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Mutations in *Bacchus* reveal a tyramine-dependent nuclear regulator for acute ethanol sensitivity in *Drosophila*

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ABSTRACT

Fruit flies and humans display remarkably similar behavioral responses to ethanol intoxication. Here we report that loss-of-function mutations in the *CG9894* gene (now named *Bacchus* or *Bacc*) attenuate ethanol sensitivity in flies. *Bacc* encodes a broadly expressed nuclear protein with a motif similar to ribosomal RNA-binding domains. The ethanol-related activity of *Bacc* was mapped to *Tdc2*-GAL4 neurons. Genetic and pharmacological analyses suggest that ethanol resistance of *Bacc* mutants is caused by increased *tyramine β-hydroxylase* (*tβh*) activity that results in excessive conversion of tyramine (TA) to octopmaine (OA). Thus, *tβh* and its negative regulator *Bacc* define a novel biogenic amine-mediated signaling pathway that regulates fly ethanol sensitivity. Importantly, elevated *tbh* activity has been shown to promote fighting behavior, raising the possibility that the *Bacc*/*tbh* pathway may regulate complex traits in addition to acute ethanol response.

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1. Introduction

Linkage and association studies have revealed the effects of genetic variations on individual susceptibility to ethanol abuse disorders (Gelernter and Kranzler, 2009; Spence et al., 2009). Genetic links also exist between alcoholism and other behavioral disorders (Dick et al., 2004; Hill et al., 2002; Lappalainen et al., 1998). However, molecular and neural mechanisms underlying such complex behavioral traits remain poorly understood.

In both flies and mammals, ethanol elicits an excitatory state at lower concentrations but induces incoordination and sedation at higher doses (Guarnieri and Heberlein, 2003). The ethanol sensitivity of *Drosophila* involves a growing number of genes that are functionally conserved in mammals (Chen et al., 2008, 2010; Corl et al., 2005; Moore et al., 1998; Rodan and Rothenfluh, 2010; Wen et al., 2005). For example, *Drosophila* neuropeptide F (NPF), like its mammalian homologue neuropeptide Y (NPY), is essential for the normal sensitivity to acute ethanol intoxication (Wen et al., 2005). In addition, at least two neurotransmitters, serotonin and GABA, have been implicated in the regulation of acute ethanol sensitivity in both rodent and fly models (Chen et al., 2010; Dzitoyeva et al., 2003; Hill, 1974; Martz et al., 1983). Together, these findings provide validations for the use of the fly model for genetic study of ethanol use disorders.

Tyramine (TA) and octopamine (OA) of insects have been suggested to be the functional homologues of epinephrine and norepinephrine, respectively, and their receptors may also be evolutionarily conserved (Roeder et al., 2003). In the fly nervous system, the enzyme tyrosine decarboxylase 2 (Tdc2) synthesizes TA from tyrosine, which, in turn, can be converted to OA by tyramine β-hydroxylase (Tβh) (Cole et al., 2005; Monastirioti et al., 1996). OA has been shown to influence diverse physiological processes and behaviors (Crocker and Sehgal, 2008; Hardie et al., 2007; Saraswati et al., 2004; Scholz et al., 2000). The $t\beta h^{nM18}$ flies deficient for OA signaling fail to display rapid ethanol tolerance, suggesting an essential role of OA in the development of rapid tolerance (Scholz et al., 2000). Although existing in a trace amount, TA has also been recognized to be a neurotransmitter (da Silva and Lange, 2008; Lange, 2009; Nagaya et al., 2002). However, unlike OA, the neurobiological significance of TA remains less understood (Lange, 2009; Roeder, 2005).

The *CG9894* gene encodes a nuclear protein expressed broadly in diverse tissues including the fly brain (Brody et al., 2002; Chintapalli et al., 2007). However, aside from observations that

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targeted disruption of *CG9894* via P-element insertion caused an enhanced fighting phenotype, its molecular function remains uncharacterized (Edwards et al., 2009). In this study, we show that loss-of-function mutations in *CG9894* (now named *Bacchus* or *Bacc*) reduces fly sensitivity to the intoxicating effect of ethanol. We also provide evidence that in *Tdc2*-GAL4 neurons, a *Bacc*-dependent nuclear mechanism negatively regulates the *tβh* transcripts level, thereby modulating the ratio between TA and OA. Our findings suggest that *Bacc* and *tβh* define a novel biogenic amine-mediated signaling pathway that regulates acute ethanol sensitivity in *Drosophila*.

2. Materials and methods

2.1. Flies

Larvae and adults were reared on apple juice agar plates with yeast paste at room temperature with exposure to natural lighting. Adult females, synchronized by collecting flies enclosed within a 12-h period, were aged for 7 days. All fly lines are in the w^{1118} genetic background by backcrossing for 6-7 generations. The $Bacc^{F2}$ and $Bacc^{537m}$ alleles were generated by a P-element insertion in this study (Toba et al., 1999). The $Bacc^{KG08597}$ was obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN). The UAS-$Bacc^{dsRNA}$ (transformant ID 35461) was purchased from Vienna *Drosophila* RNAi Center (Vienna, Austria). A p[acman] vector containing *Bacc* (CH322-08H15) was purchased from BACPAC Resources Center (Oakland, CA) for genomic rescue (Venken et al., 2009). This vector was inserted at 65B2 on the third chromosome (BestGene Inc, Chino Hills, CA). The *elav*-GAL4 is a pan-neuronal driver. *Tdc2*-GAL4 labels neurons expressing tyrosine decarboxlyase (Cole et al., 2005). The $t\beta h^{nM18}$ mutant, which has excessive TA but lacking OA, was a gift from Henrike Scholz (University of Cologne) (Monastirioti et al., 1996). The $Tdc2^{RO54}$ mutant lacking both TA and OA was a gift from Jay Hirsh (University of Virginia) (Cole et al., 2005).

2.2. Behavioral and pharmacological tests

The procedures for behavioral assays were described previously (Wen et al., 2005). At least 3 independent trials were performed per experiment. TA, OA, yohimbine, and citalopram were purchased from Sigma (St. Louis, MO). Flies were fed the mixture of drugs and autoclave killed yeast. The drug concentration (w/w) was 5% tyramine, 3% octopamine, 1% yohimbine, or 0.1% citalopram in food. Flies were fed with drugs for 3 d prior to behavioral assays.

2.3. Quantitative RT-PCR (qRT-PCR)

RNAs were extracted from intact fly heads. The procedures of RNA extraction, first-strand cDNA synthesis, and qRT-PCR were described previously (Chen et al., 2008). The total *Bacc* transcripts were quantified using TaqMan or SYBR green-based qRT-PCR. Primers used include TaqMan probe Dm01800963_m1 (Applied Biosystems Inc, Foster City, CA), a forward primer (CCCCCGCAAAGGAATCCGTGAAAG) and a reverse primer (GGGGATGTCGTACTCGTCGCTCTC). Two primers (CGAATTGTTCTCTATATAACGCAG and CTCGATGATTTCCTCCAGAG) were used for quantification of *Bacc* transcript isoform RA, and two primers, CAGTCTTGTACGAACCGTCG and CTCGATGATTTCCTCCAGAG, were used for RB and RC. *Tdc2* transcripts were detected by primers TCAAAGA-TAAGCGCTTCGAGA and GTCCCAGGCGTAGTCAATGT, and *tbh* transcripts were detected by primers TTATGCCAGTGATGCTGCTC and TGAAAGCATTCTGCAAGTGG. The relative RNA quantification was normalized against *gapdh2* (Dm01843776_s1) for TaqMan-based qRT-PCR or *rpS17* (forward primer: CCTGCAACTTGATGGAGATACC; reverse primer: CGAACCAAGACGGTGAAGAAG) for SYBR green-based qRT-PCR.

2.4. Biogenic amine quantification

OA in fly brains were quantified by HPLC according to a method developed by Biomarkers Core Laboratory, Yerks National Primate Research Center at Emory University (Atlanta, GA). The samples were prepared according to a published protocol (Hardie and Hirsh, 2006). Briefly, twenty 7d-old female adult brains were dissected, and eye pigments were completely removed. Brains were placed into 100 μl of ice cold 50 mM citrate/acetate buffer (pH 4.5), homogenized and centrifuged at 4 °C to remove debris. 3, 4-dihydroxybenzylamine was added in samples as the internal standard for HPLC. The HPLC system consists of Dionex AS50 autosampler, GP50 gradient pump, and ED50 detector with potentials set at 900 mV. Chromatography was performed with a Supelco Discovery C18 column (Bellefonte, PA). Samples in 20 μl volume were injected in duplicates. The mobile phase was composed of 42 mM citric acid, 0.16 mM sodium EDTA, 64 mM sodium acetate, 1.5 mM sodium heptanesulfonate monohydrate, and 8% HPLC grade methanol (pH 4.5), and the flow rate was 1.00 ml/min for HPLC. OA and 5HT were detected and analyzed at 900 mV. The calibration curves were generated based on OA standards from Sigma with concentrations 5, 10, 25, 50, 75 and 100 nM. A mutant fly line $Tdc2^{RO54}$ lacking both OA and TA was used as the negative control. The biogenic amines in each fly line were quantified based on 6–12 replicates.

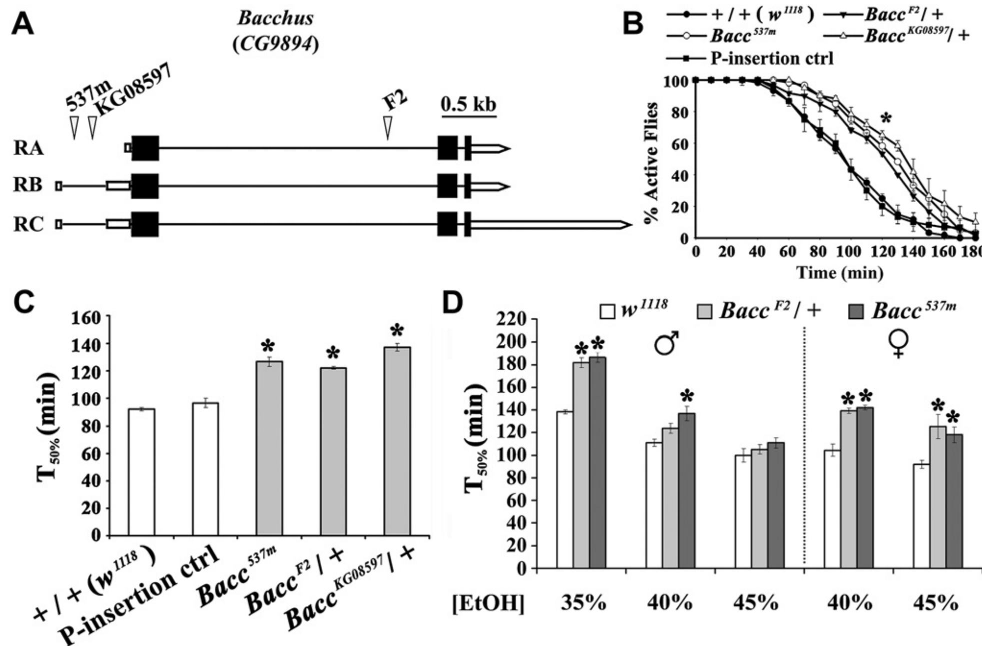

Fig. 1. Downregulation of *Bacc* reduced ethanol sensitivity. (A) Structural organization of the *Bacc* (*CG9894*) gene. Exons are represented by boxes, and coding regions are black. Although two initiation sites give rise to RA, RB and RC transcripts, they share an identical protein coding sequence. The P-element insertion sites for three *Bacc* alleles are shown. (B) The time courses showing reduced ethanol sensitivity of homozygous $Bacc^{537m}$, heterozygous $Bacc^{F2}$ and $Bacc^{KG08597}$ flies. (C) Comparison of $T_{50\%}$ values of the same flies. Asterisk in above panels, $P < 0.001$. (D) Analysis of sexually dimorphic effects of *Bacc* mutations. *Bacc* mutant males showed significantly reduced ethanol sensitivity in the presence of 35% and 40% ethanol solution. The *Bacc* mutant females displayed increased ethanol resistance that is dose-sensitive. However, unlike *Bacc* mutant females, males showed no detectable difference in ethanol sensitivity when ethanol solution was 45%, suggesting that the behavioral effects of *Bacc* mutations are sexually dimorphic. Asterisk, $P < 0.05$. Bars indicate SEM in all figures. $n = 3$ trials.

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