescence intensity (Macrophage)
P = 0.0198
P < 0.0001
P < 0.0001
P = 0.0103
P = 0.9993
P = 0.0111
P = 0.4236
P = 0.9588
P = 0.5819

Fig. 4 Suppressive effects of SKF on proinflammatory changes in injured brain tissues. **A** Experimental timeline for qPCR, and ELISA. **B** IL-1β and TNFα mRNA expressions were increased in the injured tissues, and SKF suppressed their expression significantly at 1 dpi as determined by qPCR. $n=8$. **C** Microglia were sorted from the injured tissues, and their mRNA expression was investigated with qPCR. SKF decreased the expression of mRNA encoding IL-1β, and TNFα in sorted microglia. $n=3$. **D** SKF did not change the expression of IL-1β, and TNF mRNA in sorted macrophages. $n=3$. **E** Experimental timeline for FCM. **F** FCM analyses revealed increased infiltration of $CD11b^{+}/CD45^{high}$ macrophages and granulocytes, and decreased percentage of $CD11b^{+}/CD45^{low}$ microglia in number. $n=6$. **G**, **H** SKF suppressed CD11b expression by microglia and macrophages. $n=6$. TBI increased CD45 expression by microglia but not macrophages, a finding that was partially reversed by SKF. Similarly, to CD45, FS values were increased only in microglia, and SKF suppressed the increase. $n=6$. A one-way ANOVA with Tukey's post hoc test

Results

Expression of Dopamine Receptors on Tissue and the Aggravating Cells of TBI Brain

Dopamine receptors D1 (DR1) and DR5 are classified as D1-like receptors, which are GPCR linked to G protein Gs. mRNAs encoding DR1 and DR5 were expressed in the striatum of control, Vcl-, and SKF-81297 (SKF)-injected TBI rats (Fig. 1B). Considering the modulatory role of inflammatory cells in the pathology of TBI brain demonstrated in our previous study (Abe et al. 2018), we sorted microglia and macrophages from TBI rat brain for qPCR and found dominant mRNA expression of D1-like receptors and D3R in those cells (Fig. 1C). Then, we prepared the primary cultures of microglia, macrophages, and neutrophils that may play potentially aggravating roles in the TBI brain and found that the D1-like receptors-specific agonist SKF decreased LPS-induced IL-1β and TNFα expression in these cells (Fig. 2). To further exclude a possible involvement of off target effects SKF, the expression of D1R was knocked down with the validated siRNA (Kopec et al. 2018) and siRNA reduced mRNA for D1R (Supplementary Fig. 1B). The D1R-knockdown partially abolished the SKF on expression of LPS-induced IL-1β and TNFα mRNA expressions (Supplementary Fig. 1C).

Ameliorative Effects of SKF on TBI

Considering the immunosuppressive effect of SKF observed in our in vitro studies, we initially conducted a dose-dependent immune response experiment using FCM analysis. Our FCM findings revealed that a dose of 10 mg/kg is the most effective in suppressing macrophage infiltration (Supplementary Fig. 2). SKF was administered to TBI rats once daily for 3 days with 10 mg/kg, and its long-term ameliorative effects were evaluated (Fig. 3). In the open-field test (OFT) performed 8 weeks after TBI, the total distance moved and mobile duration decreased in vehicle-administered TBI rats, but administration of SKF reversed these TBI effects (Fig. 3B). TBI has been shown to disturb cognitive and learning/memory functions (Abe et al. 2018). The MWM test with platform showed that the success rate for reaching platform increased in SKF-treated TBI group (Fig. 3Ca). Moreover, in the MWM test without platform, SKF increased the frequency of entering the target quadrant in the pool, where the platform was used to be placed during the trial sessions of the test (Fig. 3Cc). This finding suggests that TBI-induced cognitive and learning abilities were ameliorated by SKF. Probably due to secondary degeneration (Corps et al. 2015; Gyoneva and Ransohoff 2015), TBI generates severe brain tissue loss on a monthly basis which greatly correlated behavioral outcome of TBI rats as shown in our previous study (Abe et al. 2018). Considering the findings of our behavioral studies, we confirmed the efficacy of SKF with a brain slice study and found that SKF suppressed brain tissue loss (Fig. 3D).

Effects of SKF on Inflammatory Responses

TBI increased expression of proinflammatory cytokines IL-1β and TNFα at mRNA and protein levels in and around the injured core (Figs. 4Ba and Bb, respectively). SKF suppressed TBI-induced elevated expression of these cytokines in the brain tissue of lesion area. Microglia and macrophages were sorted from injured tissues at 1 dpi, and processed for qPCR. SKF administration decreased the mRNA expression of IL-1β and TNFα in sorted microglia (Fig. 4C) but not in macrophages (Fig. 4D). The phagocytic activity of microglia and macrophages may be responsible for tissue restoration, as injury-induced accumulation of degenerated materials prevents restoration (Matsumoto et al. 2008). On the other hand, it has also been insisted that the phagocytes accelerate neuronal death by eliminating viable neurons (Neher et al. 2014). This aggravating process is called phagoptosis and is mediated by the opsonization of damaged cells with complement C1q, suggesting that phagocytosis may have both beneficial and detrimental effects on injured brain (Brown and Neher 2014). TBI increased the expression of CD68, a marker for phagosomes, and complement C1qb, and SKF reduced the increased CD68 and C1qb expression in the injured brain tissues (Supplementary Fig. 3).

FCM analyses showed that the percentage of microglia decreased in injured brain tissues, while macrophages and granulocytes increased (Fig. 4F). Besides, our immunohistochemical staining with the cell cycle marker Ki67 showed macrophages identified as $CD45^{high}$ and $Iba1^{+}$ were $Ki67^{+}$, whereas microglia identified as $CD45^{low}$ and $Iba1^{+}$ are $Ki67^{-}$, indicating that macrophage but not microglia were proliferative at lesion area of TBI brain (Supplementary

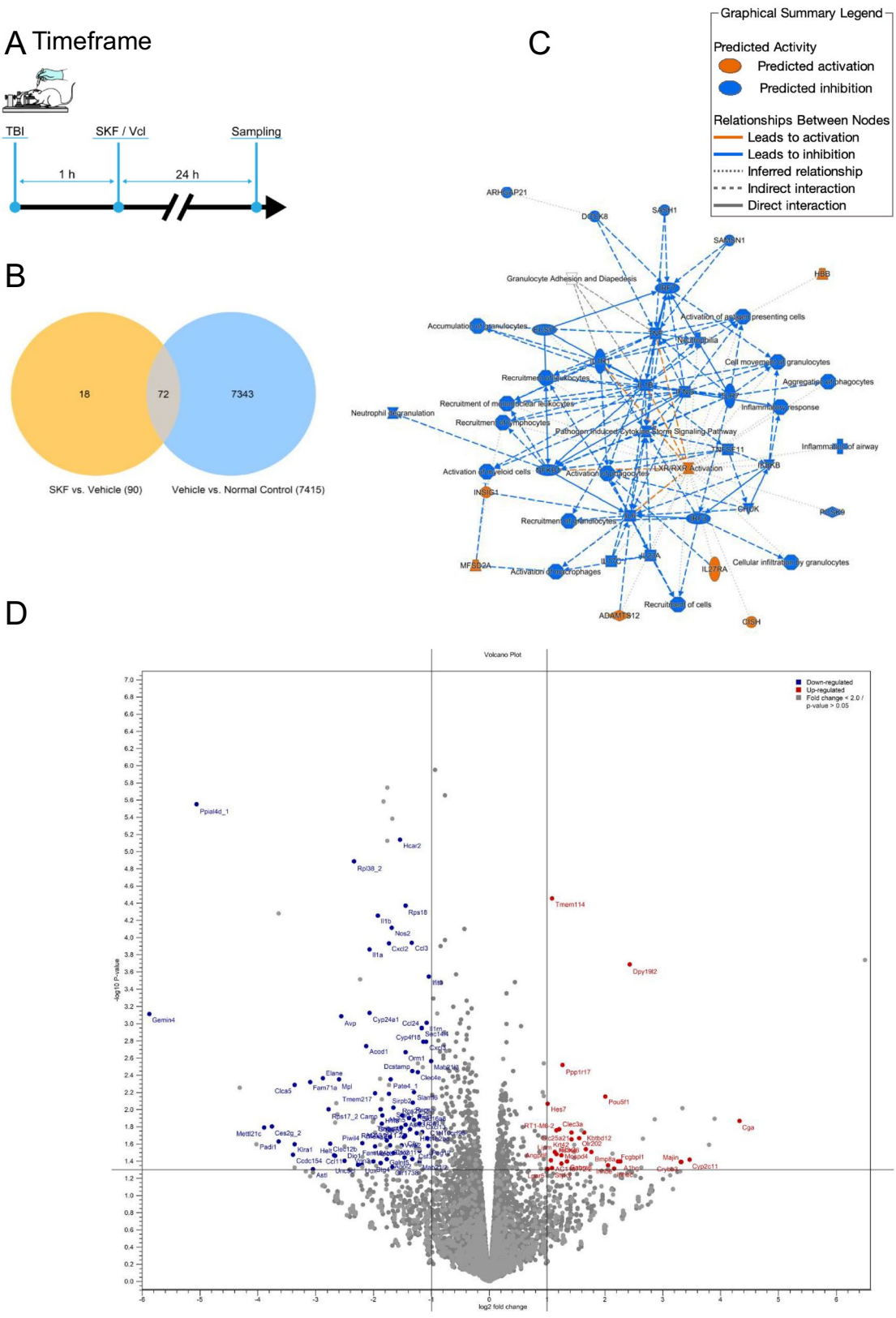


Fig. 5 Effect of SKF on comprehensive gene expression. **A** Experimental timeline **B** Venn diagram of RNAseq results. A total of 7415 genes had variable expression compared to normal subjects by TBI, 72 of which were affected by SKF. $n=8$. **C** Volcano plot showing that SKF treatment decreased the expression of many proinflammatory factors, such as IL-1β, IL-1α, and NOS2. $n=8$. **D** A gene expression network analysis, showing SKF-induced changes in gene expression, mostly of pro-inflammatory factors such as IL-1β. $n=8$

Fig. 4). SKF reversed TBI-induced changes in the $CD11b^+$ cell population. The microglia and macrophages were further characterized using FCM. SKF decreased CD11b expression in both microglia and macrophages (Fig. 4G, H), whereas CD45 expression decreased in microglia but not in macrophages (Fig. 4G, H). Forward scatter (FS) values also decreased only in microglia (Fig. 4G, H).

Effects of SKF on Comprehensive Gene Expression in the Injured Brain Tissues

RNAseq was conducted to comprehensively investigate gene expression, and the results are presented in a Venn diagram, illustrating the expression changes of 7,343 genes induced by TBI. The expression of 72 genes was altered by SKF treatment. The primary genes with altered expression were a group of proinflammatory mediators, such as IL-1 (Fig. 5B). A pathway analysis was performed to identify the specific pathways through which SKF exerted its effects. The analysis revealed that SKF inhibited pathways that promote immune cell activation and leukocyte infiltration into the brain (Fig. 5C). A volcano plot demonstrated that the expression of genes encoding many proinflammatory factors, such as IL-1β and NOS2, was decreased by SKF treatment (Fig. 5D).

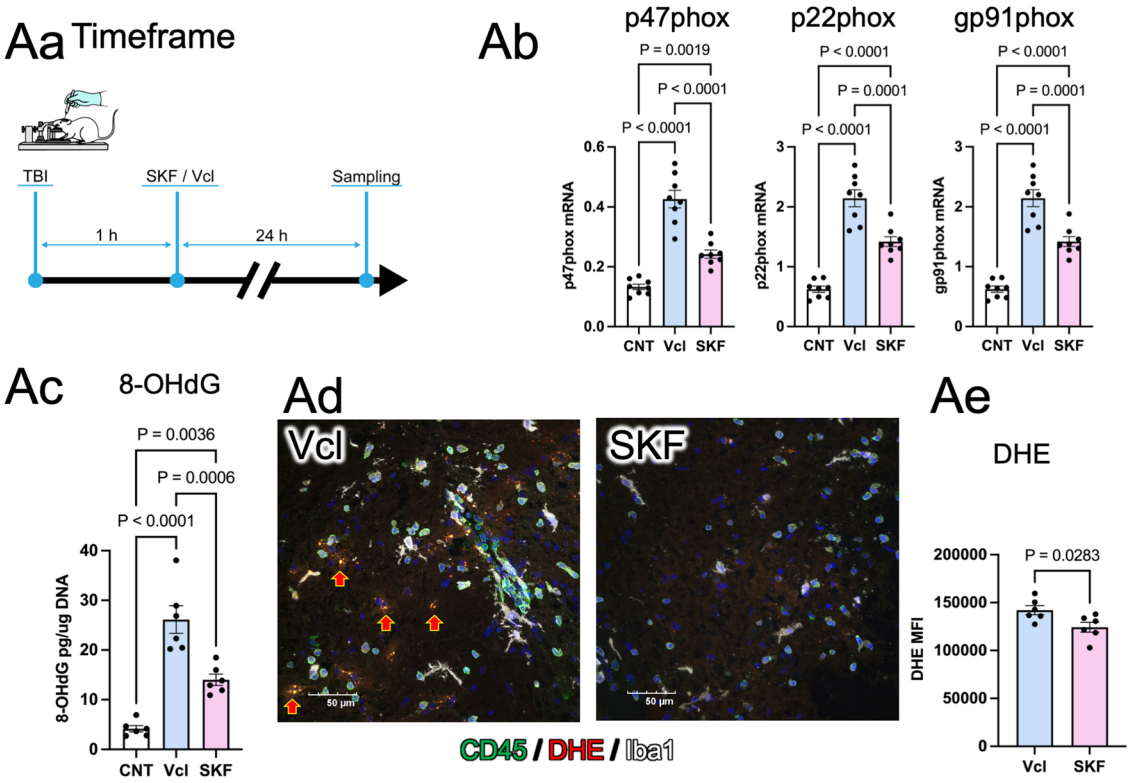
Suppression of Oxidative Stress and Cellular Energy Metabolism by SKF

Considering the effects of SKF suppressing the infiltration of macrophage and granulocytes that can cause oxidative stress, expression of the relevant enzymes was investigated by qPCR (Fig. 6Ab). The expression of gp91phox, a subunit of NADPH oxidase 2 (NOX2), was upregulated in Vcl-treated injured tissues, and this upregulation was reversed by SKF administration (Fig. 6Ab). The expression of p47phox and p22phox, subunits necessary for NOX2 activity, was also increased by TBI and suppressed by SKF. Then, we confirmed the effects of SKF against ROS by 8-OHdG ELISA as ROS causes the generation of 8-OHdG, a marker of oxidative damage to DNA. TBI significantly increased 8-OHdG levels, which were reduced by SKF (Fig. 6Ac). DHE is oxidized to emit red fluorescence from ethidium (Abe et al. 2018). An immunohistochemical analysis showed that most $Iba1^+/CD45^-$ ramified microglial cells did not exhibit red fluorescence, and $Iba1^+/CD45^+$ round macrophages were weakly positive for DHE-derived fluorescence (Fig. 6Ad, Supplementary Fig. 5C, Supplementary Video. 1). Furthermore, the study suggests that $Iba1^-/CD45^-$ cells were the major source of ROS. Compared to Vcl-treated brain sections, SKF-treated sections displayed much weaker red fluorescence (Fig. 6Ad, Supplementary Video. 2), suggesting that SKF might have suppressed ROS generation by not only myeloid cells but also neuroectodermal cells, such as neurons (Abe et al. 2018). Additionally, $CD11b^+$ cells were sorted using MACS and analyzed by flow cytometry to measure oxidized DHE-derived fluorescence (Fig. 6Ae). The mean fluorescence intensity was reduced in $CD11b^+$ cells sorted from SKF-treated rat brains compared to vehicle (Vcl)-treated rat brains, suggesting that $CD11b^+$ cells were responsible for ROS generation.

To reveal the mechanisms by which SKF reduced oxidative injury, we examined mitochondrial metabolism using the Seahorse Mito-Stress Test, as mitochondria are the major source of ROS (Jitschin et al. 2014). SKF reduced glycolysis and mitochondrial respiration in primary cultured microglia, macrophages, and neutrophils as revealed by reduced OCR and ECAR (Fig. 6B, Supplementary Fig. 6). Pro-inflammatory responses in immune cells are correlated with metabolic changes as shown in previous studies (Abe et al. 2018; Kawasaki et al. 2017; Takeda et al. 2023a). Cells can mitigate oxidative stress not only by suppressing ROS generating mechanisms but also by increasing the activities of anti-oxidative enzymes and/or factors (Takeda et al. 2021). Yet, SKF reduced expression of genes encoding glutathione peroxidase 4 (GPX4), Cu/ZnSOD, and catalase (Supplementary Fig. 7).

Suppression of NFκB-Mediated Proinflammatory Pathways by SKF

ROS have been shown to induce NFκB activation, a critical transcription factor that elevates the expression of various proinflammatory factors, such as IL-1β and TNFα (Gloire et al. 2006; Morgan and Liu 2011). Immunoblotting data of our previous study revealed that LPS induced NFκB p65 localization in the nuclear fraction of rat primary cultured microglia (Islam et al. 2018). In the present study, we employed the same approach and found that SKF prevented LPS-induced nuclear localization of p65 (Fig. 7A). Phosphorylated IκB kinase (pIKK) induces degradation of IκB, resulting in nuclear localization of p65 (Ishii et al. 2015). To examine whether or not SKF suppressed the TBI-induced nuclear localization of p65 in injured brain tissues, immunoblotting for pIKK and IKK were performed with injured tissues. SKF administration suppressed phosphorylation of IKK in the injured tissues (Fig. 7B). To determine the cell type bearing pIKK in and around injured tissues, an immunohistochemical study was conducted. Figure 7Bd shows that the $pIKK^+/CD11b^+$ cells with morphological characteristics of infiltrated macrophages that are a round morphology without apparent ramified processes (Islam et al. 2018). In contrast, ramified $CD11b^+$ cells or microglia appeared to be $pIKK^-$. In SKF-treated rat tissues, the number of


Aa Timeframe
TBI
SKF / Vcl
Sampling
1 h
24 h
Ab
p47phox
p22phox
gp91phox
P = 0.0019
P < 0.0001
P < 0.0001
P < 0.0001
P = 0.0001
P < 0.0001
P < 0.0001
P = 0.0001
P < 0.0001
p47phox mRNA
p22phox mRNA
gp91phox mRNA
CNT Vcl SKF
Ac 8-OHdG
P = 0.0036
P = 0.0006
P < 0.0001
8-OHdG pg/ug DNA
CNT Vcl SKF
Ad
Vcl
SKF
50 µm
CD45 / DHE / Iba1
Ae
DHE
P = 0.0283
DHE MFI
Vcl SKF

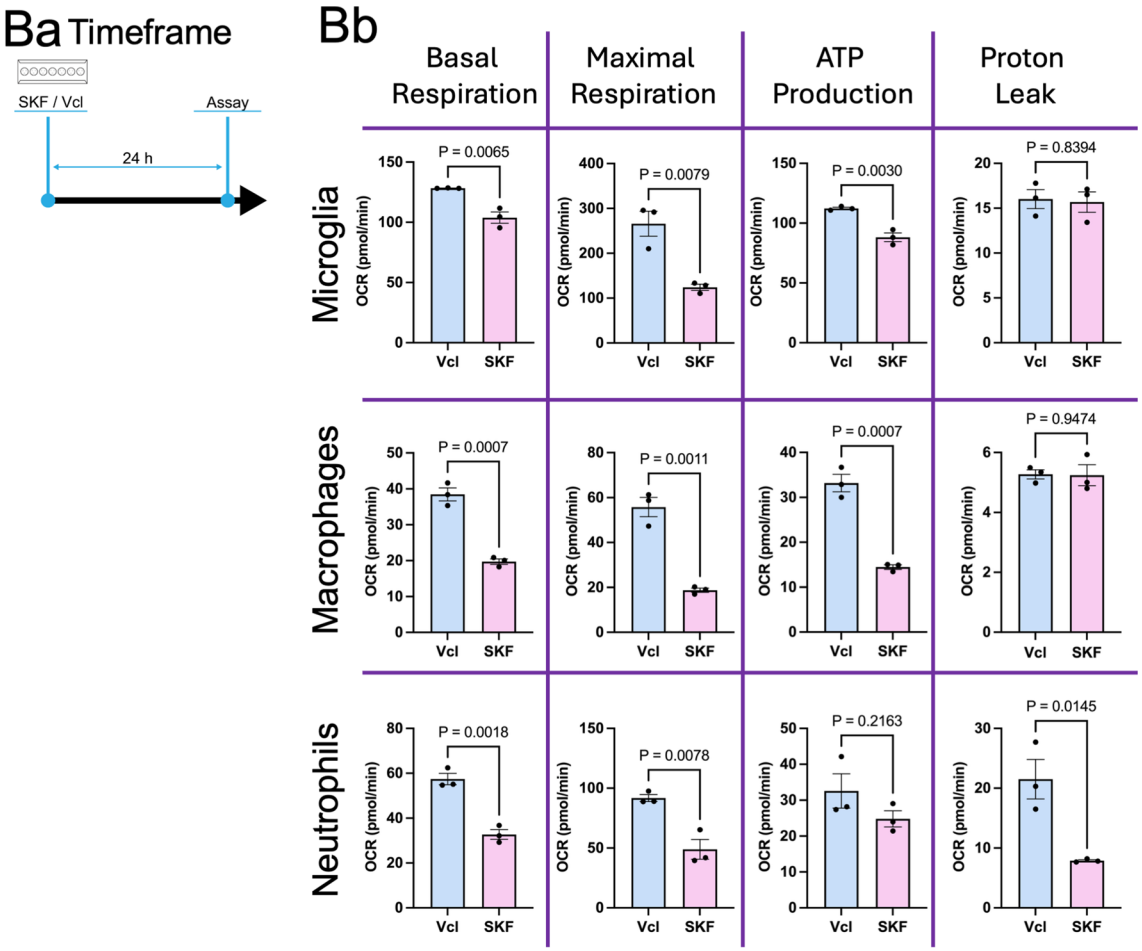

Ba Timeframe
SKF / Vcl
Assay
24 h
Bb
Basal Respiration
Maximal Respiration
ATP Production
Proton Leak
Microglia
Macrophages
Neutrophils
OCR (pmol/min)
P = 0.0065
P = 0.0079
P = 0.0030
P = 0.8394
P = 0.0007
P = 0.0011
P = 0.0007
P = 0.9474
P = 0.0018
P = 0.0078
P = 0.2163
P = 0.0145
Vcl SKF

Fig. 6 SKF reduced oxidative stress in the injured tissues and cellular energy metabolism. **Aa** Experimental timeline for in vivo study. **Ab** mRNA encoding p47phox, p22phox, and gp91phox was increased in the injured tissues and SKF decreased the expression. $n=7$. **Ac** 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was increased in the amounts in the injured tissues as revealed by an ELISA, while SKF suppressed the increase. $n=6$. **Ad** Immunohistochemical identification of cells bearing DHE-derived red fluorescence. The strong red fluorescence was found in $CD45^{-}$/$Iba1^{-}$ cells (arrowheads). SKF reduced the fluorescence bearing cells. Please refer to Supplementary Fig. 3C, Supplementary Video 1, and Supplementary Video 2. **Ae** Fluorescence intensity of dihydroethidium (DHE) reflecting from ROS generation by $CD11b^{+}$ cells isolated from SKF-treated brains was weaker than that by the cells from Vcl-treated brains. $n=6$. **Ba** Experimental Timeline for in vitro studies with primary cultured cells. **Bb** SKF significantly suppressed oxygen consumption rate (OCR) of basal respiration [(last rate measurement before rotenone/antimycin A injection)—(non-mitochondrial respiration rate)], maximal respiration [(maximum rate measurement after FCCP injection)—(non-mitochondrial respiration)], ATP production [(last rate measurement before oligomycin injection)—(minimum rate measurement after oligomycin injection)], and proton leak [(minimum rate measurement after oligomycin injection) – (non-mitochondrial respiration)], in rat primary cultured microglia, macrophages, and neutrophils. n=3

$pIKK^{+}$ cells was much lower than that in Vcl-treated rat tissues (Fig. 7Bd, Supplementary Video. 3 and 4). In addition to microglia/macrophages, an abundant number of $CD11b^{-}$ cells appeared to be $pIKK^{+}$ (Supplementary Fig. 5D, Supplementary Video. 3).

Effects of the β 2AR-Specific Agonist Clenbuterol (CLB) on TBI

The effects of CLB, a BBB-permeable β2AR-specific agonist (Qian et al. 2011), on TBI were investigated. b2AR is a Gs-coupled GPCR identical to dopamine D1-like receptors (Rasmussen et al. 2011). CLB ameliorated motor activity of TBI rats and tissue lost volume (Fig. 8Ab and 8Ac). CLB had an inhibitory effect on macrophage and granulocyte accumulation (Fig. 8Bb). A qPCR analysis of gene expression around injured tissues revealed that CLB suppressed the mRNA expression of IL-1β, TNFα, and p22phox but did not affect Cu/ZnSOD expression (Fig. 8Cb).

Effects of SKF on Neuroprotective Responses

IGF1 is a neuroprotective factor involved in the restoration of injured brains, and blood-borne infiltrating macrophages may be a source of IGF1 in neuroinflammatory disease (Smirkin et al. 2010; Matsumoto et al. 2015; Montivero et al. 2021). SKF did not increase IGF1 mRNA expression following 24 h after TBI, but SKF induced changes in IGF1 mRNA levels in TBI brains at 7 dpi (Fig. 9). Likewise, SKF increased expression of neuroprotective factors basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) only at 7 dpi (Fig. 9). These data indicate that the neurotrophic effects of SKF are attributable only in the subacute or chronic phases of brain injury.

Discussion

TBI is still an intractable condition, for which there are still no specific and effective interventions. TBI has a high mortality rate, and most survivors suffer from neurological and psychological sequelae, which often severely disturb their well-being in daily life. Although microglia themselves may not be thought to have strong deleterious effects on the brain (Abe et al. 2018, 2020), they can induce infiltration into the injured tissues of circulating leukocytes, especially monocytes, which are the precursor cells of brain macrophages (Corps et al. 2015; Gyoneva and Ransohoff 2015; Masel and DeWitt 2010). The infiltrated leukocytes, in particular monocyte-derived macrophages, play aggravating effects on injured brains through several mechanisms: releasing pro-inflammatory mediators, such as proinflammatory cytokines/chemokines; generating ROS causing oxidative injuries in brain tissues and cells as well as eliminating viable cells by phagocytosis (Abe et al. 2018, 2020; Matsumoto et al. 2008, 2015; Neher et al. 2014; Butler et al. 2021). Thus, microglia are thought to play a central role in the pathogenesis of TBI.

Although both microglia and macrophages express D1R and D5R, SKF may act more strongly on microglia than macrophages, and it more markedly suppresses the pro-inflammatory activation of microglia than macrophages (Tanaka et al. 2024). SKF reduced the infiltration of monocytes and granulocytes, probably due to the reduced expression of proinflammatory mediators by microglia, resulting in the improvement of TBI pathogenesis. SKF ameliorated the oxidative damage in and around the injured tissue caused and ameliorating the chronically progressive loss of brain tissue and cognitive dysfunction and motor inactivity. These results strongly suggest that pharmacological intervention to activate D1-like receptor-mediated signaling in TBI pathology may improve the prognosis.

RNAseq results showed that SKF suppressed the expression of proinflammatory mediators, such as IL-β1, whose expression is driven by NFκB (Ishii et al. 2015; Islam et al. 2018). SKF may suppress translocation of NFκB into the nuclei to prevent proinflammatory responses in the injured brain. SKF reduced oxidative stress-induced injury, which was likely correlated with decreased proinflammatory reactions. SKF suppresses NOX2 enzyme subunit expression. The present immunohistochemical observations suggest that $CD11b^{-}$ cells may be a critical source of ROS. Neurons express NOX2 during inflammation, chronically causing neurodegeneration (Tu et al. 2023). In addition to suppression of NOX2 expression, SKF suppressed mitochondrial activities, presumably

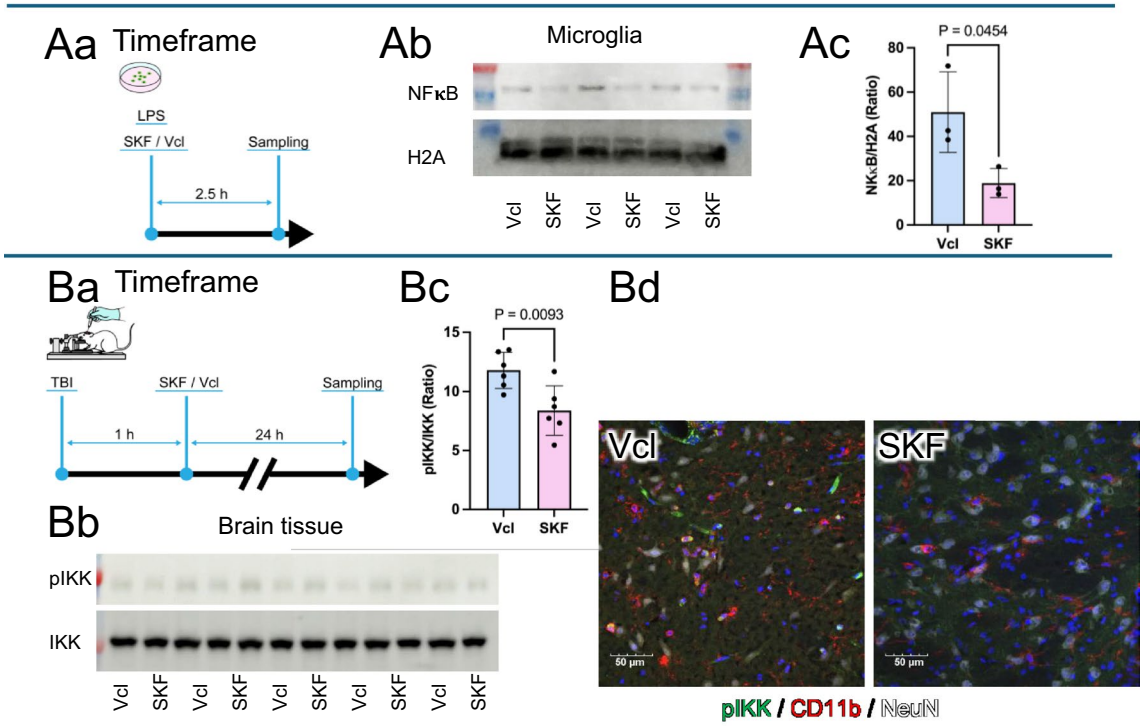


Fig. 7 SKF inhibits NFκB signaling. **Aa** Experimental timeline for in vitro study. **Ab** SKF prevented the accumulation of NFkB in the nuclei of LPS-treated primary cultured microglia as revealed by immunoblotting of nuclear fractions. Blots images and statistical analyses on $n=3$ data are shown. **Ba** Experimental timeline for in vivo study. **Bb-c**) Phosphorylated IkB kinase (pIKK) was detected in the injured tissues and the ratio of pIKK-immunoreactivity to IKK-immunoreactivity in immunoblotting was reduced by SKF. Representative blots and statistical analysis on $n=5$ data are shown. **bd** Immunohistochemically stained tissues around the injured cores using antibodies to pIKK, CD11b, and NeuN. $pIKK^{+}$ cells were frequently present in the Vcl-treated brain but such cells were much less in the SKF-treated brain. Please refer to Supplementary Fig. 3**D**, Supplementary Video 3, and Supplementary Video 4

leading to suppression of mitochondrial ROS generation (Abe et al. 2018). To mitigate oxidative stress, it is important not only to suppress the mechanisms of ROS generation, but also to enhance the expression or production of antioxidant enzymes and antioxidant factors. However, SKF reduced the expression of antioxidant enzymes such as Cu/ZnSOD. These results suggest that although SKF suppressed NFκB, it is unlikely to increase the activity of the transcription factor NF-E2-related factor 2 (Nrf2), which increases the expression of antioxidant enzymes/factors with enhancing anti-inflammatory effects (Takeda et al. 2021).

Regarding the mechanism of suppression of NFκB activity by SKF, it is well known that when intracellular cAMP concentration is elevated by β2AR agonists (Mori et al. 2002) or phosphodiesterase inhibitors (Zhang et al. 2002; Nishikawa et al. 2023), nuclear translocation of NFκB is prevented. As both D1-like receptors and β2AR are Gs-coupled GPCRs (Beaulieu and Gainetdinov 2011), it is likely that they also inhibit the nuclear translocation of NFκB via an increase in intracellular cAMP concentration. In fact, this study showed that the β2AR agonist CLB exerted anti-inflammatory effects similarly to SKF, suggesting that the ameliorating effects of SKF on TBI were mediated by its cAMP-elevating effects on microglia and macrophages (Patel et al. 2013).

Microglia and infiltrating macrophages are not always detrimental to injured brains as they release several neuroprotective cytokines and growth factors, such as IGF1, BDNF, and bFGF (Smirkin et al. 2010; Islam et al. 2018; Matsumoto et al. 2015). They also secrete anti-inflammatory cytokines, including TGFβ1, which have strong and sustained effects in suppressing the proinflammatory activation of infiltrating macrophages (Islam et al. 2018). Thus, microglia and macrophages are often described as a "double-edged sword" (Patel et al. 2013). A previous study with brain ischemic model rat, showed CLB increase neurotrophic factor nerve growth factor (NGF), bFGF, and transforming growth factor-β1 (TGF-β1) mRNA in cortical and hippocampal tissue (Culmsee et al. 1999). Likewise, SKF increased several neuroprotective cytokines and growth factors, such as IGF1, BDNF, and bFGF at 7dpi. However, stimulative effects of SKF on the expression of neuroprotective factors were not observed at 1dpi and 3dpi. Therefore, it is probable that SKF improves the prognosis of TBI by suppressing the proinflammatory response by inhibiting the nuclear localization of NFκ B at the acute stage. The suppression of

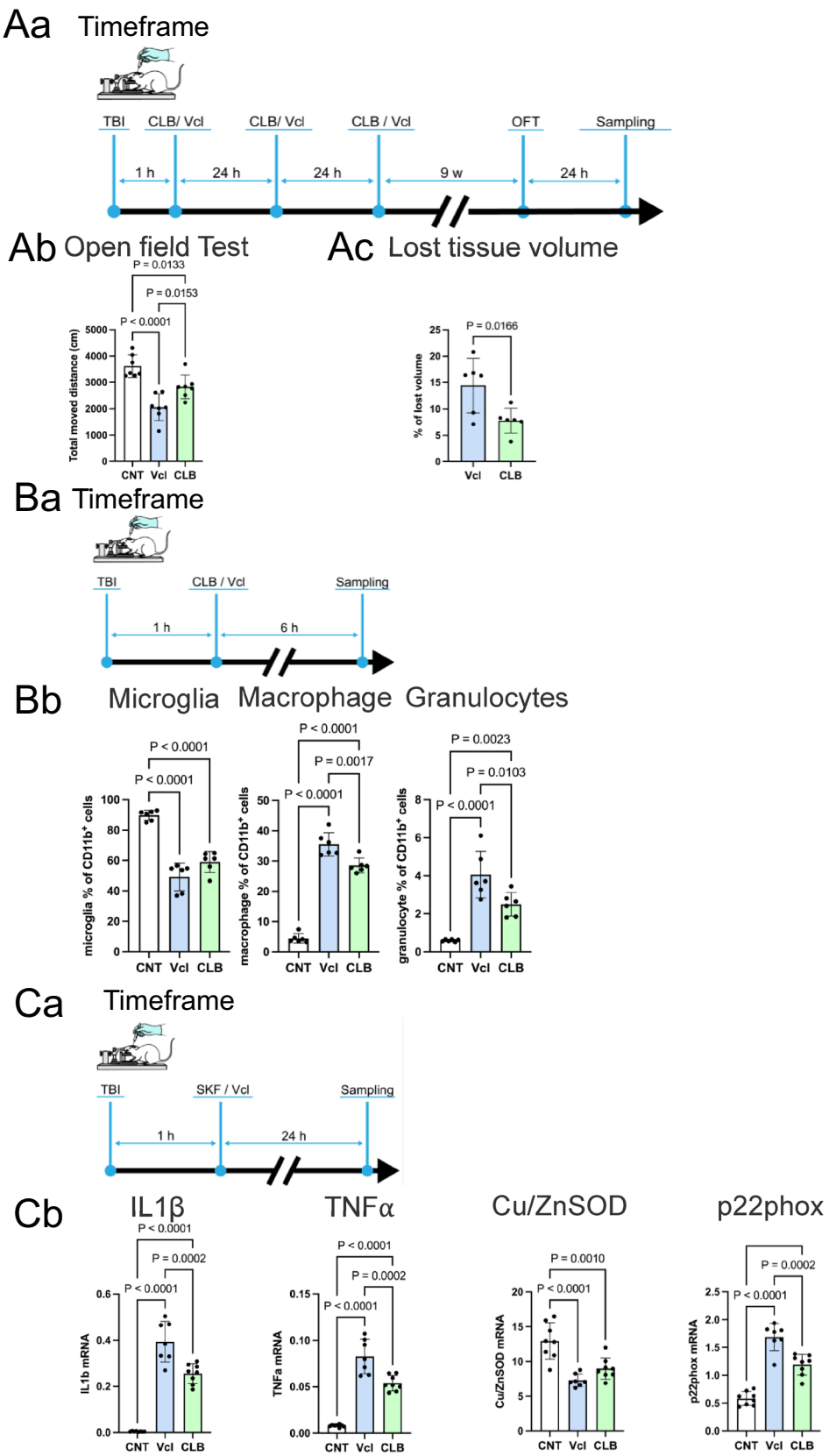


Fig. 8 Effects of an adrenergic β2AR agonist, clenbuterol (CLB). **A** The effect of CLB on motor activity of TBI rats. CLB improved moved distance of rats in OFT. n = 7. **B** The effects of CLB on accumulation of leukocytes in the injured brains. CLB also showed an inhibitory effect on the accumulation of macrophages and granulocytes. n = 6. **C**, **D** Similar to SKF, CLB also showed suppression of CD11b (**C**) and CD45 (**D**) expression in microglia but not in macrophages around the injured tissue. n = 6. **E** The results of qPCR examining gene expression around the injured tissue. Like SKF, CLB suppressed the expression of IL-1β, TNFα, and p22phox, and did not affect the expression of Cu/ZnSOD. n = 7

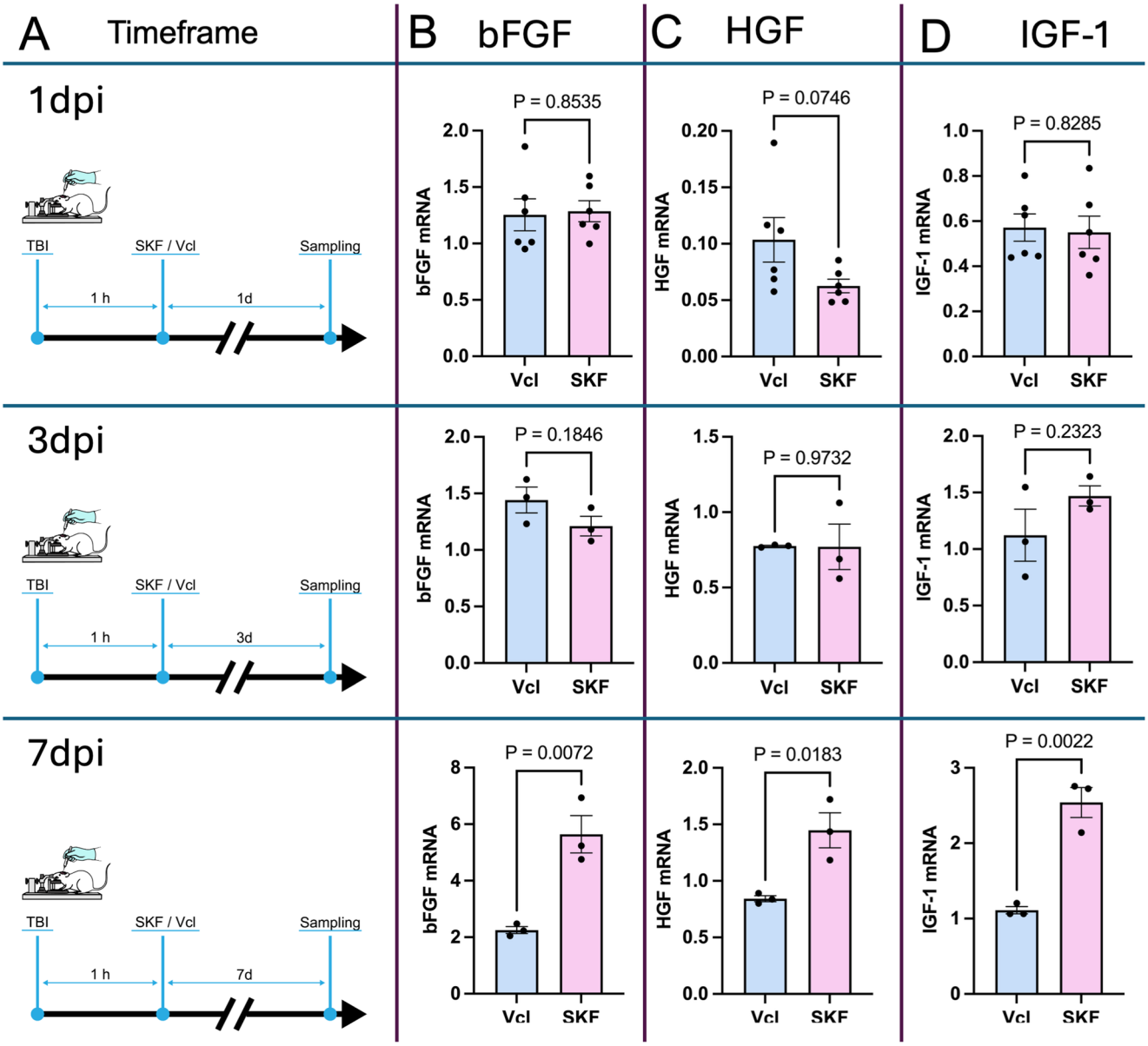


Fig. 9 SKF enhanced neurotrophic factors in the brain tissue of TBI rats. **A** Experimental Timeline for in vivo Studies. SKF failed to increase neurotrophic factors following 1 day and 3 days after TBI. However, it significantly elevated neurotrophic factors bFGF (**B**), HGF (**C**), and IGF-1 (**D**) at 7 days following treatment. n = 3–6

proinflammatory responses at the acute phase may lead to elevated expression of neurotrophic factors at subacute or chronic stage in the damaged brain tissue.

In conclusion, SKF, a blood–brain barrier-permeable dopamine D1-like receptor agonist, suppressed brain inflammation and improved the functional prognosis in a rat TBI model. These results suggest that D1-like agonists are effective for the treatment of brain injury, a refractory pathology. This action was thought to be associated with an elevation of the intracellular cAMP concentration resulting in the inhibition of the expression of proinflammatory mediators.

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MEC, AT, MS, HY(Yamamoto), HY (Yamauchi), KS, EK, AK, NT, KT; Methodology: MEC, NA; Project administration: MEC, JT; Resources: MN, TN, TK; Supervision: TN; Validation: MN, TN, TK; Visualization: MEC, JT; Writing – original draft: MEC, JT; Writing – review & editing: MEC, JT.

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Data Availability The RNA-seq datasets generated and/or analysed during the current study are available in the Gene Expression Omnibus repository, https://www.ncbi.nlm.nih.gov/geo/, and GSE298938. Other datasets used and/or analysed in the present study are available on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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