



Published in final edited form as:

*Physiol Behav.* 2012 December 5; 107(5): 663–665. doi:10.1016/j.physbeh.2012.04.010.

## Reduced sulfate plasma concentrations in the BTBR T+tf/J mouse model of autism

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### Abstract

Clinical studies have shown that children diagnosed with autism show abnormal sulfate chemistry, which is critical for cellular and metabolic processes. To determine if the inbred BTBR T+tf/J mouse shows autism-relevant aberrations in sulfate chemistry, the present study examined plasma sulfate concentrations in BTBR T+tf/J, inbred C57BL/6J, and outbred CD-1 mice. Results showed that the BTBR T+tf/J mouse exhibits significantly lower plasma sulfate concentrations in comparison to both C57BL/6J and CD-1 mice. These results suggest that the BTBR mouse shows autism-relevant abnormalities in sulfate chemistry and may serve additional utility in examining the role of sulfate and sulfate-dependent systems in relation to autism-relevant behavioral aberrations.

### Keywords

Autism spectrum disorders; Animal model; BTBR; Sulfate

## 1. Introduction

Autism spectrum disorders (ASD) appear to be increasing in prevalence [1, 2], and their etiology is poorly understood, in part reflecting the lack of accepted biomarkers for these disorders. ASD are diagnosed according to behavioral deficits in social interaction and communication along with repetitive, stereotypical behavior [3]. These criteria emphasize the need for relevant ASD animal models [4], leading to extensive effort in the development of autism-relevant behavioral tests and identification of candidate strains of mice that exhibit an ASD behavioral phenotype [5–9].

The BTBR T+tf/J (BTBR) mouse has garnered increasing support as a strong behavioral animal model of ASD [10, 11]. Studies have consistently shown that BTBR mice exhibit an autism-relevant behavioral phenotype capturing the diagnostic triad. BTBR mice show decreased social interaction [6, 12], impaired communication [13, 14], and restricted stereotypical behavioral patterns [15, 16]. Additional research has characterized potentially autism-relevant aberrations [17–20] in the BTBR mouse, including corpus callosum alterations [21], disturbed hippocampal commissure [22], and aberrant immune system [23].

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To further validate the BTBR as a model of ASD, additional features associated with ASD need to be examined.

Physiological and metabolic abnormalities are widely implicated in ASD [24]. An intriguing physiological abnormality that has been reported in ASD is alterations in sulfate chemistry [25–28]. Sulfate is an inorganic anion essential to normal human growth and development [29]. Moreover, sulfate regulates various cellular and metabolic processes including the biosynthesis and detoxification of endogenous and exogenous compounds [30]. Clinical research has consistently reported substantially decreased concentrations of plasma sulfate for children diagnosed with autism in comparison to age matched controls [25, 28, 31, 32]. While these reductions have many potential implications for mechanisms in the biology of ASD, this connection has not been extensively investigated.

The purpose of the present study was to determine if the BTBR mouse model of autism displays decreased plasma sulfate concentrations, providing a parallel to the reductions seen in autistic individuals. In experiment one, concentrations of plasma sulfate were examined in adult BTBR, C57BL/6J (B6: a frequently used inbred comparison strain in studies of mouse models of ASD: [4]), and the outbred CD-1 strain. In experiment two, these measures were replicated in additional groups of BTBR and B6 mice.

## 2. Material and Methods

### 2.1 Animals

Animals were naïve male BTBR T+tf/J (BTBR), C57BL/6J (B6), and CD-1 mice ( $n = 7$  per group/experiment) from stock originally obtained from Jackson Laboratories (Bar Harbor, ME) and Charles River (Hollister, CA, USA). BTBR, B6, and CD-1 mice were 10–12 weeks old in experiment one and BTBR and B6 were 6–8 weeks old for experiment two. Animals were bred at the University of Hawaii animal housing facility and group housed (3–4 per cage) with same sex littermates in ventilated polypropylene cages (35.5 cm L  $\times$  20 cm W  $\times$  13 cm H) under controlled environmental conditions (12:12 light/dark, lights on at 0600;  $21 \pm 3$  °C temperature; 55–58% humidity). PicoLab Rodent Diet 20 food (LabDiet, Cucamonga, CA) and water were provided *ad libitum*. Experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Animals and approved by the University of Hawaii Institutional Animal Care and Use Committee.

### 2.3 Procedure

Plasma sulfate concentrations were determined using a turbidimetric sulfate determination kit (Bioassay Systems, Hayward, CA) with a sensitivity between 0.02 mM and 2 mM according to the manufacturer's instructions for plasma samples. For plasma collection, trunk blood was collected between 1000 and 1300 h in 1.5 ml centrifuge tubes containing EDTA. Blood samples were centrifuged at 5000 rpm for 5 min and the supernatant was pipetted into new epi tubes. Plasma samples were stored at 4 °C and assayed as duplicates within 8 hours in a 96 well plate. Sample wells optical density was read at 600nm on a VersaMax Elisa microplate reader (Molecular Devices, Sunnyvale, CA).

### 2.4 Statistical Analysis

Sulfate concentrations in BTBR, B6, and the outbred CD-1 plasma samples were analyzed using a one-way ANOVA followed by Dunnett's post hoc comparisons using the BTBR mouse as the comparison group. The replication experiment for BTBR and B6 samples was analyzed using a two-tailed Student's t-test. Statistical significance was set at  $p < .05$  for all statistical tests.

### 3. Results

A one-way ANOVA showed significant differences in plasma sulfate between strains of mice,  $F(2,18) = 4.69$ ,  $p < .05$  (Figure 1A). Post hoc Dunnett's analysis showed BTBR were significantly lower in plasma sulfate concentrations compared to B6 and CD-1 mice. BTBR mice showed half the amount of plasma sulfate of B6 and CD-1 mice. We replicated these results with another set of BTBR and B6 animals and found plasma sulfate concentrations were again significantly lower for BTBR compared to B6 mice,  $t(12) = 2.806$ ,  $p < .05$  (Figure 1B).

### 4. Discussion

This study demonstrates that a leading behavioral animal model of ASD, the BTBR mouse, exhibits significantly lower plasma sulfate concentrations in comparison to either the inbred B6 mouse strain, a commonly used comparison strain to the BTBR, or to the outbred CD-1 mouse strain. Taken together, these results suggest that the level of plasma sulfate in adult BTBR mice is reduced by about 50%, a figure that is also typical of data from children with autism [25, 32]. Notably, the plasma sulfate concentrations of the B6 mice, from about 0.4 mM to 0.6 mM, were in agreement with previous data [33].

Concentrations of BTBR plasma sulfate, at about 0.2 mM were similar to those reported for a sodium-sulfate cotransporter (Nas1) knockout mouse (Nas1<sup>-/-</sup>) [34]. The authors of that study suggested that decreased serum sulfate in the Nas1 mice may be attributed to impaired absorption and/or reabsorption of sulfate from the intestine and kidney, suggesting the value of determining if the BTBR exhibits alterations in the Nas-1 sulfate transporter system and if impaired absorption/reabsorption of sulfate underlies the BTBR's decreased plasma sulfate levels. Deficits in sulfate availability for autistic individuals have also been attributed to increased urinary excretion [28], altered metabolism of amino acids into sulfate [32], or abnormal transport of sulfate [35].

Sulfate concentrations in blood are dependent upon diet, gender, circadian rhythm, and age [36]. The current study controlled for diet, gender, age, and time of blood collection for each experiment; therefore, the reported differences should be attributable to the strain of mouse. A limitation of the current study is that we did not determine if sulfate differences for the BTBR extend to prenatal ages, or to females. Previous research that maternal concentrations of sulfate substantially increase to supply the dependent fetus [29], indicate the potential importance of sulfate reductions in prenatal development, and suggest that this should be examined for the BTBR mouse.

Sulfate deficiencies may alter sulfation capacity, the addition of sulfate to another molecule. Impaired sulfation may contribute to the deficits in detoxification, inactivation of catecholamines, neural growth abnormalities, and gastrointestinal problems typically characterized in autism [37]. Abnormal sulfation capacity [31, 38, 39] and decreased processing of paracetamol through sulfate conjugation has been consistently reported in low functioning autistic children [26]. Our lab has recently reported additional evidence for impaired sulfation for the BTBR mouse, with decreased N-sulfated heparan sulfate within the subventricular zone of the lateral ventricles [10, 40]. As this area is one of two neurogenic zones in adult mammals, and also very active in development, while heparan sulfate modulates the activity of an array of growth and guidance factors [41], this finding, compatible with the results of the present study, may provide additional possibilities with regard to mechanisms involved in autism. Additionally, deficits in sulfation may account for reported alternations in serotonin and serotonin receptor function in BTBR mice [42] that parallel alterations reported for autistic individuals [43]. Since previous work has suggested that blood sulfate concentration may determine the rate of sulfation [36], we hypothesize

that BTBR deficiencies in plasma sulfate may disturb processes related to properly sulfated glycoaminoglycans and metabolism of neurotransmitters.

These results add reductions in plasma sulfate to the list of biological aberrations of BTBR mice (e.g. agenesis or thinning of the corpus callosum and hippocampal commissure; aberrant immune functioning: [17–20]) that may provide parallels to characteristics of some or many autistic individuals. This suggests that the value of the BTBR mouse as an animal model of autism may extend well beyond behavior, providing a model for experimental studies investigating the association of sulfate aberrations and autism-relevant behavioral characteristics. Indeed, some clinical treatments of ASD have already been based on administration of sulfate [28]. A recent study examined the effects of a vitamin/mineral supplement on children and adults with autism and found behavioral improvements were associated with increases in total sulfate and sulfation measurements [44]. Use of an appropriate animal model would greatly facilitate experimental evaluations of these and other possible treatments of ASD

In summary, our experiments demonstrate substantially decreased concentrations of plasma sulfate in a leading behavioral mouse model of autism, suggesting that the BTBR mouse exhibits abnormalities in sulfation capability. These results propose another utility of the BTBR mouse, to examine the role of sulfate and sulfate-dependent systems in relation to autism-relevant behavioral aberrations.

## Acknowledgments

The present study was supported by the National Institute of Mental Health(NIMH) grant MH081845-01A2 to RJB.

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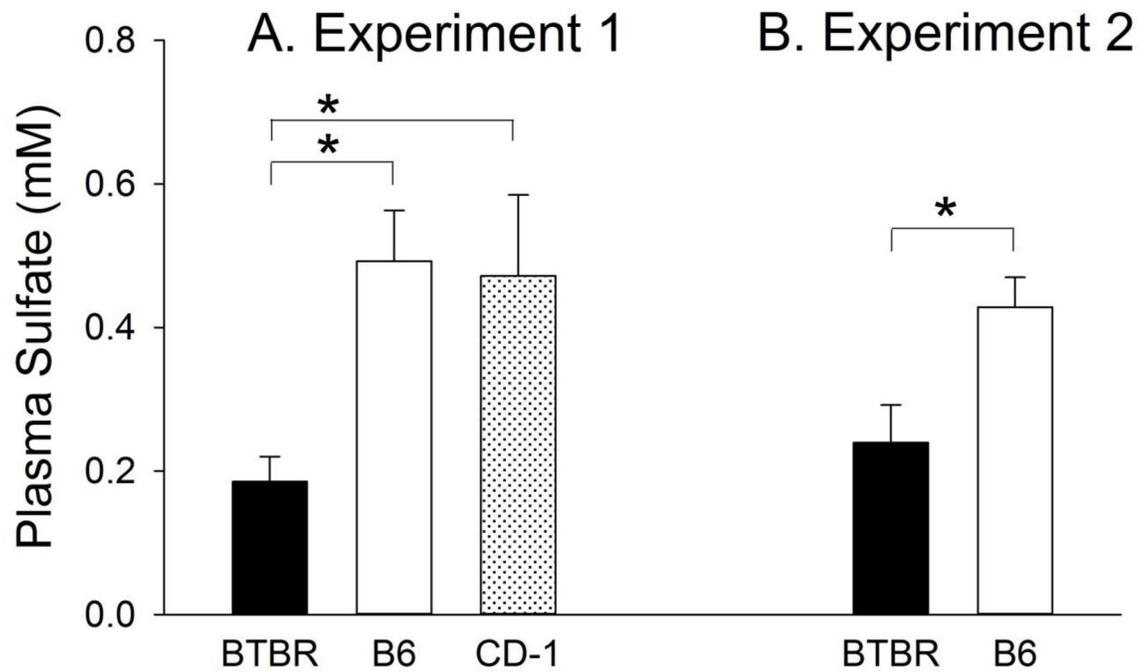
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### Highlights

- We examine autism-relevant aberrations in sulfate chemistry in a leading behavioral animal model of autism spectrum disorders, the BTBR mouse.
- BTBR mice show significantly decreased plasma sulfate in comparison to B6 and CD-1 mice.



**Figure 1.** Plasma sulfate concentration (mM) for BTBR T+tf/J (BTBR), C57BL/6J (B6), and CD-1 strains in Experiment 1 (n=7 per strain); BTBR and B6 (n=7 per strain) in Experiment 2 (means  $\pm$ SEM) \*  $p < 0.05$ .