UC Davis
UC Davis Previously Published Works

Title
Blood—Brain Barrier KCa3.1 Channels

Permalink
https://escholarship.org/uc/item/5d3512nd

Journal
Stroke, 46(1)

ISSN
0039-2499

Authors
Chen, Yi-Je
Wallace, Breanna K
Yuen, Natalie
et al.

Publication Date
2015

DOI
10.1161/strokeaha.114.007445

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Blood Brain Barrier KCa3.1 Channels: Evidence for a Role in Brain Na Uptake and Edema in Ischemic Stroke

Authors:

Yi-Je Chen, DVM, PhD, Department of Pharmacology, University of California, Davis, CA

Breanna K. Wallace, PhD, Department of Physiology and Membrane Biology, University of California, Davis, CA

Natalie Yuen, PhD, Department of Physiology and Membrane Biology, University of California, Davis, CA

David P. Jenkins, PhD, Department of Pharmacology, University of California, Davis, CA

Heike Wulff, PhD, Department of Pharmacology, University of California, Davis, CA

Martha E. O'Donnell, PhD, Department of Physiology and Membrane Biology, University of California, Davis, CA

Corresponding author:

Yi-Je Chen, DVM, PhD,

Assistant Professor

Department of Pharmacology, UC Davis, School of Medicine

University of California, Davis

451 Health Sciences Drive, GBSF 3409

Davis, CA 95616

email: ijchen@ucdavis.ucdavis.edu

phone:1- 530-754-7400

fax: 1-530-752-7710

Figure number: 6

Table number: 0

Key words:

blood-brain barrier; brain edema; cerebral ischemia; stroke; KCa3.1

Subject codes:

[44] Acute Cerebral Infarction

Number of words:

Title page: 181

Abstract: 250

Main body of text: 4418

Acknowledgments: 0

Sources of funding: 48

Disclosures: 49

References: 1781

Figure legends: 594

Abstract

Background and Purpose: KCa3.1, a calcium-activated potassium channel, regulates ion and fluid secretion in the lung and gastrointestinal tract. It is also expressed on vascular endothelium where it participates in blood pressure regulation. However, the expression and physiological role of KCa3.1 in blood-brain barrier (BBB) endothelium has not been investigated. BBB endothelial cells transport Na^+ and Cl^- from the blood into the brain transcellularly through the cooperation of multiple co-transporters, exchangers, pumps and channels. In the early stages of cerebral ischemia, when the BBB is intact, edema formation occurs by processes involving increased BBB transcellular Na^+ transport. This study evaluated whether KCa3.1 is expressed on and participates in BBB ion transport. **Methods:** The expression of KCa3.1 on cultured cerebral microvascular endothelial cells (CMEC), isolated microvessels and brain sections was evaluated by Western blot and immunohistochemistry. Activity of KCa3.1 on CMEC was examined by K^+ flux assays and patch-clamp. Magnetic resonance spectroscopy and imaging were used to measure brain Na^+ uptake and edema formation in rats with focal ischemic stroke following TRAM-34 treatment. **Results:** KCa3.1 current and channel protein were identified on bovine CMEC and freshly isolated rat microvessels. In situ KCa3.1 expression on BBB endothelium was confirmed in rat and human brain sections. TRAM-34 treatment significantly reduced Na^+ uptake, and cytotoxic edema in the ischemic brain. **Conclusions:** BBB endothelial cells exhibit KCa3.1 protein and activity and pharmacological blockade of KCa3.1 appears to provide an effective therapeutic approach for reducing cerebral edema formation in the first 3 hours of ischemic stroke.

Introduction:

KCa3.1, a Ca^{2+}-activated potassium channel, regulates ion and fluid secretion in the kidney, intestine, airway, pancreatic duct, and in colonic epithelial cells.[1-4] In these secretory epithelia, KCa3.1 recycles K^+ out of the cell following accumulation of Cl^- through the Na-K-Cl transporter and thus facilitates Cl^- secretion by maintaining a favorable electrochemical gradient for Cl^- efflux.[5, 6] Accordingly, inhibition of KCa3.1 has been proposed as a therapeutic strategy for diarrhea,[1] while KCa3.1 activators have been considered for increasing fluid secretion in cystic fibrosis.[7] KCa3.1 is also known to be expressed on peripheral vascular endothelial cells where it participates in endothelium-derived hyperpolarization (EDH) for blood pressure regulation.[8, 9] Even though there is a large body of literature on K^+ channel expression in peripheral blood vessel endothelial cells, little is known about the expression and physiological role of KCa3.1 in endothelial cells of the blood-brain barrier (BBB). BBB endothelial cells tightly regulate transcellular movement of ions and other solutes between blood and brain via transporters and channels that are asymmetrically distributed between luminal and abluminal membranes, much like secretory epithelial cells. In the healthy normoxic brain BBB endothelial cells secrete Na^+, Cl^- and water into the brain, producing up to 30% of brain interstitial fluid and also remove K^+ from the brain as needed to maintain an appropriately low brain extracellular K^+ concentration. [10-12] Much is still unknown about the ion transporters and channels that accomplish this although there is evidence that Na^+, Cl^- and water secretion into the brain involves luminal Na-K-Cl cotransport (NKCC) and Na/H exchange (NHE), together with the abluminal Na/K ATPase and abluminal Cl^-

channels.[13-18] During the early hours of ischemic stroke, cerebral edema forms in the presence of an intact BBB as transendothelial secretion of Na^+, Cl^- and water into the brain is greatly increased.[10-12, 17, 18] Previous studies have provided evidence that this involves ischemia stimulation of luminal NKCC and NHE activity and that inhibition of these transporters by intravenously administered bumetanide or HOE642 effectively reduces edema and brain Na^+ uptake[17]. The primary anion accompanying Na^+ in ischemia-induced BBB secretion is Cl^-.[19] In Na^+ and Cl^- secreting epithelia, K^+ channels serve the important role of maintaining an outwardly directed electrochemical gradient for Cl^- to support Cl^- efflux. Further, NKCC is highly sensitive to inhibition by elevation of intracellular Cl^- concentration via a Cl^- sensitive kinase.[20] In this regard, BBB K^+ channel activity is predicted to also support transendothelial secretion of Na^+ and Cl^- by supporting luminal NKCC activity. The present study was conducted to investigate whether KCa3.1 is expressed on BBB endothelial cells and participates in ischemia-induced cerebral edema formation.

Materials and Methods

Cell Culture

Bovine cerebral microvascular endothelial cells (CMEC) were maintained in DMEM containing 5 mM D-glucose, 1 mM Na-pyruvate, 2 mM L-glutamine, 50 μg/ml gentamicin, 1 ng/ml bovine basic fibroblast growth factor, 5% calf serum, and 5% horse serum (DMEM growth medium) in an atmosphere of 95% humidified air with 5% CO_2 at 37°C, as described previously.[16] Cells were grown to confluence on collagen- and attachment factor-coated (Cell Systems, Kirkland, WA) 6-well plates or coverslips. Cells were re-fed fresh DMEM growth medium every 48 hours until 2 days prior to the experiments, when medium was replaced with a 50:50 mixture of DMEM growth medium and astrocyte-conditioned medium (ACM) to all CMEC culture. ACM was prepared by exposing primary cultured rat neonatal astrocytes to DMEM containing 10% FBS for 48 hours and added to the CMEC cultures before functional assays since multiple previous studies had shown that ACM promoted a BBB phenotype in the cultured cells [21].

Electrophysiology

Bovine CMEC grown for 48 h in ACM containing medium were studied in the whole-cell configuration of the patch-clamp technique as described.[22] Briefly, the holding potential in all experiments was −80 mV. For measurement of KCa3.1 currents, the internal pipette solution contained (in mM): 145 K^+ aspartate, 2 $MgCl_2$, 10 HEPES, 10 K_2 EGTA, and 8.5 $CaCl_2$ (3 μM free Ca^{2+}), pH 7.2, 290–310 mOsm. External solution (in mM): 160 Na^+ aspartate, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4, 290–310 mOsm. KCa3.1

currents were elicited by 200-ms voltage ramps from −120 mV to 40 mV applied every 10 s and the reduction of slope conductance at −80 mV by drug taken as a measure of channel block. TRAM-34, charybdotoxin (ChTX), apamin, and iberiotoxin were used to identify KCa3.1 channels. TRAM-34 was synthesized in the Wulff laboratory as previously described.[22] ChTX, apamin and iberiotoxin were from Bachem Americas (Torrance, CA).

Immunocytohistological (ICC) and Immunofluorescence (IF) staining

CMEC monolayers grown on collagen-coated glass slides in the presence of ACM were subjected to ICC and IF staining. HEK-293 cells stably expressing hKCa3.1 were used as positive control in IF staining.[23] KCa3.1 channel protein was identified with anti-KCa3.1 antibody (AV35098, rabbit polyclonal, 1:3000, Sigma-Aldrich, St. Louis, MO) as described.[24] Bound primary antibodies were detected by either: a) Biotin-SP-conjugated secondary antibodies (111-065-144, Goat anti-rabbit igG (H+L), 1:500, Jackson ImmunoResearch Laboratories), followed by a horseradish peroxidase-conjugated avidin complex (PK-6100, VECTASTAIN® Elite® ABC System, Vector Laboratories) and peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB) (SK-4100, DAB Peroxidase (HRP) Substrate Kit, Vector Laboratories). Staining results were evaluated by regular bright filed microscopy; or b) Alexa Fluor® 488-conjugated secondary antibodies (A-11009, 1:500, Life Technologies). Sections were mounted in Fluoromount-G (SouthernBiotech) with DAPI and imaged with a Zeiss LSM-510 confocal microscope. In situ expression of KCa3.1 was evaluated in rat and human brain sections by double labeling immunofluorescence with the rat BBB endothelial cell-

specific marker (Clone: SMI-71, mouse monoclonal, 1:1000, Covance), Von Willebrand factor (vWF) for human endothelial cells (Clone: RFF-VIII R/1, mouse monoclonal, 1:100, Serotec), and KCa3.1. Staining was visualized by Alexa Fluor® 405 (A-31556, Goat anti-mouse, 1:500, Life Technologies) and Alexa Fluor® 647 conjugated secondary antibodies. (A-20991, Goat anti-rabbit, 1:500, Life Technologies). The results were analyzed by confocal microscopy. Human brain samples were provided by the Alzheimer's Disease Center at the University of California Davis, funded by NIH/NIA (P30 AG10129). The tissue procurement was approved by the Institutional Review Board. Informed consent to share research tissue after death was obtained from all patients or a representative prior to their death.

Western Blot

Bovine CMEC whole cell lysates from cells grown for 48 h in ACM containing medium and freshly isolated rat brain microvessel lysates were subjected to Western blot analysis. Rat brain microvessels were isolated as described.[25] Five mg protein from each sample was denatured with 50 mM dithiothreitol (DTT) in SDS sample reducing buffer (NP0004, Life Technologies) and heated at 70°C for 10 min. Protein samples were then separated on 12% Tris-HCl gel (No.5952, PAGEr™ Gold Precast Gel, Lonza) using electrophoresis and electrotransferred to polyvinylidene fluoride membranes (BioRad XCell SureLock, Hercules). Membranes were blocked with 7.5% milk in PBS with 0.1% Tween-20 (PBST) for 1hr, incubated with anti-KCa3.1 antibody (AV35098, rabbit polyclonal, 1:3000, Sigma-Aldrich, Louis, MO) in milk/PBST overnight at 4°C, washed with PBST, and incubated with horseradish peroxidase-conjugated secondary

antibody (A16096, goat anti-rabbit, 1:2000, Life Technologies) in milk/PBST for 1 hr at room temperature. After washing with PBST, bound antibody was detected using enhanced chemiluminescence kit (RPN2133, ECL plus; GE Healthcare) and visualized on a Fuji Film LAS-4000 Imaging Machine (Medford, UK).

KCa3.1 Activity Assay

KCa3.1 activity was assessed by Ca^{2+}-sensitive and KCa3.1 channel blocker-sensitive K^+ efflux using ^{86}Rb as a tracer for K^+ as described previously.[7, 16] Bovine CMEC monolayers grown for 48 h in ACM containing medium on 24-well plates were placed in a hypoxia chamber (COY Laboratory Products; Grass Lake, MI) preset to 37°C with an atmosphere of 5% CO_2 (normoxia). Cells were incubated in DMEM with ^{86}Rb (1 μCi/mL) for 2 hrs. Media was removed and cells were washed three times with Low-Ca^{2+} Ringers (138 mM NaCl, 10 mM HEPES, 4.5 mM KCl, 1 mM $MgCl_2$, 250 μM $CaCl_2$). Treatment media was added to the cells for 10 minutes: High-Ca^{2+} Ringers (20 mM $CaCl_2$, 120 mM NaCl, 20 mM HEPES) or High-Ca^{2+} Ringers with Paxillin (1 μM) (Sigma-Aldrich, Louis, MO), NS8593 (10 μM), NS6180 (250 and 1000 nM) or TRAM-34 (100 nM, 250 nM, 500 nM or 1 μM). NS6180 and NS8593 were synthesized at NeuroSearch A/S (Denmark). After treatment, supernatant was collected at 10 minutes for ^{86}Rb quantitation by liquid scintillation analysis (Tri-Carb 2500 TR liquid scintillation counter). ^{86}Rb efflux was calculated by raw radiation Counts per Minute (CPM).

Middle Cerebral Artery Occlusion in Rats

This study was approved by the University of California, Davis, Animal Use and Care Committee and conducted in accordance with the guidelines for survival surgery in rodents. Adult male Sprague Dawley rats weighing 110 to 130 g (Charles River, Wilmington, MA, USA) were acclimatized to the new vivarium for 5 to 7 days and used for the surgery when they weighed 190 to 200 g. Rats were anesthetized using box induction with 5% isoflurane in medical grade oxygen and then maintained on 0.5 to 1.5% isoflurane via a facemask. To assure consistent reduction of cerebral blood flow (CBF) throughout the procedure, a Laser Doppler (Moor Instruments, Wilmington, DE, USA) was used to monitor CBF throughout the MCAO surgery. Permanent focal cerebral ischemia was then induced by occlusion of the left middle cerebral artery (MCA) as previously described.[18, 24] Briefly, the left common carotid artery was surgically exposed, the external carotid artery was ligated distally from the common carotid artery, and a silicone rubber-coated nylon monofilament with a tip diameter of 0.43±0.02 mm (Doccol Corp., Redlands, CA) was inserted into the external carotid artery and advanced into the internal carotid artery to block the origin of the MCA. Vehicle (Miglyol 812 neutral oil, 1 µl/g, Spectrum Chemicals, Gardena, CA) or TRAM-34 (40 mg/kg, intra-peritoneal injection) was given 20 min before MCAO.

Magnetic Resonance Chemical Shift Imaging of Brain Na^+

Magnetic resonance Chemical shift Imaging (CSI) was used to determine extravascular brain Na^+ content of rat brains with the NMR chemical shift/relaxation reagent Dysprosium triethylenetetraminehexaacetic acid (DyTTHA) as described previously.[17, 18]

Following ligation of both sides of the renal artery and vein, 250 mmol/L of DyTTHA (Sigma-Aldrich, Louis, MO) was infused intravenously at 0.3 mL/min to achieve a final dose of 1.5 mmol per liter per kg and allowed to equilibrate across the various body compartments for 20 minutes before MCAO. A 7-T Bruker Biospec MRS/MRI system (Bruker) with a double tuned $^{1}H/^{23}Na$ probe (Doty Scientific, Columbia, SC) was used for Na^+ CSI imaging. Two-dimensional Na^+ CSI images were acquired via the standard Bruker CSI protocol with Paravision 2.1 software (Bruker Biospin GmbH, Rheinstetten, Germany) and each ^{23}Na CSI data set was acquired in 21 minutes. Na^+ CSI spectra were further analyzed using MATLAB 2011b (MathWorks, Natick, MA) to integrate over the unshifted extravascular Na^+ peak and that of an external standard to calculate the extravascular Na^+ concentration in the core of the infarct up to 170 min after MCAO.

Magnetic Resonance Diffusion Weighted Imaging of Edema Formation

Resonance Diffusion Weighted Imaging Analysis of Apparent Diffusion Coefficient Values was performed to evaluate edema formation using a 7-T Bruker Biospec MRS/MRI system as described previously.[17, 18] Briefly, rats were placed into a 72-mm radio frequency probe inside the 7-T magnet and spin echo images (2 mm slices) were then acquired at 38 to 236 minutes following occlusion of the MCA. Apparent diffusion coefficient (ADC) values were determined for selected penumbral region of interest (ROI) using four gradient strengths and Paravision 2.1 software. ADC values for anatomically corresponding ipsilateral (left) and contralateral (right) hemisphere ROI were compared and ratios of L/R ADC values were calculated.

Statistics Analyses

Data were analyzed for significance using analysis of variance or by Student's *t*-test. *P* values < 0.05 were considered to indicate significant difference.

Results:

KCa3.1 is Expressed in Isolated Rat Brain Microvessels and Cultured Bovine CMEC

To test the hypothesis that the KCa3.1 channel participates in edema formation at the BBB during ischemic stroke, we first evaluated KCa3.1 protein expression on the BBB endothelium using Western blot and ICC staining. A KCa3.1 protein specific band at ~47 KDa was detected in whole cell lysates of freshly isolated rat brain microvessels and cultured bovine CMEC (Figure 1A). ICC and IF further confirmed the expression of KCa3.1 channel protein on bovine CMEC (Figure 1B (left) and 1C (right)). The specificity of the polyclonal KCa3.1 antibody was confirmed by staining HEK-293 cells stably expressing hKCa3.1 (Figure 1C left). We next used the whole-cell patch-clamp technique to assess the magnitude of the KCa3.1 current in bovine CMEC and determine whether the cells expressed any other significant K^+ conductances. Voltage ramps from -120 to +40 mV in the presence of 3 μM free Ca^{2+}, a concentration that fully activates KCa3.1 currents, in the patch pipette elicited Ca^{2+}-activated K^+ currents that exhibited the biophysical and pharmacological properties of KCa3.1 in the majority of cells. The current was voltage-independent, reversed around -80 mV and was not visible in the absence of Ca^{2+} (not shown). The current was sensitive to the "classical" but not KCa3.1 selective scorpion venom peptide charybdotoxin (Figure 2A), insensitive to the KCa2 blocker apamin and the KCa1.1 blocker iberiotoxin (data not shown), and dose-dependently inhibited by the KCa3.1 selective small molecule KCa3.1 blocker TRAM-34 (Figure 2B). In addition to KCa3.1, CMEC also expressed small inward-rectifier currents in roughly 50% of cells (Figure 2A), but no significant voltage-

dependent conductance, demonstrating that KCa3.1 is the major K^+ channel in BBB endothelial cells.

Rat and Human BBB Endothelium Express KCa3.1 *in situ*

In these studies we also evaluated KCa3.1 expression in BBB in situ using rat and human brain sections and confocal immunofluorescence microscopy. BBB endothelial cells were identified in rat brain sections by co-labeling with anti-IST-57 antibody which stains rat BBB endothelial cells (Figure 3, upper panel). Anti-Von Willbrand Factor antibody was used to identify BBB endothelial cells in human brain sections (Figure 3 lower panel). In both cases KCa3.1 was clearly expressed on the same cells labeled with the endothelial markers.

KCa3.1 Blockers Inhibit K^+ Flux on Bovine CMEC

In order to determine whether KCa3.1 is involved in K^+ movement across the BBB we tested the effect of KCa3.1 inhibitors in a well-established ^{86}Rb flux assay, which was previously used to evaluate activity of the Na-K-Cl cotransporter and Na/H exchange activity in CMEC.[26] All experiments were performed in the presence of 20 mM Ca^{2+} and results were ratioed to ^{86}Rb flux in DMSO treated control cells. In cells exposed to 0.25 mM external Ca^{2+} the ratio dropped to 0.84 demonstrating that the K^+ efflux is sensitive to changes in external Ca^{2+} concentration and thus to the resulting changes in Ca^{2+} driving force for Ca^{2+} entry. In CMEC exposed to 20 mM Ca^{2+} application of the KCa3.1 blockers NS1680 and TRAM-34 reduced K^+ efflux in a dose-dependent manner. As expected, the KCa1.1 blocker paxilline (1 μM) and the KCa2.2 inhibitor NS8593 (10 μM)

did not significantly reduce K^+ efflux (Figure 4). We exclusively used small molecule inhibitors instead of peptides for this assay because they are more membrane permeable and less “sticky” and therefore much easier to handle in the flux experiments.

TRAM-34 Reduces Na^+ Accumulation and Edema Formation in a Rat MCAO Model

In order to test our hypothesis that KCa3.1 inhibition can attenuate edema formation we evaluated the effects of TRAM-34 on brain Na^+ and water uptake in the rat permanent MCAO model of focal ischemic stroke. Following induction of MCAO extravascular Na^+ accumulation in the brain was visualized and quantitated by Na MRS Chemical Shift Imaging (CSI) over a time course of 60 to 170 min from the onset of occlusion. The NMR software used converts the integrated signals into pseudo-color (orange in this case) correlating the Na^+ concentration to color intensity (higher intensity represents higher concentration). Rats administered TRAM-34 immediately before induction of MCAO showed significantly lower brain Na^+ uptake compared to vehicle treated rats. Figure 5A shows representative brain Na^+ images obtained at 104 and 170 min following onset of MCAO in vehicle- and TRAM-34- treated rats. Figure 5B depicts the quantitated changes in brain extravascular Na content. In vehicle treated rats subjected to MCAO, the extravascular Na^+ content rose linearly, reaching 1.92 ± 0.06 by 170 min. For rats given TRAM-34 (40 mg/kg i.p.) and then subjected to pMCAO, the increase in brain Na^+ content ratio was significantly attenuated at all time points up to 170 minutes in pMCAO (Figure 4A and B; ratio = 1.45 $\pm$ 0.4 at 170 min). The ratio of ipsilateral to

contralateral extravascular Na^+ content in rats with sham surgery was 1.0 (data not shown).

Edema formation was evaluated in a separate group of animals by diffusion weighted imaging (DWI) analysis of apparent diffusion coefficient (ADC) values over a time course of 38 to 236 min from the onset of permanent MCAO. Figure 5C shows representative DWI images from vehicle- and TRAM-34-treated rats at 104 and 236 min after induction of MCAO. Lower ipsilateral to contralateral ADC ratios indicate greater edema formation. We found that TRAM-34 treatment attenuated the MCAO-induced fall in ADC ratios. In vehicle treated rats ADC ratios fell from 1.0 (Sham rats) to 0.69 ± 0.08 at 38 min, and 0.54 ± 0.03 at 236 min; whereas ADC ratios fell to only 0.84 ± 0.09 at 38 min and 0.65 ± 0.15 at 236 min in TRAM-treated rats. This attenuation of edema formation by TRAM-34 was sustained throughout the nearly 4-hr experiment. Statistically significant differences in ADC ratios of vehicle- vs TRAM-treated rats were observed from 38 to 148 min from the start of MCAO (Figure 5D)

Discussion

In this study we demonstrate that the Ca^{2+}-activated K^+ channel KCa3.1 is expressed on bovine, rat and human BBB endothelial cells and is a significant participant in K^+ fluxes at the BBB. Moreover, we show that the KCa3.1 blocker TRAM-34, a compound that is highly selective for KCa3.1[22, 27], significantly attenuates edema formation and Na^+ accumulation in the brain of rats in the early stage of ischemic stroke, suggesting that KCa3.1 constitutes a new therapeutic target for reducing brain edema. K^+ channels have been proposed to participate in K^+ homoeostasis of the brain for decades; however, very few studies have investigated K^+ channels on BBB endothelial cells. Most of those studies were performed before KCa3.1 was cloned in 1997 [28, 29] and were conducted with very limited tools, such as antibodies or specific channel blockers. In 1991 Hoyer et al. reported Kv and inward rectifier (Kir) currents in freshly isolated and primary cultured porcine brain microvascular cells.[30] In 1995 Van Renterghem et al. described a KCa current with biophysical and pharmacological properties resembling a KCa3.1 channel in cultured rat brain capillary endothelial cells.[31] More recently, Millar et al. described Kv1 and Kir family channels in cultured rat brain endothelial cells by electrophysiology and demonstrated the presence of mRNA for Kv1.3, Kir2.1 and Kir2.2 channels by RT-PCR.[32] In the present study we have confirmed the observations of Van Renterghem, who described an intermediate-conductance KCa current (but could not assign it a molecular identity because KCa3.1 had not yet been cloned), and now show that KCa3.1 is one of the major K^+ channels in BBB endothelial cells and is involved in ischemia-induced K^+ fluxes and in edema formation in vivo. In the electrophysiology experiments conducted in this study we also observed Kir currents

with properties resembling Kir2.1 and Kir2.2 but did not study them further because we do not consider Kir2.1/2.2 realistic targets for stroke treatment.

In this study, we have shown for the first time that inhibition of BBB KCa3.1 reduces edema formation during the first 4 hours of permanent MCAO. In the early stage of stroke when the BBB is still intact, the activity of a luminal membrane BBB NKCC is stimulated by ischemic factors and, together with the abluminal Na/K ATPase that mediates active extrusion of Na^+ at the abluminal membrane, contributes to secretion of Na^+, Cl^- and water from the blood into the brain and formation of brain edema.[33] Our previous studies have shown that the K^+ content of the BBB cells does not increase for several hours despite the fact that both NKCC and the Na/K ATPase keep bringing K^+ into the BBB endothelial cells during ischemia.[33] This lack of K^+ increase might be caused by K^+ channel activity, most likely KCa3.1, allowing extrusion of K^+ from the cells and thereby maintaining an appropriate intracellular K^+ concentration and membrane potential (Figure 6).

The KCa3.1 channel regulates Cl^- secretion across secretory epithelia by maintaining the electrochemical driving force for Cl^- efflux through apical Cl^- channels. In airway, gastrointestinal and pancreatic duct epithelia, inhibition of KCa3.1 channels slows down Cl^- efflux and increases intracellular Cl^- ($[Cl^-]i$).[2, 34-37] Since NKCC activity is very sensitive to $[Cl^-]_i$ through a Cl^--sensitive kinase signaling mechanism, with elevated $[Cl^-]_i$ inhibiting and reduced $[Cl^-]_i$ stimulating NKCC, increased $[Cl^-]_i$ inhibits not only Cl^- influx, but also Na^+ and K^+ influx.[20] In addition to the regulation of ion secretion, KCa3.1 plays an important role in regulating Ca^{2+} signaling and membrane potential in different cells, such as T cells and microglia.[38-40] K^+ efflux through KCa3.1 channels

helps to maintain a negative membrane potential for Ca^{2+} influx through inward-rectifier type Ca^{2+} channels.[41] In vascular endothelial cells, Ca^{2+} similarly plays a crucial role in initiating signal transduction cascades with the transient receptor potential (TRP) channels TRPC3, TRPV3 and TRPV4 serving as the major type of Ca^{2+}-permeable channels in cultured human, rat and mouse microvessel endothelial cells.[42] In in-vitro studies, intracellular Ca^{2+} increases in 10 to 15 seconds after ischemic factor stimulations. Both intracellular-stored and extracellular Ca^{2+} are crucial for the Ca^{2+} elevation.[43-45] Considering the evidence that blockade of KCa3.1 slows down intracellular Ca^{2+} elevation, increases $[Cl^-]_i$,and reduces NKCC activity, we hypothesize that blocking KCa3.1 will elevate BBB $[Cl^-]_i$ and reduce intracellular Ca^{2+}, which both reduces NKCC activity in BBB endothelial cells, and should therefore reduce Na^+ secretion in to the brain in ischemic stroke (Figure 6) as indeed observed in the MRI experiments following KCa3.1 blockade.

While K^+ channels may participate in edema formation and transport of K^+ across the BBB, it should be recognized that K^+ channels also participate in the regulation of cerebrovascular tone. In cerebral capillaries that form the BBB, the endothelium is surrounded by astrocyte endfeet. However, in larger vessels, where vascular smooth muscle cells underlie the endothelium, K^+ channels are involved in endothelium-dependent hyperpolarization (EDH)-induced vasodilations.[46-48] KCa2.3 and KCa3.1 channels open in response to increases in intracellular Ca^{2+} and hyperpolarize the endothelium. This hyperpolarization is then transmitted to the smooth muscle cells by direct electrical coupling through myoendothelial junctions and/or by the accumulation of K^+ ions in the intercellular myoendothelial space.[47, 49, 50] EDH-mediated vascular

responses have been observed in human, rat and guinea-pig cerebral arteries, [51-53] and several studies have reported that EDH-mediated responses remain intact during hypoxia, [51, 54] and even become more prominent during ischemia/reperfusion.[55] In the middle cerebral artery, combined inhibition of KCa2.3 and KCa3.1 is required to abolish EDH responses; however, blocking KCa3.1 alone is sufficient to abolish EDH when NO synthase (NOS) is inhibited.[47, 56] We are therefore of course aware of the fact that blocking KCa3.1 might affect EDH responses in the brain vasculature; however, the in vivo effects and physiological roles of EDH in ischemic stroke are currently not well understood and we therefore are unable to determine how partial or complete inhibition of EDH responses contributes to the effects on edema formation we report here.

Besides the potential benefit of reducing edema in the early stage of stroke, we have also recently reported that TRAM-34 reduces infarct area, microglia activation and neurological deficits in rat MCAO with 7 days of reperfusion even if treatment was started at 12 hours after reperfusion.[24] In this case TRAM-34 inhibited microglia activation and decreased neuroinflammation through acting on KCa3.1 channels on microglia. Since our experiments were performed between 38 min and 3 hours after MCAO, we do not think microglia activation is involved at this early time point. Since these experiments showed that TRAM-34 effectively penetrates into the brain[24] we also cannot completely exclude that in addition to acting on the endothelium in early stroke, TRAM-34 could have affected astrocyte functions. Whether KCa3.1 is expressed or functionally important on astrocytes is currently not completely clear. Three groups cloning KCa3.1 did not detect mRNA for KCa3.1 (which was called hSK4, hIK1 or hKCa4 at that time) in the brain in 1997 when they evaluate the distribution of KCa3.1 in

different type of tissue.[28, 29, 57] When we performed IHC staining of KCa3.1 in brain sections from rats at 7 days after reperfusion ischemic stroke, we only observed staining on activated microglia and the vascular endothelium.[24] However, other studies have suggested that KCa3.1 might be expressed on astrocytes in some pathological conditions such as spinal cord injury, glioblastoma and astrogliosis.[47, 56, 58] Longden et al reported that KCa3.1 might express on astrocyte endfeet.[59] More experiments with higher detecting resolution, like immunoelectron microscopy, will be needed to clarify this question. It will further need to be investigated whether pericytes express KCa3.1.

On average, a stroke occurs every 40 seconds in the United State and 87% of these cases are ischemic strokes.[60] Cerebral edema is a major cause of neuronal death in stroke. Yet acute therapies for stroke induced cerebral edema are currently quite limited. This is the first study evaluating a link between BBB endothelial K^+ channels and edema formation in the brain and it might open up a new therapeutic strategy for the prevention or treatment of ischemia induced cerebral edema. Other very useful applications for our findings that KCa3.1 blockers reduce brain edema would be the treatment of traumatic brain injury or premedication of patients undergoing brain surgeries. In this context we would like to point out that pharmacological KCa3.1 blockade has been shown to be safe and well tolerated in various mouse, rat, pig and sheep models of autoimmunity[61] and that senicapoc, a compound structurally very similar to our TRAM-34, was safe and well tolerate in a Phase-1 clinical trial in healthy volunteers[62]. Senicapoc was afterwards found to significantly reduce hemolysis and increase hemoglobin levels in a 12-week, multicenter, randomized double-blind Phase-2 study in sickle cell disease patients[63]. However, in a subsequent Phase-3 study, which

was designed to compare the rate of acute vaso-occlusive pain crisis occurring in sickle cell disease patients, senicapoc failed to reduce this desired clinical endpoint despite again reducing hemolysis and increasing hemoglobin levels and not inducing any significant adverse events[64]. Senicapoc was subsequently deposited in the NIH NCATs library and would therefore theoretically be available for investigator initiated clinical trials.

Sources of Funding:

This work was supported by NIH RO1 GM076063 to H. Wulff, NIH RO1 NS039953 to M. O'Donnell, the Alzheimer's Disease Center at the University of California Davis funded by NIH/NIA (P30 AG10129) and a postdoctoral fellowship from the American Heart Association Western States Affiliate (09POST2260973) to Yi-Je Chen.

Conflict of Interest / Disclosure:

H.W. is an inventor on a University of California patent claiming TRAM-34 for immunosuppression. However, since no pharmaceutical companies expressed any interest in the subsequently filed disclosure claiming TRAM-34 for ischemic stroke the University of California decided not to file an addendum to the TRAM-34 patent with this indication.

References:

1. Rufo PA, Merlin D, Riegler M, Ferguson-Maltzman MH, Dickinson BL, Brugnara C, et al. The antifungal antibiotic, clotrimazole, inhibits chloride secretion by human intestinal T84 cells via blockade of distinct basolateral K^+ conductances. Demonstration of efficacy in intact rabbit colon and in an in vivo mouse model of cholera. *J Clin Invest*. 1997;100:3111-3120
2. Wang J, Haanes KA, Novak I. Purinergic regulation of CFTR and Ca^{2+}-activated Cl^- channels and K^+ channels in human pancreatic duct epithelium. *Am J Physiol Cell Physiol*. 2013;304:C673-684
3. Warth R, Barhanin J. Function of K^+ channels in the intestinal epithelium. *J Membr Biol*. 2003;193:67-78
4. Devor DC, Singh AK, Frizzell RA, Bridges RJ. Modulation of Cl^- secretion by benzimidazolones. I. Direct activation of a Ca^{2+}-dependent K^+ channel. *Am J Physiol*. 1996;271:L775-784
5. Bernard K, Bogliolo S, Soriani O, Ehrenfeld J. Modulation of calcium-dependent chloride secretion by basolateral SK4-like channels in a human bronchial cell line. *J Membr Biol*. 2003;196:15-31
6. McCann JD, Welsh MJ. Basolateral K^+ channels in airway epithelia. II. Role in Cl^- secretion and evidence for two types of K^+ channel. *Am J Physiol*. 1990;258:L343-348
7. Singh S, Syme CA, Singh AK, Devor DC, Bridges RJ. Benzimidazolone activators of chloride secretion: Potential therapeutics for cystic fibrosis and

chronic obstructive pulmonary disease. *J Pharmacol Exp Ther*. 2001;296:600-611

8. Feletou M, Vanhoutte PM, Weston AH, Edwards G. EDHF and endothelial potassium channels: IKCa and SKCa. *Br J Pharmacol*. 2003;140:225

9. Wulff H, Kohler R. Endothelial small-conductance and intermediate-conductance KCa channels: An update on their pharmacology and usefulness as cardiovascular targets. *J Cardiovasc Pharmacol*. 2013;61:102-112

10. Bradbury MW, Stulcova B. Efflux mechanism contributing to the stability of the potassium concentration in cerebrospinal fluid. *J Physiol*. 1970;208:415-430

11. Cserr HF, DePasquale M, Patlak CS, Pullen RGL. Convection of cerebral interstitial fluid and its role in brain volume regulation. *Annals of the New York Academy of Sciences*. 1989;481:123-134

12. Keep RF. Potassium transport at the blood-brain and blood-CSF barriers. *Frontiers in Cerebral Vascular Biology: Transport and its Regulation*. 1993:43-54

13. Betz AL, Ennis SR, Ren X, Schielke GP, Keep RF. *Blood-brain barrier sodium transport and brain edema formation*. New York: Plenum Press; 1995.

14. Betz AL, Firth JA, Goldstein GW. Polarity of the blood-brain barrier: Distribution of enzymes between the luminal and antiluminal membranes of the brain capillary endothelial cells. *Brain Research*. 1980;192:17-28

15. Ennis SR, Ren X, Betz AL. Mechanisms of sodium transport at the blood-brain barrier studies with in situ perfusion of rat brain. *J Neurochem*. 1996;66:756-763

16. Foroutan S, Brillault J, Forbush B, O'Donnell ME. Moderate to severe ischemic conditions increase activity and phosphorylation of the cerebral microvascular

endothelial cell Na-K-Cl cotransporter. *Am J Physiol Cell Physiol*. 2005;289:C1492-C1501

17. O'Donnell ME, Chen YJ, Lam TI, Taylor KC, Walton JH, Anderson SE. Intravenous HOE-642 reduces brain edema and Na uptake in the rat permanent middle cerebral artery occlusion model of stroke: Evidence for participation of the blood-brain barrier Na/H exchanger. *J Cereb Blood Flow Metab*. 2013;33:225-234

18. O'Donnell ME, Tran L, Lam T, Liu XB, Anderson SE. Bumetanide inhibition of the blood-brain barrier Na-K-Cl cotransporter reduces edema formation in the rat middle cerebral artery occlusion model of stroke. *J Cereb Blood Flow Metab*. 2004;24:1046-1056

19. Betz AL, Keep RF, Beer ME, Ren X. Blood-brain barrier permeability and brain concentration of sodium, potassium, and chloride during focal ischemia. *J Cereb Blood Flow Metab*. 1994;14:29-37

20. Russell JM. Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process. *J Gen Physiol*. 1983;81:909-925

21. Sun D, Lytle C, O'Donnell ME. Astroglial cell-induced expression of Na-K-Cl cotransporter in brain microvascular endothelial cells. *Am J Physiol*. 1995;269:C1506-C1512

22. Wulff H, Miller MJ, Hänsel W, Grissmer S, Cahalan MD, Chandy KG. Design of a potent and selective inhibitor of the intermediate-conductance Ca^{2+}-activated K^+ channel, IKCa1: A potential immunosuppressant. *Proc Natl Acad Sci USA*. 2000;97:8151-8156

23. Coleman N, Brown BM, Olivan-Viguera A, Singh V, Olmstead MM, Valero MS, et al. New positive KCa channel gating modulators with selectivity for KCa3.1. *Mol Pharmacol*. 2014; 86:342-357

24. Chen Y-J, Raman G, Bodendiek S, O'Donnell ME, Wulff H. The KCa3.1 blocker TRAM-34 reduces infarction and neurological deficit in a rat model of ischemia/reperfusion stroke. *J Cereb Blood Flow Metab*. 2011;31:2363-2374

25. Lam TI, Wise PM, O'Donnell ME. Cerebral microvascular endothelial cell Na/H exchange: Evidence for the presence of NHE1 and NHE2 isofroms and regulation by arginine vasopressin. *Am J Physiol Cell Physiol*. 2009;297:C278-C289

26. Foroutan S, Brillault J, Forbush B, O'Donnell ME. Moderate-to-severe ischemic conditions increase activity and phosphorylation of the cerebral microvascular endothelial cell Na^+-K^+-Cl^- cotransporter. *Am J Physiol Cell Physiol*. 2005;289:C1492-1501

27. Strobaek D, Brown DT, Jenkins DP, Chen YJ, Coleman N, Ando Y, et al. NS6180, a new KCa3.1 channel inhibitor prevents T-cell activation and inflammation in a rat model of inflammatory bowel disease. *Br J Pharmacol*. 2013;168:432-444

28. Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J. A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci USA*. 1997;94:11651-11656

29. Joiner WJ, Wang LY, Tang MD, Kaczmarek LK. HSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc Natl Acad Sci USA*. 1997;94:11013-11018

30. Hoyer J, Popp R, Meyer J, Galla H-J, Gögelein J. Angiotensin II, vasopressin and GTP[gamma-s] inhibit inward-rectifying K^+ channels in porcine cerebral capillary endothelial cells. *J Membrane Biol*. 1991;123:55-62

31. van Renterghem C, Vigne P, Frelin C. A charybdotoxin-sensitive, Ca^{2+}-activated K^+ channel with inward rectifying properties in brain microvascular endothelial cells: Properties and activation by endothelins. *J Neurochem*. 1995;65:1274-1281

32. Millar ID, Wang S, Brown PD, Barrand MA, Hladky SB. Kv1 and Kir2 potassium channels are expressed in rat brain endothelial cells. *Pflugers Arch*. 2008;456:379-391

33. Brillault J, Lam TI, Rutkowsky JM, Foroutan S, O'Donnell ME. Hypoxia effects on cell volume and ion uptake of cerebral microvascular endothelial cells. *Am J Physiol Cell Physiol*. 2008; 294: C88-96

34. Halm ST, Liao T, Halm DR. Distinct K^+ conductive pathways are required for Cl^- and K^+ secretion across distal colonic epithelium. *Am J Physiol Cell Physiol*. 2006;291:C636-648

35. Linley J, Loganathan A, Kopanati S, Sandle GI, Hunter M. Evidence that two distinct crypt cell types secrete chloride and potassium in human colon. *Gut*. 2014;63:472-479

36. Al-Hazza A, Linley JE, Aziz Q, Maclennan KA, Hunter M, Sandle GI. Potential role of reduced basolateral potassium (IKCa3.1) channel expression in the pathogenesis of diarrhoea in ulcerative colitis. *J Pathol*. 2012;226:463-470

37. Sheikh IA, Koley H, Chakrabarti MK, Hoque KM. The EPAC1 signaling pathway regulates Cl^- secretion via modulation of apical KCNN4c channels in diarrhea. *J Biol Chem*. 2013;288:20404-20415

38. Kaushal V, Koeberle PD, Wang Y, Schlichter LC. The Ca^{2+}-activated K^+ channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide-dependent neurodegeneration. *J Neurosci*. 2007;27:234-244

39. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev*. 2011;91:461-553

40. Cahalan MD, Chandy KG. The functional network of ion channels in T lymphocytes. *Immunol Rev*. 2009;231:59-87

41. Ohana L, Newell EW, Stanley EF, Schlichter LC. The Ca^{2+} release-activated Ca^{2+} current (I(CRAC)) mediates store-operated Ca^{2+} entry in rat microglia. *Channels (Austin)*. 2009;3:129-139

42. Wong CO, Yao X. TRP channels in vascular endothelial cells. *Adv Exp Med Biol*. 2011;704:759-780

43. Song C, Al-Mehdi AB, Fisher AB. An immediate endothelial cell signaling response to lung ischemia. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L993-1000

44. Hu Q, Ziegelstein RC. Hypoxia/reoxygenation stimulates intracellular calcium oscillations in human aortic endothelial cells. *Circulation*. 2000;102:2541-2547

45. O'Donnell ME, Duong V, Suvatne S, Foroutan S, Johnson DM. Arginine vasopressin stimulation of cerebral microvascular endothelial cell Na-K-Cl cotransport activity is V1 receptor- and [Ca]-dependent. *Am J Physiol Cell Physiol*. 2005;289:C283-C292

46. Gillham JC, Myers JE, Baker PN, Taggart MJ. Regulation of endothelial-dependent relaxation in human systemic arteries by SKCa and IKCa channels. *Reprod Sci*. 2007;14:43-50

47. Marrelli SP, Eckmann MS, Hunte MS. Role of endothelial intermediate conductance KCa channels in cerebral EDHF-mediated dilations. *Am J Physiol Heart Circ Physiol*. 2003;285:H1590-1599

48. Si H, Heyken WT, Wolfle SE, Tysiac M, Schubert R, Grgic I, et al. Impaired endothelium-derived hyperpolarizing factor-mediated dilations and increased blood pressure in mice deficient of the intermediate-conductance Ca^{2+}-activated K^+ channel. *Circ Res*. 2006;99:537-544

49. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol Sci*. 2002;23:374-380

50. Feletou M, Vanhoutte PM. EDHF: New therapeutic targets? *Pharmacol Res*. 2004;49:565-580

51. Petersson J, Zygmunt PM, Hogestatt ED. Characterization of the potassium channels involved in EDHF-mediated relaxation in cerebral arteries. *Br J Pharmacol*. 1997;120:1344-1350

52. Petersson J, Zygmunt PM, Brandt L, Hogestatt ED. Substance P-induced relaxation and hyperpolarization in human cerebral arteries. *Br J Pharmacol*. 1995;115:889-894

53. McNeish AJ, Garland CJ. Thromboxane A2 inhibition of SKCa after no synthase block in rat middle cerebral artery. *Br J Pharmacol*. 2007;151:441-449

54. Feletou M, Girard V, Canet E. Different involvement of nitric oxide in endothelium-dependent relaxation of porcine pulmonary artery and vein: Influence of hypoxia. *J Cardiovasc Pharmacol*. 1995;25:665-673

55. Marrelli SP. Altered endothelial Ca^{2+} regulation after ischemia/reperfusion produces potentiated endothelium-derived hyperpolarizing factor-mediated dilations. *Stroke*. 2002;33:2285-2291

56. McNeish AJ, Sandow SL, Neylon CB, Chen MX, Dora KA, Garland CJ. Evidence for involvement of both IKCa and SKCa channels in hyperpolarizing responses of the rat middle cerebral artery. *Stroke*. 2006;37:1277-1282

57. Logsdon NJ, Kang J, Togo JA, Christian EP, Aiyar J. A novel gene, HKCa4, encodes the calcium-activated potassium channel in human T lymphocytes. *J Biol Chem*. 1997;272:32723-32726

58. Cipolla MJ, Smith J, Kohlmeyer MM, Godfrey JA. SKCa and IKCa channels, myogenic tone, and vasodilator responses in middle cerebral arteries and parenchymal arterioles: Effect of ischemia and reperfusion. *Stroke*. 2009;40:1451-1457

59. Longden TA, Dunn KM, Draheim HJ, Nelson MT, Weston AH, Edwards G. Intermediate-conductance calcium-activated potassium channels participate in neurovascular coupling. *Br J Pharmacol*. 2011;164:922-933

60. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics--2012 update: A report from the American Heart Association. *Circulation*. 2012;125:e2-e220

61. Wulff H, Castle NA. Therapeutic potential of KCa3.1 blockers: Recent advances and promising trends. *Expert Rev Clin Pharmacol*. 2010;3:385-396

62. Ataga KI, Orringer EP, Styles L, Vichinsky EP, Swerdlow P, Davis GA, et al. Dose-escalation study of ICA-17043 in patients with sickle cell disease. *Pharmacotherapy*. 2006;26:1557-1564

63. Ataga KI, Smith WR, De Castro LM, Swerdlow P, Saunthararajah Y, Castro O, et al. Efficacy and safety of the gardos channel blocker, senicapoc (ICA-17043), in patients with sickle cell anemia. *Blood*. 2008;111:3991-3997

64. Ataga KI, Reid M, Ballas SK, Yasin Z, Bigelow C, James LS, et al. Improvements in haemolysis and indicators of erythrocyte survival do not correlate with acute vaso-occlusive crises in patients with sickle cell disease: A phase III randomized, placebo-controlled, double-blind study of the gardos channel blocker senicapoc (ICA-17043). *Br J Haematol*. 2011;153:92-104

Figure Legends

Figure 1

KCa3.1 protein expression in cultured bovine CMEC: A) Western blots of lysates from freshly isolated rat brain microvessel endothelial cells and cultured bovine CMEC were conducted using a KCa3.1-specific primary antibody. The representative Western blot shows a ~47 KDa band consistent with KCa3.1 protein for both freshly isolated microvessels (single lane on the left) and cultured bovine CMEC (duplicate lanes on the right; the second lane is empty). B) Bovine CMEC grown on collagen-coated glass slides were subjected to ICC staining with anti-KCa3.1 antibody (left panel). Bound antibodies were visualized by DAB (brown) or secondary Ab only (right panel) as control slide. C) HEK-293 cell (left panel) stably expressing human KCa3.1 (used as positive control) and bovine CMEC (right panel) were subjected to IF staining with anti-KCa3.1 antibody.

Figure 2

KCa3.1 current in cultured bovine CMEC: A) Current recorded from a CMEC with a ramp-pulse from -120 to +40 mV and 3 μM free Ca^{2+} in the patch-pipette. After blocking the KCa current with 100 nM charybdotoxin (ChTX) a small Kir current (red) remains.; B) The KCa current is blocked by the KCa3.1-specific inhibitor TRAM-34 (IC_{50} 20 nM) but is insensitive to the KCa2 blocker apamin or the KCa1.1 blocker iberiotoxin (not shown).

Figure 3.

KCa3.1 is expressed on BBB endothelial cells *in situ*. Sections of rat brain (upper panels) and human brain (lower panels) were stained with rat BBB endothelial cell-specific marker (SMI-71), Von Willebrand factor (vWF) for human endothelial cells, and KCa3.1 antibody. Representative images are shown.

Figure 4

Bovine CMEC exhibit a TRAM-34 sensitive K^+ flux. TRAM-34 and NS6180 reduced K^+ efflux from bovine CMEC in a dose-dependent manner whereas neither Pax nor NS8593 significantly altered K^+ efflux from the cells. K^+ efflux in the presence of inhibitors relative to 20 mM Ca^{2+} control efflux was 0.86 ± 0.05, p<0.01 for 1 μM NS1680, and 0.9 ± 0.057 (p<0.01), 0.76 ± 0.086 (p<0.01), 0.63 ± 0.198 (p<0.01), and 0.58 ± 0.269 (p<0.01) for TRAM-34 at 100 nM, 250 nM, 500 TRAM-34, and 1 μM significantly reduced $^{86}Rb^+$ efflux compared to the vehicle group (0.84 ± 0.09, p<0.01, n=5). Lower doses of NS1680 250 nM (0.93 ± 0.14, p=0.052) reduced $^{86}Rb^+$ efflux but the effect was not statistically significant. ***Significantly different from Ringer with 20 mM Ca^{2+}, p<0.01.

Figure 5

TRAM-34 reduces brain Na^+ accumulation and edema formation in a rat MCAO model. A) Representative brain Na^+ uptake images in vehicle- and TRAM-34 treated rats after pMCAO. Na^+ chemical shift imaging (CSI) MRS with DyTTHA was used to

measure extravascular Na^+ content as described in Materials and Methods. Brighter orange indicates higher extravascular Na^+ content; B) Quantitated extravascular Na^+ content in the infarct core up to 170 min after induction of MCAO. n = 7 rats; C) Representative T2-weighted images for brain edema evaluation in vehicle- and TRAM-34 treated rats subjected to pMCAO. Brighter intensity indicates more edema; D) Quantitated Ipsilateral/Contralateral ADC ratios for Vehicle- and TRAM-34 treated rats up to 236 min from onset of MCAO, n=7. Values are mean ± SD. * significantly different than Vehicle, $p<0.05$.

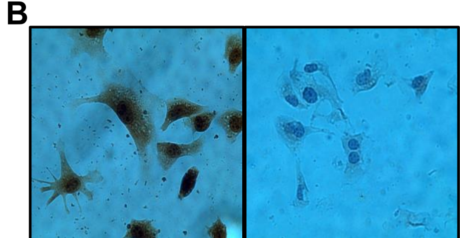
Figure 6

Hypothesized role of BBB KCa3.1 channels in edema formation during cerebral ischemia. During cerebral ischemia, a BBB luminal NKCC works with an abluminal Na/K pump and a Cl^- channel to cause transport of Na^+ and Cl^- (with water following) from blood in to brain. This secretion is greatly increased during ischemia by vasopressin (VP), hypoxia and aglycemia, three prominent factors present during cerebral ischemia. Blocking KCa3.1 might reduce NKCC activity by reducing intracellular Ca^{2+} and increasing intracellular Cl^- concentration.

Figure 1

A

B



C

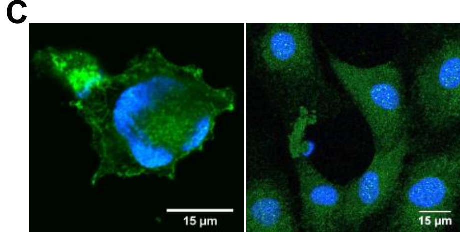


Figure 2

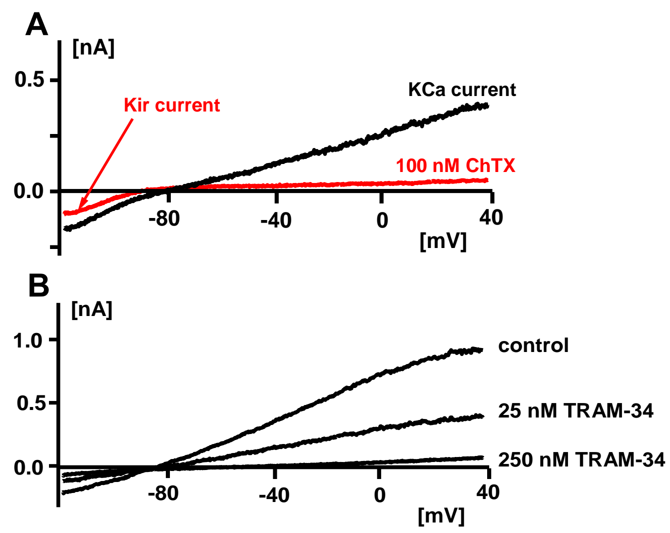


Figure 3

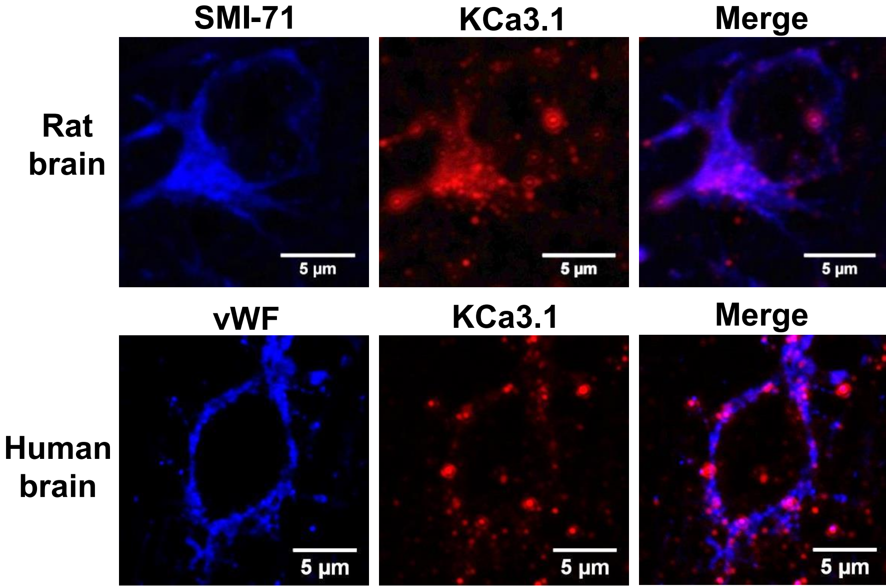


Figure 4

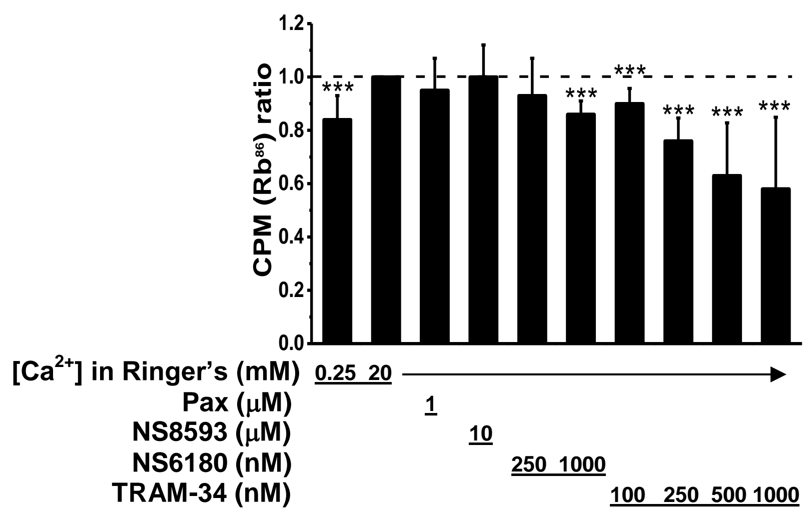


Figure 5

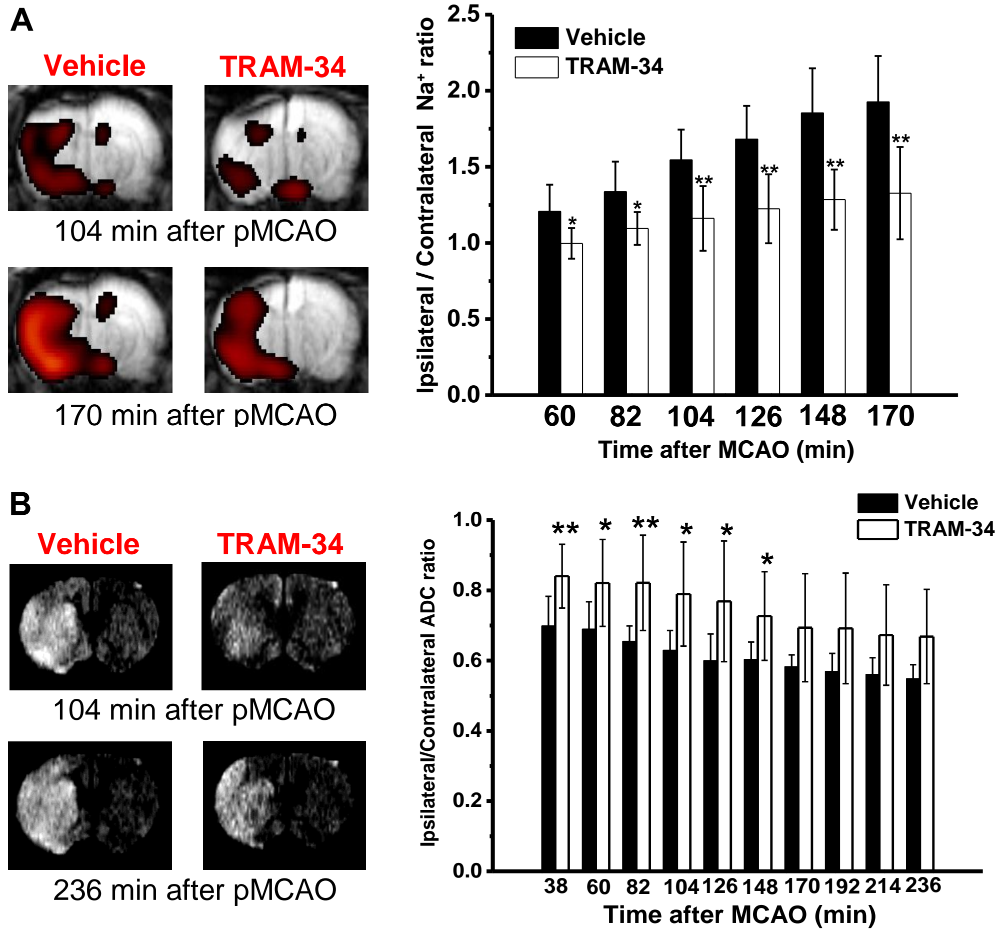


Figure 6