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Original Article

Neuroprotective effects of volume-regulated anion channel blocker DCPIB on neonatal hypoxic-ischemic injury

Ammar ALIBRAHIM[1, 2], Li-yan ZHAO[5], Christine You-jin BAE[2], Andrew BARSZCZYK[2], Christopher LF SUN[2], Guan-lei WANG[5, *], Hong-shuo SUN[1, 2, 3, 4, *]

[1]Departments of Surgery, [2]Physiology and [3]Pharmacology, [4]Institute of Medical Science, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8; [5]Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China

Aim: To evaluate the role of swelling-induced activation of volume-regulated anion channels (VRACs) in a neonatal hypoxic-ischemic injury model using the selective VRAC blocker 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on5-yl) oxobutyric acid (DCPIB).
Methods: Cerebral hypoxic-ischemic injury was induced in 7-day-old mouse pups with Rice-Vannucci method. Prior to the onset of ischemia, the animals were *ip* administered DCPIB (10 mg/kg). The animals were sacrificed 24 h afterwards, coronal sections of the brains were cut and the areas of infarct were examined using TTC staining and an image-analysis system. Cultured PC12 cells were subjected to oxygen-glucose deprivation (OGD) for 4 h. The cellular viability was assessed using Cell Counting Kit 8. Intracellular chloride concentration $[Cl^-]_i$ was measured using 6-methoxy-N-ethylquinolinium iodide.
Results: DCPIB-treated mice showed a significant reduction in hemispheric corrected infarct volume (26.65%±2.23%) compared to that in vehicle-treated mice (45.52%±1.45%, $P<0.001$). DCPIB-treated mice also showed better functional recovery as they were more active than vehicle-treated mice at 4 and 24 h post injury. In cultured PC12 cells, DCPIB (10 μmol/L) significantly reduced OGD-induced cell death. Moreover, DCPIB (20 μmol/L) blocked hypotonic-induced decrease in $[Cl^-]_i$ in PC12 cells of both control and OGD groups.
Conclusion: The results further support the pathophysiological role of VRACs in ischemic brain injury, and suggest DCPIB as a potential, easily administrable agent targeting VRACs in the context of perinatal and neonatal hypoxic-ischemic brain injury.

Keywords: neonatal stroke; neonatal hypoxic-ischemic injury; neuroprotection; volume-regulated anion channel; chloride channel; ClC-3; DCPIB; oxygen-glucose deprivation; PC12 cell

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Introduction

Perinatal and neonatal hypoxic-ischemic brain injury often leads to cerebral palsy, which is a major cause of acute mortality and chronic neurological morbidity in infants and children[1–5]. Cerebral palsy is a general term given to a group of cognitive, perceptive and motor disorders that are caused by abnormal development or damage to the parts of the brain that control motor function and activities. Cerebral palsy can affect various neural functions such as learning, thinking, hearing, seeing and movement in children. The perinatal cerebral hypoxic-ischemic brain damage and the neurological entities of cerebral palsy, epilepsy and mental retardation were originally described in the Little's Classic Treatise (Little WJ 1861)[6, 7]. Statistically, the prevalence of asphyxia is approximately 0.2% (2–4 per 1000) in full-term or near full-term newborn infants, and reaches 60% in premature or low-birth-weight newborn infants. The perinatal hypoxic-ischemic encephalopathy has a very high mortality rate, as about 20% to 50% of asphyxiated newborn infants actually terminate as early as during the newborn period. Among the survivors, up to 25% of asphyxiated newly born infants show permanent neuropsychological handicaps in the form of cerebral palsy either with or without epilepsy, learning disability and/or mental retardation[1, 7]. Depending on the severity of the disorder, an early diagnosis can be difficult depending on the severity of the disorder and currently there is no cure for cerebral palsy. Potential treatments and therapies are aimed at helping

* To whom correspondence should be addressed.
E-mail hss.sun@utoronto.ca (Hong-shuo SUN);
wangglei@mail.sysu.edu.cn (Guan-lei WANG)
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cerebral palsy patients to reach their fullest individual potential and capacity of independency; these often require frequent and lifelong rehabilitation. Thus, disabilities in afflicted survivors of infant hypoxic-ischemic brain injury directly affect quality of life of these children and further have significant social and economic impacts on our society. The lifetime costs of cerebral palsy to the healthcare system were estimated at 11.5 billion dollars, according to 2003 US statistics[8, 9]. Thus, finding the causes and eventual cure for this childhood disease is an important goal for our medical researchers.

Neonatal encephalopathy is associated with neonatal hypoxic-ischemic injuries or neonatal stroke, and affects 1-6 per 1000 live full-term births[10]. Neonatal encephalopathy results in disturbed neurological functions and activities in newborn infants due to problems in initiating and maintaining respiration. It is clinically defined as a syndrome of disturbed neurological functions in the early days of newborn infant life with apparent evidence in difficult starting and maintaining breathing, poor awareness and consciousness, inadequate and declined reflexes and muscle tone, and even seizure activities. Along with these, neonatal encephalopathy increases the chances of developing neurodevelopmental disabilities, such as cerebral palsy, after birth. Depending on severity, neonatal encephalopathy can cause the development of cognitive disorders or death.

Perinatal cerebral hypoxic-ischemic brain damage is one of the major causes of acute mortality and chronic disability in infants and children. However, the underlying mechanisms of the neonatal stroke are still largely unclear. There are few animal models available in studying the neonatal hypoxic-ischemic brain injury and neonatal stroke[11, 12]. With the understanding of what we have learned from the stroke mechanisms in adult animal models, we investigated one of the non-glutamate mechanisms[13], the volume-regulated anion channels (VRAC), in neonatal stroke using mouse neonatal hypoxic-ischemic injury model[11, 12]. We are interested in developing prevention and therapeutic strategies of neuroprotection for neonatal stroke and thus its subsequent disorder, cerebral palsy.

Chloride (Cl^-) is the most abundant anion under physiological conditions. It permeates the cell membrane through several types of Cl^- channels that have different gating characteristics, including ligand-, voltage-, and Ca^{2+}-gated channels[14]. An important class of Cl^- channels is the volume-regulated anion channel (VRAC). Principally, VRACs are responsible for mediating the swelling-induced Cl^- current, which also plays an important role in a cell's regulatory mechanism for decreasing cell volume during osmotic perturbations. In the brain, the VRACs are widely expressed and have been proposed to play an important role in cerebral ischemia[13]. VRAC is ubiquitously expressed and mediates Cl^- efflux when a cell faces hypo-osmotic swelling. VRAC-mediated Cl^- efflux is normally paired with that of K^+, bringing along osmotically-obliged water and consequently giving rise to a regulatory volume decrease. However, the molecular identities and the *in vivo* roles of the VRACs in ischemia and stroke are not yet clear[15].

ClC-3 channel has been proposed as one of the candidates for VRACs[16, 17]. Studies have shown that ClC-3 channel plays an important role in myocyte volume regulation during heart ischemia *in vivo* using *ClCn3*$^{-/-}$ mice[18, 19] as well as in redox signalling and vascular remodelling in vascular cells[20, 21]. ClC-3 channels are expressed in the brain[22, 23] and the recent evidence suggests that ClC-3 may also engage in neuronal excitability[22], but their pathophysiological role in cerebral ischemia and stroke has not yet been investigated.

There is a rich pharmacology for different types of Cl^- channels. 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and tamoxifen, which are both potent but non-selective Cl^- channel blockers, have been extensively studied. Although the molecular identities of VRACs remain elusive, a study[24] showed that 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) is a novel selective blocker for the swelling-induced Cl^- current and therefore of VRACs as well.

In conditions of cerebral ischemia, a prolonged excitotoxicity — the overstimulation of neurons by the neurotransmitter glutamate — causes an excessive depolarization, which drives water inflow and consequently cell swelling. Persistent depolarization caused by excitotoxicity results in Cl^- influx through VRACs (rather than its normal efflux as part of the regulatory volume decrease), which further contributes to swelling and eventually necrotic neuronal death[25]. In a recent study, the specific VRAC inhibitor DCPIB showed a neuroprotective effect in reducing the infarction and improving neurobehavioral scores in a rat transient middle cerebral artery occlusion (MCAO) model only when the drug was delivered intracisternally[26]. Here we report that the selective VRAC blocker DCPIB showed a promising neuroprotective effect in a mouse neonatal stroke model as well as some supporting evidence from *in vitro* study.

Methods and materials

Materials and drugs

Timed-pregnant CD1 mice were from Charles River Laboratories (Sherbrooke, Quebec, Canada) and were used in the study. Only one nursing female mouse and her litters were allowed per cage with a free access to food and water, in a room with an ambient temperature of 20±1 °C and a 12:12 h light/dark cycle. All experiments using these animals strictly followed the guidelines of the Canadian Council on Animal Care (CCAC protocol) in science and all animal experimental procedures were approved by the local Animal Care and Use Program Committee, Office of the Research Ethics at the University of Toronto.

Drugs used were from Sigma-Aldrich Canada: 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB), Sigma catalogue # D4068, was dissolved first in 0.2% dimethyl sulfoxide (DMSO) then diluted in 1% phosphate buffered saline (PBS) for the final concentration of 10 mg/kg.

Surgical preparation

Mouse pups were anesthetized with isoflurane and skin in

the neck was cleaned with iodine followed by 75% alcohol. The midline ventral incision was made in the anterior neck. DCPIB was delivered intraperitoneally. The body temperature was monitored and maintained using a Harvard Apparatus temperature control and heating blanket system.

Drug administration

Twenty minutes prior to the onset of ischemia, DCPIB (10 mg/kg)[27] with 0.2% dimethyl sulfoxide (DMSO) (treatment group) and 0.2% DMSO alone (control group) were diluted in 1% phosphate buffered saline (PBS) before intraperitoneally administering to the mouse pups. Total volume injected per animal was 0.1 mL.

Hypoxic-ischemic injury model

Postnatal seven-day-old (P7) CD1 wild-type mouse pups of either sex were used in the study. The Rice-Vannucci[28] neonatal adaptation of Levine[29] procedure with some modifications was used to induce cerebral hypoxic-ischemic injury in the neonatal mice. Appropriate measures were taken to ensure that discomfort and pain were minimized. P7 mice weighing 5 to 5.5 g were anesthetized with 3% isoflurane in balance of oxygen for 3 min as induction, followed by 2% isoflurane for maintaining during the procedure. A stereo dissecting microscope (SMZ-2B Nikon, Japan) with fiber-optic bifurcation and ring lens illumination was used. After a 0.5-cm midline cervical incision and a separation of the muscles covering the frontolateral neck with a fine-tip forceps, the right common carotid arteries were exposed, separated from accompanying vagus and sympathetic nerves, and occluded by bipolar electrical coagulation (Vetroson V-10 Bi-polar electrosurgical unit). For the model development, the local cerebral blood flow was measured using the PeriMed PeriScan System PIM II Laser Doppler Blood Perfusion Imager (PeriMed, Stockholm, Sweden) to ensure the sufficient local cerebral blood flow reduction in the ipsilateral hemisphere. Normal body temperatures were maintained by using a homeothermic heating blanket. The procedure took approximately 10 min for each pup to complete. After the surgical procedure, the wounds were closed and the pups were placed in an incubator at 37 °C for 10 min until fully awake, after which they were returned to their dam for at least 90 min to completely recover and feed.

For the hypoxic component of the insult, the pups were transferred into an airtight, transparent chamber (A-Chamber A-15274 with ProOx 110 Oxygen Controller/E-720 Sensor, Biospherix, NY, USA) and perfused with a humidified gas mixture that contained 7.8% oxygen balanced with 92.2% nitrogen. Gas flowed at a constant rate for 50 min and oxygen concentration was regulated by a compact oxygen controller (ProOx 110 controller, Biospherix, NY, USA), to which a compressed nitrogen gas source (Linde, Mississauga, ON, Canada) was attached. One pup was monitored to ensure that body temperature did not exceed 36.5 °C by using homeothermic blanket control unit (K-017484 Harvard Apparatus, Massachusetts, USA). After the hypoxia exposure, the mouse pups were recovered on a heating pad (33 °C) for 30 min and later returned to their mother in the dam.

Measurement of infarct volume

After twenty-four hours, the animals were then sacrificed and the brains were removed and cut coronally into approximately 1 mm sections. The sections were then immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 1% phosphate buffered saline (PBS) at 37 °C in a dark place for 20–30 min[30].

After TTC staining, the brain slices were scanned and the areas of infarct were measured. The area of ipsilateral hemisphere and that of the contralateral hemisphere were measured with an image-analysis system (NIH ImageJ software) for each section. The volumes were calculated by summing the representative areas in all sections and multiplying by the slice thickness. After correcting for edema, the volumes of infarction were calculated as follows:

Corrected infarct volume (CIV), (%)=[contralateral hemisphere volume–(ipsilateral hemisphere volume–infarct volume)]/ contralateral hemisphere volume×100 [31].

Cell culture and reagents

PC12 cells were cultured as previously described[32]. PC12 (rat pheochromocytoma) cells were cultured in RPMI-1640 (Invitrogen, USA) supplemented with 10% heat-inactivated horse serum (Invitrogen, USA) and 5% heat-inactivated fetal calf serum (Invitrogen, USA) in a humidified incubator containing 5% CO_2 at 37 °C. PC12 cells were cultured on collagen-coated plates or dishes when different experiments were performed.

Oxygen-glucose deprivation (OGD)

OGD was implemented using methods as described[32]. Briefly, 24 h after PC12 cells were seeded in 96 wells plates, the culture medium was changed to the glucose-free DMEM or glucose-free DMEM containing 10 μmol/L DCPIB. Cells were cultured under normoxic incubating conditions for 30 min, followed by being placed in an anaerobic chamber that was flushed with 5% CO_2 and 95% N_2 (*v/v*). The anaerobic chamber was kept in a humidified incubator at 37 °C. Four hours later, the culture medium was changed to the normal medium containing the same concentration of DCPIB and the plates were returned to the normoxic incubating conditions.

Cellular viability

Cellular viability was assessed using Cell Counting Kit 8 (Beyotime, China) according to the instruction from the company. Briefly, 18 h after OGD, 10 μL CCK8 solution was added into 100 μL culture medium in the 96 well plate, wells without cells acted as control. The plates were then placed in a humidified incubator at 37 °C with 5% CO_2 and 95% air (*v/v*) at 90% humidity for 2 h. Finally, the spectrophotometric absorbance was measured using a microplate reader at an absorbance wavelength of 450 nm.

Measurement of $[Cl^-]_i$

Intracellular chloride concentration $[Cl^-]_i$ was measured using 6-methoxy-N-ethylquinolinium iodide (MEQ) as previ-

ously described in our publication[33]. Briefly, dihydro-MEQ was freshly prepared before the measurement. Cells were incubated, at room temperature in the dark for 30 min, with 100–150 μmol/L dihydro-MEQ in a Ringer's buffer containing (mmol/L): 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose, and pH 7.4. At room temperature, the MEQ reduction was performed by adding 32 μmol/L sodium borohydride to MEQ solution under flowing nitrogen in the dark for 30 min. In cytoplasm, dihydro-MEQ was quickly oxidized to MEQ, which is sensitive to $[Cl^-]_i$, and the fluorescence quenching induced by Cl^- was measured by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) with 350-nm excitation wavelength and 435-nm emission wavelength. Percentage decrease of $[Cl^-]_i$ with the exposure to hypotonic solution was calculated from the equation of $([Cl^-]_{i\ iso}-[Cl^-]_{i\ hypo})/[Cl^-]_{i\ iso}*100\%$; percentage inhibition of $[Cl^-]_i$ by DCPIB was calculated from the equation of $([Cl^-]_{i\ DCPIB}-[Cl^-]_{i\ hypo})/([Cl^-]_{i\ iso}-[Cl^-]_{i\ hypo})*100\%$.

Statistics and data analysis

Data are presented as means±SEM. Statistical analysis was carried out using SigmaStat (3.0, Jandel Scientific). Student's *t*-test was performed to assess the statistical significance of the difference in two groups between control group and DCPIB treated group. In multiple groups, one-way ANOVA with Bonferroni test were used. Significance was defined by the probability level of lower than 0.05 ($P<0.05$).

Results and discussion

In vivo study: effects of DCPIB on neonatal hypoxic-ischemic brain injury in mice

The Rice-Vannucci method of neonatal hypoxic-ischemic injury produced an area of brain injury confined from the ipsilateral brain hemisphere to the common carotid occlusion as shown in the TTC stain, which is in white color (Figure 1A). The contralateral hemisphere has no demonstrable brain injury with the TTC stain, which is in red color (Figure 1A). The mean percentage of right hemispheric corrected infarct volume was 45.5%±1.45% (n=5) in vehicle treated animals, which was significantly reduced to 26.65%±2.23% (n=5) in DCPIB treated animals (P=<0.001) (Figure 1B). Individual coronal sections of both vehicle and DCPIB treated groups were also plotted and graphed (Figure 2); the DCPIB also significantly reduced the infarctions in individual stereotaxic coronal plates in comparison to that of the vehicle treated group (Figure 2). In addition, the hypoxic-ischemic mouse pups treated with DCPIB recovered better than that of the vehicle treated control group. The DCPIB treated group was more active from 4 and 24 h after the hypoxic-ischemic injury. Thus the selective VRAC blocker DCPIB reduced the neonatal hypoxic-ischemic brain injury and improved the behavioural outcomes in the 24 h after the insults.

In vitro study: effects of DCPIB on OGD and intracellular chloride concentration

From Stern-Volmer equation, the resting $[Cl^-]_i$ in PC12 cells of control group and OGD group were 24.71±1.28 mmol/L and 40.49±2.44 mmol/L, and there were significant differences between the two groups (P<0.01, n=30, Figure 3). After perfusion with hypotonic solution, the $[Cl^-]_i$ in control and OGD

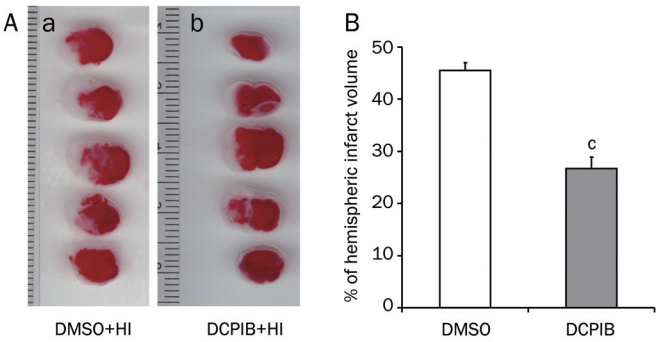

Figure 1. Effects of DCPIB in reducing infarction size in mouse neonatal hypoxic-ischemic injury model. (A) Representative infarcts in 2% 2,3,5-Triphenyltetrazolium Chloride (TTC) stained coronal sections from vehicle (DMSO) (A) and DCPIB (B) treated groups at 24 h after the neonatal hypoxic-ischemic injury in P7 mice. (B) Bar graph shows the effects of intraperitoneal (ip) treatment with DCPIB (ip 10 mg/kg in 0.2% DMSO) on brain hemispheric infarct volume compared to that of the vehicle DMSO treated animals. DCPIB given 20 min before initiation of ischemia reduced infarct volume significantly. (^cP<0.01, n=5 per groups, Student *t*-test).

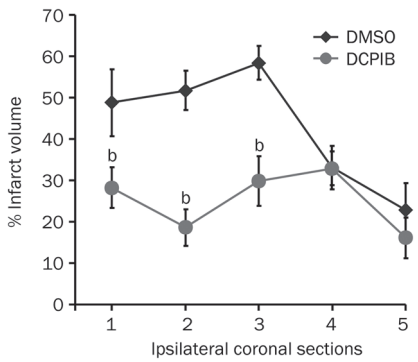

Figure 2. Individual coronal sections of ipsilateral brains also show reduction of the infarction in mouse neonates treated with DCPIB in comparison to that of vehicle DMSO treated group. (n=5 per groups, ^bP<0.05).

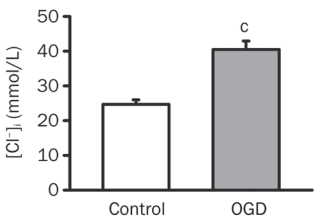

Figure 3. Intracellular Cl^- concentration ($[Cl^-]_i$) of PC12 cells in isotonic solution from control and OGD groups. Bar graph shows that OGD treated cells had significant higher resting $[Cl^-]_i$. (n=30, ^cP<0.01).

groups were decreased to 21.47±1.19 mmol/L and 36.55±2.181 mmol/L, respectively, which resulted in 13.66%±0.91% and 9.60%±0.45% decrease in $[Cl^-]_i$ as compared with their isotonic controls, and there were significant differences (P<0.01, n=30, Figure 4). The percentage inhibition of reduction in $[Cl^-]_i$ by DCPIB (20 μmol/L) in PC12 cells from control and OGD groups are 12.99%±1.54% and 11.80%±1.48%, there was no significant difference between the two groups (n=7, P>0.05, Figure 5), indicating that DCPIB could block hypotonic-induced decrease in $[Cl^-]_i$ change in both control and OGD groups.

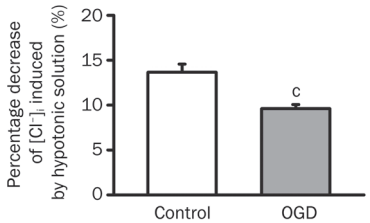

Figure 4. The percentage decrease of $[Cl^-]_i$ induced by hypotonic perfusion in PC12 cells from control and OGD groups. Bar graph shows that the percentage decrease was significantly lower in OGD treated cells. (n=30, ^cP<0.01).

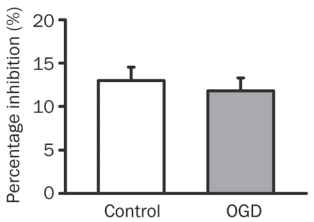

Figure 5. The percentage inhibition of reduction in $[Cl^-]_i$ by DCPIB (20 μmol/L) in PC12 cells from control and OGD groups. Bar graph shows that there is no significant difference between the two groups. (n=7, P>0.05).

We further tested the *in vitro* effect of DCPIB on cell viability in PC12 model of OGD. OGD in PC12 cell line has been used as a rapid and sensitive *in vitro* model of ischemic stroke in searching for potential neuroprotective agent. After 4 h of OGD, incubation with DCPIB (10 μmol/L) for 18 h significantly attenuated cell death induced by OGD and increased cell survival (P<0.001, Figure 6). DCPIB alone at the given concentration did not show any toxic effects on cultured cells. The data from *in vitro* study thus suggested that DCPIB at the concentration of 10 μmol/L was able to block OGD-induced injury in PC12 cells.

In summary, we studied the neuroprotective effect of this selective VRAC blocker DCPIB in neonatal hypoxic-ischemic injury model in P7 mice. This drug has been shown to be effective in reducing the infarct volumes in the adult MCAO model[26]. In our study, intraperitoneally administered DCPIB effectively and significantly reduced the brain damages induced by the neonatal hypoxic-ischemic injury in P7 mice,

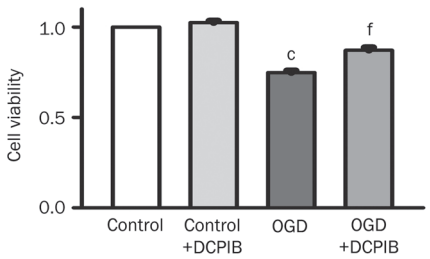

Figure 6. Effect of DCPIB on cell viability in PC12 model of OGD. Bar graph shows the effect of treatment with DCPIB (10 μmol/L) on the survival of cultured PC12 cells at 18 h after 4 h of OGD. The cell viability was determined by Cell Counting Kit 8 assay and indicated by OD values. Treatment with DCPIB attenuated OGD-induced cell injury. Values are mean±SEM. (^cP<0.01 *vs* control group, ^fP<0.01 *vs* OGD group, n=6/groups, one way ANOVA with Bonferroni test).

and improved the neurobehavioral outcomes. This implied that the blood-brain barrier is less developed in the perinatal and neonatal mice. In addition, the DCPIB was able to block, partially but significantly, the apparently VRAC mediated cell death *in vitro* under OGD condition. The DCPIB also reversed the changes in the intracellular Cl^- concentration under hypotonic condition in the cultured cells. Collectively, the findings strongly suggest that the volume-regulated anion channel or volume-regulated chloride channel, which is one of the newly recognized non-glutamate mechanisms, is involved in triggering intracellular ion imbalance and cell death in ischemia. Even the molecular identities of the VRAC are not yet fully understood, studies have suggested chloride ClC-3 channels may be one of the VRAC channels[16, 17]. Further studies will be needed to provide evidence for the VRAC identity and blocking of Cl^- current using DCPIB should be confirmed using electrophysiological tests. In conclusion, we demonstrated that 1) the VRAC is one of the non-glutamate mechanisms in cerebral ischemia and potential therapeutic target for stroke, and 2) the selective VRAC blocker DCPIB has the potential for prevention and treatment of human neonatal stroke and its subsequent neurological disorders including cerebral palsy. Further study will be needed to test the pharmacology of this drug.

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Author contribution

Hong-shuo SUN and Guan-lei WANG designed and coordinated the overall project. Ammar ALIBRAHIM carried out *in vivo* work. Li-yan ZHAO carried out *in vitro* experiment. Christine You-jin BAE, Andrew BARSZCZYK, and

Christopher LF SUN assisted experiment, data analysis and manuscript preparation. Hong-shuo SUN, Guan-lei WANG, Ammar ALIBRAHIM, Li-yan ZHAO, Christine You-jin BAE, and Christopher LF SUN wrote the paper. All authors discussed the results and commented on the manuscript.

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