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Research paper

Genetic regulation analysis reveals involvement of tumor necrosis factor and alpha-induced protein 3 in stress response in mice

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ABSTRACT

In order to study whether *Tnfaip3* is related to stress response and further to find it's genetic regulation, we use C57BL/6J (B6) and DBA/2 (D2) mice to built the model of chronic unpredictable mild stress. RT-PCR, Western blotting and immunohistochemistry were used for studying the expression variation of *Tnfaip3* in hippocampus tissue of B6 and D2 mice after being stressed. We found that the expression of *Tnfaip3* was more remarkably increased in chronic unpredictable stress models than that in untreated mice ($P < 0.05$). It is indicated that *Tnfaip3* might take part in the process of stress response. The expression of *Tnfaip3* is regulated by a cis-acting quantitative trait locus (cis-eQTL). We identified 5 genes are controlled by *Tnfaip3* and the expression of 64 genes highly associated with *Tnfaip3*, 9 of those have formerly been participate in stress related pathways. In order to estimate the relationship between *Tnfaip3* and its downstream genes or network members, we transfected SH-SY5Y cells with *Tnfaip3* siRNA leading to down-regulation of *Tnfaip3* mRNA. We confirmed a significant influence of *Tnfaip3* depletion on the expression of *Tsc22d3*, *Pex7*, *Rap2a*, *Slc2a3*, and *Gap43*. These validated downstream genes and members of *Tnfaip3* gene network provide us new insight into the biological mechanisms of *Tnfaip3* in chronic unpredictable stress.

1. Introduction

It has been well recognized that stress has become an important environmental factor which affects the life of individuals, families and social groups (Saxe et al., 2005). Stress response is a complex trait which is regulated by many genes (Desbonnet et al., 2012) and its definite mechanisms remain elusive. Tumor necrosis factor, alpha-induced protein 3 (*Tnfaip3*) gene is induced by the tumor necrosis factor (TNF) and it is a negative regulator of tumor necrosis factor (TNF)-induced signaling pathways (Dai et al., 2013). In current study, we found there is a very close relationship between neuroinflammation and stress. The relationship is very complex. We have found that there is an increase in inflammation facto revels after stress in the cortex and hippocampus such as TNF-α, IL-6 (Jiang et al., 2013). But it is reported that anti-inflammatory pathways are also activated in response to stress in brain (Garcia-Bueno et al., 2008). It is may endogenous mechanism against excessive inflammation of defense. We also found *Tnfaip3* highly expresses in hippocampus which is a main brain region related to stress response, and using phenotype database of the BXD RI strains (www.genenetwork.org), we can find the expression of *Tnfaip3* significantly associated with some stress related phenotypes on the basis of our genetic correlation analysis. (Table 1) So we inferred that *Tnfaip3* may be implied in stress response and choose it for further study.

At present, DBA/2J (D2), C57BL/6J (B6) and their recombinant inbred (RI) progeny, BXD RI strains, are used to study the genetic regulation of *Tnfaip3* and to identify the member of its gene network. The BXD population has been extensively phenotypes for thousands of behavioral traits and gene expression level profiles have been generated for many brain regions including stress related brain parts. In addition, this family has been densely genotyped and both parental genomes are fully sequenced and known to be segregating at ~5 million sequence variants. This extensive set of molecular, genetic and phenotypic data make the BXD panel of strains particularly well suited for system genetic analysis of sequence variants, expression differences and linkage to behavioral traits (Tarricone et al., 1995; Peirce et al., 2003). Parental strains of the BXD family have previously been shown to display

Abbreviations: B6, C57BL/6J; D2, DBA/2; PCR, polymerase chain reaction; cDNA, DNA complementary to RNA; cis-eQTL, cis-acting quantitative trait locus; eQTL, expression quantitative trait locus; TNF, tumor necrosis factor; RI, recombinant inbred; CUMS, Chronic Unpredictable Mild Stress; WB, Western blotting; SH-SY5Y, Human neuroblastoma; BDNF, brain derived neurotrophic factor.

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Table 1
Genetic correlation of *Tnfaip3* expression with 16 behavioral phenotypes in GeneNetwork's published phenotypes database.

Record ID	Phenotype	Sample r*	Max LRS location (Chr: Mb)	N	Sample p(r)	Reference	PMID
12338	Anxiety assay, baseline untreated control, time in open quadrants using an elevated zero maze in 60 to 120-day-old females only during 10 min	−0.4216	Chr10: 19.098322	58	0.00085	Melloni Cook et al. (2009)	Unpublished
12336	Anxiety assay, baseline untreated control, time in open quadrants using an elevated zero maze in 60 to 120-day-old females only during first 5 min	−0.4060	Chr10: 19.098322	58	0.00146	Melloni Cook et al. (2009)	Unpublished

(Values of Record 1433699_at in the Hippocampus Consortium M430v2 (Jun06) RMA database were compared to all 4545 records in the BXD Published Phenotypes database, $P < 0.01$.).
Note: *Pearson product-moment correlation was performed. PMID denotes the PubMed ID and unpolished represents the data set has not been published but been used in GeneNetwork.

fundamental genetic differences in stress response and have been used to identify genetic modifiers of stress response (Mozhui et al., 2010).

In this study, we associate linkage analysis and molecular biology techniques with gene expression profiling in the hippocampus area of the BXD mice resource to identify genetic regulation of *Tnfaip3* gene expression and gene network of *Tnfaip3*. Our findings will provide us new insight into the biological role of *Tnfaip3*, which is involved to stress research.

2. Materials and methods

2.1. Animals

A total of 32 adult DBA/2J (D2) and C57BL/6J (B6) male mice aged 25 days were used for the experiment. We used 10 adult male mice (5 B6 and 5 D2) in the step of immunohistochemistry, 10 adult male mice (5 B6 and 5 D2) in the step of Western blotting, 12 adult male mice (6 B6 and 6 D2) in the step of RT-PCR. The animals were adapted to the new environment before experiments for one week. All mice were housed in the same-sex standard cages (42 cm × 27 cm × 16 cm), 6 mice per cage until the time of experimenting. All mice were kept under conditions (free access to standard laboratory food and water, temperature: 20 to 24 °C, 12:12 h light/dark cycle). The experimental methods was performed in accordance with animal research guidelines and all protocols involving in brain dissection, mice tissue collection and the chronic unpredictable mild stress model were approved by and the Institutional Animal Care and Use Committee of Medical College of Nantong University, China.

2.2. Chronic unpredictable mild stress (CUMS)

The methods of CUMS model was set up according to Willner et al. (1992) with modifications (Willner et al., 1992; Huang et al., 2010). The CUMS protocol in our experiment lasted 8 weeks and consisted of 10 different mild stressors. The stressors were: 24-h of water or food deprivation, 5-min cold swimming in 4 °C water, cage tilt (45°) for 4 h, cage rocking (5 times per second) for 15 min, restraint for 12 h, alterations of the dark and light cycle for 24 h, noise (85 dB), elevated temperature (45 °C) for 15 min. During a period of 56 days, one of the stressor was selected randomly and applied to the mice at 9:00 AM every day so that the mice were not able to expect the stimulus. Every stressor used 4 or 6 times in total for each mice within 56 days and consecutive selection of the same stressor was avoided.

2.3. RT-PCR

Total RNA form cultured SH-SY5Y cells and hippocampus was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Hippocampus tissues were stored at −80 °C until assayed. RNA were reverse transcribed into cDNA by using a commercial RT-PCR kit (Fermentas, Vilnius, Lithuania) following the manufacturer's instructions. The primer sequences were as follows: *Tnfaip3* (sense) 5′-AGTTTTGCCCACAGT-3′ and (antisense) 5′-TCCCATTCGTCATTC-3′; *GAPDH* (sense) 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and (antisense) 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. PCR products separated on a 1.5% agarose/TAE gel were visualized by staining with ethidium bromide. The densitometric analysis of the data was normalized to GAPDH. The intensity of bands was determined using the Image-Pro Plus 6.0 software.

2.4. Western blotting

Fresh hippocampus was got from the D2 and B6 mice. Brain tissues were stored at −80 °C until assayed. Total hippocampus protein was isolated in a lysis buffer, resolved by 10%.

SDS–PAGE and transferred onto PVDF (polyvinylidene fluoride) membranes by electro blotting. *Tnfaip3* protein was detected using mouse monoclonal antibody against human *Tnfaip3* (1:500 sc-166692, Santa Cruz, USA). The HRP-conjugated secondary antibody was provided with the Western Dot kit. The bands were scanned using Alpha Imager HP and the intensity of each band was determined using Alpha View Imaging Software (version 3.0.3.0, AlphaInnotech Corp, Santa Clara, CA, USA). The optical density was normalized against of the GAPDH (1:1000,sc-365062, Santa Cruz, USA).

2.5. Immunohistochemistry

The mice were first anesthetized by pentobarbital sodium and then the brains of the mice were first perfuse with Physiological saline and then perfuse with 4% paraformaldehyde. Then mice were killed by cervical dislocation. Their brains tissue were rapidly removed and further fixed in the 4% paraformaldehyde at 4 °C for 1 day. Brain tissue were cut into 4-um-thick transverse sections after washing in phosphate buffer and immersion in sucrose (first 20% and then 30%). Immunohistochemistry was accomplished according to our routine method (Yamada et al., 2008). Firstly, the sections were incubated with mouse monoclonal antibody against human *Tnfaip3* (sc-166692, Santa Cruz, USA) diluted 1:100 in PBS with 1% BSA (bovine serum albumin) in PBS at 4 °C for 24 h. After it, the tissue sections were incubated for 2 h at room temperature with biotinylated goat anti-rabbit IgG (a secondary antibody) diluted 1:100 in PBS. Then the tissue sections were washed and incubated with avidin-biotin-horseradish peroxides (ABC; Vector Laboratories) diluted 1:200 in PBS-BSAT at room temperature for 30 min. After a final wash, the tissue sections were developed in 0.02% DAB (3,3-diaminobenzidine tetra hydrochloride), 0.6% ammonium nickel sulfate and 0.005% hydrogen peroxide in 0.05 M Tris–HCl buffer solution for 5 min. Tissue sections were then mounted, dehydrated, cleared, and covered with a cover slip with Malinol (Muto Pure Chemicals, Tokyo, Japan).

2.6. Gene expression data sets

The expression of hippocampus data set used in our study, comes from “Hippocampus Consortium M430v2 (Jun06) RMA”. The sets can be accessed from www.genenetwork.org. The data contains detailed information, for example RNA extraction, tissue acquisition, array hybridization methods, normalization methods and data processing can be found in the “Info” page of the data sets in geneNetwork.