Role of SPAK-NKCC1 signaling cascade in the choroid plexus blood-CSF barrier damage after stroke

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Running title: **SPAK-NKCC1 complex in the blood-CSF barrier**

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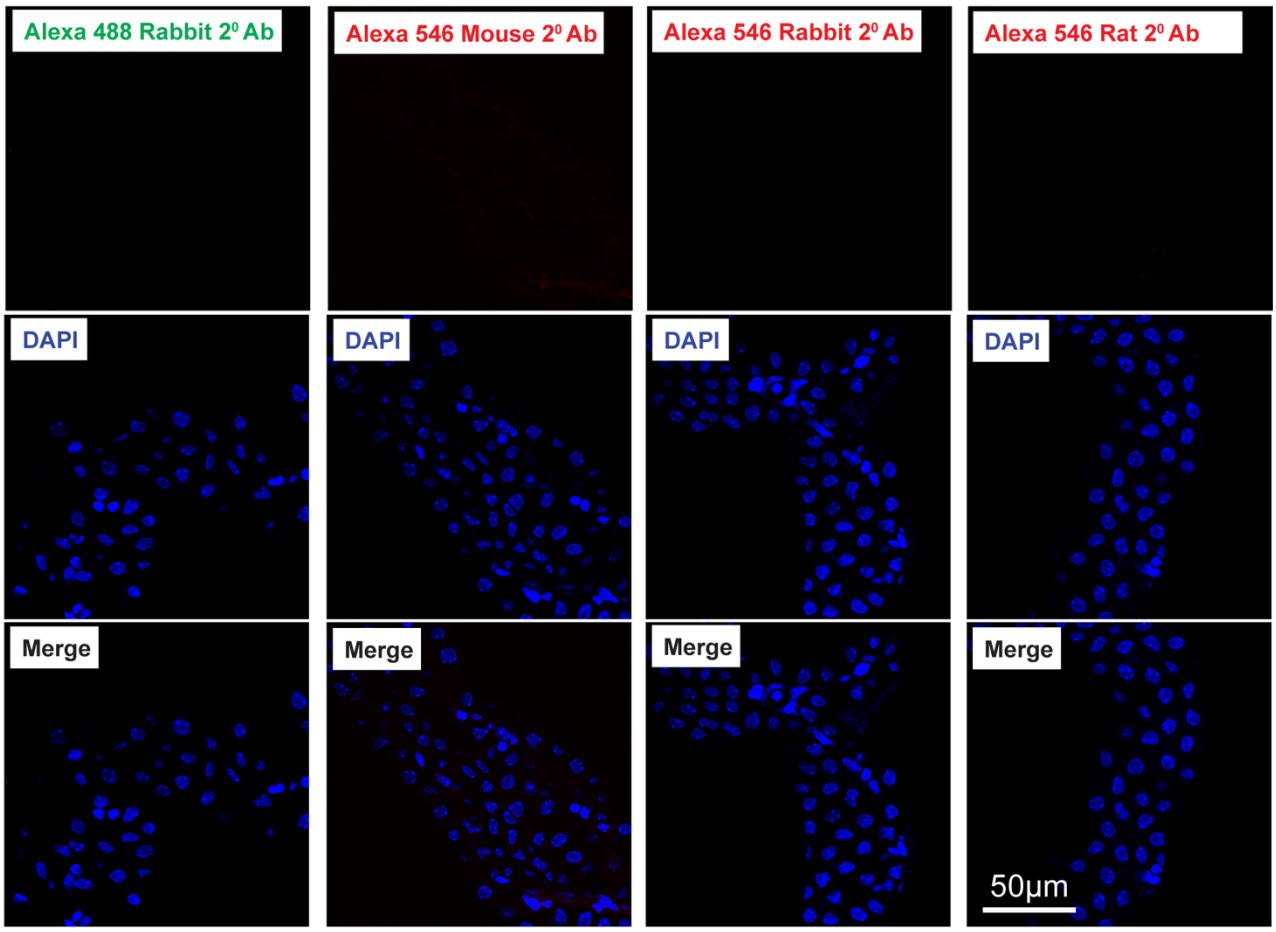
Supplementary Table 1. List of antibodies used for immunofluorescence (IF), western blot (WB) and flow cytometry (Flow).

Antibody	Host	Dilution	Company	Catalog No.	Application
SPAK	Rabbit	1:200	Cell Signaling Technology	2281	IF
pSPAK	Rabbit	1:200	EMD Millipore	07-2273	
NKCC1	Rabbit	1:200	Abcam	ab59791	
pNKCC1	Rabbit	1:200	EMD Millipore	ABS1004	
pT58 NCC	Rabbit	1:500	N/A	N/A	
(pThr211 NKCC1)	Rabbit	1:200	Wako Bioproducts	019-19741	
Iba1					
ZO-1	Rabbit	1:200	Invitrogen	40-2200	
Claudin-1	Rabbit	1:200	Invitrogen	51-9000	
Claudin-5	Mouse	1:200	Invitrogen	35-2500	
Cytokeratin	Mouse	1:200	Sigma	C2562	
MAP2	Mouse	1:200	EMD Millipore	MAB3418	
CD8	Rat	1:100	Invitrogen	14-0081-82	
SPAK/OSR1	Rabbit	1:300	N/A	N/A	WB
pSPAK/pOSR1	Rabbit	1:300	N/A	N/A	
NKCC1 (T4)	Mouse	1:3000	DSHB	T4	
pNKCC1	Rabbit	1:300	N/A	N/A	
pNF-κB p65	Rabbit	1:500	Cell Signaling Technology	3031S	
NF-κB p65	Rabbit	1:1000	Santa Cruz	SC-372	
MMP9	Rabbit	1:500	Abcam	ab283575	
GAPDH	Rabbit	1:5000	Cell Signaling Technology	2118S	
β-actin	Rabbit	1:5000	Cell Signaling Technology	4970S	
BUV395-CD11b	Rat	250	BD Biosciences	56353	Flow
APC-CD45	Rat	250	BioLegend	103111	
PerCP/Cy5.5-Ly6G	Rat	250	BioLegend	127615	
PE-Cy7-Ly-6C	Rat	250	BD Biosciences	560593	
BV421-CD3	Armenian Hamster	250	BioLegend	100336	
FITC-CD206	Rat	250	BioLegend	141704	
PE-Ym-1	Rabbit	2500	Abcam	Ab211621	

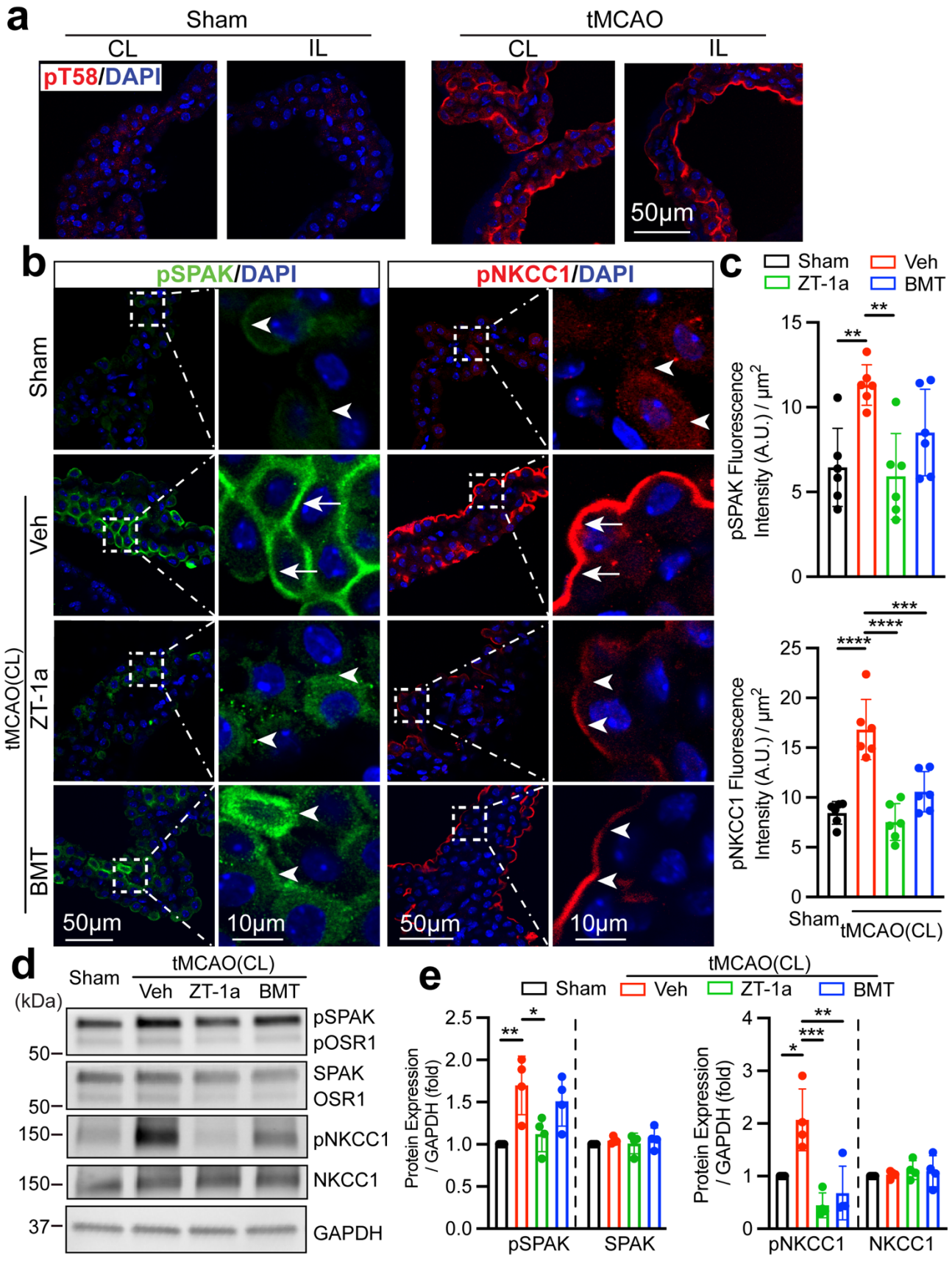
Supplementary Figure 1



Supplementary Figure 1. Immunostaining negative control.

Representative images of secondary antibody staining in the mouse LVCP with the following anibodies: Goat anti-rabbit Alexa 488, Goat anti-mouse Alexa 546, Goat anti-rabbit Alexa 546 and Goat anti-rat Alexa 546 at 1:200, images were taken under a 40x oil-immersion objective with identical setting.

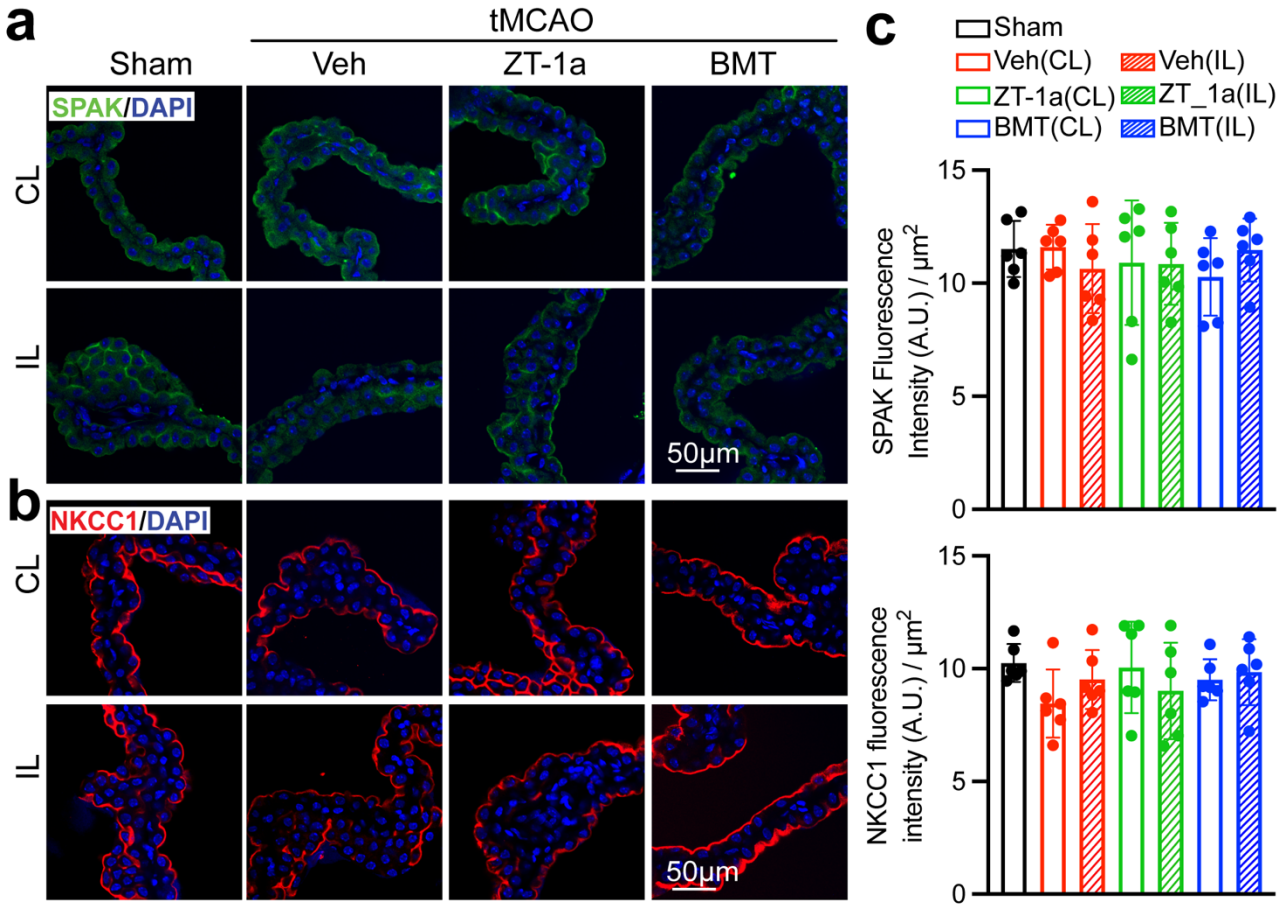
Supplementary Figure 2



Supplementary Figure 2. Activation of SPAK-NKCC1 complex in choroid plexus post ischemic stroke.

a. Representative immunostaining images of a phosphorylated species (active) of pT58 in Sham or stroke ChP. **b.** Representative immunofluorescent images of pSPAK and pNKCC1 staining of lateral ventricle choroid plexus (LVCP) in the contralateral (CL) hemispheres in Sham, stroke Veh-control, ZT-1a or BMT-treated brains. Arrowheads: low level pSPAK and pNKCC1 expression. Arrows: elevated pSPAK or pNKCC1 expression. **c.** Quantification summary. Data is represented by mean ± SD (n = 6, 4 male, 2 female), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The Sham pSPAK and pNKCC1 data in panel **c** are the same as the Sham data presented in **Fig 3e**. **d.** Western blot analysis of SPAK-NKCC1 cascade expression in the CL of LVCP in Sham, Veh-control, ZT-1a- or BMT-treated mice at 24 h Rp after ischemic stroke. ChP tissue lysates were prepared and subjected to immunoblotting with the indicated antibodies. **e**. Immunoblot summary in ChP. Data are expressed as mean ± SD (n = 4, 2 male, 2 female). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. One-way ANOVA.

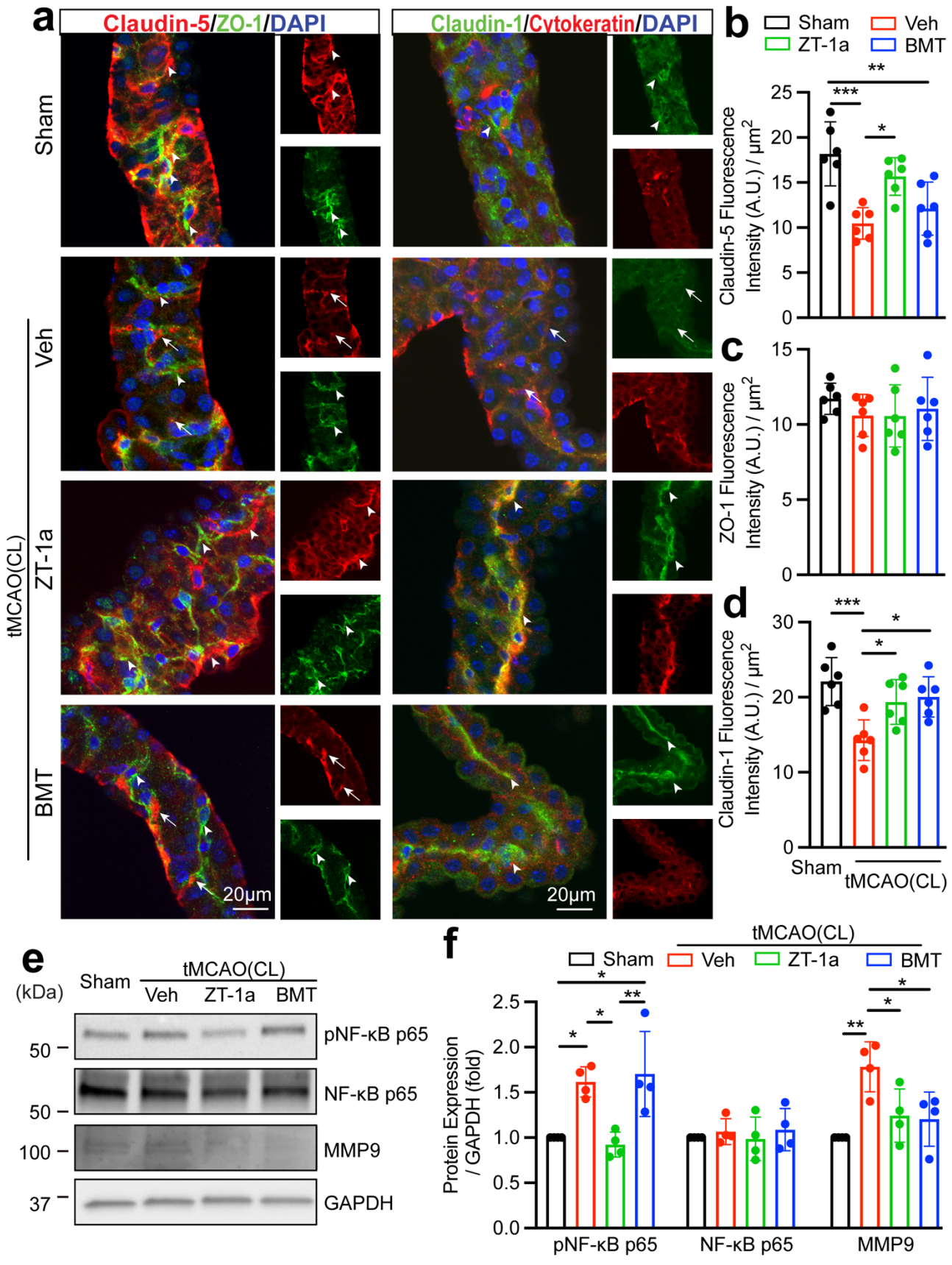
Supplementary Figure 3



Supplementary Figure 3. Pharmacology block SPAK-NKCC1 cascade restore SPAK protein expression at the apical membrane of CPECs.

a and **b.** Representative confocal images of SPAK and NKCC1 protein expression in the contralateral (CL) and ipsilateral (IL) of lateral ventricle choroid plexus (LVCP) post-stroke. **c.** Summary data of SPAK and NKCC1 fluorescence intensity. Data is represented as mean ±SD (n = 6, 4 male, 2 female).

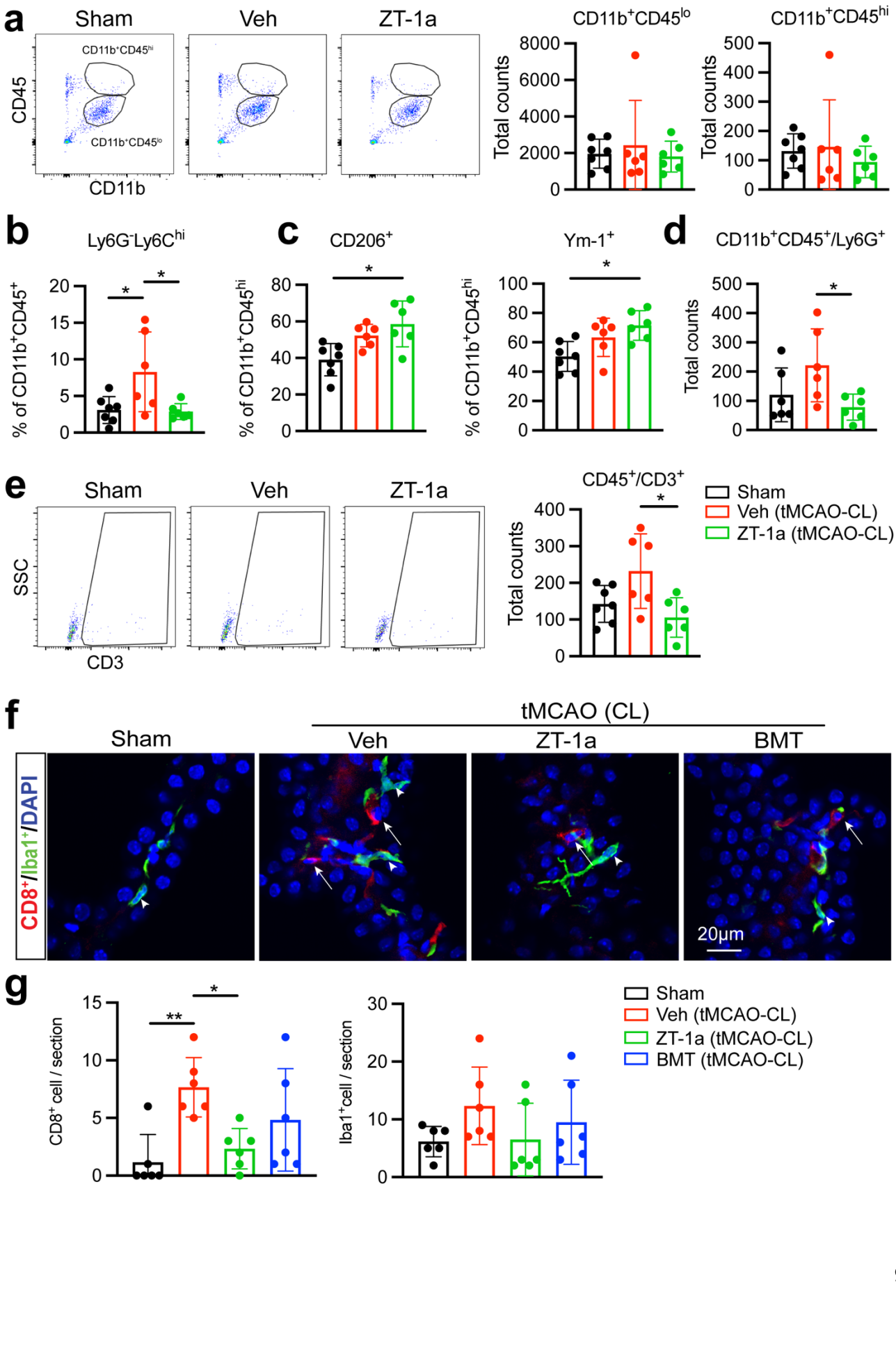
Supplementary Figure 4



Supplementary Figure 4. Blocking SPAK-NKCC1 cascade increased ChP tight junction integrity after ischemic stroke.

a. Representative confocal images of tight junction proteins (Claudin-5, ZO-1, Claudin-1) as well as the epithelial marker cytokeratin in the lateral ventricle choroid plexus (LVCP) of contralateral (CL) hemispheres of in Sham, stroke Veh-control, ZT-1a- or BMT-treated brains. Arrowheads: expression of Claudin-5, ZO-1 or Claudin-1. Arrows: Low level expression of Claudin-5 and Claudin-1. The Sham data in panel **b** - **d** are the same as the Sham data represented in **Fig. 4b** - **d**. Data are represented as mean ±SD (n = 6, 4 male, 2 female). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **e.** Western blot analysis of pNF-Kb, NF-κB and MMP9 protein expression in the CL LVCP of Sham, stroke Veh-control, ZT-1a- and BMT-treated mice at 24 h reperfusion after stroke. ChP tissue lysates were prepared and subjected to immunoblotting with the indicated antibodies. **f.** Immunoblot quantitation. Data are means ± SD (n = 4, 2 male, 2 female). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. One-way ANOVA.

Supplementary Figure 5



Supplementary Figure 5. Pharmacological inhibition of SPAK-NKCC1 cascade reduced immune cell infiltration in ChP.

a. Representative flow cytometric plots of $CD11b^{+}CD45^{lo}$ or $CD11b^{+}CD45^{hi}$ myeloid cells from the isolated CL 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 ± SD (n = 6-7). *$p < 0.05$. One-way ANOVA. **f.** Representative images of $CD8^{+}$ T cells (arrows) and Iba1^{+} microglia cells (arrowheads) of CL LVCP in Sham, stroke Veh-control, ZT-1a- or BMT-treated stroke mice at 24 h Rp. **g.** Summary. Data are mean ± SD (n=6, 4 male, 2 female). *$p < 0.01$, **$p < 0.001$. One-way ANOVA.