

Low-Level Neonatal Thimerosal Exposure: Further Evaluation of Altered Neurotoxic Potential in SJL Mice

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Ethylmercury in thimerosal-preserved childhood vaccines has been suggested to be neurotoxic and to contribute to the etiology of neurodevelopmental disorders, including autism. Immune system function may be an important factor influencing vulnerability of the developing nervous system to thimerosal. This possibility is based in part on a report by Hornig *et al.* (2004, *Mol. Psychiatry* 9, 833–845) of neurodevelopmental toxicity in SJL/J mice that develop autoantibodies when exposed to organic mercury. The present study reexamined this possibility by injecting neonatal SJL/J mice with thimerosal, with and without combined HiB and DTP vaccines. Injections modeled childhood vaccination schedules, with mice injected on postnatal days 7, 9, 11, and 15 with 14.2, 10.8, 9.2, and 5.6 $\mu\text{g}/\text{kg}$ mercury from thimerosal, respectively, or vehicle. Additional groups received vaccine only or a 10 times higher thimerosal + vaccine dose. Low levels of mercury were found in blood, brain, and kidneys 24 h following the last thimerosal injection. Survival, body weight, indices of early development (negative geotaxis, righting) and hippocampal morphology were not affected. Performance was unaffected in behavioral tests selected to assess behavioral domains relevant to core deficits in neurodevelopmental disorders such as autism (i.e., social interaction, sensory gating, anxiety). In an open-field test the majority of behaviors were unaffected by thimerosal injection, although thimerosal-injected female mice showed increased time in the margin of an open field at 4 weeks of age. Considered together the present results do not indicate pervasive developmental neurotoxicity following vaccine-level thimerosal injections in SJL mice, and provide little if any support for the hypothesis that thimerosal exposure contributes to the etiology of neurodevelopmental disorders.

Key Words: thimerosal; mercury; SJL mice; locomotor behavior; social behavior; hippocampus; stereology.

Thimerosal (sodium ethylmercury thiosalicylate) is an antimicrobial preservative used since the 1930's in numerous vaccines and medicinal preparations (Magos, 2001; Pless and Risher, 2000). It is 49.6% mercury by weight and rapidly dissociates to ethylmercury and thiosalicylic acid in aqueous solutions (Reader and Lines, 1983; Tan and Parkin, 2000). Ethylmercury poisoning has occurred in humans, resulting in renal and neurotoxicity in the affected populations (Cinca *et al.*, 1980; Damluji, 1962; Hilmy *et al.*, 1976; Zhang, 1984). However, the possible effects of exposure to lower levels of ethylmercury, such as those levels associated with immunization with thimerosal-preserved vaccines, are poorly understood. Initial risk assessments for ethylmercury were based on studies of oral methylmercury toxicity (Clarkson *et al.*, 2003; Myers and Davidson, 2000), but recent data indicate that the kinetics of tissue disposition and metabolism differ substantially for these two forms of organic mercury (Burbacher *et al.*, 2005; Clarkson and Magos, 2006; Harry *et al.*, 2004; Magos, 2003; Magos *et al.*, 1985; Pichichero *et al.*, 2002; Stajich *et al.*, 2000; Suzuki *et al.*, 1973). This makes the assumptions underlying risk assessment different for each form of mercury.

Thimerosal has been removed from mandatory childhood vaccines in the United States due to concern that expanded immunization regimens could result in increased organic mercury exposure and elevated body burdens in children (Ball *et al.*, 2001). However, thimerosal-preserved vaccines are still used outside of the United States where the advantages of multiuse vials of vaccine requiring an antimicrobial preservative take precedence over perceived mercury hazards (WHO, 2002). Removal of an antimicrobial preservative or identifying an alternative to thimerosal is not trivial, and could result in increased risk of microbial contamination or exposure to unknown toxicants. As a result, thimerosal-preserved influenza vaccines continue to be widely used both within and outside the United States, and the potential toxicity of thimerosal remains a question for human health (Pichichero *et al.*, 2002; Stratton and McCormick 2001).

Concern remains about the possible contribution of exposure to low levels of ethylmercury via childhood vaccinations with

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thimerosal-preserved vaccines to the etiology of neurodevelopmental disorders, including autism (Bernard *et al.*, 2002). There is no direct evidence at the present time supporting an association between thimerosal exposure and autism (Davidson *et al.*, 2004; Stratton and McCormick 2001; Thompson *et al.*, 2007), and the majority of epidemiological studies published to date have not supported such a relationship (Thompson *et al.*, 2007). However, a study by Hornig *et al.* (2004) examined the neurotoxicity of thimerosal in SJL/J mice and suggested that toxicity may be dependent on an organism's underlying immune status. Neonatal injections of thimerosal in SJL/J mice produced abnormal brain development, delayed growth, and altered locomotor behavior. SJL/J mice have the mouse major histocompatibility complex H-2^s associated with increased susceptibility to autoimmunity, and develop autoantibodies, including antinucleolar antibodies to fibrillarin (Havarinasab *et al.*, 2005), when exposed to high levels of methyl and ethylmercury (Havarinasab *et al.*, 2005; Havarinasab and Hultman, 2005; Hultman and Hansson-Georgiadis, 1999). Thimerosal effects were not found in C57BL/6J (H-2^b) or Balb/c (H-2^d) mice that are less susceptible to autoimmunity. Hornig *et al.* (2004) suggested that the H-2^s haplotype, and altered immune system function in general, might confer heightened susceptibility to neonatal thimerosal exposures in mice. Although antinucleolar antibodies have not been found in autistic children (Singh and Rivas, 2004), the findings in SJL/J mice raised the possibility that children with altered immune system function could be particularly vulnerable to the neurotoxicity of thimerosal, and that the SJL/J mouse strain may provide a sensitive model for thimerosal developmental neurotoxicity studies. These findings are also intriguing because, if confirmed, they would mean that the neurotoxicology of thimerosal, and possibly other forms of mercury, would need to be recast within the framework of immune system status and function. Thus, we reexamined thimerosal neurotoxicity in SJL/J mice, using experimental procedures that closely followed the original report by Hornig *et al.* (2004). Specifically, we examined the somatic growth, locomotor behavior, the structure of the hippocampus, and immunoreactivity of the kainic acid receptor subtype 2 (e.g., KA2) in the hippocampus of SJL/J mice following neonatal thimerosal injections. The distribution of the KA2 subunit was of particular interest because of the importance of the kainate receptor for excitatory transmission in the hippocampus and its role in synaptic plasticity (Contractor *et al.*, 2001). As an extension of the previous study by Hornig *et al.* (2004) we also included a higher mercury dose (i.e., 10 times higher), measured tissue mercury levels (blood, brain, and kidney), used unbiased stereological techniques for assessing the numbers of hippocampal CA1 pyramidal and dentate granule cell numbers, and included tests of social interaction, sensory gating, and anxiety. These behaviors were selected because they assess behavioral domains that are considered relevant to core deficits in neurodevelopmental disorders such as autism (Crawley, 2004; Ricceri *et al.*, 2007).

MATERIALS AND METHODS

Subjects

Timed pregnant (i.e., 11- to 14-day gestation) SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) arrived at UC Davis in nine shipments of two to eight dams, approximately 2 weeks apart. Shipments were based upon availability from the breeding colony at Jackson Labs. Dams were individually housed in plastic cages (11" × 7" × 5½") with ~3 cm of bedding (Carefresh, Absorption Corp., Ferndale, WA). Dams constructed nests and maintained their litters in small transparent red Plexiglas mouse enclosures within each cage (Bioserve, Frenchtown, NJ). Cages and bedding were changed once per week. Purina mouse breeder chow or Purina mouse chow (reported mercury levels < 25 ppb; Nestle Purina, St Louis, MO) and water were available *ad libitum*. The day of birth was considered postnatal day 0 (PND 0). On postnatal day (PND) 1, pups were sexed, marked with an identifying nontoxic foot tattoo (Ketchum Manu, Inc., Brockville, ON, Canada), and returned to their litter. Pups were weighted and injected on PNDs 7, 9, 11, and 15 as described below. At weaning on PND 21, the dam was removed and the male and female littermates were housed together for an additional week. On PND 28 the males and females in each litter were separated and group housed with a maximum of four mice per cage. Pups from 38 litters were used for behavioral experiments and 13 additional litters were used for blood and brain mercury measurements.

Thimerosal Exposure Procedures

Pups were injected on PNDs 7, 9, 11, and 15 with one of five solutions: phosphate-buffered saline (PBS, pH 7.4) vehicle (VEH), 1× thimerosal (1×), 1× thimerosal + vaccine (1× + VAC), 10× thimerosal + vaccine (10× + VAC), or vaccine alone (VAC). The 1× injections provided a cumulative dose of 39.8 µg/kg mercury over the four injections. This represents the maximum mercury exposure, on a µg/kg basis, to which a child could have been exposed from vaccination if each of the following vaccines were preserved with thimerosal: hepatitis B, diphtheria tetanus pertussis (DPT), and hemophilus influenza B (HiB). The 10× injection is a 10-fold higher dose (390 µg/kg mercury) and was included to increase the ability to detect possible adverse effects. Injections were made into the hindlimbs (side alternated) in order to match the intramuscular (im) injection procedure described by Hornig *et al.* (2004). However, the small size of the mouse pups and the limited muscle development at these ages, particularly on PND 7 (3.4 g ± 0.04 SE), necessarily resulted in injections that were a combination of im and subcutaneous routes. Injections were delivered using a sterile 30 gauge × ½" hypodermic needle (BD, Franklin Lakes, NJ) and 100-µl Hamilton microsyringe (Model 51115 with Luer ground glass tip, Hamilton, Reno, NV). The injection volumes averaged (mean ± SE) 48.2 ± 0.6, 46.3 ± 0.5, 43.7 ± 0.5, and 37.6 ± 0.3 µl on injection days, PNDs 7, 9, 11, and 15, respectively. The timing of injections, dose, and relation to childhood vaccinations shown in Table 1 were chosen to be the same as in Hornig *et al.* (2004), with PND 7 in rodents approximating development of a full-term human infant at 37–40 weeks of age (Dobbing and Sands, 1979; Roohey *et al.*, 1997). Extreme care was taken in dosing to ensure a slow and accurate delivery and to minimize vascular involvement and damage to the hindlimb.

Experimental Design

The time line for the experiments is shown in Figure 1. A within-litter design was used in which pups within each litter were randomly assigned to be injected with one of the five solutions. This design was used to minimize confounding by possible litter effects, as well as to reduce the total number of animals needed for the study. Because thimerosal-injected and VEH-injected mice were reared in the same litter with this design, the possible cross-contamination of mercury was examined in a pilot study prior to the initiation of the main study. As compared with experimentally naïve mice, VEH-injected mice housed with thimerosal-injected mice showed no statistically significant

TABLE 1

(A) Human Weight-for-Age Vaccination Schedule upon which the Mouse Injection Schedule (B) was based (after Hornig *et al.*, 2004); (C) Summary of the 5 Experimental Mouse Groups Used

(A) Human					
Age (months)	2	4	6	12	Total
Weight (kg) ^a	4.4	5.8	6.8	9.0	
Maximum Hg in vaccines (μg) ^{b,c}					
DPT × 4 doses	25	25	25	25	100 μg
Hib × 4 doses	25	25	25	25	100 μg
Hepatitis B × 3 doses	12.5	12.5	12.5	—	37.5 μg
Total Hg (μg)	62.5	62.5	62.5	50	237.5 μg
Total Hg dose/body weight (μg/kg)	14.2	10.8	9.2	5.6	39.8 μg/kg
(B) Mouse (SJL/J)					
Age (days)	7	9	11	15	Total
Total Hg dose/body weight (μg/kg)	14.2	10.8	9.2	5.6	39.8 μg/kg
(C) Injection summary					
Groups	Total Hg (μg/kg)				
VEH	0				
VAC	0				
1×	39.8				
1× + VAC	39.8				
10× + VAC	398.0				

^aBased on 10th percentile for males from the weight-for-age percentiles from the National Center for Health Statistics, 4/20/01.

^bImmunization schedule based on U.S. recommended childhood immunization schedule, December 2000 (<http://www.cdc.gov/MMWR/preview/MMWRhtml/mm4902a4.htm>).

^cVaccine mercury levels from Ball *et al.* (2001).

differences in mercury levels in blood (naïve 0.12 ± 0.05 ppb vs. VEH 0.09 ± 0.06 ppb, $p = 0.75$) or brain (naïve 0.61 ± 0.12 ppb vs. VEH 0.59 ± 0.16 ppb, $p = 0.89$) demonstrating the absence of measurable cross-contamination between mice within litters.

Solutions

Thimerosal (F.W. 404.82, $C_9H_9HgNaO_2S$, Lot # 053K0090) was purchased from Sigma-Aldrich (Atlanta, GA) and stored at room temperature in the dark. Injection solutions were made from a concentrated stock solution of 2.016 mg thimerosal/ml PBS, which is equivalent to 1.0 mg/ml mercury (i.e., thimerosal is 49.6% mercury). The stock solution was diluted in PBS 1:100 for the 10× mercury injection and 1:1000 for the 1× injection solution.

The diphtheria and tetanus toxoids and acellular pertussis vaccine (DAPTACEL, Lot C2103AA/Exp Sep 29, 2006) and hemophilus b conjugate vaccine (tetanus toxoid conjugate, ActHIB, Lot UE646AA/Exp 20 Dec 06) were purchased from Aventis Pasteur, Lyon, France. Vaccines were diluted per the manufacturer's instructions.

The VAC dosing solution was made by adding 100 μl of DTaP and 100 μl of Hib to 9.8 ml of sterile PBS. The 1× + VAC and 10× + VAC solutions were made by adding 100 μl of each vaccine to 9.8 ml of the 1× or 10× thimerosal solutions, respectively. All solutions were made and stored in Teflon containers (Greenwood and Clarkson, 1970), with Teflon syringe septa for removing solutions by hypodermic needle (Saville, Minnetonka, MN).

Behavioral Testing Procedures

Group sizes for behavioral testing at PNDs 8 and 10 and at 4 weeks of age were VEH = 37, VAC = 37, 1× = 38, 1× + VAC = 37, and 10× + VAC = 38, with the number of male and female mice in each group approximately equal. Subsets of male and female mice were sacrificed at 5 weeks of age for histology. The remaining mice were retested at 10 weeks of age in the rotarod and open field (VEH = 26, VAC = 25, 1× = 24, 1× + VAC = 25, 10× + VAC = 26).

Locomotor behaviors were tested using four TruScan Photo Beam open-field activity arenas for mice (Coulbourn Instruments, Allentown, PA). The locomotor apparatus (28 × 28 cm) generates a grid (x, y, and z planes) of infrared beams, and movement in each of the three planes is detected by a beam break. The time of day when testing occurred was counterbalanced across groups. Each mouse was placed in the apparatus and activity was recorded online by the TruScan software. Measures included horizontal and vertical movements, distance, and location summaries. Repetitive beam breaks, labeled as "stereotypy" by the TruScan software, were also recorded. However, as stated in the Coulbourn Instruments application notes, stereotypy measurements collected by the TruScan system only measure repetitive beam breaks (three or more less than 2 s apart), and cannot be used reliably to discriminate between ambulation and those behavioral measures that characterize stereotypy (e.g., repetitive turning, ear scratching, grooming, acrobatic flips, gnawing, head bobbing). Pups were tested for 2 min on PNDs 8 and 10 (i.e., defined as protoambulatory behavior in Hornig *et al.*, 2004). The floor of the arena was elevated by 1 cm to allow for detection of movements of the small pups by the

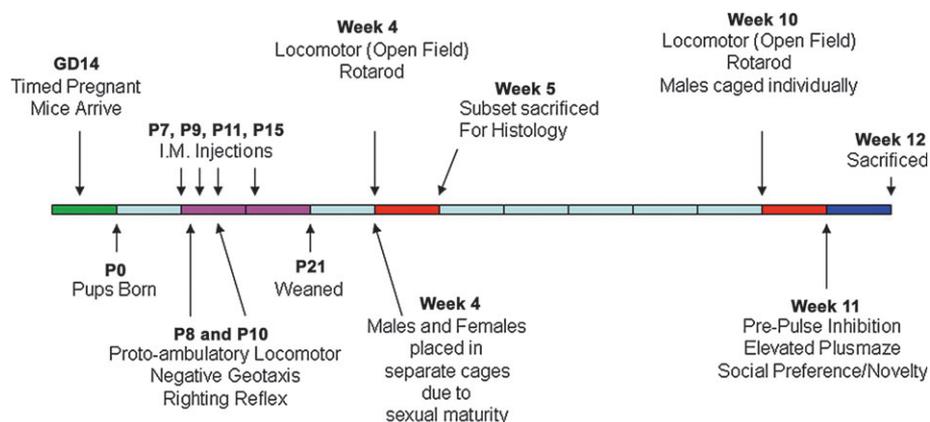


FIG. 1. Flow chart of experiments.

photocell devices. Each animal was later retested for locomotor behavior at PND 28 (week 4) using the same procedures described above, except the test period was 90 min and the floor of the chamber was not raised. Pups from the first 12 litters were sacrificed at 5 weeks of age for histological analysis. The remaining 26 litters were retested for locomotor behavior at 10 weeks of age for 90 min.

Negative geotaxis was also tested on PNDs 8 and 10 by placing each pup on a wire mesh screen (4" × 8") at a 45° angle with head down (start position). The time to turn around to 90° and 180° from start position or time to fall was recorded. Righting response was tested by placing each animal on its back and scoring the response as 1 = righting attempted but belly did not reach surface; 2 = righting > 5 s, or one or more paws remain under body; or 3 = pups rights with all four paws free within 5 s.

Motor coordination was tested at 4 weeks and retested at 10 weeks of age using a rotarod apparatus (Rota-rod 7600, Ugo Basile Biological Research Apparatus, Malvern, PA). On day 1 mice were given two 2-min test sessions 2 h apart using a speed of 16 r.p.m = $4.67 \times 10^{-3} \times g$. The following day a single 2-min test trial was conducted at 20 r.p.m = $7.29 \times 10^{-3} \times g$. Time on the rod, time to first fall and number of passive rotations around the rod (i.e., mouse holds on to and rotates with rod) were recorded.

Acoustic startle and prepulse inhibition (PPI) were tested at 11 weeks of age in the same animals used for the 10-week locomotor test. PPI of the auditory startle response was measured to assess sensorimotor gating (Dulawa *et al.*, 2000), which is in turn a measure of the ability of the nervous system to inhibit a motor response to sensory input (Geyer *et al.*, 2002). Mice were individually placed into a tubular Plexiglas restrainer and then placed in an auditory startle apparatus housed in a sound-attenuating recording chamber (SR-LAB, San Diego Instruments, San Diego, CA). Programmed auditory stimuli (20 ms duration) were delivered from a speaker in the roof of the chamber. Movements during the recording were transduced into startle amplitude, digitalized, and stored in the SR-LAB software. Each animal was tested individually in one 20-min session without prior training. Sessions began with a 5-min acclimation period followed by 10 blocks of five pseudorandomized test trials. Test trials were separated randomly by 5- to 20-ms intertrial intervals in steps of 5 ms, and auditory pulses were presented in the following combinations: (1) 120 dB (analog level 690) startle alone, (2) 120 dB startle with 74 dB (analog level 410) prepulse, (3) 120 dB startle with 82 dB (analog level 455) prepulse, (4) 120 dB startle with 90-dB (analog level 505) prepulse, and (5) no stimulus (background white noise only). Each trial started with a 50-ms null period and ended with a 65-ms recording period. Both periods had background white noise at 70 dB (analog level 390). All prepulse stimuli were 20 ms long and were presented 100 ms before the 120 dB startle.

Anxiety was assessed in the elevated plus-maze (File, 2001; Lister, 1987). The white Plexiglas apparatus had two open (30 × 5 × 0.25 cm) and two closed (30 × 5 × 6 cm) arms emanating from a central platform (5 × 5 cm) forming a plus shape. The maze was elevated 60 cm above the floor. A low, narrow border (0.25 × 0.25 cm) along the edges of the open arms reduced falls and encouraged exploration (Rodgers and Johnson, 1995). Mice were placed onto the central platform and allowed 5 min of free exploration (without acclimation). Movement was video recorded (Sony Digital Handicam) under dim red illumination (4 × 60 W indirect), and analyzed using the SMART tracking system (San Diego Institute, San Diego, CA). Distance traveled and number of entries into each arm and the central platform were recorded. Percent time and number of entries into the open arms were calculated. The ratios of open to closed arm time, distance, and entries were calculated and used for statistical analyses ($[\text{OPEN}/(\text{OPEN} + \text{CLOSED})] \times 100\%$). Increased time in the open arm indicates decreased fear and anxiety (File, 2001).

Sociability and preference for social novelty were subsequently tested (Moy *et al.*, 2004). Sociability was tested by placing an injected test mouse into the empty middle chamber of a three-chambered clear Plexiglas apparatus (20 cm L × 40 cm W × 22 cm H) and measuring the time spent in either an adjacent empty chamber or an opposite adjacent chamber containing a caged, experimentally naïve, unfamiliar mouse (same sex, same strain). "Sociability" is demonstrated when mice spend more time with the unfamiliar mouse than in the empty chamber. This preference was quantified as percent time with the

unfamiliar mouse (M1) versus time in the empty (E) chamber (i.e., $[(M1)/(M1 + E)] \times 100\%$). Animals were videotaped using an automated tracking system (Smart Tracking System, San Diego, Instruments) that recorded numbers of entries into and amount of time in the chambers, as well as proximity to the caged mouse. The test trial was 5 min and sides were counterbalanced. When given a new choice between the now familiar mouse (M1) and a new unfamiliar mouse (M2) in the adjacent chamber, mice prefer the chamber with the unfamiliar mouse (i.e., social novelty test). Preference for social novelty was quantified as percent time with the new (M2) mouse versus the now familiar (M1) mouse (i.e., $[(M2)/(M2 + M1)] \times 100\%$).

Mercury Measurements

Levels of total mercury in blood, brain, and kidney were measured 24 h (six litters) and 7 days (six litters) after the last thimerosal injection. Mice were anesthetized (Euthasol, 100 mg/kg, ip, Delmarva Laboratories, Inc, Midlothian, VA.) and 100 µl of blood was collected from the left ventricle in a heparinized capillary tube. Pups were then transcardially perfused with 0.9% sodium chloride solution and coded brains and kidneys were collected, frozen, and shipped to the commercial contractor (Research Triangle Institute, RTP, NC) for mercury analysis.

Total mercury in blood was measured using a Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer designed for direct mercury analysis (DMA-80, Milestone, Inc., Pittsburgh, PA, EPA Method 7473 protocol, 1998). Fifty microliters of blood samples was placed in quartz sample boats and 25 µl of dH₂O and 25 µl of 1% HNO₃ were added to each sample. Samples were dried in the DMA-80 at 300°C for 90 s, decomposed on a catalytic column at 850°C for 300 s, and mercury vapor was collected on a gold amalgamation trap and subsequently desorbed for quantification. Mercury content was determined using atomic absorption spectrometry at 254 nm, and compared with external mercury reference standards (Certiprep Hg standard, SPEX CertiPrep, Inc., Metuchen, NJ). The lower limit of detection (LOD) was 0.05 ng total mercury. Mercury content in each of the thimerosal injection solutions (i.e., 1×, 1× + VAC, and 10× + VAC) was also verified using these procedures.

Total mercury in kidney and brain was measured by cold vapor atomic fluorescence spectrometry using previously published methods (Levine *et al.*, 2002). Briefly, a constant aliquot, by weight, from each sample was placed into an acid-washed vial. Each aliquot was treated with a 30/70 (vol/vol) mixture of sulfuric and nitric acids and subjected to muffle oven digestion. Following digestion, samples were allowed to cool to room temperature prior to the addition of hydrochloric acid and a potassium bromide/potassium bromate oxidizing solution. After 2 h, excess oxidant was removed from the samples by the addition of hydroxylamine hydrochloride. Total mercury in the kidney and brain was calculated on the basis of tissue concentration (total micrograms mercury/g tissue). The tissue to blood ratio was calculated to determine possible accumulation of mercury.

Histological Procedures

Animals in the first 12 litters were sacrificed for histological analysis at 5 weeks of age. The remaining mice were sacrificed at ~11 weeks of age after the completion of all behavioral testing. Mice were anesthetized (100 mg/kg, ip, Euthasol, Delmarva Labs, Inc.) and transcardially perfused with PBS, followed by buffered 4% paraformaldehyde in 0.1M PBS. Coded brains were postfixed in 4% paraformaldehyde for 20 h and then shipped overnight in 20% sucrose to a commercial histology contractor who was blinded to treatment assignments for sectioning (FD Neurotechnology, Inc., Baltimore, MD; www.fdneurotech.com). Serial cryostat sections (50 µm) were cut coronally through the cerebrum containing the whole hippocampus. These sections were then used for unbiased stereology of cresyl violet stained sections, cupric acid silver staining for neurodegeneration, and immunohistochemistry for KA2.

Amino Cupric Silver Staining

Silver staining was used as a sensitive marker of neuronal degeneration (DeOlmos and Ingram, 1971; Switzer, 2000). Silver impregnation for neuronal

degeneration was conducted on 50- μ m cyrostat sections from the brains of injected mice. Approximately, 15 sections representative of the entire hippocampus were collected from each animal, and processed by Amino Cupric Silver stain (FD Neurosilver TM kit, FD NeuroTechnologies, Inc., Ellicott City, MD). Fiber degeneration was diagnosed by the presence of punctate to linear black profiles, and neuronal degeneration was diagnosed when nuclei of neurons were shrunken, densely black, and the perikaryon was stippled with dense black deposits. All 15 sections were examined from six mice per treatment condition.

Unbiased Stereology

Stained sections were shipped to Dr Peter R. Mouton at the Stereology Resource Center (Chester, MD) for computerized stereology analysis by a trained technician blind to treatment group. Total numbers of cresyl violet stained neurons in two reference spaces, granule cells in DG and pyramidal cells in Ammon's horn sector 1 (CA1), were quantified using computerized stereology (Stereologer, Stereology Resource Center, Inc.) as previously reported (Lei *et al.*, 2003; Long *et al.*, 1998; Mouton *et al.*, 2002). The total number of pyramidal cells and granule cells were quantified through the entire rostrocaudal extent of CA1 and DG, respectively, using the rat stereotaxic atlas of Paxinos and Watson, (2004) as previously detailed (Duffy *et al.*, 2007; Lei *et al.*, 2003; Long *et al.*, 1998; Mouton *et al.*, 2002). Sampling fractions for the optical fractionator method (West *et al.*, 1991) were (1) section sampling fraction (the number of sections sampled divided by the total number of sections); (2) the area sampling fraction (the area of the sampling frame divided by the area of the x - y sampling step); and (3) the thickness sampling fraction (the height of the disector divided by the section thickness). The DG and CA1 on each section was outlined under low power magnification (4 \times objective), and neurons in granule cell and pyramidal cell layers, respectively, were counted under oil immersion magnification (60 \times objective). The counting criteria were the presence of a distinct neuronal phenotype, including the presence of a nuclear membrane, nucleolus, cytoplasm, and cell membrane; and placement within the 3D virtual counting probe (disector) or touching the inclusion planes, according unbiased counting rules (Gundersen, 1977). A guard volume of 2–3 μ m was used to avoid artifacts (e.g., lost caps) at the sectioning surface. Stereological analysis was carried out to a high level of sampling stringency (coefficient of error [CE] < 0.10) on a set of 8–12 sections sampled through the entire rostrocaudal extent of each brain in a systematic-random manner, that is, every fifth with a random start on sections 1–5 (for further details, see Mouton, 2002).

KA2 Immunostaining

Based upon the previous report from Hornig *et al.* (2004) we looked for a possible increase in immunostaining for the KA2 excitatory glutamate receptor subunit in the hippocampus. Approximately ten 50- μ m sections were stained representing the entire hippocampus, with six mice used per treatment condition. Immunostaining was carried out using a polyclonal anti-KA2 antibody that recognizes the N-terminal region of KA2 (N-17, sc-8914, Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, endogenous peroxidase activity was inactivated with hydrogen peroxidase, and sections were incubated with avidin and biotin solutions (Vector Lab, Burlingame, CA) to block nonspecific binding of endogenous biotin, biotin-binding protein, and lectins. Sections were then incubated free-floating in 0.1M PBS (PBS, pH 7.4) containing 1% normal blocking serum, 0.3% Triton X-100, and the goat anti-KA2 IgG (1:500) for 3 days at 4°C. The immunoreaction product was visualized according to the avidin-biotin complex method of Hsu *et al.* (1981) using the Vectastain elite ABC kit (Vector Lab) and 3',3'-diaminobenzidine as a chromogen. After thorough washes sections were mounted on gelatin-coated slides, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount. Specificity of immunostaining was verified via separate control sections by 1) replacement of the specific antibody with the isotype control IgG; and 2) preabsorption of the specific antibody with the antigen (Santa Cruz Biotechnology, Inc., sc-8914P).

Although Hornig *et al.* (2004) reported decreased immunostaining of NR1 and NR2b glutamate receptors, in our hands antibodies from the same commercial source (Chemicon, Temecula, CA) did not provide an acceptable

level of specificity for immunohistochemistry in mouse brain. For example, NMDAR2b antibody at a high concentration (1:50 dilution) stained subpopulations of neurons, for example, hippocampal neurons in the subiculum proper and the CA3. The NMDAR1 antibody was tested at various dilutions and at higher concentrations (1:50 and 1:100) and most neurons also showed immunoreactivity. In both cases specificity of the staining could not be confirmed and data were therefore not considered to be valid and reportable. All immunostaining was conducted on coded sections by FD NeuroTechnologies, Inc.

Statistical Analysis

Locomotor activities were measured on PNDs 8 and 10, and at 4 and 10 weeks of age. The coded data files collected by TruScan Photo Beam Activity analysis package (Coulbourn Instruments) were sent to statistical contractors (Constella Group, LLC, Research Triangle Park, NC) for analysis. Data files remained coded through the entire analysis so that the analysts were blinded to the experimental treatments given and group assignments for the mice. A total of 37 locomotor variables generated by the TruScan apparatus were summarized and analyzed statistically (see Supplementary data, Suppl. 1 for a list of locomotor variables). Data for each variable were examined for homogeneity of variance using the Levene test, and when assumptions were met, an analysis of variance (ANOVA) was used to compare treatments, sexes, and the treatment \times sex interaction (SAS, Cary, NC). Litter was included as a random factor if litter effects were found to be significant using the likelihood ratio test. *Post hoc* analyses were carried out when appropriate, using the Tukey-Kramer test for multiple comparisons. When assumptions of homogeneity of variance were not met data were analyzed using a Kruskal-Wallis nonparametric test with sexes combined, as well as individually for each sex. Individual *post hoc* comparisons were carried out using a Mann-Whitney *U* adjusted for multiple comparisons. Sex differences were found for the majority of locomotor variables and sex differences were therefore analyzed separately.

Acoustic startle response, elevated plus-maze activity, sociability and preference for social novelty, negative geotaxis, and righting response were also analyzed with a two-factor (treatment \times sex) ANOVA. PPI was analyzed by repeated measures ANOVA (treatment \times sex \times startle intensity). The stereology data were analyzed by a one-factor (treatment) ANOVA. *Post hoc* individual group comparisons were carried out using a Tukey-Kramer test, with the minimum levels for statistical significance set at $p < 0.05$ for all statistical analyses. Data in figures are expressed as means \pm standard error (SE) of the mean.

RESULTS

Tissue Levels of Mercury

Mercury levels in blood, brain, and kidney are presented in Table 2. Mercury levels were at or below the LOD in the blood (LOD = 0.90 ppb), brain (LOD = 0.43 ppb), and kidney (LOD = 6.6 ppb) of VEH-injected control mice at 24 h and 7 days after the last injection. There was a detectable, dose-related concentration of mercury in blood, brain, and kidney for the 1 \times and 10 \times thimerosal doses at 24 h, but no statistically significant differences between mercury levels with or without vaccine. Based on tissue to blood ratios presented in Table 2, the concentration of mercury was 9.9- and 8.0-fold higher in brain compared with blood for the 1 \times and 1 \times + VAC groups, and 3.5- and 4.6-fold higher for the 10 \times and 10 \times + VAC groups. Mercury levels were also more than 100-fold higher in the kidney than in the blood for 10 \times and 10 \times + VAC groups. These brain to blood and kidney to blood ratios were even

TABLE 2
Tissue Mercury Concentration (ppb) 24 h and 7 days after the Last Thimerosal Injection

Sample time	Group ^a	Blood (ppb)	Brain (ppb)	Kidney (ppb)	Brain/blood ratio	Kidney/blood ratio
24 h	VEH (<i>n</i> = 4,4,4)	LOD ^b	LOD	LOD	NA	NA
	1× (<i>n</i> = 5,5,5)	1.03 ± 0.12	9.36 ± 0.44	133.62 ± 6.43	9.9 ± 1.9	141.4 ± 25.9
	1× + VAC (<i>n</i> = 4,5,5)	1.17 ± 0.22	8.52 ± 0.17	135.78 ± 9.58	8.0 ± 1.4	125.1 ± 18.6
	10× (<i>n</i> = 4,4,4)	23.81 ± 1.96	82.56 ± 04.3	1274.90 ± 156.34	3.5 ± 0.4	53.5 ± 4.9
	10× + VAC (<i>n</i> = 7,7,7)	23.34 ± 0.95	107.46 ± 06.95	1308.29 ± 50.18	4.6 ± 0.4	56.4 ± 2.3
7 days	VEH (<i>n</i> = 4,4,4)	LOD	LOD ^c	LOD	NA	NA
	1× (<i>n</i> = 7,7,7)	LOD	6.72 ± 0.54	100.94 ± 7.22	NA	NA
	1× + VAC (<i>n</i> = 8,7,8)	LOD	6.79 ± 0.36	117.56 ± 4.15	NA	NA
	10× (<i>n</i> = 6,6,6)	5.39 ± 0.50	60.16 ± 1.68	965.58 ± 45.33	11.6 ± 0.9	185.6 ± 15.9
	10× + VAC (<i>n</i> = 6,6,6)	5.66 ± 0.38	59.5 ± 2.36	1225.00 ± 53.59	10.9 ± 1.1	222.1 ± 19.2

^aNumber of samples for blood, brain, and kidney, respectively.

^bBelow LOD.

^c*n* = 4 with one outlier removed.

higher at 7 days due to the rapid elimination of mercury from the blood. By 7 days mercury in the blood for the 1× dose was no longer detectable, and mercury levels for the 10× dose had decreased by approximately 75%. Mercury levels in brain and kidney were also lower at 7 days, but the decrease was only by approximately 24 and 15%, respectively.

Hippocampal Cytoarchitecture

Cresyl violet staining indicated a normal pattern of cellular morphology in the hippocampus (Fig. 2). No evidence was found for disruption of the gross cytoarchitecture of the hippocampus in SJL/J mice exposed neonatally to thimerosal + vaccine. Unbiased stereology supported the observation that the numbers of neurons and regional volumes of the CA1 and DG hippocampal subregions did not differ significantly between the VEH, 1× + VAC, and 10× + VAC treatment groups (Fig. 3). The overall staining pattern was similar between VEH and 1× + VEH groups of mice for other surrounding brain regions (e.g., cortex, striatum) contained within the plane of sectioning (data not shown). Examination of similar brain sections using an amino cupric acid silver stain also showed no evidence of neuronal degeneration within the hippocampus (Fig. 4). An example of positive silver staining of degenerating hippocampal pyramidal neurons is shown in Supplementary data (Suppl. 2).

Immunohistochemistry for KA2 in the hippocampus showed no distinct differences between controls and thimerosal-injected mice (Fig. 5). Immunoreactivity for KA2 within the hippocampus (Fig. 5) showed a specific neuropilar staining pattern within the axonal pathways and within the CA pyramidal cell layer. This pattern is consistent with previous reports on the localization of KA2 receptors within the pyramidal cell layer (Darstein *et al.*, 2003; Petralia *et al.*, 1994). Immunoreactivity was present in the polymorphic layer

of the DG, in the stratum lucidum, and in the area of infrapyramidal projections from the dentate granule cell layer (DG). We did not detect specific staining of the soma of the CA pyramidal neurons (see Supplementary data, Suppl. 3). Specificity of the staining was confirmed by the lack of staining following incubation in the absence of primary antibody and following antigen preabsorption (see Supplementary data, Suppl. 4). The pattern and intensity of staining was not altered in mice exposed to 1× + VAC as compared with the VEH control group (Fig. 5A). High magnification of the CA1 pyramidal cell layer (insert) showed a similar staining pattern between both groups of mice with no specific staining of the pyramidal neuron cell body (Fig. 5B). These results contrast with those of Hornig *et al.* (2004) who reported increased KA2 immunostaining in CA1 neurons of thimerosal-treated SJL/J mice.

Developmental Indices

Average litter size at birth was 6.1 ± 1.0 and percent male versus female pups was 52.4 and 47.6%, respectively. Treatment groups did not differ significantly in body weight on injection days 7 and 15 (Fig. 6A), or at 4 or 10 weeks of age (Fig. 6B). There were sex differences in body weight with males weighing significantly more than females at 4 and 10 weeks of age (data not shown). Before the start of injection three complete litters died, along with six individual female and four male pups. Over the four injections, one male 1×, one male 10×, one male 1× + VAC, and one female VEH died. There was no obvious relationship between treatment condition and loss of pups due to the injection procedure, and no animal died during or immediately after an individual injection (i.e., within ~1 h). There was no evidence of cannibalism or self-mutilation by the mice during the period of group housing (i.e., after 5 weeks of age). The general health of all mice appeared to be good, and no

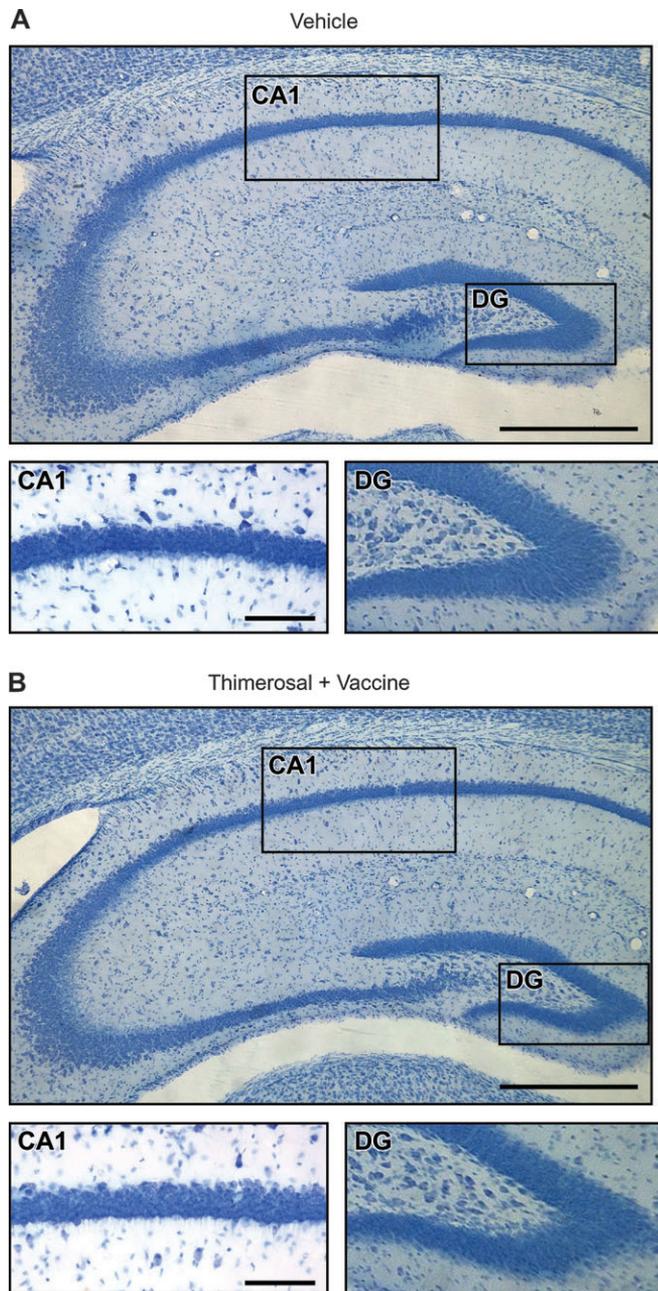


FIG. 2. Representative ($n = 6/\text{group}$) cresyl violet stained coronal sections of dorsal hippocampus ($5\times$ objective) from (A) VEH control and (B) thimerosal + vaccine ($1\times + \text{VAC}$) injected mice at 5 weeks of age. Insets show CA1 pyramidal neurons and granule cells in the DG at higher magnification ($16\times$ objective). No morphological differences were found between groups. Scale bars: small = $100\ \mu\text{m}$, large = $300\ \mu\text{m}$.

mice showed obvious movement or gait anomalies, lethargy, or signs of distress or illness at any time during the study.

Locomotor Behaviors

There were no significant sex differences in locomotor behavior on PND 8 or 10. However, statistically significant sex

differences were observed at 4 weeks in 27 of 37 variables and 23 of 37 variables at 10 weeks of age. These sex differences are consistent with previous findings (Voikar *et al.*, 2001), and were found across the range of locomotor behaviors measured by the TruScan open-field activity apparatus.

PNDs 8 and 10

There were no significant treatment or sex differences in measures of negative geotaxis or righting response (all $p > 0.05$). Of 37 measures of open-field locomotor activity on each of PNDs 8 and 10, a single significant group difference in PND8 ambulatory movement time was found ($F_{4,141} = 2.57$, $p = 0.041$). Specifically, female VEH-injected control mice showed significantly ($p = 0.011$) less total movement time than $1\times + \text{VAC}$ mice over the 2-min observation period (data not shown), representing a 17% difference between the two groups. No other treatment effects were statistically significant.

Postnatal Week 4

In the rotarod test, there were no significant group differences in the number of falls during the two training trials. There were also no significant differences in time to first fall during the two training trials. However, at the 24-h test ($\chi^2 = 13.6$, $\text{df} = 4$, $p < 0.01$), mice in the $1\times + \text{VAC}$ fell significantly sooner than mice in the $1\times$ group ($p = 0.022$). This difference was not statistically significant when sexes were analyzed separately. There were no other significant differences in time to fall. No significant group differences were found for the number of passive rotations (i.e., rotating with the rod) on the first training trial or during the 24-h test. However, there was a significant group difference in the number of passive rotations (i.e., rotating with the rod) on training trial 2 ($\chi^2 = 13.1$, $\text{df} = 4$, $p = 0.01$), with the VEH group showing more passive rotations than the $1\times$ group ($p = 0.029$). This difference was not statistically significant for males or for females when sexes were analyzed separately, and no other group comparisons reached statistical significance. Rotarod data are shown in Supplementary data (Suppl. 5).

Four of 37 interrelated locomotor variables collected by the TruScan open-field apparatus showed significant group differences. These variables were (Fig. 7A) center entries ($F_{4,176} = 3.74$, $p < 0.01$ (Fig. 7B) ambulatory center distance/center distance (almost identical measures, $\chi^2 = 16.62$, $\text{df} = 4$, $p < 0.01/\chi^2 = 16.22$, $\text{df} = 4$, $p < 0.01$) (Figs. 7C and 7D) center time/margin time (reciprocal measures, $\chi^2 = 15.12$, $\text{df} = 4$, $p < 0.01$) and (Fig. 7E) vertical plane (VP) entries ($F_{4,139} = 2.45$, $p = 0.049$). Further analysis showed that female $1\times$ mice entered the center of the open-field fewer times than female VEH controls (Fig. 7A, $p = 0.045$), traveled a shorter distance in the center (ambulatory center distance, $p = 0.033$), spent less time in the center (center time, $p = 0.049$), and consequently increased their time in the margin (margin time, $p = 0.049$) of the apparatus compared to female VEH controls (Figs. 7B–D).

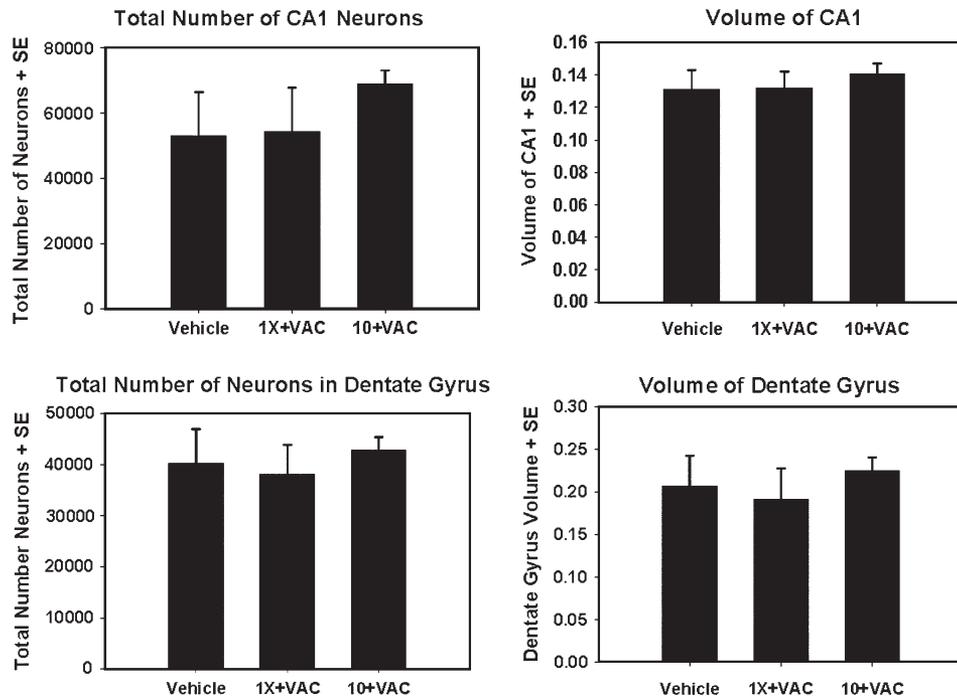


FIG. 3. Stereological analysis of total numbers of pyramidal neurons and volume (mm^3) in the CA1 subregion of the hippocampus at 5 weeks of age. There were no significant differences among groups (VEH $n = 6$; $1\times + \text{VAC}$ $n = 6$, $10\times + \text{VAC}$ $n = 3$).

Female $1\times$ mice also differed significantly from female $10\times + \text{VAC}$ mice in center distance, center time, and margin time. Center time and margin time are reciprocal measures because mice can only be in either the center or the margin regions of the open-field apparatus. Also note that ambulatory center distance equals center distance minus stereotypy movement distance. These two variables are highly correlated ($r = 0.985$) and measure essentially the same locomotor behavior. This overall pattern of locomotor behavior reflects the fact that $1\times$ -injected female mice spent more time in the margin next to the walls of the locomotor chamber (i.e., thigmotaxis) than VEH-injected controls. Female $1\times$ mice also showed a reduced number of VP entries compared with VAC-injected mice, and this difference approached statistical significance (Fig. 7E, $p = 0.056$). Performance of $1\times + \text{VAC}$ female mice in these open-field behaviors was similar to that of $1\times$ females, but apparent differences did not reach statistical significance. There were no statistically significant group differences for male mice for any locomotor variable, although male mice in the $1\times$ group showed a trend for decreased time in the center and increased time in the margin of the open field. None of the measures of repetitive beam breaks (i.e., circling, stereotypy) or rearing showed significant treatment effects for either sex.

Postnatal Week 10

There was a significant sex \times group interaction for distance traveled in the margin of the open field ($F_{4,116} = 3.76$, $p < 0.01$) and for ambulatory margin time ($F_{4,116} = 3.64$,

$p < 0.01$). As shown in Figure 7F, males in the $10\times + \text{VAC}$ traveled significantly further (and hence spent more time) in the margin of the open field compared with the VAC group ($p = 0.032$). No other comparisons reached statistical significance for locomotor behavior or for the rotarod test.

Acoustic Startle, Sensory Gating (PPI), Anxiety, Sociability, and Preference for Social Novelty.

Figure 8A shows the baseline activity in the absence of a startle tone, and maximum startle amplitude to a 120-dB tone for the five treatment groups. There were no significant treatment differences at baseline ($F_{4,115} = 0.07$, $p = 0.99$) or in startle response ($F_{4,115} = 1.16$, $p = 0.33$). There was a significant sex difference in baseline activity ($F_{1,115} = 10.13$, $p = 0.001$) and in startle response ($F_{1,115} = 10.62$, $p = 0.001$). Females showed uniformly lower response amplitudes compared with males as has been previously reported (Plappert *et al.*, 2005). Individual analyses for each sex were carried out but did not reveal any statistically significant differences between thimerosal exposure groups.

Figure 8B shows the magnitude of PPI (% inhibition of startle response) to a 120-dB tone produced by 74-, 82-, and 90-dB prepulses for each treatment group. As expected, the magnitude of PPI significantly increased with increasing tone intensity ($F_{2,230} = 172.2$, $p = 0.0001$). No differences were found between treatment groups ($F_{4,115} = 1.17$, $p = 0.33$). There was a significant sex difference ($F_{1,115} = 9.164$, $p = 0.003$), but separate analyses for males and females failed to show any significant thimerosal treatment effects.

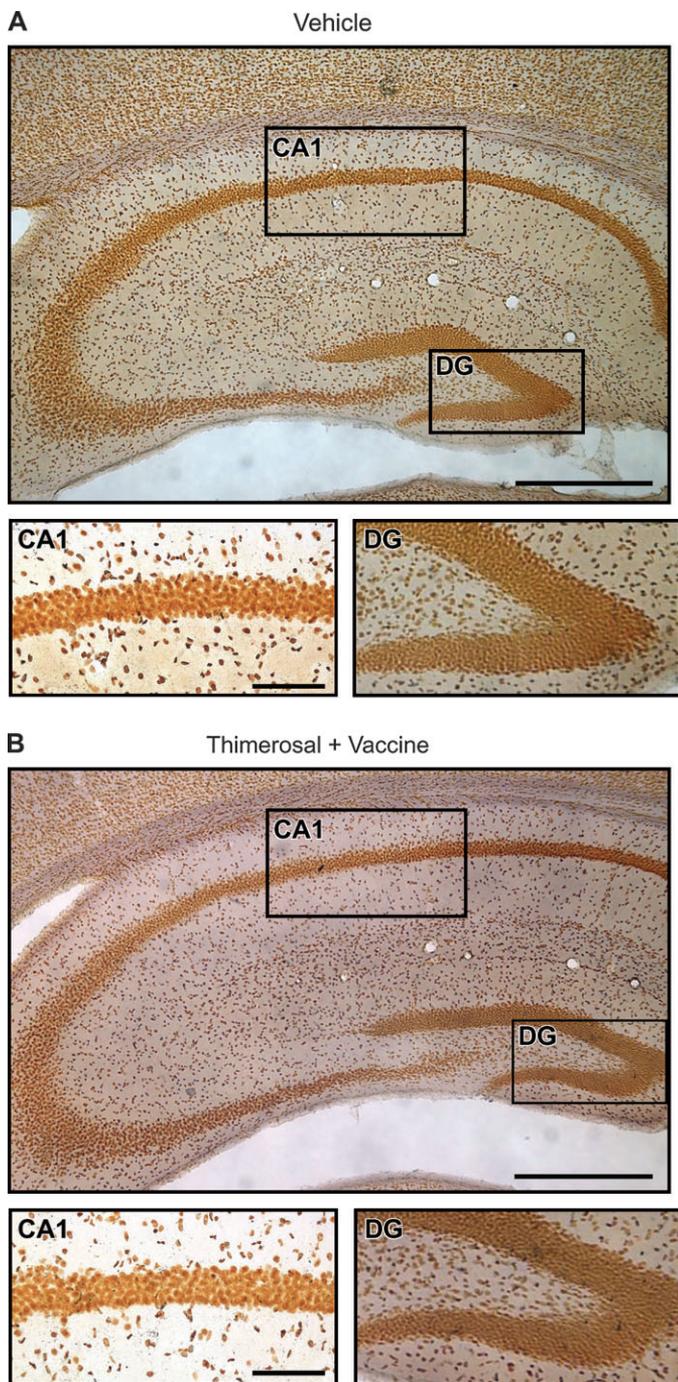


FIG. 4. Representative sections of silver degeneration staining in the hippocampus at 5 weeks of age for (A) VEH and (B) 1 \times + VAC at equivalent plane of cut (5 \times objective; scale bar = 300 μ m). Insets: higher magnification of the CA1 pyramidal cell layer and dentate granule cells (16 \times objective; scale bar = 100 μ m).

Figure 9 shows the time spent, distance traveled, and number of entries into the open arm of the elevated plus-maze. Statistical analyses did not show any significant differences among treatment groups in the ratio $([\text{open}/(\text{open} + \text{closed})] \times 100)$ of time in the open arm ($F_{4,116} = 0.81, p = 0.52$), distance

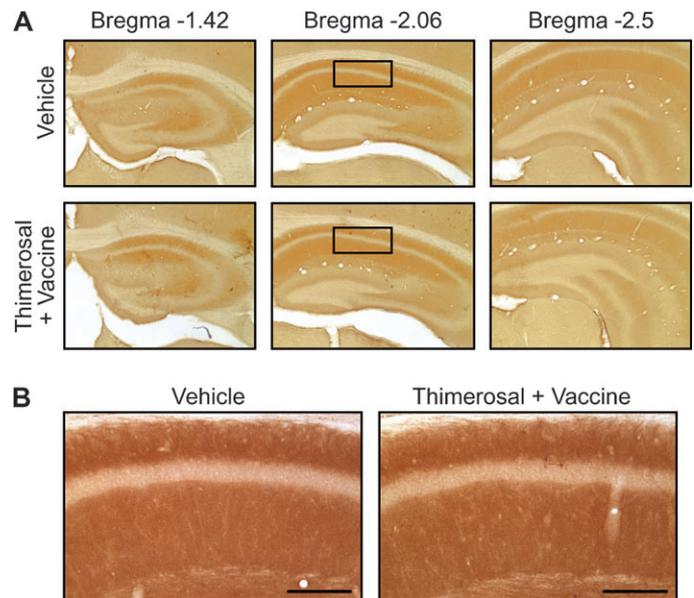


FIG. 5. (A) Representative sections of KA2 immunohistochemistry at three distinct stereotaxic coordinate sites demonstrate the similar staining pattern and gross hippocampal structure in control and thimerosal + vaccine mice (5 \times objective; scale bar = 300 μ m). (B) Inset: higher magnification of the CA1 pyramidal cell layer (16 \times objective; scale bar = 100 μ m).

traveled in the open arm ($F_{4,116} = 0.93, p = 0.45$), or number of open arm entries ($F_{4,116} = 0.75, p = 0.56$). Sex differences and treatment \times sex interactions were also not statistically significant. The SJL/J mice showed few anxiety-related behaviors in this test, consistent with a previous report in this mouse strain (Griebel *et al.*, 2000), possibly due to poor vision that could interfere with this behavioral assay (Chang *et al.*, 2002; Wong and Brown, 2006).

Sociability, measured as percent time spent by injected mice in the chamber with the stranger mouse, is shown in Figure 10A. There was a significant difference across groups in the amount of time spent in the chamber with the stranger mouse compared to the empty chamber across treatment groups ($F_{1,87} = 77.0, p = 0.001$), but no difference between treatment groups ($F_{4,87} = 0.77, p = 0.55$). There were no significant sex differences ($F_{1,87} = 0.07, p = 0.80$) and the treatment \times sex interaction was not significant ($F_{4,87} = 1.53, p = 0.20$). Preference for social novelty (Fig. 10B), was calculated as the ratio between time spent with the novel mouse compared with time with the familiar mouse. This analysis also failed to reveal significant treatment effects ($F_{4,85} = 1.91, p = 0.12$), sex differences ($F_{1,85} = 0.22, p = 0.64$), or a treatment \times sex interaction ($F_{4,85} = 0.97, p = 0.43$).

DISCUSSION

Tissue Mercury Levels

Blood mercury levels in VEH-injected mice at 24 h or 7 days after the last of the four injections were below the LOD,

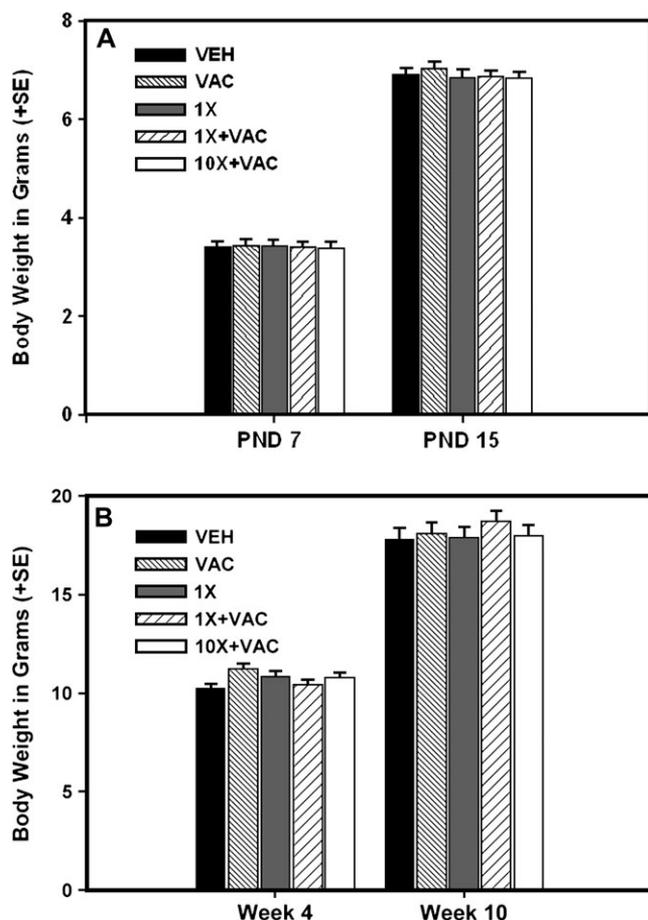


FIG. 6. (A) No significant differences in average body weight between groups before the start of thimerosal injections (PND7), at the end of injections (PND15), or (B) at 4 weeks or 10 weeks of age.

demonstrating that background mercury exposure (i.e., not from injections) was minimal, and that cross-contamination between littermates did not occur. In contrast, mercury levels were elevated in blood, brain, and kidney 24 h after the last injection for 1X and 1X + VAC groups, with blood mercury levels more than 20 times higher for the 10X and 10X + VAC groups (Table 2). At 7 days mercury was no longer detectable in the blood in the 1X and 1X + VAC groups, and had decreased by approximately 75% in the 10X and 10X + VAC groups.

The biological half-life for elimination of mercury from blood ($T_{1/2}$) for the 10X and 10X + VAC groups was 2.9 days (single compartment model), although this estimate was based on only two time-points (i.e., 24 h and 7 days) and should be viewed with caution (Magos, 1987). This rapid elimination of mercury from blood appears to be somewhat faster than that previously observed in CD1 mice (~4 days) following an im injection with thimerosal (Harry *et al.*, 2004), as well as in infant monkeys (6.9 days) (Burbacher *et al.*, 2005) and human infants (7 days) (Pichichero *et al.*, 2002) following im injection with thimerosal-preserved vaccines. It is also considerably shorter than the clearance rate for methylmercury,

supporting the conclusion that the toxicokinetics of ethylmercury cannot be accurately predicted from data on methylmercury (Burbacher *et al.*, 2005; Clarkson and Magos, 2006; Harry *et al.*, 2004; Magos, 2003).

The 1X and 1X + VAC injections were designed to model the maximum possible exposure that could have occurred following childhood vaccinations. Blood levels of mercury in these two groups 24 h after the last injection were approximately 1 ppb. This mercury level in SJL mice is lower than that reported in full-term infants (2.24 ± 0.58 ppb) 48–72 h following a single im injection of thimerosal-preserved hepatitis B vaccine (Energix, Smith Kline Biol., Rixensart, Belgium) (Stajich *et al.*, 2000). It is also lower than that found in the blood of 2-month-old (8.2 ± 4.9 SD ppb) and 6-month-old (5.5 ± 1.2 SD ppb) infants 3–28 days following vaccination with thimerosal-preserved vaccines (Pichichero *et al.*, 2002). These differences are likely due to the more rapid elimination of mercury from the blood of SJL/J mice, compared with humans. Therefore, the 1X and 1X + VAC ethylmercury doses may not be optimal for modeling human vaccine-associated mercury exposure. However, the higher 10X and 10X + VAC thimerosal injection doses resulted in blood mercury levels that were approximately 23 ppb at 24 hr after the last injection. This approximates the highest blood level (i.e., 20.55 ppb) observed by Pichichero *et al.* (2002) in human infants 5 days after immunization with vaccines containing a total of 37.5 μ g mercury (Pichichero *et al.*, 2002).

The total mercury concentration in brain was approximately 8.0- to 9.9-fold higher than blood mercury 24 h after the last injection with 1X or 1X + VAC thimerosal, and 3.5- to 4.6-fold higher for 10X and 10X + VAC groups, respectively (Table 2). At 7 days blood mercury levels were below the LOD for the 1X and 1X + VAC groups and brain to blood ratios could therefore not be calculated, but ratios had increased for the 10X and 10X + VAC groups due to the rapid elimination of mercury from blood. This higher brain to blood ratio is consistent with earlier observations in infant monkeys by Burbacher *et al.* (2005), who reported that brain mercury levels in thimerosal-exposed infant monkeys were between 2.4- and 4.6-fold higher than blood mercury levels, with the ratio increasing over time following the last dose (Burbacher *et al.*, 2005). The estimated $T_{1/2}$ for elimination of total mercury from the brain in 10X and 10X + VAC thimerosal-injected mice was approximately 15.4 days, compared with 24.2 days reported for infant monkeys (Burbacher *et al.*, 2005). Finally, the highest mercury levels were found in kidney at both 24 h and 7 days. This is consistent with earlier mouse studies using higher exposure levels (Harry *et al.*, 2004), reflecting the accumulation of mercury in the kidney during metabolism and elimination. Similar to brain, the kidney to blood ratio increased over 7 days due to the more rapid clearance of mercury from blood. No measurable mercury was found in the brain or kidney of VEH-injected mice 24 h or 7 days after the last injection. The general findings for blood, brain, and kidney

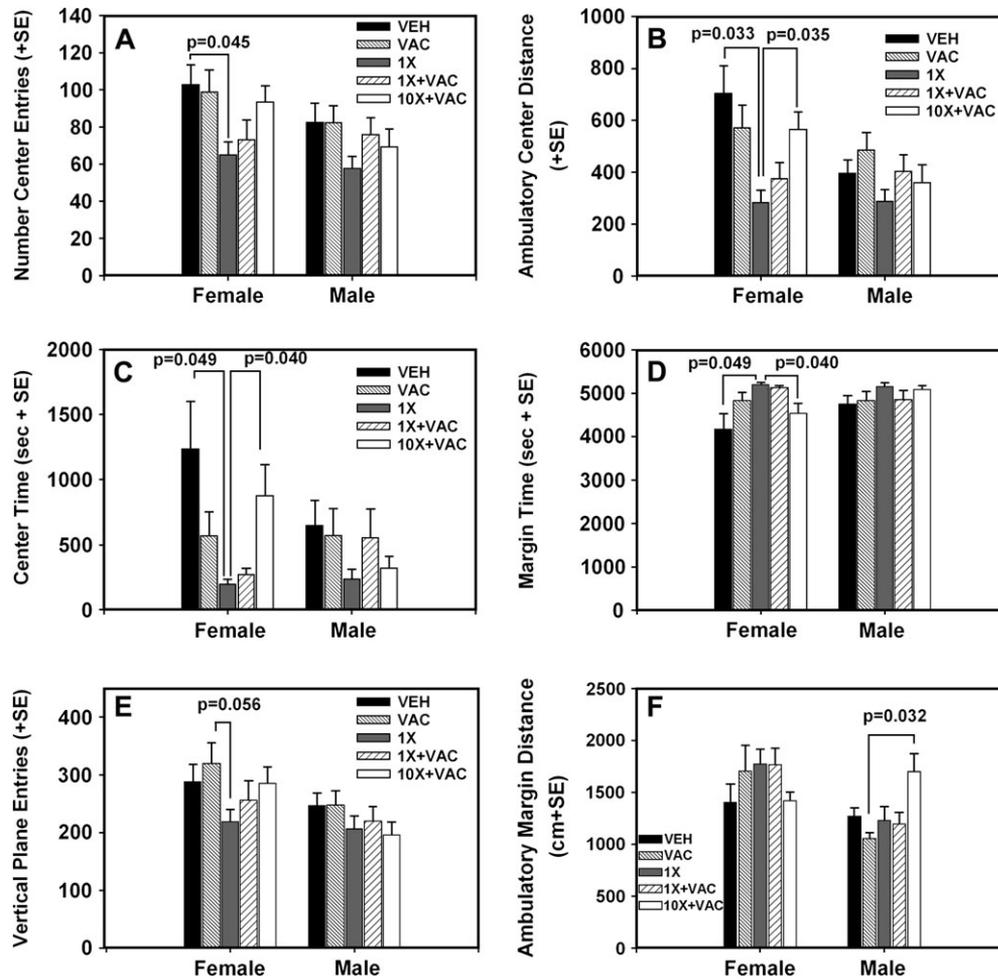


FIG. 7. Open-field locomotor effects of neonatal thimerosal exposure at 4 weeks of age (A–E). (A) Female 1× mice entered the center of the open field significantly fewer times than female VEH controls. Female 1× mice also traveled a shorter distance (B) and spent less time (C) in the center than female VEH control and 10× + VAC mice. (D) Female mice consequently showed increased time in the margin of the open field than female VEH control and 10× + VAC mice. (E) Female 1× mice showed fewer VP entries than VAC-injected mice (marginally significant, $p = 0.056$). (F) At 10 weeks of age, male 10× + VAC-injected mice traveled further in the margin of the open field than VAC-injected mice (4 weeks: VEH $n = 37$; VAC $n = 37$, 1× $n = 38$, 1× + VAC $n = 37$, 10× + VAC $n = 38$; 10 weeks: VEH $n = 26$; 1× VAC $n = 25$, 1× $n = 24$, 1× + VAC $n = 25$, 10× + VAC $n = 26$).

mercury levels in SJL mice are similar to those reported earlier in monkeys and humans, although the elimination appears to be more rapid in mice.

Hippocampal Cytoarchitecture

In the earlier study by Hornig *et al.* (2004), gross morphological changes in the dorsal hippocampus were reported at 5 weeks of age in thimerosal-injected SJL/J mice, but not in BALB/c mice. Changes included increased numbers and density of neurons in the CA1, CA2, and dentate gyrus (DG) of the hippocampus, overall enlargement and distortion of hippocampal morphology, and hyperchromatic pyramidal cells in CA1 and CA2 pyramidal neurons. These authors concluded that thimerosal exposure, patterned after childhood vaccination schedules, altered the morphology of the hippo-

campus in autoimmune susceptible SJL/J mice. The histological data from the present study do not support these earlier conclusions. Specifically, the current study did not find alterations in volume or numbers of neurons in DG or CA1, in staining intensity or in the general morphology of the hippocampus.

Methodological differences must be considered when comparing the present study with those of Hornig *et al.* (2004). Morphological data presented in the Hornig *et al.* (2004) study were based on a small number of tissue sections and did not use stereological procedures. In contrast, the present study estimated neuronal numbers and hippocampal brain regional volumes using well-accepted unbiased stereological procedures as previously detailed (Calhoun *et al.*, 1998; Lei *et al.*, 2003; Long *et al.*, 1999; Mouton, 2002; Mouton *et al.*, 2002). Specifically, 8–12 sections were selected in

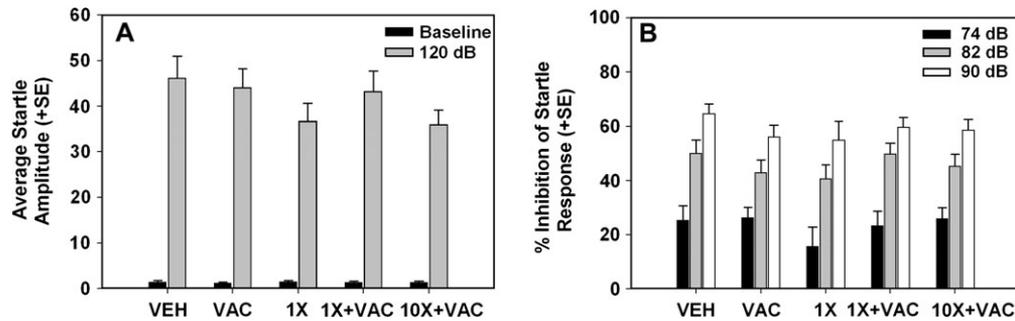


FIG. 8. (A) Baseline motor activity and acoustic startle to a 120-dB tone. (B) PPI of the startle response following either a 74-, 82-, or 90-dB prepulse tone. There were no significant differences among groups in startle amplitude or PPI (VEH $n = 27$; VAC $n = 26$, $1\times n = 24$, $1\times + VAC n = 23$, $10\times + VAC n = 25$).

a systematic-random manner to avoid bias in selection of tissue sections for analysis. We quantified total neuron numbers using the optical fractionator, which avoids the well-known problem (i.e., reference trap) associated with using neuronal density to estimate total numbers of neurons. In addition, neuroanatomical reference spaces for cell counting in the DG and CA1 were based on unambiguous anatomical criteria. We also used well-defined counting criteria, including the presence of a distinct nuclear membrane, nucleolus, cytoplasm, and cell membrane, and Gundersen's unbiased 3D counting rules (Gundersen, 1977) to quantify total neuron numbers. Finally, sampling within each brain continued until the mean CE for each group was between 5 and 10%. This is critical because it means that $> 90\%$ of the variance in neuronal number and regional brain volume is of biological origin rather than the result of methodological error, allowing us to accept the lack of differences between treatment groups with a high degree of confidence. This is in contrast to the analysis of the density of neuronal profiles (profiles per unit area) reported by Hornig *et al.* (2004).

Immunostaining for the KA2 receptor subunit was carried out to assess the effects of neonatal exposure to thimerosal on the distribution of glutamatergic kainate receptors in the hippocampus. The immunostaining pattern for KA2 in the present study was similar to that reported by Darstein *et al.* (2003), with prominent staining in the neuropil of the hilus and in the stratum lucidum and projection areas of the mossy fibers. In contrast, the staining pattern reported by Hornig *et al.* (2004) showed prominent immunoreactivity on CA1 pyramidal neurons that increased with thimerosal + vaccine treatment. The Hornig *et al.* (2004) paper showed an increase in CA1 neuronal soma immunostaining for SJL mice exposed to thimerosal + vaccine, whereas the current study showed no effect of thimerosal exposure (i.e., $1\times + VAC$) on the KA2 immunoreactive staining pattern. Although the antibodies were purchased from the same commercial source, the differences in the patterns of immunoreactivity may be due to different levels of antisera selective for KA2 or different tissue preparations. However, variability of antisera levels or differences in tissue preparation would not be expected to explain the absence of

changes in the relative immunostaining intensity in the stratum radiatum and stratum pyramidale of hippocampal CA layer with thimerosal exposure in the present study.

Although Hornig *et al.* (2004) provided evidence for a disruption of NR1 and NR2b glutamate receptors, in our hands antibodies from the same commercial source (Chemicon, Temecula, CA) did not provide an acceptable level of specificity for immunohistochemistry in mouse brain. For example, the NR2b antibody at a high concentration (1:50 dilution) stained subpopulations of hippocampal neurons in the subiculum proper and the CA3 and the NR1 antibody stained most of all neurons in the brain. In both cases, the staining was not specific and could not be blocked by preincubation with the antigen peptides.

Behavioral Studies

In the rotarod test, the number of falls, the time to first fall and the number of passive rotations were measured as an index of overall motor coordination and balance (Golub *et al.*, 2004). There were no significant differences among treatment groups

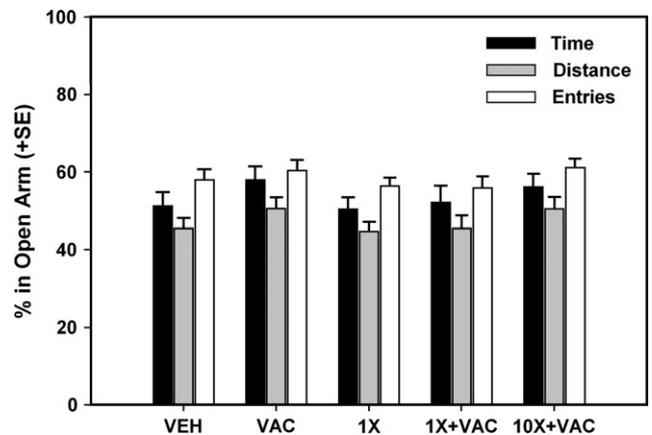


FIG. 9. Elevated plus-maze performance. Percent time, distance, and number of entries into the open arm of the elevated plus-maze as a measure of fear and anxiety. There were no significant differences among groups (VEH $n = 28$; VAC $n = 29$, $1\times n = 25$, $1\times + VAC n = 21$, $10\times + VAC n = 23$).

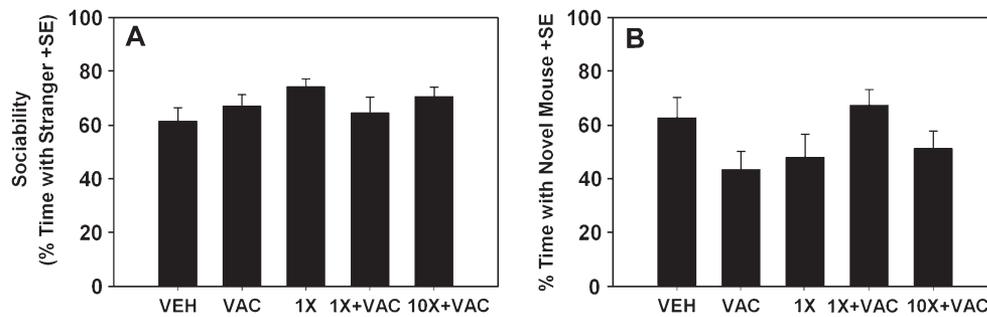


FIG. 10. Performance in the (A) sociability test and (B) recognition of social novelty test for the five treatment groups. There were no significant differences among treatment groups in either test (VEH $n = 22$; 1 \times VAC $n = 21$, 1 \times $n = 16$, 1 \times + VAC $n = 18$, 10 \times + VAC $n = 20$).

in the number of falls during the two training trials. For the time to first fall, mice in the 1 \times + VAC group fell significantly sooner than mice in the 1 \times group during the 24-h test but not during the two training trials. Importantly, performance (i.e., time to fall) of these two groups did not differ from that of any other treatment group, including the VEH and VAC control groups. For passive rotations, the VEH group showed more rotations than the 1 \times group, but only on training trial 2, and not during the first training trial or during the 24-h test. In general, passive rotations were infrequent, typically less than one per session, making it difficult to interpret any group differences as evidence of a motor deficit. The lack of marked or consistent differences between treatment groups in the rotarod test indicates that motor performance was intact in the vaccine-treated and thimerosal-treated mice.

Thirty-seven interrelated locomotor endpoints were analyzed in the automated open-field test on postnatal days 8 and 10, and at 4 and 10 weeks of age. The majority of locomotor behaviors, including total movements, total movement time, rotations, and stereotypy, did not show statistically significant effects associated with thimerosal injections. However, female mice injected with 1 \times thimerosal showed a pattern of increased time in the margin of the open field compared to VEH controls. Similar behavior was seen in female mice in the 1 \times + VAC group but the effects did not reach statistical significance; nor did they reach statistical significance for male mice in any treatment group. Sex differences in open-field behavior following gestational and preweaning exposure to methylmercury have been reported previously, with female, but not male C57BL/6 mice showing decreased locomotor activity when tested at 9 weeks of age (Goulet *et al.*, 2003). Within this context it should be noted that autism is predominantly found in males at approximately a 4:1 male:female ratio (Fombonne, 2002; Kanner, 1954), whereas the locomotor effects found in the present study were predominantly in female mice. The explanation for these motor deficits is unclear, but a large body of previous research indicates that for methylmercury the most common developmental effects of exposure in rodents are motor deficits (Rice, 1996).

It is notable that at 4 weeks of age no differences were found in the open field among groups injected with VEH, VAC alone,

or the higher 10 \times thimerosal dose (i.e., 10 \times + VAC). Therefore, thimerosal effects on locomotor behaviors did not appear to exhibit any simple dose-response relationship. Nonmonotonic dose-response relationships and “inverted-U” shaped dose-responses have been observed for several biological functions at the extremes of physiological conditions. For example, several recent studies have shown an “inverted-U” dose-response on working memory following stimulation of DA receptors in prefrontal cortex (Vijayraghavan *et al.*, 2007). A similar phenomenon has been observed in mice (Lidow *et al.*, 2003), rats (Zahrt *et al.*, 1997), monkeys (Cai and Arnsten, 1997), and humans (Gibbs and D’Esposito, 2005).

When behavioral performance was examined using more complex tasks designed to assess social interaction, social recognition, anxiety, and sensory gating (i.e., PPI), no differences were found among treatment groups. The first three tasks have been used to phenotype inbred mouse strains using behaviors that have been suggested to be relevant to the core deficits in autism (Crawley, 2004; Moy *et al.*, 2007). The latter behavioral test, PPI, is a measure of the ability of the nervous system to gate sensorimotor processing, and has been reported to be abnormal in autistic adults (Perry *et al.*, 2006). Specifically, adults with autism show reduced PPI that correlates with restricted and repetitive behaviors on the Autistic Diagnostic Inventory-Revised assessment. Perry *et al.*, (2006) suggested that the reduced PPI may reflect a failure of inhibitory processing associated with perseverative and repetitive behaviors in autism. The observation that neonatal thimerosal injections did not alter performance in these behavioral assays suggests that the effects of thimerosal in SJL/J mice may be quite restricted, and possibly limited to mainly locomotor phenomenon.

Thimerosal-exposed mice did not differ from VEH controls in tests of social interaction or preference for social novelty. Although there are clear limitations when comparing rodent behaviors with those of humans (Ricceri *et al.*, 2007), the absence of effects in these behavioral domains does not provide support for the argument that thimerosal plays a role in the etiology of neurodevelopmental disorders, such as autism. Although no thimerosal-related differences were noted in the preference for social novelty, it should be noted that previous

studies have demonstrated substantial mouse strain differences in this behavioral test. For example, C3H/HeJ, AKR/J, A/J, and 129S1/SvImJ mouse strains display a muted preference for social novelty (Moy *et al.*, 2007). Based upon the present results, the SJL/J mouse strain may also be included in this group of weakly responding strains.

In the earlier Hornig *et al.* (2004) study, it was suggested that the observed reduced distance traveled within the center of the open-field apparatus represented an increase in the anxiety level of the animal and a decreased willingness to explore a novel environment. However, there are many other possible interpretations of these data, and before an appropriate interpretation of the data can be made, additional tests are needed to assess motor function, sensory function, and anxiety/fear response in the experimental animals. In the current study, we identified a marginal effect on locomotor behavior at 4 weeks of age in female mice that did not exhibit a simple dose-response relationship. In order to interpret these effects, additional tests were run to assess sensorimotor processing and anxiety in the mice. In the elevated plus-maze there was no evidence that early thimerosal exposure increased anxiety in SJL mice. The SJL/J mice, regardless of thimerosal treatment, distributed their time evenly between the open and closed arms of the maze, suggesting generally low anxiety levels in this task. This finding is consistent with reports that the SJL inbred mouse strain generally shows low anxiety in the plus-maze compared with several other commonly used strains such as C57BL/6, DAB/2, and NZB (Griebel *et al.*, 2000). However, a lower basal level should allow for an increased ability to detect any increased anxiety associated with developmental thimerosal exposure. The fact that no alterations in anxiety were detected in the elevated plus-maze in thimerosal-exposed mice does not support the interpretation that changes in open-field locomotor activity are anxiety related. Although no differences between treatment groups were found in the plus-maze, it should be noted that SJL/J mice are homozygous for the retinal degeneration allele *Pde6b^{rdl}* (i.e., *rdl* mutation) and are functionally blind as early as 4 weeks of age (Chang *et al.*, 2002; Wong and Brown, 2006). This presents a clear limitation to the use of the SJL/J mouse in neurobehavioral studies, especially in tasks such as the open field that rely heavily on the visual system.

Altered sensorimotor processing was studied using the acoustic startle response and PPI. Deficits in PPI have been found for a number of psychiatric and developmental disorders, including adult autism (Perry *et al.*, 2006). Given its clinical relevance to autism and the suggestion that thimerosal exposure through childhood vaccinations may contribute to the etiology of autism, we included this measurement in our assessment of thimerosal-exposed SJL mice. Neither the acoustic startle response nor PPI was significantly affected by neonatal thimerosal exposure, this was true even in mice exposed to a relatively high dose of thimerosal (i.e., $10\times + \text{VAC}$).

CONCLUSIONS

No evidence was found that exposure to vaccine-associated levels of thimerosal, whether or not in combination with vaccine, resulted in abnormal somatic growth or altered the normal development or structure of the hippocampus. No deficits were observed in tests of complex behaviors that have been considered to be particularly relevant to the study of neurodevelopment and its disorders, including social interaction, sensory gating, or anxiety (Crawley, 2004; Ricceri *et al.*, 2007). Only limited locomotor effects were observed, and these were primarily in female SJL mice in the open field at 4 weeks of age. Considered together, the overall pattern of results of the present study does not indicate marked or pervasive neurotoxicological deficits in neonatal SJL/J mice following injections of vaccine-associated levels of thimerosal. Particularly relevant to human health concerns, the current data do not provide support for the inference that neonatal thimerosal exposure is involved in the etiology of neurodevelopmental disorders that alter social behaviors such as autism. Although the SJL mouse strain carries the major histocompatibility complex H-2^S and develops autoantibodies when exposed to high levels of methyl and ethylmercury (Havarinasab *et al.*, 2005; Havarinasab and Hultman, 2005; Hultman and Hansson-Georgiadis, 1999), the strain does not appear to show dramatic neurohistological or neurobehavioral deficits when exposed to low-level ethylmercury. This mouse strain may therefore not represent an unusually sensitive murine model of thimerosal neurotoxicity. However, the hypothesis that genetic factors may predispose an organism to higher risk from toxicant exposure, including various forms of mercury, is still important and needs to be thoroughly investigated in order to better define the critical biological factors that determine safe human exposure levels to toxic agents. This is particularly true as exposure to mercury from other environmental sources remains an important health issue (Clarkson and Magos, 2006).

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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