Original article

# Prenatal exposure to organomercury, thimerosal, persistently impairs the serotonergic and dopaminergic systems in the rat brain: Implications for association with developmental disorders

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## Abstract

Thimerosal, an organomercury compound, has been widely used as a preservative. Therefore, concerns have been raised about its neurotoxicity. We recently demonstrated perturbation of early serotonergic development by prenatal exposure to thimerosal (Ida-Eto et al. (2011) [11]). Here, we investigated whether prenatal thimerosal exposure causes persistent impairment after birth. Analysis on postnatal day 50 showed significant increase in hippocampal serotonin following thimerosal administration on embryonic day 9. Furthermore, not only serotonin, striatal dopamine was significantly increased. These results indicate that embryonic exposure to thimerosal produces lasting impairment of brain monoaminergic system, and thus every effort should be made to avoid the use of thimerosal.
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## 1. Introduction

Thimerosal, an organomercury compound, has been widely used as a preservative [1]. Thimerosal is metabolized first to ethylmercury and further to inorganic mercury, both of which accumulate in the brain and other organs and have neurotoxic activity [2,3]. Accordingly, use of thimerosal such as vaccines is of great concern, particularly on infants and fetuses [4,5], and therefore, efforts have been made to reduce thimerosal from vaccines [6].

The adverse effects of thimerosal after neonatal administration include impaired pain sensitivity [7], hippocampal neurodegeneration [8], and changes in the dopamine system with subsequent behavioral disorders [9]. In addition, thimerosal was shown to affect neurite extension of neuroblastoma cells *in vitro*, therefore, it is evident that thimerosal leads to neurological abnormalities [10]. However, little is known regarding the prenatal effects of thimerosal. We recently reported that exposure of pregnant rats at gestational day 9 (E9) to thimerosal increased the number of serotonergic neurons in the lateral portion of the caudal raphe in E15 rat hindbrain and thus prenatal thimerosal exposure impaired early serotonergic development [11]. We have also demonstrated that prenatal exposure at E9 to thalidomide or valproic acid (VPA) specifically caused long-term effects on the normal development of serotonergic neuronal systems [12,13], accompanied with behavioral abnormalities that mimicked human

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autism [14]. Although a relationship between autism and thimerosal has not been confirmed yet [15,16], we need to know whether prenatal thimerosal exposure effects can persist into adulthood. Here, we investigated serotonin and dopamine content in the brains of postnatal day 50 (P50) adult rats following prenatal treatment of thimerosal.

## 2. Materials and methods

Pregnant Wistar rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Thimerosal (Sigma–Aldrich, St. Louis, MO) was dissolved in saline, and was administered to pregnant rats on E9 in volume of 50 μl by intramuscular injection into the *glutei maximi*. Thimerosal doses per injection were: 1, 0.1 and 0.01 mg Hg/kg. For the control group, saline was administered in the same manner. Three dams for each group were examined. All animal experiments were authorized by the Animal Research Committee of Mie University.

Measurement of concentration of serotonin (5-HT), dopamine (DA) and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were performed as described previously [12]. Because brain 5-HT levels are influenced by estrus cycle in females, we used the tissues only from male animals. Each offspring was deeply anesthetized on P50 and then decapitated. The hippocampus and striatum were immediately removed on ice, collected into a tube, frozen with liquid nitrogen, and stored at $-80\,°C$ until assay. The tissues were homogenized using an ultrasonic homogenizer (NR-50M; Microtec, Chiba, Japan) in 5 volumes of a mixture of 0.2 M perchloric acid, 100 μM EDTA, and 200 ng of isoproterenol hydrochloride as an internal standard and incubated on ice for 30 min. After centrifugation (20,000$g$, 20 min, 4 °C), the supernatant was adjusted to pH 3 with 1 M sodium acetate and filtered through a 0.45-μm pore size membrane filter (Millex-LH; Millipore, Billerica, MA). A part of the aliquot was separated by high performance liquid chromatography (HPLC) with an electrochemical detector (HTEC-500; Eicom, Kyoto, Japan) and an Eicompak SC-5 ODS column (3.0 mm × 150 mm, Eicom). The mobile phase (0.1 M sodium acetate–citrate acid buffer, pH 3.5, 17% methanol, 190 mg/L sodium 1-octanesulfonate, and 5 mg/L EDTA) allowed for the separation of 5-HT and DA, and their metabolites. Consistent results were obtained from three independent experiments. Statistical evaluation was carried out by grouped *t*-test.

## 3. Results

To evaluate the potential effects of embryonic exposure to thimerosal on postnatal brain monoamine content, different doses of thimerosal (1, 0.1, and

0.01 mg Hg/kg) were administered to E9 pregnant rats, and then allowed to have pups. When exposed to 1 mg Hg/kg thimerosal, most of the pups were dead soon after birth. On the other hand, in the 0.1 and 0.01 mg Hg/kg thimerosal-exposed groups, no major anomalies, growth retardation, or reduced number of delivered pups were observed in the two groups. Therefore, for monoamine content analysis, thimerosal doses of 0.1 and 0.01 mg Hg/kg were used. Concentrations of hippocampal 5-HT and striatal DA on P50 were measured by HPLC. As shown in the Fig. 1, a significant increase in hippocampal 5-HT levels was observed in the thimerosal-exposed groups (0.01 mg Hg/kg, $266.2 \pm 22.2$ ng/g weight, $p < 0.05$ vs. control; 0.1 mg Hg/kg, $307.0 \pm 7.2$ ng/g weight, $p < 0.01$ vs. control; control group, $177.8 \pm 27.8$ ng/g weight). Striatal DA concentrations were also significantly increased in the exposed groups (0.01 mg Hg/kg, $7039 \pm 448$ ng/g weight,
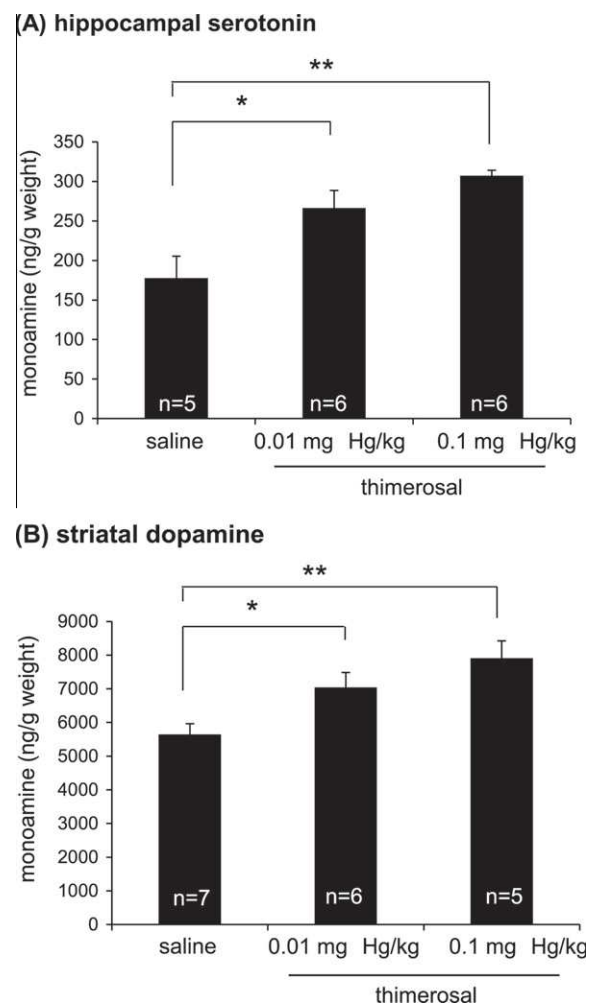


Fig. 1. Monoamine levels in control vs. thimerosal-exposed rats (ng/g weight). Different doses of thimerosal (0.1, and 0.01 mgHg/kg) were administered to E9 pregnant rats, and then allowed to have pups. On P50, concentrations of hippocampal serotonin and striatal dopamine were measured by HPLC. Values are mean ± SEM. $^*p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control.

$p < 0.05$ vs. control; 0.1 mg Hg/kg, $7905 \pm 520$ ng/g weight, $p < 0.01$ vs. control; control group, $5643 \pm 323$ ng/g weight). Hippocampal 5-HIAA, a metabolite of 5-HT, was also increased in thimerosal-exposed groups compared to control (0.01 mg Hg/kg, $457.8 \pm 30.8$ ng/g weight, not significant to control; 0.1 mg Hg/kg, $477.4 \pm 20.2$ ng/g weight, $p < 0.05$ vs. control; control group, $388.2 \pm 35.3$ ng/g weight), but its increase was not as much as that of 5-HT. Subsequently, the ratio 5-HIAA/5-HT was decreased (control, $2.32 \pm 0.26$; 0.01 mg Hg/kg, $1.75 \pm 0.11$, $p < 0.05$ vs. control; 0.1 mg Hg/kg, $1.56 \pm 0.06$, $p < 0.01$ vs. control). Striatal DOPAC and HVA, a metabolite of DA, was not changed statistically in thimerosal-exposed groups compared to control (0.01 mg Hg/kg, $2082 \pm 152$ ng/g weight, not significant to control; 0.1 mg Hg/kg, $2225 \pm 100$ ng/g weight, not significant to control; control group, $1997 \pm 103$ ng/g weight). The ratio (DOPAC + HVA)/DA was also decreased (control, $0.356 \pm 0.011$; 0.01 mg Hg/kg, $0.295 \pm 0.007$, $p < 0.01$ vs. control; 0.1 mg Hg/kg, $0.283 \pm 0.010$, $p < 0.01$ vs. control). These results indicate that prenatal exposure to thimerosal on E9 affects levels of 5-HT and DA, and their metabolites in the adult brain.

## 4. Discussion

In this study, we demonstrated that prenatal exposure to thimerosal on E9 caused a significant increase in 5-HT and DA content in the brains of adult rats. This finding indicates that prenatal thimerosal exposure may cause lasting neurochemical impairments to the serotonergic and dopaminergic systems.

Prenatal exposure to thimerosal has been shown to alter early embryonic development of 5-HT in our previous study [11]. These findings, together with those of the present study, suggest that a single prenatal exposure to thimerosal causes irreversible and critical effects to the brain serotonergic system. Persistent effects caused by a single prenatal exposure to chemicals are not, however, surprising because we have previously reported that prenatal exposure on E9 to thalidomide or VPA, chemicals known to induce autism when exposed at E9 [17,18], also induced increased hippocampal 5-HT in the adult brains of rats at P50 [12,13]. In thalidomide or VPA experiments, behavioral abnormalities in rats closely mimicked human autism [14]. Importantly, we also showed that abnormalities in 5-HT content caused by prenatal thalidomide exposure were time-specific (i.e., on E9). Therefore, the present result that exposure to thimerosal on E9 caused 5-HT abnormalities is consistent with previous findings. Because the possible link between thimerosal and autism is still controversial [15,16], further experiments are necessary to resolve this issue.

Prenatal exposure to thimerosal also seems to cause persistent changes in the striatal dopaminergic neuron of the brain. Faro et al. demonstrated that ethylmercury and methylmercury increased the *in vivo* release of DA from the striatum in free-moving adult rats [19]. Olczak et al. demonstrated that early postnatal administration of thimerosal caused persistent changes of the dopamine system in rats [9]. Therefore, mercury can cause short- and long-term effects on the dopaminergic system. However, to the best of our knowledge, our present report is the first to demonstrate that the effects from prenatal exposure to thimerosal persisted through the P50 dopaminergic and serotonergic systems. Because both dopaminergic and serotonergic neurons are known to be fated to develop from precursors starting from about E9, with help from the sonic hedgehog and fibroblast growth factor 8 genes [20], exposure to thimerosal at E9 is thought to cause irreversible effects on serotonergic and dopaminergic neurons. Further experiments are necessary to determine how thimerosal perturbs the normal development of both neurons.

Hippocampal 5-HIAA, a metabolite of 5-HT, were also increased in thimerosal-exposed groups compared to control, but its increase was not as much as that of 5-HT. Subsequently, the ratio 5-HIAA/5-HT was apparently decreased. Striatal (DOPAC + HVA)/DA ratio was decreased. We are not sure whether this decreased ratio means the true change of neurotransmitter metabolism, i.e., change of monoamine oxidase (MAO) level. However, in any case, the fact that thimerosal-dependent increase of 5-HT was more or less accompanied by 5-HIAA increase and that thimerosal-dependent increase of DA suggests that fetal exposure to thimerosal causes somehow lasting change of neurotransmitter metabolism. Further study to lead the conclusion of the effects of thimerosal on neurotransmitter metabolism such as by analyzing MAO activity will be published elsewhere.

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