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Article

Blockade of the swelling-induced chloride current attenuates the mouse neonatal hypoxic-ischemic brain injury *in vivo*

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Abstract

Activation of swelling-induced Cl^- current ($I_{Cl,swell}$) during neonatal hypoxia-ischemia (HI) may induce brain damage. Hypoxic-ischemic brain injury causes chronic neurological morbidity in neonates as well as acute mortality. In this study, we investigated the role of $I_{Cl,swell}$ in hypoxic-ischemic brain injury using a selective blocker, 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl) oxybutyric acid (DCPIB). In primary cultured cortical neurons perfusion of a 30% hypotonic solution activated $I_{Cl,swell}$, which was completely blocked by the application of DCPIB (10 μmol/L). The role of $I_{Cl,swell}$ in neonatal hypoxic-ischemic brain injury *in vivo* was evaluated in a modified neonatal hypoxic-ischemic brain injury model. Before receiving the ischemic insult, the mouse pups were injected with DCPIB (10 mg/kg, ip). We found that pretreatment with DCPIB significantly reduced the brain damage assessed using TTC staining, Nissl staining and whole brain imaging, and improved the sensorimotor and vestibular recovery outcomes evaluated in neurobehavioural tests (i.e. geotaxis reflex, and cliff avoidance reflex). These results show that DCPIB has neuroprotective effects on neonatal hypoxic-ischemic brain injury, and that the $I_{Cl,swell}$ may serve as a therapeutic target for treatment of hypoxic-ischemic encephalopathy.

Keywords: neonatal stroke; neonatal hypoxic-ischemic brain injury; volume regulated anion channels; chloride channels; cortical neurons; whole-cell recording; DCPIB; neurobehavioural tests; neuroprotection

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Introduction

Neonatal hypoxic-ischemic (HI) brain injury is a condition that occurs when the brain is deprived of adequate oxygen[1,2]. In infants and children, HI brain damage is a major cause of acute mortality and chronic neurological disability, such as hypoxic-ischemic encephalopathy (HIE)[3]. The leading cause of HI brain injury is birth asphyxia, which causes 23% of all neonatal deaths worldwide[4,5]. Although the prevalence of asphyxia in full-term newborn infants is ~0.2% (2–4 per 1000), it reaches ~60% in premature or low birth weight newborns. Mortality rate is high at ~20% to 50% of those afflicted. Furthermore, survivors suffer from severe motor impairments and learning disabilities[2,5]. This is in part because HI brain injury often results in HIE and/or cerebral palsy, a generic term describing a group of perceptive, cognitive, and motor disorders resulting from damage to or abnormal development of the brain[6]. Children with cerebral palsy have affected neurological functions (*eg* vision, auditory, *etc*), and depending on the severity, early detection of cerebral palsy can be challenging[6-8]. Currently, there is no cure available; standard therapeutic strategies are aimed at assisting patients with cerebral palsy to develop into their full potential of independency[7,8]. However, lifelong rehabilitation is often necessary[7,8]. Therefore, survivors of infant HI brain injury suffer lasting neurological damage, which negatively influences their quality of life. Moreover, the lifetime cost to the healthcare system is ~$1.5 million per person in Canada[9]. Thus, HI brain injury and HIE represent a serious socioeconomic burden, and there is urgent need to elucidate the underlying mechanisms in order to develop a more effective prevention and treatment.

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However, the mechanisms underlying neonatal HI remain ill-defined.

An important cellular role of the volume-regulated anion channels (VRACs), a major class of ubiquitously expressed Cl^- channels, is the regulation of cell volume[10]. This is accomplished through the efflux of Cl^- when the cell experiences hypo-osmotic swelling[10,11]. Consequently, VRACs mediate the swelling-induced Cl^- current $I_{Cl,swell}$. This has major implications on cell volume homeostasis (*ie* to decrease cell volume during osmotic perturbations). VRACs are highly expressed in the brain, and the swelling-induced chloride current $I_{Cl,swell}$ has been suggested to be involved in cerebral ischemia[10,12]. Under physiological conditions, hypo-osmotic swelling activates swelling-induced chloride current $I_{Cl,swell}$ via mechanosensory mechanisms (that is still poorly understood), which results in Cl^- efflux. This efflux is coupled with K^+ efflux, which leads to the expulsion of water osmotically[13]. As a result, cell volume is reduced, thus retaining homeostasis. Nonetheless, it should be noted that the molecular identities of the channels underlying the swelling-induced Cl^- current $I_{Cl,swell}$ remain controversial[13]. Despite this, however, there is evidence demonstrating that 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) is a selective antagonist that potently inhibits the swelling-induced Cl^- current $I_{Cl,swell}$ [14]. Under pathological conditions, prolonged neuron overstimuation (*eg* by glutamate) results in excitotoxicity that causes excessive depolarization[15]. This, in turn, drives water inflow and consequently results in cell swelling[15]. Due to the persistent depolarization, there is influx of Cl^- (rather than efflux) through the swelling-induced chloride current $I_{Cl,swell}$ (*ie*, opposite phenomenon compared to the normal regulatory volume decrease mechanism under physiological conditions). Consequently, a positive feedback for further swelling is established, and ultimately necrotic neuronal death is induced.

Because swelling-induced Cl^- current $I_{Cl,swell}$ is normally inactive[15], the rationale is that pharmacologically reducing swelling-induced Cl^- current $I_{Cl,swell}$ activity would not affect normal brain function. Thus, swelling-induced Cl^- current $I_{Cl,swell}$ is a suitable potential drug target for treatment of HI brain injury, and inhibition of swelling-induced Cl^- current $I_{Cl,swell}$ is a feasible therapeutic strategy. Previous publication described the role of the swelling-induced Cl^- current $I_{Cl,swell}$ in mediating adult stroke damage[16]. The authors found that pharmacologically inhibiting the swelling-induced Cl^- current $I_{Cl,swell}$ with DCPIB endowed neuroprotection by reducing infarction and improving neurobehavioural scores in a rat transient middle cerebral artery occlusion (MCAO) model when the drug was delivered intracisternally[16]. In our laboratory, we previously described the pharmacological role of DCPIB in reducing HI brain injury *in vivo*[17], and suppressing glioblastoma cellular functions *in vitro* by inhibiting the swelling-induced Cl^- current $I_{Cl,swell}$[18]. Additionally, we also reported that pharmacologically inhibiting the swelling-induced Cl^- current $I_{Cl,swell}$ *in vitro* with DCPIB decreased the hypotonic-inducing intracellular Cl^- concentration, and reduced the oxygen and glucose deprivation (OGD)-induced cell death in PC12 cell line[17]. Moreover, we showed preliminary evidence that the swelling-induced Cl^- current $I_{Cl,swell}$-dependent mechanism was involved partially in neonatal HI brain injury[17]. However, there is currently no *in vivo* behavioural assessment demonstrating whether inhibition of the swelling-induced Cl^- current $I_{Cl,swell}$ by DCPIB can confer neuroprotection at the level of neurobehavioural performance. Here, we show that: 1) *in vitro*, DCPIB inhibits the swelling-induced Cl^- current $I_{Cl,swell}$ in cortical neurons; and 2) *in vivo*, inhibiting the swelling-induced Cl^- current $I_{Cl,swell}$ with DCPIB treatment results in reduced brain damage, and improved short-term neurobehavioural performance following the HI insult. Thus, the swelling-induced Cl^- current $I_{Cl,swell}$ -mediated outcomes represent a non-glutamate stroke mechanism that plays critical role in both adult and neonatal variations of multiple brain diseases. There is broad implication because, ultimately, the goal of our work is contribution towards the development of effective therapeutic neuroprotective agents for reducing HI brain injury and its related HIE.

Materials and methods

Reagents

All reagents, unless specified otherwise, were from Sigma-Aldrich (Canada). DCPIB (Sigma catalogue # D4068) was first dissolved in 0.2% dimethyl sulfoxide (DMSO), and then diluted in 1% phosphate buffered saline (PBS) for the final concentration of 10 mg/kg.

Animals

CD1 timed-pregnant mice were purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada). Each cage only housed one nursing female mouse and her litter. Food and water were readily accessible, and maintained in a room with an ambient temperature of 20±1°C and a 12:12 h light/dark cycle. Experiments involving animals followed the guidelines of the Canadian Council on Animal Care (CCAC) protocol. All animal experimental protocols were approved by the local Animal Care and Use Program Committee, Office of the Research Ethics at the University of Toronto.

Surgical preparation

Prior to surgery, mouse pups were anesthetised with isoflurane. The skin was cleaned with iodine followed by 75% alcohol before incision. A midline ventral incision was conducted at the anterior neck. DCPIB was given via intraperitoneal administration. A temperature control and heating blanket system (Harvard Apparatus) was used to monitor/maintain body temperature.

Hypoxic-ischemic brain injury model

Seven-day-old (P7) postnatal CD1 wild-type mouse pups of either sex were used. As previously described[17,19], a modified Rice-Vannucci neonatal adaptation of the Levine procedure was used to induce cerebral HI injury in the mouse pups. Appropriate protocols were undertaken to minimize discomfort and pain of animals throughout the common carotid artery occlusion (CCAO) procedure. In brief, P7 mice (weigh-

ing ~5 to 5.5 g) were anesthetised with 3% isoflurane for 3 min as induction, and then followed by 2% isoflurane for maintenance during the procedure. We used a stereo dissecting microscope containing ring lens illumination and fiber-optic bifurcation (SMZ-2B Nikon, Japan). After conducting the midline cervical incision (0.5 cm) and separating the muscles at the frontolateral neck, the right common carotid arteries become exposed. These were separated from the associated sympathetic and vagus nerves, and then occluded via bipolar electrical coagulation (Vetroson V-10 Bi-polar electrosurgical unit, Summit Hill Laboratories, Tinton Falls, NJ, USA). Animal body temperature was maintained with a homeothermic heating blanket. For each pup, the procedure took ~5 min for each pup. After surgery, wounds were closed and the pups recovered in a 37 °C incubator for 10 min (or until regaining consciousness). Afterwards, pups were returned to their dam (at least 90 min) for further recovery.

To induce hypoxia, pups were placed into an airtight chamber (A-Chamber A-15274 with ProOx 110 Oxygen Controller/E-720 Sensor, Biospherix, NY, USA), into which a humidified gas mixture (7.8% oxygen balanced with 92.2% nitrogen) was perfused. Flow of gas was constant for 60 min. A compact oxygen controller (ProOx 110 controller, Biospherix, NY, USA) with a compressed nitrogen gas source (Linde, Mississauga, ON, Canada) was used to regulate oxygen concentration. A homeothermic blanket control unit (K-017484 Harvard Apparatus, Massachusetts, USA) monitored and maintained animal body temperature at 36.5 °C. After exposure to hypoxia, pups recovered on a heating pad and then returned to their dam.

Drug administration

Mouse pups weighing ~5 g were randomly assigned into sham group, control HI group or DCPIB-treated HI group. DCPIB (10 mg/kg) with 0.2% DMSO (DCPIB-treated HI group) or 0.2% DMSO alone (control HI group) was diluted in 1% PBS before intraperitoneal injection into the mouse pups. Drug injection was done ~20 min before CCAO for all tests listed below except for the TTC test (~1 h after CCAO). The total volume injected per mouse pup was 0.1 mL.

Neurobehavioral tests

Two neurobehavioral tests (*ie* geotaxis, cliff avoidance reaction) were used to assess the recovery outcomes of the mouse pups on Day 1, Day 3, and Day 7 after the HI insult. These tests are well-documented[20], and were carried out as previously described[21-27]. In brief, geotaxis reflex is an automatic, stimulus-bound orientation movement. It is considered to be diagnostic of vestibular and/or proprioceptive function. Here, pups were placed head facing down in the middle of a plane inclined at an angle of 45°, and the latency time for the pup to rotate 180° (*ie*, head facing up) was recorded. Cliff avoidance reaction evaluates the integration of exteroceptive input and locomotor output. For this test, pups were placed on the edge of a platform, and the latency time for the pup to remove both paws from the edge by turning away from the cliff was recorded.

Measurement of infarct volume

TTC staining: 24 h after the HI episode, mouse pups were sacrificed and the brains were extracted and coronally cut into ~1 mm sections, which were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 1% PBS at 37°C in the dark (20–30 min). TTC is a redox indicator that can indicate cellular respiration for differentiating between metabolically active and inactive tissues. After stained with TTC, brain slices were scanned and infarct areas were measured, as previously described[24-27]. An image analysis system (NIH ImageJ) was used to quantify the ipsilateral and contralateral hemisphere area for each section. Volumes were determined by summing the representative areas in all sections and then multiplying by slice thickness. Edema correction was conducted. Infarction volume was calculated with the following formula: CIV (%)=[CHV-(IHV-IV)]/CHV*100; where CIV is corrected infarct volume, CHV is contralateral hemisphere volume, IHV is ipsilateral hemisphere volume, and IV is infarct volume.

Nissl staining/whole brain imaging: Seven days after the HI episode, whole brain tissue was collected and imaged to reveal morphological changes between the groups, as previously described[24-27]. At this point, the infarct areas have undergone liquefactive necrosis. The severity of the brain damage was quantified. The brains were sliced into ~100 μm coronal sections and stained with 1% Cresyl violet (Nissl) to indicate histological damage. Infarct area was imaged using ImageJ, and was calculated as follows: IV (%) = IV/CHV*100.

Primary mouse neuron culture

Primary cortical neurons were cultured from E16 CD1 mice, as previously described [28]. In brief, cortices were isolated and then digested using 0.025% trypsin/EDTA (37 °C; 15 min). An Improved Neubauer hemocytometer was used to measure cell density. Cells were plated at 1.0×10^4 on poly-*D*-lysine coated glass coverslips (Sigma; 12 mm No 1 German Glass, Bellco cat. No 1943-10012). Neurons were maintained in serum-free culture Neurobasal medium supplemented with 1.8% B-27, 0.25% Glutamax, and 1% antibiotic-antimyocotic. Incubation of cells was in 5% CO_2 at 37 °C.

Patch clamp electrophysiology

Recordings were carried out in the whole-cell configuration using the Axopatch 700B (Axon Instruments, Inc), similar to our previous studies[18,29-31]. The ramp protocol was from -100 to +100 mV (400 ms; -70 mV holding; 5 s interval at 2 kHz; digitized at 5 kHz). Data acquisition and analyses were via Clampex 9.2 and Clampfit 9.2. All experiments were performed at room temperature. The pipette (intracellular) solution contained (in mmol/L): 145 CsMSF, 8 NaCl, 55 KCl, 10 HEPES, 1 MgATP, and 10 EGTA (pH adjusted to 7.2 with CsOH). After filling with solution, pipette resistance was 8–13 MΩ. The bath (extracellular) solution contained (in mmol/L): 140 NaCl, 5 KCl, 2 $CaCl_2$, 20 HEPES, and 10 glucose (pH adjusted to 7.4 with NaOH; osmolarity to ~300 mOsm with sucrose).

Statistic analysis

GraphPad Prism 6 (GraphPad Software, Inc) was used for statistical analysis. The statistical significance between control and DCPIB-treatment group was assessed with the Student's *t*-test. In multiple groups, one-way ANOVA with Bonferroni multiple comparison test was used. Significance was defined as $P<0.05$. Data are shown as mean±SEM.

Results and discussion

Since the molecular identities of the swelling-induced Cl^- channels remain elusive, we first measured the electrophysiological properties of the swelling-induced Cl^- current $I_{Cl,swell}$ in primary neurons and pharmacologically verified with DCPIB (Figure 1). When exposed to 30% hypotonic solution, there was activation of swelling-induced Cl^- current $I_{Cl,swell}$, with a reversal potential that is consistent with what we previously reported[18]. In the continued presence of the hypotonic solution, application of DCPIB (10 μmol/L) inhibited the activated current (Figure 1A and 1B). As shown in the summary graph (Figure 1C), perfusion of the hypotonic solution activated a swelling-induced Cl^- current $I_{Cl,swell}$, which was fully inhibited by subsequent application of DCPIB (*ie*, hypotonic solution increased current density to 4.05±0.49 pA/pF from 1.76±0.17 pA/pF, $P<0.01$; DCPIB administration abolished this increase by reducing current density back to 1.87±0.11 pA/pF, $P<0.01$). Therefore, the swelling-induced Cl^- current $I_{Cl,swell}$ is present in primary neurons and sensitive to inhibition by DCPIB.

Next, we provide the first *in vivo* evidence demonstrating the neuroprotective effects of a selective swelling-induced Cl^- current $I_{Cl,swell}$ antagonist DCPIB on improving behavioural functional outcomes after HI brain injury. As shown in the schematic in Figure 2A, we used a well-documented mouse hypoxic-ischemic (HI) model (Rice-Vannucci method) with modifications[17,19]. The key events throughout the procedure have been noted on the timeline in Figure 2B. Our model of neonatal HI brain injury formed a brain infarct that is confined from the ipsilateral brain hemisphere to the common carotid occlusion, as shown from the TTC stain in Figure 3A (white section of the brain; extent of damage 24 h following HI). On the contrary, the contralateral hemisphere did not demonstrate brain injury (red section). This finding is consistent with our previous report[17], noting the reliability of our HI model. The right hemispheric corrected infarct volume was 46.38%±3.76% ($n=23$) in animals treated with vehicle (control HI group; Figure 3B). When DCPIB was injected ~1 h after common carotid artery occlusion (CCAO), the corrected infarct volume was significantly reduced to 31.75%±5.14% (DCPIB-treated HI group; $P<0.05$). This suggested that inhibition of the swelling-induced Cl^- current $I_{Cl,swell}$ with DCPIB can reduce the severity of brain damage.

Seven days after the HI, the brains of mouse pups were extracted to examine the extent of morphological and histological damage. As shown in Figure 4, integrity of brain morphology in the control HI group is severely compromised seven days after HI brain injury. However, for animals in the DCPIB-treated HI group, the morphology of these brains is qualitatively more comparable to the brains in the sham group. Consistent with this observation, Nissl staining to assess histological damage also shows a similar trend. That is, the DCPIB-treated HI group showed significantly reduced infarction volume from 41.79%±3.23% to 10.21%±2.08% ($P<0.05$; $n=4$/group; Figure 4). This is the first evidence to suggest the lasting neuroprotective effect of DCPIB against HI brain injury, as even only one administration of DCPIB ~20 min before CCAO elicited an improvement one week after the HI.

We further quantitatively assessed recovery outcomes of the HI by employing short term neurobehavioural tests (*ie* geotaxis reflex, and cliff aversion reflex). These reflex tests were chosen because they are representative of the earliest development stages in mice, and thus are good indicators of sensorimotor function[20]. Specifically, the geotaxis reflex assesses vestibular and proprioceptive functions, whereas the cliff aversion reflex measures the maladaptive impulse behav-

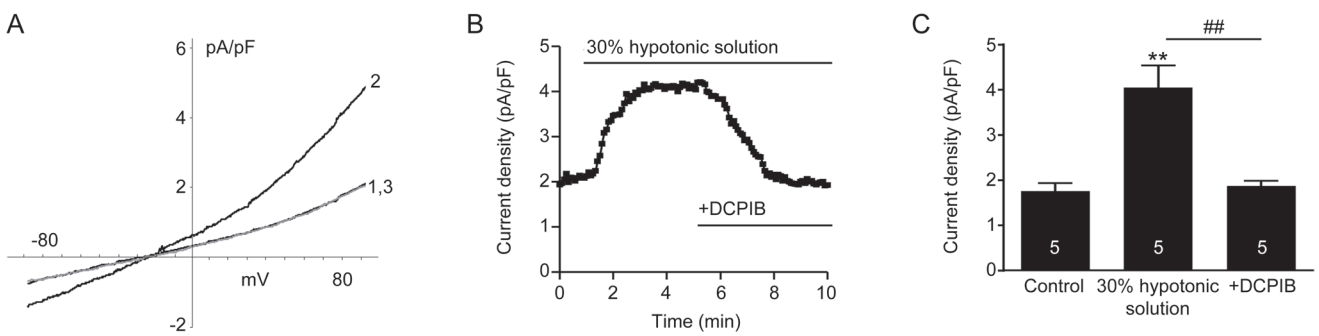

Figure 1. In primary cortical neurons, the swelling-induced Cl^- current $I_{Cl,swell}$ is inhibited by DCPIB. (A) Current density-voltage trace where: 1 is bath solution; 2 is with perfusion of 30% hypotonic solution; and 3 is with application of DCPIB (10 μmol/L) in the continued presence of hypotonic solution. Holding potential was -70 mV. (B) Representative time course plotting the outward current at +80 mV. (C) Comparison summary chart of the outward current (at +80 mV) of: control bath solution, activation of swelling-induced Cl^- current $I_{Cl,swell}$ with hypotonic solution, and subsequent inhibition with DCPIB in the continued presence of hypotonic solution. $^{**}P<0.01$ *vs* control. $^{\#\#}P<0.01$ *vs* 30% hypotonic solution, respectively ($n=5$/group; Repeated-measure One-way ANOVA with Bonferonni multiple comparison test).

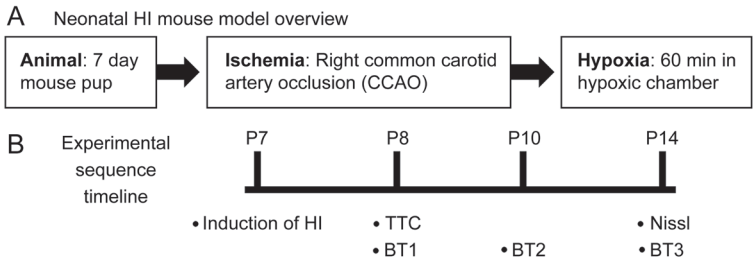

Figure 2. Schematic of the *in vivo* neonatal hypoxic-ischemic (HI) mouse model to examine the effects of DCPIB. (A) Overview of the process involved in developing the model, which contained two main parts: ischemia and hypoxia. First, using postnatal day 7 (P7) mice, ischemia was induced by isolation of the right common carotid artery and then subsequent ligation with a bipolar electrocoagulation device (common carotid artery occlusion; CCAO). Following recovery from the surgery, hypoxia was then induced by placing operated pups in a 37 °C chamber perfused with a gas mixture of 7.5% oxygen and 92.5% nitrogen for 60 min. (B) Timelines of the experimental sequences for model development as well as the subsequent histological, morphological, and behavioural assessments. The timeline was carried out across three groups: sham, control HI, and DCPIB-treated HI. The "control HI group" represents HI-induced animals administered with vehicle only; "DCPIB-treated HI group" represents HI-induced animals treated with DCPIB. Infarct volume measurement with TTC was carried out 24 h after HI (*ie*, when pups were P8), whereas measurement with Nissl was 7 days after (*ie* when pups were P14). Neurobehavioural performance (BT; behavioural test) was assessed at three separate time points; at one, three, and seven days after HI (*ie*, when pups were P8, P10, and P14, respectively).

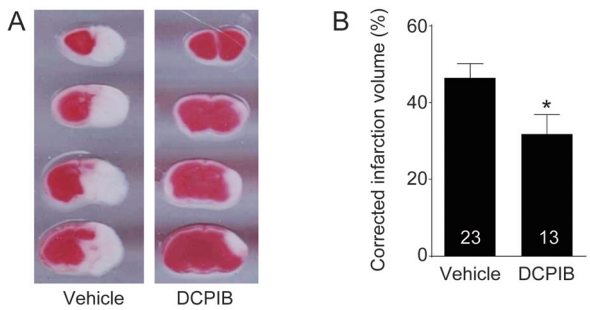

Figure 3. Treatment with DCPIB reduced brain infarct volume of neonatal hypoxic-ischemic (HI) brain injury *in vivo*. (A) Representative 2,3,5-triphenyltetrazolium chloride (TTC) images of the infarcts of the coronal sections of P8 mouse pup brains treated with vehicle (control HI group) or 10 mg/kg DCPIB (DCPIB-treated HI group). (B) Column chart summarizing that animals in the DCPIB-treated HI group (n=13) had significantly reduced brain infarct volume compared to those in the control HI group (n=23; $^{*}P$<0.05; Student's t-test).

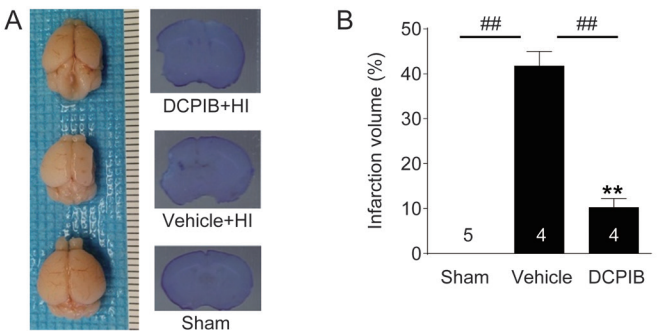

Figure 4. DCPIB-treated animals showed less morphological and histological damage following HI. (A) Left panel shows representative whole brain images of P14 mouse pups in either the sham group (bottom), control HI group (middle), or DCPIB-treated HI group (top). Note that morphological integrity is severely compromised for the control group brain, when compared to the treatment and sham group brains. Right panel displays the corresponding representative images of Nissl staining. (B) Summary column chart showing that intraperitoneal (ip) treatment of DCPIB (10 mg/kg in 0.2% DMSO) 20 min before induction of HI significantly reduced infarct volume measured seven days following the HI episode ($^{**}P$<0.01 *vs* Sham. $^{\#\#}P$<0.01 *vs* vehicle, respectively; n=4/group; One-way ANOVA with Bonferonni multiple comparison test), thus suggesting the lasting neuroprotective effectiveness of DCPIB.

iour[20]. These neurobehavioral tests determine the functional recovery of animals after the insult. A longer time indicates slower reflex, which is considered a negative score (*ie*, lower time is better). Each test was conducted one, three, and seven days after the HI. Animals in all groups (*ie*, sham, control HI, and DCPIB-treated HI) were subjected to these tests (Figure 5). On Day 1, pups in the control HI group had the slowest reflex for both geotaxis and cliff avoidance (at 3.61±0.17 and 7.42±0.18 s, respectively; P<0.05 for both). Treatment with DCPIB improved reflex speed of animals in the DCPIB-treated HI group (to 2.86±0.06 and 5.94±0.51 s, respectively), which is not significant compared to animals in the sham group (1.93±0.27 and 4.76±0.31 s, respectively). On Day 3, control HI group animals still had the slowest cliff avoidance reflex, at 6.30±0.93 s (P<0.05), which was improved with DCPIB treatment (3.80±0.38 s; note that this score was not significantly different compared with the animals in the sham group at 2.99±0.58 s). Although pups in the control HI group did not significantly have a slower geotaxis reflex on Day 3, it was clearly gravitating towards this trend. One possible explanation is that at Day 3, mouse pups become P10, which is when their eyes begin to open. The sudden acquisition of vision can potentially confer drastic improvement to vestibular and proprioceptive performance despite suffering HI. This remains to be tested. Nevertheless, by Day 7, geotaxis reflex of the control HI group had markedly worsened. This was ameliorated with inhibition of the swelling-induced chloride current $I_{Cl,swell}$ by DCPIB (from 3.15±0.54 s in the control HI group to 1.44±0.14 s in the DCPIB-treated HI group; P<0.05). However,

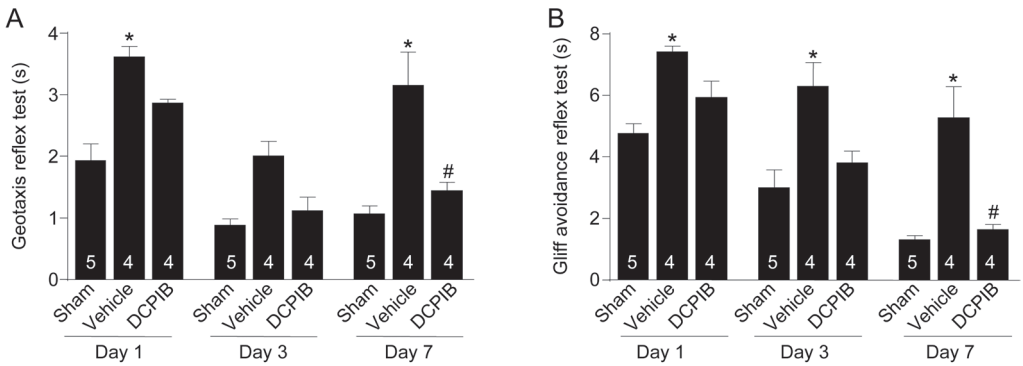

Figure 5. Neurobehavioural performance after HI is improved with DCPIB treatment. (A) Summary of geotaxis reflex test on Day 1, Day 3, and Day 7 (when mouse pups were P8, P10, and P14, respectively) across animals from all groups: sham (n=5), control HI (n=4), and DCPIB-treated HI (n=4). Animals in the control HI group consistently had a slower reflex, either significant ($^*P$<0.05) or clearly trending towards significance. Note that comparisons were made between groups on each respective day (compared to the sham group). The general trend is that DCPIB-treatment improves geotaxis reflex towards that of sham animals. (B) Summary of cliff avoidance reflex test on Day 1, Day 3, and Day 7 (when mouse pups were P8, P10, and P14, respectively) across animals from all groups: sham (n=5), control HI (n=4), and DCPIB-treated HI (n=4). Animals in the control HI group consistently had a significant slower reflex ($^*P$<0.05). Note that comparisons were made between groups on each respective day (compared to the sham group). The general trend is that DCPIB-treatment improves cliff avoidance reflex towards that of sham animals. (One-way ANOVA with Bonferonni multiple comparison test).

note that at this time point, DCPIB-treated HI group animals still performed worse than sham group animals (at 1.07±0.13 s; P<0.05); nevertheless, the difference was minimal and the trend of improvement was clearly present. Similarly, animals in the DCPIB-treated group also demonstrated improvement in cliff avoidance reflex at Day 7; reflex time was reduced from 5.27±1.02 s (control HI group) to 1.64±0.17 s (DCPIB-treated HI group; P<0.05). However, although evidently trending towards the same reflex levels as animals in the sham group (at 1.31±0.14 s), pups in the DCPIB-treated HI group remained significantly slower (P<0.05). Nevertheless, we provide the first evidence that a single injection of DCPIB ~20 min prior to CCAO can elicit a lasting neuroprotective effect that ultimately improves neurobehavioural performance.

In our previous study, we focused on the *in vitro* effects of DCPIB by demonstrating the effects of DCPIB on OGD and intracellular chloride concentration[17]. Specifically, we showed that the resting $[Cl^-]_i$ significantly increased following OGD in the PC12 cell line, and that DCPIB (10 μmol/L) reduced this influx. Furthermore, we demonstrated that treating PC12 cells with DCPIB significantly reduced cell death induced by OGD, and ultimately increased cell survival. In addition, DCPIB alone did not show any toxicity on cultured cells, which was suggestive of safety[17]. This *in vitro* work gave evidence that inhibition of the swelling-induced Cl^- current $I_{Cl,swell}$ can ameliorate OGD-induced injury. Recently, we showed that DCPIB inhibited the swelling-induced Cl^- current $I_{Cl,swell}$, and consequently suppressed the malignant cellular functions (*ie*, proliferation, migration, and invasion) of glioblastoma cell lines (*ie*, U87, U251)[18]. This suggests that DCPIB is an effective swelling-induced Cl^- current $I_{Cl,swell}$ antagonist across multiple cell types. In our present study, we examined the *in vivo* effects of DCPIB using a neonatal mouse HI model. Specifically, we assessed the behavioural, histological, and morphological aspects of the HI insult with or without DCPIB treatment. This is unknown in the literature and represents a knowledge gap, because inhibition of the swelling-induced Cl^- current $I_{Cl,swell}$ can potentially be exploited as an effective therapeutic strategy for neonatal HI brain injury. Moreover, DCPIB has been reported to be effective in reducing the infarct volumes of adult MCAO model[16], which further articulates the broad implications and therapeutic feasibility of DCPIB in ischemia. However, in adult brain ischemia, DCPIB only demonstrated neuroprotection when given intracisternally, but not intravenously. This was in part due to an impedance of drug delivery by the developed blood brain barrier (BBB)[16]. In our previous[17] and current studies, however, we showed evidence that intraperitoneal administration of DCPIB was able to cross the immature BBB of the perinatal or neonatal brain. That is, DCPIB treatment exerted neuroprotection during HI insults by significantly reducing brain damage and improving neurobehavioural outcomes. The BBB in neonates may be immature and undergoing developmental changes, thus favouring drug penetration into the neonatal brain, especially during ischemic conditions which can disrupt the BBB integrity[27]. Here, we showed critical *in vivo* evidence for the pathophysiological role of the swelling-induced Cl^- current $I_{Cl,swell}$ in the HI brain injury. Moreover, we provided important findings for potential drug development of swelling-induced Cl^- current $I_{Cl,swell}$ antagonists such as DCPIB as a readily administrable drug for prevention and treatment of the HI brain injury. In conclusion, we established that the effects mediated by the swelling-induced Cl^- current $I_{Cl,swell}$ represent a non-glutamate mechanism of the neonatal HI brain injury (Figure 6). Thus, the swelling-induced Cl^- current $I_{Cl,swell}$ is exploitable as a drug target (by using selective potent antagonists; *eg*, DCPIB) in order to prevent and treat human neonatal HI brain injury in addition to its consequential neurological disorders such as

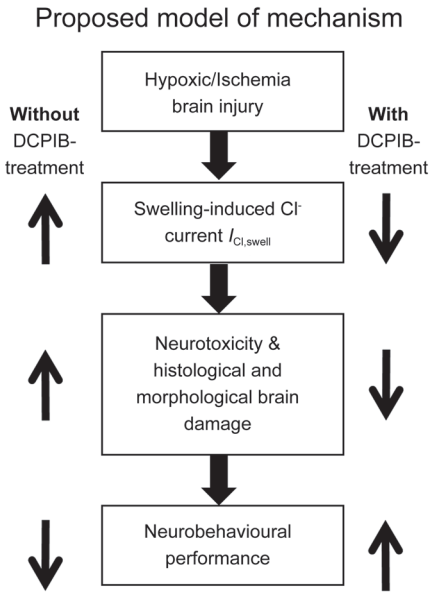

Figure 6. Schematic of proposed model of mechanism underlying the swelling-induced Cl^- current $I_{Cl,swell}$ in HI brain injury. Following hypoxic and ischemic insult, there is aberrant activity of the swelling-induced Cl^- current $I_{Cl,swell}$. This can lead to neuronal death and result in brain damage. Ultimately, neurobehavioural performance may be negatively affected. However, this outcome can be reversed with treatment using the specific swelling-induced Cl^- current $I_{Cl,swell}$ antagonist DCPIB. Aberrant swelling-induced Cl^- current $I_{Cl,swell}$ activity is reduced. Consequently, there is less neuronal death and brain damage, which would contribute to improved neurobehavioural performance.

hypoxic-ischemic encephalopathy and cerebral palsy.

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Author contribution

Hong-shuo SUN and Zhong-ping FENG developed the concepts and designed the study. Raymond WONG, Ahmed ABUSSAUD, Joseph WH LEUNG, Bao-feng XU, Sammen HUANG, and Fei-ya LI performed experiments and analyzed the data; Raymond WONG and Hong-shuo SUN wrote the manuscript; all authors discussed the results and commented on the manuscript.

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