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Research report

Fractone-associated N-sulfated heparan sulfate shows reduced quantity in BTBR T+tf/J mice: A strong model of autism

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ABSTRACT

BTBR T+tf/J (BTBR) mice show abnormal social, communicatory, and repetitive/stereotyped behaviors paralleling many of the symptoms of autism spectrum disorders. BTBR also show agenesis of the corpus callosum (CC) suggesting major perturbations of growth or guidance factors in the dorsal forebrain [1]. Heparan sulfate (HS) is a polysaccaride found in the brain and other animal tissues. It binds to a wide variety of ligands and through these ligands modulates a number of biological processes, including cell proliferation and differentiation, migration and guidance. It is aggregated on fractal-like structures (fractones) in the subventricular zone (SVZ), that may be visualized by laminin immunoreactivity (LAM-ir), as well as by HS immunoreactivity (HS-ir). We report that the lateral ventricles of BTBR mice were drastically reduced in area compared to C57BL/6J (B6) mice while the BTBR SVZ was significantly shorter than that of B6. In addition to much smaller fractones for BTBR, both HS and LAM-ir associated with fractones were significantly reduced in BTBR, and their anterior–posterior distributions were also altered. Finally, the ratio of HS to LAM in individual fractones was significantly higher in BTBR than in B6 mice. These data, in agreement with other findings linking HS to callosal development, suggest that variations in the quantity and distribution of HS in the SVZ of the lateral ventricles may be important modulators of the brain structural abnormalities of BTBR mice, and, potentially, contribute to the behavioral pathologies of these animals.

1. Introduction

Diagnoses of autism spectrum disorders (ASD) have become increasingly common in the past decade and now may involve nearly 1% of young children [2–5]. Although there is considerable evidence for a strong genetic factor in autism [6], it appears to be polygenic, with hundreds of contributing loci [7,8]. In addition, a range of environmental factors including fetal exposure to teratogens appear to influence rates of ASD [9,10]). The defining symptoms of ASD are behavioral, and, while a number of neural features are known to be associated with these disorders [11–13] it is questionable whether these features are sufficiently robust and specific as to suggest underlying neural mechanisms.

The definition of ASD in terms of three major symptom clusters: social interaction deficits, communication deficits, and ritualistic–repetitive behaviors; all typically detectable in early childhood but continuing throughout life [14–16], has put an extraordinary emphasis on the development of animal models displaying behaviors that are relevant to these symptoms. In this context, the inbred BTBR T+tf/J (BTBR) mouse has emerged as a particularly strong model. Adult BTBR mice display low levels of social behavior [17–19], including specific avoidance of oriented nose-to-nose contact [20]; poor social learning in the transmission-of-food-preference assay [18]; lower levels of vocalization [21] and higher levels of repetitive self-grooming [18,22–24] and cognitive stereotypies [25,26], compared to other mouse strains tested. They also show an unusual repertory of pup separation ultrasonic vocalizations [27] as well as adult ultrasonic calls [21]. These changes in BTBR mice may be relatively specific, as they are not attributable to low locomotor activity [28] or a general enhancement in emotional responsivity [18,19,22,29–31].

The BTBR mouse also displays some consistent neuroanatomical differences from most other mouse strains, including total absence of the corpus callosum (CC) and reductions in the hippocampal commissure [1,32]). As surgical lesions of CC at postnatal day 7 (PND7) in C57BL/6J (B6) mice do not produce juvenile or adult social changes, or grooming enhancement, the autism-relevant behaviors displayed by BTBR mice are not a direct consequence of callosal damage after this time [22], although it is possible that the lack of a CC between embryonic day 15 (E15) and PND7 might be

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relevant. However, the magnitude and consistency of these structural changes in BTBR suggest some major perturbation of cell genesis or guidance factors in this strain, and these perturbations may have effects on autistic-like behaviors that do not directly involve the CC.

Heparan sulfates (HS) are a highly diverse and complex family of sulfated glycans responsible for regulation of a wide range of biological processes including growth factor signaling, enzyme activity and cell adhesion [33,34]. HS proteoglycans (HSPGs) consist of HS chains linked to core proteins. They are located on the cell surface and extracellular matrix of mammalian cells where they exert vital roles in regulating cell behavior. HS side chains bind to a broad range of growth factors, cytokines and morphogens during cell proliferation and differentiation, co-operatively regulating ligand binding and signal transduction. In the subventricular zone (SVZ) of the anterior lateral ventricle (LV), one of two neurogenic zones in adult brain, HS are frequently associated with extracellular matrix structures named fractones. The fractal structure of the latter may facilitate contact with neural stem cells and their progeny [35].

Since HS synthesizing and modifying enzymes are crucial for development of a normal CC [36,37] we hypothesized that there might be some abnormality in the HS system in the brains of acallosal BTBR mice. Moreover, recent studies indicated that mice with conditional knockout of the HS synthesizing enzyme EXT1 (*CaMKII-Cre;Ext1$^{flox/flox}$* [38]) display abnormal social behaviors. The aim of the current study was to describe HS system abnormalities by evaluation of the density of both HS and LAM associated with fractones in the SVZ of the LV in BTBR and B6 mice. Notably, the ventricles of BTBR mice are distorted, especially in the neurogenic zone of the LVs, in comparison to those of B6 [39], and most other mouse strains. Our analyses therefore included determination of differences in terms of the normal anterior–posterior distribution patterns of HS and LAM, as well as measurement of both LV area and the length of the LV SVZ in the neurogenic zone.

2. Materials and methods

2.1. Animals

Naïve, 10–17-week-old male mice were used in this study. Inbred BTBR ($n = 5$) and B6 ($n = 5$) mice were bred at the University of Hawaii Laboratory Animal Services from stock originally obtained from Jackson Laboratory (Bar Harbor, ME). Animals were euthanized with an overdose of Avertin (2,2,2-tribromoethanol, Sigma–Aldrich, St. Louis, MO, i.p. at approx. 330 mg/kg) and their brains were removed from the cranial cavity. The brains were instantaneously frozen in isopentane cooled at −70 °C and subsequently stored at −20 °C. The animal experimental protocol followed NIH guidelines and was approved by the Institutional Animal Care and Use Committee at the University of Hawaii, protocol # 09-786-2.

2.2. Analysis of N-sulfated heparan sulfate and laminin immunoreactivity

2.2.1. Immunocytochemistry

N-Sulfated heparan sulfate- and laminin-immunopositive material was localized in the subventricular zone of the lateral ventricles of B6 and BTBR mice using single and dual immunofluorescence cytochemistry as described before (Mercier et al., 2002 [40]).Briefly, the immunostaining was performed on serial 25 μm-thick brain sections (cut with CM1900 Leica cryostat) mounted on poly-L-lysine (P4707; 1/5; Sigma–Aldrich) coated microscope slides. The serial coronal sections were stored at −20 °C until the time of fixation (2 min) in cooled (−60 °C) acetone. Then, the slices were incubated with the primary antibodies directed against N-sulfated heparan sulfates (1/500 antibody 10E4, Seikagaku-Cape Cod, East Falmouth) and laminin (1/1000 L9393, Sigma–Aldrich, Saint Louis, MO). Goat anti-rabbit conjugated to Alexa-Fluor647, goat anti-mouse IgM conjugated to Alexa-Fluor488 (Molecular Probes/Invitrogen), and donkey anti-rabbit conjugated to Alexa-Fluor 488 were used as secondary antibodies (all diluted at 1/400).

2.2.2. Image collection and analysis

The ventricle cross-sectional area and SVZ length were measured in all subjects on images (1392 × 1040 pixels) taken at 10× magnification using a digital DFC350FX Leica camera mounted on a Leica DMIL epifluorescence microscope set to visualize heparan sulfate immunoreactivity. The area of the LV was measured in pixels with the use of ImageJ (NIH) software by following the ependymal wall of the ventricle with the freehand selection tool. The length of the SVZ was measured using the freehand selection tool along the midline of the HS-positive fractone zone. The results were averaged across at least 3 brain slices (measurements taken bilaterally) per animal, from the same antero-posterior (AP) points (identified with the use of mouse brain atlas, [41]) in all animals ($n = 5$/group). The AP points of interest were AP + 1.0, +0.5, 0.0, −0.5 and −1.0, which cover the extent of the neurogenic zone of the LVs. To visualize the differences in LVs area we have performed additional staining of brain sections from the relevant AP levels with cresyl violet (Fig. 1).

Area and intensity of laminin and heparan sulfate immunoreactivity in individual fractones were analyzed for two randomly selected subjects from each group (BTBR or B6) in images taken using a Zeiss Pascal confocal laser scanning microscope at 20× magnification. Each picture was composed of 3 channels (red (650 nm) for LAM; green (505–620 nm) for HS; and a blank blue channel). Partial images (unmodified, 2048 × 2048 pixels) were merged to show complete ventricles using Photoshop software (v 7.1; Adobe Systems, Mountain View, CA) and subjected to channel separation (ImageJ software) after merging. The area of individual fractones was measured (in pixels) using the freehand selection tool (ImageJ) on the LAM channel. The intensity of LAM and HS immunostaining were measured on LAM and HS channels (both represented in grayscale, in an arbitrary 1–256 scale) respectively for each fractone. The parameters were scored separately for brain slices representing distinct AP points. All parameters were measured by a single, trained observer.

2.3. Statistical analysis

The normal distribution and homoscedasticity of data was tested (with the use of Shapiro–Wilk and Levene tests) before analysis of variance was applied. For assessment of main effects of mouse strain and anterior–posterior distribution on LV area, SVZ length, and individual fractone area and LAM and HS immunoreactivity one-way ANOVA (strain serving as between-subject factor) with repeated measures for different AP points (within-subject factor) was performed with subsequent post-hoc Tukey's range test for direct comparisons of values. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Ventricle area and SVZ length

The quantification of differences in the size and shape (Figs. 1 and 2) of LV showed a strikingly reduced volume of LV cavity, and a reduction of about 40% in the length of the SVZ measured in the midline of HS-ir zone in BTBR vs. B6 mice. The BTBR LVs were smaller ($F_{1,4} = 465.10$, $p < 0.001$) and this effect was also AP dependent ($F_{4,12} = 6.62$, $p < 0.01$) with a significant interaction between strain × AP ($F_{4,12} = 6.79$, $p < 0.01$). Post-hoc comparison showed that at AP points 0.0 and −0.5, the LV cavities of B6 were significantly larger than those of BTBR mice (Fig. 2A, $p < 0.05$ and $p < 0.001$ respectively, indicated with #'s). Within strain comparisons showed that the area of LVs significantly increased in B6 mice from AP + 1.0 to 0.0 and −0.5 (Fig. 2A, $p < 0.05$ and $p < 0.001$, respectively, indicated with *'s) and AP + 0.5 to −0.5 (Fig. 2A, $p < 0.05$, indicated with a *) and then dropped at AP − 1.0, compared to AP − 0.5 ($p < 0.05$, indicated with a *), while in BTBR mice LV area remained constant, increasing (nonsignificantly) only at AP − 1.0.

Although the effect of mouse strain on the length of the SVZ was significant ($F_{1,4} = 9.92$, $p < 0.05$), the effect of AP and the interaction between the two factors was not. Therefore no detailed post-hoc analysis could be performed to establish if there were any AP-dependent differences in SVZ length between the two strains.

3.2. Size of individual fractones and densities of N-sulfated heparan sulfate (HS) and laminin (LAM)

The three parameters recorded using ImageJ (size of individual fractones, the intensity of laminin and heparan sulfate staining in fractones) as well as the HS/LAM ratio all yielded significant effects of mouse strain.

The total number of fractones identified and measured was $n = 1080$ in 2 B6 brains, and $n = 345$ in 2 BTBR brains. The mean area of fractones (measured in pixels, Fig. 3A and B) was greater in B6 mice than in BTBR ($F_{1,185} = 10.42$, $p < 0.001$, Fig. 3A, indicated