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Prenatal exposure to antibodies from mothers of children with autism produces neurobehavioral alterations: A pregnant dam mouse model

Harvey S. Singer [a,*], Christina Morris [a], Colin Gause [a], Matthew Pollard [a], Andrew W. Zimmerman [a,b], Mikhail Pletnikov [c]

[a] Departments of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[b] Kennedy Krieger Institute, Baltimore, MD, United States
[c] Departments of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, United States

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ABSTRACT

A pregnant mouse model was used to compare the effect of IgG, administered E13-E18, from mothers of children with autistic disorder (MCAD), to controls (simple- and IgG-) on behavioral testing in offspring. Mice, exposed in-utero to MCAD-IgG, as adolescents, were more active during the first ten minutes of central field novelty testing and, as adults, displayed anxiety-like behavior on a component of the elevated plus maze and had a greater magnitude of startle following acoustic stimulation. On a social interaction paradigm, adult mice had alterations of sociability. Pilot studies of immune markers in MCAD IgG-exposed embryonic brains suggest evidence of cytokine and glial activation. These studies demonstrate that the transplacental passage of IgG from MCAD is capable of inducing long-term behavioral consequences.

1. Introduction

Autism is a neurodevelopmental disorder characterized by core clinical deficits in the areas of socialization, verbal and nonverbal communication, and restricted and repetitive patterns of behavior. The disorder is complex, encompasses a broad range of pervasive developmental disabilities, and in the majority of cases there is no defined etiology. Suggestions of an immune abnormality as a contributing factor in autism were initially based on the presence of a family history of autoimmune dysfunction (Comi et al., 1999) and the identification of serum antibodies targeting brain epitopes in affected children (Plioplys et al., 1989; Singer et al., 2006; Singh et al., 1997, 1993; Todd and Ciaranello, 1985; Zimmermann, 1993). Subsequent studies, however, have shifted the hypothesis from a primary autoimmune abnormality to one of potential transplacental antibody exposure in utero. More specifically, several investigators have identified the presence of unique serum antibodies against human fetal brain tissue in mothers with offspring having the diagnosis of autistic spectrum disorders (Braunschweig et al., 2008; Croen et al., 2008; Singer et al., 2008). A correlation between maternal antibodies against fetal brain proteins in the 36–39 kDa range and the presence of developmental regression in affected offspring has been used to support a functional association (Braunschweig et al., 2008; Singer et al., 2008).

Although antibody detection in mothers of children with autistic disorders (MCAD) raises the possibility that placentally transferred antibodies interfere with fetal brain development, their presence alone is insufficient to define causality. For example, autoantibodies are present in healthy individuals as well as mothers of typically developing offspring and may even have a regulatory role (Archelos and Hartung, 2000). Hence, the confirmation of a pathogenic role for maternal antibodies in neurodevelopmental disorders or autism requires: demonstrating that the passive transfer of maternal IgG can induce a behavioral disorder in offspring; showing that immunoglobulins are present at the pathological site; and defining the mechanism(s) by which passively transferred IgG alters brain development.

The pregnant dam (mouse) model provides a valuable system for investigating the IgG placental transfer hypothesis. The putative pathological antibody(ies) can be injected during the gestational period and offspring evaluated for changes in behavior. Similar approaches have identified behavioral abnormalities in offspring following exposure of pregnant dams to serum antibodies from mothers of dyslexic children and from a mother with anti-Purkinje antibodies who had an autistic child (Dalton et al., 2003; Vincent et al., 2003). In addition, mouse behavioral paradigms are available to evaluate overactivity, anxiety, and sociability, clinical deficits that occur in children with neurodevelopmental disorders (Crawley, 2007; Moy et al., 2007). While studies in more advanced species, e.g., monkeys, (Martin et al., 2008) might provide a broader social repertoire, the mouse model is more economical, permits

* Corresponding author. Johns Hopkins Hospital, Rubenstein Child Health Building, 200 N. Wolfe Street, Suite 2158, Baltimore, MD 21287, United States. Tel.: +1 410 955 7212; fax: +1 410 614 2297.
E-mail address: hsinger@jhmi.edu (H.S. Singer).

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testing in larger numbers, and provides the ability to analyze embryonic brain for inflammation and structural alterations.

The goal of this exploratory study was to use the pregnant mouse model to establish whether the intraperitoneal injection of IgG during gestation, obtained from mothers of children with autistic disorder, produces postnatal behavioral effects in offspring. Behavior was compared to that in mice whose dams received IgG from mothers of typically developing children (IgG-controls) and simple-controls. Basic observations of neurobehavioral and neuromotor development and weight gain were recorded during the first 21 days of life. Behavioral paradigms, used in adolescent and adult mice, included measures of open field behavior and activity, anxiety-like behavior, acoustic startle response, pre-pulse inhibition, and sociability. Pilot investigations in embryonic mouse brains were designed to confirm a maternal-to-fetal transfer of human IgG, to assess for gross neuroanatomical alterations, and to identify an immune reaction by use of cytokine measurement, immunohistochemical staining for microglial reactivity, and ELISA assay of brain derived neurotrophic factor (BDNF). It was hypothesized that behavioral differences would be identified in adolescent and adult mice prenatally exposed to MCAD-IgG and that evidence of immune reactivity would be found in the embryonic brain.

2. Materials and methods

2.1. Animals

C57BL/J6 male and female mice were purchased from Jackson Laboratory. Mice were maintained and mated in the certified animal facility of the Johns Hopkins Broadway Research Building. All investigations in offspring were performed without regard to gender.

2.2. Isolation of IgG from human sera

IgG was isolated from pooled serum samples of 63 mothers of children with autistic disorder (MCAD) and 63 mothers of unaffected children (IgG-controls), participants in a previously published study (Singer et al., 2008) (see Table 1). Autistic disorder was diagnosed in children by an expert in the field (AZ) based on the presence of abnormalities in social and communication development, marked repetitive behavior, and limited imagination using the Diagnostic and Statistical Manual for Mental Disorders-IV (DSM-IV) and Autism Diagnostic Observation Schedule-Generic (Lord et al., 2000) or Childhood Autism Rating Scale (CARS) (American Psychiatric Association, 1994; Schopler et al., 1986). All were judged clinically to have moderate to severe adaptive deficits, or cognitive deficits (IQ, <70) by formal testing. Children with diagnoses of Asperger syndrome, Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) and those with established genetic or metabolic causes of autism were excluded. An equal quantity of previously frozen, never re-frozen, serum from each subject in the cohort, 225 µL, was pooled, filtered, and the IgG isolated by passage over a Protein A cartridge (Sigma, St Louis, MO) as per manufacturer protocol. The most commonly identified reactivity bands for MCAD and IgG-control cohorts are shown in Table 2. IgG was eluted from the column using acid (Elution buffer) and then immediately passed over a desalting cartridge to set the eluate to physiological pH in phosphate buffered saline (PBS). Purified IgG was then filtered (0.22 µm) and maintained at 4 °C. The pooled IgG concentration was determined by rate nephelometry using the Beckman IMMAGE 800 nephelometer in the Johns Hopkins Hospital Clinical laboratory; MCAD 279 mg/dL, IgG-control 245 mg/dL.

Table 1
Maternal demographics.

	MCAD	IgG-controls
Mother's age (mean ± SD) (range)	40 ± 6.5 yrs (26–65)	43 ± 5.6 yrs (27–65)
Maternal age at 1st pregnancy (mean +SD)	28.3 ± 6.4 yrs	29.7 ± 4.1 yrs
Parity, mean and (range)	3.3 (1–9)	3.2 (1–7)
Number mothers with 1 AD child	57 AD: 1st born = 25; 2nd born = 16; 3rd born = 10; 4th born or later = 6	
Number mothers with 1AD child and 1 PDD/asperger	2	
Number mothers with 1 AD child and 2 PDD/asperger	1	
Number mothers with 2 AD children	3	
Mean interval between birth of child with AD and blood draw mean (range)	7.6yrs (2–21)	
Number AD children with regression	25	

AD = autistic disorder, PDD = pervasive developmental disorder, ASD = autistic spectrum disorder.

Table 2
Frequently identified reactive bands identified in MCAD and IgG-control cohorts (% containing).

	IgG-control	MCAD		IgG-control	MCAD		IgG-control	MCAD
138 kDa	8	13						
110 kDa	29	24	64 kDa	43	40	39 kDa*	16	14
100 kDa	44	43	61 kDa*	32	30	36 kDa	2	10
91 kDa	30	38	58 kDa	10	10	33 kDa	10	10
83 kDa	29	21	53 kDa	16	10	31 kDa	24	24
77 kDa	10	16	47 kDa	29	24	28 kDa	10	11
72 kDa	24	24	43 kDa	11	10	22 kDa	6	11

*At 61 and 39 kDa MCAD peak heights were significantly greater (Singer et al., 2008).

2.3. Intraperitoneal injections and controls

26 pregnant mice received daily intraperitoneal injections of 0.5 mL of purified IgG from either MCAD (13 pregnant mice) or IgG-controls (13 pregnant mice) on gestational days E13 through E18, or day of sacrifice if earlier. Seven litters each were allocated to behavioral testing and the remainder to fetal brain analyses. Saline-controls used in embryonic brain and neonatal activity investigations were saline exposed embryos (from eight litters). In contrast, simple-controls used in adolescent and adult behavioral testing were not exposed to intraperitoneal injections. Embryonic day 1 represents the date a vaginal plug is first identified.

2.4. Developmental testing in offspring

On the day of delivery, postnatal day 1 (P1), all offspring were checked for gross abnormalities (Dierssen et al., 2002). Formal developmental testing over the first three weeks was performed on 10 MCAD-IgG exposed pups (two litters), 13 IgG-control exposed pups (two litters), and 14 saline-exposed pups (two litters). From P1 through P21, pups were weighed daily and a variety of developmental landmarks were observed, including pinna detachment and incisor eruption. Neurobehavioral development was tracked by observing the righting response, forelimb/hindlimb place and grasp, cliff drop aversion, and negative geotaxis. Comparisons were made based on the postnatal day that all litter pups for the MCAD, IgG-control, or saline-control groups achieved the skill. Neuromotor development was evaluated on P10 and P14 by measuring pivoting locomotion (number of 90° pivots within a 60 s period) and latency for walking a distance equivalent to the pup's body length. On P14, a homing test was performed by timing how long it took the pup to reach sawdust from its own cage in a novel environment.

2.5. Behavioral tests

The number of animals participating in each behavioral task and the number of litters from which they were derived is presented in Table 3. Animals were tested either as adolescents (4–6 weeks of age)

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Table 3
Number of animals participating in each behavioral task.

	P1-21 Dev	Adolescent				Adult			
		NOF	Startle	Social	EPM	NOF	Startle	Social	EPM
MCAD	10 (2)*	12 (3)	12 (3)	10 (3)	10 (2)	17 (4)	17 (4)	17 (5)	10 (2)
IgG-control	13 (2)	12 (3)	12 (3)	12 (3)	12 (2)	11 (4)	15 (4)	18 (5)	12 (2)
Simple-controls[a]	14 (2)	12	12	12	12	12	12	12	12

*X (Y) = number of animals per task (number of litters from which these animals were acquired). Seven MCAD and Control-IgG litters were bred for behavioral studies.
Abbreviations: Dev, developmental testing; NOF, novelty open field; Social, social interaction; EPM, elevated plus maze.
[a] Simple-controls: saline-exposed for P1-21 testing; non-exposed for adolescent and adult behavioral testing. The majority of non-exposed simple-controls were obtained from separate litters.

or as adults (4–8 months of age), the one exception being the elevated plus maze, in which the same IgG-exposed animals were tested as both adolescents and adults. The standard order of testing in each mouse was the open field test, sociability, acoustic startle test, and elevated plus maze. A one-week interval was maintained between each test in order to minimize any potential stressful effects.

2.5.1. Novelty-induced locomotion

Locomotor activity and emotionality are evaluated by placing a mouse in an open field area under a standardized room lighting condition. This paradigm measures the tendency of the mouse to explore a novel environment, but to avoid a brightly lit open area. Novelty-induced activity in the open field is assessed over a 60-min period using activity chambers with infrared beams (San Diego Instruments, Inc., San Diego, CA). Horizontal and vertical (rearing) activities, stereotypic activities, and time spent in the center (central activity) or along the walls of the chamber (peripheral activity) are automatically recorded. Results are influenced by a tendency to remain close to walls (thigmotaxis), emotionality, and anxiety (Eikelis and Van Den Buuse, 2000; Karl et al., 2003; Treit and Fundytus, 1988).

2.5.2. Acoustic startle response

A startle response is an unconditioned reflexive activity that follows a sudden environmental stimulation. This startle can be gated by a weak prestimulus or prepulse (prepulse inhibition) that, when given prior to the established stimulating acoustic stimuli, causes the animal to flinch less to the startle stimulus. Deficits have been identified in patients with schizophrenia and other neuropsychological disorders and interpreted to indicate an underlying dysfunction of the inhibitory mechanism in neuronal systems used for sensory gating (Swerdlow et al., 2000). Two identical startle chambers (San Diego Instruments, Inc., San Diego, CA) were used for measuring startle reactivity and prepulse inhibition. Acoustic stimuli are controlled by SR-LAB software (San Diego Instruments, Inc., San Diego, CA) and interface system, which rectifies, digitizes, and records responses from the accelerometer. The maximum voltages within 100-ms reading windows, starting at stimulus onset, are used as measures of startle amplitudes. Sound levels are measured inside the startle cabinets by means of the digital sound level meter (Realistic, Tandy, Fort Worth, TX, USA).

The experimental session consists of a 5-min acclimatization period to a 70-dB background noise (continuous throughout the session), followed by the presentation of ten 40-ms 120-dB white noise stimuli at a 20-s inter-stimulus interval (the habituation session). Upon the completion of the habituation session, each mouse is left in the enclosure for 5 min without presentations of any startle stimuli. Immediately after, the pre-pulse inhibition (PPI) session was begun. During each PPI session, a mouse is exposed to the following types of trials: pulse-alone trial (a 120-dB, 100-ms, broadband burst); the omission of stimuli (no-stimulus trial); and five pre-pulse-pulse combinations (pre-pulse–pulse trials) consisting of a 20-ms broadband burst used as a pre-pulse and presented 80-msec before the pulse using one of the five pre-pulse intensities: 74; 78; 82; 86 and 90 dB. Each session consists of six presentations of each type of the trial presented in a pseudorandom order. PPI is assessed as the percentage scores of PPI (% PPI): 100 × (mean startle amplitude on pulse-alone trials *minus* mean startle amplitude on pre-pulse trials / mean startle amplitude on pulse-alone trials) for each animal separately. The percentage of PPI for each animal was used as the dependent variable in statistical analysis.

2.5.3. Social interaction test

Sociability was assessed as previously described (Moy et al., 2007). Testing is performed in a standard testing box separated into three chambers by partitions, with two larger chambers on each side and a smaller middle area. In the sociability paradigm, one large chamber contained a cylindrical wire-mesh enclosure with a conspecific (an individual of the same species, stranger 1) whereas the opposite large chamber contained a similar, but empty, enclosure. The mouse to be tested is placed in the central smaller chamber and allowed to habituate to the experimental environment for 5 min. The partitions are then removed and for the next 10 min the time the mouse spends exploring (i.e., actively sniffing and/or staying within 1 cm) the enclosure with a live adult mouse of the same sex or the empty enclosure is recorded. Typically, mice prefer exploring the cage containing the conspecific. The action of sniffing is considered a more precise indication of socialization than time in area. Time spent around each enclosure is automatically registered by a video tracking system "SMART" (San Diego Instruments, Inc., San Diego, CA). Social and nonsocial stimuli are varied between the sides and the testing box is cleaned between subjects.

2.5.4. Elevated plus-maze

This test was added to evaluate levels of anxiety-like behavior. The elevated plus-maze is an approach–avoidance conflict test sensitive to anxiolytic drug treatment (Cruz et al., 1994) and is based on a mouse's tendency to explore new areas and avoid open runways. The maze is in the shape of a plus ("+"), arms are elevated 1 m above the floor and extend out from a central platform. Two alternative arms are closed while two are open. The mouse is placed on the central platform and observed for 5 min. The number of total arm entries (transitions) is indicative of general motor activity. Anxiety-like behavior is measured by the percent of time spent on the open arm [calculated as 100 × (time spent on the open arms / (time on the open arms + time on the closed arms)]. Anxious mice are expected to spend less time on the open arms.

2.6. Brain tissue preparation

2.6.1. Brain fixation and sectioning for histopathological and immunohistochemical assays

Animals were euthanized with EUTHASOL upon completion of behavioral testing (Diamond Animal Health, Inc. IA). Adolescent and adult mice were perfused with ice-cold phosphate buffered saline (PBS, pH = 7.4) followed by 4% paraformaldehyde. Brains were removed, post-fixed for 4 h in 4% paraformaldehyde (PFA), cryoprotected in 10% and 30% sucrose, frozen in 2-methylbutane, and kept at −80 °C until sectioning. For embryonic brains (E16 and E18), immersion fixation was used for those designated for histochemical study. Brains were maintained in 4% PFA overnight, then transferred to PBS, cryoprotected, frozen in 2-methylbutane, and kept at −80 °C until sectioning. One brain (or entire head) per time-point and treatment group was fixed and sectioned. Adolescent and adult brains were sectioned at 40 µm on a microtome and whole embryonic heads were sectioned on a cryostat at 20 µm. Microtome sections were saved in the cryoprotection medium at −20 °C, and cryostat sections were kept at −80 °C until staining.

2.6.2. Homogenization for BDNF and cytokine analysis

Embryonic brain tissue from 2 (E18) or 3 (E16) pups from each litter was homogenized together producing a single test sample.

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Adolescent brains were homogenized individually. Brains were homogenized on ice in 0.9% normal saline (2.5 g tissue/10 mL saline) containing protease inhibitors (1 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride) in a Teflon-glass homogenizer on ice. Homogenized tissue was centrifuged for 30 min at 12,000 ×*g*. The supernatant fraction was collected, and aliquots were stored at −80 °C. Five samples from each treatment group and timepoint (i.e., MCAD, $n=5$; IgG-control, $n=5$; saline-controls, $n=5$; E16, E18, and adolescent) were used in assays.

2.7. Anatomy/histological studies

2.7.1. Embryonic, adolescent, and adult mouse brain

Embryonic, adolescent, and adult mouse brain sections from each experimental group were Nissl stained with cresyl violet for gross anatomical comparisons.

2.7.2. Anti-human IgG staining in embryonic mouse cortex

Slides from E16 and E18 mice exposed *in utero* to MCAD IgG, IgG-controls, or saline were washed in PBS for 5 min with shaking. Each slide was then overlaid with blocking solution (3% normal rabbit serum, 0.4% Triton X-100 in PBS) and incubated in a dark, damp chamber at room temperature for 1 h. After washing, each slide was overlaid with rabbit anti-human IgG/FITC (Dako Cytomation, Carpinteria, CA) diluted 1:250 with 3% normal rabbit serum and 0.1% Triton X-100 in PBS, and incubated as above for 24 h. After washing in PBS, MOWIOL mounting medium (Calbiochem, La Jolla, California) was applied to each slide and specimens were held at 4 °C until viewing. One brain sample was analyzed from each time-point and treatment group for IgG binding around lateral ventricles.

2.7.3. Microglia staining in embryonic brain

Microglia were identified by immunofluorescent Iba1 staining. E16 and E18 brain sections from embryos exposed *in utero* to MCAD IgG, IgG-control, and saline were thawed for 5 min washed in PBS and blocked with 5% normal goat serum (NGS) and 0.04% Triton in PBS. Sections were then stained with rabbit anti-Iba1 (Wako, Richmond, VA) diluted 1:400 in 5% NGS and 0.01% Triton in PBS and incubated at room temperature for 3 h. After washing in PBS, secondary antibody, Cy3 labeled anti-rabbit IgG (Chemicon, Temecula, CA) diluted 1:400 in 5% NGS and 0.01% Triton in PBS, was added and slides were incubated for 1 h at room temperature. After another wash, 4',6-diamidino-2-phenylindole (DAPI; fluorescent DNA stain) was added at 1:2000 in PBS for 2 min at room temperature and washed briefly. Slides were mounted with MOWIOL and viewed on a fluorescent microscope at 20× magnification. Iba1 staining to microglial cells was confirmed by double staining with FITC-labeled lectin diluted 1:400.

2.8. Brain Derived Neurotrophic Factor (BDNF) and cytokine assays

2.8.1. BDNF measurement in embryonic and adolescent brain

Brain BDNF levels were assayed in 96-well microtiter ELISA plates using an EMAX immunoassay BDNF ELISA kit (Promega, Madison, WI). Plates were pre-coated with mouse anti-BDNF polyclonal antibody. Plates were washed and then blocked with provided Blocking Buffer for 1 h at room temperature (RT). Homogenized embryonic (E16, $n=5$ preparations; and E18, $n=5$ preparations) and 1 month old ($n=5$) mouse brain tissues from MCAD-IgG, IgG-controls, and saline exposed controls were serially diluted and incubated for 2 h at RT. After washing with TBS-T, secondary antibody, polyclonal chicken anti-BDNF IgY diluted 1:500, was added and incubated for 1 h at RT with agitation. After exposure to tertiary antibody, anti-IgY HRP conjugate diluted 1:200 for 1 h at RT and washing, plates were developed with tetramethylbenzidine solution. Brain BDNF concentrations were measured by optical density at 450 nm on an automated Bio-Rad Model 680 microplate reader. Standards were used to determine the slope of optical density (OD; absorbance) vs. BDNF concentration. OD values (total reading minus tissue blank) were determined for each sample before statistical analysis.

2.8.2. Cytokine measurements in embryonic brains

Assays were performed in duplicate with a Beadlyte mouse multi-cytokine detection system 2 kit (Upstate Biological, Charlottesville, VA) according to vendor protocol using brain homogenates obtained on embryonic days E16 and E18 from MCAD, IgG-controls, and saline-controls ($n=5$ preparations per group at each time point). The protein concentration of each sample was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL), followed by dilution of samples in order to standardize the assay. Analyses included cytokines interferon-gamma (IFNγ), interleukin-12 (IL-12) tumor necrosis factor-alpha (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10), and the immunomodulatory cytokine interleukin-1beta (IL-1β).

2.9. Statistical analysis

Open field novelty-induced hyper-reactive behaviors in MCAD, IgG-control, and simple-controls were compared using a two-way repeated measures analysis of variance (ANOVA), with Group and time of testing (Interval) as independent variables. In those with significant differences, Holm–Sidak post-hoc tests were applied. Elevated plus maze and acoustic startle results were evaluated by one-way ANOVA, with post-hoc testing for elevated plus maze using the Holm–Sidak method, and for acoustic startle a Bonferroni t-test. Pre-pulse inhibition analyses were performed using a 2-way repeated measures ANOVA, with the group and pre-pulse intensity as independent variables. Data for chamber time and sniffing components of the social interaction testing (sociability) was analyzed using two-way ANOVA with factors being Group and Chamber side. A Group effect indicates a difference in the total activity in the entire test box, irrespective of side, among groups. A Chamber effect indicates a difference in measured activity between the two sides of the test box, irrespective of group. A Group x Chamber interaction indicates a difference in the combination of Group and Chamber. When an overall ANOVA was significant, a Holm–Sidak post-hoc analysis was used to compare individual group means. The effects of prenatal treatment on expression of cytokines and BDNF were analyzed using ANOVA, and if significant, a Tukey post-hoc comparison. For all comparisons, significance was set at $p<0.05$.

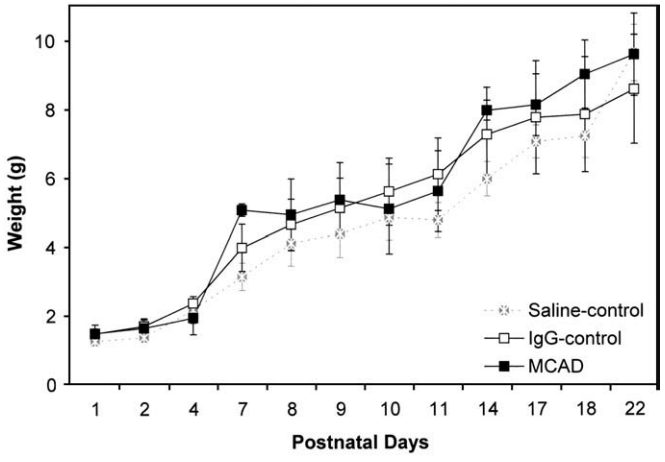

Fig. 1. Body weight (mean ± SD) for days 1-22 in saline-control (▨), IgG-control (□), and MCAD (■) mice.

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3. Results

3.1. Animal health, numbers, and gender

Following intraperitoneal injections, no pregnant dam experienced any evidence of illness, decreased activity, or change in dietary intake. Litter sizes in MCAD, IgG-control, and saline-control groups were equivalent. All pups were in good general health and body weights from P1-21 days (Fig. 1) remained equivalent.

The number of animals participating in each behavioral task analysis is shown in Table 3. Gender ratios for the adolescent and adult behavioral tests were as follows: MCAD 45% male, 55% female; IgG-controls 62% male, 38% female; and simple-controls 50% male, 50% female.

3.1.1. Neurodevelopmental tests (birth to 21 days)

MCAD ($n=10$), IgG-control ($n=13$), and saline-control ($n=14$) pups were carefully followed over the first 21 days of life. Developmental milestones (Table 4), determined by the time 100% of the cohort attained the milestone, were variable with each group attaining some skills before the other. Hindpaw placement and cliff aversion development were slowest in the saline exposed pups. On all other tests, no consistent impairments or trends were observed. Neuromotor development was also equivalent, except for latency (seconds) before walking after being placed in a cage: MCAD mice took a longer time on P14.

3.1.2. Activity in a novel open field environment

i) Adolescent mice (12 simple-controls, 12 IgG-controls, 12 MCAD)
   a) Central novelty-induced activity (Fig. 2a): a two-way repeated measures ANOVA of adolescent activity in the center of the chamber, expressed as activity in beams broken, showed a Group x Interval interaction ($p=0.012$, $df=22$, $F=1.85$) and an Interval effect ($p<0.001$, $df=11$, $F=31.0$). Post-hoc Holm-Sidak analysis showed that activity following placement in the open field was greater in the first 5 and 10 min of testing in mice prenatally exposed to MCAD as compared to IgG- and simple-control mice; at 5 min MCAD > IgG-control, p = 0.004, MCAD > simple-control, $p<0.001$; at 10 min MCAD > IgG-control, $p=0.008$, MCAD > simple-control, $p=0.010$.
   b) Peripheral novelty-induced activity (Fig. 2b): Statistical analysis of peripheral activity, expressed as activity in beams broken, showed only an Interval effect ($p<0.001$, $df=11$, $F=11.47$).
   c) Rearing: the mean number of beams broken in 5 min intervals over a 60 min testing period (Fig. 2c) showed only an Interval effect ($p<0.001$, $df=22$, $F=13.0$).
   d) Fine (stereotypic) movement: no differences among groups were identified in the number of movements observed in five-minute intervals over 60 min.

ii) Adult mice: (12 simple-controls, 11 IgG-controls, 17 MCAD)
   a) Central novelty-induced activity: a two-way repeated measures ANOVA analysis of adult novelty-induced activity in the center of the chamber, expressed as activity in beams broken, showed an Interval effect ($p<0.001$, $df=11$, $F=18.0$).
   b) Peripheral novelty-induced activity: an analysis of peripheral activity expressed as activity in beams broken showed an interval effect ($p=0.008$; $df=11$; $F=2.36$).
   c) Rearing: statistical analysis showed an Interval effect ($p<0.001$, $df=11$, $F=6.60$).

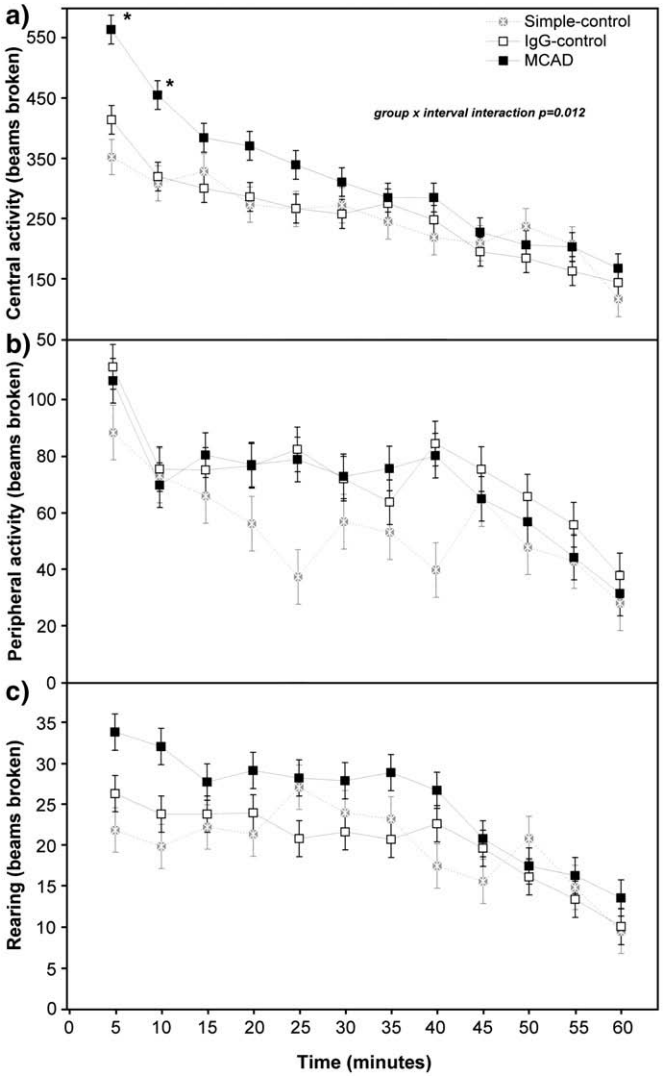

Fig. 2. Activity in novel open field environment: Adolescent mice simple-control (▒), IgG-control (□), and MCAD (■) mice. a) Central activity, b) Peripheral activity, c) Rearing. Data shown are mean (±SEM) values at each 5-minute interval. Post-hoc Holm-Sidak central activity analysis showed a significant difference between groups in the first five and ten minutes of testing. *Indicates post-hoc differences (see text).

Table 4
Early developmental assessment of pups (P1-21).

Developmental milestone	Postnatal day*	% of saline control population (n=n =14; 2 litters)	% of IgG-control population (n=n =13; 2 litters)	% of MCAD population (n=n =10; 2 litters)
Pinna detachment	P7	100%	100%	100%
Incisor eruption	P7	78%	77%	100%
Forepaw grasp	P1	100%	100%	70%
Hindpaw grasp	P3	100%	100%	60%
Forepaw place	P1	89%	100%	70%
Hindpaw place	P4	39%	100%	60%
Cliff aversion	P2	44%	100%	70%
Righting response	P6	94%	54%	100%
Negative Geotaxis	P11	72%	100%	80%
Neuromotor test	**Postnatal Day**	**Saline control mean time (s) ± SD**	**IgG-Control mean time (s) ± SD**	**MCAD mean time (s) ± SD**
Walking latency	P10	11.6 ± 9.6	9.2 ± 4.5	8 ± 6.6
	P14	4.5 ± 4.0	3.2 ± 1.9	8.1 ± − 12.9[a]
Pivoting	P10	9.1 ± 8.2	6.5 ± 3.9	8.1 ± 7.8
	P14	7.6 ± 4.1	5.1 ± 2.9	6.5 ± 3.8
Homing	P14	29.8 ± 43.8	31.5 ± 46.6	54.4 ± 57.4

*Postnatal day on which 100% of either Control or MCAD population achieved milestone.
[a] Marked difference in Walking latency at P14.

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d) Fine (stereotypic) movements: no differences among groups were identified in the number of movements observed in five-minute intervals over 60 min.

3.1.3. Elevated plus-maze testing

3.1.3.1. Percent time on open arm. The elevated plus-maze test was used to evaluate for the presence of anxiety-like responses. Fig. 3a shows the percentage of session time spent on the open arms for both adolescent and adult groups. One-way ANOVA showed differences in both the adolescent ($p=0.012$; $df=2$; $F=5.12$) and adult ($p<0.001$; $df=2$; $F=20.8$) groups. Post-hoc testing in the adolescent cohorts xshowed MCAD mice spent significantly less time on the open arm of the maze ($n=10$; mean ± SEM; 49.4 ± 3.8% of time) than IgG-controls ($n=12$; 63.6 ± 3.1% of time; $p=0.004$), but not simple-controls ($n=12$, 54.6 ± 2.7% of time; $p=0.262$). In adults, post-hoc testing showed MCAD mice spent significantly less time on open arms than controls: MCAD, $n=10$; 28.5 ± 2.3% of time; IgG-controls, $n=12$; 47.6 ± 4.3% of time; $p<0.001$; simple-controls $n=12$; 55.9 ± 2. 9% of time, $p<0.001$, suggesting a persistence of greater anxiety-like behavior.

3.1.3.2. Number of transitions. In adolescent mice only, one-way ANOVA showed a difference ($p=0.018$; $df=2$; $F=4.6$; see Fig. 3b) in the number of transitions between closed and open arms — a finding indicative of enhanced general motor activity; MCAD (28.3 ± 2.7, mean ± SEM) > IgG-controls (20.4 ± 2.7; $p=0.008$), MCAD > simple-controls (21.6 ± 1.0, $p=0.021$).

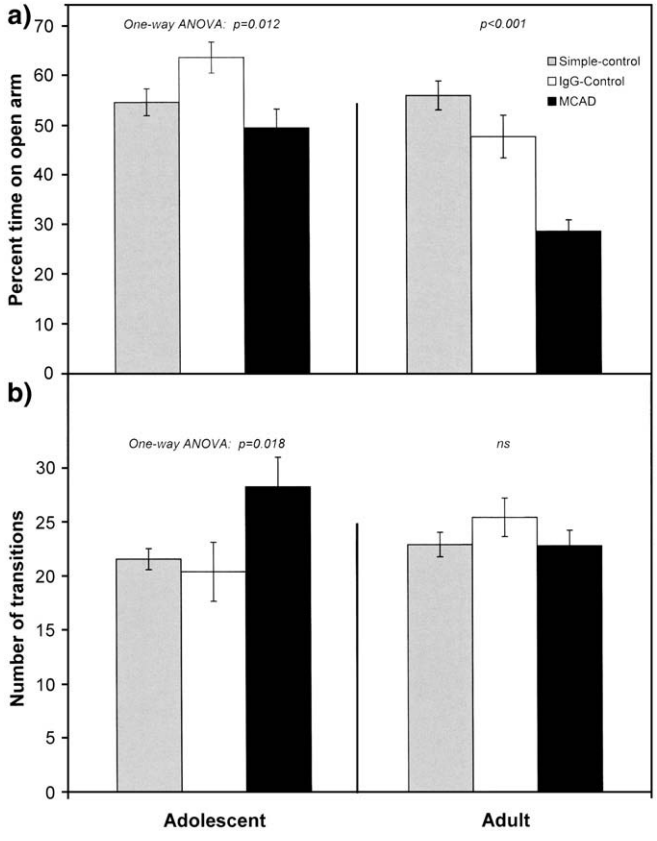

Fig. 3. Performance in the elevated plus maze test. Simple-control (■), IgG-control (□), and MCAD (■) mice.

a). Data shown are the mean ± SEM for percent time spent on the open arm for each group. Significant differences for one-way ANOVA are shown. See text for post-hoc results.

b). Data shown are the mean ± SEM for number of transitions between test arms for each group. Significant differences for one-way ANOVA are shown. See text for post-hoc differences.

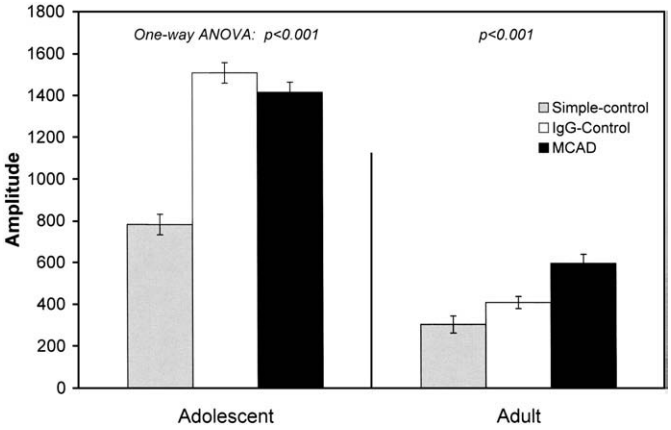

Fig. 4. Amplitude of startle activity in adolescent and adult Simple-control (■), IgG-control (□), and MCAD (■) mice. Data shown are mean (±SEM) for each group. Significant differences for one-way ANOVA are shown. See text for post-hoc differences.

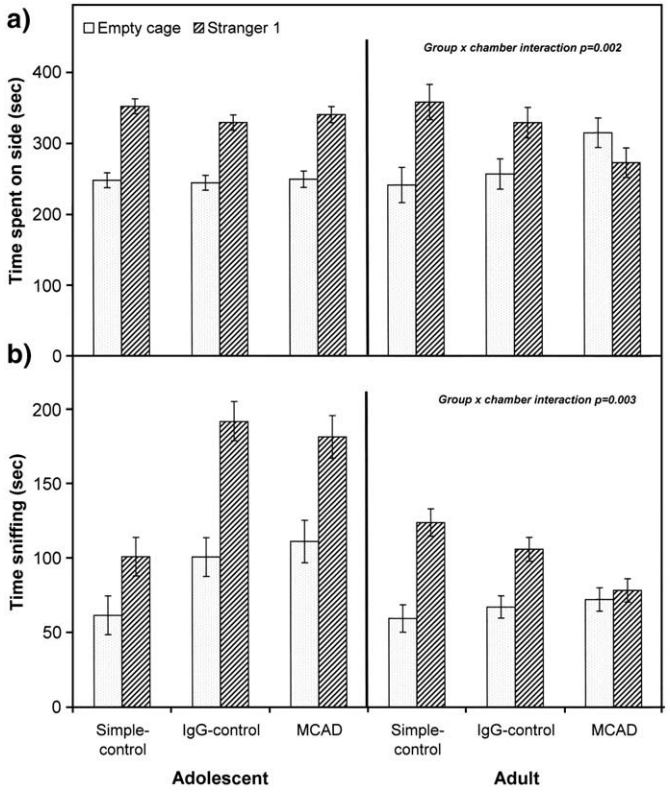

Fig. 5. Sociability test.

a). Duration of time spent on side with an empty cage or stranger 1. Data shown are mean (±SEM) for each group. Two-way ANOVA findings are presented. On within-group comparisons it is expected that "normal" animals will show a statistically greater amount of time with a live conspecific (stranger 1) versus an empty cage. In adult animals, the MCAD group spent more time with the empty cage than with stranger 1. See text for Group × Chamber interaction post-hoc Holm-Sidak comparisons.

b). Time sniffing an empty cage or stranger 1. Data shown are mean (±SEM) for each group. Two-way ANOVA findings are presented. On within-group comparisons it is expected that "normal" animals will show a statistically greater amount of time sniffing a live conspecific (stranger 1) versus an empty cage. In adult animals, the MCAD group spent equal time sniffing the empty cage and stranger 1. On within-group comparisons in adolescent animals showed greater time with or sniffing stranger 1 than the empty cage. See text for Group × Chamber interaction post-hoc Holm–Sidak comparisons.

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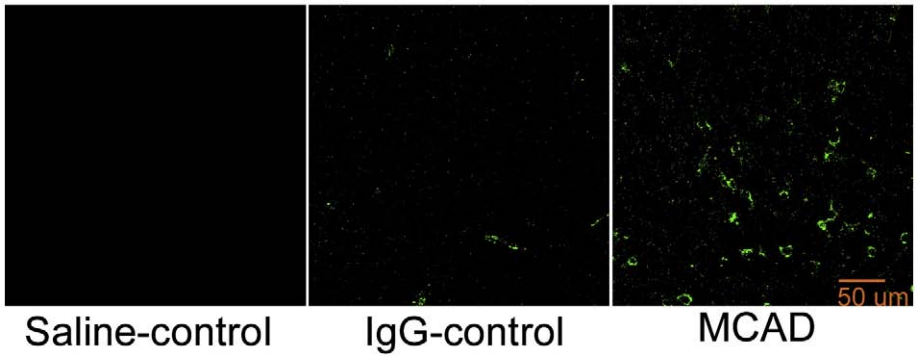

Fig. 6. Secondary staining for human IgG in E18 brain. Sections from saline-control, IgG-control, and MCAD groups. 40× magnification.

3.1.4. Acoustic startle and pre-pulse inhibition

3.1.4.1. Acoustic startle. One-way ANOVA comparisons of startle response to a strong auditory stimuli (30 ms 120 dB noise) showed differences among both the adolescent ($p<0.001$; $df=2$; $F=65.1$) and adult ($p<0.001$; $df=2$; $F=16.0$) groups (Fig. 4). Post-hoc testing in the adolescent cohorts showed MCAD mice ($n=12$; (mean ± SEM) 1415.5 ± 48.9 relative units) had a great startle response than simple-controls ($n=12$; 782.3 ± 48.9; $p<0.001$), but not IgG-controls ($n=12$; 1508.6 ± 48.9; $p=0.497$). In adults, ANOVA comparisons were significant ($p<0.001$). Post-hoc testing in the adults showed MCAD mice to have a greater magnitude of startle response than both control groups: MCAD, $n=17$; 595.1 ± 34.3 relative units; IgG-control, $n=15$; 408.2 ± 36.5 relative units; $p<0.001$; simple-controls, $n=12$; 303.4 ± 40.9 relative units, $p<0.001$.

3.1.4.2. Pre-pulse inhibition of the acoustic startle. In both adolescent and adult groups, controls (IgG and simple) and MCAD cohorts demonstrated comparable levels of pre-pulse startle reduction at the 5 different intensities (74; 78; 82; 86 and 90 dB) of pre-pulse (data not shown).

3.1.5. Sociability

Fig. 5 presents the individual group results for duration of time spent in each chamber (with empty cage or stranger 1) and time sniffing (empty cage or stranger 1).

i) Adolescents:
   a) Time with: in adolescent mice, MCAD ($n=10$), IgG-controls ($n=12$), and simple-controls ($n=12$), two-way ANOVA analysis showed only a Chamber effect ($p<0.001$; $df=1$; $F=110.7$). All groups spent significantly more time on the side with stranger 1, as is normal (Crawley, 2007; Moy et al., 2007).
   b) Sniffing: two-way ANOVA showed a Group effect ($p<0.001$; $df=2$; $F=16.2$) and a Chamber effect ($p<0.001$; $df=1$; $F=37.0$), but no Group × Chamber interaction.

ii) Adults:
   a) Time with: in the adult cohorts, MCAD ($n=17$), IgG-controls ($n=18$), and simple-controls ($n=12$), two-way ANOVA analysis showed a Chamber effect ($p=0.009$; $df=1$; $F=7.2$) and Group × Chamber interaction ($p=0.002$; $df=2$; $F=6.9$). Post-hoc analysis of Group × Chamber interaction showed that adult mice in the IgG-control and simple-control groups spent significantly more time on the side with a live mouse, which is considered normal (Crawley, 2007; Moy et al., 2007): IgG-control live mouse (mean ± SEM) 329.1 ± 21.4 vs. empty cage 256.6 ± 21.4, $p=0.019$; and simple-control, live mouse, 358.0 ± 24.8 vs. empty cage 241.0 ± 24.8, $p=0.001$. In contrast, adult MCAD spent *less* time with the live mouse, 272.6 ± 20.8, than with the empty cage, 314.9 ± 20.8, $p=0.15$, though this difference in the opposite direction was not significant.
   b) *Sniffing*: Two-way ANOVA showed a Chamber effect ($p<0.001$; $df=1$; $F=29.0$) and a Group × Chamber interaction ($p=0.003$; $df=2$; $F=6.0$). Post-hoc analysis of the Group × Chamber interaction showed adult mice in IgG-control and simple-control groups spent significantly more time sniffing a live mouse, which is considered normal (Crawley, 2007; Moy et al., 2007): IgG-control: live mouse (mean ± SEM) 106.1 ± 8.0 vs. empty cage 67.3 ± 7.5, $p=0.001$; and simple-control: live mouse, 124.0 ± 9.2 vs. empty cage 59.5 ± 9.2, $p>0.001$. In contrast, MCAD mice spent equal time sniffing the live mouse, 78.5 ± 7.8, and the empty cage, 72.4 ± 7.8.

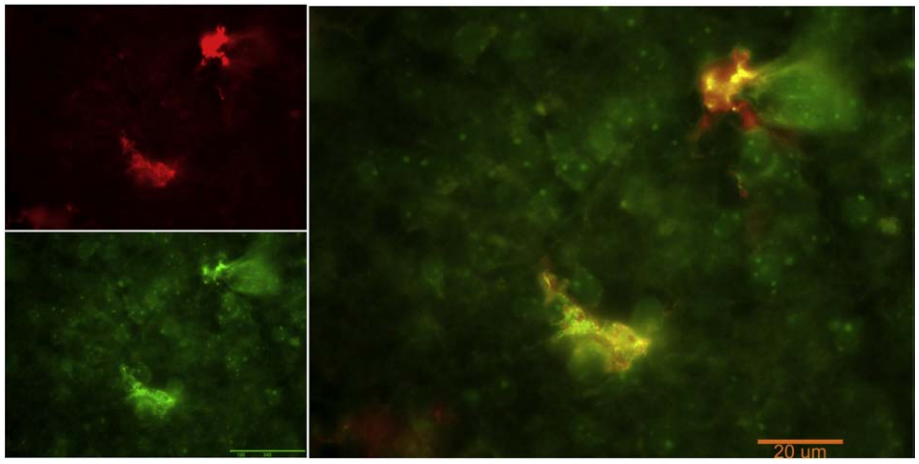

Fig. 7. Microglia staining in E18 MCAD fetal brain. Left upper insert, Iba1 stained; left lower insert, lectin stained; right insert, merged. 100× magnification.

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Table 5
BDNF and cytokine measurements during development.

	Saline-control	IgG-control	MCAD
E16			
BDNF (pg/mg)	10.2 ± 1.3	12.5 ± 4.0	11.4 ± 2.2
IL12 (pg/mL)	nd	nd	8.2 ± 2.9 (4/5)
TNFα (pg/mL)	1.3 ± 0.2 (5/5)	1.9 ± 0.2 (5/5)	2.2 ± 0.1 (5/5)
IL2 (pg/mL)	3.5 (1/5)	5.4 ± 1.8 (5/5)	7.7 ± 3.4 (5/5)
E18			
BDNF	14.8 ± 2.4	13.7 ± 4.5	9.9 ± 1.4
IL12	nd	8.8 (1/5)	6.5 ± 1.9 (2/5)
TNFα	1.7 ± 0.5 (3/5)	1.2 ± 0.1 (2/5)	1.7 ± 0.6 (4/5)
IL2	1.9 ± 0.8 (2/5)	3.7 ± 2.6 (2/5)	1.5 (1/5)
Adolescent			
BDNF	10.8 ± 1.2	10.7 ± 0.9	14.5 ± 3.4

BDNF measured in picogram per milligram total brain protein; Cytokines measured in picogram per milliliter homogenized brain tissue.
nd; not detectable. Numbers in parenthesis next to cytokine values indicate the number of individual samples having a detectable value.

3.2. Fetal brain evaluations

3.2.1. Nissl staining

No gross anatomical abnormalities were observed in fixed sections among the three treatment groups (saline-controls, IgG-controls, MCAD) in either embryonic or adult brain.

3.2.2. Anti-human IgG staining of mouse sections

Anti-human IgG staining was absent in embryos from pregnant dams injected with saline, but present in those from dams receiving IgG from MCAD and controls (Fig. 6). No specific binding pattern was identified.

3.2.3. Microglia staining

No microglial staining was present in E16 embryos. On E18, on gross observation there appeared to be a greater number of Iba1 positively stained cells, confirmed by lectin binding, in MCAD embryos as compared to both IgG-controls and saline-controls (see Fig. 7).

3.2.4. BDNF sandwich ELISA

Brain BDNF levels were measured on E16, E18, and in adolescent mice in five samples from MCAD, IgG-controls, and saline-controls. Values, expressed as mean ± SD pg BDNF per mg protein, are presented in Table 5. One-way ANOVA was significant only in adolescents ($p = 0.028$; $df = 2$; $F = 4.9$). Post-hoc Tukey showed: MCAD>IgG-controls, $p = 0.044$; MCAD>simple-controls, $p = 0.048$.

3.2.5. Cytokines

Results of cytokine measurements on 5 fetal brain tissue samples per group at E16 and E18 were limited because many values fell below the detectable range (Table 5). On *E16*: IL-12 was only detected in MCAD samples. One-way ANOVA of TNFα was $p < 0.001$ ($df = 2$; $F = 47.4$). Tukey post-hoc analyses showed that both MCAD and IgG-control mice had higher TNFα values than simple-control mice ($p < 0.001$), but did not differ from each other. IL-2 was not statistically analyzed because of the limited number of positive saline controls. On *E18*, there were insufficient values to permit meaningful comparison, i.e. data were available in only 3 or fewer samples.

4. Discussion

The goal of this study was to investigate the hypothesis that placentally-transferred antibodies are pathogenic in neurodevelopmental disorders. More specifically, we sought to determine whether, in a pregnant mouse model, the transfer of MCAD IgG could induce a behavioral disorder that mimics some symptoms seen in autism. Although many human behavioral signs and symptoms cannot be replicated in mice, evaluations of activity, social interaction, anxiety, response to sensory stimuli, and motor stereotypies have been used to identify abnormal behaviors in genetic mouse models of autism (Andres, 2002; Moretti et al., 2005; Moy et al., 2007; Ricceri et al., 2007).

Measures of behavior in mice prenatally exposed to MCAD IgG differed from controls (IgG-control and simple-control). In adolescent MCAD-exposed mice, activity, as determined by novelty-induced reactive movements in the central region of the open field and by the transition component of the elevated plus-maze testing, was increased. Anxiety-like behavior, indicated by a reduction of time spent in the open-arm of the elevated plus-maze, was present in the adult MCAD-exposed mice. This finding of anxiety-like behavior was not confirmed by measurement of time spent in the periphery of the open field, however, the latter is generally believed to be a less accurate indicator (Crawley, 1999). Hence, it appears unlikely that the underlying mechanism for increased central activity in the open field is related to anxiety-like behavior. A greater magnitude of startle following acoustic stimuli in MCAD adult, but not adolescent, mice could represent enhanced excitability, excessive fear, or an altered emotional state (Lang et al., 1990). In contrast, the normal response to pre-pulse inhibition in all groups provides evidence for an intact inhibitory sensory gating capability (Chen and Toth, 2001).

The assessment of social interaction provides insight into core symptoms seen in autism as well as other developmental disorders. In this pregnant dam model, sociability, i.e., the tendency to spend time with someone similar to oneself (conspecific), was used in the evaluation of social behavior (Moy et al., 2007). Adult mice that were prenatally exposed to MCAD-IgG showed differences from IgG- and simple-controls. We speculate that changes in social behavior identified only in adult MCAD-exposed mice represent an age-related alteration. The concept of emerging symptoms appearing with maturation is similar to that found in neurodevelopmental disorders in humans and animal models of intrauterine inflammation. Examples of the latter include the late onset of impairment in learning and memory in a lipopolysaccharide-exposed mouse model (Golan et al., 2005) and the development of abnormal social interactions in offspring of pregnant rodents injected with poly(I:C) (Shi et al., 2003). Whether anxiety had any role in altering adult mouse social behavior will require further study.

Rodent and monkey models have previously shown that maternal IgG is capable of altering development in offspring. The injection of serum containing anti-Purkinje cell antibodies from a mother of an autistic child into gestating mice caused the exposed offspring to develop a variety of behavioral changes and alteration of stationary rod performance (Dalton et al., 2003). Serum samples from mothers with two or more children with dyslexia injected into pregnant mice caused the offspring to have deficits in motor tests that correlated with changes of cerebellar metabolites (Vincent et al., 2002). In a rhesus monkey model, animals exposed on gestational days 27, 41, and 55 (of an estimated 165 day gestation) to pooled maternal IgG, from mothers of children with autistic spectrum disorders, developed higher levels of stereotypic motor activity after being removed from their normal environment and placed in a novel setting (Martin et al., 2008). In both the rhesus monkey and our pregnant dam model, the use of pooled maternal IgG samples negates the ability to determine whether each contributing mother is providing a behavioral-altering antibody. Further, it is unknown whether every animal, or human fetus, exposed to a putative triggering antibody would develop behavioral symptoms. We hypothesize that rather than a direct association, there is a complex relationship between maternal anti-fetal brain antibodies and intrauterine genetic, metabolic, and environmental factors.

In mouse models of maternal immune activation, whether by the injection of influenza, double-stranded RNA poly(I:C), or bacterial lipopolysaccaride, the resulting disruption of fetal brain development and behavior is proposed to be secondary to the induction of cytokines (Ashdown et al., 2006; Gayle et al., 2004; Meyer et al., 2006; Ozawa et al., 2006; Smith et al., 2007). The precise pathological mechanism for

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behavioral findings in the pregnant dam model is unknown. Exploratory investigations performed on MCAD IgG-exposed E16, but not E18, brain tissue showed an elevation of IL-12 as compared to both IgG-control or saline-control samples. TNFα was greater in both IgG-exposed groups as compared to saline-controls. IL-12 is produced by dendritic cells and macrophages and acts as a regulator of cell-mediated immunity (Trinchieri, 2003). TNFα is a T helper1-secreted proinflammatory cytokine associated with neuroinflammation and tissue damage (Locksley et al., 2001). Further confirmation of microglial activation (Iba1 staining) in MCAD IgG-exposed animals would support the possibility of immune involvement. Lastly, it is intriguing to note that initial findings of elevated concentrations of BDNF in MCAD-exposed adolescent mouse brain are consistent with reports of higher levels in the basal forebrain (Perry et al., 2001) and blood (Connolly et al., 2006; Nelson et al., 2001) of autistic patients. BDNF has a major role in serotonergic function, by promoting the survival and maturation of serotonergic neurons and their axons (Djalali et al., 2005; Grider et al., 2005; Mamounas et al., 1995).

This preliminary report provides further evidence for the possibility of an immune mediated mechanism affecting the brain in some patients with neurodevelopmental disabilities. It is premature, however, to conclude that the transplacental passage of maternal antibodies is the etiology for autism, even in a subgroup of children with the disorder. As an initial report, this study has several limitations including the behavioral evaluation of a relatively small number of offspring obtained from 13 MCAD-IgG and 13 IgG-control injected pregnant mice, the lack of longitudinal assessment of the same exposed animals during early adolescent and adult developmental stages, the absence of a saline-control for behavioral testing, differences in total IgG exposure in experimental and control groups, and the future need to perform additional tasks designed to evaluate further social behavior, learning, memory, and communication skills. The possibility of a gender-specific behavioral and immunological alteration (Schneider et al., 2008) also requires investigation. Nevertheless, this study does confirm that maternal antibodies from MCAD can cause symptoms in offspring that are similar to those seen in childhood neurodevelopmental disorders. Further investigations should lead to a greater understanding of the biological mechanism, the establishment of screening assays and development of preventive therapies.

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