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LOW STRESS REACTIVITY AND NEUROENDOCRINE FACTORS IN THE BTBR T^{+}tf/J MOUSE MODEL OF AUTISM

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Abstract—Autism is a neurodevelopmental disorder characterized by abnormal reciprocal social interactions, communication deficits, and repetitive behaviors with restricted interests. BTBR T+tf/J (BTBR) is an inbred mouse strain that displays robust behavioral phenotypes with analogies to all three of the diagnostic symptoms of autism, including low social interactions, reduced vocalizations in social settings, and high levels of repetitive self-grooming. Autism-relevant phenotypes in BTBR offer translational tools to discover neurochemical mechanisms underlying unusual mouse behaviors relevant to symptoms of autism. Because repetitive self-grooming in mice may be a displacement behavior elevated by stressors, we investigated neuroendocrine markers of stress and behavioral reactivity to stressors in BTBR mice, as compared to C57BL/6J (B6), a standard inbred strain with high sociability. Radioimmunoassays replicated previous findings that circulating corticosterone is higher in BTBR than in B6. Higher basal glucocorticoid receptor mRNA and higher oxytocin peptide levels were detected in the brains of BTBR as compared to B6. No significant differences were detected in corticotrophin releasing factor (CRF) peptide or CRF mRNA. In response to behavioral stressors, BTBR and B6 were generally similar on behavioral tasks including stress-induced hyperthermia, elevated plus-maze, light ↔ dark exploration, tail flick, acoustic startle and prepulse inhibition. BTBR displayed less reactivity than B6 to a noxious thermal stimulus in the hot plate, and less immobility than B6 in both the forced swim and tail suspension depression-related tasks. BTBR, therefore, exhibited lower depression-like scores than B6 on two standard tests sensitive to antidepressants, did not differ from B6 on two well-validated anxiety-like behaviors, and did not exhibit unusual stress reactivity to sensory stimuli. Our findings support the interpretation that autism-relevant social deficits, vocalizations, and repetitive behaviors are not the result of abnormal stress reactivity in the BTBR mouse model of autism. Published by Elsevier Ltd on behalf of IBRO.

Key words: autism, mouse models, BTBR.

Autism is a complex neurodevelopmental disorder affecting approximately 1 in 150 children (Landa, 2008). The etiology of autism is currently unknown but evidence for strikingly high heritability is abundant (Abrahams and Geschwind, 2008; Happe and Ronald, 2008). Linkage and association studies have identified large numbers of de novo and familial candidate genes that may be responsible for susceptibility to autism (Persico and Bourgeron, 2006; Abrahams and Geschwind, 2008; Bourgeron, 2009; Buxbaum, 2009; Lintas and Persico, 2009). Animal models offer opportunities to test genetic hypotheses and evaluate proposed treatments. One strategy utilized with success in mice is the forward genetics approach of identifying inbred strains of mice with phenotypes relevant to the symptoms of a human disease, using multiple well-validated tasks. Forward genetics strain distribution analyses by our group and others identified several inbred strains of mice with low levels of social interaction (Brodkin et al., 2004; Moy et al., 2004, 2007, 2008b; Nadler et al., 2004; Sankoorikal et al., 2006; Bolivar et al., 2007; Crawley, 2007; Panksepp et al., 2007, 2008; Panksepp and Lahvis, 2007; Yang et al., 2007a,b, 2009; Fairless et al., 2008; McFarlane et al., 2008; Scattoni et al., 2008; Chen et al., 2009; Roullet et al., 2010; Silverman et al., 2010; Wohr et al., 2010). Of particular interest is BTBR T+tf/J (BTBR), an inbred strain which exhibits lower levels of play soliciting behaviors as juveniles and lacks sociability in the adult social approach task (Bolivar et al., 2007; Moy et al., 2007; Yang et al., 2007a,b; McFarlane et al., 2008), emits fewer ultrasonic vocalizations in various social settings (Scattoni et al., 2008, 2009; Roullet et al., 2010; Wohr et al., 2010) and displays high levels of repetitive self-grooming throughout their lifespan (Yang et al., 2007b; McFarlane et al., 2008; Pearson et al., 2010; Silverman et al., 2010). These behaviors are relevant to all three core symptom domains of autism. Normal scores on measures of general health, motor functions, and sensory abilities including olfaction (Moy et al., 2007, 2008a; McFarlane et al., 2008) support an interpretation of remarkably specific autism-relevant abnormalities in BTBR.

The mechanism underlying the high level of repetitive self-grooming in BTBR is unclear. Rodent self-grooming behavior is an innate behavior elicited in both comforting and stressful situations (van Erp et al., 1994; Moyaho and Valencia, 2002; Kalueff and Tuohimaa, 2004) with ethologically different patterns emerging for each type. A normal grooming pattern includes a cephalo-caudal progression beginning with licking and washing the paws, then the nose and face, head, body, fur, legs, genitals and tail (Berridge and Aldridge, 2000a,b). More frequent bursts of rapid grooming characterize stress-evoked grooming (Kalueff and Tuohimaa, 2004, 2005a). BTBR display the nor-

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Abbreviations: CRF, corticotrophin releasing factor; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; PVN, hypothalamic paraventricular nucleus; THP, 3α, 5α tetrahydroprogesterone.

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mal full sequence of grooming, with high numbers of self-grooming bouts and excessively long durations of self-grooming bouts, often exceeding 1 min of continuous self-grooming. Since stress provoking situations in mice are accompanied by heightened grooming behavior, a critical behavioral adaption to stress (Kametani, 1988; Sachs, 1988; Spruijt et al., 1992; van Erp et al., 1994; Kalueff and Tuohimaa, 2005b), and since hyperreactivity to stressors could be the cause of high self-grooming in BTBR, we investigated the possibility that the development and expression of BTBR's unique autistic-relevant phenotype may be the result of a stress-reactive phenotype, generalized neuroendocrine differences in stress hormones, or the high circulating corticosterone that was previously reported (Benno et al., 2009; Frye and Llaneza, 2010).

The hypothalamic-pituitary-adrenal axis neuroendocrine factors that elicit high levels of self-grooming when administered to rodents include adrenocorticotropic hormone (ACTH) and corticotrophin releasing factor (CRF) (Ferrari, 1958; Gispen et al., 1975; Morley and Levine, 1982; Dunn et al., 1987; Dunn and File, 1987; Sherman and Kalin, 1987; Matsuzaki et al., 1989; Monnikes et al., 1992). Other neuropeptide transmitters also evoke high levels of self-grooming when centrally administered to rodents, including vasopressin, prolactin, substance P, somatostatin, cholecystokinin and oxytocin (Drago et al., 1981, 1986; Meisenberg, 1981, 1988; Elliott and Iversen, 1986; Kaltwasser and Crawley, 1987, Van Wimersma Greidanus et al., 1987; Pedersen et al., 1988; Stivers et al., 1988; Kaltwasser and Andres, 1989; Van Erp et al., 1993; Amico et al., 2004). To expand the existing neurochemical data in BTBR, we conducted a baseline comparison of several relevant neuroendocrine factors in BTBR and B6, a standard inbred strain with high sociability, low self-grooming, and relative resilience to stressors (Moy et al., 2004, 2007; Yang et al., 2007a,b; McFarlane et al., 2008). Neuroendocrine measures, including corticosterone, CRF, glucocorticoid receptor and oxytocin were chosen based on rodent literature indicating their roles in stress responsivity as well as grooming via specific regional activation in rats and mice (Stenzel-Poore et al., 1994; McCarthy et al., 1996; Dunn and Swiergiel, 1999; Swiergiel and Dunn, 1999; Tronche et al., 1999; Anisman et al., 2001; Ridder et al., 2005; Ring et al., 2006; Roy et al., 2007; Yoshida et al., 2009; Cohen et al., 2010).

To comprehensively characterize basal stress reactivity, we assayed BTBR and B6 mice on two tests for anxiety-like behaviors, four parameters of sensory reactivity, and two depression-relevant tasks. Tasks were chosen both as standard measures of stress-related behaviors in mice, and for relevance to the literature indicating anxiety, high reactivity to stressors, hyperreactivity to sensory stimuli, upset to change, and elevated neurochemical markers of stress in some people with autism (Tordjman et al., 1997, 2009; American Psychiatric Association, 2000; Lord et al., 2000; Rogers et al., 2003; Dawson et al., 2004; Rogers and Ozonoff, 2005; Corbett et al., 2006; Lam et al., 2006; Lord and Spence, 2006; Perry et al., 2007; Matson and Shoemaker, 2009; Reaven, 2009; Volkmar et al., 2009; Zwaigenbaum et al., 2009).

EXPERIMENTAL PROCEDURES

Mice

Adult mice of the inbred strains BTBR and B6 were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in a conventional mouse vivarium at the National Institute of Mental Health (NIMH), Bethesda, MD, USA using harem breeding trios. After 2 weeks with a male, females were separated into individual cages before delivery. Pups were kept with the dam until weaning at postnatal day 21. After weaning, juveniles were housed by sex and strain in standard plastic cages in groups not exceeding four per cage. Mice were housed in a conventional animal facility on a 12h-12h light-dark cycle (lights on from 0700 hr to 1900 hr). Cages were housed in ventilated racks in colony rooms maintained at ~20 °C temperature and ~55% humidity. Standard rodent chow and tap water were available *ad libitum*. In addition to standard bedding, a Nestlet square and a cardboard tube were provided in each cage. Male BTBR and B6 were utilized for all neurochemical assays and behavioral experiments described below. All procedures were conducted in strict compliance with the NIH guidelines for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Neurochemical assays

Naive mice were individually taken from their home cages to a procedure room 10 feet away, to minimize corticosterone surges from extraneous handling or movement. Three separate cohorts of BTBR and B6 mice were utilized for neurochemical assays: one for corticosterone, one for CRF and oxytocin, and one for glucocorticoid receptor mRNA.

Corticosterone radioimmunoassay. Three behaviorally naive cohorts of BTBR ($n=12$) and B6 ($n=12$) mice were removed from their cages beginning at 1600 hr and immediately sacrificed by rapid decapitation. Trunk blood was collected in 1.5 ml plastic microcentrifuge tubes. Serum was obtained the following day by centrifugation and was stored frozen. Serum concentrations of corticosterone were determined by radioimmunoassay (MP Biomedicals, Solon, OH, USA). The sensitivity threshold for this commercial assay was 5 ng/mL. Intra- and interassay coefficients of variance were less than 10%.

CRF and oxytocin radioimmunoassay. Brains of BTBR and B6 mice were rapidly removed, frozen on powdered dry ice and subsequently stored at −70 °C. Frozen brains were cut into 1 mm thick sections containing the hypothalamic paraventricular nucleus (PVN) on a cryostat (Leica, Bannockburn, IL, USA). The sections containing the PVN were placed on microscope slides and the PVN was harvested using a 1 mm micropunch (Stoelting, Kiel, WI, USA). Punches were placed into 150 μl of 1 N acetic acid and boiled for 20 min. Acetic acid extracts were frozen on dry ice and stored at −70 °C (Hooi et al., 1989). Subsequently, extracts were thawed and aliquoted into 12 mm×75 mm glass tubes and were dried in a Sorval SpeedVac concentrator. The dried extracts were reconstituted in assay buffer and tissue CRF (BTBR $n=10$ and B6 $n=9$) or oxytocin (BTBR $n=9$ and B6 $n=10$) concentrations were determined by radioimmunoassay according to the manufacturer's specifications (Phoenix Pharmaceuticals, Burlingame, CA, USA). The sensitivity of both the CRF and oxytocin assays were 1 pg and the coefficients of variation were less than 10%.

In situ hybridization histochemistry. Brains of BTBR ($n=5$) and B6 ($n=5$) mice were rapidly removed, frozen on powdered dry