Research report

Persistent behavioral impairments and alterations of brain dopamine system after early postnatal administration of thimerosal in rats

Mieszko Olczak[a,d], Michalina Duszczyk[a], Pawel Mierzejewski[b], Ksenia Meyza[c], Maria Dorota Majewska[a,e,*]

[a] Marie Curie Chairs Program at the Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, 02-957 Warsaw, Poland
[b] Department of Pharmacology and Physiology of the Nervous System, Institute of Psychiatry and Neurology, 02-957 Warsaw, Poland
[c] Department of Neurophysiology, Nencki Institute of Experimental Biology, PAS, Pasteur 3 Str., 02-093 Warsaw, Poland
[d] Department of Forensic Medicine, Medical University of Warsaw, Oczki 1 Str., 02-007 Warsaw, Poland
[e] Department of Biology and Environmental Science, University of Cardinal Stefan Wyszynski, Wóycickiego Str. 1/3, 01-815 Warsaw, Poland

ABSTRACT

The neurotoxic organomercurial thimerosal (THIM), used for decades as vaccine preservative, is a suspected factor in the pathogenesis of some neurodevelopmental disorders. Previously we showed that neonatal administration of THIM at doses equivalent to those used in infant vaccines or higher, causes lasting alterations in the brain opioid system in rats. Here we investigated neonatal treatment with THIM (at doses 12, 240, 1440 and 3000 μg Hg/kg) on behaviors, which are characteristically altered in autism, such as locomotor activity, anxiety, social interactions, spatial learning, and on the brain dopaminergic system in Wistar rats of both sexes. Adult male and female rats, which were exposed to the entire range of THIM doses during the early postnatal life, manifested impairments of locomotor activity and increased anxiety/neophobia in the open field test. In animals of both sexes treated with the highest THIM dose, the frequency of prosocial interactions was reduced, while the frequency of asocial/antisocial interactions was increased in males, but decreased in females. Neonatal THIM treatment did not significantly affect spatial learning and memory. THIM-exposed rats also manifested reduced haloperidol-induced catalepsy, accompanied by a marked decline in the density of striatal D_2 receptors, measured by immunohistochemical staining, suggesting alterations to the brain dopaminergic system. Males were more sensitive than females to some neurodisruptive/neurotoxic actions of THIM. These data document that early postnatal THIM administration causes lasting neurobehavioral impairments and neurochemical alterations in the brain, dependent on dose and sex. If similar changes occur in THIM/mercurial-exposed children, they could contribute do neurodevelopmental disorders.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Thimerosal (THIM; sodium ethyl-mercurithiosalicylate; containing approximately 49% of mercury (Hg) by weigh), has been added to pediatric vaccines as a preservative since the 1930s (and still is in many developing countries), without being adequately tested for safety in developing organisms. In the body THIM is metabolized first to ethyl-mercury and further to inorganic mercury compounds, which accumulate in the brain and other vital organs [1,2]. With increasing numbers of vaccines injected to progressively younger infants (some only a few hours old), a legitimate concern emerged that Hg from vaccines accumulating in infant brains might contribute to the epidemics of neurodevelopment disorders in children [3–9]. This issue is a subject of hot debates, but still remains controversial.

Concerns related to use of THIM in pediatric vaccines stem primarily from its neurotoxicity, analogous to that of other mercurials. THIM has been shown to kill neurons by apoptosis and necrosis in vitro at nanomolar and low micromolar concentrations, which might be reached in the brain after vaccination [10–15]. The molecular mechanisms of THIM-induced neurotoxicity involve DNA breakage [10,16], depolarization and damage of mitochondrial membranes [11,15], generation of reactive oxygen species, release of cytochrome and apoptosis inducing factors from mitochondria to cytosol, and activation of caspases 9 and 3, known to participate

in programmed cell death following chemical or mechanical insult [13,15,16].

In vivo, the toxic effects of THIM on neurons depend not only on tissue concentrations of this mercurial and its metabolites, but also on additional modulatory factors, which might mitigate or augment THIM's toxicity. The potential endogenous neuroprotective factors include neurotrophins [16], low molecular weight thiols [15,17], or metallothioneins, which have been shown to be induced in the mouse brain by pharmacologically relevant doses of THIM [18]. The brain concentrations of these neuroprotective agents depend on animal age, sex, diet, genetic makeup and environment. On the other hand, coexposure to various chemical or metabolic stressors, e.g. other toxic metals such as aluminum (present in most vaccines), environmental lead [19,20], or to bacterial lipopolysaccharides and toxins (from infections or vaccines) may enhance the neurotoxic effects of THIM by augmenting production of proinflammatory cytokines [21,22].

Developing mammalian organisms may be particularly sensitive to the neurotoxic effects of THIM. The study of Hornig et al. [23] documented numerous neurological deficits in autoimmune disease-sensitive mice treated at the neonatal stage with THIM doses equivalent to those used in infant vaccines. Laurente et al. [24] described the neurotoxic effects of THIM in developing hamsters, and Hewitson et al. [25,26] reported that infant monkeys injected with THIM-containing vaccines manifested a delay in the acquisition of vital neonatal reflexes and abnormal brain development. Also, documented by Minami et al. [18], the induction of mRNA for metallothionein (a sensitive surrogate marker of brain exposure to toxic metals), in the cerebellum and cerebrum of mice injected with vaccine-level doses of THIM, is indirect evidence of the neurotoxicity of this organomercurial. Only the study of Berman et al. [27] did not show neurotoxic effects of THIM in developing mice.

In order to assess the potential role of THIM in neurodevelopmental disorders, we have investigated its neurodisruptive/neurotoxic effects in a series of behavioral, neurochemical and neuropathological studies in a rat model. Our previous research revealed impairment of pain sensitivity, dependent on endogenous opioids, and abnormalities in brain mu opioid receptors accompanied by neuropathological changes in neurons and glia in brains of rats exposed to THIM at the early postnatal stage [14,28,29]. Some of the observed behavioral, neurochemical and neuropathological alterations in THIM-treated rats resemble the symptoms and pathological features of autism spectrum disorders (ASD).

ASD are diagnosed based on characteristic neurobehavioral patterns including stereotypy and impairment of movement [30,31], increased anxiety [32] and deficient social skills [33,34], hence in this study we focused on the analysis of similar behaviors in rats. We evaluated the effects of early neonatal rat exposure to THIM on locomotor and exploratory activity in the open field test, social interactions, and spatial learning and memory in the water maze test. Because disrupted dopamine activity has been found in some autistic patients and is implicated in certain autistic behaviors, especially those related to emotional processing, attention and movement disturbances [35–38], we also assessed the activity of the brain dopaminergic system in neonatally THIM exposed rats by evaluating haloperidol-induced catalepsy [39]. In addition, we measured by immunohistochemical staining the density of D_2 receptors in the striatum, which is richly innervated by dopaminergic inputs, where dopamine receptors participate in the control of movement, emotions, reward, salience and motivation [40–43].

2. Materials and methods

2.1. Experimental animals and drugs

The experiments were conducted on young adult Wistar rats, which received four postnatal injections of THIM at doses 12, 240, 1440, or 3000 μg Hg/kg per injection. The wide range of THIM doses was chosen to compare qualitatively and quantitatively the effects of pharmacologically relevant doses with those of higher doses, having also in mind the generally lower sensitivity of rats than humans to toxins. The lowest dose is in the range of doses still used in pediatric vaccines in some developing countries [8,23,28] and the higher doses are its multiples. Thimerosal was purchased from Sigma–Aldrich, Poland. The Hg content in THIM was analyzed by atomic absorption spectrometry as previously described [28]. According to the analysis, the Hg content in THIM was 48%.

Pregnant Wistar females were supplied by a breeder (Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland). The animals were kept in a room under standard environmental conditions (22 ± 1 °C, relative humidity of 60%, 12 h–12 h light-dark cycle. Standard laboratory chow (LabofeedH, WPIK, Kcynia, Poland) and tap water were available *ad libitum*. All experiments were conducted according to the ethical standards laid down in respective Polish and European (directive No. 86/609/EEC) regulations. All procedures were reviewed and approved by the ethics committee on animal studies.

2.2. Experimental procedures

2.2.1. Study design: neonatal THIM injections

Thimerosal, at different doses dissolved in saline, was injected into newborn rats on postnatal days 7, 9, 11 and 15 in four equal doses (per kg) in a volume of 50 μl, i.m. into the *glutei maximi*, alternating muscle for injection. This schedule was originally used by Hornig et al. [23] in mice to mimic the infant immunization scheme. In all data THIM dose is expressed as μg Hg/kg. Rats from one litter received the same treatment. Average litter size was ($11.8 + 0.7$; $N = 235$). There were 5 general cohorts of experimental animals: 4 cohorts, which received THIM at different doses per injection (12, 240, 1440, or 3000 μg Hg/kg) and a control cohort, which received saline injections following the same scheme. Four litters per each THIM treatment group were used. Each animal was weighed before THIM injection and the amount of drug injected was adjusted for its weight. The experimental groups for different tests consisted of pooled rats from different litters, with 2–4 animals from each sex selected per litter. Pups were weaned on the 28th postnatal day and at that time they were separated into male and female cages. Every week they handled.

2.2.2. Monitoring of pups' development

To evaluate general physical development the pups were weighed during the first 21 postnatal days (on PND: 7, 9, 11, 15, and 21). Weights of THIM-treated animals were compared with those of controls, choosing equal size litters ($n = 12$/litter). Two litters for each THIM dose and control were analyzed ($N = 24$ per group).

2.2.3. Open field tests

On the 30th postnatal day rats were tested in an open field. The test was performed in a soundproof chamber under dim light and continuous white noise (65 dB) without previous habituation. The animals were initially accustomed to the experimental room for 15 min prior to the experiment. The open field test chambers used in the experiment were black, octagonal, 90-cm in diameter with 30-cm high walls. The central field was defined as all area 20-cm away from the walls. During the 15-min registration period, parameters such as: total distance, central area time, and central area visits were monitored with a VideoMot2 – Video Activity Measuring System (TSE Systems). The test was carried out according to the protocols of Crawley et al. [44]. The TSE VideoMot2 was connected to a PC and the counting of animal activity in different parameters was fully automated (the experimenter placed the animal in the experimental chamber, switched the system on and left the room). The experimental groups included control rats and those which received postnatal THIM injections at doses of 12, 240, 1440 and 3000 μg Hg/kg of both sexes ($N = 122$). The numbers of animals in each experimental group are shown in Fig. 1 legend.

2.2.4. Social interaction test

The social interaction test was carried out on animals in the 8th postnatal week during the dark phase of 12 h/12 h dark/light cycle. There were 6 experimental groups of animals of both sexes: controls and those treated with THIM 1440 and 3000 μg Hg/kg. Each experimental group consisted of 8 pairs of same sex rats; the group of males that received 1400 μg Hg/kg consisted of 9 pairs or rats ($N = 98$). Pairs of animals were matched pseudo-randomly in a way which provided minimal weight difference (up to 50 g). Every pair consisted of two animals from different litters, which had never met before, paired control to control and THIM to THIM, to avoid contamination of data with the influence of the activity of control animals on THIM-injected animals (which we hypothesized might display deficient social behavior).

Before testing all animals were housed individually for 5 days (food and water *ad libitum*). Social interactions were recorded for 5 min in a black square-shaped open field arena (67 cm × 67 cm × 45 cm) under HU conditions (Highlight 300-lx, Unfamiliar arena – without habituation) and continuous white noise (65 dB). Each animal from a tested pair was placed in the opposite corner of the arena and social interactions were traced using a Panasonic NV-DS60 digital camera. After the test both animals were removed, the arena was cleaned with 80% ethanol and wiped dry. Social interactions were monitored and manually counted using BehaViev software [45] by two blinded readers. The following parameters were monitored: prosocial interactions: social sniffing, crawling under/over, following; asocial/antisocial:
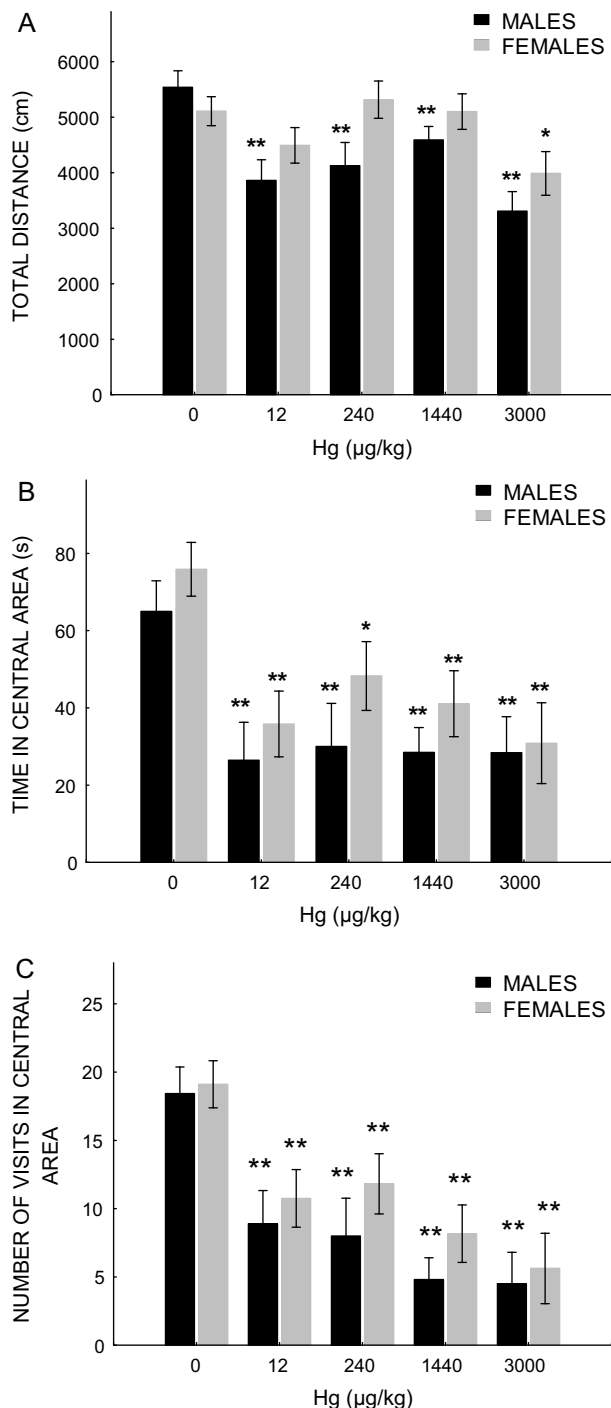
Fig. 1. Effect of early postnatal THIM administration on locomotor activity in the open field test in Wistar rats. Data represent means ± SEM. Stars denote statistically significant differences in comparison to controls; (*p*) levels <0.05 marked with (*); (*p*) levels <0.01 marked with (**). Experimental groups for males (M) and females (F) consisted of: Ctr (0 μg Hg/kg) – M14, F16; 12 μg Hg/kg – M9, F12; 240 μg Hg/kg – M14, F11; 1440 μg Hg/kg – M14, F12; 3000 μg Hg/kg – M10, F10 (N = 122).

freezing (immobilization), self-grooming, avoiding, and aggressive-defensive. Also locomotor activity was monitored during this test. The arena was divided into 9 (3 × 3) quadrants. Transitions between the squares were counted "offline" and used to evaluate the locomotor activity. The test was carried out according to the protocols of Crawley et al. [44].

2.2.5. Morris water maze (spatial learning and memory) test

The spatial memory test was conducted in the 10–11th postnatal week. The test was performed in a soundproof, lightsome chamber with continuous white noise (65 dB) and visual cues on its walls. The water maze consisted of a round, black metal pool (200 cm in diameter, 50 cm depth) filled with water (25 ± 1.0 °C). The pool was divided into four quadrants of equal area arbitrarily called northeast (NE), southeast (SE), southwest (SW) and northwest (NW). A square platform (10 cm × 10 cm) made of transparent Plexiglas was submerged 1 cm below the water surface, in the middle of NE quadrant (the target quadrant). One day before the experiment animals were accustomed to the water environment in a separate training pool by 1-min swimming.

The animals were tested daily for 4 days. Each subject was placed by the tail into the water, facing the perimeter, at one of the basic compass points (NW, SE, SW, but not at target quadrant), and then was allowed a maximal time of 60 s to locate the platform. After remaining on the platform for 30 s the subject was gently picked up, returned to its home cage, and allowed to warm up and dry. If the subject failed to find the platform during the 60 s swim it was placed onto it for 30 s, and returned to its home cage. After 5–7 min the animals were placed back in a different quadrant of the pool. After each subject in the testing group had completed one trial, the next trial began (from a different start point). The entire procedure took 4 consecutive days, each subject having 4 training trials per day. By the end of each experimental day, each subject had been placed into the pool an equal number of times from each starting point. During the first minute registration period parameters such as: total distance, latency to reach the platform and average speed, were monitored with a TSE VideoMot2 – Video Activity Measuring System. The memory test was carried out on the 5th experimental day, when each subject was placed into the water diagonally opposite the target quadrant and allowed 60 s to search the area, from which the platform had been removed. During the first minute registration period, parameters such as: crossings above the platform area and percent of time spent in the target quadrant were monitored with a TSE VideoMot2 – Video Activity Measuring System. The motivation test was carried on the 6th day. The platform was suspended 1 cm above the water surface in the SE quadrant (not the target quadrant) and its edges were covered with white plaster to make it more visible. Each subject was placed into the water diagonally opposite to the SE quadrant. During a first minute registration period, latency to reach the platform was automatically monitored with a TSE VideoMot2 – Video Activity Measuring System. The test was carried according to Crawley et al. protocols [44]. We started the experiment by first testing rats of both sexes treated with the two highest THIM doses (1440 and 3000 μg Hg/kg). Because in most parameters (except for swimming speed) there appeared to be no significant THIM effect in this test, we did not test animals treated with lower THIM doses. There were 6 experimental groups of animals of both sexes: controls and animals treated with THIM doses 1440 or 3000 μg Hg/kg (N = 57). The numbers of animals in each experimental group are shown in Fig. 5 legend.

2.2.6. Haloperidol catalepsy test

Male rats were tested in the 12th week of postnatal life. There were 3 experimental cohorts of animals: controls and those which received neonatal THIM injections 1440 and 3000 μg Hg/kg; each consisting of 14 animals (N = 42). Before the evaluation of the dopamine receptor-associated responses, the animals were repeatedly familiarized with a horizontal wooden bar. Each rat was placed on a clean, smooth table with the wooden bar suspended 10 cm above the working surface. The animal's front paws were gently placed over the bar. The length of time (in seconds) the animal touched the bar with both front paws was measured up to a pre-set cut-off time of 180 s [28,46]. One day before the experiment the animals were treated with the above described procedure using saline. On the test day they were injected *i.p.* with 0.15, 0.3, or 0.6 mg/kg haloperidol (HP) solutions (injectable formulation, Polfa, Poland). The same animals received all three HP doses 0.15, 0.30 and 0.6 mg/kg subsequently, with 4 day space between each HP injection. This made 12 treatment groups. Catalepsy was measured at times: 0, 30, 60, 90, and 120 min after HP administration.

2.2.7. Brains acquisition

A separate group of experimentally naïve 8-week old rats was used for histological and immunohistochemical staining, and for analysis of density of D_2 receptors and neurons in the striatum. Animals were sacrificed with an overdose of *i.p.* pentobarbital injections, then decapitated. The brains were removed within 30 s and placed in a buffered formaldehyde–water solution (10%) for 24 h. Brain tissues were fixed through increasing concentration ethanol solutions (60–100%), xylene, and finishing with paraffin. Paraffin blocks were stored in a refrigerator at a temperature of 3.0 °C until sectioning. Brains were sectioned using a microtome and sections were gently placed on basic glasses covered previously with L-silane. The section thickness was 4 μm. Sectioned tissue was placed in an incubator set at 56 °C for 3 h. There were 5 animals of both sexes per experimental group (for THIM doses 0, 12, 240 μg Hg/kg). Specimens (two subsequent sections from each brain) were used for H&E and D_2 receptor staining and for counting of D_2 receptor and neuron density. Hematoxylin–eosin staining

Specimens were fixed with xylene, ethanol, water, then treated with hematoxylin (5 min), rinsed with water (10 min) treated with eosin (1 min), rinsed with water (2 min), then fixed with ethanol and xylene, finally immersed in Histofluid (Marienfield) using cover glasses. There were 5 animals per experimental group (THIM dose 0, 12, and 240 μg Hg/kg) and sex.

2.2.9. Immunohistochemical staining for D_2 receptors

Tissue specimens were stained using Anti-Dopamine D_2 Receptor Rabbit Polyclonal Antibody (Millipore). Specimens were fixed through the following stages: deparaffination (xylene, ethanol, water), antigen uncovering by heating (microwave, 15 min, 1200 W) in citrate buffer (0.01 M, pH 6), methanol and 3% hydrogen peroxide solution (20 min), 0.05 M TRIS buffer in saline (15 min, pH 7.6), serum swine (60 min) (Daco, Poland), primary antibody Anti-Dopamine D_2 Receptor Rabbit Polyclonal Antibody (1:100, overnight) (Millipore), TRIS buffer (15 min, pH 7.6), secondary antibody JgG Rabbit (1:800, 45 min) (Sigma, Poland), TRIS (15 min, pH 7.6), extravidine (1:200, 60 min) (Sigma), TRIS (15 min, pH 7.6), diaminobenzidine and hydrogen peroxide solution (3 min), ethanol, xylene, finally immersed with Histofluid using cover glasses.

2.2.10. D_2 receptor counting

To evaluate the density of D_2 receptors, sections showing the caudate putamen (NE quadrant) of behaviorally naïve, control and THIM treated animals were analyzed. The position of the regions was determined according to a rat brain atlas (around Bregma 1.20) [47]. Microphotographs of each region were taken from both cerebral hemispheres with a magnification of 400× (area 35452.23 μm^2). The density of D_2 receptors was counted as an area fraction in the analyzed region, with ImageJ 1,41o software and used for statistical analysis. Stained area fraction was automatically counted with the "threshold" function of the ImageJ program, which marks all the pixels of chosen gray value and counts all the groups of marked pixels (stained receptors) within the selected area [48]. Two homonymous areas each, from opposite hemispheres per animal were analyzed. Receptor density from each side was averaged for each animal and used for statistical analysis. Experimental groups consisted of 5 animals of each sex for THIM doses 12 and 240 μg Hg/kg and of 4 animals of each sex for controls ($N = 28$).

2.2.11. Microscopic photography

The microphotographs of the brain sections were taken with an Olympus BX41 microscope and Olympus DP25 digital camera. Images were saved in TIFF format. For each experiment microphotographs were taken with the same light level for all sections.

2.2.12. Counting of neuron density

To evaluate the density of neurons in brain areas where D_2 receptor density was measured, corresponding sections were H.E. stained. Microphotographs of corresponding regions were taken from both cerebral hemispheres with a magnification of 20× (area 141808.92 μm^2) and analyzed using the cellF program. The density of the nuclei was manually counted by a blinded researcher. Nuclei density from each side and structure was averaged for each animal and used for statistical analysis. Experimental groups consisted of 5 animals of each sex for THIM doses 12 and 240 μg Hg/kg and for controls ($N = 30$).

2.3. Statistical analysis

The STATISTICA software package for Windows (StatSoft, Tulsa, OK, USA) was used to analyze all data. An analysis of variance (ANOVA) was used to compare groups of rats. The LSD test was employed for individual post hoc comparisons. Probability (p) levels less than 0.05 were considered significant.

3. Results

3.1. Pup development

General pup development was monitored by systematic recording of their weight from PND7 to PND21. Experimental groups composed each from two equal size litters ($n = 12$/litter) were chosen for analysis. We have not observed abnormal developmental weight deficits or increases in THIM treated rats, as animals from all experimental groups showed regular and proportional weight gains (Table 1). Between postnatal days 7 and 21 the control group of rats increased their weight on average by 185% ($n = 24$), rats which received postnatal THIM dose of 1440 μg Hg/kg increased their weight by approximately 172% ($n = 24$), and those which received THIM dose of 3000 μg Hg/kg increased their weight by 193% ($n = 24$). The differences in weight gain between experimental groups were not statistically significant ($p > 0.05$). These data indicate that at doses tested THIM does not markedly influence general growth of Wistar rats.

3.2. Open field test

To evaluate general locomotor activity and (indirectly) anxiety, the open field test was carried out on 30th postnatal day in rats. The results in Fig. 1 show that in male rats treated with THIM doses from 12 to 3000 μg Hg/kg, general locomotor activity (measured as total distance traveled) was significantly reduced. In females, a similar effect was noted only at the highest THIM dose. This indicates that males are more sensitive to the neurotoxic or neurodisrupting effects of THIM than females. The two-way ANOVA (sex × Hg dose) revealed a significant effect of sex: [$F(1,112) = 5.815$; $p = 0.01$]; a significant effect of Hg dose: [$F(4,112) = 7.548$; $p < 0.001$] and a non-significant interaction sex × Hg dose: [$F(4,112) = 1.752$; $p = 0.14$]. The post hoc (LSD) analysis confirmed the significance of the Hg dose effect for males for THIM doses of 12, 240, 1440, 3000 μg Hg/kg ($p < 0.05$), and for females for a THIM dose of 3000 μg Hg/kg ($p = 0.05$) (Fig. 1A).

THIM-treated male and female rats also manifested markedly reduced exploratory activity in the central field (visits and time spent in central area), suggestive of increased anxiety or neophobia. Because animals from both sexes were nearly equally affected, this indicates that anxiety parameters are largely independent from general locomotor activity (Fig. 1B). For the time spent in the central area, the two-way ANOVA (sex × Hg dose) revealed a non-significant effect of sex [$F(1,112) = 3.551$; $p = 0.06$]; a significant effect of Hg dose [$F(4,112) = 9.02$; $p < 0.001$] and a non-significant interaction sex × Hg dose: [$F(4,112) = 0.163$; $p = 0.95$]. The post hoc (LSD) analysis confirmed the significance of the Hg dose effect for both sexes: for all THIM doses in both sexes $p < 0.01$, for females at a THIM dose of 240 μg Hg/kg, $p < 0.05$.

For number of visits to the central area, the two-way ANOVA (sex × Hg dose) revealed a non-significant effect of sex: [$F(1,112) = 2.378$; $p = 0.12$]; a significant effect of Hg dose: [$F(4,112) = 14.67$; $p < 0.001$] and a non-significant interaction sex × Hg dose: [$F(4,112) = 0.207$; $p = 0.93$]. The post hoc analysis confirmed the significance of the Hg dose effect for all THIM doses in both sexes ($p < 0.01$)(Fig. 1C).

3.3. Haloperidol (HP)-induced catalepsy

The activity of endogenous DA system in experimental animals was evaluated in the 12th postnatal week using haloperidol to induce catalepsy. Catalepsy in control rats was compared to that in animals, which received postnatal THIM injections at doses of 1440 and 3000 μg Hg/kg. To prevent the interference from female sex hormones, only males were used.

Catalepsy was observed only in animals, which received HP injections, but not in those injected with saline. HP-induced catalepsy was significantly shorter in THIM-exposed rats; the effect was dependent on the dose of administered THIM (Fig. 2). The three-way ANOVA for repeated measurements (Hg dose × HP treatment × time) revealed a significant effect of Hg dose: [$F(2,156) = 15.58$; $p < 0.001$]; HP treatment [$F(3,156) = 107.78$; $p < 0.001$] and interaction Hg dose × HP treatment: [$F(6,156) = 2.834$; $p = 0.012$]. Other significant effects were on observation time:[$F(3,468) = 15.96$; $p < 0.001$], Hg dose × observation time: [$F(8,264) = 3.63$; $p < 0.001$], HP treatment × observation time: [$F(9,468) = 5.356$; $p < 0.001$].

The post hoc analysis confirmed that THIM treatment resulted in significant attenuation of HP catalepsy, with higher THIM dose generally producing more pronounced effect (Fig. 2A–C). This effect was particularly evident in rats, which received lower dose of HP (0.15 mg/kg). Overall, the results of this experiment show that early postnatal THIM administration makes animals less sensitive to HP-induced catalepsy.

Table 1
Effect of postnatal THIM treatment on developmental weight gain in Wistar rats.

THIM dose (μg Hg/kg)	PND 7Weight (g)	PND 21Weight (g)	% increase	# animals	# litters
0 (control group)	14.7 ± 0.1	42.0 ± 1.4	185	24	2
1440	17.4 ± 1.0	47.4 ± 1.0	172	24	2
3000	15.3 ± 0.3	44.8 ± 0.8	193	24	2

Weight of experimental animals was monitored in control animals and those, which received the highest two doses of THIM between the PND7 and PND21. Results show regular and proportional weight increase in all three experimental groups of animals. There were no statistical differences in weight gain between control and THIM treated groups ($p > 0.05$).

3.4. Dopamine (D_2) receptors staining

The experiment with HP-catalepsy suggested increased activity of DA, or other changes in the brain DA system in THIM-exposed rats. To confirm this with another type of experiment, we examined the density of striatal D_2 receptors with the immunohistochemical staining technique. A separate group of experimentally naïve, 8-week old rats of both sexes, treated at the neonatal stage with THIM
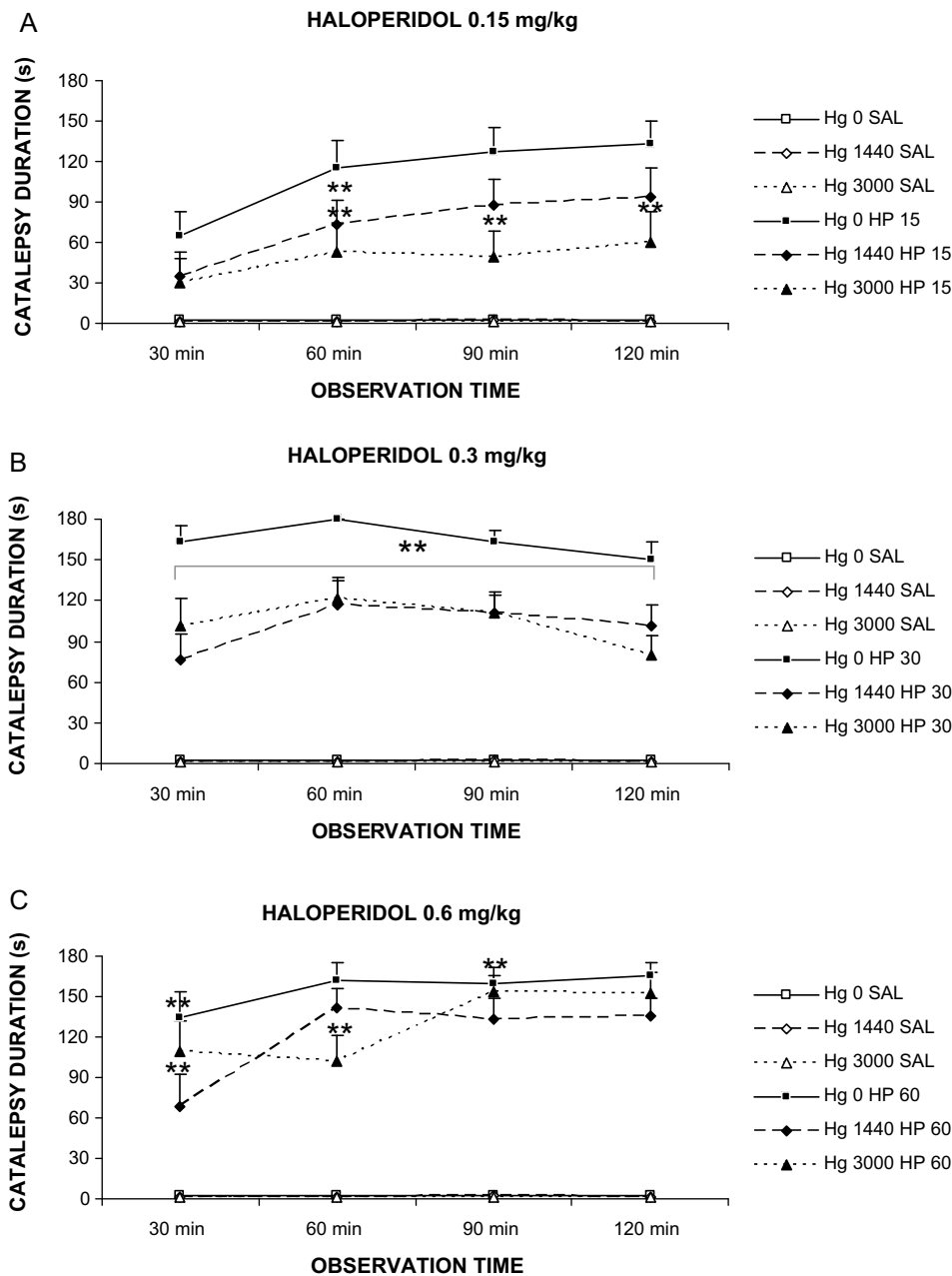

Fig. 2. Effect of early postnatal THIM administration on haloperidol (HP)-induced catalepsy at different observation timepoints in 12-week old rats. A: HP dose 0.15 mg/kg; B: HP dose 0.3 mg/kg; C: HP dose 0.6 mg/kg. Data represent means ± SEM (s). Stars denote statistically significant differences in comparison to controls; (p) levels <0.05 marked with (*); (p) levels <0.01 marked with (**). THIM-exposed rats revealed shortened duration of HP-induced catalepsy after HP administration. Experimental groups consisted of 14 animals per group/treatment.

doses of 12 and 240 µg Hg/kg were used in this experiment. The receptor densities were appraised by counting the area of positive anti-D_2 receptor reaction. We also calculated neuronal density in the corresponding striatal sections.

The results in Table 2 show markedly diminished D_2 receptor densities in the striata from THIM-treated male and female rats. The two-way ANOVA (sex × Hg dose) revealed a significant effect of Hg dose: [$F(2,22) = 6.88$; $p = 0.004$]; a non-significant effect of sex: [$F(1,22) = 0.211$; $p = 0.65$] and a non-significant interaction sex × Hg dose: [$F(2,22) = 1.689$; $p = 0.207$]. The post hoc analysis confirmed significantly reduced D_2 receptor density in the striatum for males, for Hg doses of 12 µg Hg/kg ($p = 0.002$) and 240 µg Hg/kg ($p = 0.003$); and for females, for Hg dose of 240 µg Hg/kg ($p < 0.006$).

In order to determine if reduced densities of D_2 receptors in THIM-exposed rats were due to loss of neurons, neuronal densities were assessed in the striata of these animals. Data in Table 3 show mean numbers of neurons ± SEM in analyzed striatal regions for the control group and for groups treated with THIM doses 12 and 240 µg Hg/kg. For neuron density in the striatum, the two-way ANOVA (sex × Hg dose) revealed a non-significant effects of: sex: [$F(1,24) = 0.109$; $p = 0.74$]; Hg dose: [$F(2,24) = 1.007$; $p = 0.38$] and non-significant interaction sex × Hg dose: [$F(2,24) = 0.167$; $p = 0.84$].

This experiment documented that neonatal rat exposure to THIM leads to a significant, dose-dependent decrease of the density of striatal D_2 receptors. Males reacted with greater receptor decline starting with the lower THIM dose (12 µg Hg/kg). There were no apparent changes in neuron density in the corresponding sections of the striatum, suggesting that diminished D_2 receptor density is independent of neuronal loss. Fig. 3 (images A and B) shows samples of D_2 receptor reaction in the striata of THIM-treated and control rats. Presented sections were stained with hematoxylin to show the cell structures.

3.5. Social interaction test

In order to find out if neonatal THIM exposure affects more complex social behaviors, the social interaction test was conducted in experimental animals. We initiated this test starting with rats, which received the two highest doses of THIM (1440 and 3000 µg Hg/kg) and controls. Because animals treated with the dose 1440 µg Hg/kg did not show significant changes in social interaction (see data below), we did not test animals treated with the lower THIM doses. The behaviors were divided into two general groups: prosocial (social sniffing, crawling under/over, following) and asocial/antisocial (freezing, self-grooming, avoiding, and aggressive-defensive). Generally, females manifested more intense prosocial interactions. In animals treated with the highest THIM dose (3000 µg Hg/kg), a decreased number of such interactions was observed in both males and females (Fig. 4A). For this parameter, the two-way ANOVA (sex × Hg dose) revealed a significant effects of: Hg dose: [$F(2,92) = 10.89$; $p < 0.001$]; sex: [$F(1,92) = 35.21$; $p < 0.001$] and a non-significant interaction sex × Hg dose: [$F(2,92) = 0.53$; $p = 0.59$]. The post hoc analysis confirmed the significance of the Hg dose effect for males (for the dose of 3000 µg Hg/kg, $p = 0.04$) and for females (for the dose of 3000 µg Hg/kg, $p < 0.01$).

For the total time of prosocial interactions the two-way ANOVA (sex × Hg dose) revealed: a significant effect of sex: [$F(1,92) = 7.523$; $p = 0.007$]; non-significant of Hg dose: [$F(2,92) = 1.648$; $p = 0.198$]; and a non-significant interaction sex × Hg dose: [$F(2,92) = 0.510$; $p = 0.6$] (Fig. 4B). There were no significant differences in total time of prosocial interactions between control and THIM treatment groups in males or females.

In asocial/antisocial interactions there were no basal differences between sexes, but divergence emerged in response to THIM treatment. Male rats treated with THIM (3000 µg Hg/kg) manifested an increased number of asocial/antisocial interactions, whereas in females treated with the same THIM dose—a reduction of such behaviors was noted. For the number of asocial/antisocial interaction episodes the two-way ANOVA (sex × Hg dose) revealed a significant effects of: Hg dose: [$F(2,92) = 3.5$; $p = 0.03$]; sex: [$F(1,92) = 13.86$; $p < 0.001$]; and interaction sex × Hg dose: [$F(2,92) = 20.49$; $p < 0.001$]. The post hoc (LSD) analysis confirmed the significance of the dose effect for both sexes for the Hg dose of 3000 µg Hg/kg ($p < 0.001$) (Fig. 4C).

In the parameter of total time of asocial/antisocial interactions similar trends were noted, but the two-way ANOVA (sex × Hg dose) revealed a non-significant effects of: dose: [$F(2,92) = 0.019$; $p = 0.98$]; sex: [$F(1,92) = 3.841$; $p = 0.053$]; and interaction sex × Hg dose: [$F(2,92) = 1.56$; $p = 0.21$] (Fig. 4D). These data suggest that the asocial/antisocial episodes altered by THIM treatment were brief.

The total number of line crossings, measuring locomotor activity during this test, was also analyzed. Only females treated with the THIM dose of 1440 µg Hg/kg manifested somewhat increased activity in this test. The two-way ANOVA (sex × Hg dose) revealed significant effects of: dose: [$F(2,92) = 18.68$; $p < 0.001$]; sex: [$F(1,92) = 65.99$; $p < 0.001$]; and interaction sex × Hg dose: [$F(2,92) = 3.54$; $p = 0.03$]. The post hoc analysis confirmed the significance of the dose effect (for the dose of 1440 µg Hg/kg) for females; $p < 0.001$ (Fig. 4E).

Overall in the social interaction test, rats which received postnatal THIM injections at a dose of 3000 µg Hg/kg showed decreased numbers of episodes of prosocial interactions and altered numbers of episodes of asocial/antisocial interactions—increased in males, but decreased in females. THIM treatment did not appear to significantly affect the total time of prosocial or asocial/antisocial interactions, indicating the brief nature of such interactions. Only the group of females that received the THIM dose of 1440 µg Hg/kg showed increased locomotor activity in this test, while other groups did not present such changes, suggesting that that changes in social interactions were not a consequence of alterations in locomotor activity.

3.6. Morris water maze test

To evaluate if neonatal THIM treatment affects learning and spatial memory, the Morris Water Maze test was conducted during the experimental animals' 10th and 11th postnatal week. In this test, rats placed in a large swimming pool are forced to find – using only spatial memory – a hidden platform in order to escape from water. Visits over the hidden platform area, time spent in the platform quadrant and distance to reach the platform, were analyzed to determine possible changes in memory. Average speed was monitored, as a measure of possible locomotor deficits. A motivation test (with the escape platform clearly visible) was also carried out to ensure that there were no visual/ophthalmic deficits among experimental groups, and to exclude that possible differences are due to those deficits.

As expected, females had more difficulties with finding the hidden platform, but there appeared to be no major differences between THIM-treated and control groups in memory test parameters such as visits over the platform area and percent of time spent in the platform area. For the parameter of visits over the platform area, the two-way ANOVA (sex × Hg dose) revealed significant effect of sex: [$F(1,51) = 10.767$; $p = 0.002$], a non-significant effect of Hg Dose: $F(2,51) = 0.0323$; $p = 0.97$] and a non-significant interaction sex × Hg dose: [$F(2,51) = 1.447$; $p = 0.24$] (Fig. 5A). For percent of time spent in the platform area during memory test, the two-way ANOVA (sex × Hg dose) revealed non-significant effect of: Hg dose: [$F(2,51) = 0.3105$; $p = 0.73$]; significant effect of sex: [$F(1,51) = 4.974$;

Table 2
Effect of postnatal THIM treatment on density of D_2 receptors in the striatum in 8-week old, behaviorally naïve Wistar rats.

Hg dose (µg/kg)	Males				N	Females				N
	AV-AF	±SEM	p	% CTRL		AV-AF	±SEM	p	% CTRL	
0	2.2856	0.912	x	100	4	2.0354	0.965	x	100	4
12	0.6540	0.312	0.002	28.61	5	1.4488	0.355	x	71.18	5
240	0.6772	0.160	0.003	29.62	5	0.5818	0.089	0.006	28.58	5

The receptor density is expressed as AV-AF – average stained area fraction of positive anti-D_2 dopamine receptor reaction; % CTRL – percent of control value; p – significance. The data show marked reduction of D_2 receptor densities in the striatum of neonatally THIM-exposed rats ($N = 28$).

Table 3
Effect of postnatal THIM treatment on numbers of neurons in the striatum.

THIM dose (µg Hg/kg)	Males Neurons' number (area 141808.92 µm²)	N	Females Neurons' number (area 141808.92 µm²)	N
0 (control)	169 ± 12	5	166 ± 4	5
12	162 ± 11	5	155 ± 6	5
240	153 ± 13	5	157 ± 3	5

Neurons were counted manually by a blinded researcher in control group and two groups of THIM-treated animals. Average neuron densities were not statistically different between experimental groups ($p > 0.05$) ($N = 30$).
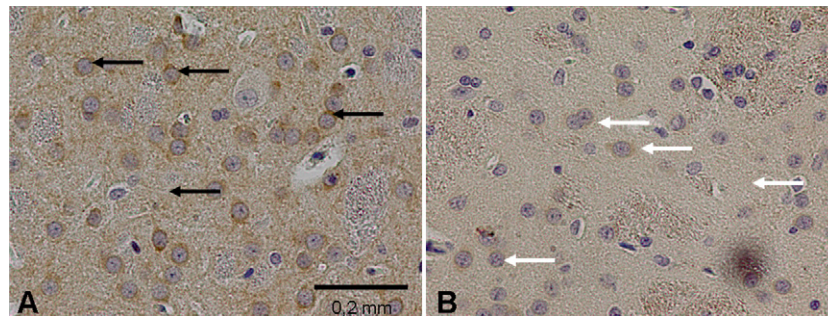

Fig. 3. Diminished anti-D_2 immunohistochemical reaction in the striatum in neonatally THIM-treated, 8-week old, behaviorally naïve male rats. Photograph illustrates loss of D_2 receptor densities in striatal neurons and neuropil. A: control group; B: Group treated with THIM dose 240 µg Hg/kg; magnification: 400×.

$p = 0.03$]; and significant interaction sex × Hg dose: [$F_{(2,51)} = 3.328$; $p = 0.04$] (Fig. 5B).

In latency to reach the platform, there were also no significant differences between control and THIM-treated animals. For this parameter, two-way ANOVA (sex × Hg dose) for repeated measurements revealed non-significant effects of: Hg dose: [$F_{(2,51)} = 1.771$; $p = 0.18$]; sex: [$F_{(1,51)} = 1.9707$; $p = 0.16$]; and a non-significant interaction sex × Hg dose: [$F_{(2,51)} = 0.923$; $p = 0.40$] (Fig. 5C). However, the group difference emerged in the parameter of distance to reach the hidden platform, where THIM treated males traveled a shorter distance to reach the platform. The two-way ANOVA (sex × Hg dose) for repeated measurements revealed a significant effects of: Hg dose: [$F_{(2,51)} = 16.60$; $p < 0.001$]; sex: [$F_{(1,51)} = 22.62$; $p < 0.001$]; and interaction sex × Hg dose: [$F_{(2,51)} = 4.6003$; $p = 0.01$]. The *post hoc* analysis confirmed the significance of the Hg dose effect for males, for Hg doses of 1440 and 3000 µg Hg/kg on days 1–3 ($p < 0.001$) (Fig. 5D).

The group differences were also seen in the parameter of swimming speed, where THIM-treated males swam noticeably slower than control males or females. The two-way ANOVA (sex × Hg dose) for repeated measurements revealed a significant effects of: Hg dose: [$F_{(2,51)} = 25.657$; $p < 0.001$]; sex: [$F_{(1,51)} = 23.346$; $p < 0.001$]; and interaction sex × Hg dose: [$F_{(2,51)} = 12.980$; $p < 0.001$]. The *post hoc* analysis confirmed the significance of the dose effect for males, for Hg doses of 1440 and 3000 µg Hg/kg on days 1–4 and for females, for a dose of 3000 µg Hg/kg on day 4 ($p < 0.001$) (Fig. 5E).

In the motivation test, the mean latencies to reach the open (visible) platform ± SEM were: for males: $32 ± 4$, $29 ± 6$, and $27 ± 5$ (s); for females: $37 ± 9$, $31 ± 2$, and $30 ± 8$ (s) for the control group

and Hg doses of 1440 and 3000 µg Hg/kg, respectively. The two-way ANOVA (sex × Hg dose) revealed non-significant effects of: Hg dose: [$F_{(2,51)} = 2.7912$; $p < 0.07$]; sex: [$F_{(1,51)} = 0.4255$; $p = 0.52$]; and interaction sex × Hg dose: [$F_{(2,51)} = 2.0472$; $p = 0.14$]. These data indicate that the water pool was an equally aversive environment for all experimental groups.

Overall, the Morris water maze experiments showed that young adult rats which received THIM injections during early postnatal life did not manifest gross visual-spatial memory impairments. The only statistically significant difference between the experimental groups was in swimming speed and distance to reach the platform, which were both lower in THIM-administered males. These data indicate that locomotor deficits in THIM-treated male rats, which were manifested in the open field test in the 4th week of life, were still present in the 10th week. This created certain difficulties in appraising the learning process in these animals. It is likely nonetheless, that a shorter distance to reach the platform in THIM-treated male rats than in controls is linked with their reduced speed of swimming, which could have improved their observation accuracy. Neonatally THIM-exposed female rats did not show locomotor and spatial memory deficits in this test.

4. Discussion

To the best of our knowledge, this is the first study documenting that neonatal THIM administration in rats produces enduring dose-dependent changes in several aspects of behavior, such as locomotor activity, neophobia/anxiety, social interactions, and response to neuroleptics. Previously, we showed lasting impair-
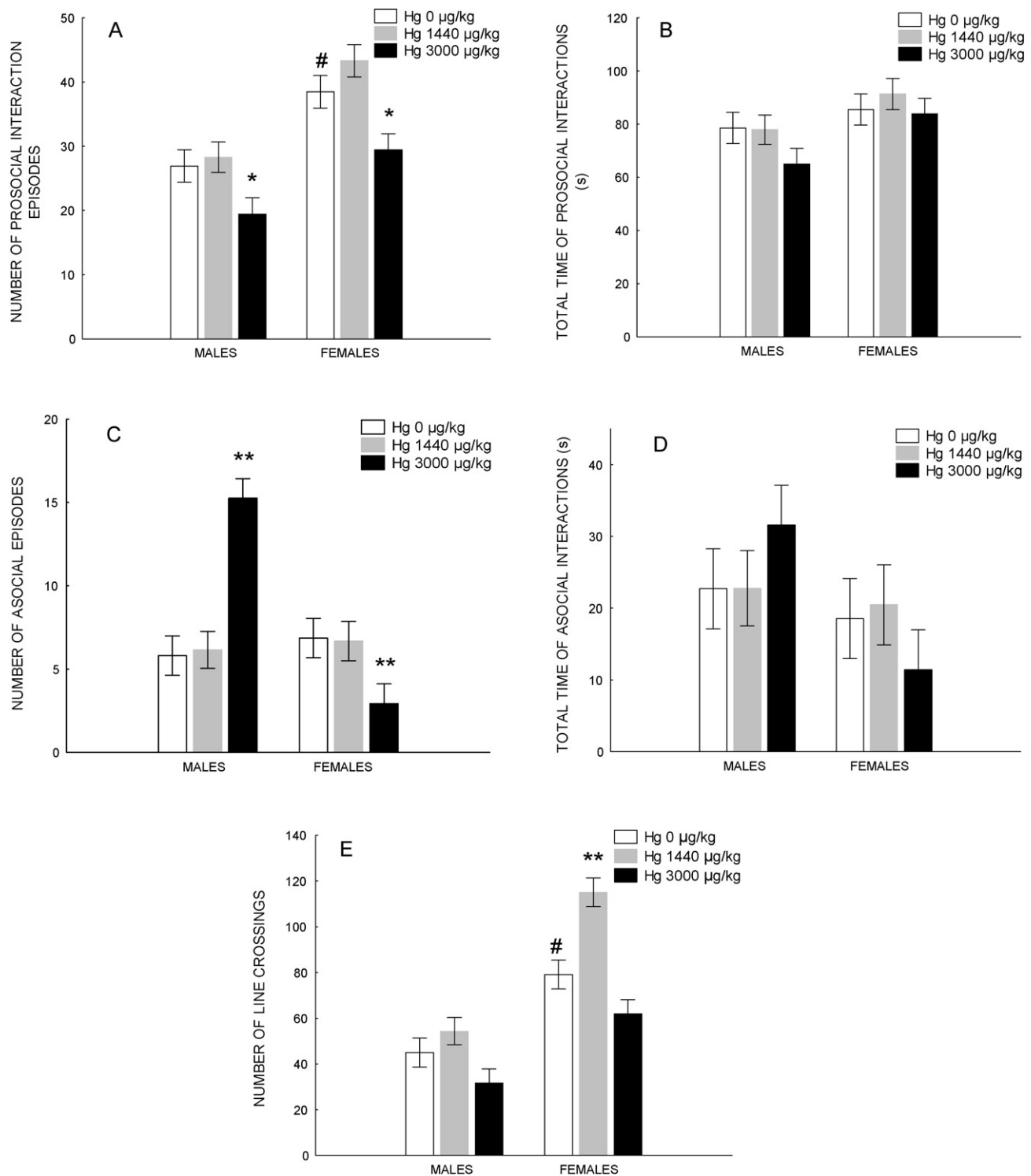
Fig. 4. Social interaction test in 8-week old control and THIM treated rats. A: total number of prosocial episodes; B: total duration of prosocial episodes; C: total number of asocial/antisocial episodes; D: total time of asocial/antisocial episodes; E: total number of line crossings (evaluation of locomotor activity during social interaction test). Bars show (means ± SEM); stars denote statistically significant differences in comparison to controls; (p) levels <0.05 marked with (*); (p) levels <0.01 marked with (**). Sign (#) denotes statistical difference between the sexes in control groups ($p < 0.05$). Each experimental group consisted of 8 pairs of same sex rats; the group of males, which received 1440 μg Hg/kg, which consisted of 9 pairs ($N = 98$).

ment of pain sensitivity and alterations in densities of brain mu opioid receptors in these animals [28,29]. Some neurobehavioral effects resulting from neonatal THIM exposure were present in both sexes, in others—pronounced sex dimorphism was observed. Certain behavioral deficiencies, such as locomotor deficits and anxiety/neophobia manifested themselves already in animals treated with the lowest doses of THIM, whereas other effects, such as impairments of social interactions and reduced sensitivity to neuroleptics, emerged only in animals treated with the highest doses of THIM.

Postnatal THIM administration did not seem to disturb general pup growth, as evidenced by proportional weight gains in THIM-
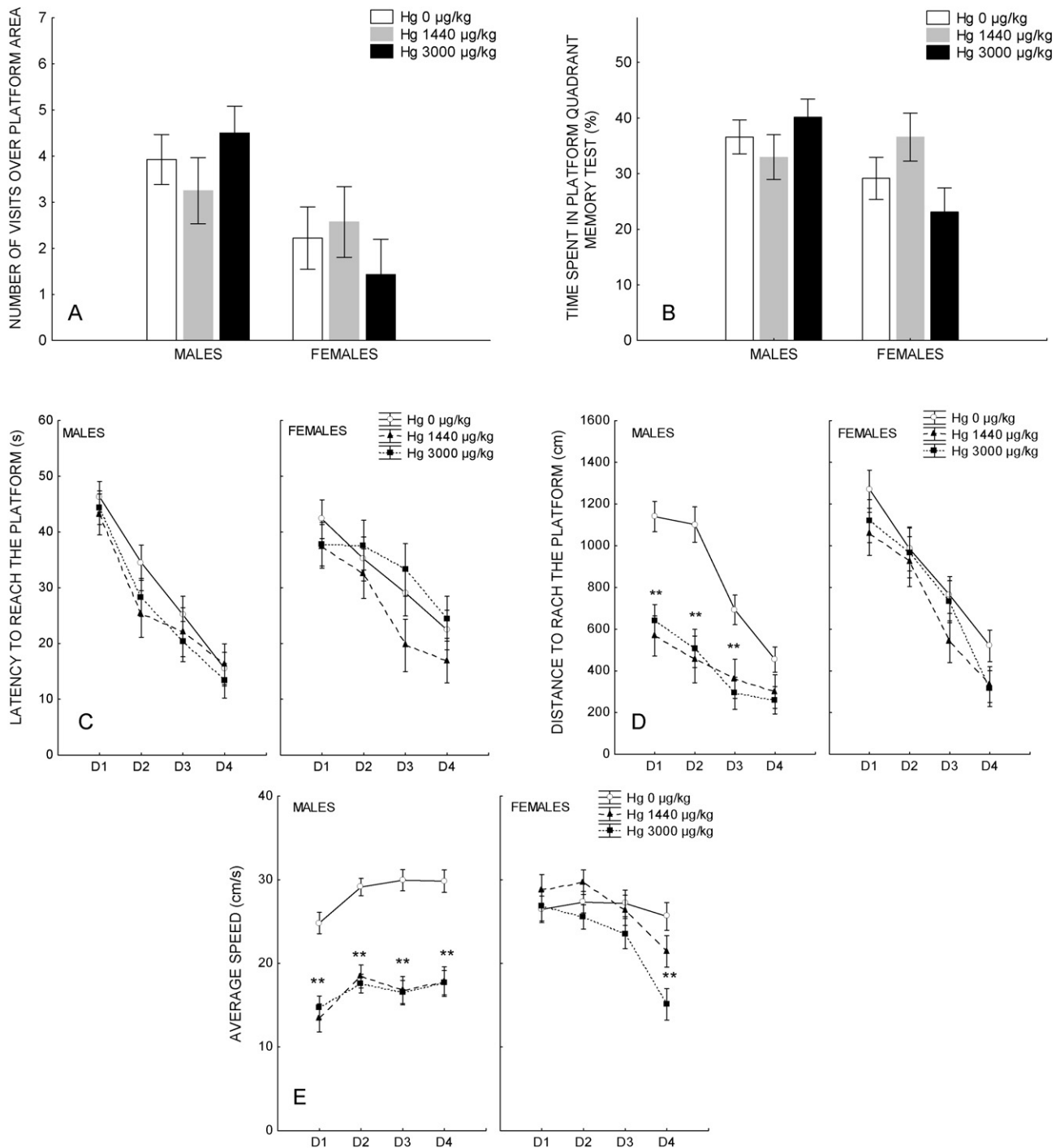
Fig. 5. Effect of postnatal THIM treatment on spatial learning and memory: Morris water maze test, in 10–11-week old rats. A: Visits over the platform area in memory test; B: percent of time spent in platform quadrant during memory test; C: mean latency to reach the platform in following test days; D: mean distance to reach the platform in following experimental days; E: average swimming speed in following days of the test. Bars show means ± SEM; stars denote statistically significant differences in comparison to controls; (p) levels <0.05 marked with (*); (p) levels <0.01 marked with (**). Experimental groups consisted of: Ctr (0 μg Hg/kg) – M14, F9; 1440 μg Hg/kg – M8, F7; 3000 μg Hg/kg – M12, F7 (N = 57).

treated animals, similar to that in controls. This effect differs from the findings of Hornig et al. [23], who observed delayed weight gain in mice after postnatal THIM administration at a dose of about 10 μg Hg/kg, although Berman et al. [27], using similar THIM doses, did not report significant changes in weight gain in THIM-treated mice.

In male rats, reduced locomotor activity was noticeable already at the lowest THIM dose tested (12 μg Hg/kg), while in females such effect was seen only at the highest dose (3000 μg Hg/kg), point-

ing to sex difference in sensitivity to neurotoxic/neurodisruptive THIM actions. The observed sex dimorphism in the neurobehavioral effects of THIM is consistent with previously reported greater vulnerability of males to mercurial toxicity, where testosterone and endogenous sulfhydryl compounds may come into play [49–52].

THIM-induced locomotor deficits in male rats persisted at least to PN week 10, as they were also observed in the water maze test, conducted at this age. Our findings are concordant with those of Hornig et al. [23], who reported lasting reduction of locomotor

activity in mice, which were treated postnatally with THIM, as well as with the study of Carvalho et al. [53], who described reduced locomotor activity accompanied with pathological changes in the Purkinje cells in mice after chronic exposure to methylmercury. In another study we also reported a marked pathology of Purkinje cells in neonatally THIM-treated rats [54], which suggests that the motor impairments in these animals may result from cerebellar pathologies.

THIM-exposed rats also showed pronounced anxiety or neo-phobia during unrestricted exploration of the open field. The biological mechanism of this effect, which was noticed in animals of both sexes treated with all doses of THIM, might be complex. On the one hand, it could be due to disruption of brain GABAer-gic systems, as mercurials were shown to reduce synthesis of GABA, decrease agonist binding to $GABA_A$ receptors and decrease GABA-evoked currents in neurons [55–57]. On the other hand, anxiogenic/proneophobic THIM action could also result from aug-mented activity of extracellular glutamate, which we observed in the prefrontal cortex of THIM treated rats (manuscript in prepara-tion).

Neonatal THIM treatment seems to also produce persistent changes in the brain dopaminergic system. In behavioral exper-iments, an attenuated catalepsy after administration of HP was observed in animals treated with the two highest doses of THIM, but significantly diminished density of striatal D_2 receptors was noted already in animals which received the lowest dose of THIM (12 μg Hg/kg). The latter effect did not seem to be due to a loss of striatal neurons. These data are concordant with several publications. Komulainen and Tuomisto [58] and Scheuhammer and Cherian [59] described reduced DA antagonist binding to D_2 receptors by mercurials in vitro, resulting from inactivation of the functional—SH groups at the receptors. Daré et al. [60] reported augmented behavioral responses to apomorphine and decreased ligand binding to DA receptors in rats exposed to methylmercury in utero, while Faro et al. [61] and Dreiem et al. [62] found that exposure to methyl mercury increases extracellular levels of DA by inhibiting the activity of DA transporters. It is likely that the THIM effect on the brain DA system is analogous to that produced by methyl mercury. Collectively, these data imply that mercurials alter DA neurotransmission in the striatum (and possibly other brain regions) in a multifaceted manner, by increasing synaptic concen-tration of DA and by bonding to thiol groups of D_2 receptors, thus affecting their function.

Neonatally THIM-exposed rats manifested characteristic, sex-ually dimorphic changes in social interactions. In males, reduced incidence of prosocial, but increased incidence of asocial/antisocial interactions was observed, although these changes were statisti-cally significant only at the highest THIM dose tested. THIM-treated females showed a diminished number of both prosocial and aso-cial/antisocial interactions. While some influence of locomotor impairments on these effects cannot be excluded, THIM effects on social interactions may be partially dissociated from them, as in males prosocial and asocial/antisocial activities were oriented in opposite directions, and there was no statistically significant THIM influence on line crossing.

Spatial learning and memory did not seem to be markedly altered by postnatal administration of THIM at doses of 1440 and 3000 μg Hg/kg in either sex or strain. This finding was rather surprising, as in the neuropathological studies we found morpho-logical changes in the hippocampal neurons in 8 week-old rats that were exposed to much lower THIM doses (12 and 240 μg Hg/kg) during the neonatal period [29,54]. Thus, in the present study we expected to see impairments in spatial learning, but no such effect was observed. One possible explanation for this apparent inconsis-tency may be the fact that THIM injections also produce a marked glutamate overflow in the rat brain (manuscript in preparation).

Since glutamate plays a key role in learning and memory [63], it could temporarily compensate for some loss of hippocampal neu-rons and synapses in young animals.

Overall, the results of this research evaluating the neurotoxic effects of THIM are concordant with several previously published studies conducted with different animal species, although there were some dissimilarities. Neurotoxic/neurodisruptive effects of low doses of THIM during early development, presented in this and in other our studies [14,28,29] are generally in agreement with the findings of Hornig et al. in mice [23], Laurente et al. in hamsters [24], and Hewitson et al. in monkeys [25,26]. Only the study of Berman et al. conducted in mice [27] did not report developmental neurotoxicity of THIM. It is difficult to speculate why some toxicological studies with apparently similar experi-mental conditions sometimes give different results. Many factors might contribute to this. One possibility (which we encountered) is inconsistent quality of reagents. Not only did we find that THIM purchased from different sources markedly differs in the content of Hg (per weight) and impurities, but we also noted significant vari-ations in the quality of different batches of THIM coming from the same source. Quality, and especially unknown impurities present in THIM, may profoundly impact the outcomes of toxicological experiments. Other possible factors may include dissimilar diets, animal breeding and housing conditions, stressors, the presence of environmental toxins, allergens, or infectious agents, which may influence vulnerability to toxins and strength of organism defense systems. It is possible that exposure to diverse environmental fac-tors (some noxious, other benign) – in addition to genetic and epigenetic causes – may contribute to individual differences in vulnerability to mercurial-induced toxicity among humans as well.

5. Conclusions

Taken together, this study documents that administration of THIM during the early postnatal period produces dose-dependent changes in rat behavior – some subtle, other robust – which include locomotor deficits, enhanced anxiety or neophobia, and impaired social interactions, accompanied by alterations in brain dopaminergic system, which persist in adult life. Vulnerability to neurotoxic/neurodisrupting effects of THIM depends on sex; males generally displayed more extensive impairments than females. While this preclinical study has obvious limitations, it may have important clinical implications. Because some behaviors, such as locomotor impairments or neophobia/anxiety were affected already by the lowest tested dose of THIM, our findings suggest that neonatal exposure to this mercurial (in THIM-containing vac-cines, which are still used in some developing countries) may lead to analogous changes in vulnerable children, potentially con-tributing to neurodevelopmental disorders. Clearly, more studies are needed to fully understand the effects of mercurials on neu-rodevelopment and behavior in different animal species, including humans. Nonetheless, already existing research calls for prudence and removal of THIM from all medicinal, cosmetic and food prod-ucts designated for children and pregnant women.

Conflict of interest

None of the authors declares a conflict of interest.

Acknowledgements

References

[1] Qvarnström J, Lambertsson L, Havarinasab S, Hultman P, Frech W. Determination of methylmercury, ethylmercury, and inorganic mercury in mouse tissues, following administration of thimerosal, by species-specific isotope dilution GC-inductively coupled plasma-MS. Anal Chem 2003;75:4120–4.

[2] Burbacher TM, Shen DD, Liberato N, Grant KS, Cernichiari E, Clarkson T. Comparison of blood and brain mercury levels in infant monkeys exposed to methylmercury or vaccines containing thimerosal. Environ Health Perspect 2005;113:1015–21.

[3] Redwood L, Bernard S, Brown D. Predicted mercury concentrations in hair from infant immunizations: cause for concern. Neurotoxicology 2001;22:691–7.

[4] Geier DA, Geier MR. A meta-analysis epidemiological assessment of neurodevelopmental disorders following vaccines administered from 1994 through 2000 in the United States. Neuro Endocrinol Lett 2006;27:401–13.

[5] Geier DA, Kern JK, Geier MR. The biological basis of autism spectrum disorders: understanding causation and treatment by clinical geneticists. Acta Neurobiol Exp (Wars) 2010;70:209–26.

[6] Mutter J, Naumann J, Schneider R, Walach H, Haley B. Mercury and autism: accelerating evidence? Neuro Endocrinol Lett 2005;26:439–46.

[7] Gallagher C, Goodman M. Hepatitis B triple series vaccine and developmental disability in US children aged 1–9 years. Toxicol Environ Chem 2008;90:997–1008.

[8] Dórea JG. Integrating experimental (in vitro and in vivo) neurotoxicity studies of low-dose thimerosal relevant to vaccines. Neurochem Res 2011. February 25 [Epub ahead of print].

[9] Majewska MD, Urbanowicz E, Rok-Bujko P, Namyslowska I, Mierzejewski P. Age-dependent lower or higher levels of hair mercury in autistic children than in healthy controls. Acta Neurobiol Exp (Wars) 2010;70:196–208.

[10] Baskin DS, Ngo H, Didenko VV. Thimerosal induces DNA breaks, caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts. Toxicol Sci 2003;74:361–8.

[11] Yel L, Brown LE, Su K, Gollapudi S, Gupta S. Thimerosal induces neuronal cell apoptosis by causing cytochrome c and apoptosis-inducing factor release from mitochondria. Int J Mol Med 2005;16:971–7.

[12] Humphrey ML, Cole MP, Pendergrass JC, Kiningham KK. Mitochondrial mediated thimerosal-induced apoptosis in a human neuroblastoma cell line (SK-N-SH). Neurotoxicology 2005;26:407–16.

[13] Herdman ML, Marcelo A, Huang Y, Niles RM, Dhar S, Kiningham KK. Thimerosal induces apoptosis in a neuroblastoma model via the cJun N-terminal kinase pathway. Toxicol Sci 2006;92:246–53.

[14] Olczak M, Duszczyk M, Mierzejewski P, Wierzba-Bobrowicz T, Majewska MD. Lasting neuropathological changes in rat brain after intermittent neonatal administration of thimerosal. Folia Neuropathol 2010;48:258–69.

[15] Zieminska E, Toczylowska B, Stafiej A, Lazarewicz JW. Low molecular weight thiols reduce thimerosal neurotoxicity in vitro: modulation by proteins. Toxicology 2010;276:154–63.

[16] Parran DK, Barker A, Ehrich M. Effects of thimerosal on NGF signal transduction and cell death in neuroblastoma cells. Toxicol Sci 2005;86:132–40.

[17] James SJ, Slikker 3rd W, Melnyk S, New E, Pogribna M, Jernigan S. Thimerosal neurotoxicity is associated with glutathione depletion: protection with glutathione precursors. Neurotoxicology 2005;26:1–8.

[18] Minami T, Miyata E, Sakamoto Y, Kohama A, Yamazaki H, Ichida S. Expression of metallothionein mRNAs on mouse cerebellum microglia cells by thimerosal and its metabolites. Toxicology 2009;261:25–32.

[19] Clarkson TW. Metal toxicity in the central nervous system. Environ Health Perspect 1987;75:59–64.

[20] Haley BE. Mercury toxicity: genetic susceptibility and synergistic effects. Med Veritas 2005;2:535–42.

[21] Prandota J. Autism spectrum disorders may be due to cerebral toxoplasmosis associated with chronic neuroinflammation causing persistent hypercytokinemia that resulted in increased lipid peroxidation, oxidative stress, and depressed metabolism of endogenous and exogenous substances. Res Autism Spectr Disord 2010;4:119–55.

[22] Abedi-Valugerdi M, Nilsson C, Zargari A, Gharibdoost F, DePierre JW, Hassan M. Bacterial lipopolysaccharide both renders resistant mice susceptible to mercury-induced autoimmunity and exacerbates such autoimmunity in susceptible mice. Clin Exp Immunol 2005;141:238–47.

[23] Hornig M, Chian D, Lipkin WI. Neurotoxic effects of postnatal thimerosal are mouse strain dependent. Mol Psychiatry 2004;9:833–45.

[24] Laurente J, Remuzgo F, Ávalos B, Chiquinta J, Ponce B, Avendaño R, et al. Neurotoxic effects of thimerosal at vaccines doses on the encephalon and development in 7 days-old hamsters. An Fac Med Lima 2007;68:222–37.

[25] Hewitson L, Houser LA, Stott C, Sackett G, Tomko JL, Atwood D, et al. Delayed acquisition of neonatal reflexes in newborn primates receiving a thimerosal-containing hepatitis B vaccine: influence of gestational age and birth weight. J Toxicol Environ Health A 2010;73:1298–313.

[26] Hewitson L, Lopresti BJ, Stott C, Mason NS, Tomko J. Influence of pediatric vaccines on amygdala growth and opioid ligand binding in rhesus macaque infants: a pilot study. Acta Neurobiol Exp (Wars) 2010;70:147–64.

[27] Berman RF, Pessah IN, Mouton PR, Mav D, Harry J. Low-level neonatal thimerosal exposure: further evaluation of altered neurotoxic potential in SJL mice. Toxicol Sci 2008;101:294–309.

[28] Olczak M, Duszczyk M, Mierzejewski P, Majewska MD. Neonatal administration of a vaccine preservative, thimerosal, produces lasting impairment

[29] of nociception and apparent activation of opioid system in rats. Brain Res 2009;1301:143–51.

[29] Olczak M, Duszczyk M, Mierzejewski P, Bobrowicz T, Majewska MD. Neonatal administration of thimerosal causes persistent changes in mu opioid receptors in the rat brain. Neurochem Res 2010;35:1840–7.

[30] Green D, Charman T, Pickles A, Chandler S, Loucas T, Simonoff E, et al. Impairment in movement skills of children with autistic spectrum disorders. Dev Med Child Neurol 2009;51:311–6.

[31] Staples KL, Reid G. Fundamental movement skills and autism spectrum disorders. J Autism Dev Disord 2010;40:209–17.

[32] Gjevik E, Eldevik S, Fjæran-Granum T, Sponheim E. Kiddie-SADS reveals high rates of DSM-IV disorders in children and adolescents with autism spectrum disorders. J Autism Dev Disord 2010. September 8 [Epub ahead of print].

[33] Schreibman L. Diagnostic features of autism. J Child Neurol 1988;3 Suppl.:S57–64.

[34] Tuchman R. Autism. Neurol Clin 2003;21:915–32.

[35] Gillberg C, Svennerholm L. CSF monoamines in autistic syndromes and other pervasive developmental disorders of early childhood. Br J Psychiatry 1987;151:89–94.

[36] Garreau B, Barthélémy C, Jouve J, Bruneau N, Muh JP, Lelord G. Urinary homovanillic acid levels of autistic children. Dev Med Child Neurol 1988;30:93–8.

[37] Salgado-Pineda P, Delaveau P, Blin O, Nieoullon A. Dopaminergic contribution to the regulation of emotional perception. Clin Neuropharmacol 2005;28:228–37.

[38] Arias-Carrión O, Pöppel E. Dopamine, learning, and reward-seeking behavior. Acta Neurobiol Exp (Wars) 2007;67:481–8.

[39] Sanberg PR. Haloperidol-induced catalepsy is mediated by postsynaptic dopamine receptors. Nature 1980;284:472–3.

[40] Carlsson A. On the neuronal circuitries and neurotransmitters involved in the control of locomotor activity. J Neural Transm Suppl 1993;40:1–12.

[41] Groenewegen HJ. The basal ganglia and motor control. Neural Plast 2003;10:107–20.

[42] Heien ML, Wightman RM. Phasic dopamine signaling during behavior, reward, and disease states. CNS Neurol Disord Drug Targets 2006;5:99–108.

[43] Hauber W. Dopamine release in the prefrontal cortex and striatum: temporal and behavioural aspects. Pharmacopsychiatry 2010;43 Suppl. 1:S32–41.

[44] Crawley J, Gerfen C, Rogawski M, Sibley D, Skolnick P, Wray S, editors. Short protocols in neuroscience, systems and behavioral methods. Hoboken, NJ: John Wiley and Sons Inc.; 2006.

[45] Meyza KZ, Boguszewski PM, Nikolaev E, Zagrodzka J. Diverse sensitivity of RHA/Verh and RLA/Verh rats to emotional and spatial aspects of a novel environment as a result of a distinct pattern of neuronal activation in the fear/anxiety circuit. Behav Genet 2009;39:48–61.

[46] Rogowski A, Rokicki D, Kostowski W, Bienkowski P. Relationship between dopamine D2 receptor-associated responses and operant ethanol self-administration in the rat: a factor analysis. Alcohol Alcohol 2003;38:305–9.

[47] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. Oxford, UK: Academic Press; 2007.

[48] Anderson BD, Nakamura T, Russell SJ, Peng KW. High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. Cancer Res 2004;64:4919–26.

[49] Muraoka Y, Itoh F. Sex difference of mercuric chloride-induced renal tubular necrosis in rats—from the aspect of sex differences in renal mercury concentration and sulfhydryl levels. J Toxicol Sci 1980;5:203–14.

[50] Clarkson TW, Nordberg GF, Sager PR. Reproductive and developmental toxicity of metals. Scand J Work Environ Health 1985;11:145–54.

[51] Branch DR. Gender-selective toxicity of thimerosal. Exp Toxicol Pathol 2009;61:133–6.

[52] Ekstrand J, Nielsen JB, Havarinasab S, Zalups RK, Söderkvist P, Hultman P. Mercury toxicokinetics—dependency on strain and gender. Toxicol Appl Pharmacol 2010;243:283–91.

[53] Carvalho MC, Franco JL, Ghizoni H, Kobus K, Nazari EM, Rocha JB, et al. Effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) on methylmercury-induced locomotor deficits and cerebellar toxicity in mice. Toxicology 2007;239:195–203.

[54] Olczak M, Duszczyk M, Mierzejewski P, Wierzba-Bobrowicz T, Majewska MD. Lasting neuropathological changes in rat brain after intermittent neonatal administration of thimerosal. Folia Neuropathologica 2010;48:258–69.

[55] Basu N, Scheuhammer AM, Rouvinen-Watt K, Evans RD, Trudeau VL, Chan LH. In vitro and whole animal evidence that methylmercury disrupts GABAergic systems in discrete brain regions in captive mink. Comp Biochem Physiol C: Toxicol Pharmacol 2010;151:379–85.

[56] O'Kusky JR, McGeer EG. Methylmercury poisoning of the developing nervous system in the rat: decreased activity of glutamic acid decarboxylase in cerebral cortex and neostriatum. Brain Res 1985;353:299–306.

[57] Wyrembek P, Szczuraszek K, Majewska MD, Mozrzymas JW. Intermingled modulatory and neurotoxic effects of thimerosal and mercuric ions on electrophysiological responses to GABA and NMDA in hippocampal neurons. J Physiol Pharmacol 2010;61:753–8.

[58] Komulainen H, Tuomisto J. 3H-dopamine uptake and 3H-haloperidol binding in striatum after administration of methyl mercury to rats. Arch Toxicol 1985;57:268–71.

[59] Scheuhammer AM, Cherian MG. Effects of heavy metal cations, sulfhydryl reagents and other chemical agents on striatal D2 dopamine receptors. Biochem Pharmacol 1985;34:3405–13.

[60] Daré E, Fetissov S, Hökfelt T, Hall H, Ogren SO, Ceccatelli S. Effects of pre-
natal exposure to methylmercury on dopamine-mediated locomotor activity
and dopamine D2 receptor binding. Naunyn Schmiedebergs Arch Pharmacol
2003;367:500–8.

[61] Faro LR, do Nascimento JL, Alfonso M, Durán R. Mechanism of action of
methylmercury on in vivo striatal dopamine release. Possible involvement of
dopamine transporter. Neurochem Int 2002;40:455–65.

[62] Dreiem A, Shan M, Okoniewski RJ, Sanchez-Morrissey S, Seegal RF.
Methylmercury inhibits dopaminergic function in rat pup synapto-
somes in an age-dependent manner. Neurotoxicol Teratol 2009;31:
312–7.

[63] Kauer JA, Malenka RC, Nicoll RA. A persistent postsynaptic modifica-
tion mediates long-term potentiation in the hippocampus. Neuron 1988;1:
911–7.