Abstract

Methylmercury (MeHg) exposure from occupational, environmental, and food sources is a significant threat to public health. Pregnancy has long been recognized as a potentially critical window of vulnerability for exposure to a variety of chemicals. MeHg poisonings in adults may result in severe psychological and neurological deficits, and in utero exposures can confer embryonic defects and developmental delays. The objective of this study was to determine whether such exposure produces deleterious effects on behavior in offspring (F1) rat, including motor/coordination abilities and overall activity. Developing rat fetuses were exposed in utero during gestational days 5 till parturition by giving pregnant female rat MeHg at a daily dose of 0.5, 1.0 and 1.5 mg/kg body weight. Dams prenatally exposed to MeHg exhibited significant deficits in motor abilities, coordination, and overall activity, as measured by rotarod, footprint analysis and open field in offspring (F1) indicate that prenatal exposure to MeHg examined to date can have long-lasting motor and cognitive consequences on offspring. Data obtained from Openfield study as well as Functional Observation Battery (FOB) also revealed impairment of neuromotor performance in offspring appeared to be gender specific. These findings have far reaching implications related to putative safe levels of MeHg ingestion, particularly during pregnancy, and increasing rates of cognitive and psychological disorders (e.g. attention hyperactivity deficit disorder, autism) in our society.

Keywords: Methylmercury; Prenatal exposure; Postnatal development; Neurobehavioral toxicity; Open-field behavior; Rat

1. Introduction

Methylmercury (MeHg) is one neurotoxic pollutant that causes much concern to toxicologists and governmental agencies dealing with environmental safety. The pernicious effects of high-level MeHg exposure are well documented by human epidemiological studies that followed poisonings in Minimata Bay (Japan) (from 1953-1960), Niigata, Japan (in 1965) and Iraq (from 1971-1972). The Japanese tragedies were associated with the consumption of MeHg-contaminated fish collected from waters receiving industrial discharge, while the Iraqi occurrence was linked to the consumption of bread that was made from grain intended for crop production and treated with a MeHg fungicide. Many deaths were linked to these incidents and affected adults initially showed signs of paraesthesia, malaise and blurred vision, which was
followed by constricted visual field, deafness, dysarthria and ataxia (Amin-Zaki et al., 1976; World Health Organization, 1976, 1990; Harada, 1997). Quite a number of chemicals polluting the environment, e.g. pesticides, heavy metals, and petroleum products, are neurotoxic. The vulnerability of the nervous system to neurotoxic insults is highest during fetal development and early childhood. Therefore, exposure during these periods, via maternal blood and/or milk, even at levels posing no risk for the mother, may result in neurological and neurobehavioral disorders in the progeny (Grandejan and Landrigan, 2006).

Although humans may be exposed simultaneously to numerous hazards, little is known about the interaction of prenatal chemical exposures with other factors, such as maternal stress, itself a modifier of fetal development. Gestational stress has been hypothesized to enhance the developmental toxicity of chemicals (Hougaard and Hansen, 2007; Gandhi et al., 2012; 2014). It has been recognized that dietary exposure to neurotoxic substances during pregnancy and breast feeding may affect the development of the child's nervous system and result in various neurological and neurobehavioral alterations later in life. One of the suspected consequences of such exposure may be an increased propensity to psychostimulant abuse and psychostimulant addiction. The developing fetus is particularly vulnerable to MeHg because of the toxicant's ease in crossing the placenta and several fold greater toxicant/accumulation relative to the adult (Bakir et al., 1973; Clarkson and Magos, 2006; Myers and Davidson, 1998; Takeuchi, 1982). Toxic levels of MeHg can display a range of symptoms that includes a loss of physical coordination, abnormal speech, and death in adults.

Methyl mercury (MeHg) is a known environmental neurotoxicant that, when ingested, results in sensory and motor deficits. Children exposed to toxic levels either pre- or postnatally display developmental defects that are associated with motor and sensory deficits and mental retardation (Clarkson and Magos, 2006). MeHg toxicity signs and symptoms of chronic exposure include paresthesia, ataxia, and constriction of the visual field, tremor, mental deterioration, and dysarthria (Bakir et al., 1973; Bakir et al., 1980; Harada, 1995). Studies using laboratory animals have also reported ataxia, tremor, paralysis, hind limb spasticity, and decreased muscle tone after adult-onset exposures to MeHg (Burbacher et al., 1987; Jacobs et al., 1977). The consequences of developmental methyl mercury exposure have generated widespread concerns. MeHg can cause severe developmental damage even in the absence of overt maternal symptoms.

Most studies have concentrated on the direct effects of high levels of prenatal MeHg exposure. Surprisingly, behavioral outcomes found in adult offspring exposed developmentally to the neurotoxic effects of chronic, low-dose mercury more akin to ingestion in humans are not well characterized. In addition, the dam is primarily determined the development of major regulatory system underlying behaviour and physiology in the neonatal rat (Huot et al., 2004). Our earlier study (Gandhi et al., 2013) reported that maternal and embryo/foetal toxicity when high dose of MeHg (2.0mg/kg/day) was given by gavages during GD5 till parturition to pregnant rats caused hundred percentage of resorption of the F1 generation offspring. So it further worsened MeHg toxicity even before birth, adding up the impact, depends on exposure, duration, route as well as form of exposure throughout life. Our goal was to develop a rat model of early life MeHg exposure through which we could identify critical windows of exposure that might result in adverse impacts on the development of the nervous system later in life. This fact raised researchers’ interest in studying effects of prolonged low-dose exposure in animal models, representing a chronic pattern of exposure in humans. Thus, the study presented here was designed to further explore the adverse developmental outcomes following early life low
dose MeHg exposure has detrimental impact on later life of development and maternal toxicity leads to change in neurobehavioral outcomes.

2. Materials and Methods

2.1 Animals
Albino rats of Wistar strain (12-14 weeks of age, 180-200 g) were procured from Animal House, National Institute of Occupational Health (NIOH), Ahmedabad, India. Before the start of experiment, animals were kept in laboratory conditions for a period of 7 days for acclimatization. After one week of acclimatization, proestrus virgin female rats, and weighted 180 ± 15 g, were mated with proven fertile male rats (2:1) overnight from our Institutional (National Institute Of Occupational Health, Ahmedabad, India) Animal House Breeding Colony. The day of mating confirmed by the presence of sperm positive vaginal smears, was designated as gestational day (GD) 0. Wistar Albino GD0 confirmed female rats were housed in individual shoebox size polypropylene cages the sterilize bedding. All individual cages were kept in a temperature controlled room at 23 ± 3°C with relative humidity of 55 ± 15% on a 12 h light/dark cycle and 10 to 15 air changes/hr. and given ad libitum free access to food pallets (Pranav Agro Industries Ltd., ISO9001 Certified Company, Maharashtra, India) and Kent RO water. Each food pallet contains: 22-23% protein; 4.20% fat; 3.50% fibre; 2.10% calcium; 1.05% phosphorus; 7.50% total ash; 8.68% moisture and 56% carbohydrate. All experiments were performed between 09.00 and 17.00hr. Pregnant animals were randomly assigned to 4 groups of 7-8 rats each in a house individually. Date of birth was designated as postnatal day (PND) 0.

2.2 Ethical issues
All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) of the National Institute of Occupational Health and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India (CPCSEA, 2003, Registration No.111/RO/c/1999/CPCSEA) and was conducted according to the Indian National Science Academy guidelines (INSA, New Delhi, India) for the use and care of experimental animals, chemical, dose and treatment schedule.

2.3 Study design
Mated female Wistar rats were dosed daily with MeHg from GD5 to till parturition. The effects of exposure on post weaning (PND21 to PND30) observations and testing such as rotilando test (PND20), the open field test (PND28), Forelimb and hindlimb grip test for neuromuscular function (PND28) and functional observation battery (FOB)- landing foot splay (PND28) during the life of Offspring (Fig.1.)

![Study design](image1)

**Fig. 1.** Study design. Mated female Wistar rats were dosed daily with MeHg from GD5 to till parturition. Behavioral parameters were assessed in pups from PND1 to PND35.
2.4 Randomization, mating and treatment

2.4.1 Pregnant rats
After one-week acclimation in the laboratory, proestrus virgin female rats were mated with males [2:1] overnight and examined the following morning for vaginal smears. Vaginal smears were taken daily between 9 a.m. to 10 a.m. from mated females rats. On the day when spermatozoa in the vaginal smear were found, was regarded as the first day of gestation (GD0). The weight of the confirmed pregnant dams (GD0) was recorded on GD0, GD5, GD10, GD15 and GD20 during gestation period. Out of thirty two GD0 females, twenty-eight confirmed pregnant females (GD0) delivered the pups; rest of the four females did not deliver. At GD0, 28 female’s pregnant animals (dams) were randomly assigned to four groups of rats, and housed individually. A summary of the distribution and fate of all mated rats of the study is given in Table 1. Date of birth was designated as postnatal day (PND) 0.

2.4.2 Preparation of dose formulation
The dose formulation for each group was prepared separately in order to maintain a constant dose volume of not more than 5-ml/kg-body weight. Methyl mercury chloride (CH3ClHg) 99.9% pure, CAS no.115-09-3; batch size 8151x, Sigma-Aldrich GmbH) was obtained from Sigma Aldrich, U.S.A. The doses were based on data showing that at this exposure level, the Hg concentration in newborn rats was comparable to that found in human infants from populations with high dietary fish consumption (Amin-Zaki et al., 1979; Rossi et al., 1997). Controls were treated with saline solution.

2.4.3 Prenatal methyl mercury exposure
Twenty eight-28; female pregnant (GD0) confirmed rats (F0 generation) were assigned to four groups, and received methylmercury orally by gavage at dose levels Vehicle Control (n=7); 0.5 mg MeHg/kg body weight/day (n= 8); 1.0 mg MeHg/kg body weight/day (n= 7); 1.5 mg MeHg/kg body weight/day (n= 6) during gestational day (GD) 5 to till parturition respectively according to a 2 (generation) X 2 (Sex) X 3 (methyl mercury exposures) experimental design. Throughout gestation, all pregnant rats were weighed and examined for signs of toxicity daily.

2.5 Open Field Behavior
An open field test was widely used to measure general locomotor and explorative activity on PND 28 (Meerlo et al., 1996). The effects of methylmercury on motor functions were analyzed by recording the locomotors in a square open field arena (50x65x6x25 mm), enclosed in a solid and sound-attenuating box (Columbus Instruments, Ohio,USA). Open-field testing consisted of monitoring the patterns of activity over a minute period when rats were placed in an empty square enclosure 46 cm across. Animal Activity Meter: Opto-Varimex-4 Auto-Track system (Columbus Instruments, Ohio, USA) was used to measure total distance traveled and dwell time spent in the 23-cm center area versus the 11.5-cm perimeter of the open field. The Auto-Track System senses motion with a grid of infrared photocells placed around the arena (typically 17.5" x 17.5" (44.5cm). 16 beams per axis sensors available with 1" (2.54cm) or 0.5" (1.27cm) beam spacing. 17.5" (44.5 cm) Cage comes with base plate of 23.25" x 23.25" (59cm) 9.5" (24 cm) comes with base plate of 12.625" (32.7 cm). Vertical motion is detected by a second array of photocells placed above the animal. The simultaneous interruption of beams along the horizontal axes (X & Y) provides coordinates that identify animal location. Vertical motion is scored and stored with the horizontal position data. Spontaneous activity was measured in an Animal Activity Monitor System (Auto Track) based on the infrared photocell. Different Spontaneous separating counts the infrared light beam interruptions created by the experiment subject associated with ambulatory activity from total activity. Total activity accumulates all
beam interruption information. Subtraction of ambulatory counts from total counts provides an indication of grooming, scratching and other non-ambulatory activities. In addition, photocell sensor array located above the subject provides two levels of vertical activity monitoring: one for rearing or jumps, the other for raised head motions (Hole poke Measurement). The following parameters were recorded by means of a keyboard: 1) exploration, 2) rearing, 3) grooming, and 4) immobility. At the end of the test, the number of faecal boli was recorded.

To minimize the influence of possible circadian changes in pup spontaneous motor activity and open-field behavior, control and experimental animals were alternated, the rats being observed at the same time of day in each session. The open-fields were washed with an alcohol-water solution (5%) before placing the animals to obviate possible biasing effects due to odor clues left by previous rats. The following data were extracted from the cage memory: i) number of ambulatory movements in seconds (horizontal shifts of the rat body, equal to or longer than 4 cm), ii) traveled distance in centimeters, iii) short-distance movements (shifts shorter than 4 cm), and iv) number of rearing. ( Interruption of at least one beam of the upper tier was counted as a rearing episode).

2.6 Rota-rod
Rota-rods (PND-20): A drum of variable diameter (10-20 cm) is rotated at about 4rpm. The rotation speed is increased until the rat falls off the drum. When the increase in velocity is controlled, the time interval from start until the animal falls becomes a measure of motor ability (Spyker and Avery, 1976; Rodier, 1978; Vorhees et al., 1979b). The motor coordination of control and prenatally treated MeHg rat was quantitatively measured using a standard rat accelerating rota-rod (model 0890R Rotamex-5, 4 Lane Rota-Rod with RS-232 and Software, Columbus Instruments, Ohio, USA). The Rotamex-5 incorporates a 32-beam optical sensor mounted slightly over the rotarod assembly. The rota-rod was 7 cm in diameter and 9.5cm-running surface constituted from grey PVC with knurled finished smoother surface for treading. Two circular plastic disks were placed at the ends of the rod and 4 disks equally spaced were placed along the length of the rod. This created 4 compartments so 4 rats could be tested at once. The disks prevented escape and served as a barrier between rats. The Rotamex-5 incorporates a 32-beam optical sensor mounted slightly over the rotarod assembly. This approach allows for more precise detection of when the animal leaves or falls from the rod assembly. The rota-rod was anchored 44.5 cm above a platform of 4 levers, one lever per compartment. Rats were placed on the rota-rod perpendicular to the long axis of the rod, with their heads facing away from the experimenter. As soon as a rat was placed on the rod, the run experiment menu allows the experimenter runs experiments. When the experiment is running the current speed of the rota-rod assembly is displayed, channels running as well as the amount of elapsed time since the experiment has started. Once an animal falls from the roto-rod, the rotational speed of the rota-rod at the time of the fall is displayed along with the amount of time the subject was present on the rod.

Four trials (10rpm; 60 Seconds duration) were run on PND20 of behavioral testing for each rat with two minutes of resting time in between trials. Each trial was recorded (in seconds), however, the first three trials of each day were training trials and only the amount of time the rat stayed on the rod on the fourth trial of each day was analyzed.

2.7 Landing Foot Splay
Gait is measured with the footprint test which indicating peripheral nerve damage (neuropathy). The animal is released from a height, and the distance between the hind feet as the animal lands is recorded. Splay (spread of limbs out and apart) values were generally
measured in millimeter (mm). Foot splay was apparently not related to body weight, since the values did not increase over time in spite of the increasing weights of the rats (Kulig and Lammers, 1992; Schallert et al., 1978).

2.8 Forelimb and hindlimb grip test for neuromuscular function (PND28)
The animal's ability to hang with forelimb, the length of time it does hang, and its activity while hanging was observed on PND 11 in either sex. Muscle strength was also measured using the grip strength meter (Columbus Instruments International Company, USA). It is employed in assessing neuromuscular function by sensing the peak amount of force an animal applies in grasping specially designed pull bar assemblies. Metering is performed with precision force gauges in such a manner as to retain the peak force applied on digital display (Meyer et al., 1979). Forelimb and hindlimb grip strength was measured on PND28.

2.9 Functional Observation Battery (FOB)
Detailed clinical observations: Detailed clinical examination included, identification of clinical signs related to: general appearance, body position and posture, autonomic nervous system function, motor coordination, ambulatory abnormalities, reaction to being handled and to environmental stimulation, nervous system (e.g., tremor, convulsion, muscular contractions), changes in exploratory behaviour, abnormal behaviour (e.g., autophagia, backward motion, abnormal vocalization) and aggression.

Neurobehavioral assessment on PND 28: FOB (Moser, 1989; Tilson and Moser, 1992) and motor activity tests were conducted on all rats assigned to each dosage level on the 4th week of the postnatal period. During each of the test periods, the behavioural tests were conducted on the rats. Dosage groups and gender (n=20 in either sex) were counterbalanced across the test sessions. The motor activity and FOB evaluations were conducted at approximately the same time of day, across all test sessions.

A single trained observer unaware of the group assignment of each rat conducted the FOB. The order in which rats from different dosage groups were tested was randomised. Evaluation of each individual rat was conducted at the home cage during handling of the rat, for a 2-min period in an open field (85 cm X 50 cm X 13 cm), and following reactivity and sensitivity testing. The FOB evaluation lasted approximately one to one and a half hours, and included the following parameters:

1. Reactions to handling and behaviour in the open field (excitability).
2. Gait pattern in the open field, severity of gait abnormalities, air-righting reaction, and visual placing response.
3. Landing foot splay (gait and sensorimotor coordination).
4. Forelimb and hindlimb grip tests.

2.10 Statistical analysis
All data were analysed by one-way analysis of variance (ANOVA) using Graph Pad Prism 5 Software. In case of significant interaction, differences between groups within successive measurements and between measurements within groups were estimated with the use of one-way ANOVA followed by post-hoc Tukey’s test. Differences were regarded as significant when the probability of the null hypothesis was 5% or less (Winter, 1992).
3. Results

3.1 Effects of exposure to methylmercury
A summary of the distribution and fate of all mated rats of the study is given in Table 1.

Table 1 The distribution and fate of all mated rats on study

<table>
<thead>
<tr>
<th>Dose</th>
<th>Control</th>
<th>0.5 mg/kg MeHg</th>
<th>1.0 mg/kg MeHg</th>
<th>1.5 mg/kg MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of vaginal smear positive females (GD 0)</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>No. of pregnant female (Day 10)</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No delivery</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Absorption and/or early or delay in delivery</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3*</td>
</tr>
<tr>
<td>Evaluated at term</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Resorbed litters</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of litters</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Live pups (Male)</td>
<td>30</td>
<td>43</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>Live pups (Female)</td>
<td>36</td>
<td>37</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td>Live pups</td>
<td>66</td>
<td>80</td>
<td>73</td>
<td>43</td>
</tr>
</tbody>
</table>

- Delay in delivery

3.2 Maternal health status and reproduction outcome
During pregnancy, the treatment groups did not differ in water and food intake, and in the rate of the body mass increase. No statistical difference in body weight was observed between control and MeHg exposed dams. The pregnant rats from GD5 to till parturition at 0.5, 1.0 and 1.5mg MeHg /kg/day produced neither maternal toxicity nor any noticeable signs or symptoms. On day 4 of gestation, the maternal body weight remained within the control range. The respective control values (g) were 227.7±16.2; 225.2±10.7; 215.4±10.0 and 182.8±3.5. On day 20 of gestation, the maternal body weight gain (g) of control, 0.5, 1.0 and 1.5mg/kg MeHg exposed dams were 111.2±4.1; 115.4±3.4; 111.0±2.0 and 79.4±2.1. Maternal weight gain of dams during gestation and weight gain during treatment was significantly reduced at 1.5mg/kg/day MeHg treatment group (Fig.2).

3.3 Open Field- testing spontaneous locomotor activity

3.3.1 Distance Travelled
The open field is a test of activity, during the PND28 of open field was analyzed. Three measures of activity were assessed in this test: distance traveled (in cm), number of rearing movements, and resting time (RT) spent in the center of the chamber (in seconds). For measure of activity 60 seconds were examined. Looking at the first few second is important in assessing animal behavior because it measures an animal’s activity when it is first introduced to a new and novel situation. Factors such as fear and unwillingness to explore a new environment are a few factors that could decrease activity in the first few seconds. Open field session is a sufficient preliminary assessment of motor activity and for the evaluation of gross abnormalities in locomotion (Crawley, 1999) also. On PND28 offspring were tested for exploratory activity in an
open field. Following parameters, considered to be indicative of spontaneous locomotion, were evaluated: distance travels, immobility or resting time and rearing. On PND28, no significant treatment-related effects were seen in male and female offspring. However, MeHg (1.0mg/kg/day) treated male increases in distance travel [F (3,76) = 3.17, p< 0.01]; whereas MeHg treated female offspring presented a significant decrease in distance travel [F (3,76) = 20.73, p < 0.001] with 1.0mg/kg/day MeHg treatment group (Fig.3).

**Fig. 2.** Effects on Maternal Gain (%) of rat (Dam) exposed to methyl mercury on gestational day 5 to till parturition. Significantly different from the control groups: ***p < 0.001.

**Fig. 3.** Effects of gestational MeHg exposure on Spontaneous locomotor activity (distance travel) in the open field on PND28. Spontaneous locomotor activity (distance travel) in the open field during 60 seconds of recording in rats. Data represents the mean ± S.E.M. (either sex, n=20). Significantly different from control, “p<0.01 and “”p<0.001.

3.3.2 Centre Time
Time spent in the center of an area is also an indicator of fear. It is thought that if an animal spends its time in the middle of an area, it is unwilling to move outward beyond what it sees and
knows. Others believe that if an animal spends its time in the periphery of an area, it is also frightened. It is thought that the animal may be looking for a way out when it actively spends its time there. Figure 4 shows the amount of time spent in the center of the open field chamber for all measurements of time. There were no gender differences within control whereas gender differences in treated groups. There were decreases in resting time with all dose levels \( [F (3,76) = 13.9, p < 0.001] \) in male offspring; in contrast, it increases with 1.0mg/kg/day MeHg treatment group \( [F (3,76) = 150.9, p < 0.001] \) in female (Fig.4.). Locomotion frequency measured as distance travels, immobility or resting time and rearing in the open field has been used as an index of both arousal (Iviniskis, 1970; Kelley, 1993) and "emotionality" (Walsh and Cummins, 1976); the decrease or absence of movement within the apparatus normally indicates a reduction in arousal or an increase in the level of emotionality (Whimbey and Denemberg, 1967). In light of the present finding, it seems reasonable to suggest that MeHg exposure during gestation did not change arousal and/or emotionality of the offspring. Altogether, the results of the present study show that MeHg causes on age, dose and sex dependent changes in spontaneous locomotors activity suggest that repeated pre-weaning handling of the animals may have subtle effect on spontaneous locomotors performance of the rats.

There were no differences in the amount of time spent in the center between control and treated groups, indicating that both treatment groups were aware of the bounds of the chamber and could see the surrounding area. Therefore, both groups were willing to explore without fear.

![Fig. 4. Effects of gestational MeHg exposure on Spontaneous locomotor activity (Resting time) in the open field on PND28. Spontaneous locomotor activity (resting/ immobility) in the open field during 60 seconds of recording in rats. Data represents the mean ± S.E.M. (either sex, n=20). Significantly different from control, **p<0.01 and ***p<0.001.](image)

### 3.3.3 Rearing Movement

In the 1st few seconds of activity, treated females tended to have less rearing movement than both the treated males and the controls. Anxiety or fear to explore a novel situation could be due to the effects of estrus and the stage of the estrous cycle the treated females were in. MeHg treatment could also have affected females more than males when initially exploring a new environment. By the end of the 60 seconds session when the activity chamber was no longer a
new environment, treated rat seemed to overcome their anxiety and the number of rearing increases in male with 1.5 mg/kg/day MeHg treatment group \[F (3,76) = 592.2, P < 0.001\], in female with 1.0 \[F (3,76) = 282.4, p < 0.05\] and 1.5 mg/kg/day MeHg treatment groups \[F (3,76) = 282.4, p < 0.001\] significantly from the controls (Fig. 5).

Fig. 5. Effects of gestational MeHg exposure on Spontaneous locomotor activity (Rearing) in the open field on PND28. Spontaneous locomotor activity (rearing) in the open field during 60 seconds of recording in rats. Data represents the mean ± S.E.M. (either sex, n=20). Significantly different from control, *p<0.05; and ***p<0.001.

3.3.4 Rota-rod test-assessment of sensorimotor coordination

The rota-rod is a test of coordination. The offspring’s motor ability was investigated using rota rod on PND20. Either sex of offspring at dose levels 0.5, 1.0 and 1.5 mg/kg/day MeHg exposed groups, significantly in male offspring \[F (3,76) = 7516, p < 0.05; p < 0.001\] and female offspring \[F (3,76) = 2790, p < 0.05; p < 0.001\] spent shorter time on rotating rod (10 RPM; cut-off time: 60s) than control (Fig. 6).

Fig. 6. Effects of gestational MeHg exposure on rota-rod test on PND20. Latency to falling (in seconds) on the rotating rod. Data are expressed as mean ± S.E.M. (either sex, n=20). *p < 0.05) and ***p < 0.001 compared to control group.
Methylmercury seems to affect learning and coordination over the course of an extended training period and is illustrated by the decreased amount of time (compared to control rat) spent by the treated mice on the rota-rod.

3.3.5 Landing Foot Splay
The Landing foot splay analysis test was the last test done to measure coordination. The treated rat had a larger foot angle than the control rat. This could indicate that control rat aren’t as stressed during the test and walked normally. Treated rats were more agitated and tended to move slowly down the walkway. As a result, foot placement may be abnormal and an increased foot angle could indicate slower and unnatural movement. Increases in the distance between hindlimb splay or base stance was affected by MeHg in either sex with 1.5mg/kg/day MeHg treatment group in male \[ F(3,76) = 46.51, p < 0.001 \] and in female \[ F (3,76) = 87.7, p < 0.001 \] offspring respectively (Fig.7).

**Fig. 7.** Effects of gestational MeHg exposure on hind limb foot splay on PND28. Data are presented in mean ± S.E.M. (either sex, n=20). Significantly different from the control group: ***p < 0.001 respectively.
Fig. 8. Effects of gestational MeHg exposure on Forelimb grip strength-pup's hanging time (in Seconds) differences between treated and control groups of either sex of offspring performed on PND11. Data are expressed as mean ± S.E.M. (either sex, n=20). **p<0.01; ***p < 0.001 compared to control group.

3.3.6 Forelimb and hindlimb grip test for neuromuscular function
The animal’s ability to hang with forelimb, the length of time it does hang, and its activity while hanging was observed on PND 11 in either sex. There were significant reductions in male forelimb hanging time (Seconds) with 1.0 and 1.5mg/kg/day MeHg [F (3,76) = 1639, p < 0.01, p < 0.001] respectively, whereas in female offspring with 1.0 and 1.5mg/kg/day MeHg treatment group [F (3,76) = 1390, p <0.01; p < 0.001] respectively (Fig.8).

3.3.7. Functional observational battery (FOB)
FOB was performed on PND28 in offspring of rat exposed to MeHg. Statistically significantly increase in CNS activity and excitability by measuring rearing in male with 1.5mg/kg/day MeHg treatment group [F (3,76) = 592.2, P < 0.001], in female with 1.0 [F (3,76) = 282.4, p < 0.05] and 1.5mg/kg/day MeHg treatment groups [F (3,76) = 282.4, p < 0.001]; decreases in neuromuscular function/measures such as forelimb grip strength with 1.5mg/kg/day MeHg treatment group in male [F (3,76) = 69.28, p < 0.01] and in female offspring [F (3,76) = 34.29, p < 0.001], hindlimb grip strength with 1.0 [F (3,76) =
Fig. 9. Effects of gestational MeHg exposure on FOB (functional Observation Battery)- Forelimb grip strength performed in offspring on PND28. Data are presented in mean ± S.E.M. (either sex, n=20). Significantly different from the control group: **p < 0.01, ***p < 0.001 respectively.

292.7, p < 0.001] and 1.5 [F (3,76) = 305.1, p < 0.001] mg/kg/day MeHg treatment groups in either sex, increases in hindlimb splay with 1.5mg/kg/day MeHg treatment group with male[ F (3,76) = 46.51, p < 0.001] as well as [F (3,76) = 87.7, p < 0.001] in female offspring (Table 2, Fig.9;10). Thus, FOB measurements revealed impairment of neuromotor performance in offspring.

Fig.10. Effects of gestational MeHg exposure on FOB (functional Observation Battery)- Hindlimb grip strength performed in offspring on PND28. Data are presented in mean ± S.E.M. (either sex, n=20). Significantly different from the control group: *p < 0.05; **p < 0.01, ***p < 0.001 respectively
Table: 2. FOB (CNS excitability and neuromuscular function/measures) in the offspring of rat exposed to methylmercury on gestation day GD5 till parturition on PND28.

<table>
<thead>
<tr>
<th></th>
<th>Rearing</th>
<th>Forelimb grip strength (g)</th>
<th>Hindlimb grip strength (g)</th>
<th>Hindlimb Splay (cm)</th>
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<tr>
<td></td>
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<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Control</td>
<td>Mean</td>
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<td>158.0</td>
<td>63.2</td>
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<td></td>
<td>S.E.M.</td>
<td>0.15</td>
<td>178.8</td>
<td>62.95</td>
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<td></td>
<td>N</td>
<td>20</td>
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<td>20</td>
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<tr>
<td>0.5mg/kg/day MeHg</td>
<td>Mean</td>
<td>5.35</td>
<td>196.30**</td>
<td>68.2*</td>
</tr>
<tr>
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<td>S.E.M.</td>
<td>0.16</td>
<td>189.1</td>
<td>68.65*</td>
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<td>20</td>
</tr>
<tr>
<td>1.0mg/kg/day MeHg</td>
<td>Mean</td>
<td>5.3</td>
<td>192.6**</td>
<td>56.5**</td>
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<td>186.0</td>
<td>56.75**</td>
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<td>20</td>
</tr>
<tr>
<td>1.5mg/kg/day MeHg</td>
<td>Mean</td>
<td>13.05***</td>
<td>102.9*</td>
<td>56.4***</td>
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<tr>
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<td>118.6***</td>
<td>55.8***</td>
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<td></td>
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<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Data are presented in mean ± S.E.M. Significantly different from the control group: *p < 0.05; **p < 0.01, ***p < 0.001 respectively.

4. Discussion

Locomotion frequency measured as distance travels, immobility or resting time and rearing in the open field has been used as an index of both arousal (Iviniskis, 1970; Kelley, 1993) and “emotionality” (Walsh and Cummins, 1976); the decrease or absence of movement within the apparatus normally indicates a reduction in arousal or an increase in the level of emotionality (Whimbey and Denemberg, 1967). Locomotors activities (locomotion, rearing, and motility) were tested in the offspring at PND28. A significant increase in spontaneous motility in male and rearing was observed in either sex of the MeHg-treated rats. Taken all together, these findings show that during development a very low dosage of MeHg exerts neurotoxic effects detectable in adulthood, and that susceptibility is gender-dependent. In light of the present finding, it seems reasonable to suggest that MeHg exposure during gestation did not change arousal and/or emotionality of the offspring. Altogether, the results of the present study show that MeHg causes on age, dose and sex dependent changes in spontaneous locomotor performance of the rats. There were no differences in the amount of time spent in the center between control and treated groups, indicating that both treatment groups were aware of the bounds of the chamber and could see the surrounding area. Therefore, both groups were willing to explore without fear.

Behavior always entails a motor act, so it is necessary to eliminate motor deficits as confounds in experiments on cognitive function. Sensory-motor function has been shown to be disrupted by MeHg exposure in humans, nonhuman primates, and laboratory rodents and pigeons, so the possibility that motor deficits confound effects on behavioural plasticity must be addressed (Beuter et al., 1999; NRC, 2000; Watanabe and Satoh, 1996). With MeHg, the pattern of sensory-motor deficit differs, depending on whether exposure is developmental or chronic and beginning in adulthood. A direct comparison of the effects of developmental and chronic, adult-onset exposure on motor skills showed dose-related effects of MeHg only after extensive, chronic exposure. Rats were exposed either chronically or prenatally (via maternal drinking
water) to 0, 0.5, or 5.0 ppm of MeHg in drinking water, approximating 0, 40, or 400 μg/kg/day (Day et al., 2005). Chronic, adult-onset exposure produced dose-related increases in grip strength, hind limb crossing when the rat is held by the tail (a marker of chronic, high-dose exposure), gait abnormalities, and diminished running wheel activity. Similar effects have been reported in other experiments, but with higher exposure levels and, consequently, more rapid onset of effects (Sakamoto et al., 1998). Developmental exposure produced none of these effects (Day et al., 2005). Since the effects on choice, fixed-ratio acquisition, or high-rate behavior were all associated with developmental exposure, it can be concluded that they did not reflect such pronounced motor deficits. There remains the possibility that subtler motor deficits associated with developmental exposure influence the effects seen, but this seems unlikely.

The results revealed the prenatal exposure of MeHg affected the motor development of offspring. These findings are consistent with results from high-exposure human studies, which revealed significant delays in aspects of motor development such as crawling, standing, and walking. In fact, pups of rat exposed to MeHg, were deficient with two muscular tone tasks (rotating rod and landing foot splay test) impairment was damaged by methylmercury and began to appear on the rotating rod on PND20. Shorter latencies before falling during the test occurred in MeHg exposure pups for all doses and were inferior to controls by far. In the current study, there was a trend for decreased motor activity, for dose-dependent MeHg exposed male and female offspring. In contrast, MeHg-induced increase in motor activity was previously observed in offspring administered similar levels of MeHg during gestation (Gimenez-Llort et al., 2001). The decreased motor activity may suggest a developmental MeHg neurotoxicity in these offspring. On the contrary, Vincente et al. 2004, did not find any difference in strength test between pups exposed prenatally for three consecutive days (PND13, 14, 15) to methylmercury and controls. These divergent results might be related to the chemical form of the metal, its doses, the time of treatment during pregnancy, and the method used for behavioural assessment.

Motor disturbances have been reported following developmental exposure to high levels of either PCBs (Harada, 1976; Rogan et al., 1988) or MeHg (Amin-Zaki et al., 1981). Developmental motor reflexes (Overmann et al., 1987; Pantaleoni et al., 1988; Rice, 1999) and general locomotor activity (reviewed in Schantz, 1999) have also been examined. For MeHg, it is clearer that high doses (2–18 mg total dose) cause motor deficits, such as impaired rotarod performance (Sakamoto et al., 1993, 2002), retarded or abnormal walking ability (Inouye et al., 1985; Watanabe et al., 1999), retarded development of swimming ability (Elsner et al., 1988; Geyer et al., 1985; Olson and Boush, 1975; Vorhees, 1985), hind-limb dysfunction (Inouye et al., 1985; Kobayashi et al., 1981; Magos et al., 1985; Sakamoto et al., 1993), and even severe movement and postural disorders (O’Kusky et al., 1988) in laboratory animals. In females, in both exposed groups, the number of ambulation episodes was smaller and, in the low dose group, the walked distance was significantly shorter than in females of the control group. In males there were no differences between groups (Fig. 1). The results of Open field test experiment (MeHg exposure) indicate that: i) both exposure levels used in the present study were effective in inducing behavioural alterations in adult progeny, ii) alterations found in females differed qualitatively from alterations found in males. Effects observed in females (reduced locomotor activity in the open field) suggest a lowered general arousal (or increased fearfulness). Regarding the effectiveness of the MeHg dosing levels, it is worth noting that in females of the low dose group the reduction of the locomotor activity was more pronounced than in females of the high dose group. In case of the open field test, the direction of the change found in the present study, i.e. a reduction of locomotor activity is the same as that observed by other authors. Inconsistency concerns the gender relationship. According to some reports,
perinatal MeHg exposure in rats results in reduced locomotor activity in males (Castoldi et al., 2008). In mice, however, females are the affected gender (Goulet et al., 2003). In all the remaining tests of present experiment, effects were found in either sex, which is contrast with observations of other authors suggesting a higher vulnerability of male fetuses to the MeHg toxic action (Rossi et al., 1997).

The principal motor deficit observed was on the rotating rod task. Motor function was examined using a rotating rod tasks upon which performance is impaired in animals with cerebellar damage (Klintsova et al., 1998). Since rotating-rod impairments were observed only in the combined exposure group (Roegge et al., 2004), it seems possible that MeHg exposure may have caused some independent damage to the cerebellar motor system. Damage to other motor systems cannot be ruled out, but MeHg is known to target the cerebellum (Eto, 1997; Leyshon and Morgan, 1991). Higher doses of MeHg are known to cause motor deficits in laboratory animals, including impaired rotarod performance (Sakamoto et al., 1993, 2002), and prenatal exposure to higher doses of MeHg also causes morphological changes in the cerebellum (Chang et al., 1977; Choi et al., 1981; Sager et al., 1984). Thus, there is a possibility that MeHg-induced cerebellar damage contributes to the motor deficits observed following MeHg exposure, but damage to other neural systems may also occur. Although the rotating rod deficits seem to be primarily PCB-driven, MeHg exposure appears to have contributed to the effect. This behavioral effect does mimic the in vitro dopamine effect observed by Bemis and Seegal (1999), in which low doses of MeHg had no effect on dopamine when given alone but accentuated the effects of PCBs on dopamine when the two compounds were given together.

MeHg is known to target the cerebellum in humans (Eto, 1997) and in rats (Leyshon and Morgan, 1991). In laboratory studies, prenatal exposure to high doses of MeHg (total dose 2–50 mg in rats and 0.01–0.4 mg in mice) can cause pathological changes in the cerebellum, such as degeneration of Purkinje and granule cells (Chang et al., 1977), decreased dendritic arborization of Purkinje cells (Choi et al., 1981), and reduced thickness of the internal granular and molecular layers (Sager et al., 1984). The dose of MeHg used in this study was significantly lower than doses used in previous motor studies, compared with 2–18 mg used in previous studies of motor abilities. For example, Sakamoto et al. (2002) found rotarod deficits using a dose of MeHg 10-fold higher than the dose employed in our study. The rotarod task is different from the rotating-rod task used here in that, in our study, the rats were required to not only stay on the moving rod but also to跨 it lengthwise. Deficits on the rotating rod in the combined PCB and MeHg rats may be indicative of cerebellar damage. Morphological damage to the cerebellum caused by early postnatal X-irradiation of the rat cerebellum has been shown to impair rotating-rod performance (Brunner and Altman, 1973; Pellegrino and Altman, 1979). More recently, postnatal alcohol exposure was found to cause both rotating-rod deficits and cerebellar damage (Klintsova et al., 1998).

High developmental exposure to MeHg can induce severe lesions in the brainstem of rats (Kakita et al., 2000; Sakamoto et al., 1998). Collectively, these data suggest that the brainstem may be a significant target of developmental MeHg neurotoxicity. Other animal dosing studies have identified gender specific neurotoxicity following gestational exposure to MeHg (Gimenez-Llortet et al., 2001). Specifically, MeHg related differences in dopamine-mediated locomotor activity were observed in male offspring, but not females (Gimenez-Llortet et al., 2001). Additionally, other potential gender effects were observed. Significant MeHg related reductions in postnatal weight gain were only observed in males while significant increases in distance traveled in open field test were only observed in males. However, in both endpoints, similar
MeHg related trends were seen in the other gender. Therefore, it is possible that these differences may have been a result of directly due to gender differences.

Learning and memory seemed to be more affected by prenatal MeHg exposure than coordination and activity (the open field, rota-rod, and foot splay analysis tests) because there were greater differences between treatment groups in the open field activity test than coordination tests. Gender also seems to affect the extent of methylmercury effects in the central nervous system as manifested in several behavioral analyses. Therefore, even though all these above-reported findings and the available bulk of literature seem to be sometimes and somewhere controversial, integrated approaches combining biochemical markers in combination with neurophysiological and behavioural assays could represent a valuable methodological approach by which human neurotoxicity assessment may become more focused and understanding the cellular mechanisms and the behavioral outcomes of methylmercury will help reduce the morbidity and dysfunction associated with methylmercury exposure by creating avenues of prevention and treatment.

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**Conflict of Interests**

No conflict of interest.

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