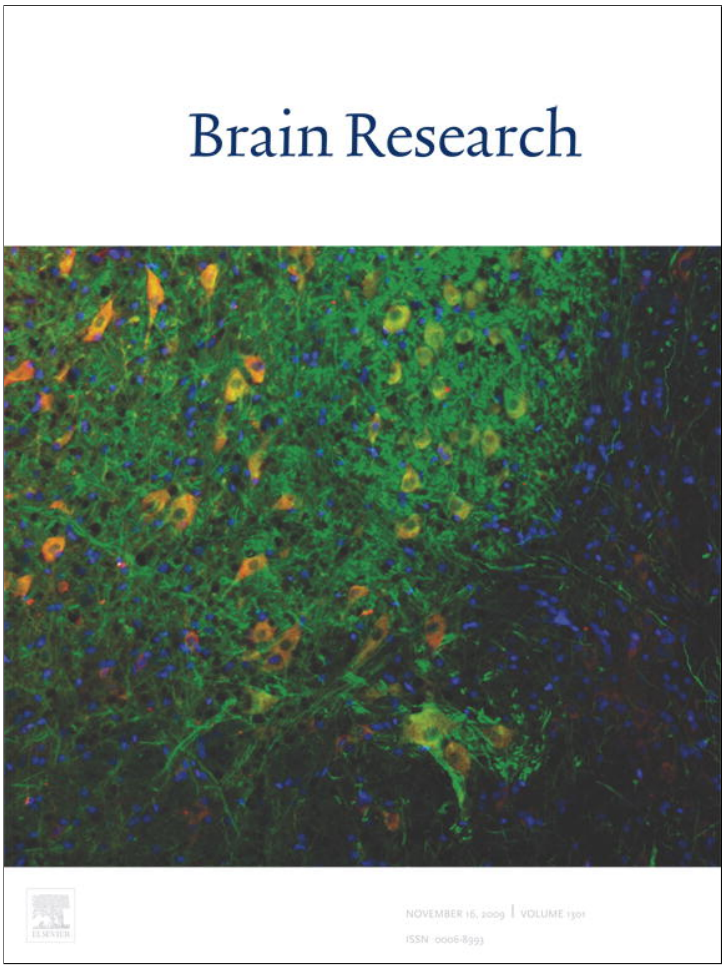

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Research Report

Neonatal administration of a vaccine preservative, thimerosal, produces lasting impairment of nociception and apparent activation of opioid system in rats

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ABSTRACT

Thimerosal (THIM), an organomercury preservative added to many child vaccines is a suspected factor in pathogenesis of neurodevelopmental disorders. We examined the pharmacokinetics of Hg in the brain, liver and kidneys after i.m. THIM injection in suckling rats and we tested THIM effect on nociception. THIM solutions were injected to Wistar and Lewis rats in a vaccination-like mode on PN days 7, 9, 11 and 15 in four equal doses. For Wistar rats these were: 12, 48, 240, 720, 1440, 2160, 3000 μg Hg/kg and for Lewis: 54, 216, 540 and 1080 μg Hg/kg. Pharmacokinetic analysis revealed that Hg from THIM injections accumulates in the rat brain in significant amounts and remains there longer than 30 days after the injection. At the 6th week of age animals were examined for pain sensitivity using the hot plate test. THIM treated rats of both strains and sexes manifested statistically significantly elevated pain threshold (latency for paw licking, jumping) on a hot plate (56 °C). Wistar rats were more sensitive to this effect than Lewis rats. Protracted THIM-induced hypoalgesia was reversed by naloxone (5 mg/kg, i.p.) injected before the hot plate test, indicative of involvement of endogenous opioids. This was confirmed by augmented catalepsy after morphine (2.5 mg/kg, s.c.) injection. Acute THIM injection to 6-week-old rats also produced hypoalgesia, but this effect was transient and was gone within 14 days. Present findings show that THIM administration to suckling or adult rats impairs sensitivity to pain, apparently due to activation the endogenous opioid system.

1. Introduction

Thimerosal (THIM; also known as thiomerosal or sodium ethylmercurithiosalicylate), which contains about 49% of mercury (Hg) by weight, has been used as a vaccine preservative for decades without rigorous studies examining its safety in developing mammalian organism, including infants. A vast body of scientific literature provides evidence that all forms of Hg are highly toxic to animals (rev. Díez, 2009; Clarkson and Magos, 2006). THIM is biotransformed in the body to ethylmercury and subsequently also into inorganic forms of Hg (Qvarnström et al., 2003). Significant amounts of

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Abbreviations: SIB, self injurious behaviors; THIM, thimerosal; Hg, mercury

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Hg have been detected in blood of human infants after inoculations with THIM-containing vaccines, with premature infants accumulating over 3 times more Hg than the mature ones (Stajich et al., 2000; Pichichero et al., 2008). Studies conducted with infant monkeys injected with THIM-containing vaccines, similar to those given to human infants, showed that after vaccination concentrations of Hg in the brain were several fold higher than those in blood and they remained markedly increased for many months (half life of Hg for in blood was about 7 days, and in the brain about 24 days) (Burbacher et al., 2005). These data suggest that Hg levels in infant brain after inoculations with THIM-containing vaccines may reach medium nanomolar concentrations, for which the neurotoxic effects have been reported in studies in vitro (Yel et al., 2005). Hence potential harm to infants from such vaccines cannot be ignored.

Many clinical and biological abnormalities (involving biochemistry, the nervous and immune systems) associated with Hg poisoning or prolonged contact resemble features of autism, hence the hypothesis was created that this neurodevelopmental disorder may result from exposure to Hg during early life (Bernard et al., 2001; Mutter et al., 2005). Several epidemiological studies linked THIM from vaccines to autism spectrum disorders, attention deficit hyperactivity disorder, learning disabilities and mental retardation (Geier and Geier 2003, 2006; Grether et al., 2004; Desoto and Hitlan, 2007; Young et al., 2008). Other studies have not found such a relationship (Hviid et al., 2003; Madsen et al., 2003), but they were criticized for major methodological flaws or an apparent conflict of interest (Mutter et al., 2005). The fact that incidence of autism and other neurodevelopmental disorders markedly increased during the 1990s, coincidentally with the introduction of several new vaccines which contained THIM (Atladóttir et al., 2007; Merrick et al., 2004), enforces the perception that these events may be connected.

Neurodevelopmental disorders are often accompanied by self-injurious behaviors (SIB) (Schreibman, 1988; Lovell and Reiss, 1993; Symons et al., 2004). The neuropsychobiological nature of such behaviors is complex and not well understood—it might include anxiety and obsessive-compulsive elements or different modes of pain expression. Whether or not SIB are related to hyposensitivity to pain, which has been anecdotally reported in some autistic and mentally disabled individuals, is a matter of debate (Tordjman et al., 1999; Messmer et al., 2008). Nevertheless, several studies reported significant reduction of SIB after therapy with the opioid receptor antagonist, naltrexone (Barrett et al., 1989; Elchaar et al., 2006; Symons et al., 2004), albeit not all studies showed such effect (Willemsen-Swinkels et al., 1995). Apparent beneficial effect of naltrexone in treating SIB is concordant with the findings showing dysregulation of endogenous opioids in autistic and mentally retarded persons (Sahley and Panksepp, 1987; Sandman, 1988; Sandyk and Gillman, 1986; Leboyer et al., 1994; Kemp et al., 2008), suggesting that this is clinically important trait at least in some neurodevelopmental disorders.

Continuing disagreements around the issue of involvement of THIM from vaccines in neurodevelopmental disorders demand further studies, both at clinical and preclinical levels. A previously published animal study by Hornig et al. (2004) revealed a selective vulnerability of autoimmune disease-sensitive mice (SJL/J) to the neurotoxic effects of THIM doses equivalent to those used in child vaccines, but lack of such an effect in mice of other strains. However, these findings were not confirmed by work of Berman et al. (2008). This discrepancy calls for additional research.

The present preclinical study is a part of broader research project, which addresses some of the existing controversies concerning potential neurotoxic effects of THIM from vaccines in developing organisms. We are testing in animal model the hypothesis that early life exposure to this organomercurial may be responsible for persistent neurodevelopmental impairments. In this study we have focused on two major issues—the pharmacokinetics of Hg in different organs after THIM injection in suckling rats and on nociception, which is expected to reflect the status of endogenous opioids. We examined sensitivity to painful stimuli and indirectly the activity of endogenous opioids in adult rats, which as neonates were treated with THIM. In order to detect possible genetic differences in vulnerability to toxic effects of THIM we conducted the experiments with rats from two strains–Wistar and Lewis–which derive from a common original stock, but differ genetically. Lewis is characterized by a deficient hypothalamic–pituitary–adrenal (HPA) axis and thus is more prone to autoimmune diseases (Oitzl et al., 1995). Finally, we examined THIM's effect on nociception after its acute administration to adult rats.

2. Results

2.1. *THIM/Hg pharmacokinetics*

The pharmacokinetic experiment measuring Hg distribution into different organs was conducted after a single THIM injection (2.9 or 5.8 mg Hg/kg, i.m.) to 10-day-old male Wistar rats. Animals were euthanized at different time points after THIM injection and organs were collected for analysis of Hg concentration. The analysis revealed that Hg from THIM accumulated in the brain, liver and kidneys (Fig. 1). In the brains of control rats there were trace amounts of Hg at the level of 0.0045 + 0.0006 μg/g (mean + SEM, $N = 3$). One day after THIM injection the brain concentration of Hg increased to 0.427 + 0.022 μg/g and 0.770 + 0.090 μg/g, for lower and higher THIM doses, respectively ($N = 4$; Fig. 1A). On day 4, brain Hg content increased further and on day 14, it declined to 53–72% of the maximal level from day 4. On day 30 Hg content in brain was at a level of 7–13% of the maximal value from day 4. Estimated $T_{1/2}$ values for THIM-Hg in brain were 6.8–8.6 days, for higher and lower THIM dose, respectively.

The liver accumulated 13–14 times more Hg than the brain per gram of tissue. On day 1, Hg levels in this organ were the highest. On day 4, Hg content in the liver declined to 74–92% of the maximal values from day 1, and on day 14 decreased to 26–29% of these values. On day 30, there remained about 3% of the maximal concentration (Fig. 1B). For the liver, estimated $T_{1/2}$ of THIM-Hg was 5.2–5.7 days (means for higher and lower Hg dose, respectively).

The kidneys accumulated largest part of injected Hg (21–32 times more than the brain) and cleared it at slowest rate (Fig. 1C). These levels declined minimally during first 14 days after THIM injection. On day 30, they were still at the level

43–56% of the concentrations from day 1. For the kidneys, THIM-Hg $T_{1/2}$ was 25.1–37.5 days (means for higher and lower doses, respectively).

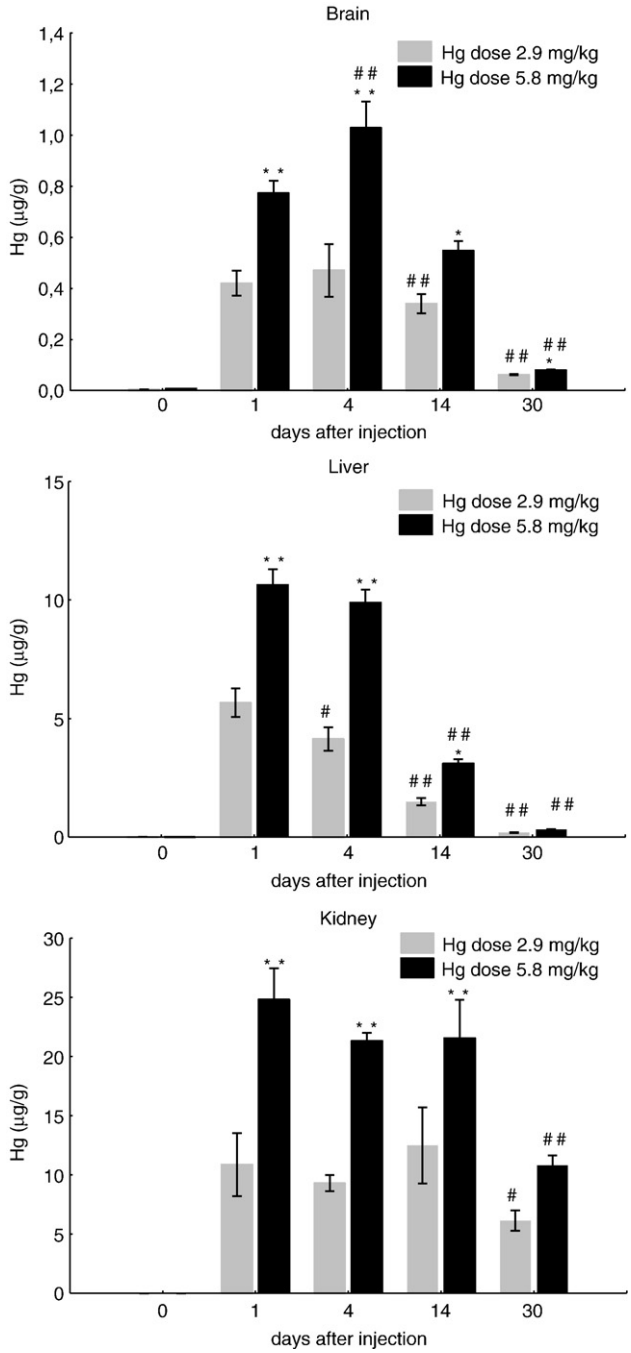
2.2. *Hot plate: neonatal THIM treatment*

Rats from both strains, which received four equal-dose THIM inoculations, i.m., on postnatal days 7, 9, 11 and 15 were examined at 6th week of life for sensitivity to nociceptive pain in the hot plate test. For Wistar rats, the THIM doses per injection were: 12, 48, 240, 720, 1440, 2160 and 3000 μg Hg/kg and for Lewis rats they were: 54, 216, 540 and 1080 μg Hg/kg. The doses were calculated based on % Hg content in THIM substance purchased from two sources. (see Experimental procedures for Hg analysis and details). The lowest used THIM dose (12 μg Hg/kg) is in the order of Hg doses still present in infant vaccines in many countries, including Poland (see Table 1 in Experimental procedures), as well as in the order of doses utilized in studies in mice (Hornig et al., 2004; Berman et al. 2008) and in the study in monkeys (Burbacher et al., 2005).

Table 1 – Calculations of Hg load, which infants may receive with vaccines in the form of THIM in many countries, including Poland, according to 2008 recommended Immunization Schedules in the USA and Poland.

Age, months	Birth	2	3–4	5–7
Vaccines	Hep B	Hep B+ DTP+Hib	DTP+MCV+Hib	DTP+Hep B+MCV+Flu
Hg load (μg)	25	75	75	100
Average infant weight (kg)	3.3	4.5	5.7	7.2
Hg dose (μg/kg)	7.6	16.6	13.1	13.8

Vaccines: Hep B—Hepatitis B, DTP—diphtheria–tetanus–pertussis, Hib–*Haemophilus influenzae* b, MCV—meningococcal conjugate vaccine. Hg load—total amount of Hg from THIM, which infants may receive. Average infant weights based on US norms for boys and girls.



Control and neonatally THIM-injected rats at the 6th week of life were assessed for nociception in hot plate test. Results show that THIM-exposed animals from both strains and sexes had reduced sensitivity to painful stimuli, as the latency of their reflexes (paw licking or jumping) was significantly prolonged. This apparent THIM induced analgesia or hypoalgesia was dose-dependent. Data in Fig. 2A show latencies to the nociceptive responses in Wistar rats and the data in Fig. 2B refer to Lewis rats.

For Wistar rats the two-way ANOVA (sex×Hg dose) revealed a non-significant effect of sex [$F(1,125)=3.103$; $p=0.08$]. There was a significant effect of Hg dose [$F(7,125)=8.701$, $p<0.001$], and a non-significant Sex×Hg dose inter-

Fig. 1 – Distribution of Hg into the brain, liver and kidneys from a single-dose THIM injection to 10-day-old rats. Data represent means+SEM, N=4. Statistics: for brain the two-way ANOVA (dose×time) revealed a significant effect of dose [$F(1,6)=81,2$; $p<0.001$], a significant effect of time [$F(3,18)=55,2$, $p<0.001$], and a significant dose×time interaction [$F(3,18)=7,76$, $p=0.001$]. For liver the two-way ANOVA (dose×time) revealed a significant effect of dose [$F(1,6)=91,3$; $p<0.001$], a significant effect of time [$F(3,18)=243,3$, $p<0.001$], and a significant dose×time interaction [$F(3,18)=17,1$, $p<0.001$]. For kidney the two-way ANOVA (dose×time) revealed a significant effect of dose [$F(1,6)=36,6$; $p<0.001$], a significant effect of time [$F(3,18)=15,2$, $p<0.001$], and a significant dose×time interaction [$F(3,18)=3,4$, $p=0,04$]. The *post-hoc* analysis: *Significant effect of dose for a given time point, $p<0.05$, **Significant effect of dose for a given time point, $p<0.01$, #Significant effect of time in comparison to the first day, $p<0.05$, ##Significant effect of time in comparison to the first day, $p<0.01$.

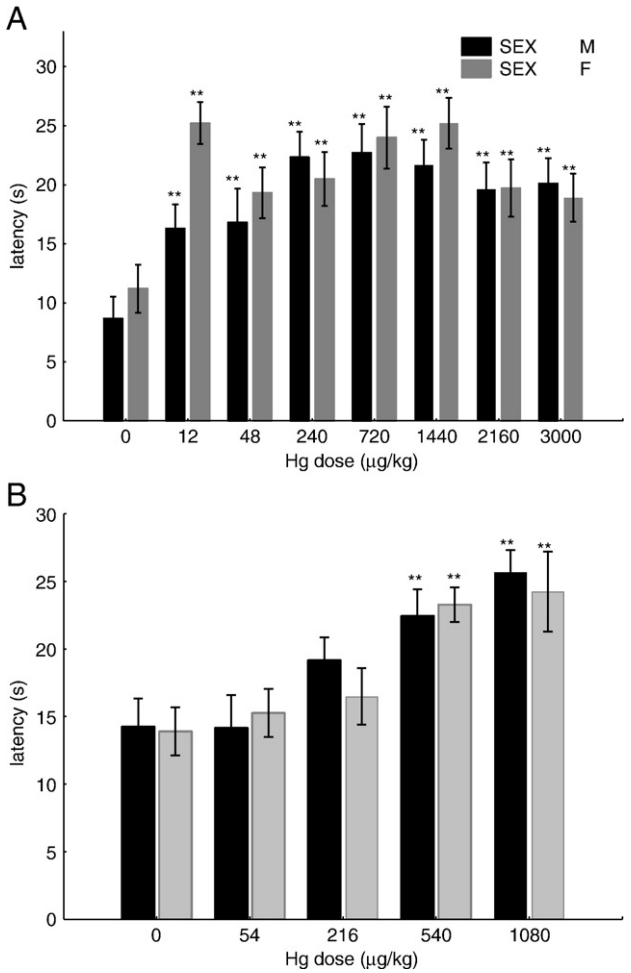


Fig. 2 – Effect of neonatal THIM administration on nociception. On X axis: THIM dose per injection, expressed as μg Hg/kg. There were four equal-dose injections per rat. On Y axis: latency to reaction on hot plate (paw licking or jumping) in seconds. Data are means + SEM; N = 8–12. (A) Wistar rats: statistically significant effects were observed in both sexes at Hg doses 12–3000 μg/kg ($p < 0.001$); (B) Lewis rats: statistically significant effects were observed in both sexes at Hg doses 540 and 1080 μg/kg ($p < 0.001$).

action [$F(7,125) = 1.377$, $p = 0.22$]. The *post-hoc* analysis confirmed a significant Hg dose effect for both sexes at doses 12–3000 μg Hg/kg (p values < 0.01).

Similarly, for Lewis rats the two-way ANOVA (sex × Hg dose) revealed a non-significant effect of sex [$F(1,93) = 0,151$; $p = 0.69$]. There was a significant effect of Hg dose [$F(4,93) = 11,063$, $p < 0.001$], and a non-significant sex × Hg dose interaction [$F(4,93) = 0.327$, $p = 0.85$]. The *post-hoc* analysis confirmed a significant Hg dose effect for both sexes at doses 540–1080 μg Hg/kg (p values < 0.01).

Whereas there were no statistically significant differences in an apparent THIM-induced analgesia/hypoalgesia between the two sexes, there were marked strain differences. Wistar rats seem to be more sensitive to this effect of THIM, because the minimal effective dose in animals from this strain was about 12 μg THIM-Hg/kg (per injection), whereas in Lewis rats, this dose was about 540 μg THIM-Hg/kg.

In search for neurobiological mechanism of persistent impairment of pain sensitivity produced by THIM we investigated the potential role of endogenous opioids in this phenomenon. We tested if this effect could be blocked by an antagonist of opioid receptors, naloxone. Wistar male and female rats, which at neonatal stage received four equal THIM injections (1440 or 3000 μg Hg/kg) were examined for pain sensitivity in hot plate test at 6th week of age. Thirty minutes before the test, they were pretreated with naloxone (5 mg/kg, i.p.) or saline. Similar doses of systemically administered naloxone were shown to block activity of endogenous opioids and produce hyperalgesia, but they are not expected per se to induce pain (Attal et al., 1989; Parsons and Herz, 1990). Result of this experiment showed that naloxone reversed the analgesic effect of THIM (Fig. 3).

The three-way ANOVA (sex × Hg dose × treatment) revealed a significant effect of Hg Dose [$F(2,103) = 19.0$, $p < 0.001$], a significant effect of naloxone treatment [$F(1,103) = 171.0$, $p < 0.001$] and a significant Hg dose × treatment interaction [$F(2,103) = 30.97$, $p < 0.001$]. All other effects and interactions were non-significant ($p > 0.05$). The *post-hoc* analysis confirmed that naloxone significantly reversed THIM analgesic effect in all rats, which received THIM injections (p values < 0.01). Moreover, in the presence of naloxone THIM-treated rats appeared even somewhat hyperalgesic comparing to controls, as evidenced by their shorter latencies to reaction, but this effect was not statistically significant.

2.3. *Catalepsy: neonatal THIM treatment*

The results of the hot plate experiment suggested that THIM treatment at neonatal stage results in protracted enhancement of activity of endogenous opioids. To verify this in another type of experiment, we examined the sensitivity of these animals to

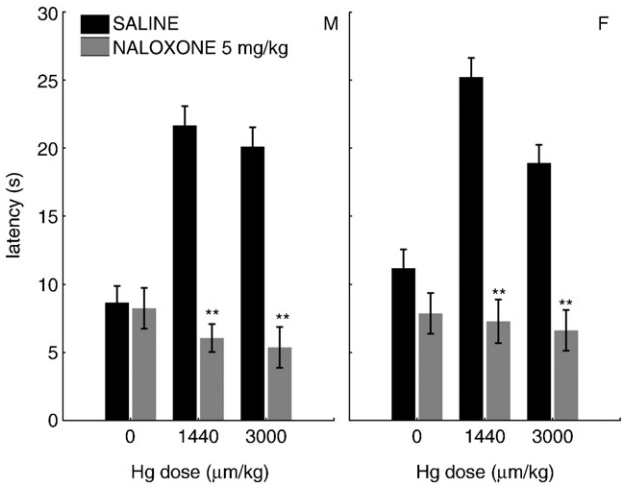


Fig. 3 – Effect of naloxone on analgesia induced by neonatal THIM administration in Wistar rats. X axis: THIM dose per injection (μg Hg/kg); rats received four equal THIM doses. Y axis: latency to reaction (paw licking or jumping) on hot plate in seconds. Data are means + SEM; N = 8. Naloxone injected before the hot plate test statistically significantly reversed analgesia induced by both doses of THIM in male and female rats ($p < 0.001$).

morphine-induced catalepsy, expecting that it might be augmented. Data shown in Fig. 4 confirm that this is the case.

The three-way ANOVA with repeated measures (Hg dose × treatment × time) revealed a significant effect of Hg dose [$F(2,66)=10.71$, $p<0.001$], a significant effect of morphine treatment [$F(1,66)=214.0$, $p<0.001$] and a significant Hg dose × treatment interaction [$F(2,66)=11.58$, $p<0.001$]. In neonatally THIM-treated (doses 1440 or 3000 μg Hg/kg) male Wistar rats catalepsy induced by morphine (2.5 mg/kg, i.p.) was statistically significantly prolonged in comparison to control group. The magnitude of this effect depended on dose of THIM administered. Moreover, there was also a significant effect of time [$F(4,264)=66.24$, $p<0.001$], a significant Hg dose × time interaction [$F(8,264)=3.63$ $p<0.001$], a significant treatment × time interaction [$F(4,264)=66.52$, $p<0.001$], and a significant treatment × Hg dose × time interaction [$F(8,264)=3.63$, $p<0.001$]. In animals treated with the lower THIM dose, statistically significant prolongation of catalepsy was observed mainly at 30 min after morphine injection ($p=0.004$), but in rats treated with the higher dose of THIM, prolongation of catalepsy was statistically significant at all time points after morphine injection ($p<0.001$).

2.4. *Hot plate: adult rats, acute THIM treatment*

In the group of rats treated with THIM at neonatal stage, delayed analgesia was measured 4 weeks after last THIM injection. The question arose: is this phenomenon due to developmental rearrangements of the central nervous system, or is it a direct analgesic effect of THIM per se? To answer it, we examined the influence of acute THIM administration on nociception in a separate group of experimentally naïve male Wistar, 6-week-old rats. First, we tested the time-course of acute THIM effects. Animals received a single injection of THIM at dose 12,000 μg Hg/kg (quadruplicate of the single highest dose used in neonates) and their pain sensitivity was measured using the hot plate test at different time points. The results in Fig. 5 show that acute THIM administration also elevated pain threshold.

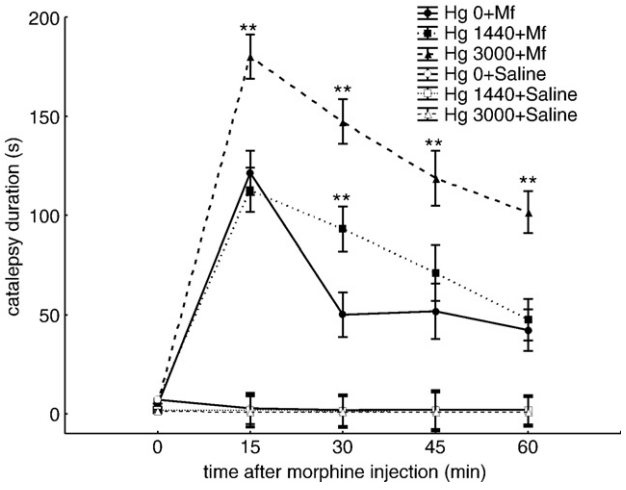


Fig. 4 – Morphine catalepsy in neonatally THIM-treated male Wistar rats. X axis: time after morphine injection. Y axis: duration of catalepsy per measurement episode (in seconds). Data are means + SEM; N = 8. Filled circles—control rats + morphine (2.5 mg/kg); filled squares—rats which as neonates received 4 THIM injections (1440 μg Hg/kg) + morphine (2.5 mg/kg); filled triangles—rats which as neonates received 4 THIM injections (3000 μg Hg/kg) + morphine (2.5 mg/kg). Open circles, squares and triangles—control rats, rats treated as neonates with THIM 1440 or 3000 μg Hg/kg, respectively, without morphine. Statistically significant prolongation of catalepsy was seen for Hg dose 3000 μg/kg at 15, 30, 45 and 60 min ($p<0.001$) and for Hg dose 1440 μg/kg at 30 min ($p<0.001$).

The two-way ANOVA with repeated measures (THIM × time) revealed a significant effect of THIM [$F(1,13)=31,86$, $p<0.001$], a significant effect of time [$F(5,65)=3.37$, $p<0.009$] and a significant THIM × time interaction [$F(5,65)=4.59$, $p=0.001$]. The analgesic effect after acute THIM treatment was transient; it emerged 1 h after drug injection, reached an apparent maximum at 4 h, lasted for at least 4 days, and was gone within 14 days. Statistically significant differences between saline and THIM-treated groups were observed at time points 1 h, 4 h, 24 h and 4 days after THIM injection ($p<0.005$).

The dose–response effect of acute THIM administration was investigated in a group of naïve 6-week-old rats, which received single injections of different doses of THIM: 48, 192, 960, and 12,000 μg Hg/kg (quadruplicates of doses administered to neonates in four separate inoculations) or saline. Their sensitivity to nociceptive stimuli was examined using the hot plate test 4 h after drug injection.

The one-way ANOVA revealed a significant effect of THIM dose [$F(1,46)=584.19$, $p<0.001$]. The *post-hoc* analysis revealed that statistically significant analgesia was produced by a single dose 192 μg Hg/kg (equivalent to 4 injections of 48 μg Hg/kg) or higher doses ($p<0.001$; Fig. 6A). The acute analgesic effect of THIM (at dose 12,000 μg Hg/kg), like the one observed in animals treated with THIM as neonates, was reversed by naloxone (5 mg/kg) (Fig. 6B). The two-way ANOVA (THIM × naloxone)

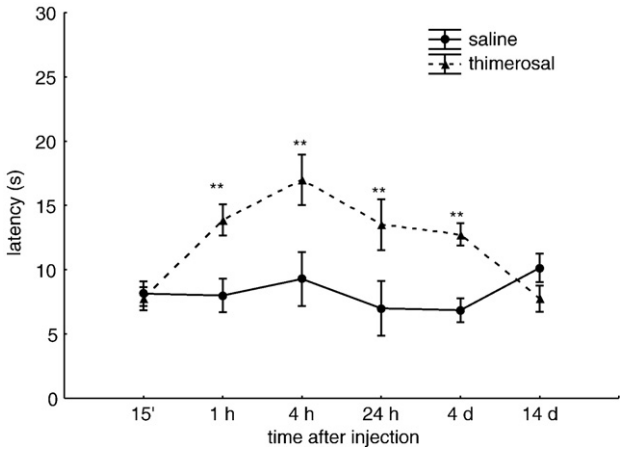


Fig. 5 – Time-course effects of acute THIM administration on nociception in Wistar male rats. X axis: time after acute single dose THIM injection (12,000 μg Hg/kg); Y axis: latency to paw licking or jumping on hot plate, in seconds. Data are means + SEM; N = 8. Animals injected with acute dose of THIM manifested significantly prolonged reaction latency in hot plate test ($p<0.001$).

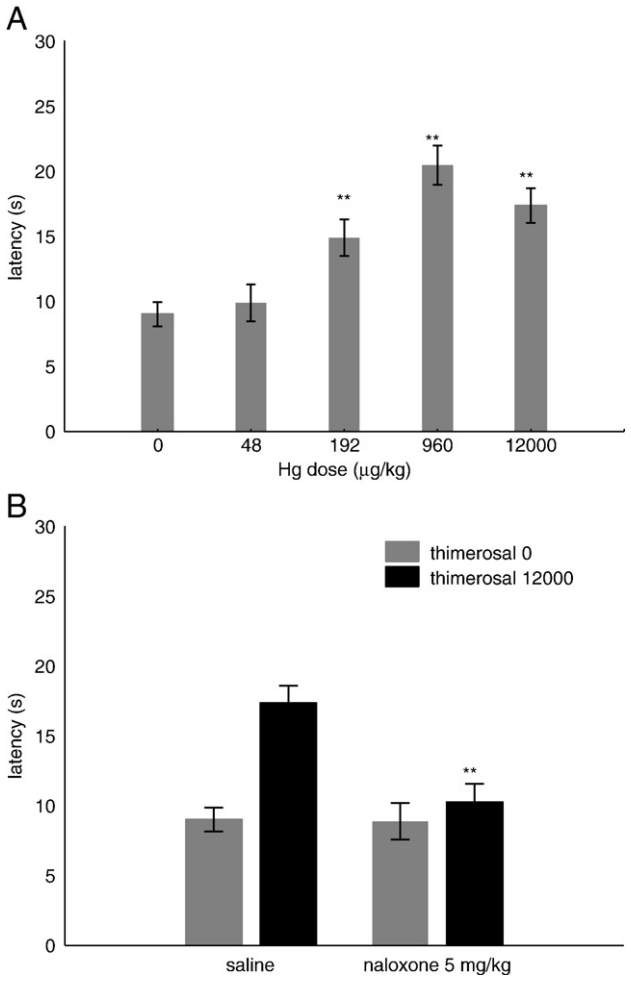


Fig. 6 – (A) Dose–response effect of acute THIM administration on nociception in male Wistar rats. The hot plate test was conducted at 4th hour after injection of single acute doses of THIM. X axis: THIM dose (μg Hg/kg). Y axis: latency to paw licking or jumping on hot plate, in seconds. Data are means + SEM; N = 8). Statistically significant prolongation of latency was seen at doses 192–12,000 μg Hg/kg ($p < 0.001$). (B) Reversal by naloxone of analgesia induced by acute THIM injection in male Wistar rats. The test was conducted at 4th hour after THIM (12,000 μg Hg/kg) injection. Data represent means + SEM; N = 8. Naloxone (5 mg/kg) reversed THIM-induced analgesia in a statistically significant manner ($p < 0.001$).

revealed a significant effect of THIM [$F(1,40) = 17.18$, $p < 0.001$], a significant effect of naloxone [$F(1,40) = 9.47$, $p = 0.004$] and a significant THIM × naloxone [$F(1,40) = 8.82$, $p = 0.005$].

3. Discussion

This is the first study, to the best of our knowledge, which shows that administration of THIM to suckling rats causes protracted impairment in pain sensitivity. This effect was dose-dependent and present in two strains of rats, Wistar and Lewis, which showed nonetheless different sensitivity to this drug. Wistar rats reacted with analgesia to lower doses of THIM (equivalent to those used in child vaccines) than Lewis rats. There were no statistically significant differences between males and females in this aspect. Analgesia/hypoalgesia produced by THIM was reversed by the opiate receptor antagonist, naloxone, suggesting that it probably involved endogenous opioids. Apparent increased activity of endogenous opioids in animals treated with THIM at the neonatal stage was confirmed in another experiment, which showed that these animals also manifested augmented catalepsy after morphine injection. Since neonatal THIM treatment somewhat decreased rats' ambulatory activity (manuscript in preparation), some input of this factor on THIM's effect on nociception cannot be ignored. However, it seems unlikely to be primarily responsible for it, because naloxone reversed the THIM effect on nociception, but its effect on locomotion, if any, is rather to slow it down (DeRossett, Holtzman, 1982), hence it would act in the same direction as THIM.

Acute administration of THIM to adult rats also produced hypoalgesia, which emerged within 60 min after the drug injection. This effect was transient however, as it disappeared within 14 days and was also reversed by naloxone. Older rats seem to be less susceptible than neonates to the neurodisrupting effects of THIM, because they required higher doses of this drug to elevate pain threshold.

THIM is converted in the body to ethylmercury, believed to be its major biologically active metabolite, and further also to inorganic mercury forms (Qvarnström et al., 2003). In its biological effects ethylmercury is similar to methylmercury (which has been studied more extensively), although there are pharmacokinetic differences between these compounds (Magos et al., 1985; Qvarnström et al., 2003; Harry et al., 2004). In rats, we detected significant amounts of Hg 1 month after THIM injection. In monkeys (and most likely in humans), Hg from i.m. THIM injections accumulates in the brain and remains there for months (Burbacher et al., 2005). Our data on antinociceptive effects of THIM are novel, hence they cannot be directly compared to those of other investigators. Nevertheless, due to chemical and pharmacodynamic similarities of ethylmercury to methylmercury, our findings may be compared–with caution–to those obtained with methylmercury. In this context, it is worth noting that elevated pain threshold in the body and extremities was also observed in patients with methylmercury poisoning, although the neuropathological nature of this phenomenon could be complex (Yoshida et al., 1992).

Our results indirectly suggest that THIM administration increases activity of endogenous opioids. The neurochemical mechanisms which lie at the basis of this effect are not clear at present. They might involve direct or indirect effects on expression, synthesis, transport or secretion of opioid peptides in the spinal and supraspinal CNS regions, decreased activity of opioid degrading enzymes, increased affinity or density of opioid receptors among others. Also indirect effects of THIM on opioid neurotransmission involving neuroanatomical and neurophysiological alterations of other neurotransmitter systems cannot be ruled out. While there are no published data on opioid peptides and THIM or ethylmercury, methylmercury has been shown to increase release of peptides linked to pain control at spinal and supraspinal

regions of the CNS (Baxter and Smith, 1998; Ortega et al., 1997). Moreover, in developmental studies, delayed upregulation of μ and δ opioid receptors in brains of rats exposed to methylmercury *in utero* was described (Zanoli et al., 1997). Using immunohistochemical assay we also found several-fold increase of density of μ receptors in the periaqueductal gray region of neonatally THIM exposed rats (manuscript in preparation).

It is intriguing that Lewis rats were less sensitive to THIM-induced analgesia (after neonatal treatment) than Wistar rats. While many biological factors might be responsible for this phenomenon, the most obvious seems to relate to inborn defects of the HPA axis in Lewis strain (Oitzl et al., 1995). Because exposure to methylmercury was shown to activate the HPA axis and increase secretion of ACTH (Ortega et al., 1997), it is likely that other organomercurials (including THIM) also activate this axis and increase secretion of β-endorphin along with ACTH. In this context, lower sensitivity of Lewis rats to THIM-induced analgesia may potentially be due to secretion of lesser amounts of β-endorphin. This concept remains to be verified.

The critical question relevant to this study is, whether the elevated pain threshold and apparently augmented activity of endogenous opioids in animals exposed to THIM at neonatal stage results from a direct neurochemical effect of ethylmercury in the CNS, or is an outcome of developmental changes in brain architecture and function. Our experiments do not give a straightforward answer to this question, but permit drawing certain inferences. The hot plate tests were conducted 4 weeks after the last THIM injection to neonates, at which point, according to kinetic data, the rat brain retains about 10% of the maximal amount of Hg reached on day 4 after the injection. The rough estimate of Hg concentration in the brain 4 weeks after THIM injection with the cumulative lowest effective dose, 48 μg Hg/kg, would be low nanomolar, assuming even compartmentalization. Such concentrations of organomercurials were shown to affect various processes in neurons, but it is not known if they also affect opioid system. High nanomolar concentrations of methylmercury were required to increase neuropeptide expression in neurons from dorsal root ganglia (Baxter and Smith, 1998), however, because these ganglia accumulate Hg (Schiønning et al., 1991), it is possible that its local concentrations in some neurons could be higher than predicted from even tissue distribution. On the other hand, in our catalepsy experiment augmented activity of endogenous opioids was evident at the 16th week of life of neonatally THIM treated rats. This would argue in favor of long-term neurodevelopmental alterations in brain organization and function.

In conclusion, our study shows that administration of THIM to suckling or adult rats causes persistent impairment of pain sensitivity, which appears to involve increased activity of endogenous opioids. Susceptibility to this effect depends on the genetic makeup and age of animals. Young rats are more sensitive to it than adult. It is premature at this time to draw firm conclusions about clinical implication of this study, which has its obvious limitations. Potential link of these changes with the neurodevelopmental disorders remains to be elucidated. Nonetheless, in view of the facts that in primates Hg stays in the brain for months after THIM injections, and autism seems to be associated with dysregulation of endogenous opioids (Brambilla et al., 1997; Leboyer et al., 1994; Sahley and Panksepp, 1987; Sandman, 1988; Sandyk and Gillman, 1986; Tordjman et al., 1997), contribution of THIM from vaccines to neuropathophysiological alterations in genetically/biologically predisposed children is plausible. Involvement in these processes of Hg derived from other sources, such as maternal amalgam or the environment is also likely.

4. Experimental procedures

4.1. *Animals and drugs*

Pregnant Wistar and Lewis rats 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 with lights on at 07:00 h). Standard laboratory chow (Labofeed H, WPIK, Kcynia, Poland) and tap water were available ad libitum. Thimerosal was purchased from Sigma-Aldrich, Poland, and from Cfm Oscar Tropitzsch, Germany (product of GIHON Laboratories), naloxone from Tocris Bioscience, Bristol, United Kingdom, morphine from (Teva Kutno S.A. Poland).

4.2. *Mercury pharmacokinetics*

The kinetics of Hg distribution in different organs after THIM administration was measured in male Wistar rats. The animals on 10th postnatal day received a single injection of THIM solution in saline, i.m., at two doses (2.9 and 5.8 mg Hg/kg) and were euthanatized by pentobarbital injection 1, 4, 14 or 30 days later. Their brains, livers and kidneys were removed and frozen to be later used for measurement of Hg content. The analysis of Hg levels in tissues by atomic absorption spectrometry was performed at the Chemical Laboratory of Multi-Elemental Analyses at Wroclaw University of Technology. A single-purpose atomic absorption spectrometer based on in situ dry washing followed by gold amalgamation cold vapor AAS method was used for analysis using Advanced Mercury Analyzer (AMA-254, ALTEC, Czech Republic). This cold vapor AAS method is one of the most widely used techniques for determination of trace amounts of total mercury in environmental materials.

4.3. *THIM treatment of neonatal and adult rats*

Different doses of THIM dissolved in saline were injected into newborn rats on postnatal days 7, 9, 11 and 15 in four equal-dose inoculations (per kg) in volume of 50 μl, i.m. into *glutei maximi*, alternating the muscle for injection. This schedule was originally used in mice to mimic the infant immunization scheme (Hornig et al., 2004). Rats from one litter received the same treatment. For Wistar rats, the THIM doses per injection were: 12, 48, 240, 720, 1440, 2160 and 3000 μg Hg/kg and for Lewis rats they were: 54, 216, 540 and 1080 μg Hg/kg. Each animal was weighted before THIM injection and the amount of drug injected was adjusted to its weight. Weighs of Wistar rats at time of drug injections on postnatal days 7, 9,

11 and 15 were, respectively: 13,5+1,7 g, 16,5+1,7 g, 21,3+2,2 g and 28+2,1 g (average+SEM), $N=124$) . For Lewis rats the weights were: 12,1+1,3 g, 15,9+3,1 g, 17,5+2,0 g and 23,6+2,8 g (Average +SEM; $N=113$) on postnatal days 7, 9, 11 and 15, respectively. There were 4 to 7 litters per dose–treatment group within each strain. The experimental groups for different behavioral tests constituted of pooled rats from different litters with maximally 2 animals of each sex selected per litter. A separate group of naïve 6-week-old Wistar rats (weighing 130–150 g) received acute (single dose) THIM injection, i.m., at doses 48, 192, 960 and 12000 μg/Hg kg, which were quadruplicates of doses used in subchronic neonatal treatment.

Wistar rats received THIM from Sigma-Aldrich and Lewis rats—the product from Tropitzsch. Mercury content in THIM substance from both sources was measured by the accredited Chemical Laboratory of Multi-Elemental Analyses at Wroclaw University of Technology by the method of atomic absorption spectroscopy. According to the analysis, Hg content in THIM from Sigma-Aldrich was 48% and in the product from Tropitzsch it was 38%. In all experimental data THIM dose is expressed as μg or mg of Hg per gram or kg. Control rats were injected with saline following the same scheme. Pups were weaned on 28th postnatal day and at that time they were divided into male and female cages.

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. All behavioral tests were performed between 14:00–18:00.

4.4. *Behavioral techniques*

4.4.1. *Hot plate tests*

Nociception was assessed in control and postnatally THIM-treated rats in the 6th week of life in the hot plate test. The hot plate consisted of an electrically heated metal surface (Hot Plate type HP OI, COTM Bialystok) kept at a constant temperature of 56 °C±0,4 °C. The latencies for paw-licking or jumping were measured for each animal using a stopwatch. The cut-off time was 30 s to prevent tissue damage. The same test was used for assessment of the effect of acute treatment with THIM in a separate group of 6-week-old Wistar rats. In order to evaluate the role of endogenous opioids in pain reactions, some animals were administered an opioid receptor antagonist, naloxone, dissolved in saline (5 mg/kg), or saline, i.p. 30 min before the hot plate test. In both developmental and adult THIM-treatment studies experimentally naïve animals were used. Rats which received naloxone were also experimentally naïve.

4.4.2. *Catalepsy test*

Morphine-induced catalepsy was tested in male Wistar rats in the 16th week of life. Before evaluation of the opiate receptor-associated responses, the animals were repeatedly familiarized with a horizontal wooden bar. On the test day they were injected s.c. with 2.5 mg/kg morphine. Catalepsy was observed at times: 0, 15, 30, 45, and 60 min after morphine injection. 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 (Rogowski et al., 2003).

4.4.3. *Statistical analysis and $T_{1/2}$ calculation*

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. LSD test was employed for individual *post-hoc* comparisons. Probability (p) levels less than 0.05 were considered significant.

The half life time ($T_{1/2}$) of Hg elimination from different organs was calculated on the basis of three last mean concentration measurements. A linear regression of log concentration of Hg in time was used to estimate kel parameter. $T_{1/2}$ was calculated as ln(2)/kel.

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