Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crybb2 associates with *Tmsb4X* and is crucial for dendrite morphogenesis

Minxuan Sun [a, *], Nafees Ahmad [b], Ruobing Zhang [a], Jochen Graw [c]

[a] Jiangsu Key Lab of Medical Optics, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou, China
[b] Institute of Biomedical & Genetic Engineering, Islamabad, Pakistan
[c] Helmholtz Center Munich, German Research Center for Environmental Health, Institute of Developmental Genetics, Neuherberg, Germany

ARTICLE INFO

Article history:
Received 24 May 2018
Accepted 29 May 2018
Available online xxx

Keywords:
Crybb2
Tmsb4X
Dendrite
Hippocampal neuron

ABSTRACT

Dendrite morphogenesis is a complex but well-orchestrated process. Various studies reported the involvement of alteration in dendrite morphology in different brain disorders, including neuropsychiatric disorders. Initially, βB2-crystallin (gene symbol: *Crybb2*/*CRYBB2*) has been described as a structural protein of the ocular lens. Mutations of the corresponding gene, *Crybb2*, lead to cataract. Recent studies in mice suggested that mutations in *Crybb2* cause alterations in hippocampal morphology and function, albeit its function in hippocampal neuron development remained elusive. In the current study, we found that *Crybb2* contributes to dendritogenesis *in vitro* and *in vivo*. Furthermore, screening of previous data on differential expression-arrays, we found *Tmsb4X* up-regulated in *Crybb2* mutants mouse brain. Additionally, *Tmsb4X* was co-expressed with *Crybb2* at actin-enriched cell ruffles. Over-expression of *Tmsb4X* in cultured hippocampal neurons inhibited dendritogenesis, which phenocopied *Crybb2* knock-down. The current study uncovers a new function of *Crybb2* in brain development, especially in dendritogenesis, and the possible interplay partner *Tmsb4X* involved in this process.

1. Introduction

In all vertebrate animals, the brain serves as the center of the nervous system. It is very well organized by neurons and glial cells, in which neurons are mainly responsible for conducting information of whole central nervous system [1]. Neurons are morphologically characterized by the presence of axons, somata, and dendrites. The shape of dendrites is the major indication how neurons integrate and process incoming information, and thus, they play a vital role in the functional properties of neural circuits [2,3]. During the last years, many studies documented considerable evidence that dendrite morphology (dendrite number and branches) is relatively dynamic. Alterations in dendrite morphology have been consistently observed in psychiatric disorders like schizophrenia [4,5]. Numerous studies have been endeavored to explore the mechanism underlying proper dendrite morphology development. The mechanism requires the tight control by neuronal signaling such as calcium as well as proteins involved in cytoskeletal rearrangement [6,7].

As one of the most prominent members of the crystallins, βB2-crystallin (gene symbol: *Crybb2*/*CRYBB2*) has been considered for a long time to be just a structural protein of the ocular lens [8]. Several clinical observations in human suggest that mutations in *CRYBB2*, especially the mutations in exon 6, are highly involved in the pathogenesis of cataract [9,10]. Different mouse mutants also showed that mutations in *Crybb2* lead to cataract (for review see Graw, 2009). Besides that, there are a few reports that βB2-crystallin has additional functions. *Crybb2* mutants have problems in fertility [11], and *Crybb*2 participates in axonal regeneration [12]. Most interestingly, βB2-crystallin also has moderate ability to bind calcium, suggesting a role as calcium buffer [13].

In addition, *Crybb2* is also found to be expressed in several regions of the mammalian brain, although its function in the brain remains mostly unknown. To uncover its function in the brain, in the previous study we combined behavioral, neuroanatomical, and physiological analyses in a *Crybb2* mouse mutant, *O377* [14,15]. Altered hippocampal morphology and neuronal functions were observed in these mutants, *O377*, as well as increased free intracellular Ca^{2+} levels and expression changes of calcium-channel

* Corresponding author. Jiangsu Key Lab of Medical Optics, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Keling Road No.88, Suzhou, 215163, China.
E-mail address: sunmx@sibet.ac.cn (M. Sun).

https://doi.org/10.1016/j.bbrc.2018.05.195
0006-291X/

related proteins (NMDA receptor).

Crybb2 was found to be clearly up-regulated in the regenerating retina compared to the unregenerated retina [12]. Overexpression of *Crybb2* in retinal ganglion cells and in hippocampal neurons increased axon formation. However, its function in dendrite morphology has not been analyzed so far. In this study, we focused on the functions of *Crybb2* on hippocampal dendrite morphology, mainly on dendrite number and dendrite branching. In addition, by analysis of previous data and further overexpression studies *in vitro*, we identified TMSB4X as an associated partner of CRYBB2 being involved in the process of dendrite formation.

2. Material and methods

2.1. Plasmid construction

To generate RNAi constructs directed against Crybb2 the following oligonucleotides were annealed and subcloned into pLentilox 3.7 (Biofeng, Shanghai, China). The oligonucleotides used in the RNAi plasmid construction is 5′- TCCCCAAGATCATCATCTTC TTCAAGAGAGAAGATGATGATCTTGGGG TTTTTTC -3′ and 5′-TCGA-GAAAAAACCCCAAGATCATCATCTTCTCTCTTGAAGAA-GATGATGATCTTGGGGA-3′.

2.2. Cell culture and immunofluorescence microscopy

COS-7 cells were maintained and transfected as described [7]. Primary hippocampal cultures were prepared and processed for immunofluorescence as described previously [7]. Transfections of neurons were performed at day 4, according to the instructions of the manufacturer (Life Technology, Shanghai, China). Neurons were fixed in 4% Paraformaldehyde (PFA) in PBS (pH 7.4) for 8 min at room temperature 2 days after transfection. Images were recorded digitally using either Nikon TS2 (Nikon, Shanghai, China) or Leica SP8 confocal microscopy (Leica, Shanghai, China) equipped with CCD camera from Diagnostic Instruments (Wholesun Equipments, Shanghai, China) and processed in compatible Software.

Morphology of transfected hippocampal neurons were measured by NIH Image Software (ImageJ: https://imagej.nih.gov/ij/). Each experiment was repeated 2–4 times with independent neuronal preparations. Neuron dendrites were identified by anti-MAP2 staining and sampled randomly for morphology analyses. The number of neurites protruding from the cell body and the number of dendritic branch from at least 80 neurons for each condition were counted and measured at day 6 in culture.

The *O377* mouse was an *Crybb2* mutant mice original from Helmholtz Center Munich, Neuherberg, Germany, and maintained as described before [14], but later transferred to Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences. Here, animals were raised for preparation of hippocampal neurons. All animal experiments were carried out in accordance with the regulations of the German or Chinese Law on Animal Protection and institutional guidelines.

2.3. In-situ hybridization

In-situ hybridization was performed as described before [14]. Briefly, mouse brains from wild types and *O377* mutants were dissected and frozen in isopentane at −30 °C and proceeded for cryosection. cRNA probes were generated from cloned inserts into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). Cryosections were fixed in 4% PFA, and *in-situ* hybridization was processed as described previously [14].

2.4. Golgi staining and neurolucida analysis

The mouse brains from wild-type and *O377* homozygotes were freshly prepared, sliced 120 μm and proceed following the instructions of manufacturer of “FD Rapid GolgiStain” kit (FD Neurotech, Columbia, MD, USA). The neuron morphology analysis was performed via neurolucida software (MBF Bioscience, Williston, VT, USA) equipped on an Olympus BX51 microscopy (Olympus, Munich, Germany). To get more homogenous neurons, only the mature neurons in the ventral dentate gyrus were selected. Classification of the mature neuron is based on previous publications [16,17]: only neurons having more than 2 primary dendrites are mature neurons.

2.5. RNA isolation, cDNA production, reverse-transcription PCR, and quantitative real-time PCR

Total RNA from mouse brain was isolated by RNA-Bee (AMS Biotechnology, Abingdon, UK). Isolated RNA was treated with DNase (Promega, Madison, WI, USA) according to the manufacturer's protocol. cDNA was synthesized by T-Primed First-strand Kit (Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA) for reverse-transcription PCR and quantitative real-time PCR. Reverse-transcription PCR was performed on a PCR thermal cycler (Eppendorf, Shanghai, China) using the Taq DNA polymerase; the PCR mixture was made following the manufacturer's instructions. Quantitative real-time PCR was performed on a step one device (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). EvaGreen® qPCR Master Mix (Solis BioDyne, Tartu, Estonia) was used according to the manufacturer's protocol. In quantitative real-time PCR, *Tuba1a* was used as a control; primers for real-time PCR were as follows: *Tmsb4X* forward: 5′-ATGTCTGACAAACCCGATATGG-3′, reverse: 5′-CGATTCGCCAGCTTGCTTC-3′; *Tuba1a* Forward: 5′-ATGTCTGACAAACCCG ATATGG-3′, Reverse: 5′-CGATTCGC-CAGCTTGCTTC-3′

3. Result

3.1. Crybb2 is expressed in dendrites and the over-expression of Crybb2 promotes dendritogenesis

To see whether CRYBB2 might play a role in dendrite formation, we checked its expression in dendrites of mouse hippocampal neurons. GFP-tagged *Crybb2* was transfected in hippocampal neurons at 4 days cultured *in vitro* (DIV4) and grew for 48 h before fixation, a key period for morphogenesis of dendrites [18]. Interestingly, CRYBB2 was found to be co-localized with the microtubule-associated protein 2 (MAP2) (Fig. 1A), a dendrite marker used previously [7].

Given the fact that CRYBB2 is expressed in dendrites, we hypothesized CRYBB2 may play a role in the establishment of neuronal dendrite morphology. To address it, GFP or GFP-tagged *Crybb2* was separately transfected in the hippocampal neurons as described above (DIV4), which were processed after for 48 h for morphological analysis. The quantitative evaluation revealed a significant increase of dendrite number and dendrite branch in neurons transfected with *Crybb2*-GFP (Fig. 1C, D, E) compared with GFP control (Fig. 1B, D, E).

3.2. Loss of CRYBB2 inhibits dendritic development

To further investigate the role of *CRYBB2* in dendrite morphology, we studied how the loss of *CRYBB2* affected the hippocampal neuron. 3 different shRNAs targeting *Crybb2* were designed and inserted into Lentilox 3.7 vector. Since no cell line we

Please cite this article in press as: M. Sun, et al., *Crybb2* associates with *Tmsb4X* and is crucial for dendrite morphogenesis, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.195