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Multiple autism-like behaviors in a novel transgenic mouse model

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Abstract

Autism spectrum disorder (ASD) diagnoses are behaviorally-based with no defined universal biomarkers, occur at a 1:110 ratio in the population, and predominantly affect males compared to females at approximately a 4:1 ratio. One approach to investigate and identify causes of ASD is to use organisms that display abnormal behavioral responses that model ASD-related impairments. This study describes a novel transgenic mouse, MALTT, which was generated using a forward genetics approach. It was determined that the transgene integrated within a noncoding region on the X chromosome. The MALTT line exhibited a complete repertoire of ASD-like behavioral deficits in all three domains required for an ASD diagnosis: reciprocal social interaction, communication, and repetitive or inflexible behaviors. Specifically, MALTT male mice showed deficits in social interaction and interest, abnormalities in pup and juvenile ultrasonic vocalization communications, and exhibited a repetitive stereotypy. Abnormalities were also observed in the domain of sensory function, a secondary phenotype prevalently associated with ASD. Mapping and expression studies suggested that the Fam46 gene family may be linked to the observed ASD-related behaviors. The MALTT line provides a unique genetic model for examining the underlying biological mechanisms involved in ASD-related behaviors.

Keywords

social behavior; mouse model; ultrasonic vocalization; autism; gene expression

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1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder currently diagnosed on a strictly behavioral basis. Autistic individuals exhibit impairments in three domains: reciprocal social interaction, communication, and the presence of stereotypic repetitive or inflexible behaviors. The high prevalence of ASD, approximately 1 in 110 persons, combined with a paucity of known causes makes ASD an important target disease for research [1,2]. While environmental factors may be responsible for some cases of ASD or increase susceptibility for populations at risk for ASD [3–5], monozygotic (MZ) and dizygotic (DZ) twin studies as well as family and sibling data provide strong evidence for genetic risk factors [6]). Depending on whether a strict or broad cognitive deficit diagnosis is considered, MZ concordance rates for ASD (60–92%) are significantly higher than DZ rates (0–31%) [7–9]. Another significant aspect of ASD is the disproportionate overexpression in males compared to females at approximately 4.3:1 [10].

It is predominantly accepted that ASD is a genetically heterogeneous disorder. A number of genetic causes or contributors to ASD have been identified from various experimental approaches including copy number variation studies of deletions and duplications, genome-wide association and linkage studies, identification of single gene mutations, and analysis of clinical populations with a high incidence of autism, including fragile X syndrome, Rett syndrome, Angelmann syndrome, and tuberous sclerosis [11–13]. However, despite the high MZ concordance rates, genetic factors identified thus far only account for approximately 20% of ASD [13,14].

It is critical to continue with efforts to identify novel models and mutations that lead to ASD-like conditions. Given that ASD diagnoses are entirely behaviorally-based with no defined universal biomarkers, one approach is to use organisms that display abnormal behaviors that model facets of ASD. Although ASD is a human syndrome and caution is always warranted when using organisms to model human disorders, model organisms have been used to study underlying central nervous system processes for other developmental disorders, such as Fragile X syndrome, Rett Syndrome, and Williams-Beuren Syndrome [15–18]. Recently, a number of researchers have developed behavioral assays that appear to capture and model aspects of ASD-like traits. Through this approach a number of studies have described deficits in social, communication, and/or stereotypic domains in inbred strains of mice [19–22] and various single-gene mutant mouse models [23–26]. However, only a few of these models have reported deficits in all three ASD-related behavioral domains.

Most of the current mouse models of ASD have used “reverse genetics”, going from an intentional and specific genetic alteration to phenotype. For instance, mouse models of synaptic genes, including Nlgn4, Nlgn3, and Neurexin-1α [23,27,28], have recently been generated based on rare-occurring mutations identified in the ASD population [29,30]. These models among others are helping to shape some of the first evidence-based molecular hypotheses regarding the pathogenesis of ASD. However, the limitation of this approach is that it requires an a priori target. One classical method for identifying unknown and potentially unpredicted genetic contributions to phenotypes is the forward genetics approach, first identifying a relevant phenotype and then elucidating the genetic underpinnings. Spontaneous mouse mutants have furthered our understanding of biological systems for more than one hundred years. The publication of the mouse genome and generation of novel mutation and screening strategies have only advanced the utility of this method.

Here we describe a mouse line generated by a forward genetics approach employing a random transgene insertion strategy. The transgenic line was generated by microinjection of a tyrosinase minigene into 1-cell stage albino FVB embryos. Expression of tyrosinase results in pigment production. Non-transgenic mice are albino, transgenic mice are pigmented. This visible reporter simplifies the maintenance of transgenic families and also the identification of families with insertional mutations. Each transgenic line was analyzed to determine whether a consistent phenotype was exhibited specifically and exclusively by the pigmented mice (dominant traits) or only by the homozygous pigmented mice (recessive mutations). The mouse line OVE876B, subsequently named “multiple autistic-like trait transgenic” (MALTT) was selected for further studies based upon the home-cage behavior of the pigmented males. We ultimately characterized this line using a number of autism-relevant assays including pup and juvenile social and communicative tasks, assessment of stereotypy, and various sensory system-related measures. Finally, through mapping and expression studies, we identified a specific molecular aberration that may play a role in the resultant behavioral anomalies.

2. Materials and Methods

2.1 Generation of MALTT transgenic line

Sequences from the 5’-end of the Moloney murine leukemia virus (MuLV) were linked to a tyrosinase minigene (described in Overbeek, et al. 1991 [31]). The minigene contains a 2.1 kb promoter linked to a 1.9 kb tyrosinase cDNA. Constructs were injected into one-cell stage FVB/N embryos. FVB/N mice are albino due to a mutation in their endogenous tyrosinase gene [32]. Expression of the tyrosinase minigene leads to melanin synthesis in the skin and fur, providing a simple phenotypic assay for transgene expression. A Kpn I to BstE II fragment of MuLV (obtained from the pS3 vector described in Faustinella, et al. 1994 [33]) was cloned upstream from the tyrosinase minigene (Fig. 1a). This fragment contains part of the R domain (30 bp), the full U5 region (85 bp), and 575 bp from the packaging domain. To remove the RU5 domain, an Eag I/EcoR I digestion was done (see Fig. 1b). Injection of the fragment without the RU5 sequences yielded 6 transgenic founders, 50% of which were pigmented. The female founder mouse for one of these families (OVE876) produced offspring with two distinctively different coat colors, indicating two independently segregating transgenic integration sites. The two branches of this line were named OVE876A and OVE876B. This manuscript describes the characterization of the 876B mice. PCR genotyping was done with a sense primer (tyroA) from exon 1 of tyrosinase and an antisense primer (tyroB) from exon 2. These primers amplify a 700 bp segment of the tyrosinase minigene.

2.2 Animal Subjects for Behavioral Studies

Mice were generated on an FVB/N background. As the transgenic inheritance pattern was determined to be X-linked, the mice were maintained by crossing heterozygous female MALTT mice with male FVB/NJ mice. Genotyping was not required because of the pigment conferred to the transgenic MALTTs by the transgene. Post-weaning female mice were housed 2–5 per cage, but male mice were housed singly from approximately postnatal day (PND) 24 onwards due to aggressive behavior (see 4.5). The housing room was maintained on a 12 hr light: 12 hr dark cycle with lights on at 6 am. Mice had ad libitum access to food and water. All animal care and testing procedures were in accordance with current NIH Guidelines and approved by the Baylor College of Medicine Animal Care and Use Committee. Except where stated otherwise, multiple novel batches of mice were assessed on each task. As the transgenic insertion site was X-linked, we expected only a partial phenotype in female hemizygous mice. Thus, hemizygous male mice were used for most tests. Females were tested on open-field activity, PPI, audiogenic seizures, and pup

USVs. Due to the high aggression observed in hemizygous males, it was not possible to generate enough homozygous females for evaluation.

3. Experimental Procedures

3.1 Insertion site characterization

Splenocytes from transgenic female MALTT mice were grown for 72h in RPMI medium with 20%FBS supplemented with 10ug/ml LPS (Sigma #L2880) and 5ug/ml concanavalin A. Metaphase spreads were prepared using standard techniques [34]. Slides were Giemsa stained and photographed to identify individual chromosomes based on G-banding patterns then destained before fluorescence in situ hybridization (FISH) analysis to detect the transgene. A minigene-specific probe was labeled by nick translation with digoxigenin-11-dUTP (Boehringer) or biotin-14-dUTP (Gibco-BRL) and detected with FITC-labeled antibody (Roche) by standard microscopy.

Genomic sequences flanking the transgene integration site were identified using PCR-based Genomewalker (Clontech) technology according to the manufacturer's instructions. Briefly, restriction enzyme digestions were performed on purified high molecular weight genomic DNA isolated from MALTT males and wild-type littermates as control. GenomeWalker Adaptors were ligated to the genomic DNA fragments and primary PCR amplification was carried using a gene-specific primer (GSP1) and an Adaptor-specific primer (AP1). A secondary nested PCR amplification was carried out using 1ul of diluted primary PCR product as template and a second gene-specific (GSP2) and Adaptor-specific primer (AP2) pair. Primer sequences are given in Supplementary Table 1. The final amplification products were resolved on an agarose gel and visible band(s) were isolated, purified, and then sequenced. Publicly available homology search programs were then utilized to identify the chromosomal region flanking the construct (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2 Non-radioactive Southern blots

Purified high molecular weight genomic DNA (gDNA) was isolated from WT and MALTT tissue using the PureGene Genomic Kit (Qiagen). Southern blot hybridizations were carried out according to standard protocol for DIG High Prime DNA Labeling and Detection Kit (Roche). Briefly, a minigene-specific probe (see Supplementary Table 1) was random primed labeled with digoxigenin-11-dUTP. Restriction digested gDNA was run out on an agarose gel and alkaline transferred to a positively charged nylon membrane (Roche). After UV-crosslinking the membrane was blocked, hybridized with probe, washed, and incubated with the provided anti-dig alkaline phosphatase conjugated antibody according to standard protocol. CSPD chemiluminescent substrate was applied and the membrane was exposed to film for 6 hrs.

3.3 Brain histology

Adult brains were fixed by immersion in 4% paraformaldehyde in PBS (pH 7.0) at 4°C for 24 hours with gentle agitation. The fixed brains were weighed, and cryoprotected in increasing concentrations of sucrose (10%, 20%, 30%) in PBS until sinking. The brains were then hemisected and frozen embedded in Tissue-Tek O.C.T. (Sakura Finetek, VWR). Right and left hemispheres were cryosectioned (15 μm) in coronal and sagittal planes, respectively. Every sixth section was stained with 1% cresyl violet (Nissl stain), dehydrated through graded ethanols and xylene, and coverslipped for microscopic examination (R.F.H.).

3.4 Activity and stereotypy measures

3.4.1 Open-field activity—Open-field activity was assessed as previously described [35]. Specifically, mice were monitored on a single day for a 10 min period. Independent sets of male and female mice from each genotype were evaluated at 17, 22, 28, and 45 days of age. Because total distance traveled has a direct effect on opportunity for rotation and MALTT mice were hyperactive (see 4.3), comparisons between genotypes were made for rotations/total distance traveled. Open-field data were analyzed using two-way (genotype × age) ANOVA utilizing Tukey's and simple effects post-hoc analysis as required.

3.4.2 Vestibular tests—Vestibular function was measured by a swim test and contact-righting test. Mice (45–60 days of age) were placed into a 24 cm × 45 cm clear plastic chamber filled with water (22–24°C) and observed for 2 min for signs of impaired swimming (non-elongated body, swimming on side, failure to keep nose above the water), immobile floating, and sinking or tumbling. For the contact-righting test mice were placed into a clear Plexiglas tube, the tube was then inverted and the subject was monitored for a righting response (normal vestibular function) or failure to right. For these experiments $n = 9$ male MALTT, $n = 10$ male WT; $n = 5$ female MALTT, $n = 11$ female WT.

3.5 Pup separation-induced vocalization measure

Ultrasonic vocalizations (USVs) were assessed on PND 3–14. Each litter was removed from its home-cage with nesting material to a holding container. Single pups were placed in a pseudorandomized order into a sound attenuating chamber for 2 min while isolation-induced USVs in the 57–77 kHz range were recorded (Noldus Ultravox, BAT Detector mini-3 with audio filter setting 6.5) and the full ultrasonic range was simultaneously monitored via a modified frequency division detector (Pettersson Ultrasound Detector D230). Plantar surface tattoos were administered post-recording on PND3. Body masses were recorded on PNDs 6, 9, and 12. Temperatures were taken on postnatal day 8 (RET-4 probe, Physitemp Instruments, Inc.) immediately following each recording. Peak period of vocalizations was defined as the three days with absolute highest number of USVs. Data were analyzed using one-way ANOVA with repeated measures factor of PND. A simple-effects analysis was used to examine PND × genotype interactions to determine (a) the number of calls between MALTT and WT mice at each age, and (b) the age when the USVs were significantly reduced from peak within each line. We operationally defined that age as two consecutive days when the number of USVs was significantly different from the peak.

3.6 Social behavior and communication measures

3.6.1 Three chamber test—A modified three chamber paradigm [36] was used to assess social interest. Mice were juvenile males age PND23–28. The Plexiglas testing apparatus consisted of two side chambers and a center chamber, each 42.5 cm × 17.5 cm × 23 cm. The outer side chambers have a secondary inner chamber of 10 cm × 17.5 cm × 23 cm for holding the object or partner, which is separated from the rest of the chamber by a perforated Plexiglas partition. Naïve partner mice were habituated to the chamber for 30 min the day before testing. Subject and partner were habituated for 10 min in separate apparatuses immediately before testing. Post-habituation, the subject was restricted to the center chamber by the closure of two doorways. An inanimate object, a 2.4 in × 2.4 in × 1.5 in light grey LEGO block, was placed behind the partition one side chamber and a partner mouse was placed behind the partition in the other side chamber of the three chambered apparatus. The doorways were then lifted and the subject was allowed to freely explore all three chambers for 10 min while movement was video-recorded and scored post-testing for duration and frequency of visits per chamber and at each partition. Data were analyzed by

two-way ANOVA with repeated measures. Frequency of entries was analyzed by one-way ANOVA.

3.6.2 Direct social interaction test—Juvenile subjects (MALTT or WT littermates) were single-housed a minimum of two days and then placed with a naïve juvenile stranger (FVB/NJ obtained directly from Jackson Laboratories) as a dyad into a clean standard cage with corncob bedding in a sound-attenuating chamber. Interactions were immediately video recorded for 10 min. In a second test, a naïve set of mice were used and both subject and "stranger" were of the same genotype, either MALTT or WT. For both tests, the subject mouse was scored post-testing by a well-trained observer blind to genotype via Psion hand-held computer in conjunction with Observer 3.0 (Noldus): active social behaviors (subject sniffing or placing paws or nose to the stranger), passive social behaviors (stranger sniffing or placing paws or nose to the subject), and nonsocial behaviors (any behavior not considered social, including grooming, digging, etc.). For the second test, total social interactions were analyzed rather than separated into active and passive as both members of the pair were experimental genotypes and there was no distinct subject and partner. Data were analyzed by one-way ANOVA for each behavioral state. .

USVs were simultaneously recorded during the direct social interaction test utilizing the same equipment and setup as described for pup USVs. The ultrasonic output from the bat detector was passed through an audio filter before registering as an event within the Ultravox software. Audio filter settings were empirically determined before any subjects were tested, such that no events were detectable from a single mouse moving around the chamber with bedding (i.e. assuring extraneous movement-based ultrasonic sounds were not captured). Directly before each set of experiments a test run with no subjects ensured that extraneous electrical ultrasonic background noise was not detected. An experienced observer also monitored the full ultrasonic range with a separate frequency division detector to ensure calls were not being missed outside of the heterodyne detector's range. On rare occasion a piece of bedding was forcefully flung against the side of the plastic cage (due to digging or darting) resulting in an ultrasonic event. This was noted by the observer and the event at that time stamp was not included in the analysis. Following testing, USV data and scored direct social behavioral data were transformed into time-event tables and analyzed for overlapping events. Total USVs were analyzed by one-way ANOVA. USVs during direct interaction were analyzed by one-way ANOVA for each behavioral state.

The same subjects were used in the three-chamber test and the first direct social interaction test. For the second direct social test, with same genotype pairs, a different set of mice were used: 7 MALTT mice and 8 WT mice had been tested previously on the olfactory discrimination task, while 11 MALTT mice and 8 WT mice were naïve.

3.6.3 Aggressive interactions—To assess aggression, one male WT and MALTT littermate were weaned as a dyad. A total of ten dyads were monitored daily for onset of aggression marks in the form of scratched, bruised, or bleeding tails. When marks appeared the insult was attributed to the partner mouse and PND of initial aggression was recorded. Standard resident intruder could not be performed because of the circling stereotypy of the MALTT mice.

3.6.4 Olfactory detection and discrimination—Olfactory discrimination for non-social and social odors was investigated using a slightly modified habituation/ dishabituation protocol [37]. The juvenile subject mouse was habituated to a non-odored cotton tip for 30 min, then given three 2 min presentations of each: water, first non-social odor, second non-social odor. Non-social odors were imitation banana and almond extracts. On the second day of testing the non-social odors were replaced by social odors (swabbed dirty cage bedding

from unfamiliar mouse home-cages). Olfactory odors were counterbalanced within each test. Time sniffing, was recorded with a Psion handheld computer in conjunction with Observer 3.0 software. The measures of interest were habituation to the previously presented odor (during the three consecutive presentations of a same odor), dishabituation of response (time spent sniffing) upon presentation of a novel odor compared to the immediately preceding familiar odor, and initial investigation of novel odors. Time sniffing during habituation and dishabituation was analyzed using three-way ANOVA (genotype × odor × presentation) with repeated measures.

3.7 Somatosensory and sensorimotor gating measures

3.7.1 Tactile sensitivity—Tactile sensitivity threshold was measured by placing adult subjects into opaque solid-walled cages with wire mesh floor allowing experimenter access to foot pads. Subjects were allowed to habituate to the testing chamber for 30 min. Plastic monofilament Von Frey hairs (Stoelting) were then applied to the plantar surface of the subject's hind paw until point of bending (reaching of target pressure). Von Frey hairs of increasing diameter (0.178, 0.203, 0.229, 0.254, 0.305, 0.356, 0.381, 0.406, and 0.432 mms) and therefore increasing pressure to bend were applied sequentially. The lowest force required to elicit a hind paw-withdrawal response was considered threshold and the response had to be observed on at least two out of three trials. Data were analyzed using one-way ANOVA to assess for a main effect of genotype on threshold values.

3.7.2 Audiogenic seizure—Mice were tested on PND 18–19. Pups were placed 2–3 at a time in a sound-attenuating chamber and allowed to acclimate. At 1 min a 140 dB noise generator is activated for 2 min. Latency to seize, seizure activity, and result of seizure (i.e. lethal or non-lethal) were recorded by visual observation in real-time. Seizure outputs were generalized tonic-clonic seizures observed as wild-running followed by body rigidity and uncontrolled jerks and convulsions. Significance was determined by Fisher's exact test.

3.7.3 Sensorimotor gating—Prepulse inhibition (PPI) analysis was carried out as described previously [38]. Percent PPI was calculated for each prepulse intensity as 100 − [(startle response on acoustic prepulse plus startle stimulus trials / startle response alone trials) × 100] and then averaged across all five prepulse sound levels. Percent PPI data were analyzed using a two-way (genotype × age) ANOVA with repeated measures. These were the same set of mice previously tested for open field activity.

3.8 mRNA expression

Whole brain from PND 0/1 and PND 40 mice was removed, cortex rapidly dissected out on cold dissecting block, and immediately immersed in RNAlater and stored at 4°C. Samples stored longer than 1 month were transferred to −20C. Total RNA from whole brain samples or cortex/forebrain was isolated and purified according to the RiboPure Kit manual (Ambion). Genomic DNA contamination was removed using Turbo DNA-free (Ambion), and RNA samples were quality-validated via NanoDrop absorbance and Agilent 2100 BioAnalyzer measurements (BCM Microarray Core Facility). Then cDNA was synthesized via SuperScript III First Strand Synthesis Supermix for qRTPCR (Invitrogen) from 1ug RNA and stored at −20C. Real-time RT-PCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) using 5ul diluted cDNA, SYBR GreenER supermix (Invitrogen), and target gene primers or reference gene primers (see Supplementary Table 1) with triplicates of each sample and samples were all run and compared within-plate. Additional controls included reverse transcriptase negative samples and melting curve analysis for each primer pair post-amplification. Ct and reaction efficiency values were generated using LinRegPCR (v12.3) program [39] and compared using the Relative

Expression Software Tool (REST) v2.0.13 (Qiagen, [40]) which employs statistical randomization tests. The REST values are reported in Tables 1 and 2.

Microarray probe signal values were obtained from the Gene Expression Omnibus repository dataset record GDS2824 (http://www.ncbi.nlm.nih.gov/sites/geo/). The data was deposited by Nishimura Y et al. and details of the study design are described fully [41]. Briefly, Nishimura et al. isolated RNA from lymphoblastoid cell lines from populations with a combination diagnosis of autism and fragile X syndrome or autism and 15q11–q13 duplication and compared to normal population. Each log ratio value was generated from the comparison of simultaneously run pooled control sample (signal 1) and subject sample (normal or affected) (signal 2). We utilized a one-way ANOVA test to compare the log ratio values from the Fam46d probe dataset.

4. Results

4.1 Identification of minigene insertion site and initial observations

The MALTT line was generated by microinjection of a tyrosinase minigene construct, which confers coat pigmentation to albino offspring upon integration. Early on, it became apparent that the OVE876B line, later termed MALTT, exhibited distinctive traits. First, the male mice were more darkly and evenly pigmented than the female mice (data not shown). Female mice showed a salt-and-pepper pigmentation pattern. Next, the male mice consistently appeared hyperactive and began fighting with their siblings at 6–7 weeks of age. Attempts to mate the transgenic males with adult FVB/NJ females were occasionally successful, although due to aggression the females could not be left with the males for any length of time. The pattern of transgene inheritance from these matings supported the hypothesis that the transgene had integrated on the X chromosome. All homozygous female offspring showed salt-and-pepper pigmentation. All male offspring were albino and were non-transgenic when assayed by PCR.

To confirm that the transgene had integrated at a single location on the X chromosome, Giemsa staining and fluorescent in situ hybridization (FISH) were performed on metaphase spreads. A biotin-labeled version of the transgene was used as a probe and indicated a single site of integration (Fig. 1a) on the mouse X chromosome. Genomic sequences flanking the transgene insertion site were isolated by PCR-based GenomeWalker technology. Sequence analysis of the junction identified the insertion site in an apparently non-coding region on band D of the X chromosome (Fig. 1b). Relative to the forward strand, the exact insertion site is 3’ of base-pair X:105,550,109 (assembly NCBI 37/ mus musculus 9) (Fig. 1b).

The construct’s right flanking sequence lies within a long interspersed nucleotide element (LINE1) repeat that occurs repeatedly throughout the genome, and the minigene itself has a large polyA run at the 3’ end. Direct PCR from predicted sequence has thus far been unsuccessful. Therefore, we used a Southern blot method to analyze the 3’ insertion site. Southern blotting with a minigene-specific probe, after digestion with BanI, resulted in two positive fragments from MALTT gDNA that were not present in WT gDNA. The larger fragment was consistent with a minigene only band (~2.9kb) expected from the presence of multiple copies in tandem. The smaller fragment was approximately 0.8kb and inconsistent with a predicted minigene plus flanking genomic sequence band (~2.0kb), based on the known 5’ insertion location and current mouse genome assembly (Fig. 1c). Thus, at this time, deletion of a portion of the last minigene copy and/or flanking X chromosomal sequence or insertion of foreign sequence at the 3’ end of integration is possible. PCR amplification with minigene-only primer sets suggested that at minimum 2 copies inserted in tandem. Southern blot hybridization with the minigene-specific probe, utilizing a restriction

enzyme not expected to cut within the construct, BbsI, resulted in an approximately 20kb band indicating a maximum of 4 copies inserted together (Fig. 1d).

4.2 Brain histology

There are currently no singularly defining brain abnormalities consistently observed across the ASD population. Preliminary histopathological examination of the adult male MALTT brain revealed no major malformations (Fig. 1e). The only obvious difference observed was significantly lower brain weight in adult MALTTs (0.474±0.011g) than for WTs (0.509±0.002g) [$F(1,4)=10.48, p=0.032$].

4.3 MALTT mice exhibit hyperactivity and developmental stereotypy

Male transgenic mice appeared to be hyperactive and display circling in the home-cage. To quantify these behaviors, the transgenic MALTT mice and WT littermates were assessed in an open-field arena assay. Overall MALTT males were hyperactive relative to WT [$F(1,84)=45.94$, $p<0.001$;] with an interaction between age and genotype [$F(3,84)=5.207, p=0.002$] (Fig. 2a). MALTT mice were more active than WT mice at each age (p's < 0.05) and MALTT males at PND 45 were more active than MALTT males at the younger ages (p's < 0.05). In contrast, activity in WT mice only differed at PND 17, with less activity at this pre-weaning age [p's <0.01] and no difference across older ages (p's > 0.05).

MALTT males additionally exhibited a circling stereotypy [$F(1,83)=24.99$, $p<0.001$] (Fig. 2b), but this behavior was only statistically different between WT and MALTT male mice at 45 days [$F(1,32)=20.60, p<0.001$]. This is a developmentally progressive behavior in MALTTs with a greater number of stereotypies exhibited on PND 45 than PND 17, 22, and 28 [p's$<.01$] (Fig. 2b,c). WT mice exhibited no differences across age [$F(3,41)=1.18, p=0.330$]. There was also a distinct directional restriction of rotation. The majority (87% ±0.04) of rotations in MALTT mice were in a single preferred direction; however, the direction of preference, anticlockwise or clockwise, differed between subjects. The spontaneous circling stereotypy continued throughout adulthood in the MALTT line (personal observation).

Female MALTTs did not develop perseverative circling but were hyperactive compared to WTs [$F(1,101)=6.92, p=0.010$] (Fig. 2d) with no genotype by age interaction [$F(3,101)=0.53, p=0.662$].

Many rodents with a circling phenotype have impaired vestibular function and/or inner ear abnormalities resulting in deafness [42,43]. To test for vestibular deficits, the MALTT mice were evaluated using a swim test and contact-righting task [44]. In both tests the MALTT mice exhibited no differences from wild-type (WT) littermates, nor did they exhibit any head-jerking or head-tilting (data not shown). To test for intact hearing ability, auditory brainstem response tests (ABR) were carried out on male mice at PND 60. There was no significant difference in mean threshold response between genotypes [$F(1,19)=0.24, p=0.878$] (Supp. Fig. 1).

These experiments indicate a developmental hyperactivity and circling stereotypy in the male MALTT line that is not attributable to vestibular dysfunction.

4.4 MALTT line pups show a prolonged ultrasonic vocalization pattern

Pup USVs are a well-defined developmentally-regulated rodent behavior. In home-cage settings, this early-age communication is ethologically relevant in eliciting a retrieval response by the dam [45]. In the current study, WT males and females showed a normal

developmental progression of USV, peaking at approximately PND5 followed by a progressive reduction in calls by PND 14. MALTT pups, however, had a shifted USV pattern (Fig. 3a,b). Overall, MALTT male pups emitted more vocalizations than WTs [$F(1,39)=13.15$, $p=0.001$]. For both genotypes the peak period of USV was PND 4–6, and average number of USVs during peak did not differ (Supp, Fig. 2a) [$F(1,39)=0.0001$,$p=0.993$]. Comparing peak period USV values to each of the following 9 days of vocalization simple-effects analysis of the significant PND × genotype interaction [$F(8,312)=2.48$,$p=0.013$] revealed that male MALTT mice emitted more calls than WT mice on PND 8–11, and 13 (p's < 0.05). Starting at PND day 7 WT mice displayed significantly fewer calls relative to their peak ($p < 0.05$), but it was not until PND 11 that male MALTT mice had significantly fewer USV calls relative to their peak ($p < 0.05$). These latter findings indicate an approximately 4 day shift in the USV developmental curve between WT and MALTT mice. To assess for an effect of repeated testing, a separate set of male mice were recorded on a single day during peak period and a single-day during the post-peak vocalization periods (Supp. Fig. 3). As observed during the everyday testing, on PND4 there was not an effect of genotype [$F(1,31)=1.079$,$p=0.307$], while on PND10 MALTT males emitted significantly more USVs than WTs [$F(1,31)=11.630$,$p=0.002$].

MALTT females also exhibited an altered pup USV phenotype (Fig. 3c). Both MALTT and WT female pups showed similar peak period vocalization levels (Supp. Fig. 2b) [$F(1,43)=0.006$,$p=0.940$] and a PND × genotype interaction [$F(8,312)=2.54$,$p=0.011$]. Female MALTT mice emitted more calls than WT mice on days 8–10 and 14 (p's < 0.05). Starting at PND day 8, WT mice displayed significantly fewer calls relative to their peak ($p < 0.05$), but it was not until PND 10 that female MALTT mice had significantly fewer calls relative to their peak ($p < 0.05$), indicating an approximately 2 day shift in the USV developmental curve between female WT and MALTT mice.

It is possible the MALTT mice called more frequently but for shorter durations, resulting in an overall similar total duration of 'calling'. Analysis of duration on PND8 revealed no difference between genotypes for average duration of calls for males (Supp. Fig 2c) [$F(2,43)=0.132$, $p=0.877$] or females (Supp. Fig. 2d) [$F(2,43)=0.009$, $p=0.991$]. Importantly, body weight and temperature regulation were also not different between WT and MALTT mice (Supp. Fig. 2e–h) (p's>0.05). The separation-induced USV results suggest that a form of a normal mouse communication is developmentally abnormal in male and female MALTT mice. Importantly abnormal isolation-induced USVs in MALTT mice are not the result of obvious physical or thermoregulatory dysfunctions.

4.5 MALTT line males have juvenile social and social-related communication deficits

To initially determine if the MALTT line exhibits abnormal social behavior, a three-chamber partition test was conducted. Investigation of a male FVB/NJ stranger mouse and novel object by either MALTT males or their WT male littermates was assessed (Fig. 4a). WTs showed a clear preference for spending time on the stranger's side relative to the side with the object (Fig. 4b) [$F(1,7)=8.83$,$p=0.021$]. WT mice also showed a preference for spending time directly at the stranger's perforated partition compared to the object's perforated partition (Fig. 4c) [$F(1,7)=9.40$, $p=0.018$]. In contrast, MALTT mice showed no preference for side (Fig. 4b) [$F(1,8)=0.39$,$p=0.548$] or partition (Fig. 4c) [$F(1,8)=4.30$,$p=0.072$], although there was a trend toward preference for stranger partition. Additionally, MALTT and WT mice spent a similar amount of time at the object partition [$F(1,15)=2.20$,$p=0.159$], but MALTTs spent significantly less time at the stranger's perforated partition than WTs [$F(1,15)=5.02$,$p=0.041$] (Fig. 4c). Frequency of entering the chamber with either the stranger or the object was not different between MALTT and WT mice, suggesting similar levels of exploration (Fig. 4d) [*stranger* $F(1,15)=0.105$,$p=0.751$], [*object* $F(1,15)=0.744$,$p=0.402$].

A direct social interaction task was employed in which each subject, the WT or MALTT mouse, was allowed to directly interact with a naïve male FVB/NJ stranger for 10 minutes in a standard cage novel to both mice (Fig. 5a). All scoring was relative to the subject mouse. MALTT males spent significantly less time engaged in active social interactions (i.e. sniffing, touching the stranger) than WT males [$F(1,15)=13.46, p=0.002$] and more time in non-social behaviors [$F(1,15)=13.46, p=0.002$] (Fig. 5a). For passive social behavior (i.e. subject mouse is being sniffed, touched, etc. by the stranger), there was no significant difference in amount of time WT or MALTT was investigated by the FVB/NJ stranger [$F(1,15)=1.03, p=0.327$]. It is important to note that the MALTT mice were not displaying any stereotypic circling at this age.

It has previously been established that juvenile mice engage in social interaction with concomitant ultrasonic vocalization [21]. Therefore, we simultaneously monitored the dyadic communication of the juveniles during the direct social interaction test. The presence of a MALTT male in the pairing had a pronounced effect on the total USVs emitted from the pair. There was an effect of genotype on mean total USVs emitted [$F(1,15)=5.37, p=0.035$] with MALTT + stranger pairs emitting fewer USVs (Fig. 5b). There was no difference between WT-containing and MALTT-containing pairs for average duration of USV [*WT pair* (84.0±8 msec), *MALTT pair* (77.3±9 msec); $F(1,15)=0.29$, $p=0.600$]. Time event data for scored social behavioral state and time event data for USVs were then overlaid (Fig. 5c). Analysis of USVs coincident with a social behavior revealed that USV events correlated almost exclusively with social interaction, where greater than 84% of USVs occurred during social interactions. When the MALTT mice were observed engaged in active social interactions with the stranger, there were fewer calls than when the WT mice were actively interacting with the stranger (Fig. 5d) [$F(1,15)=8.66, p=0.010$]. However, the amount of calls were similar when the strangers were engaged in active interactions with either the MALTT or WT subject (Fig. 5c) [$F(1,15)<0.01, p=0.987$]. This indicates that fewer USVs were emitted specifically during MALTT active investigation of FVB/NJ stranger compared to during WT active investigation of FVB/NJ stranger.

Examination of exemplary time-event plots of social interactions and USVs (Fig. 5c) clearly indicates that for the MALTT:FVB/NJ dyads almost all the calls were made during the time when the MALTT mice were in the ‘passive state’, i.e. when the FVB/NJ stranger mice were actively investigating the MALTT mice. These observations and the results above suggest an effect of MALTT presence on social USV. We hypothesized that if the MALTT mouse was explicitly responsible for the lack of or decrease in calls, then MALTT-only pairs should emit even fewer vocalizations. MALTT-only pairs were recorded and compared to WT-only pairs (Fig. 6a), and, although social interactions occurred, MALTT-only pairs emitted fewer vocalizations (45.3 ± 39.7 calls) than WT only pairs (585 ± 53.9 calls) [$F(1,15)=67.05, p<0.001$]. In fact, only 2 pairs of MALTTs emitted any USVs (Fig. 6a). As both mice in the pair were identical genotypes, total social interactions were also quantified per pair and again confirmed decreased social interactions in the MALTT pairs (Fig. 6b) [$F(1,15)=181.02, p<0.001$]. These findings demonstrate that MALTT mice emit few to no calls during social interactions and that the USVs recorded during the direct social interaction test are primarily emitted from the mouse that is actively interacting with its partner.

Olfactory cues are an important component of rodent social investigation [46,47]. A habituation/dishabituation olfactory-discrimination task was used to assess olfaction (Fig. 7a,b). For the nonsocial-odor test, the only effect was of odor [$F(1,22)=85.14$, $p<0.001$], with no difference between genotypes in ability to differentiate between odors (Fig. 7a) [$F(1,22)=0.604, p=0.445$]. All mice also habituated to the odors [$F(1,22)=113.54$, $p<0.001$], with no effect of genotype[$F(1,22)=2.88, p=0.104$] (Fig. 7a).

For the social odor test both genotypes detected the novel odors [*novel odor 1* [F(1,20)=34.32, p<0.001]; *novel odor 2* [F(1,24)=173.83, p<0.001]]. However, WT mice, but not MALTT, mice showed an interaction between presentation set and novel odor [F(1,24)=39.67, p<0.001]. WT mice showed increased interest relative to MALTT mice upon first presentation of a social odor [F(1,22)=11.18,p=0.003], but not for the first presentation of the second novel social odor [F(1,22)=0.24,p=0.627]. Despite the initial heightened response of WT mice to the initial presentation of a social odor, both genotypes habituated to the same degree by the third presentation of the novel odors (Fig. 7b) [F(1,22)=0.001,p=0.974]. This result indicates intact olfaction in the MALTT mice and also supports that MALTTs show a decreased interest in a social stimulus odor.

Overall, MALTT pairs exhibited decreased social interest, decreased active social investigation, and increased non-social behaviors compared to WT littermates. MALTTs also vocalized less than WTs during direct social interactions. These deficits were not attributable to olfactory dysfunction.

Heightened aggression or irritability is another common feature in ASD [48,49]. MALTT males displayed heightened aggression toward littermates. Due to the MALTT line's stereotypy at the adult age, the standard resident-intruder test for aggression was uninformative. Therefore, aggression was quantified by assessing inter-male home-cage behavior during the age before stereotypic circling emerges. A male WT and a male MALTT littermate were housed together post-weaning and assessed for aggression. In all cases the WT male exhibited tail bite mark(s) on its tail. This result was consistent and all MALTT males recorded engaged in tail-biting behavior beginning at 35 days ± 2.6(SEM). There were no instances where this behavior was exhibited by WT littermates toward the MALTT mouse (data not shown).

4.6 The MALTT line exhibits non-core symptom ASD endophenotypes

There are behavioral traits that persons with ASD frequently exhibit in addition to the core symptoms but which are not requisite for a diagnosis of ASD. Seizure activity and sensory dysfunctions, including elevated responsivity to sensory stimuli, are two features commonly associated with ASD [2,50–53]. To characterize an observed hypersensitivity or tactile-defensiveness, tactile withdrawal threshold was measured, and MALTT mice had a lower response threshold than WTs [F(1,13)=4.663,p=0.050], indicating increased sensitivity to paw-touch (Fig. 8a).

The pan-mammalian behavior of prepulse inhibition (PPI), a measure of sensorimotor gating in which a weak prepulse presented just before a startling sensory stimulus will inhibit the startle response, is reported as impaired in humans with a variety of cognitive disorders including Asperger's syndrome and autism [54,55]. Across a range of ages (17–45 days) male MALTT mice exhibit a PPI deficit [F(1,80)=47.13, p<0.001] (Fig. 8b) but no difference in acoustic startle response [F(1,80)=1.76,p=0.189] (Supp. Fig. 4a). Similarly, across the same age range female MALTTs displayed impaired PPI [F(1,101)=4.58,p=0.035] compared to WTs (Fig. 8c) and no difference in acoustic startle response [F(1,101)=0.23,p=0.634] (Supp. Fig. 4b).

Figure 6f also displays the results showing that young (19–21 days old) MALTT mice are significantly more likely to have a seizure [*males* p<0.001; *females* p=0.008] when subjected to a loud sound stimulus compared to WT littermates. No spontaneous seizure activity was observed in either male or female MALTTs.

4.7 Aberrant X chromosomal gene upregulation in the MALTT line

Initial experiments described above indicate that integration of the transgene did not interrupt a coding region. Numerous studies have characterized long-distance alteration of gene expression resulting from deletions, rearrangements, and transposon insertions (reviewed [56]). To determine if regional gene expression was altered, expression levels of cortical mRNA were analyzed for genes located both upstream and downstream of the insertion site (see Fig. 9, Table 1). Five genes of closest proximity to the insertion site (both proximal and distal) showed differential expression in adult WT and MALTT cortex. Three genes showed low-level upregulation, and one gene, Gm732, undetectable in WT, was induced in MALTT. The most striking expression difference was observed for Fam46D. Fam46D is minimally expressed in WT cortex and expressed approximately 380 fold higher in MALTT cortex [$p<0.001$]. Expression levels in cortices from newborn pups were also analyzed (see Table 2) and Fam46d was again found to be upregulated by at least 32 fold. Gm732 was again detectable but expressed at a very low level. These were the only nearby genes in which differences were observed between WT and MALLT mice at PND1.

Even though transgenic vectors commonly carry a tyrosinase minigene for coat color tagging, we wanted to verify the increase in Fam46d expression was not an indirect effect of the tyrosinase transgene. Tyrosinase and Fam46d levels were analyzed in a second independent tyrosinase minigene-containing transgenic line (see Supplementary Table 2). While both the MALTT and secondary non-MALTT lines expressed tyrosinase in mouse cortex, the secondary line does not show elevated levels of Fam46D expression. The secondary transgenic line additionally does not exhibit the home-cage behavioral abnormalities observed in the MALTT line.

These results indicate that the MALTT line has altered X chromosomal gene expression. The largest expression change detected was overexpression of Fam46d. Data from human autism populations analyzed by RNA microarray indicates Fam46d may be in elevated in a subset of individuals with coincident autism and Fragile X syndrome [41]. Our statistical analysis of the data found in the Gene Expression Omnibus repository shows Fam46d was significantly elevated in the subset of individuals with Fragile X syndrome who also met the diagnostic criteria for autism as compared to the normal population [$F(1,21)=5.221, p=0.033$]. Further investigation is needed to determine the specific relationship between the MALTT line's aberrant behavioral repertoire and the alterations in X chromosome gene expression.

5. Discussion

Patients with ASD manifest behavioral abnormalities in three core areas: social, communication, and stereotypic behaviors. In the present study we have shown that the MALTT male mice display consistent deficits in all three domains – reduced social interest and interactions, altered USV communication during separation and during social interactions, and stereotypic circling. The MALTT line displays additional abnormalities consistent with variable features of ASD, including increased sensitivity to audiogenic seizures, impaired sensorimotor gating, enhanced tactile sensitivity, and hyperactivity. The MALTT mice have a transgene integrated on the X chromosome, which continues to be implicated in ASD based on a disproportionate (approximately 4:1) occurrence in males versus females and also evidence from linkage studies [57–59]. However, some studies suggest the skewed ratio does not necessarily mean X chromosome insults are directly responsible for all cases of ASD, and certainly non-X chromosomal ASD-related mutations have been identified [13]. The site of transgene integration also lies specifically within a region of mouse X chromosome syntenic to regions of human X chromosome implicated in autism susceptibility loci studies [58,59]. Together our findings suggest a non-coding region

insult on the X chromosome results in robust autism-relevant behaviors that fall within each of the primary ASD diagnosing criterion and within some of the variably associated symptom groups.

Clearly defining a mouse line as a model for ASD possesses multiple challenges because of the complex nature of the social, communicative, and perseverative abnormalities present in individuals with ASD. A number of researchers have identified various behavioral assessments for studying traits in mice that parallel aspects of the three core features of ASD [26,60,61]. In the present study we used a number of these approaches to examine and characterize the MALTT line.

Pup separation-induced USVs have been used to assess early communication abilities in mice. There are no consistent reports of altered vocalizations in infants although automated tools for early-age vocal analysis in humans are being developed [62]. Thus, this measure is not intended to directly parallel the human infant state. However, pup USVs are a well-defined measure for potentially identifying early neurodevelopmental and communicative abnormalities in mice. MALTT males and females respectively showed a four- and two-day extension of the normal WT USV pattern. For example, MALTT males did not persistently decrease their vocalizations from peak levels until PND 11 compared to PND 7 in WTs. Our results are consistent with several other studies of mouse models of neurodevelopmental disorders associated with ASD including the Tcs62 down syndrome model, a Rett syndrome mouse model, a chromosome 15q11–13 duplication model, and in the BTBR inbred strain autism mouse model [24,63–65] indicating increased levels of vocalization for affected pups in multiple ASD mouse models. It is important to note that decreased vocalizations have been observed in other ASD mouse models [66–68], suggesting that impaired separation-induced USV in ASD models could be reflected as either overall decreased number of calls or, as in the current study, a change in the normal developmental USV call pattern.

To further characterize USV communication in the MALTT mice, we studied the emission of USVs from older mice during the social interaction test. For each of the social interaction tests, all mice were juveniles so the social interactions were less influenced by reproductive motivations [69], and this young age allowed us to avoid the potentially confounding stereotypies that begin in the MALTT line during adulthood. In the three-chamber test, WT mice clearly preferred to spend more time on the side with the social stimulus, a stranger mouse, and more time at the actual partition with the stranger mouse compared to the side with the object. In contrast, the MALTT mice did not show a preference for the stranger relative to a novel object for either measure. It is interesting that while the MALTT mice show a social interest deficit as measured by both time in side and sniffing at the partition, several other ASD mouse model lines exhibit a deficit in social preference when time in a social-paired chamber is considered but exhibit a normal preference when time directly (i.e. sniffing) investigating a social target is considered [19,70]. Even the MALTT line trends toward a preference for stranger partition, but not stranger side. It is not clear what a disassociation between time on the side of the social stimulus mouse and actual time sniffing/investigating indicates, but it could reflect a spectrum of social preference in mice with a deficit in direct sniffing being the most sensitive indicator of disinterest in social cues.

Direct social interactions were then analyzed for a pair of mice allowed to freely interact. In our first experiment, a WT or MALTT mouse was paired with a novel standard partner FVB/NJ. This method ensured that the partner mouse for either genotype would have a similar baseline level of social behavior, allowing us to more clearly identify differences in WT and MALTT responses to a standardized social stimulus. MALTTs showed decreased direct social investigation of a FVB/NJ partner and increased nonsocial behaviors relative to

WT littermates. During these social investigations USVs were simultaneously recorded. The presence of a MALTT mouse in the pair dramatically reduced the overall USVs emitted from the pair. In the second experiment, when pairs of only MALTT mice were analyzed the majority of pairs emitted no vocalizations at all. This suggests that the MALTT mouse was responsible for the lack of vocalizations during the social interactions when a “normal” partner mouse was present. Both pup and adolescent vocalizations have been analyzed where specific patterned frequency waveforms were identified [21,65]. It will be of interest to determine if the MALTT line’s USVs are aberrant with regard to vocalization frequency pattern in addition to the alteration in ‘call number’.

Social interactions rely heavily on olfactory input [47]. Data from the olfaction test clearly demonstrate that MALTT mice have intact olfactory function and can discriminate between non-social and social odors. However, the MALTT mice do appear to have a reduced “interest” in a first social odor. We believe this observation is consistent with reduced social interest and interactions of MALTT mice in the three-chamber and direct social interaction tests.

In addition to the social and communication deficits in the MALTT line, the males exhibit a clear and developmentally progressing circling stereotypy. In humans the repetitive feature in ASD may present as motor mannerisms, including whole body movements such as rocking. A clinical study also describes that children with ASD may “enjoy spinning or whirling their bodies” [71]. In the MALTT line, a circling stereotypy typically develops between 3–6 weeks of age in the MALTT males and lasts throughout adulthood. The stereotypy appears spontaneously in the home-cage and is not a constant trait, but rather it seems to be exacerbated when the mice are disturbed (personal observation). Vestibular dysfunction does not appear to play a role in this behavioral abnormality, as is the case for many rodents that exhibit a rotation behavior. In addition, the MALTT mice do not have impaired hearing as measured using the ABR test. MALTT mice were also hyperactive in the open-field even prior to the age of onset for the stereotypic responding. Although it is unclear why the MALTT mice are more active, hyperactivity is consistent with an impairment in response inhibition. We are currently evaluating other potential assays for assessing stereotypic responses and response inhibition in the MALTT mice.

It is important to consider how secondary traits apparent in the MALTT line might have contributed to the observed social deficits and low vocalization levels. The MALTT line is hyperactive and it is possible that MALTT mice were so active during the direct social interaction test that it interfered with interactions. For example, if increased activity of MALTT mice interfered with interactions then the level of ‘passive’ interactions (i.e. those interactions initiated by the WT partner) in the MALTT:WT dyad would have been significantly lower compared to the ‘passive’ interactions in the WT:WT dyad; however, the levels of this type of interaction were similar in both types of dyads suggesting that the activity of the MALTT mice did not confound the interactions in the direct interaction test. In the three-chamber test, although the MALTTs spent less time at the partner partition than WTs, they spent an equivalent amount of time at the object partition, and the number of entries into both chambers was equivalent between MALTT and WT mice indicating that their activity levels were similar during this type of social interaction test. Aggression and stereotypical circling behaviors could also affect social interactions. Therefore, all social tasks were performed using juveniles between 23 and 28 days of age. While the MALTTs showed a clear circling stereotypy at 45 days, there were no significant genotype differences observed at 17, 22, or 28 days of age. In addition, we observed no circling behavior during either the direct social interaction test or the three-chamber test. Similarly, the tests for social interaction were performed prior to the age-of-onset for aggressive behaviors in the MALTT mice, and we did not observe aggressive responses during the juvenile direct social

interaction test. Finally, although isolated housing is routinely utilized prior to a variety of social tests, it is still possible that MALTTs differentially responded to the isolated housing than WTs. At this point it cannot be eliminated as a contributing factor, although, since the single-housing was limited to only a few days, we believe the differential impact, if any, may have been minimized.

A wide range of additional behaviors are associated with ASD including cognitive impairments, hyperactivity, abnormal anxiety, seizures, aggressiveness, and odd responses to sensory stimuli, including oversensitivity to sounds or being touched [2]. MALTT male mice exhibit increased audiogenic seizures, reduced sensorimotor gating, and increased sensitivity to tactile stimulation. Sensory function is involved in all three of these behaviors and suggests that the MALTT line may be especially amenable to understanding a link or common pathology between sensory abnormalities and ASD core feature symptoms. One juncture at which the sensory hypersensitivity may intersect the observed social abnormalities is the heightened aggression. Elevated aggressive responses have been observed in the BTBR mouse line, and it was suggested that after prolonged investigation by a partner mouse the BTBR mouse may have experienced a “sensory overload” resulting in aggressive attack [20]. It is possible that a similar explanation might underlie the MALTT line aggression. In a third sensory-mediated test 100% of MALTT males tested exhibited full erratic seizures in response to a loud auditory stimulus. Audiogenic seizures rarely occur in humans, but epileptic spontaneous seizures occur in the ASD population at a rate as high as 30% [53]. Interestingly, similar sensory abnormalities, including increased tactile sensitivity, heat sensitivity, and susceptibility to PTZ-seizure were recently described in the Gabrb3 mouse model of ASD [72]. In the Pten condition null mouse model of ASD, reciprocal social interaction deficits, occurrence of spontaneous seizure, and impaired PPI have been reported [73]. Similarly, susceptibility to audiogenic seizure and abnormal PPI response occurs in the Fragile X mouse model, currently being investigated for insights into ASD due to an overlap in clinical populations [74,75]. ASD mouse models exhibiting both core and secondary phenotypes provide a tool for examining the extent to which a similar underlying pathology is responsible for the concomitant features.

We believe that the behavioral pattern observed in the MALTT line of mice suggest that they represent a novel mouse model with abnormalities in assays for each of the core features of ASD, in addition to multiple other phenotypes consistent with secondary abnormalities observed in some individuals with ASD.

The MALTT line was generated by a random insertional mutation rather than based on a single human population-identified genetic abnormality. This novel mouse line highlights the fact that although it is currently common to discount non-coding region duplications and deletions, they may not necessarily be benign. Certainly, insertions, rearrangements, duplications, and deletions do not only cause gene copy number effects, but they can affect coding region and non-coding region chromatin structure and status and nearby and long-distance transcription factor binding [76]. Direct analysis of the effects of these types of changes is difficult in the human disease population because the effects may largely be at the level of RNA expression and quality samples are rare and challenging to obtain. However, with the establishment of the AGRE database these types of studies are now more readily being undertaken [41,77] in human samples from lymphoblast cell lines. Currently, rodent models are especially amenable to these types of analysis because of the ease of access to preserved chromatin status and RNA expression patterns.

Results from our molecular studies have identified several genes that may be involved in the autism-related phenotypes seen in the MALTT line. In the MALTT mice, the strongest gene expression change is the dramatic upregulation of Fam46d expression observed in both adult

and newborn cortex. Fam46d is a protein of unknown function. However, it is a proposed cancer-testis antigen [78], and homology and protein-protein interaction studies suggest FAM46 family proteins may be crucial for cellular signaling and potentially involved in the TGF-β signaling pathway [79–81]. Importantly, Fam46d probes were included in an RNA expression microarray screen of lymphoblastoid cells from a population of humans with fragile X also meeting the criteria for autism, and Fam46d was found to be upregulated [41]. A close homologue, Fam46c was also found to be differentially expressed in the autism-affected versus control populations. Taken together our data and those of Nishimura et al. support the hypothesis that this gene family, and specifically Fam46d, may in some cases be involved in the expression of ASD-related behaviors. Our data also suggest low-level upregulation of the nearby genes 2610002M06Rik, Gm732, Brwd3, and Hmgn5 occurred in MALTT male cortex, although this upregulation relative to WT was not observed in newborn cortex. Whether these genes contribute to the murine behavioral output will require future systematic analyses. While the striking upregulation of Fam46d, which is located near the transgene insertion site, is compelling, future studies will be necessary to directly manipulate Fam46d to better understand its' role in the types of ASD-related responses we have documented in the MALTT mice.

Finally, the brain morphological survey indicated that MALTT male brain weights were significantly lower in adults. A wide range of autism studies have described varying morphological abnormalities in numerous brain regions, and it should be noted that no clear and consistent abnormalities have thus far been identified. However, post-mortem and imaging studies have most frequently described the frontal lobes, amygdala, and cerebellum as differing in ASD populations compared to controls [82]. Our preliminary histopathological examination of the brain revealed no major malformations. Future detailed studies will be necessary to assess for cellular organization and composition.

The molecular mechanisms underlying ASD still remain elusive. ASD is an incredibly heterogeneous disorder, and it will undoubtedly require a variety of models to elucidate common downstream mechanisms resulting in similar behavioral outcomes. Mouse models, such as the MALTT mice, which replicate the behavioral phenotypes of ASD are an important tool for identification of novel genetic abnormalities and molecular mechanisms that might improve our understanding of the etiology of ASD.

6. Conclusions

Our lab has identified a novel mouse line, MALTT, with X-linked inheritance of a strong ASD-related phenotype. The MALTT line was created with a random transgene insertion strategy resulting in a heritable transgenic mutation. We have initially identified a significant repertoire of ASD-relevant behaviors in the MALTT mice. This line exhibits consistent deficits in social and communicative tasks, including pup isolation-induced ultrasonic vocalizations (USVs), juvenile social interest tasks, juvenile direct social interaction tasks, and juvenile USVs correlated with social interaction. We also observe a robust rotational stereotypy and several associated but non-core ASD-like traits, including early age susceptibility to audiogenic seizure, tactile hypersensitivity, and impaired sensorimotor gating. We have also identified upregulated gene expression of the gene Fam46d, located near the site of transgenic integration. This gene family has been implicated previously in an ASD-population RNA expression study [41]. It is our anticipation that further analysis of the MALTT line will uncover novel genetic factors contributing to the ASD phenotype or converge with other studies' findings to support a common underlying mechanism.

Research highlights

- The MALTT line shows multiple social behavior deficits and a progressive circling stereotypy.
- Juvenile MALTT mice exhibit minimal ultrasonic communication during a social assay.
- A variety of sensory-related abnormalities are observed in the MALTT line.
- Fam46d expression is strongly upregulated in the MALTT line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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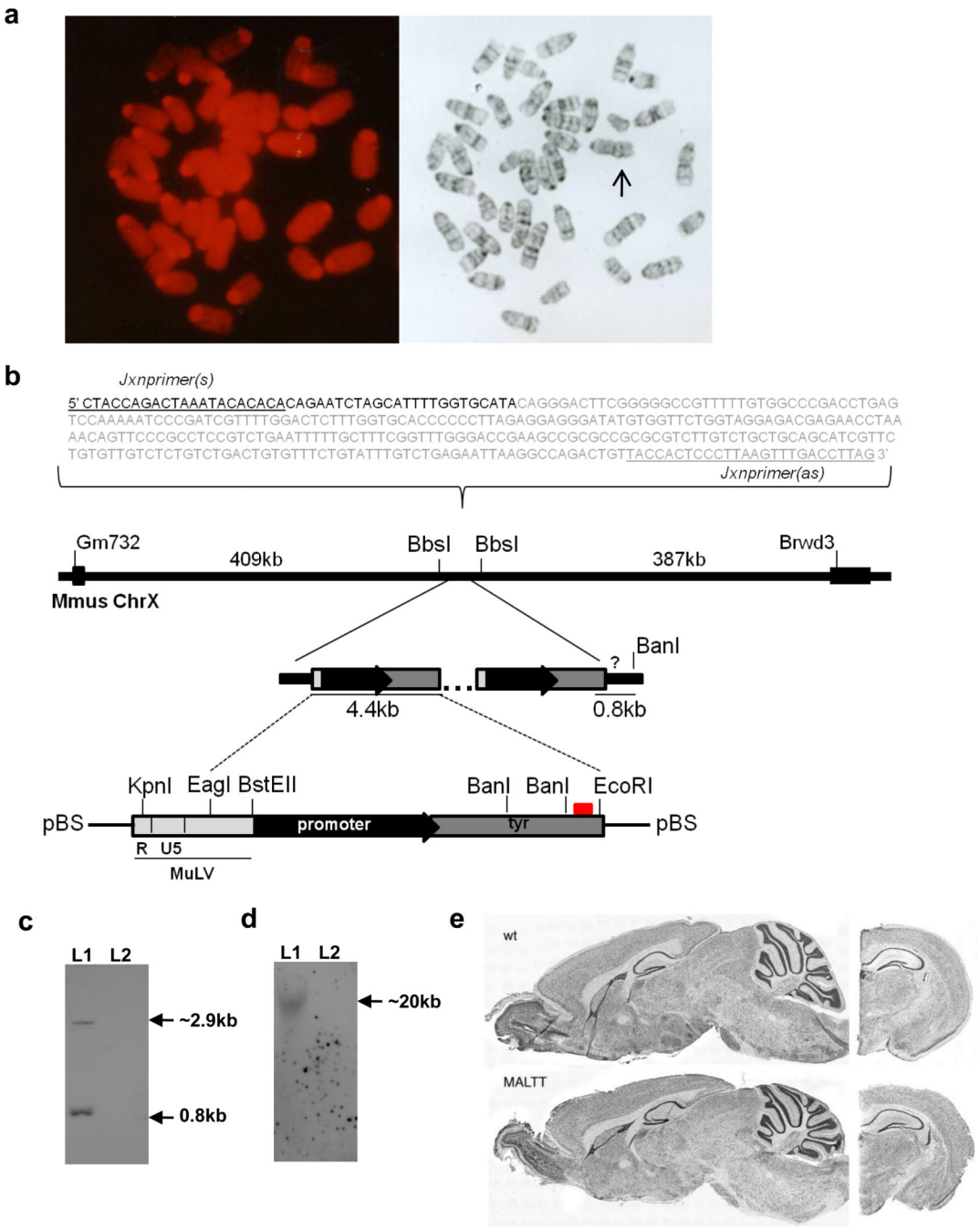

Figure 1. Initial characterization of the MALTT transgenic line

(**a**) A fluorescently labeled (FITC) anti-digoxigenin antibody indicated the location of the TyBS minigene insertion (left) and Geimsa staining indicated (black arrow) the labeled chromosome was X (right). (**b**) A schematic diagram of the mouse X chromosome indicates at minimum two copies of 4.4kb minigene construct (0.4kb MuLV sequence+1.8kb promoter region+2.2kb tyrosinase (tyr) cDNA) inserted in the non-coding region between genes Gm732 and Brwd3, as well as the 5' integration sequence (black lettering: X chromosome, gray lettering:minigene). A Southern blot probe against the minigene is indicated in red. (**c**) A Southern hybridization image indicates 2 minigene positive bands (0.8kb, ~2.9kb) apparent in MALTT (lane 1) but not WT (lane 2) BanI-digested gDNA

(left). (**d**) A single band (minimum 20kb) was apparent in MALTT (lane 1) but not WT (lane 2) BbsI-digested gDNA (right). (**e**) Exemplary sagittal and coronal Nissl-stained images of WT and MALTT brain sections are shown.

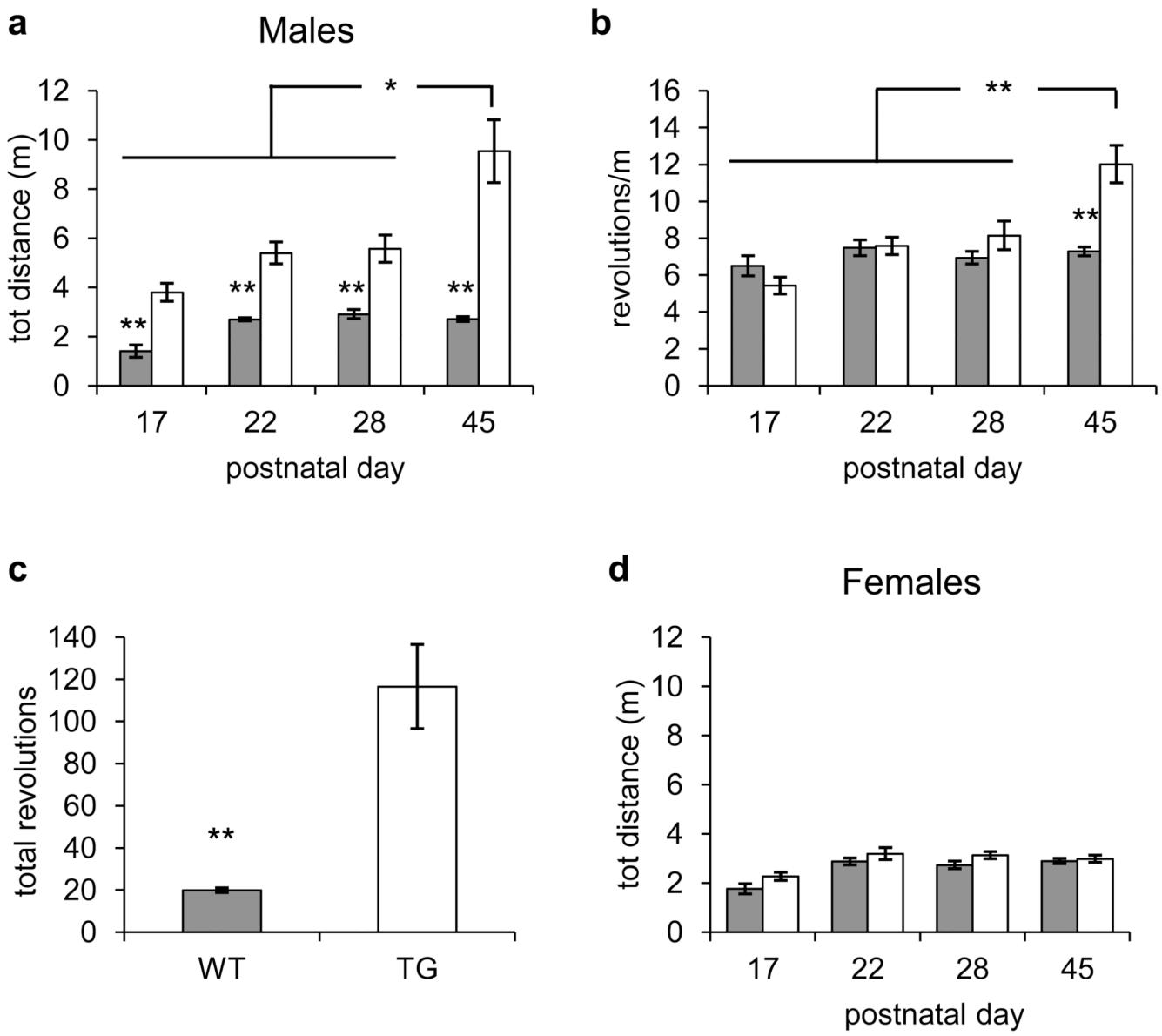

Figure 2. MALTT mice are hyperactive and exhibit a rotational stereotypy

Total distance was measured during 10 minutes of open field exploration for mice aged 17, 22, 28, and 45 days. (**a**) Male total distance is shown. (**b**) Stereotypies in males were measured as total revolutions/total distance traveled for male mice. (**c**) Average absolute numbers of revolutions for males on PND45 are shown. (**d**) Female total distance is shown. All data are presented ± SEM. Males: WT *n* = 9–17, TG *n* = 9–13; females: WT *n* = 12–17, TG *n* = 11–17; * $p \leq 0.05$, ** $p \leq 0.01$

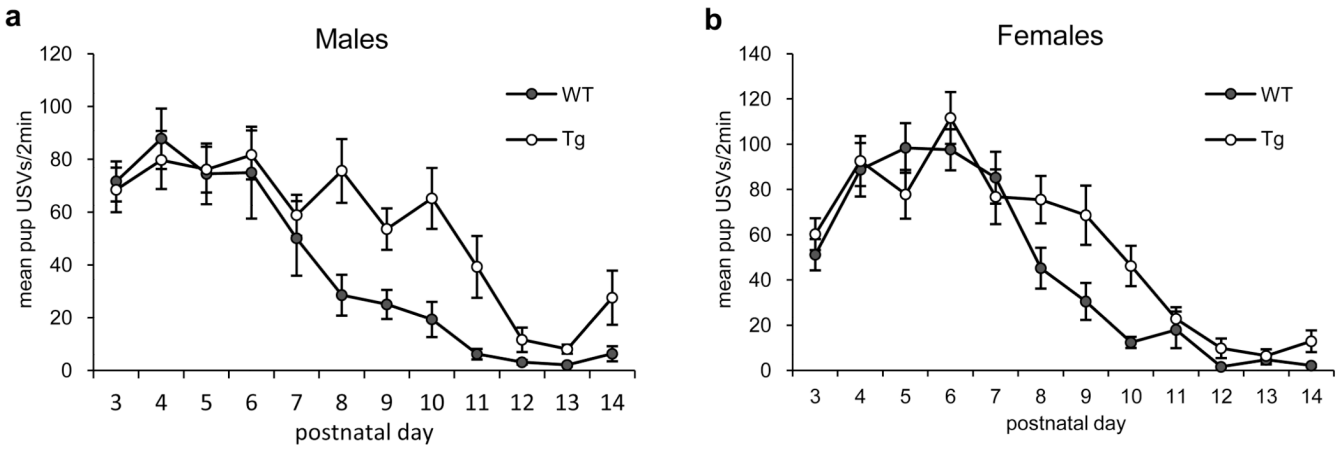

Figure 3. MALTT mice show altered pup ultrasonic vocalization patterns

Pup ultrasonic vocalizations (USVs), when separated from mother and littermates, were assessed on PND 3–14. (**a**) Male pup USV development curve is shown, WT *n* = 21; TG *n* = 24. (**b**) Female pup USV development curve is shown, WT *n* = 19; TG *n* = 22. All data are presented ± SEM.

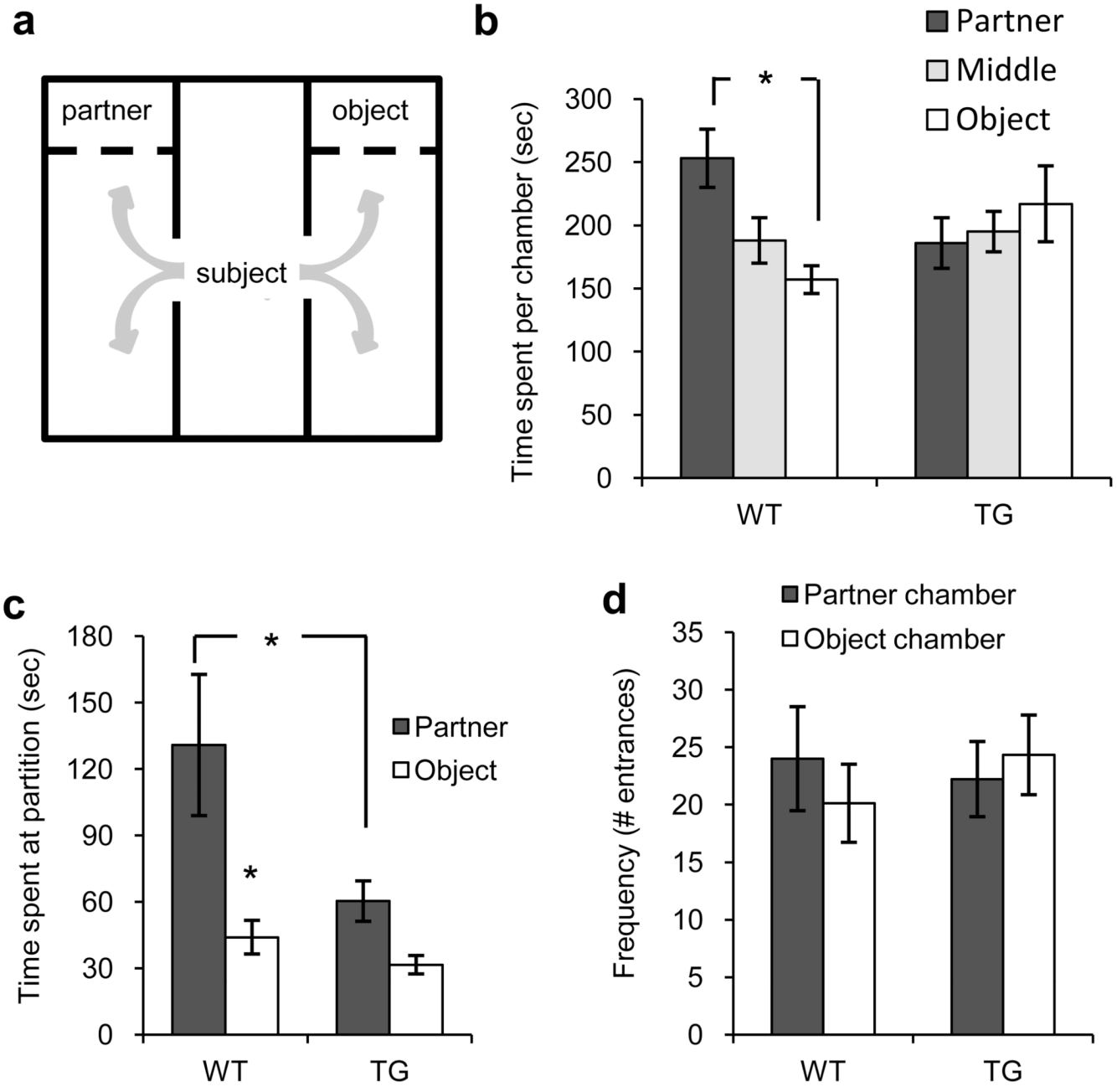

Figure 4. Juvenile MALTT mice show decreased social interest

Three-chamber partition task: (**a**) Diagram of the testing apparatus with two outer side chambers, each housing a novel object or stranger mouse behind perforated Plexiglas, and the center chamber where the subject is started. Two doorways allowed the subject to move freely between all chambers. (**b**) A MALTT or WT subject was allowed to explore the apparatus. Mean total duration each subject spent per chamber (including time at partitions within side chambers) is shown. choose to spend time investigating a stranger mouse or novel object through separate partitions, WT $n = 8$; TG $n = 9$. (**c**) Mean total duration at stranger or object partition is indicated. (**d**) Frequency of subject entry into each side chamber from the center chamber is shown. All data are presented ± SEM. * $p \leq 0.05$

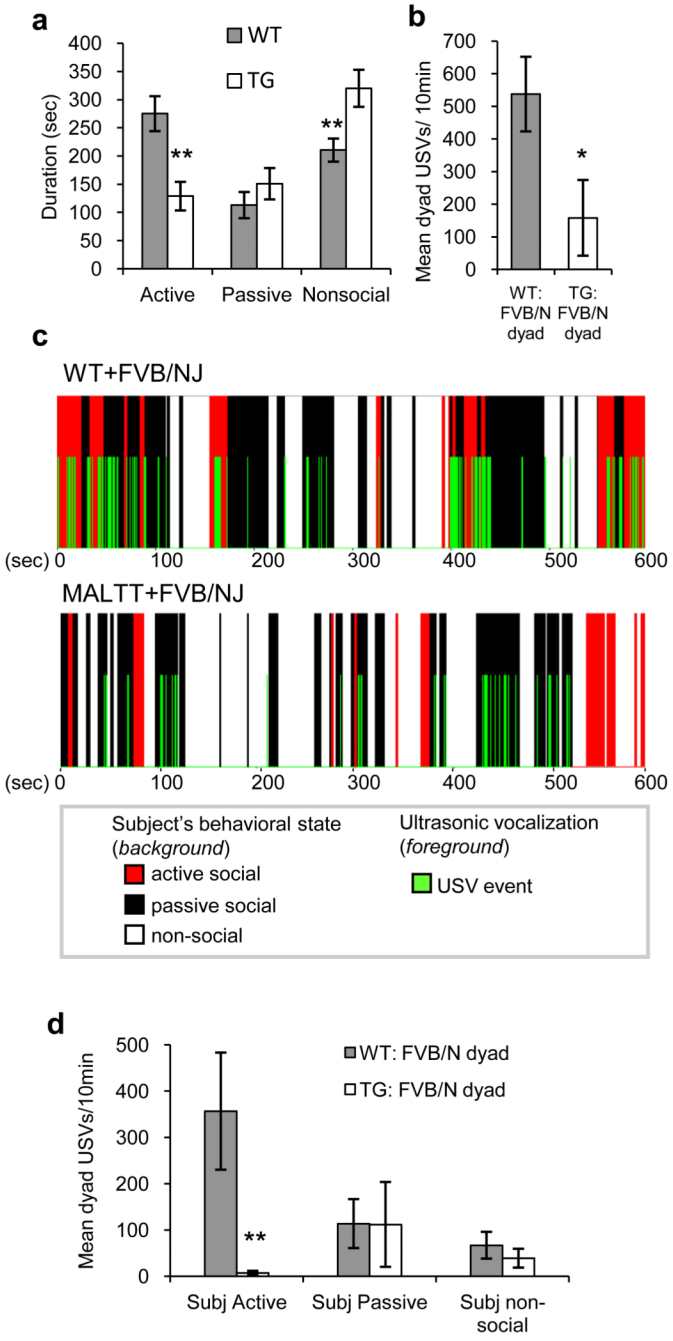

Figure 5. Juvenile MALTT mice show social and social communication-related deficits

Direct social interaction task one: Two stranger mice were allowed to directly interact for 10 minutes while USVs were concomitantly recorded. (**a**) Mean duration of active, passive, and nonsocial behaviors for the subject mouse within a pair is shown. The subject of the pair was a WT in a WT + FVB/NJ stranger dyad, $n = 8$ pairs, or a TG in a TG + FVB/NJ stranger dyad, $n = 9$ pairs; (**a–d**). (**b**) Mean total USVs emitted by pairs is shown. (**c**) Representative event plot indicating (foreground) a pair's USV events as they overlapped the subject mouse's behavioral state (background). (**d**) Shown is the mean number of USVs emitted from the pair during the WT or TG subjects' respective behavioral states. All data are presented ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$

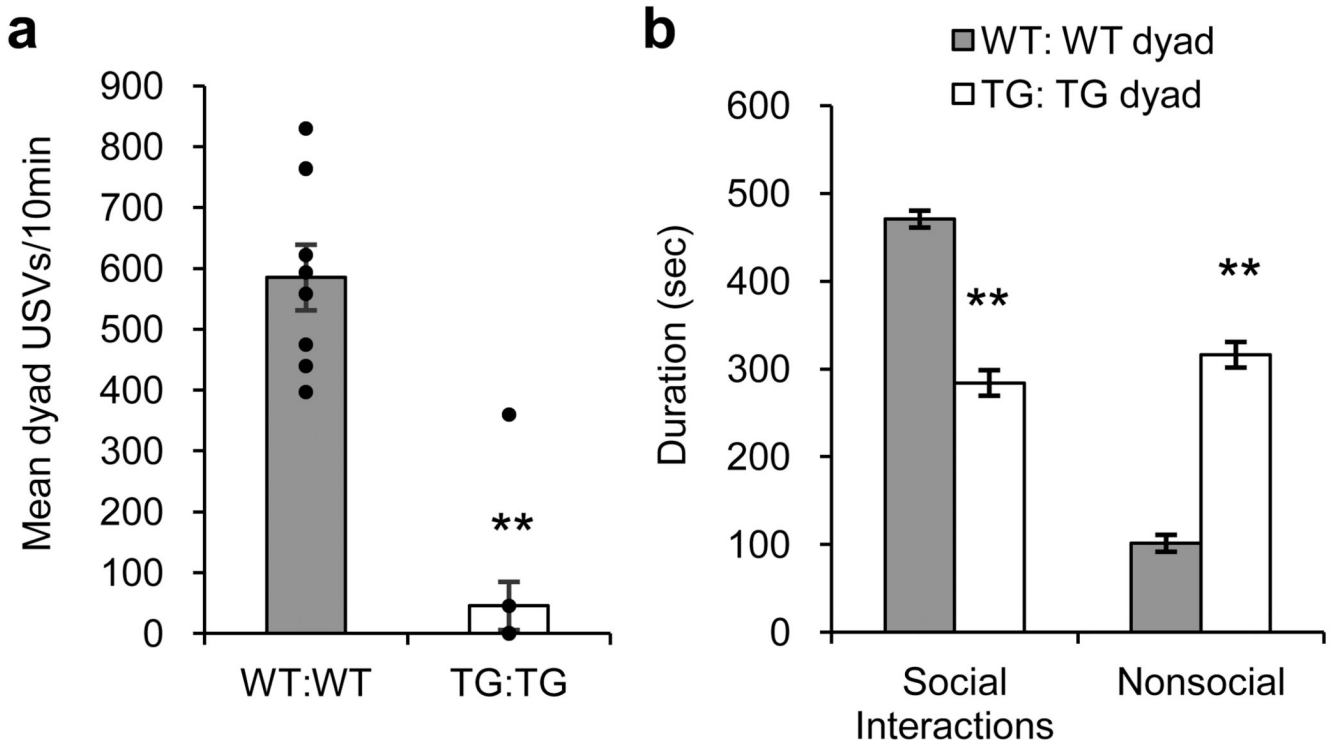

Figure 6. Juvenile MALTT mice show minimal vocalization in a social pair

Direct social interaction task two: MALTT + MALTT partner pairs or WT + WT partner pairs were analyzed for USVs during direct social interaction; MALTT $n = 9$ pairs; WT $n = 8$ pairs. (**a**) Mean total USVs emitted from same genotype pairs is shown. Individual pair data is indicated (black circles), with the exception of TG:TG data point "0" which includes 7 pairs' values. (**b**) Mean duration of social and nonsocial behaviors for same genotype pairs is shown. All data are presented ± SEM. ** $p \leq 0.01$

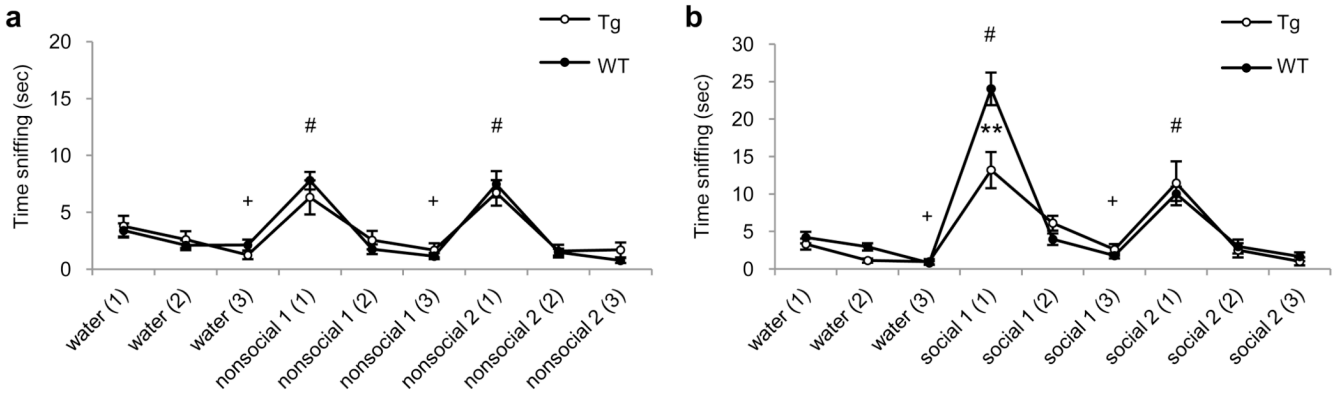

Figure 7. Juvenile male MALTT mice can detect and differentiate nonsocial and social odors

A habituation/ dishabituation olfactory test was used to assess sense of smell. WT *n* = 13; TG *n* = 11. (**a**) Nonsocial odor 1 (banana or almond) was presented repeatedly followed by a second nonsocial odor (banana or almond) and mice were assessed for dishabituation on first presentation of novel odor and habituation by the third presentation of the odor. (**b**) Same task as (**a**) but nonsocial odors were replaced with social odors. For both tasks odor 1 and odor 2 were alternated pseudorandomly between subjects. All data are presented ± SEM. + denotes p≤0.05 for habituation to odor for both genotypes, # denotes p≤0.05 for detection of novel odor for both genotypes, open circle denotes Tg, closed circle denotes WT, ** p ≤ 0.01 for a between-subjects effect

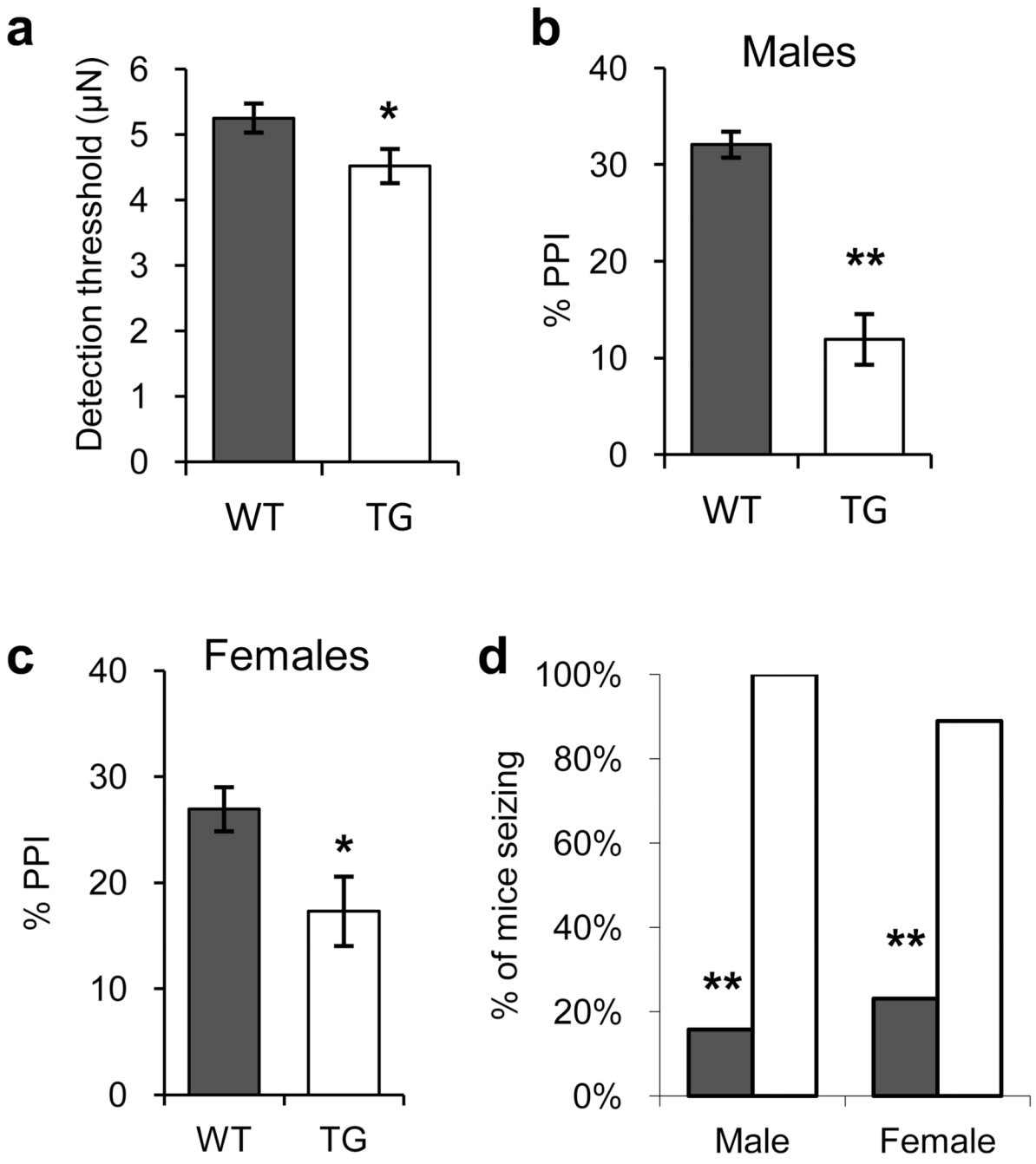

Figure 8. MALLT mice show aberrant secondary ASD-associated behaviors

(**a**) A tactile sensitivity assay indicates minimum threshold for hind paw plantar surface detection of force for male mice, WT *n* = 8; TG *n* = 7. (**b**) Mean percent prepulse inhibition (PPI) response for male mice is shown. Data is collapsed over ages 17, 22, 28, 45 days. The same subjects tested for OFA were tested second on PPI. (**c**) Mean percent PPI response for female mice is shown collapsed over ages 17, 22, 28, 45 days. (**d**) Percent of mice exhibiting seizure activity in response to an audiogenic stimulus is shown for males WT *n* = 11, TG *n* = 19; females WT *n* = 9, TG *n* = 13. All data are presented ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$

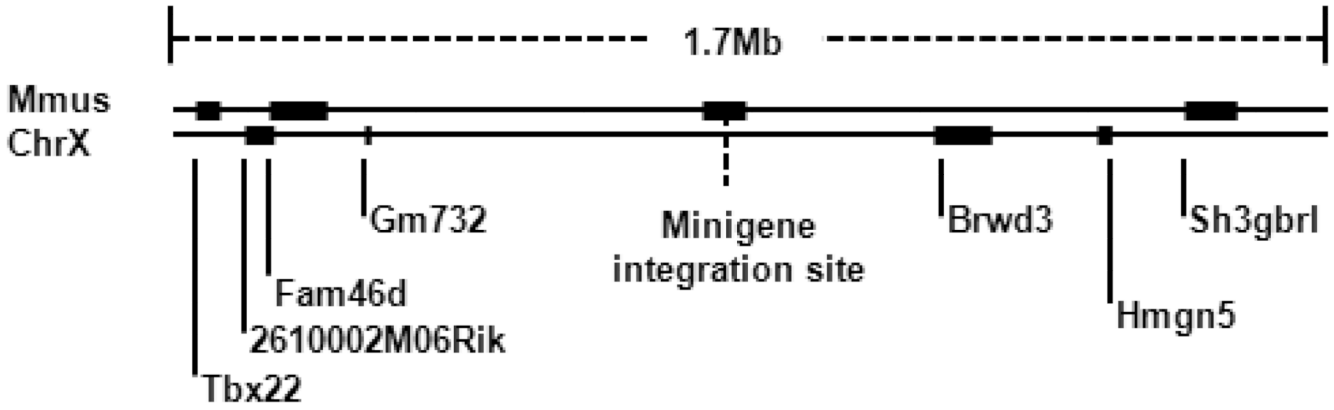

Figure 9. Genes analyzed in the vicinity of the TyBS minigene integration site

TABLE 1

Male MALTT and WT adult gene expression values

Gene	Location from TyBS insertion	fold change	p-value
Tbx22	+687kb; fwd strand	1.38	0.501
2610002M06Rik	+572kb; rev strand	**2.16***	0.001
Fam46d	+562kb; fwd strand	**379.51***	< 0.001
Gm732	+409kb; rev strand	*not detected in WT; novel expression in MALTT*	
Brwd3	−387kb; rev strand	**1.21***	0.040
Hmgn5	−650kb; rev strand	**2.13***	0.008
Sh3gbrl	−741kb; fwd strand	0.75	0.083
Pou3f4	−2,460kb; fwd strand	1.13	0.209

Values are expressed compared to WT as fold change and significance factor, except for gene Gm732, which was not detectable in WT cortex and therefore no direct comparison is possible. All genes are located on the X chromosomes and their positions relative to the identified TyBS minigene insertion site is given as distal (+) or proximal (−) to insertion site and strand location is indicated. Per genotype $n = 6$. For all subjects cortical tissue was taken between 5–6 weeks of age.

* denotes statistical significance

TABLE 2

Male MALTT and WT pup gene expression values

Gene	Location from TyBS insertion	fold change	p-value
2610002M06Rik	+572kb; rev strand	1.23	0.446
Fam46d	+562kb; fwd strand	**32.27***	0.002
Gm732	+409kb; rev strand	*not detected in WT;very low level expression in MALTT*	
Brwd3	−387kb; rev strand	1.09	0.659
Hmgn5	−650kb; rev strand	0.86	0.540
Sh3gbrl	−741kb; fwd strand	1.03	0.943

Values are expressed compared to WT as fold change and significance factor, except for gene Gm732, which was not detectable in WT cortex and therefore no direct comparison is possible. Per genotype $n = 5$. For all subjects cortical tissue was taken at PND1.

* denotes statistical significance