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Autism-relevant social abnormalities in mice exposed perinatally to extremely low frequency electromagnetic fields

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ABSTRACT

The incidence of autism spectrum disorders (ASD) has been rising, but the causes of ASD remain largely unidentified. Collective data have implicated the increased human exposure to electromagnetic fields (EMF) in the increasing incidence of ASD. There are established biological effects of extremely low-frequency (ELF) EMF, but the relation to ASD is not investigated enough. In this study we examined the effects of perinatal exposure to ELF EMF on some ASD-relevant behavioral parameters in mice. The EMF was delivered via a Helmholtz coil pair. Male BALB/C mice were used and divided into exposed and control groups ($n = 8$ and $n = 9$, respectively). Tests were used to assess sociability, preference for social novelty, locomotion, anxiety, exploratory behavior, motor coordination, and olfaction. The examined mice were all males and exposed to EMF during the last week of gestation and for 7 days after delivery. The exposed mice demonstrated a lack of normal sociability and preference for social novelty while maintaining normal anxiety-like behavior, locomotion, motor coordination, and olfaction. Exposed mice also demonstrated decreased exploratory activity. We concluded that these results are supportive of the hypothesis of a causal link between exposure to ELF–EMF and ASD; however, replications of the study with further tests are recommended.

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1. Introduction

The incidence of autism spectrum disorder (ASD) has significantly and progressively increased during the last few decades (Centers for Disease Control and Prevention, 2014). Some studies have shown that there might be a component of an actual rise in the incidence of ASD rather than being entirely a result of changes in the diagnostic practices (Fombonne, 2003; Grether et al., 2009; Newschaffer et al., 2005). This potentially implicates an environmental factor, which remains unidentified. Many injurious events during gestation and perinatal periods have been recognized as risk factors for developing ASD (Lathe, 2008).

The rise in the incidence of ASD can be seen to coincide with increased human exposure to extremely low frequency electromagnetic fields (ELF EMF) from electrical appliances and cables (Thornton, 2006). The increased exposure to EMF is possibly one of the most dramatic and progressively escalating changes humans have had, in terms of environmental exposure, in the last few decades. Several papers have been published to demonstrate the plausibility of a causal link between the increased exposure to electromagnetism and the rise in the incidence of ASD (Kane, 2004; Lathe, 2008; Mariea and Carlo, 2007). A large number of studies have demonstrated that ELF EMF can have some biological (Giudice et al., 2007; Salehi et al., 2012; Tasset et al., 2012) and biochemical effects (He et al., 2013; Sadeghipour et al., 2012; Ulku et al., 2011), as well as effects on the behavior (Al-Maliki and Al-Rawi, 1992; Lai et al., 1998; Seppia et al., 2003; Sienkiewicz et al., 1998) and brain function (Choleris et al., 2001; Reyes-Guerrero et al., 2006; Sakhnini et al., 2012; Vázquez-Garcı′a et al., 2004) of rodents. Some studies also examined the effects of EMF on human behavior and brain (Cook et al., 2002; Legros and Beuter, 2005; Legros et al., 2006; Marino et al., 2004); however, there has been no experimental study addressing the possible causal link between EMF and ASD.

In this study, we investigated the effects of exposure to ELF EMF during the perinatal period on some measures of social behavior attributed to ASD.

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2. Methodology and materials

2.1. EMF exposure

The EMF was delivered via a Helmholtz coil pair. Each of the coils is 40 cm in diameter, has 154 turns, and the coils are separated at a distance of 20 cm. The coils were energized with a generator of standard signals, providing stability of voltage and frequency with a precision of significant digits. A sinusoidal current (50 Hz) was passed through the magnet, producing relatively homogenous alternating magnetic field with an average (B) of 1 mT (measured by a PHYWE Tesla meter probe, PHYWE System GmbH & Co. KG, Germany). Magnetic force lines were parallel to the horizontal component of the local geomagnetic field. The background magnetic field of 50 Hz did not exceed 1 nT as measured using a Multidetector II (Gewerbegebiet Aaronia AG, Germany) with a sensitivity of 1 nT.

2.2. Animals

Male BALB/C mice were used. The animals were divided into two groups. The exposed group of mice ($n = 8$, from 4 different mothers) were exposed continuously to the ELF EMF from the last week of gestation and continued for one week after birth. The control group ($n = 9$, from 4 different mothers) were placed in the same boxes and experimental setup, but no EMF was applied. All behavioral tests were performed when the animals were 8–11 weeks of age. All experimental procedures abided by animal care ethics of Arabian Gulf University/Manama/Bahrain.

2.3. The tests

Behavioral tests were conducted between 9:00 and 18:00. All behavioral tests were conducted and scored blindly. All sessions, except the rotarod and olfaction test, were videotaped and scored accordingly. Each parameter was scored by one observer for all mice to eliminate any potential inter-observer variability. Each mouse was tested a maximum of one trial per day.

2.3.1. Three-chambers social apparatus (Crawley's sociability and preference for social novelty test)

Sociability and preference for social novelty was assessed using the Three-chambers social apparatus, as described previously (Kaidanovich-Beilin et al., 2011; Nadler et al., 2004). The apparatus comprised a rectangular, three-chambered box. Each chamber was 20 cm × 40 cm × 22 cm. The walls were made from clear Plexiglas. The dividing walls (made from the same material) had small square openings (5 cm × 3 cm) allowing access into each chamber. Each chamber contained a circular wire cage which was 11 cm high, with a bottom diameter of 9 cm and bars spaced 0.5 cm apart. The subject mouse was first placed in the middle chamber to habituate for 5 min. Session 1 was started when an unfamiliar male (stranger 1) that had no prior contact with the subject mouse was placed inside the wire cage in one of the side chambers while the subject mouse was allowed to explore the entire apparatus freely. The placement of stranger 1 in the left or right side chambers was systematically alternated between trials. Session 1 continued for 10 min, and the time spent in each chamber as well as the number of chamber entries were recorded. Immediately after the 10 min of session 1 finished, session 2 started with a second unfamiliar mouse being placed in the wire cage inside the chamber that had been empty during the first session. The test mouse had a choice between the chamber containing the already investigated mouse (stranger 1), and the one containing the novel unfamiliar mouse (stranger 2). The same parameters recorded for session 1 were recorded for session 2. The apparatus was cleaned with 70% ethanol and water between subjects. Three trials were done for each mouse. Session 1 tests for sociability (or social motivation/affiliation), which is spending significantly more time in the chamber containing a mouse than in the empty chamber. Session 2 tests for preference for social novelty, which is spending significantly more time in the chamber containing the novel mouse than the one containing the already investigated mouse.

2.3.2. Open field test

Locomotor activity and anxiety-like behavior were measured using an open field test, as mentioned previously (Gould et al., 2009). Each mouse was placed in next to the wall of the open field apparatus (44 cm × 44 cm × 32 cm), facing away from the experimenter. The chamber of the test was illuminated at 150 lux (measured in the center of the apparatus). The arena was divided into 16 even squares. Distance traveled (number of squares crossed) and time spent in the central zone (the central four squares) were recorded. Distance traveled was the measure for locomotor activity, and reduced time spent in the central zone was indicative of elevated level of anxiety. Test duration was 10 min. Three trials were done for each mouse. Test chambers were cleaned with 70% ethanol between subjects.

2.3.3. Elevated plus maze test

The elevated plus maze test is for anxiety-like behavior (Komada et al., 2008); it consisted of two open arms (25 cm × 5 cm) and two enclosed arms of the same size with 15-cm-high walls. The arms were elevated 55 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 3-mm-high walls surrounded the sides of the open arms. Arms of the same type were located opposite from each other. Each mouse was individually placed in the central square of the maze (5 cm × 5 cm), facing one of the closed arms and allowed to freely explore the apparatus. Mouse behavior was recorded during a 10-min test period. The number of entries into an arm and the time spent in the open and enclosed arms were recorded. Percentage of entries into open arms, time spent in open arms (s), and total number of entries were analyzed. Entering the open arms less frequently and spending less time in them were indicative of anxiety-like behavior. The apparatus was cleaned with 70% ethanol between subjects. An open- or closed-arm entry was defined as all four paws in an arm. The numbers of open- and closed-arm entries were combined to yield a measure of total entries, which reflected general activity during the10 min test.

2.3.4. Hole-board

Exploratory activity was estimated by a hole-board test. The apparatus consisted of a wooden board measuring 40 × 40 with 16 evenly spaced holes (3 cm in diameter). The board was surrounded by 25-cm-high wooden wall and was raised 10 cm above the floor. Each subject mouse was individually placed in the center of the board, facing away from the experimenter, and left to move freely for 10 min. The experiments were videotaped, and the videos were watched by a blind observer recording the number of times the mice dipped their heads into the holes to the level of their eyes. Head dipping was thought to reflect exploratory behavior.

2.3.5. Olfactory habituation/dishabituation

Olfactory abilities were assessed using the olfactory habituation/dishabituation assay (Yang and Crawley, 2009). Prior to the start of testing, each mouse was placed into a clean standard cage containing fresh bedding and a plain cotton swab tip suspended from the cage lid. After a 45 min acclimation period, olfactory testing began. Subjects were tested for time spent sniffing cotton swab tips saturated with familiar and unfamiliar odors, with and without social valence. Sequences of three identical swab tips assayed

habituation to the same odor. A different odor presented on the swab tip assayed dishabituation, i.e. recognition that an odor is new. Swab tips were dipped in (1) distilled water, (2) almond extract (McCormick, Hunt Valley, MD; 1:100 dilution), and (3) banana flavoring (McCormick, Hunt Valley, MD; 1:100 dilution) to represent a range of non-social odors. Swabs were wiped across the bottom surface of a plastic cage that contained (4) four sex-matched unfamiliar mice of a different strain, 129S1/SvImJ, and (5) four sex-matched unfamiliar mice of another different strain, C57BL6/J, to represent two distinct social odors. Each swab was presented for a 2 min period, immediately following the last swab presentation, for a total session length of approximately 30 min per mouse.

2.3.6. Rotarod

Motor coordination and balance were assessed using an accelerating rotarod. Mice were placed on a cylinder that rotates at pre-assigned speeds. Each mouse was tested for three trials on three speeds; 40, 50, and 60. Latency to fall from the rotating rod was recorded with a maximum trial length of 300 s. The two groups of mice were tested within the same experiment to allow comparison of baseline motor performance. All testing was done on the same day.

2.4. Statistical analysis

All data are presented as Mean ± SEM. Differences in the performances between the groups were tested by using *t*-test, and, for the experimental tests that have multiple trials, two-way repeated measures ANOVA was used to calculate the *p*-value for the difference in performance between the two sample groups. Effect size is given for the statistically significant differences, using eta squared. Statistical analysis was conducted using SPSS package (version PASW Statistic 18.0.3).

3. Results

3.1. Lack of sociability and preference of social novelty in exposed mice

In the three-chambers social apparatus, the control mice demonstrated normal sociability by spending more time in the chamber containing the mouse than in the chamber containing an empty cage in session 1 (Fig. 1A; ANOVA, $F_{(1,49)} = 24.8894$, $p < 0.0001$, $\eta^2 = 0.4$). In session 2, the control mice demonstrated normal preference for social novelty by spending more time in the chamber containing the novel mice than in the chamber containing the already investigated mouse (Fig. 1B; ANOVA, $F_{(1,49)} = 16.9065$, $p < 0.001$, $\eta^2 = 0.18$). On the other hand, exposed mice did not spend significantly different time in the chambers in both session 1 (Fig. 1A, ANOVA, $F_{(1,49)} = 0.00023$, $p = 0.988$) and session 2 (Fig. 1B, ANOVA, $F_{(1,49)} = 1.8$, $p = 0.186$).

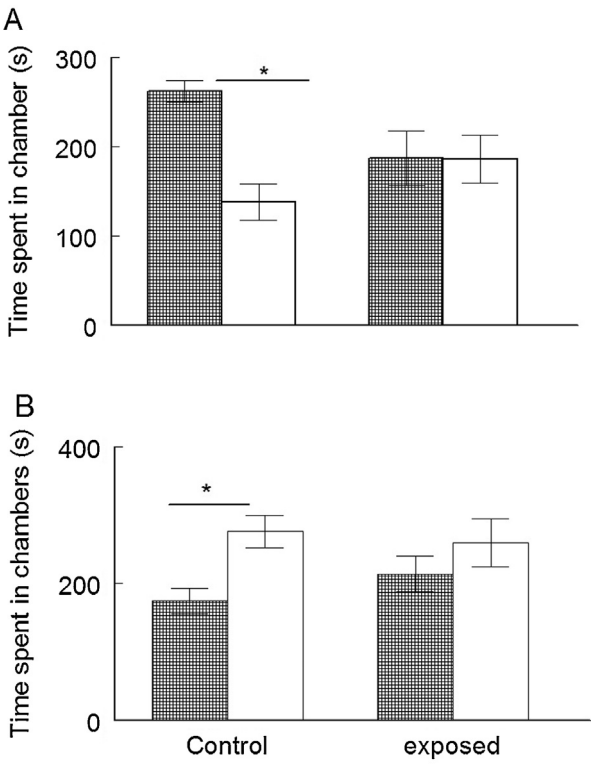

Fig. 1. (A) Time spent in the side chambers in session 1. Control mice (the two bars on the left) showed normal sociability, spending more time in the chamber containing the mouse (striped bar) than in the chamber containing empty cage (white bar). Exposed mice (the two bars on the right) showed deficit in sociability, failing to spend more time in the chamber containing the mouse than in the chamber containing empty cage. (B) Time spent in the side chambers in session 2. Control mice (the two bars on the right) showed normal preference for social novelty, spending more time in the chamber containing the novel mouse (white bar) than in the chamber containing the mouse that was already-investigated in the previous session (striped bar). Exposed mice did not show normal preference for social novelty, failing to spend more time in the chamber containing novel mouse than in the chamber containing the mouse that was already-investigated in the previous session.

3.2. Normal locomotion in exposed mice

Locomotion was examined using the open field test. No significant difference was found between exposed and control mice in total distance traveled in the open field test (Fig. 2A, ANOVA, $F_{(1,49)} = 1.165$, $p = 0.285$). There was also no significant difference in distance traveled in the first 5 min of the test (ANOVA, $F_{(1,49)} = 0.0003$, $p = 0.985$, data not shown).

3.3. Exposed mice do not display anxiety-like behavior

Anxiety-like behaviors were assessed using the open field test and the elevated plus maze test. No significant difference was detected between exposed and control mice for time spent in the central zone of the open field test (Fig. 2B, ANOVA, $F_{(1,49)} = 1.06$, $p = 0.3$) or percentage of locomotion in the central zone (Fig. 2C, ANOVA, $F_{(1,49)} = 0.936$, $p = 0.338$). In the elevated plus maze test, no significant difference was detected for the duration of time spent in the open arm (Fig. 2D, *t*-test $p = 0.368$), percentage of entries to the open arm (Fig. 2E, *t*-test $p = 0.336$), or total entries into the open and closed arms (Fig. 2F, *t*-test $p = 0.977$).

3.4. Exposed mice show decreased exploratory behavior

Exposed mice demonstrated significantly fewer head-dips than control group in the hole-board test (Fig. 3, ANOVA, $F_{(1,49)} = 7.02$, $p < 0.01$, $\eta^2 = 0.226$).

3.5. Normal olfactory abilities in exposed mice

Olfaction was assessed using the habituation/dishabituation olfactory test (Fig. 4). Both exposed and control mice displayed olfactory habituation as indicated by the decline in time spent sniffing on repeated exposures to water, non-social odor 1, non-social odor 2, social odor 1, and social odor 2. Both exposed and control mice displayed dishabituation upon presentation of a new odor as indicated by increase in time spent sniffing from presentation of water to non-social odor 1, non-social 1 to non-social odor 2,

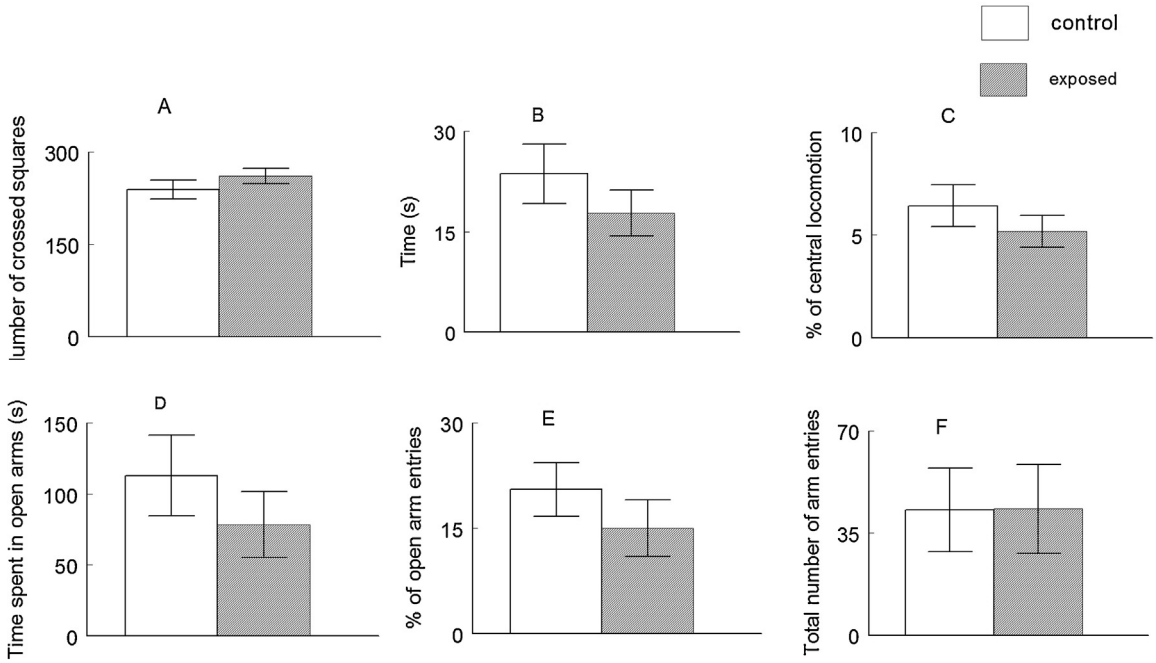

Fig. 2. Normal locomotion and anxiety-like behavior in exposed mice. (A–C) Open field test: total distance traveled (A), time spent in the center of the apparatus (B), and percentage of central locomotion (C) were recorded. (D–F) Elevated plus maze test: time spent on open arms (D), percentage of entry into open arms (E), and number of arm entries (F) were recorded.

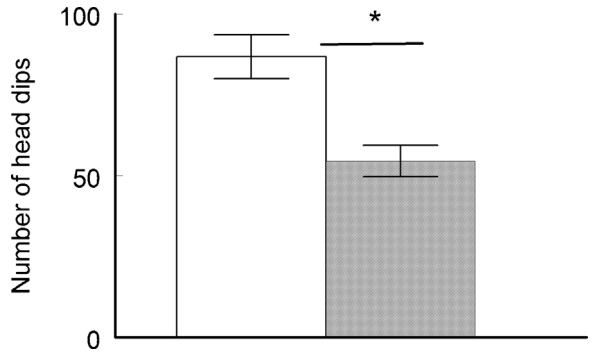

Fig. 3. Exposed mice (gray bar) displayed decreased frequency of exploratory head dips in Hole-Board test.

non-social odor 2 to social odor 1, and social odor 1 to social odor 2.

3.6. Normal motor coordination in exposed mice

There was no significant difference in latency to fall in accelerated rotarod test at speeds 40 (ANOVA, $F_{(1,49)} = 2.8$, $p = 0.59$), 50 (ANOVA, $F_{(1,49)} = 0.49$, $p = 0.82$), and 60 (ANOVA, $F_{(1,49)} = 1.5$, $p = 0.7$). Data not shown.

4. Discussion and conclusion

This study was conducted in order to investigate whether the exposure to ELF EMF of mice in the perinatal period could affect the social behavior of these animals in adulthood. It is known that the final important steps in brain development of mice occur 29.7

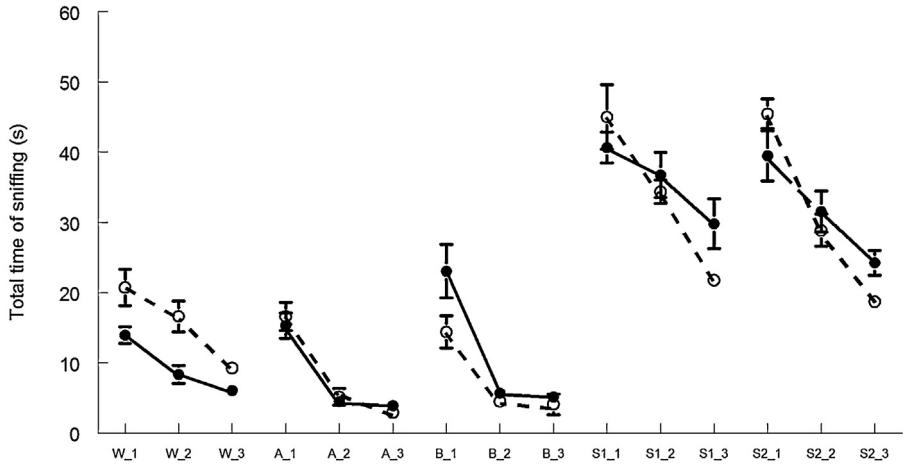

Fig. 4. Normal olfaction displayed by both control (continuous line) and exposed mice (interrupted line) in olfactory habituation/dishabituation test. A decline in sniffing to repeated presentations (habituation) of water (W), two non-social odors (A and B) and two social odors (S1 and S2) was observed in both control and exposed mice. An increase in sniffing upon the first presentation of novel odor (dishabituation) was also observed in control and exposed mice.

days after conception, or postnatal day 12, and the body growth is completed at about age postnatal day 50, which is the reasonable standard for an adult (Finlay and Darlington, 1995).

Mice exposed perinatally to ELF EMF demonstrated a lack of sociability and preference for social novelty when compared to the non-exposed control mice. On the other hand, tests for anxiety-like behaviors, locomotion, olfaction, and motor coordination showed no significant differences from the performance of the control non-exposed mice. Since olfaction is crucial for the social behavior of mice, we tested olfaction to confirm its intactness. If the motor system were affected by the independent variable, it would impact the behavior in the other tests; therefore, locomotion and motor coordination were assessed. Anxiety may inhibit sociability and preference for social novelty as well as exploratory behavior. We included tests that specifically measure anxiety to exclude it from being a confounding factor for the measured difference in social behavior. Disruptions in olfaction, motor system, or anxiety, are not characteristic features of ASD.

The exposed animals showed decreased exploratory behavior of head-dipping in the hole-board test. Such behavior has been regarded in some studies to reflect anxiety-like behavior (Brown and Nemes, 2008; Gao et al., 2011), but since other tests for anxiety in our study showed no significant difference between the exposed and control mice, the significantly decreased number of head-dips is rather likely to reflect decreased exploratory drive than increased anxiety. It was shown that ASD has reduced exploratory behavior (Pierce and Courchesne, 2001). These results are consistent with the hypothesis of a causal link between the increase in the incidence of ASD and increased ELF EMF exposure (Thornton, 2006).

The rationale for exposing the animals to EMF in the perinatal period is that it is a crucial period for brain growth and development, as well as that ASD becomes apparent during the first three years of life, implicating a genetic and/or environmental influence very early in life. The perinatal period is thus the stage during which any influence of EMF would be more likely to cause an early, permanent, and drastic effect (such as that of ASD).

The evidential strength for the link between Autistic behavior and exposure to ELF EMF as a causative factor in this experiment is suggestive but not confirmative; the sample size is small, and confounding factors may not have been entirely excluded. On top of that, the impaired communication and aberrant repetitive behavior (the core features of ASD other than impaired social behavior) were not tested.

Assays for repetitive behavior include observing and recording the frequency and duration of stereotypical motor behaviors (circling, jumping, backflips, self-grooming), and measuring resistance to change in reversal learning tasks using T-maze or Morris water maze (Moy et al., 2006; Silverman et al., 2010). There has been criticism stating that neither of those methods is a proper and legitimate model or assay for autism-relevant repetitive behavior and cognitive rigidity (Belzung et al., 2005). The assay used in the literature to test for communication deficit is measuring ultrasonic vocalization, such as from a pup upon separation from the nest. This was not available in our facility.

There are numerous published papers concerned with studying the potential biological effects of low frequency EMF and investigating possible mechanisms (Ravera et al., 2011; Reyes-Guerrero et al., 2010; Wang et al., 2008). One hypothesis we have chosen to explain our results is that the effects of the perinatal EMF exposure may be manifested in changes in the levels of the neurotransmitter serotonin. Levels of serotonin are partially mediated by Monoamine Oxidase A (MAOA), an enzyme of the mitochondrial outer membrane involved in the metabolism of catecholamine and indolamine neurotransmitters. In one study, the alleles regulating levels of MAOA were found to correlate with ASD symptom severity, with the lowest expressing alleles associated with lower IQs and more severe autistic behavior (Cohen et al., 2003). The MAOA-deficient mice also demonstrate increased freezing in a conditioned fear task (Kim et al., 1997), hyper-responsivity to acoustic stimuli, and reduced exploration in a hole-board test (the latter is reported in our exposed group of mice). All of these are considered to be indicative of a mouse model for ASD.

EMF exposure was described to cause diverse complications including nervousness, stress, anxiety and sleep disturbances (Grahame-Smith, 1992; Price et al., 1990; Siever et al., 1991). These illnesses resemble the "Serotonin syndrome" (Jacobs, 1976). The serotonin receptor 5-HT_{1B} was documented to be involved in behavioral response (Clark and Neumaier, 2001; Groenink et al., 2003; Olivier and van Oorschot, 2005; Olivier, 2004). In addition, 5-HT_{1B} receptor activity was reported to be modulated by exposure to a 50-Hz magnetic field at field strength above 0.6 mT (Espinosa et al., 2006; Massot et al., 2000). In a later report (Masuda et al., 2011), however, a lack of effect of 50-Hz magnetic field exposure on the binding affinity of serotonin for the 5-HT_{1B} receptor was demonstrated.

Autistic behavior was suggested by other mechanisms as well. It was demonstrated in animal models of ASD by perinatal exposure to valproate that there is altered glutamate metabolism in the hippocampus (Silvestrin et al., 2013) or in the endocannabinoid system (Kerr et al., 2013), as well as altered serotonin metabolism (Dufour-Rainfray et al., 2010).

The data presented here may shift the attention towards a potential hazard that humans are living with. The extremely low frequency (50–60 Hz) magnetic field is not hindered by intervening materials such as walls or body tissues, and it induces electric current within the body. Very small distance (cm) from household appliances induce magnetic field strength that may vary from 20 to few hundred μT (Crasson and Legros, 2005). Earth magnetic field, although stronger than magnetic fields produced by many of the appliances, is primarily a Direct Current (static) field rather than time-varying field, which is what is created from the Alternative Current (AC) generated and used by humans. In addition, the effect of earth's magnetic field, if any, has been present unchanged throughout the existence of humans, and the man-made magnetic fields are an emergent additional environmental exposure. Whether this new exposure, which vary considerably in amount, does or does not have an influence on the human body, and whether that influence is potentially detrimental or not, is a matter that needs to be investigated.

This study does not conclude that exposure to ELF EMF is a risk factor or a cause of ASD. This data is not sufficient to support such a conclusion, but it warrants further investigation into the matter. In addition, parameters like social affiliation and motivation are not ASD-specific and may be related to other conditions such as social anxiety disorder, depression, and schizophrenia. Replication of the study, to exclude any confounding factor, with a greater sample size and more behavioral tests relevant to ASD, as well as molecular tests, are recommended.

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