Role of SPAK-NKCC1 Signaling Cascade in the Choroid Plexus Blood-CSF Barrier Damage After Stroke

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Research Article

Keywords: bumetanide, choroid plexus, H₂O₂, Na⁺-K⁺-Cl⁻ cotransporter, SPAK, ZT-1a

Posted Date: December 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1189368/v1>

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Version of Record: A version of this preprint was published at Journal of Neuroinflammation on April 12th, 2022. See the published version at <https://doi.org/10.1186/s12974-022-02456-4>.

Abstract

Background: The mechanisms underlying dysfunction of choroid plexus (ChP) blood-cerebrospinal fluid (CSF) barrier and lymphocyte invasion in neuroinflammatory responses to stroke are not well understood. In this study, we investigated whether stroke damaged the blood-CSF barrier integrity due to dysregulation of major ChP ion transport system Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) and regulatory Ste20-related proline-alanine-rich kinase (SPAK). **Methods:** Sham or ischemic stroke was induced in C57Bl/6J mice. Changes of the SPAK-NKCC1 complex and tight junction proteins (TJs) in the ChP were quantified by immunofluorescence staining and immunoblotting. Immune cell infiltration in the ChP was assessed by flow cytometry and immunostaining. Cultured ChP epithelium cells (CPECs) and cortical neurons were used to evaluate H₂O₂-mediated oxidative stress in stimulating the SPAK-NKCC1 complex and cellular damage. In vivo or in vitro pharmacological blockade of the ChP SPAK-NKCC1 cascade with SPAK inhibitor ZT-1a or NKCC1 inhibitor bumetanide were examined. **Results:** Ischemic stroke stimulated activation of the CPECs apical membrane SPAK-NKCC1 complex, NF- κ B, and MMP9, which was associated with loss of the blood-CSF barrier integrity and increased immune cell infiltration into the ChP. Oxidative stress directly activated SPAK-NKCC1 pathway and resulted in apoptosis, neurodegeneration, and NKCC1-mediated ion influx. Pharmacological blockade of the SPAK-NKCC1 pathway protected the ChP barrier integrity, attenuated ChP immune cell infiltration or neuronal death. **Conclusion:** Stroke-induced pathological stimulation of the SPAK-NKCC1 cascade caused CPECs damage and disruption of TJs at the blood-CSF barrier. The ChP SPAK-NKCC1 complex emerged as a therapeutic target for attenuating ChP dysfunction and lymphocyte invasion after stroke.

Background

The choroid plexus (ChP), a highly vascularized tissue in each ventricle of the brain, is a main secretory source for producing cerebrospinal fluid (CSF), and simultaneously functions as the blood-CSF barrier to prevent toxic metabolites and immune cells from entry into the central nervous system (CNS) (1, 2). Like other brain barriers, tight junction proteins (TJs) are present between the adjacent choroid plexus epithelium cells (CPECs) in the ChP blood-CSF barrier and are an essential prerequisite for maintaining barrier integrity (3, 4). Despite the fact that this barrier plays an important role in development, homeostasis, and maintenance of the CNS (5, 6), it remains a relatively understudied brain structure. Impaired ChP-CSF structure and function are implicated in development of several neurological diseases, including excessive CSF accumulation in posthemorrhagic hydrocephalus (7), infiltration of leukocytes in the autoimmune diseases (e.g., Multiple Sclerosis), neuroinflammation in stroke, morphological changes of CPECs with altered CSF production in Alzheimer's disease (AD) and other neurodegenerative diseases (8-10). Moreover, new studies show that the ChP is one of the major infiltration routes for lymphocyte invasion into the post-stroke brains and plays a role in regulating neuroinflammatory responses (11). However, the cellular and molecular mechanisms underlying ChP structural and functional changes in the neurodegenerative diseases are not well understood.

Ion transporters that mediate vectorial ion transport across basolateral and apical membranes of CPECs, accompanied by transport of water through aquaporins, produce approximately 500 ml of CSF per day in adult human brains (1, 12, 13). The cation-chloride cotransporter family protein $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter isoform 1 (NKCC1) is located at the apical membrane of the CPECs (14) and contributes approximately half of the CSF secretion in the adult brains (12). Activation of NKCC1 via the upstream regulatory serine-threonine kinases, the WNK (Kinase with no lysine (K))- SPAK (Ste20-related proline-alanine-rich kinase)/OSR1(oxidative stress-responsive kinase-1), play crucial roles in regulating cell volume and maintaining ionic homeostasis in the CNS (15). However, during early postnatal brain development, it has been shown that elevated expression of NKCC1 protein in the CPECs increased CSF K^+ clearance, cerebral compliance, and reduced circulating CSF in mice (16). In experimental posthemorrhagic hydrocephalus, toll-like receptor 4 (TLR4)- and nuclear factor kappa B (NF- κ B)-dependent inflammatory responses stimulate ChP NKCC1-mediated CSF hypersecretion (7). Moreover, overstimulation of SPAK-NKCC1 complex activity is well documented to be involved in ischemic brain damage through NKCC1-mediated influx of Na^+ and Cl^- , leading to the blood brain barrier (BBB) damage, cytotoxic edema, and neural excitotoxicity (17, 18). These findings lead us to investigate whether SPAK-NKCC1 cascade in the CPECs is altered in stroke brains and has impact on the blood-CSF barrier function and brain inflammation.

We reported here that ischemic stroke stimulated phosphorylation activation of SPAK-NKCC1 cascade at the apical membrane of CPECs, which was associated with upregulation of NF- κ B, MMP9 and loss of TJs integrity as well as immune cell infiltration in the ChP. H_2O_2 -mediated oxidative stress directly activated the SPAK-NKCC1 pathway in either cultured CPECs or cortical neurons. Blocking of the SPAK-NKCC1 pathway with the novel SPAK inhibitor ZT-1a or NKCC1 inhibitor bumetanide protected ChP against the stroke-induced damage and oxidative stress-induced apoptosis. We concluded that pathological stimulation of the SPAK-NKCC1 cascade causes damages of CPECs, disruption of TJs, and immune cell infiltration of the ChP. Thus, the SPAK-NKCC1 cascade emerges as a therapeutic target for ChP dysfunction and brain inflammation after stroke.

Methods

Chemicals

Hydrogen peroxide (H_2O_2 , Cat# H325) and Calcein AM (Cat# C3100MP) were purchased from Thermo Fisher Scientific (Waltham, MA). Bumetanide (BMT, Cat# B3023) and Propidium Iodide (PI, Cat# P4864) were purchased from Sigma-Aldrich (St. Louis, MO). Free radical scavenger Ebselen (Cat# 70530) was from Cayman Chemical Company (Ann Arbor, MI). Novel SPAK inhibitor ZT-1a was developed as described before (19). Rabbit anti-pSPAK/pOSR1 (pSer383 SPAK/pSer325 OSR1), rabbit anti-SPAK/OSR1, rabbit anti-pNKCC1 (pThr206) were developed by Dr. Yang at Taiwan National University (20, 21), mouse monoclonal anti-NKCC (T4) was from the Developmental Studies Hybridoma Bank (Iowa City, IA). Novel rabbit anti-pT58 antibody was developed as described before (22), which recognizes residue pT58 in $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC) and cross-reacts with a nearly identical phospho-epitope in NKCC; in mouse

NKCC1 this site includes pThr211 NKCC1 (23). Collagenase type IV and terminal deoxy-nucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay kit was purchased from Thermo Scientific (Thermo Scientific, Cat# C10617, Rockford, IL).

Animals

All animal studies were approved by the University of Pittsburgh Medical Center Institutional Animal Care and Use Committee, which adhere to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and reported in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines 2.0 (24). The C57Bl/6J strain mice (male and female, 2-3 month old) used in the study were purchased from Jackson laboratories (Bar Harbor, ME). Mice were housed in a temperature-controlled room on a 12-h light/12-h dark cycle with standard mouse diet and water *ad libitum*.

Ischemic stroke model

Ischemic stroke was induced by transient middle cerebral artery occlusion (tMCAO) and reperfusion as previously described (18). Briefly, mice were anesthetized with 3% isoflurane vaporized in N₂O and O₂ (3:2) for induction and 1.5% isoflurane for maintenance. The left common carotid artery (CCA) was exposed through a midline pre-tracheal incision under an operating microscope. The CCA were isolated and ligated. The external carotid artery (ECA) was dissected further distally and permanently ligated. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve. To occlude the left middle cerebral artery (MCA), a rubber silicon-coated monofilament suture (size 6-0, diameter with coating 0.21 ± 0.02 mm; coating length 5-6 mm) was introduced into the ECA and advanced along the ICA 8–9 mm from the bifurcation of the carotid artery and blocked MCA flow for 60 min. The silk suture around the ECA stump was tightened around the intraluminal rubber suture to prevent bleeding. For reperfusion (Rp), the suture was gently withdrawn to restore blood flow. The sham surgery mice received the same operation, but the monofilament suture was not inserted. Body temperature was maintained for the duration of the experiment between 36.5 ± 0.5°C with a small-animal heating pad. Post-surgery, softened mouse food and water were provided to accelerate recovery.

Drug treatment

Post-stroke mice were randomly assigned to receive either vehicle (Veh, 100% DMSO, 2 ml/kg body weight/day), novel SPAK inhibitor ZT-1a (5 mg/kg body weight/day) or NKCC1 inhibitor bumetanide (BMT, 10 mg/kg body weight/day) via intraperitoneal injection (i.p.), with an initial half-dose administered at 3 h and the second half-dose at 8 h Rp as shown in **Fig. 3a**.

Immunofluorescent staining and image analysis

Mice were anesthetized with 3% isoflurane and transcardially perfused with ice-cold normal saline, followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M PBS as described before (25). Brains were dissected and kept in 4% PFA for 24 h before being transferred to a 30% sucrose solution for

cryoprotection. Coronal brain sections (25- μ m thick, at +0.62 to -0.82 mm posterior from Bregma) were selected and incubated with blocking solution (10% normal goat serum (NGS) + 0.5% Triton X-100 for 1 h at room temperature (RT)) followed by incubation with primary antibodies (**Supplementary Table 1**) in the blocking solution (3% NGS and 0.3 % Triton X-100 in PBS) for overnight at 4°C. On the following day, brain sections were washed three times in PBS and then incubated for 1 h at RT with respective secondary antibodies: goat anti-rabbit Alexa 488-conjugated IgG (1:200), goat anti-rabbit Alexa 546-conjugated IgG (1:200), goat anti-mouse Alexa 546-conjugated IgG (1:200), goat anti-rat Alexa 546 (1:200). After washing for 3 x 10 min, nuclei were stained with DAPI (1:1000, in the blocking solution) for 15 min at RT. Sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For primary antibody negative controls, brain sections were stained with the secondary antibodies only (**Supplementary Fig. 1**). Fluorescent images were captured with Olympus IX81 inverted microscope with a FV1000 laser scanning confocal system using a 40x oil-immersion objective. Identical digital imaging acquisition parameters were used and analyzed by a blinded observer throughout the study. Fluorescence images were quantified with Fiji (NIH) software.

For CPEC cultures, CPECs grown on coverslips were fixed in 4% PFA in PBS for 15 min. After rinsing with PBS, cells were incubated with a blocking solution (10% normal horse serum (NHS), 2% BSA and 0.25% Triton X-100 in 0.1 M PBS) for 1 h at RT followed by incubation with primary antibodies (**Supplementary Table 1**) overnight at 4°C. After rinsing in PBS, slides were incubated with appropriate goat or mouse Alexa fluor 546/488 secondary antibodies (1:200, Invitrogen) for 1 h at RT. Fluorescence images were captured as described above.

Brain infarct volume measurement

Loss of microtubule associated protein 2 (MAP2) as a marker for neurodegeneration was analyzed for infarct volumetric assessment as described before (25). Brain sections at different levels (0.62, 0.14, -0.34, -0.82 mm posterior from Bregma) from each brain were selected. Fluorescent images of MAP2 were captured using a 4x objective with a Nikon Eclipse Ti epifluorescent microscope (Nikon, Tokyo, Japan) and processed with NIS-Elements Advance Research microscope imaging software (version 4.30.02, Nikon). The ischemic area for each brain slice was calculated by subtracting the non-infarct area in the ipsilateral (IL) hemisphere from the total area of the contralateral (CL) hemisphere with Image J.

Immunoblotting

ChP tissue or cultured cells were harvested and incubated in RIPA buffer containing one pill of phosSTOP and 2 mM protease inhibitors as described before (26). Protein concentration was measured with the bicinchoninic acid (BCA) assay kit by using a 96-well microplate reader (Spectra Max 190; Molecular Devices). Protein samples (30 μ g cellular lysates, or 7 μ g ChP tissue homogenates) were boiled in sample buffer (Thermo Scientific, Rockford, IL) for 7 min, resolved by 4-15% sodium dodecyl sulfate polyacrylamide-gel electrophoresis and electrotransferred onto a polyvinylidene difluoride (PVDF) membranes (19, 27). The membranes were blocked in 5% BSA or 7.5% nonfat dry milk in Tris-buffered saline-T (TBS-T, 0.05% Tween-20) for 1 h at RT and then incubated with appropriate primary antibodies

(**Supplementary Table 1**) overnight at 4°C. Blots were then washed six times with TBS-T and incubated with secondary horseradish peroxidase conjugated antibodies (1:1000 or 1:2000) in 5% nonfat dry milk in TBST for 1 h at RT. Protein bands were visualized with ECL agents. The densities of protein bands were analyzed with Image J. Expression of GAPDH or β -actin was used as a protein loading control.

ChP sample preparation and flow cytometric analysis

Mice were euthanized with CO₂ and transcardially perfused with ice-cold PBS (25). Lateral ventricle choroid plexus (LVCP) were carefully isolated under a microscope and placed in 1.5 ml tube with ice-cold PBS. CL or IL ChP were enzymatically digested in collagenase type IV (400 U/ml in PBS, 45 min, 37° C), and then triturated several times using a pipette (28). Cells were stained with antibodies (**Supplementary Table 1**) for 20 min at 4°C in the dark. Samples were acquired using an LSRFortessa flow cytometer (BD Biosciences, USA) equipped with FACS Diva software and a minimum of 30,000 events for each sample was recorded. Data were analyzed using the Flow Jo (BD Biosciences, USA) software.

Primary cultures of CPECs

C57Bl/6J mice (2-3 months old) were deeply anesthetized with 3% isoflurane and transcardially perfused with ice-cold PBS (without Ca²⁺ and Mg²⁺). The brain was removed and immediately submerged in the ice-cold PBS. The ChP tissues (both lateral ventricles, third ventricle and fourth ventricle) were dissected out under a microscope and washed with ice-cold HBSS. The ChP tissues were incubated with trypsin (0.25 mg/mL) at 37°C for 20 mins and cells were dissociated by pipetting. The cell suspension was washed in culture medium (DMEM/F12) supplemented with 10% heat-inactivated FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, 5 μ g/mL insulin, 20 μ M Ara-C, 5 ng/mL sodium selenite, and 10 ng/mL human EGF as described before (29). The cells were plated on a glass coverslips (coated with poly-L-lysine, 24-well plate, $\sim 2 \times 10^5$ cells/well) and cultured at 37°C, 5% CO₂, and refeed with the fresh media on the following day. CPEC cultures for 5-6 days *in vitro* (DIV) were used in this study.

Primary cortical neuron cultures

Embryonic day 14 to 18 pregnant mice (C57Bl/6J background) were euthanized with CO₂ with a secondary method of euthanasia by cervical dislocation as described previously (30). Fetuses were removed and decapitated. Using a dissection microscope, the cortices were extracted from the brain while submerged in ice-cold HBSS and treated with 0.125 mg/ml trypsin at 37°C for 20 mins. Once the trypsin was neutralized with normal DMEM, the cells were centrifuged at 1200 RPM for 5 mins at 20°C. The cells were washed once in normal DMEM and centrifuged. The cells were then suspended in neurobasal medium containing B-27 supplement (2%), 100U/mL GlutaMAX and 0.1mg/mL penicillin/ streptomycin. The cells were seeded in 6/24-well plates (5×10^5 or 1.5×10^5 cells/well) or on glass coverslips (coated with PDL) and incubated at 37°C in an incubator with 5% CO₂ and atmospheric air. Neuronal cultures 7 to 10 DIV were used in this study.

***In vitro* oxidative stress induction and drug treatment**

Cultured CPECs in 24-well plates were treated with either the normal medium, H_2O_2 (200 μM), H_2O_2 (200 μM) + Ebselen (1 μM), H_2O_2 (200 μM) + ZT-1a (1 μM), or H_2O_2 (200 μM) + BMT (10 μM) for 24 h. For cultured neurons, cells were treated with either the normal medium, H_2O_2 (20 μM), H_2O_2 (20 μM) + Ebselen (100 nM), H_2O_2 (20 μM) + ZT-1a (1 μM), or H_2O_2 (20 μM) + BMT (10 μM) for 24 h. Cultures were collected for imaging or immunoblot experiments.

TUNEL assay of cultured CPECs

The CPEC cultures were fixed in 4% PFA in PBS for 15 min. Oxidative stress-induced apoptotic cell death was detected by TUNEL staining according to the manufacturer's instructions. The double-labeled cells were imaged with Olympus IX81 inverted microscope with a FV1000 laser scanning confocal system under 40x objective. Four independent areas were quantified from each coverslip and apoptotic cell death rate in each experiment was expressed as the ratio of TUNEL⁺/Cytokeratin⁺ cells and Cytokeratin⁺/DAPI⁺ cells.

Measurement of neuronal survival

Cell viability of cultured neurons was assessed by retention of Calcein-AM (live) and Propidium Iodide (PI) uptake (damaged) cells as described previously (31). Under the Nikon TiE inverted epifluorescence microscope, at least four independent areas at 20x magnification were quantified from each coverslip with ImageJ (18). Cell viability was expressed as the ratio of calcein⁺ neurons to the sum of calcein⁺ and PI⁺ neurons.

Cellular rubidium (Rb^+) influx and intracellular Na^+ concentration measurement

After $\text{H}_2\text{O}_2 \pm$ drug treatment, neuronal cells were rinsed with an isotonic Rb^+ free wash buffer (310 mOsm, containing 134 mM NaCl, 2 mM CaCl_2 , 0.8 mM NaH_2PO_4 , 5 mM glucose, 25 mM HEPES and 1.66 mM MgSO_4 , PH 7.4) as described before (26). Cells were exposed to isotonic buffer (310 mOsm, containing 5.36 mM Rb^+) solutions with or without BMT (10 μM) for 10 min at 37°C. To terminate Rb^+ influx, cells were washed with wash buffer (Rb^+ free) and lysed with 0.15% SDS (200 ml/well) to release the intracellular Rb^+ . The intracellular Rb^+ concentration in cell lysates were measured using an automated atomic absorption spectrophotometer (Ion Channel Reader, ICR-8000; Aurora Biomed, Vancouver, Canada). Total protein of cell lysates was measured by BCA assay. NKCC1-mediated Rb^+ influx assay was determined by subtracting Rb^+ influx value in the presence of BMT from one in the absence of the BMT. Rb^+ influx rate was calculated and presented as mg Rb^+ /mg protein/min.

Intracellular Na^+ concentration ($[\text{Na}^+]_i$) was measured with the fluorescent dye SBFI/AM as described previously with some modifications (32). Cells grown on coverslips were loaded with 30 μM SBFI/AM added with 0.02% pluronic acid. The coverslips were placed in the open bath imaging chamber and

incubated with HEPES-MEM buffer at 37 °C. The Nikon Ti Eclipse inverted epifluorescence microscope and a ×40 oil immersion lens was used. The samples were excited at 340 and 380 nm and the ratios of 340/380 were analyzed with the MetaFluor image-processing software. Absolute $[Na^+]_i$ was detected for each cell by performing an *in situ* calibration as described previously (33).

Statistical analyses

A total of 130 C57Bl/6J male and female mice were used in this study. All data assessments were performed by an investigator who was blind to treatment groups and/or experimental group assignments. Student's two-tailed t-test was used to compare between two experimental groups. For three or more groups, one-way ANOVA were conducted for multiple comparisons. *N* values represent the number of independent experiments. Data were expressed as mean and standard deviation (SD). A $p < 0.05$ was considered statistically significant. Data was graphed using GraphPad Prism 8 (GraphPad Software, Inc., CA, USA).

Results

Ischemic stroke triggered phosphorylated activation of the SPAK–NKCC1 cascade pathway in the ChP.

We assessed changes of SPAK-NKCC1 complex expression in the lateral ventricle choroid plexus (LVCP) in Sham or stroke brains at 24 h after ischemic Rp (**Fig. 1a**), a peak time for stroke-induced ChP damage (34). In the Sham brains, abundant SPAK and NKCC1 proteins were detected in the apical (luminal) membranes of CPECs (arrowheads, **Fig. 1b**), but rarely in the basolateral membrane, consistent with previous literature reports (12, 35). Ischemic stroke did not significantly alter the expression of SPAK or NKCC1 proteins in the non-lesion contralateral (CL) LVCP ($p > 0.05$, **Fig. 1b** and **c**). In the case of the phosphorylated protein species SPAK (pSPAK, Ser373) and NKCC1 (pNKCC1, Thr212/Thr217), Sham brains showed low expression of both proteins as reported before (7), particularly at the apical membrane of CPECs. In contrast, ischemic stroke triggered upregulation of pSPAK and pNKCC1 expression at the apical membranes of CPECs in both hemispheres of stroke brains (arrows, **Fig. 1d** and **e**, $p < 0.05$). Elevation of pNKCC1 was further validated with a different antibody (pT58 NCC, which cross-reacts with the NKCC1 pThr211 phosphorylation site, **Supplementary Fig. 2a**). These findings demonstrated that ischemic stroke altered SPAK–NKCC1 complex at the ChP by increasing their phosphorylatory stimulation as well as increasing the polarized protein trafficking to the apical membrane. These changes may subsequently impact the ChP blood-CSF barrier structures and function.

Stroke-induced ChP TJ damage is correlated with phosphorylatory stimulation of SPAK-NKCC1 signaling cascade.

We then examined whether upregulation of the SPAK-NKCC1 signaling pathway in CPECs is associated with changes in the TJ integrity in ischemic brains. As shown in **Fig. 2a**, abundant TJ proteins Claudin-5 (red) and ZO-1 (green) were detected in the healthy ChP from Sham brains (arrowheads). In contrast, stroke brains displayed pronounced reduction of Claudin-5 protein expression (arrows) in both LVCP ($p <$

0.01) with no significant loss of ZO-1 protein expression (arrowheads, **Fig. 2a - c**, $p > 0.05$). We next conducted Pearson's correlation analysis to evaluate the relationships between stroke-induced activation of SPAK-NKCC1 cascade and changes of Claudin-5 protein. We found that the fluorescence intensity of pSPAK ($r = -0.413$, $p < 0.05$) and pNKCC1 ($r = -0.479$, $p < 0.05$) was negatively correlated with Claudin-5 expression fluorescence intensity in ChP (**Fig. 2d**). Taken together, these findings suggest that activation of SPAK-NKCC1 cascade pathway is closely associated with TJ damage in ChP after ischemic stroke.

Pharmacological inhibition of SPAK-NKCC1 cascade reduced stroke-induced infarct volume and ChP damage.

Our recent study shows that pharmacological inhibition of SPAK protein activity with its inhibitor ZT-1a attenuated ischemic stroke brain injury or ChP CSF hypersecretion in experimental hemorrhagic hydrocephalus (19). In addition, inhibition of the ChP NKCC1 with its inhibitor bumetanide (BMT) reduced traumatic brain injury (TBI)-induced brain edema, cerebral contusion, and neuronal death (36). **Fig. 3a, b and c** demonstrated that, in comparison to the Veh-treated stroke control group, systematic administration of SPAK inhibitor ZT-1a or NKCC1 inhibitor BMT significantly decreased total infarct volume ($\sim 30\%$ or 27% respectively, $p < 0.05$), and cortical infarct volume by $\sim 42\%$ or 40% , respectively ($p < 0.05$). We then examined efficacy of ZT-1a or BMT on decreasing stroke-induced damage of ChP. As shown in **Fig. 3d and e**, in the Veh-treated stroke control brains, expression of pSPAK and pNKCC1 at the IL LVCP was significantly elevated (arrows, $p < 0.05$). In contrast, ZT-1a, but not BMT, decreased expression of pSPAK protein by $\sim 54\%$ (arrowhead, **Fig. 3d and e, top panel**, $p < 0.001$). ZT-1a or BMT treatment also significantly reduced expression of pNKCC1 in the IL LVCP by $\sim 50\%$ or $\sim 36\%$, respectively (**Fig. 3d and e, bottom panel**, $p < 0.001$). Consistent with our immunofluorescent staining, quantification of pSPAK and pNKCC1 expression via immunoblotting analysis clearly shows that stroke-induced elevation of pSPAK and pNKCC1 expression in the ChP was blocked by ZT-1a or BMT treatment (**Fig. 3f and g**, $p < 0.05$). Similar patterns of changes were detected in CL LVCP (**Supplementary Fig. 2b - e**). Moreover, both drug treatments restored the SPAK protein expression in the apical membrane of IL LVCP (**Supplementary Fig. 3a and c**). No significant changes of SPAK or NKCC1 expression among the three groups were detected via immunoblotting (**Fig. 3f and g**, $p > 0.05$) or immunofluorescent staining (**Supplementary Fig. 3**). These findings clearly demonstrate that pharmacological blockade of the SPAK-NKCC1 complex prevented stroke-induced phosphorylatory activation of the complex in the ChP.

Pharmacological inhibition of SPAK-NKCC1 complex preserved ChP blood-CSF barrier integrity.

We subsequently investigated whether pharmacological inhibition of SPAK-NKCC1 complex attenuates stroke-induced ChP blood-CSF barrier dysfunction. As shown in **Fig. 4a** (left panel), in comparison to the Veh stroke brains, ZT-1a-treated stroke brains exhibited preservation of Claudin-5 protein expression in the ChP (**Fig. 4a and b**, $p < 0.05$). This contrasts with the reduced Claudin-5 protein expression detected in the Veh-control stroke or the BMT-treated stroke ChP (**Fig. 4a and b**, $p < 0.05$). Consistent with the findings in **Fig. 2**, ischemic stroke did not cause detectable changes in ChP ZO-1 protein expression in all groups (**Fig. 4a and c**, $p > 0.05$). Since epithelial cells express Claudin-1 to a higher degree in comparison to

Claudin-5 (37), we further assessed changes of Claudin-1 expression in ChP in relationship to a CPECs marker protein cytokeratin. **Fig. 4b** (right panel) displayed abundant Claudin-1 expression in the Sham brain CPECs. The Veh-treated stroke mice exhibited loss of Claudin-1 expression in IL LVCP but pharmacological inhibition of the SPAK-NKCC1 complex prevented down-regulation of Claudin-1 expression (**Fig. 4a** and **d**). It was reported that pro-inflammatory cytokine-induced activation of matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) mediated stimulation of the NF- κ B signaling are involved in TJ degradation at the BBB and vasogenic edema in stroke brains (38, 39). We then determined whether these signaling mechanisms play a role in stroke-mediated damage at the ChP-CSF barrier. The ChP isolated from the Veh-treated stroke brains showed upregulated expression of the activated NF- κ B (pNF- κ B p65) by $\sim 50\%$, compared to the Sham ChP (**Fig. 4e** and **f**, $p < 0.0001$). There were no changes in the total NF- κ B protein expression (**Fig. 4e** and **f**, $p > 0.05$). Interestingly, SPAK inhibitor ZT-1a treatment, but not NKCC1 inhibitor BMT, blocked NF- κ B activation (**Fig. 4e** and **f**, $p < 0.0001$). Moreover, compared to the Sham ChP, a significant increase in the expression of MMP9 protein was detected in the Veh-treated stroke ChP (**Fig. 4e** and **f**, $p < 0.001$). Pharmacological inhibition of SPAK-NKCC1 complex with ZT-1a or BMT reduced MMP9 protein expression ($\sim 32\%$ or by $\sim 42\%$, respectively, **Fig. 4e** and **f**, $p < 0.05$). Moreover, CL LVCP exhibited similar changes under above conditions (**Supplementary Fig. 4**). Together, this data suggests that NF- κ B and MMP9 signaling pathways are activated in ChP after stroke and play a role in damaging the blood-CSF barrier. Blocking SPAK-NKCC1 complex attenuated NF- κ B and MMP9 signaling activation and ChP TJ degradation.

Pharmacological inhibition of SPAK-NKCC1 complex reduced immune cell infiltration in the ChP after stroke.

ChP regulates immune cells infiltration to the CNS (28) and is a route for lymphocyte invasion into the brains after acute ischemic stroke and neuroinflammatory responses (11). We next assessed whether pharmacological inhibition of SPAK-NKCC1 cascade has an impact on circulating leucocyte infiltration in the ChP. With the flow cytometric analysis of the immune cells in the isolated ChP at day 3 post stroke, CD11b⁺CD45^{lo} myeloid cells were unchanged between Sham, Veh-treated or ZT-1a-treated ChP, while the number of CD11b⁺CD45^{hi} myeloid cells increased by 65% in the Veh-treated ChP, compared to the Sham ChP (**Fig. 5a**, $p < 0.01$). ZT-1a treatment reduced the number of CD11b⁺CD45^{hi} cells by 50% (**Fig. 5a**, $p < 0.05$). CD11b⁺CD45⁺Ly6G⁻Ly6C^{hi} inflammatory cells were significantly increased in the Veh-control ChP (**Fig. 5b**, $p < 0.05$). However, ZT-1a-treated mice displayed reduced infiltration of CD11b⁺CD45⁺Ly6G⁻Ly6C^{hi} inflammatory cells in the ChP (**Fig. 5b**, $p < 0.05$). In addition, the CD206⁺ and Ym-1⁺ anti-inflammatory myeloid cells were increased by 20% in the ZT-1a-treated ChP, compared to the Veh-control ChP (**Fig. 5c**, $p < 0.05$). A 2.8-fold increase of CD11b⁺CD45⁺/Ly6G⁺ neutrophils and a 2-fold increase in CD3⁺ T cells were detected in the IL ChP of the Veh-treated stroke mice, compared to the Sham ChP (**Fig. 5d** and **e**, $p < 0.01$). ZT-1a-treated mice displayed reduced infiltration of neutrophils and CD3⁺ T cells by 65% and 47%, respectively (**Fig. 5d** and **e**, $p < 0.05$). Similar flow cytometry results were observed in CL LVCP tissues (**Supplementary Fig. 5a - e**). Moreover, the cytotoxic CD8⁺ T cells and Iba1⁺ microglia were increased in the LVCP in stroke Veh-control brains at 24 h Rp, compared with Sham brain (**Fig. 5f** and **g**, p

< 0.01). Pharmacological inhibition of SPAK-NKCC1 cascade suppressed CD8⁺ T and Iba1⁺ infiltration (**Fig.5f** and **g**, $p < 0.05$), consistent with the preserved ChP blood-CSF barrier integrity shown in **Fig.4**. CL LVCP exhibited similar changes of CD8⁺ cells under above conditions (**Supplementary Fig. 5f** and **g**). Taken together, these data further shows that dysregulation of SPAK-NKCC1 complex plays a role in the ChP blood-CSF barrier integrity and immune cell infiltration.

Oxidative stress is a mechanism in stimulating SPAK-NKCC1 cascade and induces apoptosis of cultured CPECs.

The underlying mechanisms in which ischemic stroke triggers activation of SPAK-NKCC1 complex in ChP remains undefined. It was reported that ROS stimulate SPAK phosphorylation and causes claudin-18 disruption in alveolar epithelium cell following hyperoxia insult (40). To determine whether the ROS-mediated mechanism upregulates SPAK-NKCC1 cascade phosphorylation activity in ChP, we established an *in vitro* model of primary cultures of CPECs which expressed abundant epithelial cell cytoskeletal protein cytokeratin as well as TJ ZO-1(**Fig. 6b**). To mimic oxidative stress, we exposed CPECs to H₂O₂ for 24 h and measured H₂O₂-incuded activation of SPAK-NKCC1 signaling pathway with immunostaining (**Fig. 6a**). H₂O₂-treated CPECs significantly stimulated SPAK-NKCC1 phosphorylation activation, but the CPECs treated with H₂O₂ plus the ROS scavenger Ebselen for 24 h failed to activate SPAK-NKCC1 signaling (**Fig. 6c – e**, $p < 0.01$). Exposing CPECs to H₂O₂ combined with ZT-1a, but not BMT, downregulated pSPAK protein expression (**Fig. 6c** and **d**, $p < 0.01$). Moreover, H₂O₂ plus ZT-1a or BMT suppressed oxidative stress-induced pNKCC1 protein expression (**Fig. 6c** and **e**, $p < 0.05$). We next assessed weather oxidative stress induced TJ ZO-1 damage, as shown in **Fig. 6f**, there were no differences in ZO-1 staining intensity in the control and H₂O₂-treated CPECs, which is consistent with our *in vivo* data. Moreover, H₂O₂-treated CPECs significantly increased TUNEL⁺ apoptotic cell counts (**Fig. 6g** and **h**, $p < 0.001$). In contrast, exposing CPEC cultures to ZT-1a or BMT in combination with H₂O₂ failed to induce apoptotic death, which is similar to H₂O₂ scavenger Ebselen effects (**Fig. 6g** and **h**, $p < 0.01$). In summary, oxidative stress activated the SPAK-NKCC1 complex and induced cell apoptosis in cultured CPECs.

Pharmacological inhibition of the SPAK-NKCC1 signaling pathway reduced H₂O₂-mediated ion influx and neuronal death.

To further strengthen our conclusion, we also assessed whether the same mechanism of H₂O₂-mediated activation of the SPAK-NKCC1 signaling pathway occurs in primary cortical neurons (**Fig. 7a**). In the control conditions, the majority of living cortical neurons stained with calcein-AM (green) and less PI (red signal, **Fig. 7b**). H₂O₂ reduced the survival rate to 75%, significantly lower than the control group (**Fig. 7b** and **c**, $p < 0.001$). However, in the presence of H₂O₂ and Ebselen, the oxidative stress-induced neuronal death was prevented, and the survival rate was maintained at 98% (**Fig. 7b** and **c**, $p < 0.01$).H₂O₂ plus ZT-1a or BMT failed to trigger H₂O₂-mediated neuronal death (**Fig. 7b** and **c**, $p < 0.01$). These results indicate that pharmacological inhibition of the SPAK-NKCC1 signaling pathway with ZT-1a or BMT is also

protective against H₂O₂-induced neuronal death. We next examined the intracellular Na⁺ concentration ([Na⁺]_i) and the impact of elevated NKCC1-mediated Rb⁺ (K⁺) influx in neurons in response to H₂O₂ insult. As predicted, exposure of neurons to H₂O₂ for 24 h significantly increased [Na⁺]_i by ~ 2.9 fold (from 10.2 ± 4.4 mM to 28.1 ± 1.3 mM, **Fig. 7d**, *p* < 0.001). In contrast, inhibition of SPAK with ZT-1a and NKCC1 with BMT prevented H₂O₂-mediated rise in [Na⁺]_i (9.9 ± 4.1 mM and 14.5 ± 5.4 mM, respectively, **Fig. 7d**, *p* < 0.01). Similarly, H₂O₂ scavenger Ebselen treatment also significantly prevented the H₂O₂-mediated rise in [Na⁺]_i by ~ 48 % (**Fig. 7d**, *p* < 0.01). In addition, exposure of neurons to H₂O₂ for 24 h significantly increased the total Rb⁺ and NKCC1-mediated Rb⁺ influx (determined in the presence of 10 μM BMT during Rb⁺ assay) by ~23% and by ~ 41%, respectively (**Fig. e** and **f**, *p* < 0.01) in isotonic conditions (310 mOsm). H₂O₂ treatment in the presence of Ebselen for 24 h failed to stimulate total or NKCC1-mediated Rb⁺ influx (**Fig. 7e** and **f**, *p* < 0.05). We further assessed whether blockade of SPAK-NKCC1 signaling pathway activity with ZT-1a or BMT would prevent H₂O₂-mediated stimulation of the Rb⁺ influx in neuronal cells. As **Fig. 7e** and **f** showed that H₂O₂ in combination with ZT-1a or BMT effectively decreased the total Rb⁺ (by ~ 20% and ~ 23%, respectively, *p* < 0.05) as well as NKCC1-mediated Rb⁺ influx (by ~ 51% and ~ 58%, respectively, *p* < 0.01). Finally, we assessed protein expression changes with immunoblotting to prove that H₂O₂ stress indeed triggered SPAK-NKCC1 activation. As shown in **Fig. 7g** and **h**, H₂O₂ exposure triggered significant increase in expression of pSPAK/SPAK and pNKCC1/NKCC1 protein in neurons, compared to the control group (*p* < 0.01). The H₂O₂-mediated activation of SPAK-NKCC1 cascade pathway is indeed mediated by oxidative stress, since concurrently exposing neurons to H₂O₂ and Ebselen failed to trigger SPAK-NKCC1 signaling pathway activation and displayed no changes from control (**Fig. 7g** and **h**). Compared to the H₂O₂ insult group, exposure of neurons to H₂O₂ combined with ZT-1a (1 mM) significantly decreased H₂O₂-mediated stimulation expression of pSPAK/pNKCC1 protein as well as upregulation of SPAK protein (**Fig. 7i** and **j**, *p* < 0.05). In contrast, exposure of neurons to H₂O₂ in combination with BMT (10 mM) only decreased expression of pNKCC1 protein (**Fig. 7i** and **j**, *p* < 0.01) and has no effect on upstream SPAK proteins compared to the H₂O₂ treated group. Taken together, these data showing that H₂O₂ not only induced phosphorylation activation of the SPAK-NKCC1 signaling complex, NKCC1-mediated K⁺ and Na⁺ influx, but also upregulated SPAK and NKCC1 protein expression in cultured neurons, which collectively contributed to neuronal death.

Discussion

Significance of SPAK-NKCC1 complex proteins in ChP structure and function.

The ChP being formed by cuboidal epithelial cells and surrounding a core of fenestrate capillaries are involved in the production of CSF (41), which plays a prominent role in regulation of brain development, neuronal function, nutrient transport and removal of metabolites (10, 12). The specialized CPECs, interconnected by the TJs at the basement membrane, form the blood-CSF barrier between the capillaries and the CSF (42). The NKCC1, expressed in the apical membrane of CPECs, transports salt and water,

contributing approximately half of the CSF production (12). SPAK, a master regulator of epithelial ion transporters and channels, is highly expressed and localized to the apical membrane of the CPECs (7, 35). Phosphorylation of SPAK-NKCC1 cascade at the CPECs apical membrane results in hyperactive CSF secretion following intraventricular hemorrhage (7). Stimulation of NKCC1 at the luminal membrane of BBB endothelial cells has also been reported to aggravate the initial edema formation and BBB disruption after ischemic stroke or TBI (43, 44). Pharmacological inhibition or a genetic deficiency of NKCC1 decreases BBB breakdown, brain edema and retains TJ protein integrity (43), but the role of NKCC1 in the ChP blood-CSF barrier after ischemic stroke has received little attention. In this study, we showed that both the SPAK and NKCC1 proteins were phosphorylated, activated at the apical membrane of the CPECs at 24 h Rp after stroke. In addition, activation of the SPAK-NKCC1 cascade was correlated with the ChP blood-CSF barrier damage and dysfunction. Our new findings in this study demonstrate that stroke-induced activation of the SPAK-NKCC1 complex at the ChP is associated with barrier dysfunction, immune cell infiltration, activation of NF- κ B and MMP9 signaling at the ChP.

Pharmacological blockade of SPAK-NKCC1 complex is protective of ChP after ischemic stroke.

SPAK can associate with the (R/K)FX(V/I) motif in the NKCC1 and phosphorylate its N terminus at Thr203, Thr207, and Thr212 amino acids, which play an important role in inflammation (7, 45). SPAK integrates and transduces environmental stress signals, including NF- κ B-dependent inflammatory signals (7). The 5'-flanking region of the SPAK gene contains two transcriptional start sites, three transcription factor Sp1-binding sites, and one transcription factor NF- κ B-binding site (45). siRNA-mediated knockdown of NF- κ B reduced SPAK protein expression in human intestinal cell line Caco2-BBE (45). Increased expression of MMP-9 through the NF- κ B signaling pathway occurred in angiostrongyliasis meningoencephalitis, leading to Claudin-5 degradation and alteration of the blood-CSF barrier permeability (46). We speculate that increased SPAK activation may exacerbate the inflammatory environment and damage of Claudin-1 and Claudin-5. In our study, we detected increased NF- κ B pathway activation and MMP9 protein expression in ChP from ischemic stroke brains, which likely contributes to damage to the ChP barrier integrity. Both the SPAK inhibitor ZT-1a and the well-established NKCC1 inhibitor BMT blocked SPAK and NKCC1 activation reduced MMP9 protein expression and exhibited preservation of TJ Claudin-1 and Claudin-5 in the ChP, suggesting that inhibition of SPAK with ZT-1a may suppress its upstream NF- κ B activation. This is supported by findings that BMT treatment or NKCC1 deficiency suppressed upregulation of MMP9 expression and prevented the BBB TJ protein degradation in TBI (43).

ChP SPAK-NKCC1 activation and immune cell infiltration in ischemic stroke brain.

The ChP structure is much simpler than BBB and as a gateway for leukocyte infiltrate into the CNS (1). Reducing blood supply to the LVCP caused the blood-CSF barrier to be disrupted as indicated by the increased [3 H]-inulin entry into CSF and hippocampus (42). Immune cells from the peripheral blood while under the influence of chemokines undergo adhesion, rolling, and diapedesis across the fenestrated capillary endothelium and pia mater of the ChP (47). The invasion of lymphocytes through the ChP is a

critical aspect of neuroinflammation following ischemic stroke, particularly due to its role in affecting initial infarct volume and thus the eventual poor clinical outcome (11). In this study, we detected that pathological stimulation of the SPAK-NKCC1 cascade after ischemic stroke causes ChP blood-CSF barrier integrity dysfunction and elevated infiltration of T cells, CD11b⁺CD45⁺/Ly6G⁺ neutrophils and CD11b⁺CD45^{hi} myeloid cells into both the CL and IL ChP, contributing to neuroinflammation. Inhibition of SPAK-NKCC1 complex not only preserved the barrier integrity, but also attenuated immune cell infiltration, emerging SPAK-NKCC1 signaling cascade as a therapeutic target for attenuating ChP dysfunction and brain inflammation after stroke.

H₂O₂ stimulates expression and activation of SPAK-NKCC1 signaling pathway in CPECs and neurons.

Free radical and ROS play important roles in ischemic reperfusion injury. Oxidative damage led to disruption of the blood-CSF barrier in sepsis-associated encephalopathy (48). The underlying cellular and molecular mechanisms are not completely understood. A recent study shows that ROS-mediated upregulation of pSPAK in alveolar epithelium cells is linked to loss of TJ Claudin-18 protein, knockdown of SPAK preserved the alveolar epithelium barrier integrity after hyperoxic stress (40). Moreover, manganese, oxidants and NO donors have been reported to increase the oxidation and nitration of NKCC1 protein and upregulation of total and phosphorylated NKCC1 protein expression in cultured astrocytes (49). Those research findings suggest that oxidative stress plays an important role in regulating SPAK-NKCC1 complex expression and post-translational modulation. However, it is unknown whether stimulation of SPAK-NKCC1 complex in the CPECs or neurons is regulated by oxidative stress. Now, we provided direct evidence that H₂O₂-mediated activation of the SPAK-NKCC1 signaling pathway stimulated NKCC1-mediated K⁺ and Na⁺ influx and cell damage. The oxidative stress-mediated changes can be blocked by Ebselen, which is an organoselenium compound with glutathione peroxidase (GPx)-like, thiol-dependent, hydroperoxide reducing activity (50). In addition, Ebselen showed neuron protective effects in our study, which is consistent with previous reports about its antioxidant properties (51, 52). Both ZT-1a and BMT alleviated damages induced by H₂O₂ in both CPECs and neurons. Together, ROS trigger SPAK-NKCC1 activation which led to intracellular ion dysregulation as well as apoptosis cell death in both CPECs and neurons.

Conclusions

The integrity of ChP blood-CSF barrier plays a crucial role in maintaining CNS homeostasis. Stroke-induced damage of CPECs and TJ structures alter the ChP blood-CSF barrier function and the CNS neuroinflammation responses. As shown in **Fig. 8**, we report here that the stroke triggered stimulation of the SPAK-NKCC1 complex, NF-κB cascade as well as MMP9 protein expression in the CPECs, which is associated with degradation of TJ proteins and dysfunction of the ChP blood-CSF barrier. H₂O₂-mediated oxidative stress directly stimulated the activation of the SPAK-NKCC1 complex through protein phosphorylation in cultured CPECs and caused apoptosis. These findings suggest that stroke-induced stimulation of the ChP SPAK-NKCC1 cascade is in part mediated by oxidative stress. Pharmacological

blockade of the SPAK-NKCC1 cascade has therapeutic potential for protection of the ChP blood-CSF barrier integrity and reducing CPEC damage as well as immune cell infiltration.

Abbreviations

ChP	choroid plexus
CSF	cerebrospinal fluid
NKCC1	Na ⁺ -K ⁺ -Cl ⁻ cotransporter 1
SPAK	Ste20-related proline-alanine-rich kinase
TJs	tight junction proteins
CPECs	choroid plexus epithelium cells
NF-κB	nuclear factor kappa B
MMP9	Matrix metalloproteinase 9
CNS	central nervous system
AD	Alzheimer's disease
WNK	Kinase with no lysine (K)
OSR1	oxidative stress-responsive kinase-1
TLR4	toll-like receptor 4
BBB	blood brain barrier
H ₂ O ₂	hydrogen peroxide
BMT	bumetanide
PI	Propidium Iodide
TUNEL	terminal deoxy-nucleotidyl-transferase-mediated dUTP nick-end labeling
tMCAO	transient middle cerebral artery occlusion
CCA	common carotid artery
ECA	external carotid artery

ICA	internal carotid artery
MCA	middle cerebral artery
Rp	Reperfusion
i.p.	intraperitoneal injection
MAP2	Microtubule associated protein 2
CL	contralateral
IL	ipsilateral
PFA	paraformaldehyde
NGS	normal goat serum
RT	room temperature
DIV	days in vitro
LVCP	lateral ventricle choroid plexus
TBI	Traumatic brain injury
ROS	reactive oxygen species

Declarations

Ethics approval and consent to participate

All animal studies were approved by the University of Pittsburgh Medical Center Institutional Animal Care and Use Committee (IACUC), which adhere to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

Supporting data and information about used material are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported in part by the University of Pittsburgh Center for Research Computing through the resources provided, National Institutes of Health grant R01 DK119252 (A.R.S.), UPMC Endowed Chair professorship for Brain Disorders Research (D.S.), VA I01BX002891 (D.S.) and VA Research Career Scientist award IK6 BX005647-01 (D.S.).

Authors' contributions

D.S. and Y.Y. designed and supervised the project; J.W., R.L., M.N.H, V.M.F., G.B., M.I.H.B., S.D. and E.L. performed research and S.F., M.C., Y.C., performed data analysis; J.W., M.C., S.F., V.M.F., S.D., K.T.K., J.Z., X.D., A.R.S., Y.Y. and D.S. wrote, edited, and proofread the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

Not applicable.

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Figures

Figure 1

Stroke-induced phosphorylated activation of SPAK-NKCC1 complex in the choroid plexus.

a. Experimental protocol. Changes of SPAK-NKCC1 protein complex in the lateral ventricle choroid plexus (LVCP) from Sham or tMCAO mice were assessed by immunostaining. Representative images of contralateral (CL) and ipsilateral (IL) LVCP stained for the NKCC1 protein was shown. **b.** Representative

confocal fluorescence images of SPAK and NKCC1 expression. Arrowheads: apical membrane expression of SPAK or NKCC1 protein. Arrows: reduced apical SPAK expression. **c.** Quantification summary. Data are mean $\pm$ SD (n = 8). **d.** Representative confocal fluorescence images of the phosphorylated species (active) of pSPAK and pNKCC1 protein. Arrowheads: low level of pSPAK and pNKCC1 in Sham groups. Arrows: elevated pSPAK or pNKCC1 expression in the post-stroke ChP. **e.** Quantification summary. Data are mean $\pm$ SD (n = 8). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. One-way ANOVA.

Figure 2

Stroke-induced reduction of choroid plexus tight junction (TJ) proteins.

a. Representative confocal fluorescence images of tight junction Claudin-5 and ZO-1 protein expression in the LVCP of Sham and tMCAO brains. Arrowheads: preserved Claudin-5 and ZO-1 expression. Arrows: reduced Claudin-5 expression in stroke brains. **b** and **c.** Quantification summary data. Data are mean $\pm$ SD

(n = 8). ** $p < 0.01$, **** $p < 0.0001$. One-way ANOVA. **d.** Pearson correlation between Claudin-5 and pSPAK or pNKCC1 fluorescence intensity in ChP. n = 24.

Figure 3

Pharmacological suppression of SPAK-NKCC1 complex phosphorylation in choroid plexus after ischemic stroke.

a. Experimental protocol and timepoint. Mice were subjected to tMCAO and randomly assigned to vehicle (Veh), ZT-1a or bumetanide (BMT) treatment group administered via intraperitoneal injection (i.p.) with a half dose at 3 h and 8 h post-reperfusion (Rp), respectively. **b.** Representative infarct images with MAP2 expression staining at 24 Rp. **c.** Infarct volume quantification summary. Data are mean $\pm$ SD (n = 6, 4 male, 2 female). $*p < 0.05$. One-way ANOVA. **d.** Representative immunofluorescent images of pSPAK and pNKCC1 staining in the Sham or ipsilateral (IL) LVCP post-stroke brains. Arrowheads: low level expression of pSPAK and pNKCC1. Arrows: elevated pSPAK and pNKCC1 expression. **e.** Quantification summary. Data are mean $\pm$ SD (n = 6, 4 male, 2 female). $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. One-way ANOVA. **f.** Western blot analysis of SPAK-NKCC1 cascade expression in the ChP of Sham, stroke Veh-control, ZT-1a- or BMT-treated mice. ChP tissue lysates were prepared and subjected to immunoblotting with the indicated antibodies. **g.** Immunoblot summary in ChP. Data are mean $\pm$ SD (n = 4, 2 male, 2 female). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One-way ANOVA.

Figure 4

Pharmacological suppression of SPAK-NKCC1 complex increased choroid plexus tight junction integrity after ischemic stroke.

a. Representative confocal images of tight junction proteins (Claudin-5, ZO-1, and Claudin-1) as well as the epithelial marker cytokeratin in the IL LVCP in Sham, stroke Veh-control, ZT-1a- or BMT-treated mice at

24 h Rp. Arrowheads: expression of Claudin-5, ZO-1 and Claudin-1. Arrows: Low level expression of Claudin-5 and Claudin-1. **b - d.** Summary data of Claudin-5, ZO-1 and Claudin-1 fluorescence intensity. Data are mean $\pm$ SD (n = 6, 4 male, 2 female). * p < 0.05, ** p < 0.01, *** p < 0.001. One-way ANOVA. **e.** Western blot analysis of pNF- κ b p65, NF- κ B p65 and MMP9 protein expression in the IL LVCP of Sham, stroke Veh-control, ZT-1a- or BMT-treated mice at 24 h Rp. ChP tissue lysates were prepared and subjected to immunoblotting with the indicated antibodies. **f.** Immunoblot quantitation. Data are mean $\pm$ SD (n = 4, 2 male, 2 female). * p < 0.05, *** p < 0.001, **** p < 0.0001. One-way ANOVA.

Figure 5

Pharmacological inhibition of SPAK-NKCC1 signaling reduced infiltration of the immune cell in ChP.

a. Representative flow cytometric plots of CD11b⁺CD45^{lo} or CD11b⁺CD45^{hi} myeloid cells from the isolated IL ChP at 3 d post-surgery with quantification of total number of CD11b⁺CD45^{lo} or CD11b⁺CD45^{hi} myeloid cells in the ChP. **b** and **c.** Percentage of CD11b⁺CD45⁺Ly6G⁻Ly6C^{hi} and CD206⁺

and Ym-1⁺ cells gated within CD11b⁺CD45⁺ cells. **d.** Total number of CD11b⁺CD45⁺Ly6G⁺ neutrophils in the ChP. **e.** Representative flow cytometric plots and the total number of CD3⁺ T cells in the ChP. Data are mean $\pm$ SD (n = 6-7). *p < 0.05, **p < 0.01. One-way ANOVA. **f.** Representative images of CD8⁺ T cells (arrows) and Iba1⁺ microglia cells (arrowheads) in the IL LVCP in Sham, stroke Veh-control, ZT-1a- or BMT-treated stroke mice at 24 h post reperfusion. **g.** Quantification of CD8⁺ T cells and Iba1⁺ microglia cells per one coronal section at the bregma level in IL hemisphere. Data are mean $\pm$ SD (n=6, 4 male, 2 female). **p < 0.01, ***p < 0.001. One-way ANOVA.

Figure 6

H_2O_2 -mediated stimulation of SPAK-NKCC1 cascade and CPEC apoptosis are blocked by ZT-1a and BMT.

a. Experiment protocol. **b.** Representative confocal images of cultured CPECs stained for cytokeratin and ZO-1. **c.** Representative confocal immunostaining images of pSPAK/pNKCC1 and epithelial marker, cytokeratin in CPECs. Cells were exposed to either normal DMEM/F12 medium (CTL), 200 μM H_2O_2 , 200 μM H+E, 200 μM H+Z, or 200 μM H+B.

$\mu\text{M H}_2\text{O}_2$ + 1 μM Ebselen (H+E), 200 $\mu\text{M H}_2\text{O}_2$ + 1 μM ZT-1a (H+Z) or 200 $\mu\text{M H}_2\text{O}_2$ + 10 μM BMT (H+B) in the culture medium for 24 h. Arrowheads: low level expression of pSPAK and pNKCC1. Arrows: elevated pSPAK and pNKCC1 expression, **d** and **e**. Summary data of pSPAK and pNKCC1 fluorescence intensity. Data are mean $\pm$ SD (n = 4). * p < 0.05, ** p < 0.01, *** p < 0.001. One-way ANOVA. **f**. Representative confocal image of ZO-1 and summary, data are mean $\pm$ SD (n = 3). **g**. Representative image of TUNEL⁺ CPECs. Arrows: TUNEL⁺/cytokeratin⁺ cells. **h**. Quantitative analysis of TUNEL⁺ cell counts. Data are mean $\pm$ SD (n = 4). ** p < 0.01, *** p < 0.001. One-way ANOVA.

Figure 7

H_2O_2 -mediated activation of SPAK-NKCC1 cascade-mediated Na^+ , K^+ (Rb^+) influx and cell death in cultured neurons.

a. Experiment protocol. **b.** Representative fluorescent images of primary cortical neuron cultures. Cells were stained with Calcein-AM and Propidium Iodide (PI) at 24 h post exposure either to control (CTL), 20

$\mu\text{M H}_2\text{O}_2$, $20 \mu\text{M H}_2\text{O}_2 + 100 \text{ nM Ebselen (H+E)}$, $20 \mu\text{M H}_2\text{O}_2 + 1 \text{ mM ZT-1a (H+Z)}$, or $20 \mu\text{M H}_2\text{O}_2 + 10 \text{ mM BMT (H+B)}$. Arrowheads: live neurons stained with Calcein-AM (Green); Arrows: dead neurons stained with PI (Red). **c.** Neuronal survival rates. Values are calculated as the number of live cells divided by the total number of live plus dead cells, multiplied by 100. Data are expressed as mean $\pm$ SD ($n = 4$), $**p < 0.01$, $***p < 0.001$. **d.** Representative intracellular Na^+ [Na^+]_i concentration using Na^+ -sensitive dye SBFI/AM at 24-hour post exposure of control (CTL) and H_2O_2 insult $\pm$ drug treatment conditions. Values are expressed as mean $\pm$ SD ($n = 4-5$), $**p < 0.01$, $***p < 0.001$. **e** and **f.** Total Rb^+ (K^+) influx and NKCC1-mediated Rb^+ (K^+) influx. Neuron cells were exposed to each condition for 24 h. Rb^+ influx into cells under isotonic (310 mOsm, pH 7.4) solutions were assayed for 10 min and determined using ICR 8000. Values are expressed as mean $\pm$ SD ($n = 4$), $*p < 0.05$, $**p < 0.01$. **g.** Representative immunoblots of primary cortical neuron cultures under conditions as in **b**. Cell lysates were subjected to SDS-PAGE and incubated with appropriate antibody. **h.** Summary. Values are expressed as mean $\pm$ SD ($n = 4$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. **i.** Representative immunoblots of primary cortical neuron cultures under conditions as in **b**. **j.** Summary. Data is expressed as mean $\pm$ SD ($n = 4$), $*p < 0.05$, $**p < 0.01$. One-way ANOVA.

Figure 8

Schematic summary of stroke-induced dysregulation of the blood-CSF barrier via SPAK-NKCC1 signaling pathway.

Ischemic stroke activation of SPAK-NKCC1 cascade in ChP. Overstimulation of SPAK-NKCC1 signaling pathway activity leads to TJs damage, ChP blood-CSF barrier dysfunction, and immune cell infiltration at the ChP. Ischemic reperfusion-mediated production of ROS can directly activate SPAK-NKCC1 cascade and MMP9 protein expression with NF-κB activation as an upstream mechanism. Pharmacological inhibition of the SPAK-NKCC1 cascade attenuate blood-CSF barrier damage and brain inflammation.

Supplementary Files

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