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Research paper

Interaction of DCF1 with ATP1B1 induces impairment in astrocyte structural plasticity via the P38 signaling pathway

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ABSTRACT

Astrocytes are known to regulate and support neuronal and synaptic functions. Changes in their size and morphology in mouse models result in mental retardation. However, the mechanism underlying these morphological changes remains unclear. In the present study, abnormal astrocyte morphology was found in the mouse brain following knockout of dendritic cell factor 1 (*Dcf1*). Immunoprecipitation-mass spectrometry (IP-Mass) identified that ATP1B1 is bound to DCF1, and co-immunoprecipitation and cell fluorescence further confirmed an interaction between these two proteins, with asparagine residue 266 of ATP1B1 being required for the interaction with DCF1. Moreover, *Dcf1* knockout in mice resulted in upregulation of ATP1B1 expression in the hippocampus. Furthermore, DCF1 interaction with ATP1B1 in astrocytes impaired their structural plasticity. Ultimately, *Dcf1* knockout increased glutamate release. Mechanism exploration proposed that *Dcf1* knockout led to significantly perturbed expression of AMPA receptors (AMPARs) and induced morphological changes in astrocytes through the P38 signaling pathway. Our data shed light on the possible mechanisms underlying changes in astrocyte morphology and provide new avenues for the exploration of proteins involved in glutamate release.

1. Introduction

Glial cells are non-neuronal cells that make up almost 80–90% of the adult rat brain (Bandeira et al., 2009). Decades ago, glial function was thought to support and protect neuronal cells and maintain the dynamic balance of their surrounding environment (Jessen and Mirsky, 1980; Kato et al., 2013). With the development of science, neurologists have found that glial cells are actually involved in many physiological processes including breathing (Gourine et al., 2010), synaptic connections between neurons (Wolosker et al., 2008), providing nutrients and oxygen to neurons (Marina et al., 2016), maintaining their surrounding environment (Ismail et al., 2017), forming myelin to maintain insulation (Schirmeier et al., 2016), destroying pathogens (Mitterreiter et al., 2017), and removing dead neurons (Ulbrich et al., 2016). Due to these important functions, glial cells exert an important role in neuro-degenerative diseases such as Alzheimer's (Gajardo-Gomez et al., 2017), Parkinson's (Leonoudakis et al., 2017), and Huntington's (Valenza et al., 2015) diseases.

Astrocytes are the most abundant subtype of glial cells in the mammalian central nervous system, which make up almost half of the adult brain, indicating their important function (Zhang et al., 2016). Astrocytes perform multiple functions crucial for brain circuitry and processing (Vasile et al., 2017), such as normal neuronal development (Liddelow and Barres, 2017; Singh and Abraham, 2017), formation of synapses and clearance of neurotransmitters (Vasile et al., 2017). Astrocytes are also found in close contact with neurons, regulating neuronal function at the synaptic and network levels, and exerting an important role under both physiological and pathophysiological conditions (Singh and Abraham, 2017). Astrocytes can respond to neural activity and release gliotransmitters, regulating LTP (long-term potentiation) and LTD (long-term depression) (Gordon et al., 2009; Jones et al., 2013). However, the function of astrocytes is not homogenous (Ben Haim and Rowitch, 2017). Recent evidence has shown that astrocytes display distinct features and functional diversities at different regions, which regulating local neural circuit function (Ben Haim and Rowitch, 2017). Moreover, astrocyte morphology has also been found to influence its function (Tavares et al., 2017). It has been reported that protoplasmic astrocytes in the human neocortex are 2.6-fold larger in diameter than their rodent counterparts, facilitating Ca^{2+} waves at a higher speed of approximately 4-fold faster (Oberheim et al., 2009). The size of astrocytes has also been shown to affect the plasticity of the brain, including the density of human cortical synapses (DeFelipe

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et al., 2002). In addition, a rapid change in astrocyte morphology in the hypothalamic nucleus affects the transmission of information between neurons (Piet et al., 2004). It has also been reported that astrocytes play an important role in learning and memory ability in rodents (Franklin and Bussey, 2013; Han et al., 2013) by affecting synaptic junction formation (Pascual et al., 2005; Rowitch and Kriegstein, 2010).

Dendritic cell factor 1 (*Dcf1*) was first discovered to exhibit high expression level in neural stem cells (NSCs) by our lab (Wen et al., 2002). Downregulation of *Dcf1* increases the differentiation of NSCs into neurons and astrocytes (Wang et al., 2008), and *Dcf1* deficiency leads to social interaction defects through the dopamine system (Liu et al., 2017). Moreover, *Dcf1* can maintain energy balance through the regulation of NPY expression (Liu et al., 2017), and also triggers dendritic spine formation, facilitating learning and memory in mice (Liu et al., 2017). These results suggested important roles of *Dcf1* in the brain. In order to establish the function of *Dcf1* in neural cells, RNA-sequencing results in wild-type and *Dcf1*-knockout mice were analyzed to compare specifically-expressed genes in neurons, astrocytes, and oligodendrocytes (Wang et al., 2012). Interestingly, it was found that the ratio of specifically-expressed genes in astrocytes was significantly changed compared with that in neurons or oligodendrocytes (Table S1). However, currently, nothing is known about the influence of *Dcf1* on astrocytes morphology in the central nervous system.

In the present paper, we show that *Dcf1* deficiency causes astrocyte morphology deficit, which increases glutamate release and interferes with the expression of glutamate receptors. Through exploring the mechanism underlying the morphology deficit, the interaction of DCF1 and ATP1B1 contributes to the impairment in astrocyte structural plasticity. Further, our results indicate that abnormal astrocyte structure appears to be mediated by the P38 signaling pathway, and provide the first evidence that *Dcf1* is an important regulator of astrocyte morphology and glutamate release.

2. Materials and methods

2.1. Materials

DMEM (Invitrogen, USA), FBS (Invitrogen, USA), Lipofectamine 2000 (Invitrogen, USA), Total RNA Extraction Kit (Promega, USA), RT Master Mix (TaKaRa, Japan), Top Green qPCR Super Mix (Yeasen, China), anti-Glur1 mouse monoclonal antibody (Santa Cruz, USA), anti-Glur2 rabbit polyclonal antibody (BBI, Canada), anti-Glur3 rabbit polyclonal antibody (BBI, Canada), anti-Glur4 rabbit polyclonal antibody (BBI, Canada), anti-P38 rabbit monoclonal antibody (Wanleibio, China), anti-Arc rabbit polyclonal antibody (BBI, Canada), anti-Creb rabbit polyclonal antibody (BBI, Canada) 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA), anti-GFAP goat monoclonal antibody (Abcam, USA), anti-S100B rabbit monoclonal antibody (Proteintech, USA), anti-myc rabbit monoclonal antibody (ABclonal, USA), anti-1D4 mouse monoclonal antibody (ABclonal, USA), anti-HIS mouse monoclonal antibody(ABclonal, USA), anti-GAPDH mouse monoclonal antibody (Abcam, USA), anti-ATP1B1 rabbit monoclonal antibody (ABclonal, USA), infrared dye 700-conjugated affinity-purified goat anti-mouse IgG secondary antibody (Zemed, USA), infrared dye 800-labeled goat anti-rabbit IgG secondary antibody (Zemed, USA) and infrared dye 800-labeled donkey anti-goat IgG (Zemed, USA), glutamic acid concentration detection kit (njjcbio, China). SMCC-7721 and HEK293 (human embryonic kidney cells) cells were maintained in our laboratory.

2.2. Methods

2.2.1. Primary hippocampal astrocytes culture

Culture of the primary hippocampal astrocytes was performed as described (Mamber et al., 2012; Schildge et al., 2013; Sun et al., 2016). Mouse pups (P0-P3) were placed under ice anesthesia, sterilized with 70% ethanol, and the brain was removed and the hippocampus separated under a stereoscope. All hippocampal tissues were collected and cut into pieces approximately 1 mm^3 in size using ophthalmic scissors. 2.5% trypsin was added and tissues were incubated at 37 °C for 5 min. Preheated culture medium (DMEM, high glucose +10% heat-inactivated fetal bovine serum +1% Penicillin/Streptomycin) was immediately added to terminate the digestion reaction. Following digestion, the samples were placed on ice for 5 min and carefully transferred the supernatant after precipitation. The samples were then centrifuged for 5 min at 800 ×*g* to collect single cells, and the supernatant discarded. The pellet was resuspended in 1 ml medium and the cells were counted using an automated cell counter. The plating density was 5×10^4 cells/cm^2. The culture plates were gently shaken approximately 20 times and placed at 37 °C, with the half of the culture medium being changed every three days. All plates were coated with poly-D-lysine.

2.2.2. Immunofluorescence

The hippocampus was excised from *Dcf1*$^{+/+}$ and *Dcf1*$^{-/-}$ mouse brains by coronal frozen sectioning. The following staining procedure was performed in a dark humidified chamber: Slices were rinsed 3 times with PBS to remove the O.C.T. The slices were dried slightly, making sure that the tissue sections did not dry out completely. Primary hippocampal astrocytes were seeded onto glass coverslips in a 24-well plate and rinsed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature (RT). The samples were permeabilized with 0.1% Triton X-100-PBS for 30 min. Subsequently, samples were blocked with 5% BSA-PBS at RT for 1 h and incubated with an anti-GFAP goat monoclonal antibody or S100B rabbit monoclonal antibody at 4 °C overnight. The following day, samples were washed 3 times with PBS, incubated with secondary antibodies for 2 h at RT, then with DAPI at RT for 30 min, and finally washed 3 times with PBS. Fluorescence was detected using a Zeiss LSM710 fluorescence microscope.

Astrocytes were cultured on glass coverslips in a 24-well plate and co-transfected with *Dcf1*-1D4 and *Atp1b1*-myc and mutant constructs. The mutant plasmids were constructed using overlap extension PCR cloning (the primer sequences are given in Table 1). The transfection was performed 24 h later and the cells were changed into culture medium without serum. 1 μg DNA of the constructs were introduced into astrocytes using 1 μl of Lipofectamine 2000 transfection reagent (Invitrogen). The transfection medium was replaced with 500 μl of fresh culture medium 8 h after transfection. 48-h post-transfection, the corresponding proteins were detected by an anti-1D4 mouse monoclonal antibody and an anti-myc rabbit monoclonal antibody, as described above. Expression of the two proteins was detected by different fluorescent secondary antibodies, and fluorescence was detected using a Zeiss LSM710 fluorescence microscope.

2.2.3. Measurement of astrocyte length

Primary astrocytes from the hippocampal region of *Dcf1*$^{+/+}$ and *Dcf1*$^{-/-}$ mouse pups (P0-P3) were cultured and stained with an anti-GFAP goat monoclonal antibody (Abcam, USA) or S100B rabbit monoclonal antibody (Proteintech, USA). Following subsequent incubation with a fluorescent secondary antibody, the cells were photographed. The outlines of confocal images of single cells without any cell–cell contacts were used for morphometric analysis. Image-Pro Plus 6.0 software (de Campos et al., 2014; Mei et al., 2016) was used for measuring the length of astrocytes. Astrocyte length has been defined as the length of a direct line between two points at the maximal distance on a cell outline passing through the cell nucleus (Levina et al., 2001) (Fig. S1). The white line represented the length of the astrocytes from *Dcf1*$^{+/+}$ and *Dcf1*$^{-/-}$ mice respectively (Fig. S1).

2.2.4. Quantitative real-time PCR

Total RNA was extracted using an RNA extraction kit (Promega), according to the manufacturer's protocol. The concentration of RNA