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Research Paper

Novel *Drosophila* model for psychiatric disorders including autism spectrum disorder by targeting of ATP-binding cassette protein A

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ABSTRACT

Autism spectrum disorder (ASD) is characterized by persistent deficits in social communication and social interactions, as well as restricted, stereotyped patterns of behavior and interests. In addition, alterations in circadian sleep-wake rhythm are common in young children with ASD. Mutations in *ATP binding cassette subfamily A member 13* (*ABCA13*) have been recently identified in a monkey that displays behavior associated with ASD. ABCA13, a member of the ABCA family of proteins, is predicted to transport lipid molecules and is expressed in the human trachea, testis, bone marrow, hippocampus, cortex, and other tissues. However, its physiological function remains unknown. *Drosophila CG1718* shows high homology to human *ABCA* genes including *ABCA13* and is thus designated as *Drosophila ABCA* (*dABCA*). To elucidate the physiological role of *dABCA*, we specifically knocked down *dABCA* in all neurons of flies and investigated their phenotypes. The pan-neuron-specific knockdown of *dABCA* resulted in increased social space with the closest neighbor in adult male flies but exerted no effect on their climbing ability, indicating that the increase in social space is not due to a defect in their climbing ability. An activity assay with adult male flies revealed that knockdown of *dABCA* in all neurons induces early onset of evening activity in adult flies followed by relatively high activity during morning peaks, evening peaks, and midday siesta. These phenotypes are similar to defects observed in human ASD patients, suggesting that the established *dABCA* knockdown flies are a promising model for ASD. In addition, an increase in satellite boutons in presynaptic terminals of motor neurons was observed in *dABCA* knockdown third instar larvae, suggesting that *dABCA* regulates the formation and/or maintenance of presynaptic terminals of motor neurons.

1. Introduction

Mutations in human *adenosine triphosphate* (*ATP*)*-binding cassette subfamily A member 13* (*ABCA13*) are associated with schizophrenia and bipolar disorder (Tomioka et al., 2012; Knight et al., 2009). ABCA13 protein is expressed in mouse and human hippocampus and cortex, which are both regions relevant to schizophrenia and bipolar disorder (Tomioka et al., 2012; Knight et al., 2009). Whole-exome sequencing analyses of children with autism spectrum disorder (ASD) have identified many autism susceptibility genes including *ABCA13* (Iossjfov et al., 2014; De Rubeis et al., 2014; Neale et al., 2012; Iossifov et al., 2012). Recent studies show that a monkey carrying the heterozygous *ABCA13* deletion displays an impaired ability to monitor others' actions, an obsession with systems, and repetitive behavior, which are most frequently associated with ASD (Yoshida et al., 2016). In general, ASD is characterized by impaired social interaction, language and communication abnormalities, and stereotypic behaviors (Yenkoyan et al., 2017). In addition, alterations in circadian sleep-wake rhythm are common in young children with ASD (Tordjman et al., 2015). Interestingly, whole-exome sequencing and copy number variation analyses identified rare coding variants that are linked to the monkey homologue of human *ABCA13*, strongly suggesting that monkey *ABCA13* is an ASD-causing gene (Yoshida et al., 2016).

ABCA13, a member of the ABCA protein family, is predicted to have

Abbreviations: ABCA, ATP-binding cassette protein A; ASD, autism spectrum disorder; CNS, central nervous system; NMJ, neuromuscular junction

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the typical structure of full-size ABC proteins, including two transmembrane domains (TMDs), each containing six transmembrane alpha-helices and two nucleotide binding domains (NBDs), the latter containing characteristic ATP-binding Walker A and B motifs. ABCA13 is the largest member of the ABC protein family with > 3500 amino acids and has a long N-terminal region. It is expressed in the human trachea, testis, bone marrow, hippocampus, cortex, and other tissues, but its physiological function remains unknown.

Phylogenetic analyses of human ABCs revealed that the ABC transporters can be divided into seven subfamilies, named as ABCA to ABCG. ABC transporters use the energy from ATP binding and hydrolysis to translocate various substrates including lipids, ions, sugars, carbohydrates, amino acids, peptides, vitamins, steroid hormones, and xenobiotics, such as anticancer drugs. In various eukaryotic cells, ABC transporters are located in the plasma membrane and in the lipid membranes of the Golgi apparatus, endosomes, endoplasmic reticulum, multivesicular bodies, peroxisomes and mitochondria. Most ABCA transporters are ubiquitously expressed in various tissues including brain. Mutations in several ABC proteins result in monogenetic disorders affecting diverse physiological systems, suggesting that ABC transporters play roles in a broad range of physiological systems (Piehler et al., 2012). *Drosophila* has a homologue for human *ABCA* family genes called *CG1718*, which is located on the 19F3 region of the fly X chromosome. Because *Drosophila* CG1718 and human ABCA family proteins including ABCA13 are highly conserved, we designated the *Drosophila CG1718* as *dABCA*.

Drosophila has been established as an excellent model for studying various human diseases, although there are some limitations with this animal model (Chow and Reiter, 2017). For instance, there is no Schwann cell in *Drosophila*, it is therefore not suitable as a model of human diseases that have defects in Schwann cells. However, considering the advantages of *Drosophila* as a useful model organism, development of *Drosophila* models for ASD will contribute to understanding of its pathological mechanism. Detailed phenotypic analyses of *Drosophila* mutants of the autism candidate gene *neurobeachin* (*rugose*) demonstrated that the *rugose* plays a role in neuronal development, synapse formation, locomotion, and adult social behavior and activity patterns (Wise et al., 2015). The phenotypic characteristics of the *rugose* mutants are reminiscent of human ASD patients, suggesting that this is a useful fly model for studying ASD (Wise et al., 2015). Another gene, *fragile X mental retardation 1* (*FMR1*) encoding FMRP, is also a causative gene for ASD. Mutations in *Drosophila FMR1* (*dfmr1*) induce neuromuscular junction (NMJ) synapse overelaboration such as overgrowth, overbranching, and excess boutons in synapse, accompanied by accumulation of developmentally arrested satellite boutons and altered neurotransmission (Siller and Broadie, 2011; Gatto and Broadie, 2008; Zhang et al., 2001). In the present study, we have established an ASD model using *Drosophila* targeting of *dABCA*. Here, we found that pan-neuron-specific knockdown of *dABCA* results in a reduction in social space and enhances the activity of adult flies. Anatomical defects at NMJs, such as an increase in satellite boutons in presynaptic terminals of motor neurons in third instar larvae, were also observed in the knockdown flies.

2. Materials and methods

2.1. Fly stocks

Flies were raised on standard food containing 0.65% agar, 10% glucose, 4% dry yeast, 5% cone flour, and 3% rice bran. Flies were entrained on a 12 h:12 h light-dark cycle at 25 °C. Canton S was used as the wild type. *w; UAS-dABCA-IR$_{997–1106}$; +* (CG1718) were obtained from the Vienna *Drosophila* RNAi Center. The RNAi of this strain was targeted to the region corresponding to amino acid residues (aa) 997–1106 of *Drosophila* ABCA. The fly lines carrying *w; +; UAS-dABCA-IR$_{666–672}$* were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. The RNAi of this strain was targeted to the region corresponding to amino acid residues 666–672 of *Drosophila* ABCA. The fly line carrying *w; P[GAL4-elav.L]^{3}* was also obtained from the Bloomington *Drosophila* Stock Center at Indiana University.

2.2. Comparison of aa sequences of human ABCA, human ABCA13, and Drosophila ABCA

The amino acid sequence of *Drosophila* ABCA was retrieved from UniProt (http://www.uniprot.org). The identity and similarity of human ABCA3, human ABCA13, and *Drosophila* ABCA were compared using FASTA and BLAST.

2.3. Production of rabbit anti-dABCA antibody

The dABCA peptide, H-CKVRRNLEALRQARLSGGYA-OH, corresponding to aa1577-aa1595 of dABCA (the underlined N-terminal residue C was an added residue) was conjugated to keyhole limpet hemocyanin. Then it was mixed with Freund's complete adjuvant to provide a suspension, which was injected subcutaneously into a rabbit (Japanese White) kept under specific pathogen-free conditions. The rabbit was then boosted with inoculations of an immunogen of the same quality once a week for 7 weeks. A terminal bleed was performed to collect the maximum amount of serum (Sigma-Aldrich). The IgG was purified from the serum with Protein G Mag Sepharose™ Xtra (GE Healthcare) following the manufacturer's instructions.

2.4. Western blot analysis

Protein extracts from the central nervous system (CNS) of *Drosophila* adults carrying *w/Y; UAS-GFP-IR/+; elav-GAL4/+*, *w/Y; +; UAS-dABCA-IR$_{666–672}$/elav-GAL4*, *w/Y; UAS-dABCA-IR$_{997–1106}$/+; elav-GAL4/+*, and *w/Y; UAS-dABCA-IR$_{997–1106}$; elav-GAL4/+* were prepared. Briefly, the CNS was excised from newly eclosed adult male fly heads. The heads were boiled at 95 °C for 2 min in 0.1 M Tris-HCl (pH 7.6) and cOmplete Mini, EDTA-free (Roche Diagnostics) and homogenized in sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue, and 1.2% β-mercaptoethanol. The homogenates were boiled at 95 °C for 5 min and then centrifuged. The supernatants (extracts) were electrophoretically separated on SDS-polyacrylamide gels containing 6% acrylamide and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The blotted membranes were preincubated with TBS/0.1% Tween 20 containing 0.3% skim milk for 1 h at 25 °C and incubated with rabbit anti-dABCA IgG (1:500) in Can Get Signal Solution-1 (TOYOBO) for 16 h at 4 °C. After repeated washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:4000) in Can Get Signal Solution-2 (TOYOBO) for 1 h at 25 °C. Antibody binding was detected using ECL-advance Western blotting detection reagents, and images were analyzed with AE-9300H Ez-Capture MG (ATTO). To ensure equal protein loading in each lane, the membranes were also probed with an anti-tubulin antibody at the same time and HRP-conjugated anti-rabbit IgG. For detection of α-tubulin, mouse anti-α-tubulin monoclonal antibody (1:8000, Sigma) and HRP-conjugated anti-mouse IgG (1:10,000, GE Healthcare) were used as the primary and secondary antibodies, respectively.

2.5. Immunostaining

Larval brains from third instar larvae were dissected in phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min at 25 °C. After washing with PBS containing 0.3% Triton X-100, the samples were blocked with 0.15% Triton X-100 and 10% normal goat serum (NGS) for 30 min at 25 °C and incubated with mouse anti-Discs Large (anti-DLG) antibody (Developmental Studies Hybridoma Bank; 1:200) and rabbit anti-dABCA IgG (1:100) in Can Get Signal

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