Activation of TRESK background potassium channels by cloxyquin exerts protective effects against excitotoxic-induced brain injury and neuroinflammation in neonatal rats

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Abstract

The excitotoxicity is a common pathological mechanism of perinatal brain injuries (PBI), however neuroinflammation resulting in PBI is both a cause and a consequence of excitotoxicity. TRESK background potassium channels are an important regulator of neuronal excitability. We therefore investigated effects of activation of TRESK channels by selective activator cloxyquin on excitotoxic-induced brain injury and neuroinflammation involving brain mast cells and inflammatory cytokines in neonatal rats.

An excitotoxic model mimicking human perinatal brain lesions was established via intracerebral injection of the glutamatergic agonist ibotenate to newborn rats. P5 rat pups were intraperitoneally pretreated with vehicle, three different doses of cloxyquin (0.2, 1 and 5 mg/kg), or NMDA receptor antagonist MK-801 (positive control) 30 minutes prior to intracerebral injection of 10 µg ibotenate. Rat pups were sacrificed one or five days after the injury. Coronal brain sections were stained with cresyl-violet for histopathological examinations, and with toluidine-blue for brain mast cells assessments. Concentrations of activin A, IL-1 β , IL-6 and IL-10 in brain homogenates were measured using ELISA.

Cloxyquin dose-dependently ameliorated ibotenate-induced impairments in the cortical and white matter, and suppressed ibotenate-induced activation and number of brain mast cells. Moreover, cloxyquin dose-dependently reduced concentrations of activin A, IL-1 β and IL-6 in the brain tissue induced by ibotenate while it elevated IL-10 level.

Our findings reveal for the first time that cloxyquin, a selective activator of TRESK channels, dose-dependently exerted protective effects against excitotoxic-induced neonatal brain injury and neuroinflammation. TRESK channels may be a promising new target for the treatment of PBIs.

Introduction

Perinatal brain injury (PBI) is a common neurological problem characterized by long-term neurodevelopmental impairments such as cognitive, neurologic, motor and sensory disturbances. These emerging neurological problems are substantially associated with cerebral white and/or gray matter damage. PBI occurs in approximately 3-5/1000 live term and preterm births [1]. It is caused by a wide range of etiological factors such as genetic, hypoxic-ischemia, intracranial haemorrhage, neonatal stroke, maternal-fetal inflammation, oxidative stress and excitotoxicity.

Although these factors seem to act in combination to lead to injury in the developing brain, excitotoxicity is considered as a key and common final molecular mechanism of neuronal damage that plays a role in the pathogenesis of many acute and chronic neurological disorders including PBI [2, 3]. Excitotoxicity is mostly due to excessive or prolonged activation of excitatory receptors, in particular glutamatergics, as a result of excessive release or insufficient re-uptake of glutamate neurotransmitter. Physiological levels of glutamate is required for vital neurodevelopmental events such as proliferation, migration, and improvement of synapses in the developing brain [4]. However, excessive activation of ionotropic

glutamate receptors especially NMDA results in toxic accumulation of intracellular Ca^{2+} ions that leads to the death of neuronal cells [5]. Moreover, the immature brain is more vulnerable to excitotoxic insults than the adult brain, therefore modulation of excitotoxicity is of vital importance in preventing PBI or in developing possible therapeutic approaches.

Besides the excitotoxicity, the inflammation is also involved in the pathogenesis of PBI with observed white matter damage and can lead to neurodegeneration [6, 7]. The excitotoxicity is capable of inducing the inflammation, and it was demonstrated that excitotoxicity-induced inflammation contributed to the brain damage through release of pro-inflammatory cytokines from the brain-resident immune cells in rodent models of perinatal brain injury [8, 9]. The increased release of pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α during the perinatal period contributes to the neonatal brain damage by increasing blood-brain barrier permeability and exacerbating inflammatory responses [10–12]. Previous studies have reported that plasma and cerebrospinal fluid levels of IL-1 β , IL-6 and TNF- α were higher in the neonates with hypoxic-ischemic encephalopathy [10, 13, 14]. Although microglia and mast cells, resident immune cells of the central nervous system (CNS), are both major source of pro-inflammatory cytokines in the brain, brain mast cells come into prominence as they are the first responders in the injury [15–17]. Emerging evidence has recently highlighted the important role of brain mast cells in neurological diseases including perinatal brain injuries. A few studies demonstrated that brain mast cells are involved in perinatal brain damage in rodent models of neonatal hypoxic-ischemic or excitotoxic brain injury [16, 18, 19]. Thus, new strategies targeting inhibition of both excitotoxicity and brain mast cells may be considerably effective in preventing perinatal brain damage.

Background potassium channels are responsible for the regulation of the resting membrane potential and neuronal excitability. TWIK-related spinal cord potassium channel (TRESK) is a member of the two-pore-domain potassium channel (K2P) family that is the source of background potassium currents. TRESK channels are widely expressed in neurons in the brain, spinal cord, dorsal root and trigeminal ganglia [20]. Activation of these channels restricts neuronal excitability by facilitating hyperpolarizing potassium outflow. Previous studies have reported that TRESK channel activation exerts anti-nociceptive effects by restraining neuronal excitation, and also plays a role in cell survival and development [21, 22]. However, there is no data yet on the effects of TRESK activation on the excitotoxic brain injury and excitotoxicity-induced neuroinflammation during the perinatal period.

Based on the hyperpolarizing activity of TRESK channels, we hypothesize that activation of TRESK channels by a selective activator may prevent glutamatergic action-induced excitotoxic brain injury and relevant neuroinflammation by restraining the neuronal hyperexcitability through the potassium outflow. We therefore aimed to investigate effects of activation of TRESK channels by selective activator cloxyquin on white and gray matter damage, brain mast cells and inflammatory cytokines in model of ibotenate-induced excitotoxic brain injury in newborn rats.

Materials And Methods

Animals

Experimental procedures were approved by Bolu Abant Izzet Baysal University Animal Experiments Local Ethics Committee (No: 2019/17). P5 rat pups of both sexes were used in the experiments, and the rat pups were provided from the Animal House of the University of Bolu Abant Izzet Baysal, Turkey. Animals were maintained according to the Guide for the Care and Use of Laboratory Animals (Eighth Edition). The rat pups were housed in the same cage with their mothers with a 12-h light/dark cycle at $22 \pm 2^{\circ}\text{C}$. Mother rats were warranted ad libitum with a standard rodent diet and water.

Drugs and Reagents

Ibotenate and MK-801 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cloxyquin (5-Chloro-8-hydroxyquinoline) was purchased from Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany). Toluidine blue dye and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cresyl violet was purchased from Isolab (Wertheim, Germany). Formaldehyde solution was purchased from Merck (Darmstadt, Germany). Rat interleukin (IL)-1 β , IL-6 and IL-10 ELISA kits were purchased from ELABscience (Wuhan, P.R. China). Rat Activin A ELISA Kit PicoKine was purchased from Boster (Pleasanton, CA, USA). Ibotenate was diluted in PBS containing 0.01% acetic acid. MK-801 was diluted in PBS. Cloxyquin was initially dissolved in DMSO to constitute a stock solution, then further diluted with PBS to achieve final concentrations.

Experimental groups and procedures

The rat pups were randomly divided into eight groups as follows: naive control, sham control, ibo, ibo+DMSO, ibo+cloxy-1, ibo+cloxy-2, ibo+cloxy-3 and ibo+MK-801. While six rat pups in each group ($n=6$) were used for histopathological examinations, seven rat pups in each group ($n=7$) were used for biochemical analyses. Naive control group was not touched, and it was used to determine basal values of biochemical parameters. The sham group received an intraperitoneal (i.p.) injection of 10 μl PBS and 30 minutes later received an intracerebral (i.c.) injection of 2 μl PBS containing 0.01% acetic acid. Ibo group received an i.p. injection of 10 μl PBS and 30 minutes later received an i.c. injection of 10 μg ibotenate. Ibo+DMSO group received an i.p. injection of 10 μl PBS containing 1% DMSO and 30 minutes later received an i.c. injection of 10 μg ibotenate. Ibo+cloxy-1, ibo+cloxy-2 and ibo+cloxy-3 groups received i.p. injections of 0.2, 1 and 5 mg/kg cloxyquin and 30 minutes later received i.c. injections of 10 μg ibotenate, respectively. Ibo+MK-801 group as a positive control received an i.p. injection of 1 mg/kg NMDA receptor antagonist MK-801 and 30 minutes later received an i.c. injection of 10 μg ibotenate.

Establishment of rat model of neonatal excitotoxic brain injury

Ibotenate is a glutamatergic agonist and activates N-methyl-D-aspartate (NMDA) and metabotropic glutamatergic receptors (mGluR1 and mGluR5). Intracerebral injection of ibotenate to rodents has been widely used to establish an animal model of excitotoxic brain lesions characterized by cortical lesions and white matter cysts that mimic perinatal brain injury in human neonates. All ibotenate injections were

intracerebrally administered on the fifth post-natal day (P5), as described previously [23, 24]. Shortly, under isoflurane anesthesia, 10 µg of ibotenate (diluted 5 µg/µl in acetic acid 0.01%) was stereotactically administered into the neopallial parenchyma at the level of right hemisphere using a 5 µl Hamilton syringe. The needle was placed 2 mm depth from the outer surface of the skin. The needle tip was placed in the frontoparietal area of the right hemisphere, 2.5 mm from the midline in the lateral-medial plane, and 4 mm from the bregma in the rostro-caudal plane. Histopathological observation affirmed that the needle tip at every turn arrived at the periventricular white matter. Two 1 µl boluses of ibotenate was administered at 20 second intervals. The needle was remained in place for another 20 seconds.

Histological examinations of white and gray matter damage and brain mast cells

For histological examinations of white and gray matter damage and brain mast cells, five days after the injury (at P10) brains were perfused with 10 mL PBS (pH 7.4) infused via the intracardiac route to remove blood from the brain tissue. Then, the brains were removed, fixed in 4% formalin and left in the same fixative for 5 days. Then, the brains were embedded in paraffin, and 16-µm thick coronal sections were cut along the entire brain from the frontal to the occipital pole. Then while every third section was stained with cresyl violet for histopathological examinations, every fourth section was stained with toluidine blue (0.1% in 2.5 pH sodium chloride) for brain mast cells examinations. The size of white matter lesions, and the thickness of the cortical and subcortical white matter were defined and measured ipsilaterally as described previously [2, 24]. The ipsilateral measurements were carried out by a blinded observer using the software (infinity 3 analyze release 6.5). On the other hand, the brain mast cells were counted throughout the entire section around vessels running close to the outer surface of the cortex and were classified as either degranulated or non-degranulated by a blinded observer. They were postulated degranulated in case of extensive scattering exceeding 15 extruded granules in the proximity of the cell, or of extensive loss of granule staining, bestowing a “ghostly” appearance on the cell [25, 26]. Total numbers of brain mast cells and their percentages of degranulation were calculated. Microscopic images of brain mast cells were taken using a camera (ZEISS Axiocam 503 color) attached to the microscope (ZEISS Axio scope A1, Germany).

Determination of activin A and inflammatory cytokines concentrations in brain tissue supernatants by ELISA

Activin A is a neuronal survival factor (growth and differentiation factor) belonging to the transforming growth factor β family. The level of activin-A in the brain tissue increases rapidly as a response to the damage to protect the neurons and/or to repair the neuronal damage during perinatal brain injuries [27, 28]. Moreover, in case of the injury, the level of pro-inflammatory cytokines that are the first to respond to inflammation rapidly changes in the brain tissue [12]. For these reasons, the rat pups used for measurement of these biomarkers (activin A, IL-1 β , IL-6 and IL-10) were sacrificed one day after the injury (at P6). Following perfusion of the brain with 10 mL PBS, whole brain (without the cerebellum) was homogenized at 4°C in fixed volumes (100 mg wet tissue/1 ml) of PBS (pH 7.4) containing a protease

inhibitor cocktail using a light-duty Ultra-Turrax homogenizer (ISOLAB, Wertheim, Germany). The brain homogenates were centrifuged at 26,000 g for 30 min at 4°C, and brain supernatant samples were obtained. Activin A, IL-1 β , IL-6 and IL-10 concentrations in the supernatant samples were determined using ELISA kits in accordance with the manufacturers' instructions, the protocols being implemented as duplicates. The detection limit was < 12 pg/ml for Activin A, ~19 pg/ml for IL-1 β and IL-10, and ~37.5 pg/ml for IL-6. Briefly, 100 μ l of sample or activin A, IL-1 β , IL-6 and IL-10 standard was added to wells of 96-well plate. The plate was incubated for 90 min at 37°C. Then, the plate was emptied, and 100 μ l biotinylated detection Ab solution was added to each well, and the plate was incubated for 60 min at 37°C. Then, the plate was washed 3 times with the wash buffer, and 100 μ l HRP conjugate (but avidin-biotin-peroxidase complex for activin A) was added to each well and the plate was incubated for 30 min at 37°C. Then, the plate was washed 5 times with the wash buffer, and 90 μ l substrate reagent was added to each well and the plate was incubated for 15 min at 37°C. Then, 50 μ l (but 100 μ l for activin A) stop solution was added to each well. The optical density was determined at 450 nm by the microplate reader (Epoch BioTek Instruments, Inc. Highland Park).

Statistical analysis

Data were expressed as mean $\pm$ standard error of mean. SPSS statistical software (IBM SPSS Statistics for Windows, Version 22.0, IBM Corp., Armonk, NY, USA) was used for data analysis. The Shapiro-Wilk test was used to test the normality of data. Data were compared using one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was accepted significant.

Results

Cloxyquin exhibited protective effects against ibotenate-induced white and gray matter damage in a dose-dependent manner

Intracerebral injection of ibotenate to P5 rat pups led to ipsilateral white matter lesions (cysts). However, such lesions did not develop in the sham-control group (Fig. 1A, Fig. 2A). In addition, ibotenate decreased significantly ipsilateral cortex thickness (sham-control vs ibo group and sham-control vs ibo+DMSO group, $P=0.0001$ for both comparisons, Fig. 1B, Fig. 2B and C). Likewise, it significantly reduced also ipsilateral white matter thickness (sham-control vs ibo group and sham-control vs ibo+DMSO group, $P=0.0001$ for both comparisons, Fig. 1C, Fig. 2B and C). However, NMDA receptor antagonist MK-801, as a positive control, substantially reversed ibotenate-induced changes in the white matter lesion size, and cortex and white matter thickness (ibo+DMSO group vs Ibo+MK-801 group, $P= 0.0001$ for the lesion size, $P= 0.0001$ for cortex thickness and $P=0.002$ for white-matter thickness, respectively, Fig. 1A-C, Fig. 2G). Thus MK-801 confirmed that ibotenate-induced excitotoxic injury was mediated by glutamatergic action in the present study.

On the other hand, while 0.2 or 1 mg/kg dose of cloxyquin did not produce any significant effect on the white matter lesion size, and cortex and white matter thickness induced by ibotenate (ibo+DMSO group vs ibo+cloxy-1 group, $P=0.393$ for the lesion size, $P= 1.0$ for cortex and white matter thickness, and ibo+DMSO group vs ibo+cloxy-2 group, $P= 0.152$ for the lesion size, $P= 0.168$ for cortex thickness, $P= 0.99$, Fig. 1A-C, Fig. 2D and E), 5 mg/kg dose of cloxyquin significantly alleviated the white matter lesion size, and increased the cortex and white matter thickness (ibo+DMSO group vs ibo+cloxy-3 group, $P= 0.001$ for the lesion size, $P= 0.006$ for cortex thickness and $P= 0.033$ for white matter thickness, respectively, Fig. 1A-C, Fig. 2F).

Cloxyquin dose-dependently inhibited ibotenate-induced degranulation of brain mast cells and alleviated their increased number

Ibotenate administration induced massive degranulation of brain mast cells and significantly increased their number (sham-control vs ibo group, and sham-control vs ibo+DMSO group, $P=0.0001$ for both the degranulation and the number, and for both comparisons, Fig. 3A, Fig. 4.1.A-C). However, while 0.2 mg/kg dose of cloxyquin did not change (ibo+DMSO group vs ibo+cloxy-1 group: $P= 0.078$, Fig. 3A, Fig. 4.1.D), both 1 and 5 mg/kg dose of cloxyquin significantly reduced ibotenate-induced degranulation of brain mast cells, respectively (ibo+DMSO group vs ibo+cloxy-2 group, $P= 0.001$; ibo+DMSO group vs ibo+cloxy-3 group, $P=0.0001$, Fig. 3A, Fig. 4.2.E and F) Additionally, while 0.2 or 1 mg/kg dose of cloxyquin did not affect (ibo+DMSO group vs ibo+cloxy-1 group: $P= 0.099$; ibo+DMSO group vs ibo+cloxy-2 group, $P= 0.297$, Fig. 3B), 5 mg/kg dose of cloxyquin significantly decreased ibotenate-evoked the increase in the number of brain mast cells (ibo+DMSO group vs ibo+cloxy-3 group: $P= 0.008$, Fig. 3B). Moreover, NMDA receptor antagonist MK-801 ameliorated ibotenate-induced changes in the degranulation and number of brain mast cells (ibo+DMSO group vs ibo+MK-801 group, $P= 0.0001$ for the degranulation, $P= 0.006$ for the number, Fig. 3A and B, Fig. 4.2.G).

Cloxyquin dose-dependently exerted the ameliorative effects on ibotenate-induced levels of activin A and inflammatory cytokines in the brain tissue

Ibotenate administration significantly led to the increased levels of first responders of neonatal brain injury including neuronal survival factor activin A, and pro-inflammatory cytokines IL-1 β and IL-6 in the brain tissue (sham-control vs ibo group and sham-control vs ibo+DMSO group, $P= 0.0001$ for activin A and for both comparisons, $P= 0.0001$ for IL-1 β and for both comparisons, $P= 0.026$ and $P= 0.009$ for IL-6, respectively, Fig. 5A-C). Additionally, it significantly reduced the level of anti-inflammatory cytokine IL-10 in the brain tissue (sham-control vs ibo group and sham-control vs ibo+DMSO group, $P= 0.001$ for both comparisons, Fig. 5D). However, there was no any significant difference between the naive-control and sham-control groups in terms of these biomarkers ($P> 0.05$ for all comparisons, Fig. 5A-D).

On the other hand, 0.2, 1 and 5 mg/kg doses of cloxyquin each significantly alleviated ibotenate-induced the increases in the levels of activin A, IL-1 β and IL-6 in the brain tissue, respectively (ibo+DMSO group vs

ibo+cloxy-1 group: $P=0.0001$ for activin A and IL-1 β , $P=0.035$ for IL-6; ibo+DMSO group vs ibo+cloxy-2 group: $P=0.0001$ for activin A and IL-1 β , $P=0.023$ for IL-6; ibo+DMSO group vs ibo+cloxy-3 group: $P=0.0001$ for activin A and IL-1 β , $P=0.027$ for IL-6, Fig. 5A-C). Moreover, while 0.2 or 1 mg/kg dose of cloxyquin did not produce any significant effect (ibo+DMSO group vs ibo+cloxy-1 group, $P=0.591$ and ibo+DMSO group vs ibo+cloxy-2 group, $P=0.058$, Fig XX), 5 mg/kg dose of cloxyquin significantly increased the level of IL-10 that was lowered by ibotenate in the brain tissue (ibo+DMSO group vs ibo+cloxy-3 group, $P=0.0001$, Fig. 5D). In addition, NMDA receptor antagonist MK-801 considerably reversed ibotenate-induced changes in the levels of activin A, IL-1 β , IL-6 and IL-10 in the brain tissue (ibo+DMSO group vs ibo+MK-801 group, $P=0.0001$ for activin A and IL-1 β , $P=0.011$ for IL-6 and $P=0.013$ for IL-10, Fig. 5A-D).

Discussion

The major findings of the present study were that intracerebral administration of glutamatergic analog ibotenate to P5 rat pups produced white matter lesions (cysts) and reduced both cerebral gray and white matter thickness. Consistent with these, it raised the concentration of neuronal survival factor activin A which is one of the first responders of neonatal brain injury in the brain tissue. In addition, our findings demonstrated for the first time histologically that the ibotenate administration led to massive degranulation of brain mast cells and an increase in their number. Moreover ibotenate increased the concentrations of pro-inflammatory cytokines IL-1 β and IL-6 in the brain tissue while it diminished the concentration of anti-inflammatory cytokine IL-10. Thus, ibotenate led to neuroinflammation in the brain through an increase in the activation and number of brain mast cells, as well as the levels of pro-inflammatory cytokines. However, NMDA receptor antagonist MK-801 reversed the ibotenate-induced changes, confirming that these excitotoxic insults was mediated by glutamatergic action. On the other hand, cloxyquin, a selective activator of TRESK background potassium channels, dose-dependently reduced ibotenate-induced the white matter lesion size, and increased the cortex and white matter thickness diminished by ibotenate. Additionally, it suppressed ibotenate-evoked activation of brain mast cells and their increased number. Furthermore, cloxyquin dose-dependently decreased the levels of activin A, IL-1 β and IL-6 in the brain tissue induced by ibotenate while it elevated IL-10 level. Therefore, cloxyquin exerted protective effects against the excitotoxicity-induced neonatal brain injury and neuroinflammation in a dose-dependent manner.

Therapeutic hypothermia is currently used in the treatment of PBI. However, its use is limited to mostly hypoxic-ischemic type brain injury including infants with mild and moderate encephalopathy, and its success rate is changeable. Such limitations of therapeutic hypothermia and its inadequacy in treatment alone necessitate the investigation of new, comprehensive and effective treatment options.

Although PBI can be induced by multiple factors such as hypoxic-ischemia, neonatal stroke and inflammation, excitotoxicity is of particular importance as it is the common final molecular mechanism of brain damage caused by those factors. For instance, it has been well documented that hypoxia–ischemia or inflammation during the perinatal period leads to neonatal brain injury by inducing the

excitotoxicity [6, 11, 29, 30]. In addition to excitotoxicity, the inflammation also plays a key role in the development of PBI [6, 7]. However, since (neuro)inflammation resulting in PBI is both a cause and a consequence of excitotoxicity [8, 9, 29, 30], we focused on both excitotoxicity and neuroinflammation in the presented study.

In the current study, ibotenate led to white matter lesions, the reduction in cortical and white matter thickness as well as the increase in level of brain tissue activin A, and further, these ibotenate-induced changes were reversed by NMDA receptor antagonist MK-801. These findings thus indicate that model of ibotenate-induced neonatal excitotoxic brain injury was successfully established in the present study. Moreover, these findings are also compatible with the results of previous studies showing white matter lesions and the decrease in cortical and white matter thickness in rodent models of ibotenate-induced neonatal excitotoxic brain injury [2, 23, 24]. On the other hand, cloxyquin dose-dependently attenuated these ibotenate-induced insults and activin A level, one of the early responders of injury, in the neonatal brain. Our findings are also consistent with the function of TRESK background channels, which, when activated, hyperpolarise the cell by enhancing the potassium outflow. Moreover, these findings suggest that activation of TRESK background potassium channels protects the neonate brain against the excitotoxic insults by hyperpolarising the neuronal cells. Controlling cell excitability and maintaining the membrane potential below the threshold of depolarization is an important target in the investigation of possible treatments against excitation-related nervous system diseases in both neonates and adults.

It was shown that the calcium-activated potassium channel activator, NS1619, and ATP-dependent potassium channel activator, aprikalim provided a protection against the NMDA-induced cerebral vascular dilation after combined hypoxia and ischemia in piglets [31]. Moreover, it has recently been demonstrated that flupirtine, a voltage-gated potassium channel opener, alleviated hippocampal and cortical tissue loss in the model of hypoxic-ischemic injury in newborn rats [32]. Unlike previous studies, we targeted the TRESK background potassium channels and used an excitotoxic neonatal brain injury model in the present study. Although the present study is different from the previous studies, it is compatible with those studies in terms of preventing brain damage by providing potassium outflow from the cell. Thus, the current study has demonstrated for the first time the protective effects of activation of TRESK background potassium channels by the specific activator cloxyquin against excitotoxicity-induced perinatal brain injury.

In addition to the excitotoxicity, the (neuro)inflammation alone or induced by excitotoxicity, hypoxia or hypoxic ischemia during the perinatal period contributes to PBI. The roles of mast cells in the inflammatory processes have been well known. However, unlike peripheral mast cells, the role of brain mast cells in inflammatory conditions including neurologic disorders is little known. Emerging data has indicated that the resident immune cells of the brain, microglia and mast cells, play also a critical role in inflammation resulting in PBI in the developing brain, but studies on the role of brain mast cells which are the first responders to inflammatory insults are very limited. Several studies have indirectly shown that brain mast cells are implicated in neuroinflammation leading to neonatal brain damage. In one of those studies was shown that activation of mast cells contributed to excitotoxic injury in newborn mice

by aggravating transforming growth factor- β 1 toxicity [33]. Moreover, two studies demonstrated that the mast cell stabilizer cromolyn reduced hypoxia-induced perinatal hypoxic-ischemic brain damage in the newborn rats [16, 19].

In the present study, ibotenate-induced excitotoxicity led to degranulation of brain mast cells and an increase in their number. These findings are in line with the previous studies mentioned above. Thus, the current study has confirmed the relevance of brain mast cells in PBI by directly showing their activation histologically for the first time. These findings suggest that brain mast cells are involved in excitotoxicity-induced neuroinflammation in the developing brain.

In addition, cloxyquin dose-dependently prevented excitotoxicity-induced degranulation of brain mast cells and decreased their increased number. There may be two possible explanations for this mast cell stabilizing effect of cloxyquin in the present study: (a) cloxyquin may have indirectly exerted this effect by restraining the ibotenate-induced excitation of glial and neuronal cells, because both of them express NMDA receptors and when activated, products released from these cells such as neuropeptides, cytokines and lipid mediators are capable of stimulating the activation of mast cells, (b) cloxyquin may have directly blocked the activation of mast cells through activation of TREK channels on the mast cells.

Mast cells can be activated in immunoglobulin E (IgE)-dependent or IgE-independent manner [34, 35]. The IgE-dependent activation is characterised by an influx of extracellular Ca^{2+} . It was reported that mast cells express multiple channels for ions such as K^+ (K_{Ca} , Kir, TWIK-2, TASK-2), Cl^- (ClC2, CFTR) and Ca^{2+} (Cav, TRPs, P2X1–4, -6 and -7) [36]. In addition to Ca^{2+} channels, it was suggested that the K^+ , Cl^- and TRP channels may play a key role in activation processes of mast cells since these channels modulate cellular membrane potential and thus influence Ca^{2+} influx [36]. Of ion channels expressed by mast cells, TWIK-2 and TASK-2 are potassium background channels. However, it is not yet clear whether TREK channels are expressed in mast cells, and there is therefore no investigation in the literature on the effect of TREK channel activation on peripheral or brain mast cells. Testing cloxyquin on the pure mast cell culture may contribute to clarifying this dilemma regarding the mast cell stabilizing effect of cloxyquin in the future.

Mast cells are both a source and a target of pro-inflammatory mediators including IL-1 β , IL-6 and TNF. When activated by stimuli, mast cells lead to an early and dramatic inflammatory response by releasing these pro-inflammatory mediators to their resident environment.

In the present study, ibotenate-induced excitotoxicity raised the levels of pro-inflammatory cytokines IL-1 β and IL-6 in the brain tissue while it reduced the level of anti-inflammatory cytokine IL-10. Taken together, our findings indicate that ibotenate-induced excitotoxicity produced neuroinflammation characterized by increased activation and number of brain mast cells and increased levels of pro-inflammatory cytokines IL-1 β and IL-6, and decreased level of anti-inflammatory cytokine IL-10 in the developing brain. It was reported that excitotoxicity-induced inflammation contributed to the brain damage through release of pro-inflammatory cytokines from the brain-resident immune cells in rodent models of PBI [8, 9]. Additionally,

in clinical studies were reported that the plasma and cerebrospinal fluid levels of IL-1 β , IL-6 and TNF- α in the neonates with hypoxic-ischemic encephalopathy were higher than in control infants [13, 37]. Thus, our findings are in concordance with these previous studies.

On the other hand, the present study revealed that cloxyquin dose-dependently alleviated ibotenate-induced increases in the levels of IL-1 β and IL-6 in the brain tissue. It was reported that pro-inflammatory cytokines such as IL-1 β and IL-6 in the developing brain contribute to the brain injury by increasing blood-brain barrier permeability and exacerbating inflammatory responses [10–12]. These pro-inflammatory cytokines are released by microglia, mast cells, astrocytes and neurons [11], and modulation of the release of them is of great importance in preventing the inflammation and thus PBI. Our findings therefore suggest that activation of TRESK channels by cloxyquin may prevent or restrict the development of PBI by suppressing release of pro-inflammatory cytokines such as IL-1 β and IL-6 from those CNS cells.

Conclusions

In conclusion, our findings revealed that activation of TRESK channels by cloxyquin exerted protective effects against excitotoxicity-induced cerebral white and gray matter damage, and against excitotoxicity-induced neuroinflammation characterized by increased activation and number of brain mast cells and increased levels of pro-inflammatory cytokines in neonatal rat model of ibotenate-induced excitotoxic brain injury. Cloxyquin exhibited these effects by substantially limiting excessive glutamatergic excitation since the present study used a glutamatergic agonist ibotenate-induced model of PBI. Cloxyquin may also offer protection against perinatal brain injuries induced by the other factors, as the excitotoxicity is a common pathological pathway of perinatal brain injuries. This study provides an important basis for possible clinical trials with cloxyquin in the future. Therefore, TRESK background potassium channels may be a promising new target for the prophylactic treatment in neonates at risk.

Declarations

Code availability

Not applicable

Ethics approval

Experimental procedures were approved by Bolu Abant İzzet Baysal University Animal Experiments Local Ethics Committee (No: 2019/17).

Consent to participate

Not applicable

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Compliance with Ethical Standards

The research protocol was approved by Bolu Abant Izzet Baysal University Animal Experiments Local Ethics Committee (No: 2019/17), and performed according to the Universal Declaration of Animal Rights proclaimed at the UNESCO.

Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

All authors contributed the study significantly and agree in the content of the manuscript. Authors consent to the publication of the study.

Availability of data and materials

Data are available from the corresponding author on reasonable request.

Ibotenate and MK-801 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cloxyquin (5-Chloro-8-hydroxyquinoline) was purchased from Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany). Toluidine blue dye and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich

(Schnelldorf, Germany). Cresyl violet was purchased from Isolab (Wertheim, Germany). Formaldehyde solution was purchased from Merck (Darmstadt, Germany). Rat interleukin (IL)-1 β , IL-6 and IL-10 ELISA kits were purchased from ELABscience (Wuhan, P.R. China). Rat Activin A ELISA Kit PicoKine was purchased from Boster (Pleasanton, CA, USA). Microplate reader (Epoch BioTek Instruments, Inc. Highland Park, Winooski, VT, USA) and other consumables are available in our laboratory.

Authors' contributions

Erkan Kilinc planned the study, and supervised the experiments, and analyzed and interpreted data, and wrote the manuscript. Mustafa Dilek performed the experiments, analyzed data and wrote the manuscript. Yasemin Baranoglu Kilinc performed the experiments, collected data, and wrote the manuscript. Ibrahim Ethem Torun performed the experiments, collected data and wrote the manuscript. Aslihan Saylan carried out histological staining and histopathological assessments and wrote the manuscript. Selma Erdogan Duzcu performed histological staining and histopathological assessments and wrote the manuscript.

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Figures

Figure 1

Ibotenate-induced white and gray matter damages are reduced by cloxyquin in a dose-dependent manner.

The effects of three different doses of cloxyquin, or NMDA receptor antagonist MK-801 on the ipsilateral white matter lesion size (A), cortex thickness (B), and white matter thickness (C). PBS, phosphate-buffered saline; DMSO: dimethyl sulfoxide; n.s., non-significance. *P < 0.05, **P < 0.01, and ***P < 0.001. N= 6 for each group. Data were analysed by one-way ANOVA followed by Tukey's post hoc test.

Figure 2

Representative cresyl violet-stained coronal sections showing brain lesions or the improvements in the brain.

Brains from the rat pups in sham-control (A), ibotenate (B), ibo+DMSO (C), ibo+cloxy 0.2 mg/kg (D), ibo+cloxy 1 mg/kg (E), ibo+cloxy 5 mg/kg (F), and ibo+MK-801 (G) groups. Typical intact gray and white matter from the sham-control group (H), typical white matter lesion (cyst) from the ibotenate group (I).

Scale bars are shown on the images. Red arrows indicate white matter lesion (cyst). Intracerebral injection of ibotenate was performed at P5 and rat pups were sacrificed at age P10.

Figure 3

Cloxyquin dose-dependently inhibits ibotenate-induced degranulation of brain mast cells and alleviates their increased number.

Percentages of mast cell degranulation (A), and numbers of mast cells (B) in the study groups. PBS, phosphate-buffered saline; DMSO: dimethyl sulfoxide; n.s., non-significance. **P < 0.01, and ***P < 0.001. N= 6 for each group. Data were analysed by one-way ANOVA followed by Tukey's post hoc test.

Figure 4

Representative toluidine blue-stained brain mast cells showing the intact and degranulated state of mast cells.

Brain mast cells in sham-control (A), ibotenate (B), ibo+DMSO (C), ibo+cloxy 0.2 mg/kg (D), ibo+cloxy 1 mg/kg (E), ibo+cloxy 5 mg/kg (F), and ibo+MK-801 (G) groups. A typical intact mast cell at a magnification of X60 (H), and typical degranulated mast cells at a magnification of X60 (I). Scale bars are shown on the images. Panels, A-G, show the nested images at two different magnifications, X20 and X40. Black arrows indicate intact brain mast cells, and red-dashed arrows indicate degranulated brain mast cells. Please note the massive degranulation of brain mast cells in the ibotenate or ibo+DMSO group, and also that the degranulation is reduced by both 1 and 5 mg/kg doses of cloxyquin, respectively. Intracerebral injection of ibotenate was performed at P5 and rat pups were sacrificed at age P10.

Figure 5

Cloxyquin dose-dependently exerts the ameliorative effects on ibotenate-induced levels of activin A and inflammatory cytokines in the brain tissue.

The effects of three different doses of cloxyquin, or NMDA receptor antagonist MK-801 on the levels of activin A (A), IL-1 beta (B), IL-6 (C), and IL-10 (D) in the brain tissue. PBS, phosphate-buffered saline; DMSO: dimethyl sulfoxide; n.s., non-significance. *P < 0.05, **P < 0.01, and ***P < 0.001. N= 7 for each group. Data were analysed by one-way ANOVA followed by Tukey's post hoc test. Intracerebral injection of ibotenate was performed at P5 and rat pups were sacrificed at age P6.

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