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Maternal and offspring methylenetetrahydrofolate-reductase genotypes interact in a mouse model to induce autism spectrum disorder-like behavior

Noa Sadigurschi[1,2] | Hava M. Golan[1,2]

[1]Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

[2]Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Correspondence
Hava M. Golan, PhD, Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Rager Ave, P.O. Box 653 Beer-Sheva 8410501, Israel.
Email: havag@bgu.ac.il

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Individuals with autism constitute a variable population whose members are spread along the autism spectrum. Subpopulations within that spectrum exhibit other conditions, such as anxiety, intellectual disabilities, hyperactivity and epilepsy, with different severities and co-occurrences. Among the genes associated with the increased risk for autism is the methylenetetrahydrofolate-reductase (*MTHFR*) 677C>T polymorphism, which impairs one-carbon (C1) metabolic pathway efficiency. The frequency of the *MTHFR*677TT homozygote is markedly higher among autism patients and their mothers than in the general population. Here, we report on the *Mthfr* heterozygous knockout (KO) mouse as a rodent model of autism that shows the contributions of maternal and offspring genotypes to the development of autistic-like behaviors. Maternal *Mthfr*-deficiency was associated with developmental delays in morphogenic features and sensory-motor reflexes in offspring. In the adult male mouse, behaviors representing core autism symptoms, such as repetitive behavior and restricted interest, were affected by maternal genotype while social behaviors were affected by both maternal and offspring genotypes. In females and males, behaviors associated with autism such as memory impairment, social aggression and anxiety were affected by both the maternal and offspring *Mthfr* genotypes, with sex-dependent differences. *Mthfr*-deficient male mice with observable impacts on behavior presented a particular laminar disturbance in parvalbumin interneuron density and innervation in superficial and deep layers of the cingulate cortex. This mouse model of autism will help to elucidate the molecular mechanisms that predispose a significant subgroup of autistic patients to abnormal development and to distinguish between the in-utero and autonomous factors involved in autism.

KEYWORDS

autism, developmental delay, in-utero, one-carbon metabolism, social behavior

1 | INTRODUCTION

Autism spectrum disorders (ASDs) are characterized by impaired social interactions, abnormal communication, restricted interest and stereotypic behavior.[1] In addition, the comorbidity of ASDs with other behavioral conditions such as intellectual disability, anxiety, epilepsy and hyperactivity is well documented.[2–8] The increasing incidence of ASD, which is currently 1:68 live births, and its variability between populations suggest multiple interactions between susceptibility

Abbreviations: ASDs, autism spectrum disorders; BS, body-sniffing; C1 metabolism, one-carbon metabolism; CO, crawl-over; CU, crawl-under; FA, folic acid; GABA, gamma-amino butyric acid; HT, heterozygote; KO, knockout; mPFC, medial prefrontal cortex; MTHFR, methylenetetrahydrofolate-reductase; NA, nose-to-anogenital; NH, nose-to-head; NN, nose-tip-to-nose-tip; P, postnatal day; PFA, paraformaldehyde; PV, parvalbumin; Wt, whisker-trimming; WT, wild-type.

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genes and environmental factors. Among the genes associated with the increased risk for ASD is methylenetetrahydrofolate reductase (*MTHFR*, Entrez Gene ID: 4524), whose activity strongly affects the C1 metabolic pathway. The activity of the enzyme, however, is depressed by the *MTHFR* 677C>T polymorphism (rs1801133), the genotype frequency of which is markedly higher among ASD patients and their mothers than in the general population (11% of non-autistic Caucasians in the United States),[9–17] see also meta-analysis by Rai.[18]

The importance of the C1 metabolic pathway to healthy human development has been shown in several studies. Support for an additive effect of C1 metabolism enzymes in the in-utero environment that promotes normal neurogenesis was shown in a large-scale case-control study, where the risk for ASD among newborns to mothers with the *MTHFR* 677TT genotype was increased by the presence of additional gene variants in C1 metabolism.[16] That study also suggested that the risk for ASD children among the same population of mothers is reduced when folic acid (FA) and prenatal vitamin supplements were taken periconceptionally and in the first trimester of pregnancy.[16,17] A meta-analysis performed by Pu et al. confirmed that the polymorphic allele *MTHFR*677T was more abundant in the ASD population compared with the general population,[15] and they also reported that FA fortification had a protective effect. In addition, regardless of genotype, a significant subgroup of children diagnosed with ASD exhibit low C1 metabolism activity compared with controls based on their C1 metabolite levels.[12,19–21] These differences may reflect the effects of genetic variations and/or of other factors, such as eating problems, which present in a large percentage of children with ASD.[22–24] We did not find evidence in the clinical literature for a similar connection between an autism-relevant phenotype and a paternal *MTHFR* polymorphism.

The neurological mechanisms suggested to underlie brain function in ASD involve a shift in the excitation-inhibition balance in neuronal circuits, in particular in cases where comorbidity with epilepsy occurs.[2,3,25] In line with this mechanism, a decrease in the size of the main GABAergic neuron population expressing parvalbumin (PV) was found in the medial prefrontal cortex (mPFC) of ASD individuals compared with those of controls.[26] This finding supports a restricted availability of gamma-amino butyric acid (GABA) in particular brain regions. In parallel with the progress that has been made in research of the GABA pathway in patients on the autistic spectrum, alterations in the GABA pathway were also described in genetic and induced murine models of autism. Failure to express PV was observed in a fraction of the interneuron populations in particular brain regions of several ASD mouse models.[27–32] Indeed, lack of PV was sufficient to induce autistic-like behavior.[27,33,34] Taken together, human and rodent data support a suppression of GABA signaling in ASD that involves GABA synthesis, levels and postsynaptic proteins and that engenders significantly impaired GABA transmission.

In the present study, we aimed to validate the *Mthfr*-KO mouse as a model of autism by using a comprehensive battery of tests to address the core domains of ASD-like behavior and associated conditions. Since a criterion for ASD is its early onset, we tested the effects of maternal and/or offspring *Mthfr* deficiency on developmental milestones during lactation and at prepubertal age. In addition, we evaluated the PV expressing interneuron population, which has a profound impact on cortical circuit processing and was implicated in ASD[26] and in rodent models of ASD.[27]

This study indeed validates the *heterozygote Mthfr*-KO mice as an ASD model. Affected mice present an ASD-like endophenotype, including developmental delay, core symptoms of ASD and associated impairment in behavioral domains such as memory and anxiety. Our findings also emphasize the critical dependence of the fetus in the maternal in-utero environment and the significant, immediate and long-lasting impacts of that environment on morphogenesis and on the core symptoms of ASD. This model can potentiate the search for the molecular pathway linking C1 metabolism and ASD and it can advance the development of treatment/preventive interventions targeted at populations with low C1 pathway activity.

2 | MATERIALS AND METHODS

2.1 | Mouse colony and study design

Mice on a Balb/cAnNCrlBR background were studied (mice kindly provided by Prof. Rima Rozen, McGill University, Montreal, QC, Canada).[35] The mouse colony was maintained in a 12:12 hour light/dark schedule, and food and water were provided ad libitum. All procedures were performed according to the guidelines of the Israeli Council on Animal Care and approved by the Animal Care and Use Committee of Ben-Gurion University of the Negev (protocol IL-16-07-14).

To assess the effect of maternal *Mthfr*+/− genotype vs offspring genotype, *Mthfr*+/+ (wild-type [WT]) and *Mthfr*+/− (heterozygote [HT]) female mice were mated with WT males to create three groups divided by genotype and maternal genotype as follows: WT offspring from WT mothers (WT:WT), WT offspring from *Mthfr*+/− mothers (HT:WT) and *Mthfr*+/− offspring from *Mthfr*+/− mothers (HT:HT). Male and female mice were tested separately, and female mice were tested regardless of their estrus cycle status. When two pups from the same litter were tested, they were analyzed as individual subjects. *Mthfr*−/− mice are not viable.

2.2 | *Mthfr* genotyping

Mothers and offspring were genotyped as described previously,[35] by using polymerase chain reaction amplification of DNA isolated from toe clips.

The primers used were as follows: sense primer 1 (5′-GAAGCA-GAGGGAAGGAGGCTTCAG-3′) in exon 3, sense primer 2 (5′-AGCCTGAAGAACGAGATCAGCAG C-3′) in the neo^r gene, and antisense primer 3 (5′-GACTA GCTGGCTATCCTCTCATCC-3′) in intron 3.

2.3 | Morphogenic and behavioral assessments

Offspring were tested at three different developmental stages representing critical periods of brain development: Neonates, postnatal day (P)4 to P17, at weaning, P30, and at the adult stage, P80 to P100.

To keep track of the pups' identities, beginning on P4, each mouse was marked daily with a nontoxic pen during testing sessions. At P10, pups were marked by toe clipping while under light anesthesia

with isoflurane. Litters were kept with the mother until weaning (P30), at which time they were grouped by sex.

2.3.1 | Developmental milestones

Pups were evaluated daily until P17 or when results in all the tests met the criteria for three consecutive days. During developmental evaluation, the mother was removed from the cage to avoid communication between dam and pups, while the newborns were left in the nest and were removed sequentially for tests. Following the tests, each pup was marked with a nontoxic pen, wiped lightly with nest material containing maternal scent, and then returned immediately to the nest.

2.3.2 | Morphogenic development

In addition to body weight, the evaluation included assessments of eyelid and ear canal opening, teething and fur growth, and for each of feature measured, the day and extent of appearance was recorded (0, 0.5 or 1 for no, partial or full appearance). The sensory-motor reflexes tested for included the following: Righting reflex was measured by the ability of a pup placed on its back to right itself on all four limbs in up to 30 seconds. Geotaxis is the ability to sense body orientation relative to the gravitational force. Healthy geotaxis behavior, which was tested by pup response when placed facing downward on a 45° slope included turning around and climbing upward in up to 60 seconds.[36] The rotarod performance test adjusted for neonates measured the ability of the pup to hold onto a rotating bar (one cycle/4 seconds) for a full cycle (360°).[37] The cliff avoidance test assessed the ability of the pup to sense a cliff and avoid walking forward by measuring the time required, in up to 60 seconds, for a pup placed on the edge of a 5-cm platform with its nose facing the edge to turn away from the edge.[37] The nest finding test measured the ability of the pup to find its way back to its nest after being placed at a distance of 5 cm from it. For all the sensory-motor reflexes tested, full accomplishment of the task was considered a success. The percent of pups that successfully accomplished each task was calculated.

2.4 | Prepubertal behavioral assessment

At P30, mice were tested for several behaviors associated with ASD, including nest building, marble burying and resident-intruder (as described below). The tests were performed in the order they are listed in the methods. Mice were separated into individual cages 2 days before the beginning of the tests and handled daily for 2 minutes by the experimenter.

2.5 | Behavioral tests of adult mice

At P80-100, mice were tested in behavioral tests selected to evaluate general ASD-like behaviors and behaviors associated with autism. A week before the experiments, the mice were placed in separate cages to avoid the effect of social hierarchy on their behavior. During that week, the mice were handled daily by the experimenter for 2 minutes to adapt them to the experimenter's presence. Each mouse underwent a single test daily between 12:00 and 18:00. Males and females were tested in separate sessions in all the behavioral tests. The testing arenas were cleaned between trials with 70% ethanol. Experiments were videotaped and analyzed offline. Open field, object preference and object recognition tests were analyzed by using "EthoVision 9" software (Noldus, the Netherlands). Sociability, social preference and social proximity tests were analyzed manually. In all cases, mice were tested in random order and coded with serial numbers to ensure that the experimenter was unable to distinguish between groups either during test sessions or subsequent analyses. The tests were performed in the order in which they are listed below.

2.5.1 | Open field

General behavior and exploration were tested in an open field arena. Mice were placed for 5 minutes in a circular arena that measured 55 cm in diameter and that had 20-cm high walls. To measure exploration, mobility and anxiety-related behaviors, distance moved was analyzed.[38] In addition, the time the mouse spent in the arena center (circle of radius 5.5 cm) and around the margin of the arena (11-cm wide ring adjacent to the arena wall) was measured. The ratio of the time the mouse spent in the center of the arena to the sum of the durations it spent in the arena's center and around its margins was calculated. Shorter times spent in the center of the arena may indicate higher anxiety.

2.5.2 | Nest building

Different features of the nest were used to assay animal welfare and repetitive behavior.[39,40] Prior to testing, mice were separated and placed in individual cages. Nest material comprised tissue paper folded into a fixed size of 5 × 7 cm and placed in each cage. The size and quality of the nests were measured 24 hours after the insertion of the bedding. Scores (0-3) were given for material processing, centralization and symmetry as follows: processing: 0—no changes in the material. 1—small portion of the material was cut, 2—half of the material was cut to pieces, 3—The entire material was processed into small pieces. Centralization: 0—no changes in the material. 1—minimal change observed in the nesting material, 2—poor nest was built, 3—the material was well organized to a round nest. Symmetry: 0—no changes in the material. 1—asymmetric nest, 2—partially symmetric nest, 3—symmetric nest. Nest quality was calculated by summing the scores for the processing, centralization and symmetry parameters (0-9). Nest volume was calculated by measuring length × width × height.

2.5.3 | Marble burying

Repetitive behavior was also evaluated by marble burying.[41] Each mouse was placed in a cage with 15 marbles organized in 5 × 3 rows for a duration of 10 minutes, at the end of which the mouse was removed from the cage. Each cage was photographed before mouse introduction into and after mouse removal from it. The number of buried marbles was counted.

2.5.4 | Object preference

To test for restricted interest, mice were evaluated for their preference for an object.[42] Mice were placed in an arena measuring 55 cm in diameter and with 20-cm high walls into which three, differently

shaped, hard shiny plastic objects (Lego-like blocks) (2 × 2 × 2 cm red square, 2 × 2 × 4 cm blue rectangle and 2 cm high × 2 cm diameter orange circle) were arranged at a fixed distance from each other. The times spent in proximity to and sniffing each object were recorded. The object that the mouse sniffed for the longest time was defined as its preferred object. The preference index was calculated as the time the mouse sniffed the preferred object (which was different for every mouse) out of the time it spent sniffing all the objects.

2.5.5 | Object recognition

Twenty-four hours after the object preference test, mice were tested in the same arena for object recognition. Two of the three plastic objects were identical to the ones used the day before (Objects 1 and 2). The third object (2 × 2 × 4 cm blue rectangle) was replaced with a novel object that was similar to the others in size but different in color (2 × 2 × 4 cm green rectangle). Recognition memory (based on percent of time) was calculated as the time the mouse spent sniffing the novel object vs the time it spent sniffing all objects.[43]

2.5.6 | Social behavior

Mouse social behavior was assessed by testing for social preference and social novelty preference. Social preference was examined in a three-chambered apparatus that comprised a rectangular arena divided into three, equally sized chambers measuring 40 × 20 × 22 cm.[44,45] Externally attached to either end of the apparatus was a small box (15 × 10 × 10 cm) to house the stimulus (stranger or familiar) mouse as described before.[38] On the first day of the test, an unfamiliar WT:WT adult mouse of the same sex, Stranger 1 (S1), was placed in the right-hand box while the other, identical box on the left-hand side of the apparatus was left empty (E) (Figure 2F). At the beginning of the test, the test subject mouse was allowed to habituate to the middle chamber for 10 minutes, after which the dividers were removed, and the test subject was allowed to freely explore all three chambers during a 10-minute test session.

Preference for social novelty was tested in the same arena 24 hours after the sociability test. The original stranger mouse (S1, now a familiar mouse) was in the same box of the apparatus and a novel WT:WT mouse of the same sex, Stranger 2 (S2), was placed in the box at the opposite end of the apparatus (Figure 2I). The number of entries by the subject mouse into each of the chambers, the total duration of time it spent in each chamber and the duration it spent sniffing each box were recorded.

2.5.7 | Resident-intruder

Aggressive social behavior was evaluated by the resident-intruder paradigm.[46,47] An intruder mouse was put in the home cage of the resident mouse, wherein the resident and intruder belonged to the same group (similar in sex, genotype and maternal genotype). Mice were recorded during a 15-minute session. The following behaviors were measured (duration and number of occurrences) as described previously[48] with some modification: nose-tip-to-nose-tip (NN); nose-to-head (NH); nose-to-anogenital (NA); body-sniffing (BS); crawl-under (CU); crawl-over (CO); upright (U); fighting (F); whisker-trimming (Wt); biting and chasing. The data presented include the total duration of each of the most frequent behaviors and the sums of those behaviors. The results are shown as the cumulative sum of the durations of all interactions at 5, 10 and 15 minutes.

2.5.8 | Olfactory function

To test olfactory function, the subject mouse was placed at one end of its home cage, and the time that it took the mouse to find some small, pea-sized croutons hidden in the cage bedding at the opposite end of the cage, where the mouse feces are located, was measured.

2.6 | Immuno-fluorescence analysis of brain tissue

Three-month-old adult male mice were anesthetized by inhalation of 30% isoflurane (Minrad Inc, New York) diluted in isopropanol (Fluka Chemie GmbH, Buchs, the Netherlands), after which they were transcardially perfused with paraformaldehyde (PFA) 4%. Brains were rapidly removed into a 4% PFA solution and stored at 4°C overnight. Each brain was then washed in phosphate buffer and transferred to a 10% sucrose solution for 2 hours or until it sank to the bottom of the solution. Afterwards, brains were transferred to a 30% sucrose solution for 24 hours at 4°C and then immersed in OCT compound embedding matrix (Tissue-Tek #4583) inside cryo standard plastic vinyl disposable molds (25 × 20 × 5 mm) placed on dry ice and stored at −80°C. The brains were sliced into 10-μm thick sagittal sections between the bregma lateral plane, 0.24 to 0.36 mm. PV expression was examined using the primary antibody, monoclonal mouse anti-PV (1:3000, Sigma-Aldrich, Saint Louis, Missouri, Cat # P 3088), and the goat anti-mouse Cy3 secondary antibody (1:600, Chemicon, Temecula, California, Cat # AP-124C). An Olympus fluorescent microscope IX-70 was used to examine the frontal cortex, cingulate cortex and hippocampus CA1 and CA3 regions. Images were captured using the PD73 Olympus CCD and CellSense software and hardware (Olympus) at a ×20 magnification. The analysis was performed using NIH Image J software by an experimenter blind to the tissue identity. For cingulate cortex analysis, cortical images from the pia to the ventricle were combined and divided into 10 bins of equal sizes for cell density and fluorescence optical density analyses. In each bin, the number of cells and the fluorescence optical density were analyzed to calculate cell density and innervation, respectively. Layers were defined as follows: Layer (L)1 (bin 1), L2 (bins 2-3), L3-4 (bins 4-6), L5 (bins 7-8), L6 (bins 9-10).

2.7 | Statistical analysis

Statistical analysis was performed using SPSS 23.0 software. Univariate two-way ANOVA analysis was used to test the effect of and interaction between the independent factors of genotype, maternal genotype and sex. Mice from each sex were then tested separately for an effect of genotype and maternal genotype. One-way ANOVA with a Bonferroni post-hoc test was used to compare the three different groups (WT:WT, HT:WT, HT:HT) and the times spent exploring the three different objects in the object preference tests. ANOVA for repeated measurement was used to test the effect and interaction between the independent factors of genotype, maternal genotype and sex, for which each variable was measured at different time points, for example, during development. Comparison of values

measured in the chambers of the social arena was assessed by the two-tailed paired Student's *t*-test for each group. Differences with *P*-values <0.05 were regarded as significant. Data are presented as means ± SEM.

3 | RESULTS

3.1 | Delayed development—contribution of maternal *Mthfr* genotype

Although autistic features are observed in early childhood in humans, researchers have invested significant effort in the identification of previously unrecognized characteristics that will enable autism diagnoses before the age of 2 years.[49–52] To capture possible developmental delay, therefore, we assessed pups for the appearance of several early developmental milestones. The distinct effects of the maternal vs the offspring *Mthfr* deficiency similarly influenced both female and male mouse physical and sensory-motor development during lactation. The results of these tests are therefore presented without reference to pup sex (Tables 1 and 2).

3.1.1 | Morphogenesis

The offspring of *Mthfr* +/− genotype mothers showed delayed weight gain compared with those of WT mothers ($F_{1,99}$ = 5.16, P = 0.025) (Table 1). Moreover, pups of *Mthfr*+/− mothers exhibited delayed morphogenesis, which was expressed as late eyelid opening, ear canal opening, and fur and teeth appearance relative to the pups of WT mothers ($F_{1,98}$ = 4.98, P = 0.028; $F_{1,98}$ = 16.21, P < 0.001; $F_{1,98}$ = 7.07, P = 0.009; $F_{1,98}$ = 21.79, P < 0.001, respectively). The observed delays, that is, one postnatal day, were found to be most prominent on the first day when more than 10% of WT:WT pups met the criteria for a given characteristic (ie, P7, P7-8, P5 and P5, respectively) (Figure 1A) and later when 90% of the WT:WT pups met the criteria for full eye and ear canal openings and the appearance of fine fur (P14, P13 and P7, respectively) (Table 1, for more details see also Table S1).

3.1.2 | Motor and sensory reflexes

As a whole, differences in reflex development were observed on the day when the majority of the WT:WT pups met the criteria for full performance of the reflex (Table 2 and an example in Figure 1B, for more details see also Table S2).

Motor reflexes were evaluated by using the righting reflex and the rotarod test. Maternal and offspring genotypes were strongly associated with reductions in the percentages of pups in whom the righting reflex functioned normally ($F_{1,99}$ = 11.6, P = 0.001 and $F_{1,97}$ = 6.57, P = 0.012, respectively). Pup performances in the neonatal rotarod test were insensitive to maternal and offspring genotypes (Table 2).

Sensory reflexes were evaluated by testing mice geotaxis, nest finding and cliff avoidance behaviors. Maternal and offspring genotypes were found to affect geotaxis ($F_{1,96}$ = 12.6, P = 0.001; $F_{1,96}$ = 8.5, P = 0.004, respectively), such that development of the reflex in the HT:WT group was delayed compared with the other two groups. Marked differences were observed on days P6 and P7, when 88% of the pups in the WT:WT group met the criteria for fully developed reflex. Similar effects were detected in nest finding ability (maternal genotype, $F_{1,104}$ = 8, P = 0.006, and offspring genotype, $F_{1,104}$ = 11.14, P = 0.001). Thus, development of nest finding ability was delayed in the HT:WT group compared with both the WT:WT and HT:HT groups (Table 2). Finally, pup ability to avoid a cliff was governed only by maternal genotype ($F_{1,98}$ = 38.9, P < 0.001).

3.2 | Early signs of ASD-like behavior in prepubertal mice

The early appearance of symptoms is central in the diagnosis of ASDs and to distinguish autism from other disorders in which some features overlap those of autism. In humans, autistic features can already be observed in various cases in toddlers. To look for the early onset of behavioral impairments, we tested 30-day old mice shortly after weaning and before sexual maturation. This developmental stage is characterized by high individual variability and dynamic behavioral changes that occur in the course of just days. Moreover, during this period sex hormone levels gradually increase until the mouse reaches sexual maturation around age P45. To capture the narrow time window of this period, we restricted this part of the study to a small set of tests that could be performed over the course of several days.

General well-being was assessed by evaluating nest building.[53] The maternal *Mthfr*+/− genotype was associated with reduced nest quality ($F_{1,96}$ = 8.74, P = 0.004), regardless of offspring genotype and sex (Figure 1C). No indication of change in repetitive behavior was obtained from analyses of either the nest material processing (data not shown) or the marble burying test results (Figure 1D). Socially aggressive behavior in the resident-intruder test was similar in the resident mice of all groups, while intruder behavior showed a sex × maternal genotype interaction ($F_{1,32}$ = 5.36, P = 0.027). All female groups presented similar behavior, whereas in males, the maternal and offspring *Mthfr*+/− genotypes were associated with increased and decreased numbers of interactions engaged in by the intruders ($F_{1,15}$ = 4.65, P = 0.048 and $F_{1,15}$ = 5.25, P = 0.037, respectively) (Figure 1E,F).

Altogether, both the *Mthfr*+/− and maternal genotypes were associated with altered offspring mice behavior at the prepubertal stage, wherein the main effect was observed in terms of general well-being and social interaction. We also found that the effect of sex was limited to mouse social behavior at the prepubertal age.

3.3 | Maternal and offspring MTHFR genotypes contribute to ASD-like behavior in adult mice

The core symptoms of ASD include restricted interest, repetitive patterns of behavior, communication deficits and social behavior impairments.[1] To assess maternal and offspring *Mthfr*+/− genotype involvement in ASD-like phenotypes in adult mice, we applied a set of behavioral tests that were previously validated as measures of the core symptom domains of autism.[54,55]

TABLE 1 Effects on early morphogenetic development by maternal MTHFR genotype

Test	Group	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Weight (g)	WT:WT (n = 41)	2.6 ± 0.0	3.1 ± 0.08	3.7 ± 0.09	4.2 ± 0.1	4.8 ± 0.12	5.4 ± 0.13	6 ± 0.14	6.5 ± 0.14	6.9 ± 0.15	7.4 ± 0.17	7.9 ± 0.18	8.3 ± 0.22
	HT:WT (n = 37)	2.4 ± 0.07	2.9 ± 0.07	3.4 ± 0.07	3.9 ± 0.09	4.5 ± 0.09	5 ± 0.1	5.5 ± 0.12	6 ± 0.13	6.5 ± 0.14	7 ± 0.15	7.3 ± 0.15	7.9 ± 0.15
	HT:HT (n = 27)	2.5 ± 0.08	3 ± 0.08	3.5 ± 0.09	4.1 ± 0.11	4.6 ± 0.1	5.1 ± 0.11	5.6 ± 0.12	6.1 ± 0.14	6.7 ± 0.12	7.3 ± 0.13	7.7 ± 0.16	8.3 ± 0.16
Weight statistics	Univariate analysis maternal genotype	$F_{1,99}$ = 3.8; P = 0.052	$F_{1,104}$ = 2.9; P = 0.087	**$F_{1,104}$ = 4.9; P = 0.028**	**$F_{1,104}$ = 5; P = 0.026**	$F_{1,104}$ = 3.1; P = 0.08	**$F_{1,104}$ = 6.3; P = 0.014**	**$F_{1,104}$ = 5.7; P = 0.019**	**$F_{1,104}$ = 6.4; P = 0.012**	**$F_{1,104}$ = 4.8; P = 0.031**	$F_{1,104}$ = 3.1; P = 0.078	**$F_{1,104}$ = 4; P = 0.046**	$F_{1,104}$ = 2.5; P = 0.11
	Repeated measure	Sex ($F_{1,99}$ = 0.26; P = 0.6), genotype ($F_{1,99}$ = 1.04; P = 0.3), **maternal genotype ($F_{1,99}$ = 5.16; P = 0.025)**											
Eye opening	WT:WT (n = 41)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.17 ± 0.03	0.36 ± 0.03	0.41 ± 0.03	0.51 ± 0.01	0.51 ± 0.01	0.51 ± 0.01	0.51 ± 0.01	0.89 ± 0.03	1 ± 0.0
	HT:WT (n = 37)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.01 ± 0.01	0.41 ± 0.03	0.44 ± 0.02	0.5 ± 0.0	0.5 ± 0.0	0.56 ± 0.02	0.56 ± 0.02	0.59 ± 0.03	0.98 ± 0.01
	HT:HT (n = 27)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.4 ± 0.03	0.4 ± 0.03	0.5 ± 0.0	0.5 ± 0.0	0.57 ± 0.03	0.57 ± 0.03	0.66 ± 0.04	0.98 ± 0.01
Eye opening statistics	Univariate analysis maternal genotype				**$F_{1,104}$ = 17; P < 0.001**	$F_{1,104}$ = 0.7; P = 0.39	$F_{1,104}$ = 0.05; P = 0.8	$F_{1,104}$ = 1.7; P = 0.19	$F_{1,104}$ = 1.7; P = 0.19	$F_{1,104}$ = 3.4; P = 0.067	$F_{1,104}$ = 3.4; P = 0.067	**$F_{1,104}$ = 36; P < 0.001**	$F_{1,104}$ = 0.9; P = 0.33
	Repeated measure	Sex ($F_{1,98}$ = 0.41; P = 0.41), genotype ($F_{1,98}$ = 0.22; P = 0.63), **maternal genotype ($F_{1,98}$ = 4.98; P = 0.028)**											
Ear canal opening	WT:WT (n = 41)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.11 ± 0.03	0.14 ± 0.03	0.2 ± 0.03	0.4 ± 0.03	0.45 ± 0.02	0.59 ± 0.03	0.9 ± 0.03	1 ± 0.0	1 ± 0.0
	HT:WT (n = 37)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.01 ± 0.01	0.24 ± 0.04	0.24 ± 0.04	0.35 ± 0.03	0.51 ± 0.04	0.52 ± 0.04	0.98 ± 0.01	1 ± 0.0
	HT:HT (n = 27)	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.01 ± 0.01	0.11 ± 0.04	0.2 ± 0.04	0.23 ± 0.04	0.38 ± 0.04	0.53 ± 0.04	0.63 ± 0.05	0.98 ± 0.01	1 ± 0.0
Ear canal opening statistics	Univariate analysis maternal genotype				**$F_{1,104}$ = 10; P = 0.001**	**$F_{1,104}$ = 9.15; P = 0.003**	$F_{1,104}$ = 0.52; P = 0.46	**$F_{1,104}$ = 9.14; P = 0.003**	**$F_{1,104}$ = 4.66; P = 0.003**	$F_{1,104}$ = 1.95; P = 0.16	**$F_{1,104}$ = 44.4; P = 0.001**	$F_{1,104}$ = 0.62; P = 0.43	
	Repeated measure	Sex ($F_{1,98}$ = 1.93; P = 0.16), genotype ($F_{1,98}$ = 0.015; P = 0.9), **maternal genotype ($F_{1,98}$ = 16.21, P < 0.001)**											
Teeth appearance	WT:WT (n = 41)	0 ± 0.0	0.24 ± 0.06	0.98 ± 0.02	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:WT (n = 37)	0 ± 0.0	0.03 ± 0.07	0.97 ± 0.02	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:HT (n = 27)	0 ± 0.0	0 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
Teeth appearance statistics	Univariate analysis maternal genotype		**$F_{1,103}$ = 9.3; P = 0.003**	$F_{1,103}$ = 0.1; P = 0.89									
	Repeated measure	Sex ($F_{1,98}$ = 0.4; P = 0.5), genotype ($F_{1,98}$ = 0.002; P = 0.96), **maternal genotype ($F_{1,98}$ = 7.07; P = 0.009)**											
Fur appearance	WT:WT (n = 41)	0 ± 0.0	0.07 ± 0.02	0.04 ± 0.02	0.46 ± 0.02	0.47 ± 0.01	0.91 ± 0.02	0.98 ± 0.01	0.98 ± 0.01	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:WT (n = 37)	0 ± 0.0	0 ± 0.0	0.02 ± 0.01	0.05 ± 0.02	0.5 ± 0.02	0.86 ± 0.03	0.97 ± 0.01	0.98 ± 0.01	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:HT (n = 27)	0 ± 0.0	0 ± 0.0	0.01 ± 0.01	0.05 ± 0.03	0.51 ± 0.04	0.92 ± 0.03	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
Fur appearance statistics	Univariate analysis maternal genotype		**$F_{1,104}$ = 7.4; P = 0.007**	$F_{1,104}$ = 0.62; P = 0.43	$F_{1,104}$ = 139; p<0.001	$F_{1,104}$ = 0.84; P = 0.35	$F_{1,104}$ = 1.06; P = 0.3	$F_{1,104}$ = 0.74; P = 0.38	$F_{1,104}$ = 0.07; P = 0.78	$F_{1,104}$ = 0.62; P = 0.43			
	Repeated measure	Sex ($F_{1,98}$ = 0.06; P = 0.8), genotype ($F_{1,98}$ = 0.8; P = 0.36), **maternal genotype ($F_{1,98}$ = 21.79, P < 0.001)**											

Early developmental milestones tested in pups daily from postnatal day (P)4 to P14 included eye opening, ear canal opening, fur and teeth appearance and were scored as follows: absent—0, half—0.5, full appearance—1. Mean ± SEM. Two way ANOVA (univariate test) results for maternal genotype effect and ANOVA for repeated measurements (repeated measures) are presented. Significant differences are highlighted in bold. Gram (g), postnatal day (P).

TABLE 2 Early reflex development

Test	Group (N)	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Righting	WT:WT(n = 41)	0.22 ± 0.06	0.37 ± 0.07	0.85 ± 0.05	0.95 ± 0.03	0.98 ± 0.02	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:WT(n = 37)	0.19 ± 0.06	0.51 ± 0.08	0.49 ± 0.08	0.86 ± 0.05	0.95 ± 0.03	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
	HT:HT (n = 27)	0.19 ± 0.07	0.48 ± 0.09	0.74 ± 0.08	0.92 ± 0.05	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0	1 ± 0.0
Righting statistics	Univariate analysis	(G)$F_{1,104}$ = 0.001; P = 0.9 (MG)$F_{1,104}$ = 0.017; P = 0.8	(G)$F_{1,104}$ = 0.2; P = 0.6 (MG)$F_{1,104}$ = 1.6; P = 0.1	**(G)$F_{1,104}$ = 6; P = 0.016** **(MG)$F_{1,104}$ = 14; P < 0.001**	(G)$F_{1,104}$ = 0.1; P = 0.6 (MG)$F_{1,104}$ = 1.4; P = 0.2	(G)$F_{1,104}$ = 1.4; P = 0.2 (MG)$F_{1,104}$ = 0.7; P = 0.3	(G)$F_{1,104}$ = 0.2; P = 0.6 (MG)$F_{1,104}$ = 1.6; P = 0.1				
	Repeat (P4-P9)	Sex ($F_{1,97}$ = 0.37; P = 0.57), **genotype ($F_{1,97}$ = 6.57; P = 0.012), maternal genotype ($F_{1,99}$ = 11.6, P = 0.001)**									
Rotarod	WT:WT (n = 41)			0.02 ± 0.02	0.17 ± 0.05	0.15 ± 0.05	0.34 ± 0.07	0.63 ± 0.07	0.85 ± 0.05	0.88 ± 0.05	0.95 ± 0.032
	HT:WT (n = 37)			0.03 ± 0.02	0.08 ± 0.04	0.16 ± 0.06	0.24 ± 0.07	0.68 ± 0.07	0.73 ± 0.07	0.86 ± 0.05	1 ± 0.0
	HT:HT (n = 27)			0.07 ± 0.05	0.11 ± 0.06	0.11 ± 0.06	0.26 ± 0.08	0.52 ± 0.09	0.78 ± 0.08	0.85 ± 0.07	0.93 ± 0.05
Rotarod statistics	Univariate analysis			(G)$F_{1,104}$ = 0.3; P = 0.5 (MG)$F_{1,104}$ = 0.3; P = 0.9	(G)$F_{1,104}$ = 0.1; P = 0.6 (MG)$F_{1,104}$ = 2.5; P = 0.1	(G)$F_{1,104}$ = 0.8; P = 0.3 (MG)$F_{1,104}$ = 0.2; P = 0.6	(G)$F_{1,104}$ = 0.2; P = 0.6 (MG)$F_{1,104}$ = 0.5; P = 0.4	(G)$F_{1,104}$ = 1.5; P = 0.2 (MG)$F_{1,104}$ = 0.3; P = 0.5	(G)$F_{1,104}$ = 0.1; P = 0.7 (MG)$F_{1,104}$ = 1.4; P = 0.2	(G)$F_{1,104}$ = 0.007; P = 0.9 (MG)$F_{1,104}$ = 0.05; P = 0.9	(G)$F_{1,104}$ = 3; P = 0.08 (MG) $F_{1,104}$ = 1.8; P = 0.1
	Repeat (P6-P13)	Sex ($F_{1,97}$ = 0.037; P = 0.84), genotype ($F_{1,97}$ = 1.15; P = 0.28), maternal genotype ($F_{1,99}$ = 0.29, P = 0.58)									
Geotaxis	WT:WT (n = 41)			0.88 ± 0.05	0.83 ± 0.05	0.29 ± 0.07	0.63 ± 0.07	0.95 ± 0.03	1 ± 0.0	0.98 ± 0.02	1 ± 0.0
	HT:WT (n = 37)			0.19 ± 0.06	0.32 ± 0.07	0.27 ± 0.07	0.89 ± 0.05	0.86 ± 0.05	0.97 ± 0.02	1 ± 0.0	0.97 ± 0.02
	HT:HT (n = 27)			0.85 ± 0.07	0.74 ± 0.08	0.33 ± 0.09	0.77 ± 0.08	0.81 ± 0.07	0.88 ± 0.06	1 ± 0.0	1 ± 0.0
Geotaxis statistics	Univariate analysis			**(G)$F_{1,104}$ = 44.5; P < 0.001** **(MG)$F_{1,104}$ = 72.1; P < 0.001**	**(G)$F_{1,104}$ = 13.3; P < 0.001** **(MG)$F_{1,104}$ = 25.4; P < 0.001**	(G)$F_{1,104}$ = 0.002; P = 0.96 (MG)$F_{1,104}$ = 0.1; P = 0.68	(G)$F_{1,104}$ = 0.2; P = 0.6 **(MG)$F_{1,104}$ = 8.2; P = 0.005**	(G)$F_{1,104}$ = 0.5; P = 0.4 (MG)$F_{1,104}$ = 1.1; P = 0.2	(G)$F_{1,104}$ = 1.1; P = 0.2 (MG)$F_{1,104}$ = 0.4; P = 0.4	(G)$F_{1,104}$ = 0.001; P = 1 (MG)$F_{1,104}$ = 1.7; P = 0.1	(G)$F_{1,104}$ = 0.9; P = 0.3 (MG)$F_{1,104}$ = 1.2; P = 0.2
	Repeat (P6-P13)	Sex ($F_{1,96}$ = 0.006; P = 0.94), **genotype ($F_{1,96}$ = 8.5; P = 0.004), maternal genotype ($F_{1,96}$ = 12.6; P = 0.001)**									
Cliff avoiding	WT:WT (n = 41)			0.95 ± 0.03	0.83 ± 0.05	0.15 ± 0.05	0.73 ± 0.07	0.9 ± 0.04	0.88 ± 0.05	0.93 ± 0.04	0.95 ± 0.03
	HT:WT (n = 37)			0.95 ± 0.03	0.86 ± 0.05	0.08 ± 0.04	0.89 ± 0.05	0.84 ± 0.06	0.92 ± 0.04	0.92 ± 0.04	0.95 ± 0.03
	HT:HT (n = 27)			0.89 ± 0.06	0.93 ± 0.05	0.11 ± 0.06	0.48 ± 0.09(##)	0.81 ± 0.07	1 ± 0.0	0.96 ± 0.03	0.89 ± 0.06
Cliff avoiding statistics	Univariate analysis			(G)$F_{1,104}$ = 0.18; P = 0.6 (MG)$F_{1,104}$ = 0.001; P = 0.9	(G)$F_{1,104}$ = 0.7; P = 0.4 (MG)$F_{1,104}$ = 0.4; P = 0.5	(G)$F_{1,104}$ = 0.001; P = 0.9 (MG)$F_{1,104}$ = 0.9; P = 0.3	**(G)$F_{1,104}$ = 14.9; P < 0.001** (MG)$F_{1,104}$ = 2.2; P = 0.13	(G)$F_{1,104}$ = 0.009; P = 0.9 (MG)$F_{1,104}$ = 0.5; P = 0.4	(G)$F_{1,104}$ = 1.2; P = 0.2 (MG)$F_{1,104}$ = 0.4; P = 0.4	(G)$F_{1,104}$ = 0.1; P = 0.7 (MG)$F_{1,104}$ = 0.09; P = 0.7	(G)$F_{1,104}$ = 0.1; P = 0.7 (MG) $F_{1,104}$ = 0.005; P = 0.9
	Repeat (P6-P13)	Sex ($F_{1,98}$ = 0.91; P = 0.34), genotype ($F_{1,98}$ = 1.8; P = 0.18), **maternal genotype ($F_{1,98}$ = 38.9, P < 0.001)**									

TABLE 2 (Continued)

Test	Group (N)	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13
Nest finding	WT:WT (n = 41)			0.07 ± 0.04	0.34 ± 0.22	0.07 ± 0.04	0.12 ± 0.05	0.39 ± 0.07	0.63 ± 0.07	0.9 ± 0.04	0.88 ± 0.05
	HT:WT (n = 37)			0.08 ± 0.04	0.32 ± 0.07	0.08 ± 0.04	0.35 ± 0.17	0.51 ± 0.08	0.32 ± 0.07	0.78 ± 0.06	0.86 ± 0.05
	HT:HT (n = 27)			0.04 ± 0.03	0.15 ± 0.07	0.07 ± 0.05	0.22 ± 0.08	0.69 ± 0.09	0.78 ± 0.08	0.74 ± 0.08	0.78 ± 0.08
Nest finding statistics	Univariate analysis			$(G)F_{1,104}$ = 0.7; P = 0.3 $(MG)F_{1,104}$ = 0.005; P = 0.9	$(G)F_{1,104}$ = 0.5; P = 0.4 $(MG)F_{1,104}$ = 0.1; P = 0.7	$(G)F_{1,104}$ = 0.1; P = 0.7 $(MG)F_{1,104}$ = 0.07; P = 0.7	$(G)F_{1,104}$ = 0.9; P = 0.3 $(MG)F_{1,104}$ = 2.2; P = 0.1	$(G)F_{1,104}$ = 3.1; P = 0.07 $(MG)F_{1,104}$ = 0.6; P = 0.4	**$(G)F_{1,104}$ = 11.14; P = 0.001** **$(MG)F_{1,104}$ = 8; P = 0.006**	$(G)F_{1,104}$ = 0.5; P = 0.4 $(MG)F_{1,104}$ = 1.7; P = 0.1	$(G)F_{1,104}$ = 0.3; P = 0.5 (MG) $F_{1,104}$ < 0.001; P = 0.9
	Repeat (P6-P13)	Sex ($F_{1,98}$ = 0.19; P = 0.6), genotype ($F_{1,98}$ = 0.25; P = 0.6), maternal genotype ($F_{1,98}$ = 0.002; P = 0.9)									

Sensory and motor reflex development tested in pups daily from postnatal day (P)4 to P14. Failure to perform or full performance of the task at the given time was scored 0 or 1, respectively. Mean ± SEM. Two way ANOVA (univariate test) results for genotype (G) and maternal genotype (MG) effects and ANOVA for repeated measurements (repeat) are presented. Significant differences are highlighted in bold.

3.3.1 | Repetitive behavior

Assessments for repetitive behavior began with the marble burying test.[41] The maternal and offspring *Mthfr*+/− genotypes had opposing effects in female groups, such that the former was associated with lower and the latter with higher numbers of buried marbles ($F_{1,36}$ = 6.53, P = 0.015; and $F_{1,36}$ = 4.52, P = 0.041). In contrast to the variation found in females, the marble burying behaviors of all male groups were similar (Figure 2A). No interactions between the fixed factors were detected.

Repetitive behavior was also evaluated based on mouse processing of the nest material.[55] Mouse sex was found to affect the extent to which they processed the nest material ($F_{1,77}$ = 8.93, P = 0.004), with all female groups showing similar levels of material processing while males with a maternal *Mthfr*+/− genotype background exhibited enhanced processing compared with WT males ($F_{1,29}$ = 2.43, P = 0.04) (Figure 2B).

3.3.2 | Restricted interest

When mouse interest in various unfamiliar objects was tested to measure possible restricted interest, we showed a sex × maternal genotype interaction ($F_{1,76}$ = 13, P = 0.001), such that in mice with a maternal *Mthfr*+/− genotype background, females showed no preference for an object whereas males did, as indicated by the greater durations of time they invested sniffing the preferred object compared with WT mice ($F_{1,38}$ = 7.64, P = 0.009; $F_{1,35}$ = 5.51, P = 0.02, respectively) (Figure 2C, see also Figure S1).

3.3.3 | Social behavior

Social behavior was assessed by using the sociability and social preference tests. In the sociability tests, females showed no preference when given the chance to explore a mouse vs an empty box. The males from both the WT:WT and HT:WT groups, in contrast, spent more time sniffing the mouse than the empty box (paired Student's *t*-test, P = 0.04 and P < 0.001, respectively). Lastly, the time spent sniffing each box by HT:HT mice of both sexes was unaffected by the presence of another mouse (Figure 2D,E). Careful evaluation of group variability showed higher within-group variability in the HT:HT mice compared with the WT:WT mice, as indicated by the larger coefficient of variance (64% and 52% for the mouse and for the empty box, respectively, in the HT:HT group, compared with 35% and 29% in the WT:WT group). No differences were found in the number of transitions between chambers.

In the preference for social novelty test, female mice of all groups showed no predilection for either the familiar or the novel mouse, indicating a lack of social novelty preference. Male mice of the WT:WT group exhibited greater interest in the novel mouse over the familiar mouse, indicated by the larger amounts of time the tested mice spent in the novel mouse chamber (paired Student's t-test, P = 0.007). Mice from the other groups, in contrast, showed no preference for either the novel or the familiar mouse (Figure 2G,H, see also Figure S2).

Insofar as mouse detection and identification of peers relies critically on healthy olfactory function, olfactory hyposensitivity may interfere with mouse ability to distinguish familiar from unfamiliar

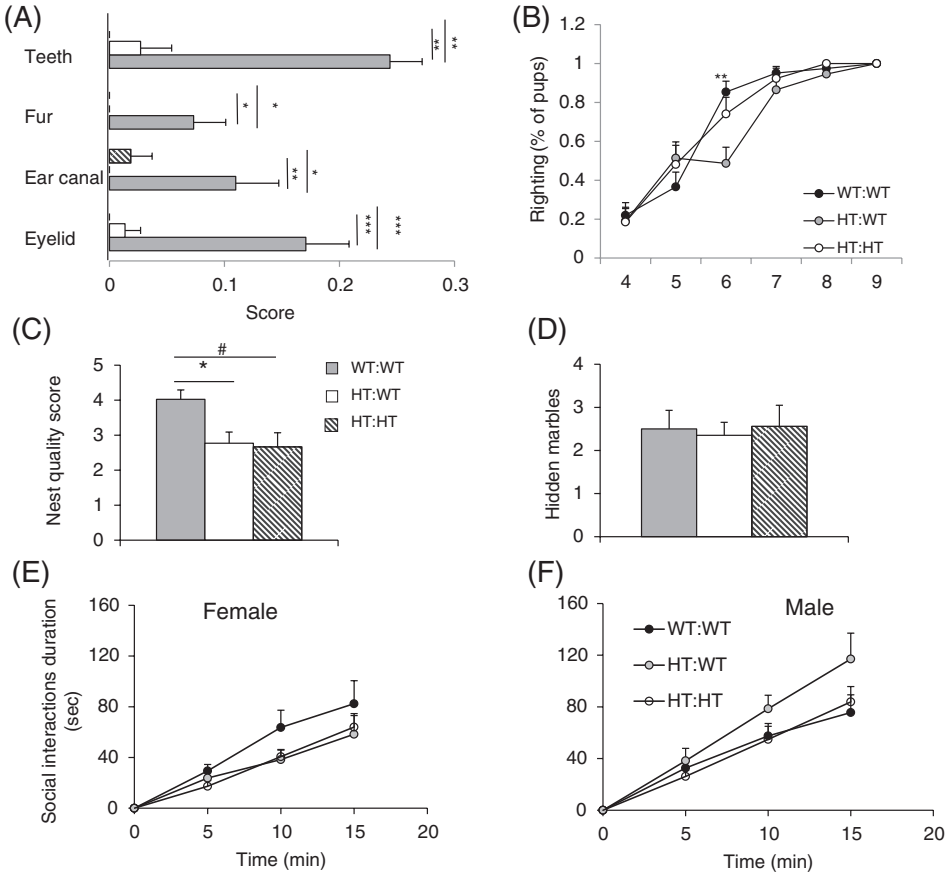

FIGURE 1 Early development and prepubertal behavior. Early developmental milestones tested in pups daily from P4 to P17 and prepubertal behavior tested at age P30. (A) *Morphogenesis*—day on which the majority of WT pups met criteria for tooth eruption, fur appearance, ear canal opening and eyelid opening. (B) *Righting reflex*—percent of pups performing the reflex. N of WT:WT = 41, HT:WT = 37, HT:HT = 27. (C-F) Prepubertal (P30) behavior: (C) *Nest building*—nest quality (score 0-9). (D) *Marble burying*—number of hidden marbles. N of WT:WT = 45, HT:WT = 45, HT:HT = 38. *Resident-intruder*—Total duration of all interactions initiated by intruder in the resident-intruder test (E) female and (F) male. N of females—WT:WT = 11, HT:WT = 11, HT:HT = 5. N of males—WT:WT = 6, HT:WT = 11, HT:HT = 9. WT:WT—black; HT:WT—gray; HT:HT—white. Mean ± SEM, one-way ANOVA with Bonferroni post-hoc test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between WT:WT and HT:WT, $^{\#}P < 0.05$ between WT:WT and HT:HT

mouse odor. Indeed, a lack of a deficiency in olfaction function was indicated and is in line with previous reports in *Mthfr*-deficient mice[38,56] (data not shown). Overall, the maternal and offspring *Mthfr* genotypes were associated with impaired social behavior in male mice.

3.4 | Behaviors associated with ASD: Effects of maternal and offspring genotypes

Several behavioral traits, among which are aggression, anxiety and hyperactivity, show comorbidity with ASD.[57] Because the *Mthfr* genotype was strongly associated with the core symptoms of autism, we tested *Mthfr*-deficient mice for the behaviors associated with those conditions.

To ensure that the tests would not erroneously indicate the presence of a broad impact, mouse general behavior and well-being were examined based on assessments of nest quality and on evaluations of distance moved in the open field arena. Both assessments showed that the different mouse groups exhibited similar behavior (Figure 3A,B).

3.4.1 | Anxiety

Exploration in the center of the open field arena was tested as a measure of anxiety. The frequency with which the mouse entered the center of the arena and the distance it walked while in the center showed sex × genotype interactions ($F_{1,73}$ = 8.21, P = 0.006; $F_{1,73}$ = 5.74, P = 0.019, respectively), where in females, but not in males, the *Mthfr* +/− genotype was associated with shorter distance walked in the center ($F_{1,35}$ = 5.54, P = 0.025). Anxiety was also expressed as the ratio of time the mouse spent in the center of the arena vs the time it spent in the center + margin, wherein a lower ratio may be indicative of higher anxiety. Sex × maternal genotype and sex × genotype interactions were found ($F_{1,67}$ = 6.02, P = 0.017; $F_{1,67}$ = 30.9, P < 0.001, respectively). The *Mthfr*+/− genotype was associated with increased anxiety (shorter times in the center of the arena) in females ($F_{1,34}$ = 9.17, P = 0.005). In males, the maternal *Mthfr*+/− genotype and the offspring *Mthfr*+/− genotype were associated with increased and decreased anxiety, respectively ($F_{1,32}$ = 8.9, P = 0.006, $F_{1,32}$ = 24.84, P < 0001, respectively) (Figure 3C).

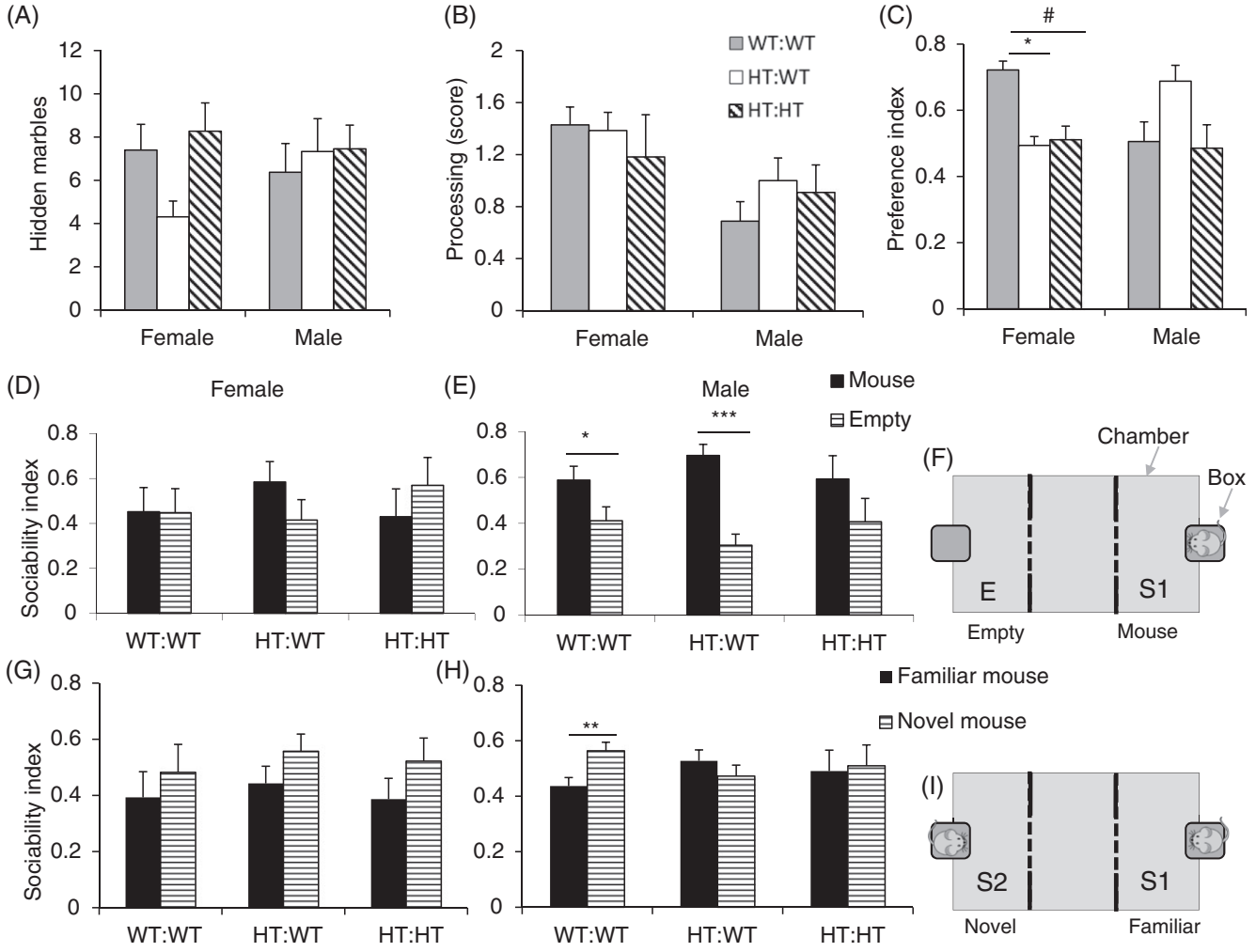

FIGURE 2 Core ASD symptoms (ASD-like behaviors) observed in adult mice (P80). (A) *Marble burying*—number of hidden marbles, (B) *Nest building*—level of nest material processing (score 0-3). (C) *Object preference*—Object preference index (time spent sniffing the preferred object/time spent sniffing all objects). WT:WT—gray; HT:WT—white; HT:HT—stripes. One-way ANOVA with Bonferroni post hoc test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between WT:WT and HT:WT, $^{\#}P < 0.05$ between WT:WT and HT:HT. (D and E) *Social preference*—percent of time spent sniffing the mouse box/percent of time spent sniffing both boxes (black), percent of time spent sniffing the empty box/percent of time spent sniffing both boxes (stripes) (D, female; E, male). (G and H) *Social novelty preference*—percent of time spent sniffing familiar mouse box/percent of time spent sniffing both boxes (black), percent of time spent sniffing novel mouse box/percent of time spent sniffing both boxes (stripes) (G, female; H, male). Schematic of the experimental setting in the social preference [F, empty (E), mouse (S1)] and social novelty preference [I, novel mouse (S2), familiar mouse (S1)] tests. N of females—WT:WT = 15, HT:WT = 14, HT:HT = 11; N of males—WT:WT = 16, HT:WT = 12, HT:HT = 11. Mean ± SEM, paired Student's *t*-test between two chambers, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

3.4.2 | Memory

Recognition memory was intact in females of the WT:WT and HT:WT groups, both of which showed greater interest, as indicated by the longer durations of time they spent sniffing the novel compared with the familiar objects (ANOVA $P = 0.007$, post hoc $P = 0.005$ between novel and familiar Object 1, ANOVA $P = 0.032$, post hoc $P = 0.027$ between novel and familiar Object 1, respectively). In contrast, female mice of the HT:HT group explored all objects similarly, indicating that the memory of previously seen objects was impaired in those mice (Figure 3D). In males, the WT:WT group spent more time sniffing the novel object than they did the familiar object (ANOVA $P < 0.001$ post hoc: $P < 0.001$ between novel object and familiar Object 2 and $P = 0.001$ between novel object and familiar Object 1), indicating that memory of the familiar objects is intact. A complete absence of recognition memory of the familiar object was found in males of the HT:WT and HT:HT groups (Figure 3E, see also Figure S3).

3.4.3 | Aggression

Aggressive behavior was assessed by the resident-intruder test.[47] The duration of the interaction initiated by the resident mouse was affected by sex, maternal genotype and genotype ($F_{1,27} = 5.23$, $P = 0.03$; $F_{1,27} = 11.01$, $P = 0.003$; $F_{1,27} = 10.84$, $P = 0.003$, respectively). In addition, sex × maternal genotype and sex × genotype interactions were found ($F_{1,27} = 7.72$, $P = 0.01$; $F_{1,27} = 5.07$, $P = 0.033$, respectively), such that the maternal and offspring *Mthfr*+/− genotypes were associated with increased and decreased durations of interactions in females ($F_{1,10} = 12.5$, $P = 0.005$; $F_{1,10} = 8.75$, $P = 0.014$, respectively). These tendencies, observed for all interaction categories

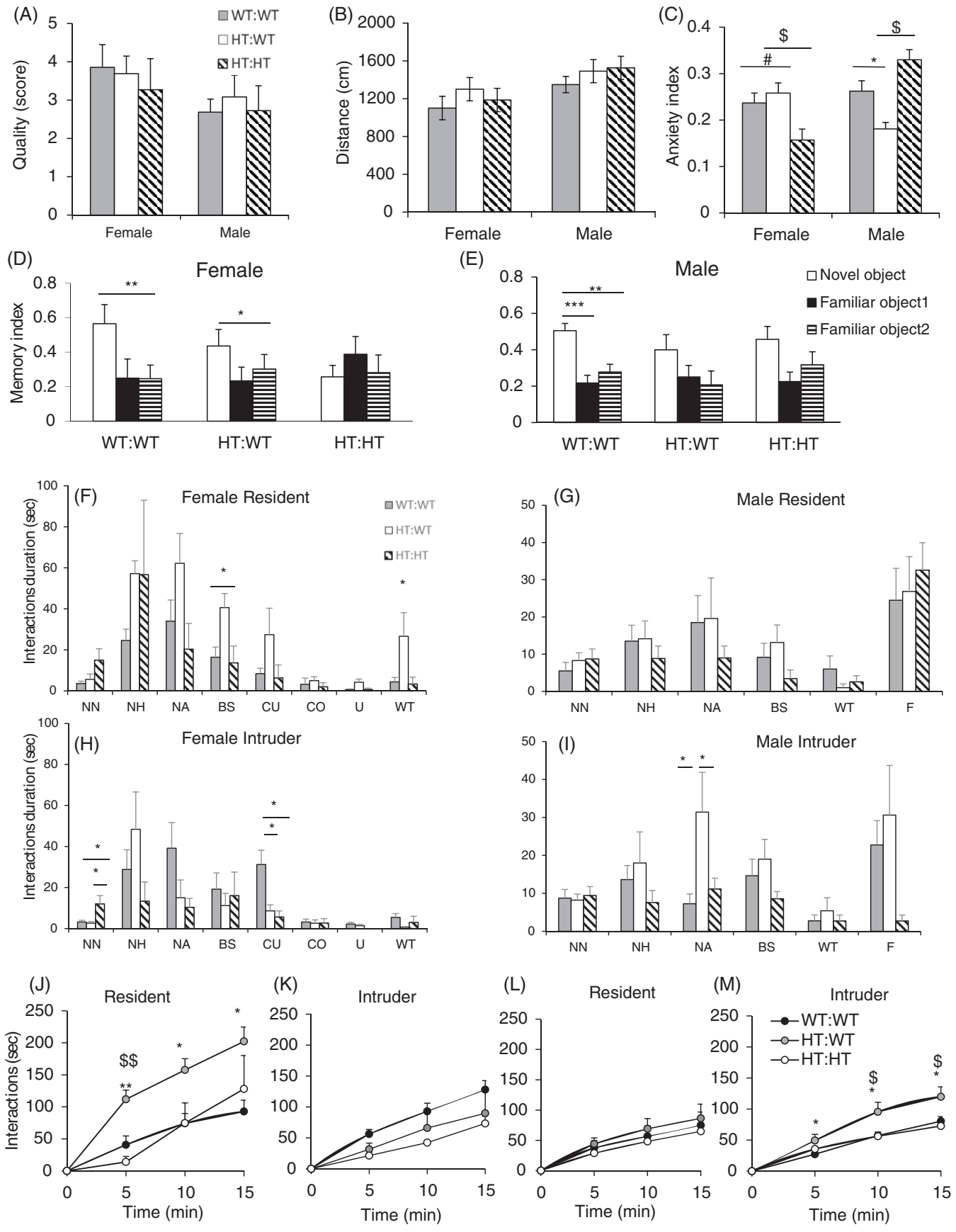

FIGURE 3 Legend on next page.

as shown in Figure 3F, amounted to a significant difference when the total number of interactions was analyzed (Figure 3J). Resident males of all groups showed similar behavior patterns (Figure 3G,L). When examining the durations of events initiated by the intruder, a sex × maternal genotype interaction was found ($F_{1,27}$ = 8.06, P = 0.008). In this case, the behavior patterns of intruder females from all groups were mostly similar with differences in particular categories of behavior (NN and CU), but no general effect (Figure 3H,K). In the males, the maternal and offspring *Mthfr*+/− genotypes were associated with increased and decreased durations of interactions ($F_{1,17}$ = 11.5, P = 0.003; $F_{1,17}$ = 10.96, P = 0.004, respectively) (Figure 3M), a result that manifested mainly in the significant differences observed in the NA behavior (Figure 3I).

Thus, from a behavioral perspective, ASD-associated behaviors, assessed via the indicators of anxiety, memory and aggression, were present in *Mthfr* deficient mice, in which maternal and offspring genotypes were involved in a sex-dependent manner. A summary of adult mice behavior in tests to detect core and associated ASD behaviors is shown in Table 3. ASD-like behavior was found to be influenced by both maternal and offspring *Mthfr*+/− genotypes. Interestingly, the results of assessments to detect the presence of core ASD symptoms in mice showed that maternal genotype had an apparently stronger effect than offspring genotype. Furthermore, male mice, in whom all the tested aspects of ASD-like behavior manifested, exhibited higher susceptibility to the effects of the maternal and offspring *Mthfr*+/− genotypes than did the females, which were not affected in all tests.

3.5 | *Mthfr*+/− affects PV cell density in the cerebral cortex

Alterations in the inhibitory system, specifically in PV cell numbers, were reported in ASD postmortem human brains and in mouse models of ASD.[29,58] We investigated PV^+ cell density in the brains of adult mice. A strong and distinctive *Mthfr*-dependent ASD-like phenotype was observed in the adult male mice that was consistent with the higher rate of ASD commonly observed among males vs females.[47,59,60] To assess the impact of the *Mthfr* deficiency, therefore, we focused our search on the PV interneuron cell population in the brains of male mice.

An example of PV^+ cells in the cingulate cortex of 3-month-old mice is shown in Figures 4A and 6B. Maternal *Mthfr*+/− genotype-specific enhancement of PV^+ cell densities were observed in the cingulate cortex ($F_{1,14}$ = 7.2, P = 0.02), as shown in Figure 4C, but not in the frontal cortex or hippocampus (Figure 4D,E). Laminar organization of distinct interneuron populations and the locations of the synaptic inputs on other neurons in the cortical circuit are fundamental for normal brain function and behavior.[61] Analysis of PV^+ cell densities in layers representing the laminar organization of the cerebral cortex indicated significant changes in superficial layer (L)2 and deep layer L6, such that maternal *Mthfr*+/− genotype increased cell density ($F_{1,14}$ = 7.33, P = 0.019; $F_{1,16}$ = 7.68, P = 0.015, respectively) and offspring *Mthfr*+/− genotype decreased cell density in the cingulate cortex ($F_{1,14}$ = 7, P = 0.021; $F_{1,16}$ = 6.08, P = 0.027, respectively), as shown in Figure 4F. Innervation, measured by PV fluorescence, was found to be potentiated by offspring *Mthfr*+/− genotype (repeated measurements ANOVA, $F_{1,9}$ = 8.26, P = 0.018), as depicted in Figure 4G. The effect was prominent in the superficial and central layers but not in the deep layers where minimal values of fluorescence were observed in all groups. No differences in innervation were found in the frontal cortex and hippocampus (data not shown).

4 | DISCUSSION

The data presented here support the *Mthfr*-deficient mouse as a valid model to study the in-utero and postnatal impacts of *MTHFR* on ASD features. As a whole, the study recapitulates the major findings in humans, where the maternal *MTHFR* genotype was shown to be associated with increased risk for ASD in children.[9–17] Moreover, the mouse model enables autistic-like features that are associated with maternal genotype to be differentiated from those that depend on offspring genotype. Delayed morphogenesis was governed solely by maternal genotype, a finding that emphasizes the dependency of newborns on the C1 metabolites supplied by the mother. The dominance of the maternal genotype was maintained at prepubertal age and into adulthood, at which stage mice behavior in tests to evaluate core ASD symptoms was impaired by maternal genotype. Thus, we suggest that the developmental programming of the neuronal circuits activated during behaviors that are situated in domains impaired in ASD relies on normal C1 metabolism levels in utero. Moreover, behaviors associated with autism that are not vital for the ASD diagnosis but that are present with high comorbidity (eg, learning and memory, anxiety) depend on either the offspring genotype (female) or on both the maternal and offspring genotypes (male) (Table 3). This finding

FIGURE 3 ASD-related behavior in adult mice (P80). (A) *Nest building*—nest quality (score 0-3). (B) *Open field*—distance moved in the arena (cm). (C) *Open field*—anxiety index—duration in arena center/duration in the arena margin. WT:WT—gray. HT:WT—white. HT:HT—stripes. $^*P < 0.05$ between WT:WT and HT:WT, $^{\#}P < 0.05$ between WT:WT and HT:HT, $^{\$}P < 0.05$ between HT:WT and HT:HT. (D and E) *Recognition memory*—memory (percent of time spent sniffing) = time spent sniffing the novel object/time spent sniffing all objects: Novel object—white, familiar object 1—black, familiar object 2—striped (D, female; E, male). N of females—WT:WT = 15, HT:WT = 14, HT:HT = 11; N of males—WT:WT = 16, HT:WT = 12, HT:HT = 11. One-way ANOVA with Bonferroni post-hoc test was used to compare the percentage of time the mouse spent with either of the familiar objects and that which it spent with the novel object, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. F-M) *Resident intruder*—durations of social interactions initiated by the resident mouse (F, G) and of those initiated by the intruder mouse (H, I). (female, F and G; male, H and I): NN, NH, NA, BS, CU, CO, upright (U), fight (F), wrestling (Wt). WT:WT—gray, HT:WT—white, HT:HT—stripes. The time course of the total durations of all social interactions initiated by the resident mouse (J, L) and of those initiated by the intruder mouse (K, M). (female, J and K; male, L and M), WT:WT—black; HT:WT—gray; HT:HT—white. N of females—WT:WT = 7, HT:WT = 7, HT:HT = 5; N of males—WT:WT = 8, HT:WT = 6, HT:HT = 5. One-way ANOVA with Bonferroni post-hoc test. $^*P < 0.05$, $^{**}P < 0.01$ between WT:WT and HT:WT, $^{\#}P < 0.05$; between WT:WT and HT:HT, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ between HT:WT an HT:HT. Mean ± SEM

TABLE 3 Summary of the effects of maternal and offspring MTHFR genotypes

Sex	Genotype	Female		Male	
Age	**Variable**	**Maternal**	**Offspring**	**Maternal**	**Offspring**
Neonatal P4-P17 Developmental milestones	Weight and morphogenesis	**Delay**	No effect	**Delay**	No effect
	Geotaxis	**Delay**	Early	**Delay**	Early
	Rotarod	No effect	No effect	**Delay**	No effect
	Nest finding	**Delay**	Early	**Delay**	Early
	Cliff avoidance	**Delay**	No effect	**Delay**	No effect
Adult—P80 ASD core symptoms	Repetitive behavior-Marble Burying and Nest building	Effect (direction not clear)	**Repetitive behavior**	**Repetitive behavior**	No effect
	Restricted interest-Object preference	No restricted interest	No effect	**Restricted interest**	No effect
	Social -Social preference	No effect	No effect	No Effect	**Social deficit**
	Social-Social novelty preference	No Effect	No effect	**Social deficit**	**Social deficit**
Adult—P80 Behavior associated with ASD	Aggressive Social interaction—Resident intruder	**Aggressiveness**	**Aggressiveness**	**Aggressiveness**	**Aggressiveness**
	Recognition memory—Object recognition	No Effect	**Poor performance**	**Poor performance**	**Poor performance**
	Anxiety—anxiety index	No Effect	**Increased anxiety**	**Increased anxiety**	Decreased anxiety

Summary of pup and adult offspring behavior. Cells in bold indicate a significant effect that is in line with the ASD-like phenotype and its associated behaviors.

suggests that the neuronal circuits that are behind the associated behaviors undergo significant postnatal development and depend on the offspring's ability to produce its own metabolites.

The brain systems that support the behaviors in which the core ASD symptoms manifest include two main areas: those responsible for the motor system (repetitive behavior), among which are the basal ganglia circuits and cortico-basal ganglia pathways, and areas of the social brain (sociability, social preference, social aggression), where the mPFC and amygdala and other limbic system areas, are involved.[62–64] Maturation of amygdala connectivity to subcortical brain areas takes place as early as P7,[65] a period during which the offspring is fully dependent on maternal metabolites. In a rat amygdala projections to and from the mPFC and other cortical regions not only exhibit a delay in their establishment, but they also undergo extensive reorganization until they reach full maturation late, during the third and fourth weeks of the rat's life and sometimes later.[65–67] These events take place during a period when cortical layer rearrangements also occur. Moreover, the third to sixth weeks of rodent life constitute a period of time when glutamatergic and GABAergic synaptogenesis in the mPFC increase markedly.[65] Late development of this pathway was also observed in humans.[62] In rodents, these critical stages in mPFC circuit maturation take place during a period when the pups begin to independently explore for their food and to rely less on maternal nutritional contributions.[68] Thus, the establishment of neuronal circuits that support social behavior may depend on certain preset conditions that have been established based on the metabolic status of the maternal in-utero environment. Thereafter, they are exposed to the metabolic status dictated by offspring genotype.

The pronounced effect of the *Mthfr* deficiency observed in males compared with the milder influence detected in females is depicted in Table 3. Besides the known difference in ASD rates among males and females,[57,59,60] in humans, gender-dependent differences in the activation of brain regions that support social behavior and in the motor circuits activated during repetitive behavior were found in autistic children.[69] These differences may lead to the gender-specific trajectories of autistic social impairment that have been observed in children and adolescents.[70] A similar difference in the autistic social phenotype was observed in mouse behavior in the resident-intruder test, which resembles an aggressive social situation.[47] In our study, although both males and females were affected by genotype, different aspects of their behavior were impaired (Figure 3F-I).

Among the behaviors associated with ASD and tested in the current study is recognition memory, which was affected by both the maternal and offspring *Mthfr* genotypes. Recognition memory depends on mature mPFC-hippocampus and hippocampal-entorhinal cortex circuitry.[71,72] The maturation of this circuitry, which in healthy mice appears to parallel the maturation of mPFC glutamatergic innervation (around the age of P30 in rodents), was previously exemplified by late onset of the ability to perform a task.[73] In males, both the maternal and offspring *Mthfr* genotypes contributed to memory impairment. Therefore, although the neuronal circuitry supporting this behavior develops postnatally, our findings indicate that the in-utero environment had long-lasting implications for the development of the neuronal foundation required for recognition memory in rodents. The crucial role played in the epigenetic programming of cortico-hippocampal memory circuits by the in-utero environment and by maternal nourishment during lactation was previously noted[74,75] and can explain both our results in mice and those of previous reports in male rats, where gestational choline supplementation enhanced recognition memory.[76] Thus, postnatal maturation of the cortico-

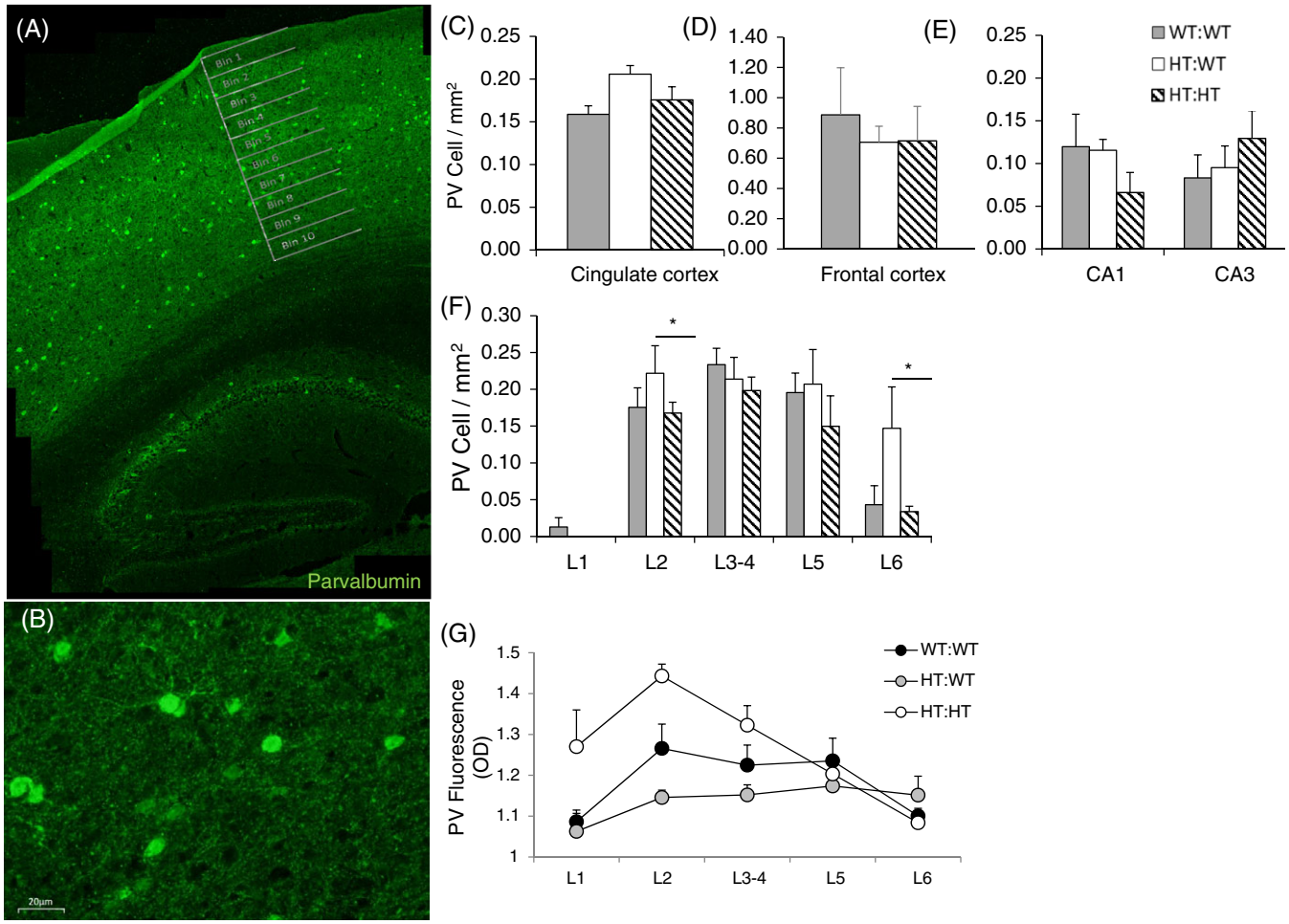

FIGURE 4 Effects of maternal and offspring genotypes on PV$^+$ cells in the adult mouse cortex. (A, B) Cingulate cortex stained with anti-PV antibody. Bins used for the analysis are overlaid on the left-hand image (magnification ×10, ×20). *Cell density* (cell/mm^2) of the (C) cingulate cortex, (D) frontal cortex and (E) CA1 and CA3 fields of the hippocampus. *Cell density* (cells/mm^2) in the cingulate cortex is presented by cortical layer: L1 (bin 1), L2 (bins 2-3), L3-4 (bins 4-6), L5 (bins 7-8), L6 (bins 9-10). (F) PV fluorescence (OD) measurement of PV immunoreactivity in cells and of processes in the cingulate cortex by cortical layer. N = 6 brains in each group; one-way ANOVA with Bonferroni post-hoc test. $*P < 0.05$. Mean ± SEM. CA, Cornu ammonis; L, layer; OD, optical density

hippocampal circuit depends on the levels of C1 metabolism in the in-utero and postnatal environments. Mouse performance in the social novelty preference test and in the object recognition test depend on the test subject mouse's memory of the familiar mouse and on the familiar objects, respectively, and the choice to interact with the novel stimulus. Although different domains of memory rely on different neuronal circuits, common brain regions participate in these two types of memory. Intriguingly, male offspring to *Mthfr+/–* dams were impaired in these two tasks (Figures 2H and 3E), suggesting possible dysregulation in the brain regions shared by the two different tasks.

Anxiety-related behavior is also associated with ASD. Similar to our previous findings, wherein offspring genotype was associated with anxious behavior in an elevated plus maze,[38] here we found an association between offspring *Mthfr+/–* genotype and stress-related variables. This study and another by K.E. Stevens' group[77] also show an association between C1 metabolism, prenatal stress and long-lasting anxiety-related behavior. A possible molecular mechanism for such an effect was described in a human study, where the level of cortisol and the methylation status of genes involved in the stress response in fetal driven tissue undergo epigenetic modification in association with higher maternal choline intake during the third trimester of pregnancy.[78] These studies also illustrate the lifespan implications of in-utero C1 metabolism.

Mouse strains vary widely in their basal social behavior.[40,44] Strains commonly used in ASD research include C57BL/6J with significant sociability and SV129 with low social approach.[27,29,31,32] In strains with low social approach, such as the Balb/c strain (used in the current study) and SV129, special attention is given to variables representing mouse behavior, such as sniffing, during the time the mouse spends in a particular chamber of the arena as suggested by the comprehensive comparative study by Moy et al.[40]

Finally, differential changes in the population of PV interneurons were observed in the offspring of *Mthfr*-deficient dams, adding to the body of evidence linking this subpopulation of interneurons to ASD-like behavior. Failure to express PV in interneurons was reported in particular brain regions of several ASD mouse models.[27–32] Indeed, lack of PV was sufficient to induce autistic-like behavior.[27,33,34] The specificity of the change to the cingulate cortex is of particular

interest in light of findings in individuals with ASD. In those individuals, a high level of connectivity is observed between the ventrolateral PFC and the cingulate cortex (which correlates with performance in tasks testing cognitive control) compared with typically developing individuals, who activate other brain regions for similar tasks.[79] A reduction in inhibition-related activation of the dorsal anterior cingulate cortex was reported in ASD individuals, in whom the severity of repetitive behavior was correlated with reduced control of response inhibition by this region.[80] Among high functioning ASD individuals, lack of activation of the cingulate cortex regions related to self-response was reported.[81] The changes observed in the current study in cingulate cortex PV neurons support a role for these cells in ASD.

Similar to the majority of cortical interneurons, PV cells originate in the ganglionic eminence and migrate during the last gestational trimester to their cortical destination via a long and complex pathway, thus rendering them susceptible to changes that occur in the molecular landscape along the migratory path.[82–84] In addition, the expression of PV protein, which appears around P14 and the protein continues to accumulate thereafter, was shown to be susceptible to pre- and postnatal conditions.[85,86] *Mthfr* deficiency was also associated with an alteration in the layer distribution of PV immunoreactivity in response to a change in the location of inhibitory input. Changes in the laminar distribution of PV innervation may alter circuit processing, as suggested previously in other psychiatric disorders.[87] In the current study, we cannot definitively determine whether the embryonic interneurons were directly affected by the *Mthfr* deficiency (which consequently induced ASD-like behavior), or whether the changes in this population were a secondary response to a common origin. This question remains open for future studies to explore.

In summary, our study provides evidence for the profound impact of a genetic deficiency in the *MTHFR* gene on the induction of autistic features. In the mouse model of ASD, this deficiency directly regulates metabolite availability and indirectly controls the environment of the developing embryonic brain. The use of a rodent model enables the contributions of maternal and self-produced C1 metabolites to be clearly differentiated from one another. The timely exposure of the brain to these factors is strongly associated with pre- and postnatal critical periods in normal neuronal circuit development. Furthermore, the heightened sensitivity of the PV interneurons of the cingulate cortex to C1 metabolites suggests that they play an essential role in the cell cycle of this population of interneurons. Finally, the time course of the MTHFR effect described here provides a good platform upon which to develop intervention strategies to attenuate the developmental impact of low C1 metabolic activity.

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CONFLICTS OF INTEREST

None.

ORCID

Noa Sadigurschi https://orcid.org/0000-0002-5797-029X
Hava M. Golan https://orcid.org/0000-0001-8661-3665

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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