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Autism-specific maternal autoantibodies produce behavioral abnormalities in an endogenous antigen-driven mouse model of autism

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Abstract

Immune dysregulation has been noted consistently in individuals with autism spectrum disorder (ASD) and their families, including the presence of autoantibodies reactive to fetal brain proteins in nearly a quarter of mothers of children with ASD versus less than 1% in mothers of typically developing children. Our lab recently identified the peptide epitope sequences on seven antigenic proteins targeted by these maternal autoantibodies. Through immunization with these peptide epitopes, we have successfully created an endogenous, antigen-driven mouse model that ensures a constant exposure to the salient autoantibodies throughout gestation in C57BL/6J mice. This exposure more naturally mimics what is observed in mothers of children with ASD. Male and female offspring were tested using a comprehensive sequence of behavioral assays as well as measures of health and development highly relevant to ASD. We found that MAR-ASD male and female offspring had significant alterations in development and social interactions during dyadic play. Although 3-chambered social approach was not significantly different, fewer social interactions with an estrous female were noted in the adult male MAR-ASD animals, as well as reduced vocalizations emitted in response to social cues with robust repetitive self-grooming behaviors relative to saline treated controls. The generation of MAR ASD-specific epitope autoantibodies in female mice prior to breeding created a model that demonstrates for the first time that ASD-specific antigen-induced maternal autoantibodies produced alterations in a constellation of ASD-relevant behaviors.

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CONFLICT OF INTEREST

Drs. Van de Water and Edmiston have a patent application involving the MAR ASD peptides described herein; all other authors have no conflicts of interest to declare.

INTRODUCTION

Autism spectrum disorders (ASD) are a set of neurodevelopmental disorders characterized by impaired verbal and non-verbal social communication accompanied by the presence of repetitive and stereotypical behaviors[1]. Current prevalence estimates suggest that ASD affects 1 in 68 children in the United States, with the average age of definitive diagnosis at 4 years of age[2]. While the etiology of this highly heterogeneous disorder remains unknown, mounting evidence suggests that both genetic and environmental risk factors contribute to the development of ASD[3–5]. One such risk factor is immune system dysregulation, which has been consistently noted in individuals with ASD as well as their family members[6]. Most notably, a sub-set of mothers of children with ASD has been reported to harbor autoantibodies reactive to fetal brain proteins by several investigators[7–10].

The maternal immune system is uniquely regulated during pregnancy under normal conditions, ensuring that a pathogen-free, non-inflammatory, environment is maintained for the developing fetus[11]. Maternal immunoglobulin G (IgG) antibodies are among the limited components of the maternal immune system that enter the fetal compartment during gestation, transferring at high concentrations via a specific receptor across the placenta beginning around mid-gestation in humans, thereby providing the immunologically naïve fetus with passive protection against pathogens[12]. In addition to immunoprotective IgG specific to external pathogens, maternal IgG autoantibodies that react to fetal 'self'-proteins' will also cross the placenta. Furthermore, while antibody access to the brain is normally inhibited by a mature blood-brain barrier (BBB), the BBB is permissive during early neurodevelopment and thus allows maternal IgG into the fetal brain[13]. Therefore, prenatal exposure to maternal autoantibodies reactive against fetal brain proteins has been suggested as a mechanism for altering the trajectory of neurodevelopment[6, 14, 15].

Our laboratory initially described the presence of a two specific patterns of maternal autoantibody reactivity to fetal proteins at approximately 37 and 73 kDa, and 39 and 73 kDa, that were uniquely found among mothers of children with ASD[7, 16]. Maternal reactivity to both patterns was also observed in prospectively collected mid-gestational blood samples of mothers who subsequently gave birth to children with ASD[17], supporting their predictive potential.

To provide support for the pathological significance of these brain-reactive maternal autoantibodies and the mechanisms by which they may exert their effects, various animal studies have been conducted in mice[18–24] and non-human primates[25, 26]. One technique often used to generate animal models of autoimmunity is passive antibody transfer, in which circulating autoantibodies from an affected individual are collected and subsequently transferred to a healthy animal, allowing researchers to determine whether the autoantibodies of interest directly exert pathogenic effects in the exposed animals[14]. Each of the previously conducted animal studies have provided evidence that introduction of human autoantibodies reactive for proteins in the fetal brain into a pregnant female during mid-gestation led to alterations in neurodevelopment and behaviors in offspring. For instance, mouse passive transfer studies have demonstrated that mice prenatally exposed to IgG from mothers of children with ASD had alterations in social and anxiety-like behaviors[18] as well

as impaired motor and sensory abilities[20]. In a 2014 study, mice receiving a single intraventricular injection on embryonic day 14 of human IgG from maternal plasma with the 37 and 73 kDa autoantibodies resulted in atypical behaviors, including stereotypical self-grooming and increased repetitive behaviors, relative to mice similarly injected with maternal IgG from mothers of neurotypical controls[23]. Two subsequent investigations using the same technique showed neuroanatomical changes in the offspring, including increased radial glial cell proliferation along with accelerated migration, reduced numbers of cortical dendritic spines, as well as increased brain and neuron size[21, 22]. A related study utilizing a passive transfer approach in rhesus macaques demonstrated higher levels of motor activity and stereotypies among in offspring of macaque dams prenatally exposed to autoantibody-positive ASD IgG, supporting potentially pathogenic IgG present in maternal circulation[25]. This study was followed by a more targeted approach that was designed to increase the duration of gestational exposure to purified IgG from mothers who tested positive for autoantibodies that recognized the 37/73 kDa proteins[26]. Macaques prenatally exposed to the 37/73 kDa maternal IgG exhibited aberrant behaviors that included rebuffed and unreciprocated social approaches and inappropriate vocalizations[26]. These animals also had increased total cerebral volume relative to control treated subjects[26], further supporting previous work in mice showing that these specific maternal autoantibodies appear to alter brain growth trajectory[20]. These findings are of great interest, as the relationship between the presence of maternal autoantibodies with increased total cerebral volume has also been observed in humans[27].

As the results from both observational and animal studies strongly suggest a role of maternal autoantibodies in the etiology of a subset of children with ASD, the identification of the targeted protein antigens for maternal autoantibody related (MAR) ASD was a critical step in advancing this area of ASD research. We recently determined the identity of the autoantigens recognized by these maternal autoantibodies in fetal brain tissue[28]. Through tandem mass spectrometry peptide sequencing, the target proteins corresponding to the 37-, 39-, and 73-kDa bands were identified as: lactate dehydrogenase A and B (LDH-A, LDH-B), stress-induced phosphoprotein 1 (STIP1), collapsin response mediator proteins 1 and 2 (CRMP1, CRMP2), and Y-box binding protein 1 (YBX1)[28]. We also discovered an additional autoantigen with a molecular weight of 44-kDA corresponding to guanine deaminase (GDA) that was not observed during the initial screen, as it was masked by the presence of the heavy chain band of IgG in the fetal brain protein preparation. All of the identified target proteins are expressed at significant levels in the human fetal brain and have established roles in neurodevelopment, such as STIP1's role in neuritogenesis[29]. Maternal autoantibody reactivity by western blot to any of the identified antigens, individually or in combination, was found to be significantly associated with an outcome of ASD in the child. Moreover, several combinations of autoantibody reactivity were identified as highly specific to mothers of children with ASD as they were not found in mothers of typically developing (TD) children. The most common pattern of combined reactivity observed exclusively in mothers of children with ASD moms was to LDH-A, LDH-B, STIP1, and CRMP1 (5% ASD mothers vs. 0% TD mothers), which coincidentally correspond to the previously described 37/73 kDa proteins recognized by these ASD-specific maternal autoantibodies[28]. When all antigen reactivity patterns were combined, a total of 23% of mothers of children

with ASD had one of the autoantibody patterns containing two or more of the target proteins relative to only 1% of control mothers[28].

While previous passive transfer animal studies provided support for the role of brain-reactive maternal autoantibodies as a risk factor for ASD, they do not reflect a constant exposure to the salient autoantibodies throughout gestation, as would be the case clinically. In contrast, the development of an antigen-driven animal model would provide a targeted and constant exposure to the relevant autoantibodies throughout gestation. We recently utilized overlapping peptide microarrays to identify the immunodominant epitope sequences recognized by MAR ASD related maternal autoantibodies for each of the seven autoantigens[30]. These MAR ASD epitopes are highly conserved between mice and humans[31], with an average sequence homology of 95.7% (LDH-A: 93.3 – 95.0%; LDH-B: 77.8 – 100.0%; STIP1: 93.3 – 94.7%; CRMP1: 86.7 – 100.0%). Thus, autoantibodies that specifically react to the human autoantibody-specific epitopes can be generated endogenously in female mice in an antigen-driven mouse model. Furthermore, the use of peptide epitopes recognized by the human maternal autoantibodies is essential in preserving the specificity of MAR ASD in an endogenous animal model, as immunization with the full-length sequences of the targeted proteins could generate autoantibodies reactive against multiple epitopes throughout the protein sequence that might not be relevant to MAR ASD. The antigen-driven approach also allows direct investigation of the cellular mechanisms related to changes in neurodevelopmental trajectory and subsequent behaviors, significantly advancing our understanding of the potential pathogenic effects exerted by the salient maternal autoantibodies. Therefore, the present study aimed to establish the first endogenous, antigen-driven animal model of MAR ASD in order to directly assess the pathologic significance of prenatal exposure to epitope-specific maternal autoantibodies in generating ASD-relevant behaviors in offspring. Considering that specific patterns of antigen reactivity were previously associated with distinct behavioral phenotypes within MAR ASD[28], the establishment of an antigen-driven model specific to one of these maternal reactivity patterns would greatly increase its translational potential as a preclinical testing platform. To create our initial antigen-driven mouse model of MAR ASD, we first focused on the combined reactivity to LDH-A, LDH-B, STIP1, and CRMP1, which was previously found to be the most selective and abundant pattern associated with MAR risk for ASD[28].

METHODS

Animals

Twenty-three sexually naïve C57BL/6J female mice were obtained from The Jackson Laboratory at four weeks of age and served as experimental dams. After weaning on postnatal day (PD) 21, experimental offspring were socially housed in groups of three to four with same-sex mice. All animals were housed in ventilated Techniplast cages in a temperature (68–72°F)- and humidity (~25%)- controlled colony room, on a 12:12 light:dark cycle with lights on at 07:00 h, and with food and water available *ad libitum*. In addition to standard bedding, a Nestlet square and shredded brown paper were provided in each cage. Animals were paw tattooed and/or ear punched for identification. All procedures were

conducted in accordance with protocols approved by the University of California, Davis Institutional Animal Care and Use Committee.

Synthesis of Multiple Antigenic Peptides (MAPs)

In order to generate the epitope-specific autoantibodies in female mice before breeding, 21 custom peptides were first synthesized and purified (>95% pure) by LifeTein (LifeTein LLC; Sumerset, New Jersey, USA). In particular, these peptide sequences correspond to the recently identified peptide sequences of the immunodominant B-cell epitopes of LDH-A, LDH-B, STIP1 and CRMP1 (Supplemental Table 1), which reflects the originally observed 37/73 kDa maternal banding pattern[30]. Peptides were synthesized as Multiple Antigenic Peptides (MAPs), in which four copies of the same peptide epitope are synthesized on a lysine-based core (MAPs-4 system). The MAPs-4 system does not require a carrier protein, as the dense packing of multiple copies of an epitope in combination with a high molar ratio produces a strong immunological response. Peptides were provided by LifeTein LLC as lyophilized powder; upon their arrival, lyophilized peptides were reconstituted into concentrated aliquots by dissolving in PBS alone, or in the event of hydrophobic peptides, PBS in combination with a small amount of DMSO. Concentrated aliquots were stored at −80°C until their use.

Treatment

Upon their arrival, sexually naïve females were randomly assigned to treatment group (MAR-ASD N=12; Control N = 11) and allowed to acclimate to the colony room for one week. All animals received a total of 5 subcutaneous immunizations, with an interval of 6 ±1 days between each immunization. MAR-ASD treated females received injections containing a mixture of the 21 custom MAPs-4 peptides in addition to Freund's complete (CFA; immunization 1) or Freund's incomplete (IFA; remaining immunization) adjuvant (Sigma-Aldrich, Saint Louis, MO) (outlined in Supplemental Table 2). Control females were treated similarly to the test dams substituting saline for the peptides (CFA/IFA/Saline). The presence of an adjuvant allows the generation of an immune response to the self-peptides.

Verification of Autoantibody Production

In order to verify the production of epitope-specific autoantibodies in treated females, maternal blood samples were first collected via saphenous vein prior to treatment (baseline) and at 4 and 9 weeks post-initial immunization. Samples were allowed to sit at room temperature for at least 1 hr, after which they were centrifuged at 10,000×g for 15 min, serum collected and stored at −80°C until use. Maternal samples were tested for reactivity to each of the 21 epitope-specific peptides administered during treatment via indirect capture enzyme-linked immunosorbant assays (ELISAs). First, NeutrAvidin coated high capacity plates (Pierce NeutrAvidin Coated High Capacity Clear 96-Well Plates; Thermo Scientific, Waltham, MA) were washed three times with PBST (Phosphate buffered saline with Tween 20). Biotinylated peptides (PEPperPRINT; Heidelberg, Germany) corresponding to the 21 MAPs treatment peptide sequences were then individually coated on the NeutrAvidin plates at 2 μg/mL in sample diluent and incubated overnight at 4°C. After incubation, non-bound peptides were removed, and the plate washed five times with PBST. Maternal serum samples (10 μg/mL) to be tested were then added to the plate and incubated for 45 min at room

temperature. Plates were washed five times, and then incubated for another 45 min in the presence of goat anti-mouse secondary antibody (InVitrogen, San Diego, CA). Following a series of four PBST and two PBS washes, plates were visualized at 450 nm following the addition of TMB substrate (3,3', 5,5' – tetramethylbenzidine; BD OptEIA, San Jose, CA). Reactivity to the intact protein antigens was additionally determined via ELISA (methods and results can be found in **Supplemental Methods and** Supplemental Table 6).

Breeding

Following confirmation of autoantibody production, MAR-ASD and control females were bred as harem trios with established C57BL/6J breeder males to generate experimental offspring. A total of at least 16 offspring (8 male, 8 female) are required to achieve optimal statistical power (80%) for each task, a total of at least 5 dams per treatment group are required. One advantage of our study design (which differs significantly from a maternal immune activation model) is that we can create the antibodies in the dam well before breeding to avoid unwanted side effects of immunization and handling stress. Thus, no immunizations were administered within 2 weeks, during, or after the time of breeding, gestation, or post-parturition. Breeding occurred in two separate cohorts (Cohorts 1 and 2), with male and female offspring from each litter randomly assigned to one of the following three behavioral testing groups: developmental milestones (Testing Group A), pup isolation-induced ultrasonic vocalizations (Testing Group B), and juvenile and adult behaviors (Testing Group C) (Figure 1).

Behavioral Assays

Behavioral assessment of experimental offspring was conducted in dedicated testing rooms during the light phase of the circadian cycle (07:00–19:00). Mice were tested in the sequence indicated in Supplemental Table 3. The order of testing was determined by the longitudinal juvenile and adult ages required for some tests, and by the principle of conducting the most stressful tests last. For all behavioral assays, procedures were employed that are consistent with best practices from the behavioral neuroscience literature, and from our previous publications[32–50]. Behavioral testing arenas were cleaned with 70% ethanol before the beginning of the first test session and after each test subject. For all non-automated assays, coded videos were scored by investigators blind to treatment status.

Developmental milestones (PD 4–14)—Pups from Cohort A (MAR-ASD: males N=8, females N = 14; Control: males N = 9, females N = 8) were tested for assays of developmental milestones and neurological reflexes every other day from postnatal days (PDs) 4–14, as previously described[20, 32, 37, 41, 42]. Parameters of physical developmental milestones included: body weight, body temperature, body and tail lengths, biparietal diameter (head width measured at top base of the pinna with a micrometer), fur development, pinnae detachment, eye opening, and incisor eruption. Parameters of behavioral developmental milestones included: righting reflex, negative geotaxis, cliff avoidance, forepaw grasping reflex, Preyer auditory startle reflex, level screen, vertical screen climbing, and bar holding.

Pup ultrasonic vocalizations during social isolation—Separation-induced ultrasonic vocalizations (USVs) emitted by Cohort B pups (MAR-ASD: males N=12, females N = 19; Control: males N = 13, females N = 10) were measured on PD 4, 6, 8, 10, and 12, as described previously[38, 41, 42]. Pups were individually removed from their dam and littermates at random and gently placed in isolation container containing clean bedding material. The isolation container was then placed in a sound-attenuating chamber and USVs were recorded for 3 min by an ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16; Avisoft Bioacoustics, Berlin, Germany). Ultrasonic calls were recorded using Avisoft Recorder software, and the sampling rate was 250 kHz, format 16 bit. At the end of the recording session, pups were returned to the nest. USV spectrograms were displayed using Avisoft SASLab Pro software. Calls were quantified manually from coded audio files by a highly-trained investigator blind to treatment status.

Juvenile reciprocal social interactions—Behavioral testing of Cohort C (MAR-ASD: males N=12, females N = 12; Control: males N = 11, females N = 11) began on PD 25 with the testing of juvenile reciprocal social interactions (JRSIs). The test was conducted in a Noldus PhenoTyper Observer 3000 chamber (25 × 25 × 35 cm^3; Noldus Information Technology Inc., Leesburg, VA). The floor of the arena was covered with a layer of approximately 0.5 cm of clean bedding. Each experimental and stimulus partner was individually housed in a clean cage for 1 hr before the test. Following this isolation period, an individual MAR-ASD or control experimental mouse was then placed in the testing arena with an age- and sex-matched juvenile C57BL/6J stimulus partner mouse. Interactions were recorded for 10 minutes under while light (~40 Lux) conditions. Frequency of social behaviors was subsequently scored from coded videos by an investigator blinded to treatment status, using Noldus Observer software (Noldus Information Technology, Leesburg, VA). Parameters of juvenile mouse social behaviors were chosen from the established literature and from our previous studies[34, 36, 40].

Elevated plus-maze—Adult offspring were tested for anxiety-like behaviors in the elevated plus-maze (EPM) test as previously described[39, 48]. Mice were individually placed in the center area of a black Plexiglas automated elevated plus-maze (Med-Associates, St. Albans City, Vermont, USA) and were allowed to freely explore the maze for 5 min. Room illumination was approximately 300 lux.

Light↔dark exploration—Subjects were tested in the light↔dark exploration task as a corroboratory measure of anxiety-like behavior, conducted in an automated chamber as previously described[38, 42]. The test began by placing the mouse in the light compartment facing away from the partition. The animal was allowed to freely explore the apparatus for 10 min. Time spent in each compartment and the number of transitions between the light (~400 lux) and dark (~3 lux) compartments were automatically recorded.

***Open field* activity—**General exploratory locomotion in response to a novel open field environment was assayed as previously described[39, 45]. Mice were individually placed in a VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, Ohio, USA) for a 30 min test session under dim lighting conditions (~30 lux).

Three-chamber social approach—Social approach was evaluated in an automated three-chamber apparatus as previously described[43, 44], using Noldus EthoVision XT videotracking software to increase throughput (version 9.0, Noldus Information Technologies, Leesburg, VA). The testing apparatus was a rectangular, three-chambered box constructed from matte white acrylic and measuring 40 × 60 × 23 cm^3. Opaque retractable doorways (12 × 33 cm^2) were designed to create optimal entryways between chamber (5 × 10 cm^2), while providing maximal division of compartments. Three zones, defined using the EthoVision XT software, detected time in each chamber for each phase of the assay. Zones to score sniffing were defined as the annulus extending 2 cm from each novel object or novel mouse enclosure (inverted wire cup, Galaxy Cup, Kitchen Plus, http://www.kitchen-plus.com); direction of the head, body and tail defined sniffing the novel object or novel mouse. A top-mounted infrared sensitive camera (Ikegami ICD-49, B&H Photo, New York, NY) was positioned directly above every two units. Infrared lighting (Nightvisionexperts.com) provided uniform, low-level illumination. The subject mouse was first enclosed in the center chamber for 5 minutes, then allowed to explore all three empty chambers for 10 minutes, then explored the three chambers containing a novel object in one side chamber and a novel mouse in the other side chamber. Novel stimulus mice were 129Sv/lmJ, a relatively inactive strain, aged 10–14 weeks old, and were matched the subject mice by sex. Stimulus mice were habituated as previously described[50]. Number of entries into the side chambers served as a within-task control for levels of general exploratory locomotion.

Male-female social interactions with USV—The male-female social interaction test was conducted as previously described[32, 47, 49]. Adult MAR-ASD and control males were group housed and sexually naïve at time of testing. Each freely moving experimental male mouse was paired with a freely moving, unfamiliar age-matched C57BL/6J female in pro-estrus or estrus. Visual observations of vaginal swelling and color were used to determine estrous state, using only those females in pro-estrus or estrus (vaginal swelling and color, open vagina with pink or reddish pink surrounding tissue) as partner mice. A 5-minute testing session was conducted in a sound-attenuating chamber under red light illumination (~10 lux). An USV microphone (Avisoft Bioacoustics, Berlin, Germany) was additionally mounted 20 cm above the testing arena and contained within the chamber; sampling frequency for the microphone was 250 kHz, and the resolution was 16 b. Bout frequency and duration of social behaviors were later scored from videos using Noldus Observer software (Noldus Observer 8.0XT; Noldus, Leeshurg, VA). USV spectrograms were displayed using Avisoft software. Numbers of vocalizations were quantified manually from coded audio files, by a trained investigator blind to treatment group.

Neurological and physical health screen—Measures of general health and neurological reflexes were measured in adult male and female mice as previously described[32]. General health was assessed by fur condition, whisker condition, body and limb tone, and body weight; muscle strength in mice was measured with a wire hang test. Neurological reflexes were assessed by righting reflex, whisker twitch, pinnae response, eyeblink response, and forepaw reaching. Behavioral reactivity was evaluated as intensity of dowel biting, and level of audible distress calls when handled.

Repetitive self-grooming—Spontaneous repetitive self-grooming behavior was scored as previously described[32, 34, 48]. Mice were individually placed in a clean, empty standard mouse cage without bedding (46 × 23.5 × 20 x cm^3). The room was illuminated at ~30 lux, and a front-mounted CCTV camera (Security Cameras Direct) was placed at approximately 1 meter from the cages to record the 20-minute testing sessions. Each mouse was first given a 10-minute (unscored) habituation period in the empty cage, then was scored for cumulative time spent grooming all body regions during the second 10 minutes of the test session. Scoring was performed from coded videos by a trained investigator, blind to treatment group.

Marble burying—Subjects were evaluated for repetitive digging behaviors using the marble burying task as previously described[45, 48]. Mice were individually placed in a standard mouse cage filled with 2 cm of bedding, in which 20 black glass marbles were arranged in a 4×5 grid on top of the bedding, under low light conditions (~30 lux). At the end of 30 minutes, the number of marbles buried (>50% covered by bedding) was counted as a measure of repetitive behavior.

Morris water maze acquisition and reversal—Spatial learning and reversal learning were assessed in the Morris water maze using procedures and equipment as previously described[32, 37]. The apparatus was a circular pool (120 cm diameter) filled 45 cm deep with tap water rendered opaque with the addition of non-toxic white paint (Crayola, Easton, PA). Distal room cues were black and white cardboard patterns on the walls, approximately 1 m from the circumference of the pool. Trials were videotaped and scored using EthoVision XT videotracking software (Noldus Information Technologies, Leesburg, VA). Acquisition training consisted of 4 trials a day for 5 days. Each training trial began by lowering the mouse into the water close to the pool edge, in a quadrant that was either right of, left of, or opposite to the target quadrant containing the platform. The start location for each trial was alternated in a semi-random order for each mouse. The hidden platform remained the same quadrant for all trials during acquisition training for a given mouse but varied across subject mice. Mice were allowed a maximum of 60 s to reach the platform; mice that failed to reach the platform in 60 s were subsequently guided to the platform by the experimenter using a wire cage lid. Mice were left on the platform for 15 s before being removed. After each trial, the subject was placed in a cage lined with absorbent paper towels and allowed to rest under an infrared heating lamp for 60 s. Acquisition training continued until the control group reached the criterion of 15 s latency to find the hidden platform. Three hours after the completion of acquisition training (day 5), the platform was removed, and mice were tested in a 60 s probe trial in order to confirm that their spatial training was acquired by using distal environmental room cues. Parameters recorded during training days were latency to reach the platform, total distance traveled, and swim speed. Time spent in each quadrant and number of crossings over the trained platform location and over analogous locations was used to analyze probe trial performance. Reversal training began 3 days after the completion of acquisition training. In reversal training trials, the hidden platform was moved to the quadrant opposite to its location during acquisition training. Procedures for reversal training and probe trial were the same as in the initial acquisition phase.

Statistical Analyses

Statistical analyses were performed using SPSS software (SPSS Version 23; IBM Corp., Armonk, NY); *p*-values < 0.05 for two-tailed tests were considered statistically significant. Data graphs were created using GraphPad Prism (Version 6; GraphPad Software Inc., La Jolla, CA); all results are presented as mean ± SEM, using statistical tests previously described[32, 36, 48]. All data were first assessed for the detection of outliers by inspection of boxplots, with outliers defined as greater than 3 box-lengths from the edge of the box in a boxplot; unless otherwise noted, no outliers were detected. Analysis of variance (ANOVA) was used to analyze juvenile reciprocal social interactions, elevated-plus maze, light↔dark exploration, repetitive self-grooming, and marble bury behaviors, as well as adult neurological and physical health measures and developmental milestones that occurred only on PD 14 (between-subject factors: treatment and offspring sex). The ANOVAs with repeated measurements (between-subject factors: treatment and offspring sex; within-subject factor: time) were used to estimate the effect of time, sex, and treatment on open field activity, Morris water maze learning, call rate for pup isolation calls, and for most of the developmental milestones. Sidak post hoc analysis was conducted to compare individual groups in cases of a significant ANOVA value. Student's unpaired *t* tests were used to compare across treatments on breeding parameters, on parameters of male-female reciprocal social interactions (MFSI), and to compare time spent sniffing novel and familiar objects in the 3-chambered social approach task.

RESULTS

Verification of MAR ASD-specific autoantibodies

In sexually naïve females immunized with the MAR ASD peptides, autoantibodies reactive against all of the targeted peptide epitope sequences were successfully produced in MAR-ASD treated females prior to breeding (**Figures 2A-D**) (Supplemental Table 4). Furthermore, these endogenously produced maternal autoantibodies persisted to circulate at detectable-levels in dams post-parturition and during near the end of lactation (**Figures 2E-H**) (Supplemental Table 5). Maternal reactivity to the intact proteins can be viewed in Supplemental Table 6.

Breeding parameters

While there were no differences in the absolute number of male pups born per litter between treatment groups ($t(21) = 0.918$, $p = 0.369$), MAR-ASD litters were found to produce significantly more female offspring than observed in control litters ($t(21) = -2.204$, $p = 0.039$). This was additionally confirmed in comparing the percentage of average female pups per litter ($t(21) = -2.058$, $p = 0.052$), with a non-significant trend for MAR-ASD litters to produce more females than produced in control litters (MAR-ASD mean ± SE = 58.93% ± 5.18%; control mean ± SE = 43.66% ± 5.31%). There was no significant difference between MAR-ASD and control litters in measuring average litter size ($t(21) = -1.249$, $p = 0.225$).

Developmental milestones and neurological reflexes

Markers of early physical development and neurological reflexes were assessed in neonatal offspring, revealing differences between MAR-ASD and control offspring in a variety of parameters measured. A significant main effect of treatment was observed in assessing body weight ($F_{1,35} = 7.627$, $p = 0.009$), with MAR-ASD offspring weighing significantly more than control offspring at all time points ($p < 0.05$) (Figure 3A). Furthermore, significant treatment × time interactions were observed for several of the physical and behavioral parameters measured. Most notably, the biparietal width (head width) of MAR-ASD offspring was found to be significantly larger relative to control offspring on PDs 10 and 12 (treatment × time interaction: $F_{5,175} = 5.853$, $p < 0.001$; Sidak-adjusted post-hoc tests: PD 10, $p = 0.037$; PD 12, $p = 0.003$) (Figure 3B). Additional measures of physical development with significant treatment × time interactions include pinnae detachment ($F_{5,175} = 8.958$, $p < 0.001$; MAR-ASD > Control on PD 8 and 12) and fur development ($F_{5,175} = 6.753$, $p < 0.001$; MAR-ASD < Control on PD 12)(Supplemental Tables 7 **and** 8), although these differences were less pronounced than the observed disparity in pup head width (biparietal diameter). Measures of behavioral development with significant time-dependent treatment effects included righting reflex ($F_{5,175} = 3.197$, $p = 0.009$; MAR-ASD offspring faster than controls on PD 6), cliff avoidance ($F_{5,175} = 2.377$, $p = 0.041$; MAR-ASD > Controls on PDs 8 and 10), and forelimb strength during vertical screen climbing ($F_{5,175} = 2.811$, $p = 0.018$; MAR-ASD > Controls on PD 10). A non-significant trend was additionally observed in measuring auditory startle responses on PD 14 ($F_{1,41} = 3.844$, $p = 0.057$), with MAR-ASD startling marginally more than control offspring. Finally, interactions of treatment × time × sex were found significant for measures of body temperature ($F_{5,175} = 2.405$, $p = 0.039$) and negative geotaxis behavior ($F_{5,175} = 2.515$, $p = 0.032$) (Supplemental Tables 7 **and** 8).

Pup USVs

In assessing the number of USVs emitted by neonatal offspring, a significant treatment × sex interaction was detected ($F_{1,50} = 4.662$, $p = 0.036$), with MAR-ASD females emitting significantly more USVs than control females (Sidak-adjusted p-value = 0.013) while MAR-ASD and control males emitted a similar number of USVs on average (Sidak-adjusted p-value = 0.610) (Figure 3C). Furthermore, a treatment × time interaction approached significance ($F_{4,200} = 2.289$, $p = 0.061$), with MAR-ASD offspring emitting significantly more USVs on PD 10 relative to controls (Sidak-adjusted p-value = 0.005) (Figure 3D). As expected, a significant main effect of day was observed ($F_{4,200} = 17.167$, $p < 0.001$), with animals emitting more calls on PDs 6 and 8 relative to PDs 4, 10, and 12. No other interactions or main effects were found to approach significance ($p > 0.15$).

Juvenile reciprocal social interactions

In assessing reciprocal social interactions in same-sex juvenile dyads, a robust behavioral profile highly relevant to ASD was observed in MAR-ASD offspring relative to controls. Specifically, MAR-ASD mice engaged in significantly fewer bouts of nose-nose sniffing ($F_{1,42} = 5.758$, $p = 0.021$), nose-anogenital sniffing ($F_{1,42} = 5.113$, $p = 0.029$), push-crawl play behavior ($F_{1,42} = 17.656$, $p < 0.001$), front approach behavior ($F_{1,42} = 24.727$, $p < 0.001$), and following behavior ($F_{1,42} = 4.372$, $p = 0.043$), indicating lower levels of social

behaviors relative to controls. Prenatal exposure to the salient maternal autoantibodies was also found to influence repetitive self-grooming behaviors in exposed mice, as MAR-ASD juvenile mice engaged in significantly more repetitive self-grooming bouts compared to controls ($F_{1,42} = 128.477$, $p < 0.001$). There was no significant effect of treatment on total arena exploration ($F_{1,42} = 0.773$, $p = 0.384$). There were no significant effects of offspring sex or interactions of treatment × sex for any of the parameters measured ($p > 0.10$) (**Figure 4;** Supplementary Video 1).

Elevated-plus maze

There were no significant differences in the percentage of time spent in the open arms ($F_{1,42} = 0.071$, $p = 0.791$), number of open arm entries ($F_{1,42} = 1.789$, $p = 0.188$, or in the number of total entries ($F_{1,42} = 2.959$, $p = 0.093$) in MAR-ASD offspring relative to controls (Supplemental Figures 1A-C).

Light↔Dark exploration

There were no significant differences across treatment groups in the latency to enter the dark chamber ($F_{1,42} = 0.129$, $p = 0.721$), time spent in the dark chamber ($F_{1,42} = 0.915$, $p = 0.344$), or number of transitions between chambers ($F_{1,42} = 0.230$, $p = 0.634$). However, a significant main effect of sex was observed for the number of transitions ($F_{1,42} = 6.886$, $p = 0.012$), with females transitioning between the chambers more frequently than males. No other significant main effects or interactions were observed ($p > 0.15$) (Supplemental Figures 2A-D).

Open field

Measures of open field locomotion were normal for MAR-ASD relative to controls in most cases. While a significant time × treatment interaction was detected in assessing both distance traveled ($F_{5,210} = 2.819$, $p = 0.017$) and horizontal activity ($F_{5,210} = 3.821$, $p = 0.002$), Sidak-adjusted post-hoc analyses revealed MAR-ASD animals engaged in similar exploration as controls except during the first 5 minutes of the task (Distance traveled, $p = 0.057$; Horizontal activity, $p = 0.040$). This seems to be driven in part by a significant time × sex interactions (Distance traveled: $F_{5,210} = 6.581$, $p < 0.001$; Horizontal activity: $F_{5,210} = 9.721$, $p < 0.001$; Vertical Activity: $F_{5,210} = 9.308$, $p < 0.001$), as males consistently explored less than females during the first 5 minutes of the 30-minute task (Supplemental Figures 3A-F). No other effects of or interactions with treatment were observed as significant.

Three-chambered social approach

Typical sociability, defined as spending significantly more time in the chamber containing the novel mouse than time in the chamber containing the novel object, was seen in both treatments and both sexes (Control males: $F_{1,10} = 27.301$, $p < 0.001$; Control females: $F_{1,10} = 19.308$, $p < 0.001$; MAR-ASD males: $F_{1,11} = 38.064$, $p < 0.001$; MAR-ASD females: $F_{1,11} = 8.414$, $p = 0.014$)(Supplemental Figure 4A). Normal sociability was also found for time spent sniffing the novel stranger versus time spent sniffing the novel object (Control males: $F_{1,10} = 76.179$, $p < 0.001$; Control females: $F_{1,10} = 15.137$, $p = 0.003$; MAR-ASD males:

$F_{1,11} = 52.708$, $p < 0.001$), although the observed difference in MAR-ASD females was reflected in a non-significant trend (MAR-ASD females: $F_{1,11} = 4.070$, $p = 0.069$) (Supplemental Figure 4B).

While no differences were observed across treatments or sex for the total number of entries to either chamber during the sociability trial (Treatment: $F_{1,42} = 1.098$, $p = 0.301$; Sex: $F_{1,42} = 2.291$, $p = 0.138$; Treatment × Sex: $F_{1,42} = 0.018$, $p = 0.894$), MAR-ASD mice did not enter one chamber more frequently than the other (MAR-ASD males: $F_{1,11} = 3.167$, $p = 0.103$; MAR-ASD females: $F_{1,11} = 2.200$, $p = 0.166$)(Supplemental Figure 4C). Control animals, however, entered the chamber containing the novel mouse significantly more frequently than entering the chamber containing the novel object (Control males: $F_{1,10} = 26.783$, $p < 0.001$; Control females: $F_{1,10} = 7.042$, $p = 0.024$). No significant main effects or interactions observed in distance traveled during social approach testing ($p > 0.05$) (Supplemental Figure 4D). There were similarly no observed main effects or interactions observed in assessing both chamber time and chamber entries during the habituation session preceding social approach testing ($p > 0.05$)(Supplemental Figures 4E-F).

Male-female social interactions and ultrasonic vocalizations

Relative to control males, adult MAR-ASD males displayed deficits on several parameters measured during reciprocal social interactions with an unfamiliar stimulus female in estrus. Prenatal exposure to the salient maternal autoantibodies produced significant reductions in the number of bouts of front approach ($t(21) = 2.586$, $p = 0.017$), duration of nose-to-anogenital sniffing (Duration: $t(21) = 2.140$, $p = 0.044$; Bouts: $t(21) = 1.829$, $p = 0.082$), duration and number of bouts of nose-to-body social sniffing (Duration: $t(21) = 2.173$, $p = 0.041$; Bouts: $t(21) = 2.890$, $p = 0.009$), and duration and number of bouts of following (Duration: $t(21) = 3.969$, $p < 0.001$; Bouts: $t(21) = 2.760$, $p = 0.012$) in adult MAR-ASD males. MAR-ASD males additionally emitted significantly fewer total USVs over the 5-minute test session (main effect of treatment: $F_{1,20} = 13.459$, $p = 0.0015$) compared to adult control males. A robust treatment effect was additionally observed in assessing repetitive self-grooming behaviors, as the duration and number of bouts spent grooming was significantly higher in MAR-ASD males relative to controls (Duration: $t(21) = -3.113$, $p = 0.0053$; Bouts: $t(21) = -5.905$, $p < 0.0001$). Prenatal maternal autoantibody exposure has no significant effect on the duration or number of bouts of either nose-to-nose sniffing (Duration: $t(21) = 0.104$, $p = 0.918$; Bouts: $t(21) = 1.357$, $p = 0.189$) or total arena exploration (Duration: $t(21) = -1.234$, $p = 0.231$; Bouts: $t(21) = -1.618$, $p = 0.1205$) in adult male mice relative to controls (**Figures 5A-H;** Supplemental Table 9; Supplementary Video 2).

Neurological and physical health screen

Adult male and female offspring were evaluated on measures of general health and neurological reflexes. MAR-ASD and control mice scored similarly on measures of fur, body tone, limb tone, lack of physical abnormalities, missing whiskers, presence of auditory startle, righting reflex, whisker twitch response, ear twitch response, corneal reflex, forepaw reaching, wire hanging, and vocalization. A treatment effect was observed in assessing the amount of dowel biting ($X^2(2) = 6.409$, $p = 0.041$), with MAR-ASD offspring engaging in

less dowel biting behaviors compared to controls. This effect was not evident in assessing male offspring ($X^2(2)=3.494$, $p = 0.174$), but was evident when assessing females only ($X^2(1)=5.282$, $p = 0.022$). As anticipated, a significant sex effect was observed in measuring body weight in adult mice ($F_{1,42} = 87.685$, $p < 0.001$). No other treatment or sex effects reached significance.

Repetitive self-grooming behaviors

A total of 3 outliers, defined as being greater than 3 box-lengths from the edge of the box in a boxplot, were identified and subsequently excluded from the following analysis of empty cage behaviors (1 control male, 1 control female, and 1 MAR-ASD male). Within the remaining 43 animals, MAR-ASD offspring were found to engage in repetitive self-grooming behaviors for a significantly more time than control mice ($F_{1,39} = 7.075$, $p = 0.011$). There was no effect of sex ($F_{1,39} = 2.790$, $p = 0.103$), or treatment × sex interaction ($F_{1,39} = 0.202$, $p = 0.656$), on repetitive self-grooming behaviors in the empty cage (Supplemental Figure 5).

Marble burying

The total number of marbles buried, reflective of repetitive digging behaviors, was not significantly different between MAR-ASD and control animals ($F_{1,42} = 0.015$, $p = 0.902$) (Supplemental Figure 6A). A non-significant trend of offspring sex was observed, with males burying moderately more marbles than female mice during the 30-minute task ($F_{1,42} = 3.619$, $p = 0.064$; treatment × sex interaction: $F_{1,42} = 0.313$, $p = 0.579$) (Supplemental Figure 6B).

Morris water maze acquisition and reversal

Evaluation of spatial learning on the Morris water maze showed that both MAR-ASD and control males and females displayed similar learning curves during acquisition training. Mice performed normally, with similar latencies to reach the hidden platform (Treatment: $F_{1,42} < 0.001$, $p = 0.991$) and distance traveled to the platform (Treatment: $F_{1,42} = 0.698$, $p = 0.408$) during hidden platform learning. A significant effect of treatment was observed in assessing swim speed during acquisition training ($F_{1,42} = 7.146$, $p = 0.011$), with MAR-ASD mice swimming at a moderately slower speed relative to controls. Selective search of the trained quadrant of the pool was significant for both MAR-ASD and control mice ($p < 0.001$), indicating normal hippocampal-dependent learning using distal cues (Treatment: $F_{1,42}=0.521$, $p = 0.475$). Time spent in the target quadrant during the probe trial was also found to be similar between MAR-ASD and control offspring, with animals spending significantly more time in the target quadrant than all other quadrants ($p < 0.020$). Performance during reversal training was additionally similar between MAR-ASD and control mice, with comparable latencies to reach the hidden platform (Treatment: $F_{1,42} = 0.335$, $p = 0.566$), distance traveled to the platform (Treatment: $F_{1,42} = 1.199$, $p = 0.280$), and swim speeds (Treatment: $F_{1,42} = 1.135$, $p = 0.293$). In the probe trial conducted 3 hours after the last reversal training trial, both MAR-ASD and control animals displayed significant selective quadrant search, spending significantly more time in the new training quadrant than time in any of the three other quadrants ($p < 0.025$)(Supplemental Figure 7).

DISCUSSION

ASD is highly heterogeneous neurodevelopmental disorder whose various etiologies remain under investigation. The identification of biological factors that provide indicators of risk as well potential pathological mechanisms are critical for achieving the future goal of more targeted and individualized therapies[51]. Therefore, the present study aimed to create the first endogenous preclinical model of MAR ASD to directly assess the pathological significance of ASD-specific maternal autoantibodies through the generation of disorder-relevant behaviors and altered developmental milestones in prenatally exposed offspring. Where previous studies employed a passive antibody approach, the current study administered specific peptide antigens to females prior to breeding to ensure a continuous exposure to the endogenously generated antibodies from conception through birth. Prenatally exposed MAR-ASD male and female offspring displayed a range of ASD-relevant behaviors throughout life, including aberrant social interactions between juvenile dyads and in adult males interacting with estrous females, higher repetitive self-grooming behaviors, reduced vocalizations emitted during social interactions, and significant alterations in developmental milestones including increased head size. Thus, we have successfully demonstrated for the first time that these ASD-specific maternal autoantibodies are directly related to behavioral changes in prenatally exposed offspring.

The assays employed in the current study expand upon those used in our prior passive IgG transfer studies[20, 23, 25, 26]. A comprehensive set of longitudinal mouse behavioral testing methods evaluated behaviors relevant to the core diagnostic and associated symptoms of ASD in both male and female offspring. In the social behavioral domain, significant decreases in reciprocal social interactions during dyadic play were observed in MAR-ASD male and female offspring relative to controls, both as juveniles (JRSI) and as adults (MFSI). However, normal social approach scores were observed in both control and MAR-ASD offspring during the three-chamber assay. The disparity between these findings is likely due to the binary yes-or-no outcome nature of the three-chambered social approach task, whereas dyadic play is a more sensitive fine-grained behavioral analysis tool[46]. Furthermore, MAR-ASD male offspring emitted significantly fewer vocalizations in response to social cues as adults (MFSI USVs) relative to controls, while MAR-ASD females emitted significantly more vocalizations in response to social isolation as pups (pup USVs). Of the behavioral findings observed from several human and animal studies of MAR ASD, the most robust and consistent finding is the marked presence of repetitive and stereotypical behaviors in MAR-ASD exposed offspring[28]. We found robust increases in repetitive self-grooming behaviors in male and female MAR-ASD offspring during dyadic play (JRSI and MFSI) and when placed in an empty cage. We did not observe any differences between treatment groups in the marble burying task; however, this finding contributes to the existing question in the behavior neuroscience field about whether marble burying in fact represents a repetitive behavior[44, 52].

While there were an extensive number of ASD-related behavioral findings, there were also several assays for which MAR-ASD male and female offspring behaved similarly to controls. For example, measures of general health and neurological reflexes were comparable between MAR-ASD and control adult mice. No anxiety-like phenotype was

detected in MAR-ASD male and female offspring, as determined via the elevated plus-maze and light↔dark exploration assays. Normal general exploratory locomotion in an open field were observed in MAR-ASD and control mice. The lack of any noticeable treatment effects on anxiety- or locomotor- relevant behaviors suggests that tasks in which treatment differences are observed are unlikely to be influenced by competing behavioral confounds, such as sedation or hyperactivity[44]. These findings correlate with what is noted clinically with MAR ASD. In addition, no cognitive deficits were detected in MAR-ASD offspring relative to controls during the Morris water maze task, which is supported by neurotypical cognitive abilities observed clinically in children with the MAR subtype of ASD[16, 28].

In assessing markers of early physical development and neurological reflexes in neonatal offspring, we observed several differences between MAR-ASD and control offspring indicative of an altered neurodevelopmental trajectory. Of these, the most striking was the increased head size of MAR-ASD male and female pups. This finding, which persisted after correcting for body size and weight, mirrors that of the clinical population. In particular, children with the MAR subtype of ASD have significantly increased cerebral volumes in comparison to not only TD control children, but children with non-MAR ASD[27]. Of additional interest, MAR-ASD litters produced significantly more females than control litters. While intriguing, further study is necessary to replicate and interpret this finding.

Other laboratories have described the presence of maternal autoantibodies associated with a diagnosis of ASD. For example, using a technical approach different than in our previous studies[7], Brimberg et al. examined maternal samples from the Simons Simplex Collection for reactivity against mouse tissue sections using immunofluorescence. They found that mothers of children with ASD were four times as likely to have circulating anti-brain antibodies, but the antibodies were not entirely specific to ASD[53]. This study led to the identification of contactin-associated protein-like 2 (CASPR2)-reactive maternal autoantibodies, and the subsequent mouse model of Caspr2-reactive antibody for risk for ASD with some behaviors reminiscent of ASD[24]. This work highlights the importance multiple investigators taking on this critical area of research while employing a variety of approaches.

While the etiology of maternal autoantibody generation in the mothers is unclear, studies that aim to gain an understanding of the pathogenic mechanisms are currently underway. One risk factor identified to be strongly associated with the presence of these ASD-specific maternal autoantibodies is a functional variant in the 5’ promoter region of the *MET* receptor tyrosine kinase gene[54]. This variant, rs1858830 or the ‘*MET* C allele,’ is a common G-to-C single-nucleotide polymorphism (SNP) that has independently been associated with increased risk of ASD[55–59]. Furthermore, the *MET* C allele was associated with decreased MET protein expression and decreased levels of interleukin-10 (IL-10) in activated peripheral blood mononuclear cells from these mothers[54]. A second identified risk factor of MAR ASD is metabolic conditions experienced during pregnancy[60]. In particular, maternal autoantibody prevalence was observed to be more prevalent amongst mothers diagnosed with hypertensive disorders, mothers diagnosed with diabetes (type 2 or gestational), and among those mothers who were moderately overweight (body mass index of 27.0 – 29.9) compared to healthy mothers[60]. Finally, in a distinct but similar study, Brimberg et al. found

that autoantibody-positive mothers of children with ASD were more likely to have an autoimmune disease such as rheumatoid arthritis and systemic lupus erythematous[9]. Thus, while the etiology of the maternal autoantibodies in humans remains unknown, through the generation of the current mouse model coupled with ongoing molecular and neuroanatomical studies in these animals, we will begin to understand the pathogenic mechanisms associated with MAR ASD.

Although our endogenous model supports a direct pathological association with one type of ASD, this is not reflective of all MAR risk for ASD, as we examined only one antigenic MAR ASD autoantibody pattern. Efforts to recapitulate our findings in a cross-species model are currently underway, as well as ongoing studies to create models for other significant autoantibody patterns of MAR ASD. Additionally, studies to examine whether the behavioral phenotype of the offspring is paralleled by ASD-relevant changes in terms of neuroanatomy and neuromorphology, as well as gene and protein expression within the developing and adult brain, are currently in progress. The establishment of endogenous, antigen-driven preclinical models is a critical step towards the advancement of this work to the clinical setting. To best ensure the translational success of these preclinical models, research is ongoing to further identify the behavioral phenotypes associated with each maternal autoantibody reactivity pattern using well-characterized clinical cohorts. Translational animal models provide the underpinnings for understanding the underlying mechanisms related to maternal autoantibodies in ASD, as well as the development of future therapeutic interventions for a subset of individuals with ASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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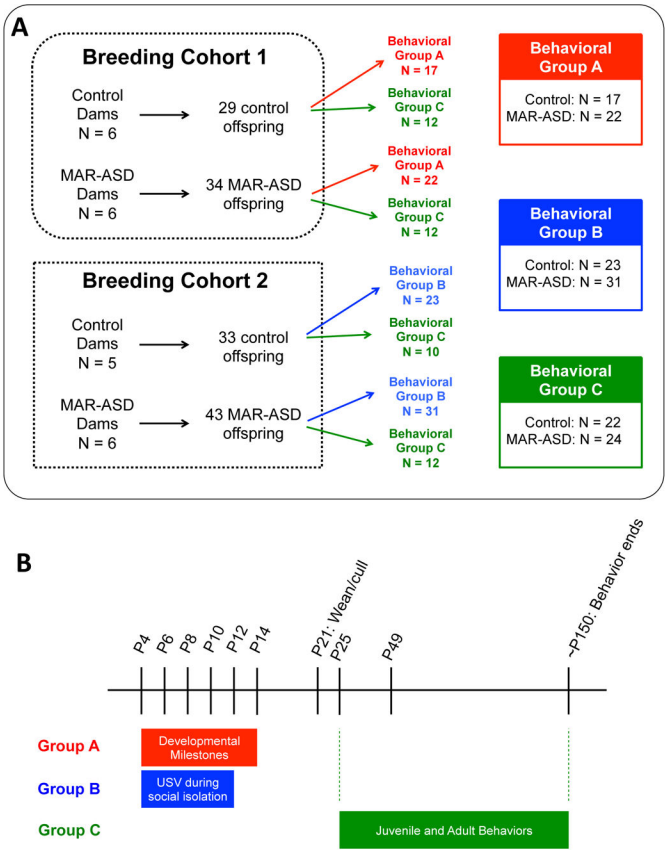

Figure 1. A depiction of the breeding strategies and behavioral testing timeline used in the present study.

A) Two cohorts of experimental dams were bred to produce the male and female experimental offspring tested in a comprehensive behavioral testing array. B) Behavioral testing timeline of the three separate groups of experimental MAR-ASD and control male and female offspring.

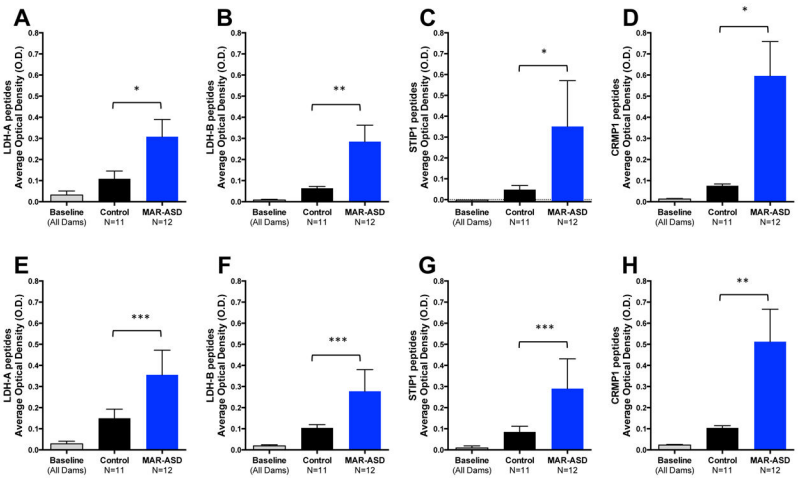

Figure 2. Production of endogenous maternal autoantibodies towards the targeted peptide epitope sequences.

Average maternal autoantibody reactivity towards epitope-specific peptide sequences at (A-D) pre-gestational and (E-H) post-parturition time points is illustrated. Average maternal reactivity towards peptides of LDH-A (A, E), LDH-B (B, F), STIP1 (C, G), and CRMP1 (D, H) are displayed (Mean ± SEM). *average peptide reactivity $p<0.05$, **$p<0.01$, ***$p<0.001$.

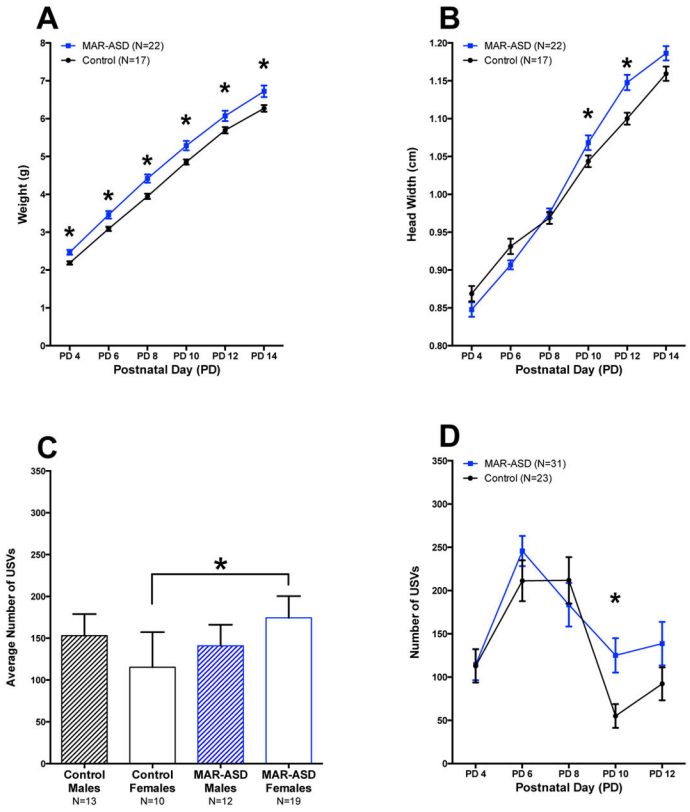

Figure 3: Developmental milestones and isolation-induced ultrasonic vocalization in pups.

Several measures of rodent developmental milestones were significantly different between MAR-ASD and control pups, such as increased body weight (A) and head width (B) in MAR-ASD male and female pups (controlled for total body weight). In measuring USVs emitted by pups in response to social isolation, MAR-ASD female pups emitted significantly more USVs than control females (C) and a non-significant treatment × time interaction trend revealed MAR-ASD male and female offspring emit significantly more USVs on PD 10 compared to control male and female pups (D). *p<0.05

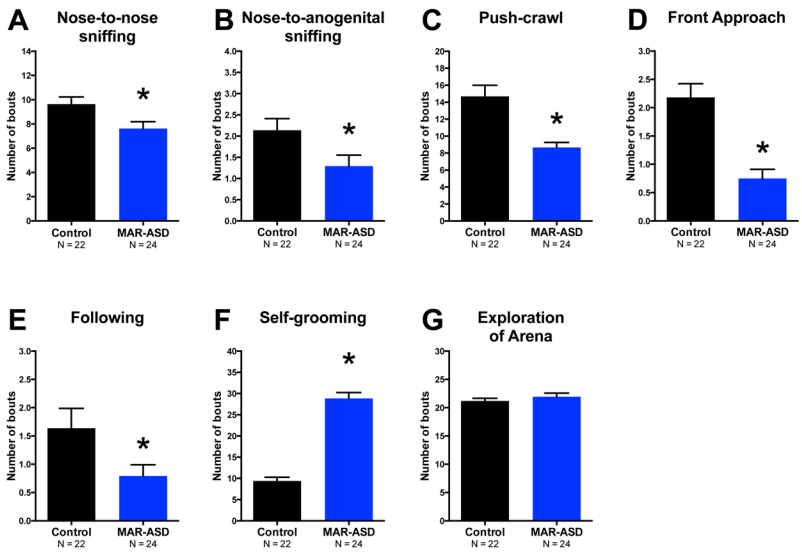

Figure 4. Juvenile reciprocal social interactions.

Male and female MAR-ASD offspring exhibited significantly less bouts of nose-nose sniff (A), nose-anogenital sniff (B), push-crawl (C), front approach (D), and following (E) during 10 minutes of dyadic play with an age- and sex- matched novel C57BL/6J stimulus partner mouse. MAR-ASD mice exhibited significantly higher levels of repetitive self-grooming behaviors (F) relative to controls but were similar to controls on exploration of the arena (G). *$p<0.05$

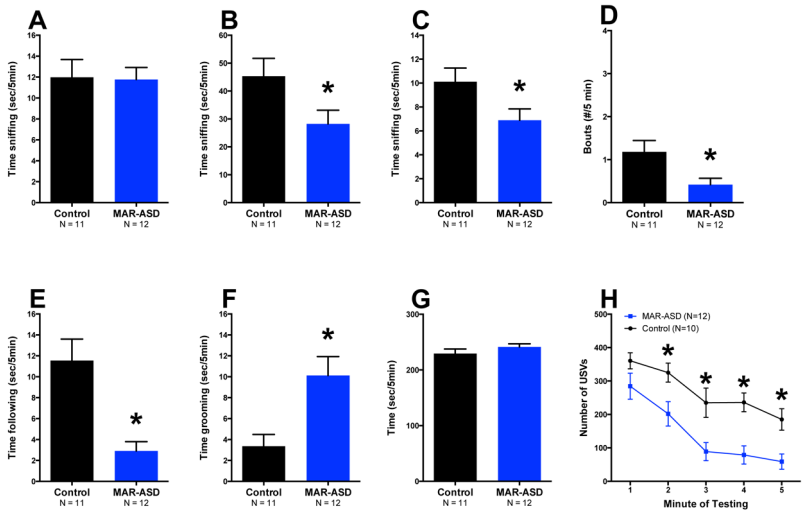

Figure 5. Male-female social interactions.

MAR-ASD adult males exhibited ASD-relevant behaviors during 5-min dyadic play with a novel female in estrus. While no significant difference was observed in nose-nose sniff (A), MAR-ASD adult males spent significantly less time engaging in nose-anogenital sniff (B), body sniff (C), front approach (D), and following social behaviors (E). MAR-ASD males also exhibited significantly higher levels of repetitive self-grooming behaviors (F) but were similar to controls in exploration of arena (G). Compared to control males, MAR-ASD adult males additionally emitted significantly fewer total USVs over the 5-min test session (H). *$p<0.05$