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Involvement of CGRP receptors in retinal spreading depression

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ABSTRACT

Background: Cortical spreading depression (CSD) is a transient propagating excitation of synaptic activity followed by depression, which is implicated in migraine with aura and is regarded as the underlying cause of migraine. Calcitonin-gene related peptide (CGRP) receptors play a crucial role in mediating the magnitude of CSD in rat cortical slice. This study aimed to examine whether CGRP receptors are involved in retinal spreading depression (RSD) in chicks.
Methods: Western blot was used for detection of calcitonin-receptor like receptor (CALCRL) and intrinsic optical imaging was used for pharmacological investigation.
Results: We found that the key component of CGRP receptor, CALCRL, is expressed in the chick retina. Using an *in vitro* migraine RSD model, we demonstrated that BIBN4096, a potent antagonist for CGRP receptors, markedly reduced the magnitude of RSD induced by K^+, but also the propagation rate.
Conclusions: The data suggest that CGRP receptors mediate RSD propagation involving neuronal mechanism and approve that RSD is an efficient *in vitro* approach for assessing anti-migraine drugs targeting CGRP receptors.

Introduction

Migraine is defined as a common recurrent neurological disorder characterized by attacks of pulsating, unilateral headache, which are often associated with certain features such as nausea, vomiting, photophobia and phonophobia. Migraine was often thought to be a neurovascular disease; however, increasing evidence supports that cortical spreading depression (CSD) is the pathophysiological basis of migraine [1–3]. CSD is a temporary excitation of synaptic activity, followed by depression that propagates slowly across the cerebral cortex, subcortical regions and retina. The molecular pathways that mediate CSD leading to the development of migraine are currently poorly understood.

Calcitonin-gene related peptide (CGRP) and CGRP receptors are widely distributed in both central and peripheral nervous system [4–6] and are valid targets for treating migraine [7,8] as both CGRP- [9] and CGRP receptor- [7,10] targeted therapeutics were shown to have the ability to abate and even prevent migraine attacks. Experimental studies also suggested that CGRP receptors in both peripheral and central nervous system play a key role in CSD as inhibition of CGRP receptors prevented CSD induced meningeal vasodilation in cats [11] and reduced the magnitude of CSD in cortical brain slices [12]. Whilst migraine pathophysiology is mainly related to both central and peripheral tissues at multiple levels [1,8,13,14], retina tissue is also associated with migraine as reduction of retina nerve fiber layer was found in chronic migraine patients [15]. Additionally, this tissue has been increasingly used for studying migraine pathophysiology related to excitatory and inhibitory neurotransmitter receptors [16–18]. However, whether CGRP receptors mediate retinal spreading depression (RSD) remains unknown.

Using chick retina, the primary aim of this study was to explore the role of CGRP receptors in regulating RSD propagation by investigating effects of BIBN4096 that is preferably selective for calcitonin-receptor like receptor (CALCRL) and receptor activity modifying protein 1 (RAMP1) binding pocket of CGRP receptors [12,19]. As retina is devoid of blood vessels, this study also allowed us to clarify whether the action of CGRP receptors in RSD involving neuronal mechanism. In order to ensure the tissue is suitable for CGRP receptor pharmacology, the key component, CALCRL of CGRP receptors, was firstly confirmed to be present in chick retina.

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Materials and methods

Seventeen male chicks (WuXi Yangzichang Ltd, Wuxi, China) were housed for at least a week before use (aged 8–28 days). Chicks were rapidly killed after neck dislocation using schedule one method. Chick retina preparation from posterior eyecup was as that described previously [17] for subsequent CALCRL detection ($n = 3$) and CGRP receptor pharmacology ($n = 14$). All animal procedures were approved by the Ethical Review Panels of Xi'an Jiaotong-Liverpool University and performed in accordance with the associated guidelines. Efforts were made to minimize animal suffering.

Western blot

Western blot was used to detect whether CALCRL are expressed in chick retina. Total protein was extracted from homogenized chick retina. The homogenization buffer was boiled for 10 min, centrifuged at 4 °C. Protein concentration was determined by BCA kit (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's protocol. Protein samples (200 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide loading buffer (NuPAGE_LDS Sample Buffer 4X, Invitrogen, Carlsbad, CA, USA), transferred onto nitrocellulose membranes. Non-specific binding was blocked with 5% milk. The membranes were then exposed to rabbit polyclonal anti-CALCRL antibody (SAB2100335, Sigma–Aldrich, St. Louis, MO, USA) and incubated overnight at 4 °C, following with 1-h incubation to β-actin antibody (4970S, Cell Signaling Technology, Danvers, USA) at room temperature. Once excess primary antibodies were washed, membranes were incubated with goat anti-rabbit horseradish peroxidase-labeled secondary antibody (Sangon Biotech, Shanghai, China). Protein bands were detected by incubating the membrane with Western Bright enhanced chemiluminescence working solution (Advansta, Menlo Park, CA, USA). The film (Kodak XBT-1, Carestream, Xiamen, China) was scanned and analyzed with Bio-rad Gel Doc XR+ with Image Lab 2.0 Software (BIO-RAD, Shanghai, China).

Intrinsic optical imaging of RSD

Tissue preparation and RSD induction were as that described previously [17]. Briefly, posterior eyecup was positioned in a chamber. Unless otherwise stated, the chamber was perfused at 0.5 mL/min with Ringer's solution. The tissue was stabilized for at least 30 min before elicitation of RSD and temperature kept at 32 °C. Ten repeated RSD episodes were induced by 0.1 μmol/L KCl with 20-min interval, the minimal but sufficient duration for tissue recovery under the experimental condition. The retina was illuminated for 25 ms every 1 Hz using a high-power LED spotlight (625 nm, SLS-0307-A, Mightex; Pleasanton, CA, USA). The reflected light was simultaneously recorded with a monochrome camera (QIC-F-M-12, Media Cybernetics, Marlow, UK). Image sequences were taken at 1 Hz over a 3-min period, started as RSD was elicited for recording the initial synaptic excitation phase of RSD. Camera exposure and illumination were synchronized using the same external trigger (TG5011, TTi, Huntingdon, UK). Image Pro Plus software (IPP 7.0; Media Cybernetics, Marlow, UK) was used for image acquisition, storage and analysis.

Experimental design for drug testing

To investigate effects of BIBN4096 on RSD propagation in chick retina, two groups were designed: (i) BIBN4096 (Tocris, Bristol, UK) at 1, 3, 10 μmol/L ($n = 6$), at which concentration, the drug was preferably selective for CGRP receptors [20] (ii) DMSO (Sigma–Aldrich, Dorset, UK) at 0.001%, 0.03% and 0.1% ($n = 8$). Ten RSD episodes were induced in each experiment, with two separate RSDs for each of the different and consecutive tests: (i) initial Ringer's control; (ii) low concentration; (iii) medium concentration; or (iv) high concentration of drug of vehicle; (v) post-treatment with Ringer's control. For each test sequence, the perfusion medium was changed immediately after the end of the 2nd, 4th, 6th, and 8th RSD recording when required.

Data presentation and statistical analysis

As reported previously [16], for each image sequence, an area of interest (AOI) parallel to RSD wave front was delineated manually (Fig. 2). For each picture within the sequence, the gray levels constituting the AOI were averaged and plotted against the time as an indicator to characterize the 1st phase of RSD that coexists with cellular swelling induced by ion distribution [18]. For each RSD wave, the area under the curve (AUC, gray levels × minute) of the transient cellular depolarization was calculated and used as an index of the magnitude of propagating RSD (Fig. 2). For each RSD wave, propagation velocity was calculated to reflect the degree of tissue excitability to RSD (Fig. 2). The calculated values within each different test were averaged and all corresponding data were given as mean ± SD in percentages of their respective baselines, that is, averaged value for the first two K^+ stimuli. Mann–Whitney test was used for comparison of the AUC and propagation rate between the drug and respective control group.

Results

CALCRL is expressed in chick retina

No band was seen in the absence of the primary antibodies in chick retina, whilst intensive bands of CALCRL and β-actin in chick retina were detected (Fig. 1).

Suppression of RSD by BIBN4096 in chick retina

In DMSO group, there was no significant change in all parameters tested over repeated RSD episodes throughout the experiment (Fig. 3, $n = 8$). Compared with respective DMSO group, the AUC of RSD were significantly suppressed by BIBN4096 in a

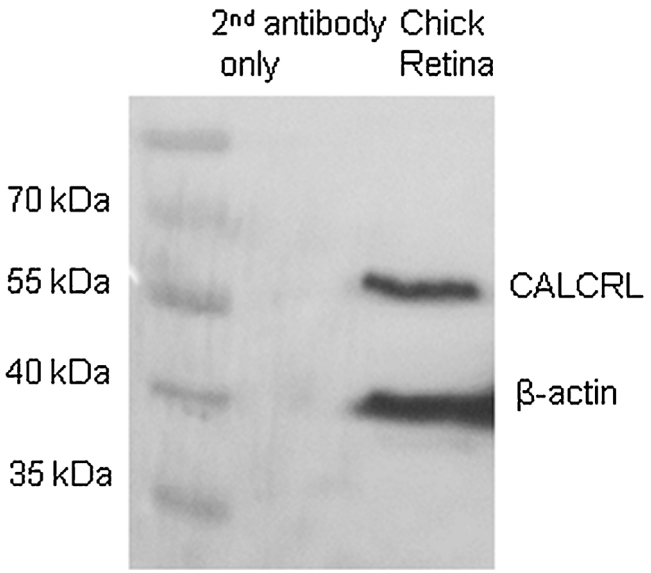

Fig. 1. Immunoblotting detection of CALCRL in the chick retina. β-Actin was also detected as the reference.