

# Transcriptomic Analyses of Neurotoxic Effects in Mouse Brain After Intermittent Neonatal Administration of Thimerosal

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Thimerosal is a vaccine antimicrobial preservative which has long been suspected an iatrogenic factor possibly contributing to neurodevelopmental disorders including autism. The association between infant vaccine thimerosal exposure and autism remains an open question. Although thimerosal has been removed from mandatory childhood vaccines in the United States, thimerosal-preserved vaccines are still widely used outside of the United States especially in developing countries. Notably, thimerosal-containing vaccines are being given to the newborns within the first 12–24 h after birth in some countries. To examine the possible neurotoxic effects of early neonatal exposure to a higher level of thimerosal, FVB mice were subcutaneously injected with thimerosal-mercury at a dose which is 20× higher than that used for regular Chinese infant immunization during the first 4 months of life. Thimerosal-treated mice exhibited neural development delay, social interaction deficiency, and inclination of depression. Apparent neuropathological changes were also observed in adult mice neonatally treated with thimerosal. High-throughput RNA sequencing of autistic-behaved mice brains revealed the alternation of a number of canonical pathways involving neuronal development, neuronal synaptic function, and the dysregulation of endocrine system. Intriguingly, the elevation of anterior pituitary secreting hormones occurred exclusively in male but not in female thimerosal-treated mice, demonstrating for the first time the gender bias of thimerosal-mercury toxicity with regard to endocrine system. Our results indicate that higher dose of neonatal thimerosal-mercury (20× higher than that used in human) is capable of inducing long-lasting substantial dysregulation of neurodevelopment, synaptic function, and endocrine system, which could be the causal involvements of autistic-like behavior in mice.

**Key words:** thimerosal; transcriptomic analyses; anterior pituitary; hormone; neurotoxicity; autistic disorder.

Thimerosal (sodium ethylmercury thiosalicylate, 49.6% mercury (Hg) by weight) has been used as an antimicrobial preservative

in many vaccines and medicinal preparations since 1930s (Pless and Risher, 2000). It rapidly metabolizes to ethylmercury and subsequently to inorganic mercury forms which accumulate in different organs/tissues including the brain for months or years (Qvarnstrom *et al.*, 2003). The neurotoxicity of ethylmercury has been well known (Zhang, 1984). Because the blood-brain barrier of newborns is not well-developed, and the developing brain is uniquely vulnerable to neurotoxic hazard exposure, thimerosal-mercurials are suspected pathogenic factors in the etiology of several neurodevelopmental disorders, including autism (Bernard *et al.*, 2001; Geier and Geier, 2003, 2005, 2006b; Hewitson *et al.*, 2010; Majewska *et al.*, 2010; Young *et al.*, 2008). However, the association between thimerosal exposure via childhood vaccinations and neurodevelopmental disorders such as autism remains an open question (Blaxill *et al.*, 2004; Kern *et al.*, 2012; Nelson and Bauman, 2003). Several independent epidemiological investigations support a hypothesis linking this disorder with postnatal exposure to mercurials (Gallagher and Goodman, 2010; Geier and Geier, 2003, 2004, 2006a,b; Mutter *et al.*, 2005; Young *et al.*, 2008), whereas the others do not support such a relationship (Heron and Golding, 2004; Hviid *et al.*, 2003; Immunization Safety Review Committee, 2004; Madsen *et al.*, 2003; Stehr-Green *et al.*, 2003; Thompson *et al.*, 2007; Verstraeten *et al.*, 2003). Nevertheless, due to concern of increased mercury exposure and elevated body burdens in children (Ball *et al.*, 2001), thimerosal has been removed from mandatory childhood vaccines in the United States (American Academy of Pediatrics and United States Public Health Service, 1999).

Thimerosal-preserved vaccines are still widely used outside of the United States especially in developing countries such as Brazil and China, where the advantages of multiuse vials of thimerosal-preserved vaccines take precedence over perceived mercury hazards (Dorea, 2007; WHO IRIS, 2002). According to 2001 United States vaccination schedule, each 1-year-old U.S. child could have been exposed to a total of 237.5 µg Hg from vaccines distributed at 2, 4, 6, and 12 months

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(Hornig *et al.*, 2004). However, the thimerosal concentrations vary among brands of vaccines (from 25 to 50  $\mu\text{g}$  Hg per 0.5 ml shot), and the immunization schedules vary depending on a country's health policy. For example, the newborns receive two shots of thimerosal-containing vaccines within the first 12–24 h after birth in China, and the vaccination schedule causes a total of 300–600  $\mu\text{g}$  of Hg exposure during the first year of life. Due to the immaturity of newborns in the excretory system, blood-brain barrier, and central nervous system (Dorea, 2007), 1-day-old newborns are supposed to be more susceptible to mercurial exposure than 2-month-old children. Unfortunately, this 1-day-old newborn vaccination schedule and the thimerosal-containing vaccines are still widely used in the majority of newborns and infants in developing countries including China and Brazil, and the possible neurotoxic effects of this early neonatal vaccination schedule on those children have never been modeled.

To examine the potential neurodevelopmental toxicity of early neonatal thimerosal exposure, we immunize FVB pups with high dose of thimerosal by mimicking the infant vaccination schedule during first 4 months of life in China. We observed that the neonatal thimerosal exposure caused the delay in neural development, behavior alternation, and the neuropathology. Moreover, we use a high-throughput comparative RNA sequencing platform to unravel the changes of functional categories and molecular pathways associated with thimerosal toxicity. These findings may help to understand the potential involvement of environmental factor such as thimerosal-mercuries in neurodevelopmental disorders.

## MATERIALS AND METHODS

### *Animals and Thimerosal Administration*

FVBN<sub>NJ</sub> mouse was chosen for its active social behavior and excellent fecundity. FVBN<sub>NJ</sub> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding of FVB mice have been described previously (Wang *et al.*, 2013). All animal experiments were reviewed and approved by the Institute of Zoology, Institutional Animal Care and Use Committee and were conducted according to the committee's guidelines. According to the recommended Chinese infant vaccination schedule, each 1-year-old child could have been exposed to a total of 300–600  $\mu\text{g}$  of EtHg from vaccines distributed at 12–24 h after birth, 1, 2, 3, 4, 5, 6, 8, and 9 months of age. Immunization during the first 4 months causes a cumulative dose of 45.7  $\mu\text{g}/\text{kg}$  EtHg (Table 1). To increase the ability to detect possible adverse effects of thimerosal exposure, the thimerosal-mercury dosages were increased up to a 20-fold higher amounts than those used in current Chinese infant immunization schedule during the first 4 months of life. FVB pups were injected with thimerosal solution or saline vehicle (0.9% NaCl solution) on postnatal day 1 (P1), P3, P5, and P9 (20 $\times$  dose: 304, 238, 196, and 176  $\mu\text{g}/\text{kg}$ , respectively; 10 $\times$  dose: 152, 119, 98, and

88  $\mu\text{g}/\text{kg}$ , respectively; 5 $\times$  dose: 76, 60, 49, and 44  $\mu\text{g}/\text{kg}$ , respectively) (Table 1). Because of the small size of the mouse pups and the limited muscle development at these ages, injections were made via subcutaneous routes. Adult male mice (8–12 weeks old) were chosen for behavioral test, neuropathological study and high-throughput RNA sequencing. For sex hormone level measurements, both male and female mice were used.

### *Behavioral Analysis*

**Sociability test.** Adult male mice (8–12 weeks old) were tested for social interaction in the three-chambered apparatus as described previously (Yang *et al.*, 2011). Animals were tested between 8 and 10 weeks of age. The social testing apparatus is a three-chamber box. Each chamber is 20 cm long, 20 cm wide, and 22 cm high. Dividing walls are made from clear Plexiglas, with small circular doors (3.5 cm in diameter) allowing access to each chamber. The chambers of the social apparatus were cleaned and fresh paper chip bedding was added between trials. Mice were habituated in the center compartment for 10 min. During social testing, mice were allowed to freely enter adjacent chambers through the doors in the dividing walls. In the test for sociability, the test mouse was given a choice to spend time with an unfamiliar mouse (stranger) versus an empty chamber. In preliminary sociability experiments with normal FVB adult mice, we found females were not keen on communicating with strangers (Supplementary fig. 1a and b). Therefore, only adult males were used for behavioral test, neuropathology assay, and RNA sequencing.

**Sucrose preference test.** Sucrose preference tests were conducted as described previously (Strekalova and Steinbusch, 2010). The individually housed animals were offered the choice to drink from two bottles, one filled with water and the other filled with 1% sucrose in water. The daily intake of water and sucrose solution was estimated by weighing the bottles every day between 8:30 and 9:00 A.M. The bottles were cleaned and refilled with water or 1% sucrose every day. To prevent bias from preference for bottle location, the relative position of the two bottles in the cage lid was switched at the midpoint of testing every day.

### *Histopathology Experiments of Mouse Brain*

Both saline control and thimerosal-treated male mice were sacrificed at  $\sim$ 12 weeks of age after the completion of all behavioral testing. Mice were anesthetized with pentobarbital sodium (8 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS (w/v). Brains were then post-fixed in 4% paraformaldehyde for 24 h and dehydrated in gradient ethanol. Serial coronal brain sections (6- $\mu\text{m}$ ) were prepared through the whole cerebrum (Leica, CM1900). The sections from prefrontal cortex (bregma 1.70 mm), temporal cortex (bregma  $-2.54$  mm) and hippocampus (bregma  $-1.46$  to  $-1.94$

TABLE 1

**Thimerosal-Mercury Dosage and Timing of Recommended Chinese Infant Immunization Schedule During the First 4 Months of Age and the Neonatal Mouse Thimerosal Administration Procedure**

Age of infant (months)	12–24 h	1–2	3	4
Ethylmercury load ( $\mu\text{g}$ )	50	50	50	50
Average boy's body weight (kg)	3.3	4.2	5.1	5.7
Ethylmercury dose ( $\mu\text{g}/\text{kg}$ )	15.2	11.9	9.8	8.8
Thimerosal from EPI vaccines ( $\mu\text{g}/\text{shot}$ )	Hep B (50)	Hep B (50)	OPV (0)	OPV (0)
nbsp;	BCG (0)	OPV (0)	DPT (50)	DPT (50)
Mouse postnatal days for thimerosal	1	3	5	9
EtHg dose for mouse 20 $\times$ dose	304	238	196	176
pups ( $\mu\text{g}/\text{kg}$ )				
nbsp; 10 $\times$ dose	152	119	98	88
nbsp; 5 $\times$ dose	76	60	49	44

EtHg, ethylmercury; Hep B, hepatitis B; BCG, Bacille Calmette-Guerin; OPV, poliomyelitis vaccines; DPT, Diphtheria-Pertussis-Tetanus

mm) were stained histologically (haematoxylin-eosin) following standard histological protocols. Microphotographs of the brain sections were taken with an Olympus IX71 microscope and Canon 60D digital camera. The number of “dark” neurons in one sample is the average number of “dark” neurons from five regions (1 mm<sup>2</sup>) in prefrontal/temporal cortex or the total number of “dark” neurons in whole hippocampus. The statistics were the mean value of “dark” neurons from three samples ( $n = 3$ ).

#### Library Preparation and High-Throughput RNA Sequencing

Total RNA was isolated from the thimerosal- and placebo-treated male mouse whole brains using Trizol reagent (Invitrogen). Thimerosal-treated mice with autistic-like behavior were chosen as thimerosal group for RNA sequencing. Sequencing libraries were prepared according to the manufacturer's instructions (Illumina). Four samples were analyzed in this manner, with two from thimerosal-treated mice and two from placebo-treated mice.

#### Bioinformatic Analysis

**Reads trimming and alignment.** The original image data were converted into sequence data (raw data or raw reads) and saved as FASTQ files. After trimming low-quality reads, the high-quality paired reads were aligned against the reference genome of *Mus musculus* (NCBIM 37; ENSEMBL release 62; <http://asia.ensembl.org/info/data/ftp/index.html>) using the Burrows-Wheeler Alignment Tool 0.5.8 (BWA) with allowing up to four mismatches and other default options. We counted the number of reads mapped to exons, introns, splice junctions, intron-exon adjacent regions of the annotated genes, and intergenic regions using in-house built pipeline wapRNA (Zhao *et al.*, 2011). Transcript expression analysis was also done using TopHat v1.0.12, which incorporates the Bowtie v0.11.3 algorithm to perform the reads alignment. The aligned reads together with mouse genome annotation data were subsequently

used for transcripts assembly to detect known and unannotated transcripts by Cufflinks v2.0.2.

**Gene annotation and expression analysis.** Gene expression levels were defined by numbers of reads per kilobase of exon model in a gene per million mapped reads (RPKM). To compare the gene expression levels between the thimerosal-treated and placebo-treated groups, differential gene expression (Massey *et al.*, 2004) analysis was done using MARS (MA-plot-based method with Random Sampling) model (Wang *et al.*, 2010), and with the Benjamini-Hochberg false-discovery rate (FDR) adjustment for multiple testing. The log<sub>2</sub> fold changes ( $\text{fc} = \text{RPKM}_{\text{thimerosal}}/\text{RPKM}_{\text{placebo}}$ ) were used to describe the gene expression differences between the two groups.

Transcripts (expressed isoform) were assembled using the aligned reads by Cufflinks, and the expression level for those transcripts were defined as the numbers of fragments per kilobase of exon model in a gene per million mapped reads (FPKM). Cuffdiff were further used to find significant changes in transcript expression, splicing, and promoter.

**Gene ontology analysis.** Differentially expressed gene lists containing gene IDs and FC values were submitted to Ingenuity Pathway Analysis (IPA) v9.0-3211 (Ingenuity Systems, Inc., Redwood City, CA) for network and gene ontology analysis. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base. These genes were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base, then generated networks based on their connectivity. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the dataset. Genes with changed expression after thimerosal-treatment were chosen for further analysis. Fisher's exact test was used to calculate a p-value determining the probability of the association between the genes dataset and the canonical pathway.

### RNA Isolation and Quantitative Real Time PCR

Quantitative real time PCR was used to validate several gene expression changes predicted by RNA sequencing. Total RNA was extracted from the whole brain of placebo- and thimerosal-treated mice ( $n = 3$ , 3 months old males). The complementary DNA (cDNA) of 1  $\mu$ g RNA sample was reverse-transcribed using the First Strand cDNA Synthesis kit (Promega). The expressions of target genes (four up-regulated and two down-regulated genes predicted by RNA-sequencing) in the three placebo- and thimerosal-treated mice brain samples were detected by Q-PCRs in triplicate. The gene expression levels were quantified relative to the expression of mouse GAPDH gene, employing an optimized comparative Ct ( $\Delta\Delta$ Ct) value method.

### Hormone Level Measurements

Serum was collected from mice treated with placebo and thimerosal (3 weeks after birth) and flash frozen. The serum from each mouse was numbered and shipped on dry ice to Sino-UK Institute of Biological Technology (Beijing) for analysis. Serum levels of anterior pituitary hormones (PRL, ACTH, LH, FSH, CGB, TSH, and GH) and testosterone, were assayed using a radioimmunoassay (ng or mIU/ml). Fold change was calculated by the hormone levels from thimerosal treated mice over that from placebo-treated mice. Both males and females were used in serum hormone measurements.

### Statistics

Data were expressed as the mean  $\pm$  standard error of the mean (SEM), and statistical significance of differences between different groups were assessed using the  $t$ -test with  $p < 0.05$ .

## RESULTS

### Neural Development and Growth of Thimerosal-Treated Pups

FVB mice were administrated subcutaneously with 20 $\times$ , 10 $\times$ , and 5 $\times$  thimerosal solution or placebo saline at P1, P3, P5, and P9 as described in Table 1. The weight and the eye opening time were then recorded. Thimerosal exposure did not affect the growth of pups, no between-group differences were found in weight gain through P12 (Fig. 1a). The time of eye opening reflects the developmental maturity of nervous system in mice. Compared with placebo group, the eye opening time of thimerosal-treated mice were apparently delayed (Fig. 1b), suggesting that early neonatal exposure to thimerosal led to a delayed neural development.

### Neonatal Exposure to Thimerosal Causes Autistic-Like Behavior in Mice

Social interaction test was performed when placebo- and thimerosal-treated pups grew up to 8–12 weeks of age, and the adult male mice were chosen for behavioral test. The sociability tests assess the general social interactions as well as behavior in novel social situations. The social behavior was measured when

mice were allowed to choose between exploring a social situation with a stranger mouse versus an empty cage (Figs. 1c and 1d). Like the normal social behavior in rodents, placebo-treated mice showed a preference for the stranger mouse than the empty chamber (Fig. 1c,  $p < 0.05$ ). On the contrary, thimerosal-treated (20 $\times$  dose of thimerosal) mice exhibited a preference for the empty chamber than the stranger mouse ( $p < 0.05$ ). When compared with placebo-treated mice, thimerosal-treated mice spent significantly less time with the stranger mouse ( $p < 0.01$ ) and significantly more time staying in empty chamber ( $p < 0.05$ , Fig. 1c). Moreover, the number of entry to visit stranger was also significantly decreased in thimerosal-treated mice than placebo-mice ( $p < 0.05$ , Fig. 1d). Exposure of 5 $\times$  thimerosal to neonatal pups did not cause significant effects on mouse social behavior. Mice pretreated with 10 $\times$  thimerosal exhibited a certain degree of social deficiency, with some criteria of sociability showing statistically significant difference between placebo and thimerosal group (Supplementary figs. 1c–f). All these behavioral changes suggested that early neonatal thimerosal exposure caused a dose-dependent social interaction deficiency in adult mice, which is the key behavior feature of autism spectrum disorders (ASD).

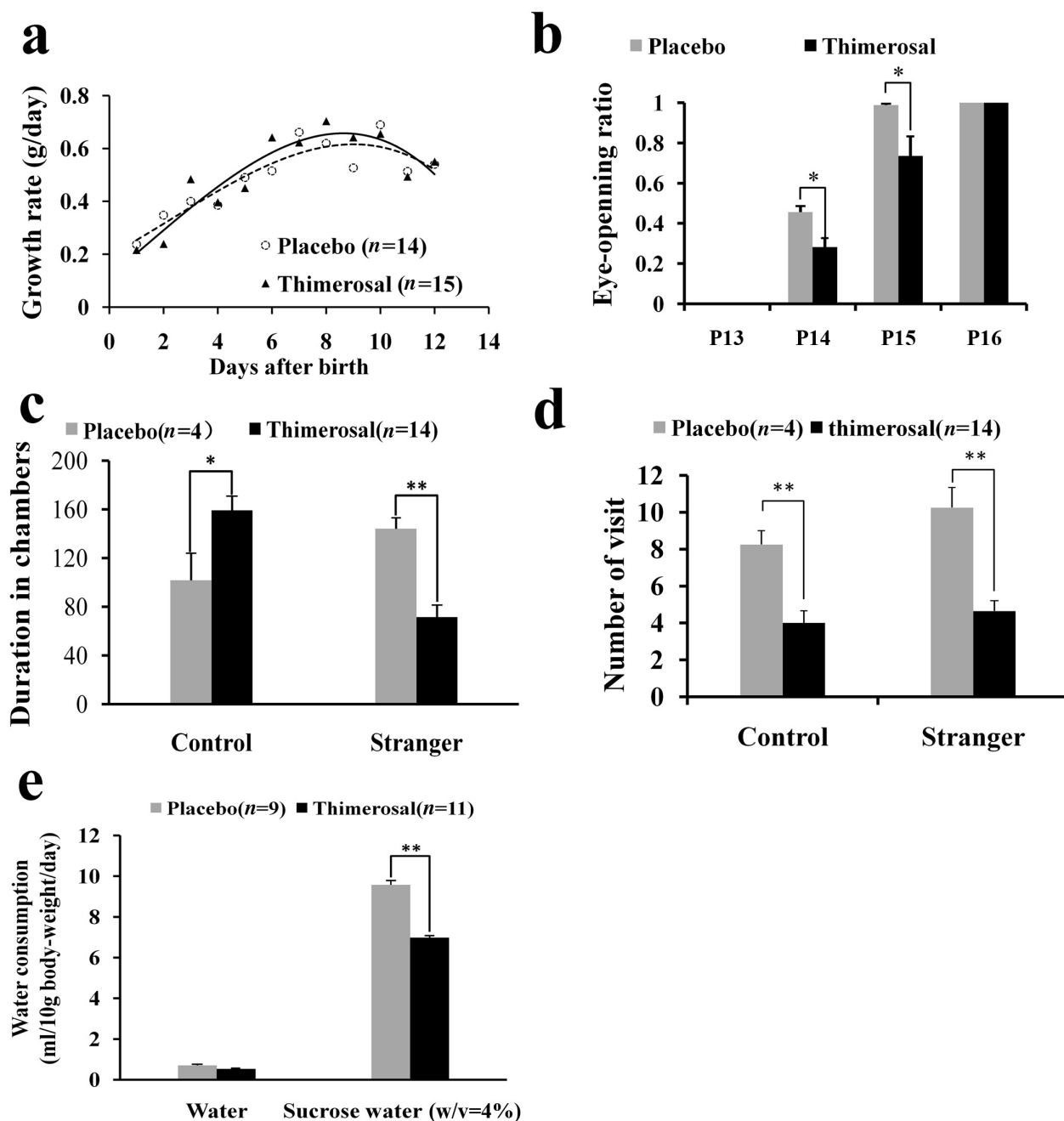
### Depression-Like Behavior of Thimerosal-Treated Mice

Sucrose preference test is frequently used as measure of anhedonia and depressive-like behavior in mice (Strekalova and Steinbusch, 2010). Normal mice establish the sucrose preference very quickly and consume much more sucrose than water. The placebo- and thimerosal-treated adult mice were housed individually and offered the choice to drink either water or 1% sucrose. The consumption of water or 1% sucrose per 10 g body weight per day was calculated. Thimerosal-treated mice drank significantly less sucrose solution than placebo-treated mice (Fig. 1E), indicating that neonatal thimerosal also caused inclination to depression in adult mice.

### Cerebrum Histopathological Assay of Thimerosal-Treated Mouse Brains

Hematoxylin and eosin (H&E) staining was used for evaluating the neuronal morphologic changes in the hippocampus, prefrontal and temporal cortex of thimerosal-treated mouse brains. Hippocampus and temporal cortex are important brain regions of temporal lobe, which are important for accurate perception and interpretation of social communication (Poirier *et al.*, 2011; Salmond *et al.*, 2005). Temporal lobe also cooperatively works together with prefrontal cortex in memory processing (Preston and Eichenbaum, 2013). The trial-unique short-term memory is necessary to guide prospective search behavior, whereas long-term potentiation (LTP) is widely believed to be one of the main neural mechanisms by which memory is stored in the brain (Guzowski *et al.*, 2000). Animal models with autistic-like behavior and autism patients show reduced LTP (Bangash *et al.*, 2011; Jung *et al.*, 2013), suggesting the possible pathological changes in hippocampus and prefrontal cortex. Prefrontal cortex as well





**FIG. 1.** Effects of early neonatal thimerosal exposure on growth, neurodevelopment, and neurological behaviors. (a) Growth of mouse pups pretreated with thimerosal and placebo. The pup body weight for each group (placebo-treated and thimerosal-treated) was recorded during the first 15 days after birth at 9:00 A.M. each day. The growth rate is depicted as body weight increase per day (g/day). The growth rate curves were regressed by a binomial. (b) Thimerosal caused eye-opening delay. Mouse pups treated with thimerosal or placebo were checked with eye-opening at P13, P14, P15, and P16 on 9:00 A.M. The number of mice with opened-eyes was recorded, respectively. The eye-opening ratio was the statistic based on three independent experiments. (c and d) Thimerosal-treated mice showed sociability deficiency. (c) Adult male mice pretreated with placebo ( $n = 4$ ) and thimerosal ( $n = 14$ ) were placed in the test room, the duration of mice staying in empty chamber and stranger chamber in 300 s were recorded, respectively. Thimerosal-treated mice spent significantly less time in the stranger mouse chamber ( $p < 0.01$ ) and significantly more time staying in empty chamber ( $p < 0.05$ ). (d) Male mice treated with placebo ( $n = 4$ ) and thimerosal ( $n = 14$ ) were placed in the test room, the total visitation frequency to empty chamber and stranger chamber in 300 s were recorded, respectively. Thimerosal-treated mice visit significantly less to the stranger mouse chamber ( $p < 0.05$ ). Data were shown as mean  $\pm$  SE. \* $p < 0.05$ ; \*\* $p < 0.01$ . (e) Sucrose water preference test of mice treated with placebo and thimerosal. Male mice treated with placebo ( $n = 9$ ) and thimerosal ( $n = 11$ ) were feeding with both water and sucrose water (w/v = 4%) for 72 h, consumption of water and sucrose water as well as animal weight was measured each day. The amount of mean ( $\pm$ SE) consumption of water and sucrose water per 10 g weight per day was calculated. Thimerosal-treated mice consumed significantly less sucrose water than placebo-treated mice. Data were shown as mean  $\pm$  SE. \*\* $p < 0.01$ .

as hippocampus pathological changes have also been reported in autism patients (Courchesne *et al.*, 2011; Gilbert *et al.*, 2008; Nicolson *et al.*, 2006; Raymond *et al.*, 1996; Rojas *et al.*, 2004; Saitoh *et al.*, 2001; Schumann *et al.*, 2004; Wallace *et al.*, 2012). Therefore, Hippocampus, temporal cortex and prefrontal cortex were evaluated for histopathological assays. We found that both the prefrontal cortex and temporal cortex of thimerosal-treated mouse brains manifested more “dark” neurons (Figs. 2a and 2b). The markedly shrunken and hyperchromatic morphology of these neurons indicate that they are undergoing necrosis or apoptosis (Kovesdi *et al.*, 2007). These dying neurons were predominantly resided in the second and third layers of the cortex (Figs. 2a and 2b). Similar morphologic changes were also observed in the dorsal hippocampus of thimerosal-treated mice (Fig. 2c). The dying neurons present in the granular layer of the dentate gyrus, CA1, and CA3 areas of thimerosal-treated mouse brains (Fig. 2c). The quantitative results showed significant increase in the numbers of the “dark” neurons in hippocampus and prefrontal cortex of the thimerosal-treated mice (Fig. 2d). It should be further noticed that 70% of thimerosal-treated mouse brains exhibited different extent of dying “dark” neuron pathology, whereas the others did not show apparent neuronal degeneration in these brain areas.

#### *Neonatal Thimerosal Causes Long-Lasting Substantially Transcriptional and Splicing Dysregulation in Adult Male Mice*

**RNA-sequencing of placebo- and thimerosal-treated mouse brain tissue.** Neonatal exposure to a higher dose of thimerosal-mercury caused autistic- and depressive-like behaviors in adult mice, suggesting the long-lasting adverse effects in mouse brains. We next sought to determine the transcriptome differences between placebo- and thimerosal-treated autistic-behaved mouse brains, using the high-throughput RNA sequencing technique.

The total number of reads produced for each brain sample ranged from 36,382,522 to 39,231,238 (Supplementary table 1). Sequence reads  $(3-4) \times 10^7$  per sample has been previously reported to deliver sufficient sequence coverage for transcriptome profiling (Aprea *et al.*, 2013). No significant difference was observed in the number of reads between placebo- and thimerosal-treated mouse brains (Student's *t*-test,  $p = 0.86$ ). 73.52–74.72% of reads were aligned to the reference genome in a unique manner (Supplementary table 1), which have met quality standards of the RNA-Seq technique.

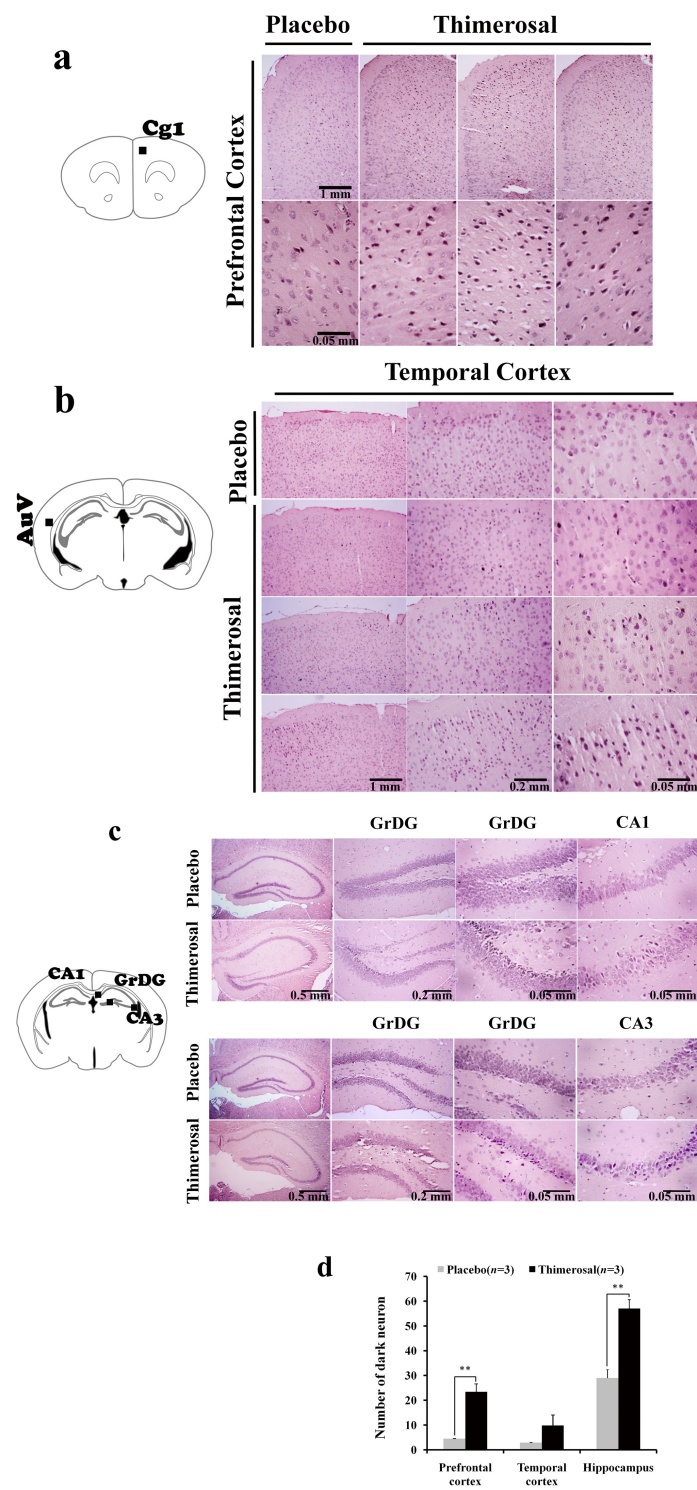
**Differentially expressed genes and isoforms.** MARs were employed to identify differentially expressed genes. Among genes expressed in thimerosal- and placebo-treated groups, 2434 genes were found to be differentially expressed in the two groups (Supplementary table 2). Compared with the placebo-treated brain samples, 26.75% (647) of the genes had increased expression, whereas 73.25% (1777) were decreased in the thimerosal-treated brain samples. Those genes were considered

as differentially expressed and potentially biologically relevant, and were then used for further biological interpretation (Supplementary table 2). The top 10 up- and down-regulated genes in autistic-behaved brain samples were presented in Figure 3a, and the expanded list of the top 30 up- and down-regulated genes were presented in the supplementary data (Supplementary tables 3 and 4).

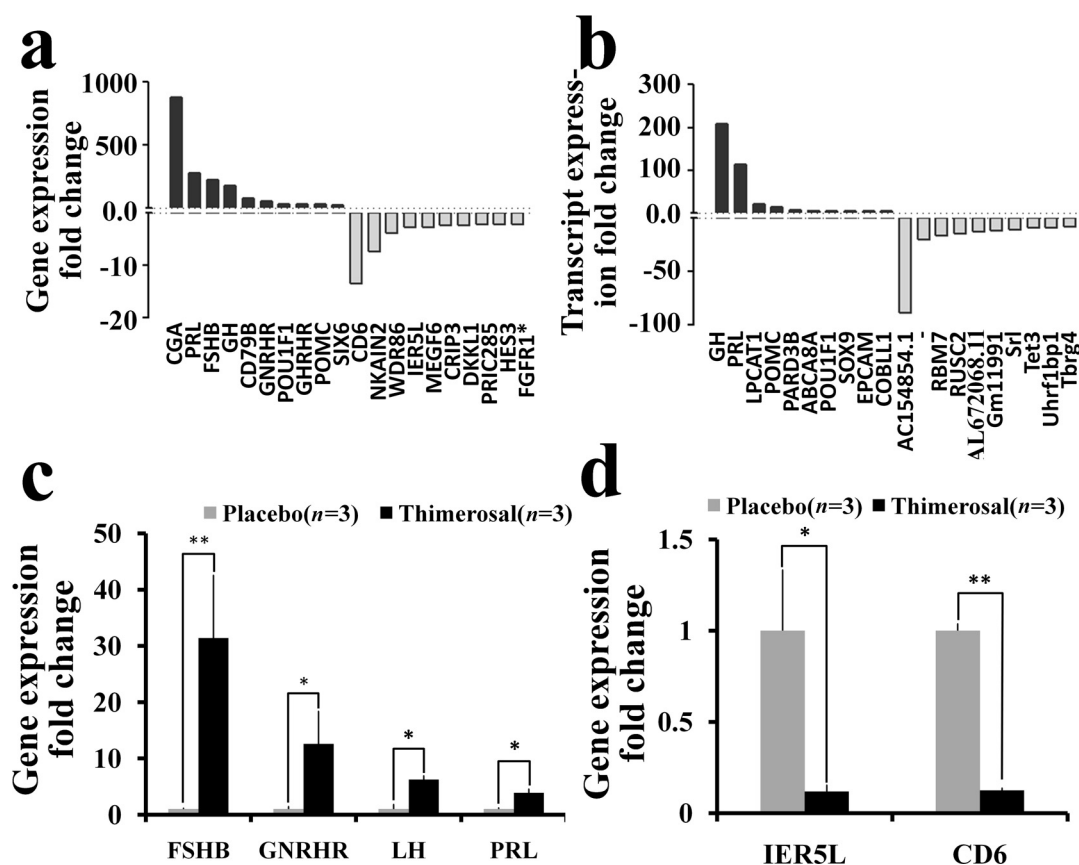
As for known isoforms, 459 known isoforms were found to be significantly differentially expressed ( $FC > 1.5$  or  $FC < -1.5$ ) (Supplementary table 2). The top 10 up- and down-regulated known isoforms were presented in Figure 3b, and the expanded list of the top 30 up- and down-regulated known isoforms were presented in the supplementary data (Supplementary tables 5 and 6). We also found novel isoforms, whose annotations were not known in the current reference gene or transcript database, were differentially expressed in the two groups.

**Validation of several important and the most differentially expressed genes by qRT-PCR.** To validate the RNA-sequencing results in this study by using an alternative approach, we performed a qRT-PCR experiment to reassess the expression level of four up-regulated and two down-regulated genes identified by RNA-sequencing. RNA sequencing data showed that the expression of *FSHB* (follicle stimulating hormone B) was up-regulated by 223.22-fold, *GNRHR* (gonadotropin-releasing hormone receptor) was up-regulated by 57.87-fold, *LH* (luteinizing hormone) was up-regulated by 178.41-fold, and *PRL* (prolactin) was up-regulated by 281.84-fold; whereas *CD6* was down-regulated by 13.55-fold, and *IER5L* (immediate early response 5-like) was down-regulated by 2.65-fold (Figs. 3a and 3c; Supplementary tables 3 and 4). In our qRT-PCR data, *FSHB*, *GNRHR*, *LH*, and *PRL* was up-regulated by 31.42-, 12.6-, 6.25-, and 3.92-fold, respectively, whereas *CD6* and *IER5L* were both down-regulated by 8.33-fold (Figs. 3b and 3d). The qRT-PCR data confirmed the coherence of the changes of the differentially genes identified by RNA-sequencing.

**Transcriptional Dysregulation of GnRH Pathway Genes and Immune Genes.** It was surprising that several gonadotropin hormone transcripts were strikingly up-regulated in thimerosal-treated adult male mouse brains. For example, neonatal exposure of thimerosal caused significant elevation of gene expression of *GNRHR*, *CGA*, *FSHB*, *LH*, and *PRL*, etc. in adult male mouse brains (Figs. 3a–c). *GNRHR* encodes gonadotropin-releasing hormone receptor that is responsible for eliciting the actions of GnRH to stimulate the production and release of FSH and LH from the pituitary. *CGA* gene encodes the alpha subunit of the four anterior pituitary secreting hormones: glycoprotein hormones chorionic gonadotropin (CG), LH, FSH, and thyroid-stimulating hormone (TSH). The specific beta subunit of these hormones confers receptor specificity and biological activity to the hormone. FSH and LH are important hormones for follicle growth/maturation and follicle luteinizing in female ovaries. *PRL* gene encodes prolactin, which is not



**FIG. 2.** Cerebrum histopathological changes in thimerosal-treated mouse brains. Placebo-treated ( $n = 3$ ) and thimerosal-treated adult male mice ( $n = 3$ , with autistic-like behavior) were selected for the histopathological examination. Hematoxylin and eosin (H&E) staining of coronal sections of cerebrum was used for inspecting the neuronal morphological changes. Ischaemic degeneration of neurons and “dark” neurons are remarkably increased in the prefrontal cortex (a), temporal cortex (b), and hippocampus (c) of thimerosal-treated mouse brains. Diagrams of coronal sections of the mouse brain and the photo area (dotted areas in diagram) were also shown in (a), (b) and (c). (a), Cg1 (Cingulate cortex, area 1) region of prefrontal cortex (bregma 1.70 mm). (b) AuV (2ary auditory cortex, ventral) region of temporal cortex (bregma  $-2.54$  mm). (c) GrDG (granular dentate gyrus), CA1 (field CA1 hippocampus), CA3 (field CA3 hippocampus) field of hippocampus (bregma  $-1.46$  to  $-1.94$  mm). (d) statistics of the “dark” neuron numbers in prefrontal cortex (cells/mm<sup>2</sup>), temporal cortex (cells/mm<sup>2</sup>), and hippocampus (“dark” neuron numbers in whole hippocampus region).



**FIG. 3.** Neonatal thimerosal caused long-lasting transcriptional dysregulation in adult mouse brains. (a and b) Top 10 up-/down-regulated genes/isoforms in thimerosal-treated mice brain. The top 10 up-/down-regulated genes (a) or isoforms (b) in thimerosal-treated male mice brain were listed. The differentially expressed genes/isoforms in thimerosal were determined by DEGseq, after Benjamini-Hochberg correction. The differentially expressed genes/isoforms were ranked on the fold change (Bultynck *et al.*, 2004). To be more intuitive, the fold change value (Bultynck *et al.*, 2004) is depicted as following: When  $fc \geq 1$ , fold change =  $fc$ ; when  $fc < 1$ , fold change =  $-1/fc$ . The full name of each gene symbol is provided in text and Supplementary tables 3 and 4, and the full name of each isoform symbol is provided in text and Supplementary tables 5 and 6. (c and d) Validation of up- and down-regulated gene expression by qRT-PCR. total RNA was extracted from the whole cerebrums of placebo- and thimerosal-treated male mice ( $n = 3$ , respectively, 12 weeks old males). The expressions of several up-regulated genes (*FSHB*, *GNRHR*, *LH*, and *PRL*) (c) and down-regulated genes (*CD6* and *IER5L*) (d) predicted by RNA-sequencing were determined by qRT-PCR which were performed in triplicate. The gene expression levels were quantified relative to the expression of mouse GAPDH gene, employing an optimized comparative Ct ( $\Delta\Delta Ct$ ) value method. The qRT-PCR data showed a good agreement with the RNA sequencing results.  $*p < 0.05$ .

only functionalized in stimulating mammary development and lactation, but also in other different biological functions: water and electrolyte balance; growth and development; endocrinology and metabolism; brain and behavior; reproduction, and immune-regulation and protection. Interestingly, up-regulations of these female reproduction-related hormones were detected in thimerosal-treated adult male mouse brain samples.

In contrast to the up-regulation of gonadotropin sex hormones, some immune genes such as *CD6* and *NKAIN2* were significantly down-regulated in thimerosal-treated adult mouse brains (Figs. 3b and 3d). *CD6* encodes a 160 kDa type 1 transmembrane glycoprotein mainly expressed on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD6 stimulation plays an important role in the maintenance of T cell activation, and its expression increased with thymocyte maturation. *NKAIN2* gene en-

codes a protein named TCBA1 (T-cell lymphoma breakpoint-associated target 1).

#### Serum Hormone Level Assays Demonstrate the Gender Bias of Thimerosal-Mercury Toxicity

Our RNA-sequencing and qRT-PCR results showed that neonatal thimerosal exposure caused significant up-regulation of pituitary hormones in adult male mouse brains. To gain a further insight into the thimerosal toxicity on endocrine system, we assayed the actual serum hormone levels by radioimmunoassay, and compared the fold change (Bultynck *et al.*, 2004) between thimerosal-treated and placebo-control group. The serum hormone assay data revealed the significant elevation of PRL ( $\log_2(FC_{PRL}) = 0.41$ ,  $p < 0.05$ ), FSH ( $\log_2(FC_{FSH}) = 0.14$ ,  $p < 0.01$ ), LH ( $\log_2(FC_{LH}) = 0.15$ ,  $p < 0.05$ ), and ACTH (adrenocorticotrophic hormone,  $\log_2(FC_{ACTH}) = 0.19$ ,  $p$



$< 0.05$ ) in thimerosal-treated male mice ( $n = 3$ ) (Fig. 4a). While in female serums of thimerosal-treated mice, only TSH was found to be significantly elevated when compared with placebo-treated group ( $n = 3$ ,  $\log_2(\text{FC}_{\text{TSH}}) = 0.41$ ,  $p < 0.05$ ) (Fig. 4b). It is interesting that pituitary hormones which are closely related to female reproductive endocrine function (such as FSH, LH; PRL) and stress-responsive hormone adrenocorticotropin (ACTH) went up in serums of thimerosal-treated male mice but not in that of thimerosal-treated female mice, indicating the gender bias of thimerosal-mercury toxicity.

#### *Implications of Aberrant Transcription on the Development of Autistic-Like Behavior in Thimerosal-Treated Mice*

We hypothesized that the genes that were differentially expressed between the thimerosal- and placebo-treated mice would represent important biological differences and could thus associate with different functional categories, which could be the causal involvements for the development of autistic-like behavior in thimerosal-treated mice. We used IPA to determine the functional annotation of the differentially expressed genes. Functional networks affected by up- and down-regulated genes/isoforms in thimerosal-treated brains were listed in Supplementary table 8. Notably, the networks involving nervous system development and function, autoimmune disease and metabolism, and endocrine system development and function were on the top 10 list of networks of differential expression enrichment (Supplementary figs. 5 and 6; table 8). Although the dysfunction of immune system has been frequently associated with ASD in human (Sweeten *et al.*, 2003; Torres *et al.*, 2002; Voineagu *et al.*, 2011), dysregulation of endocrine system has not been reported.

Canonical pathways analysis identified the IPA canonical pathways for which the differentially expressed genes were most significantly enriched. Top canonical pathways affected by up- and down-regulated genes/isoforms in thimerosal-treated brain were shown on Figure 5A. In concordance with the network observations, the most affected pathways in thimerosal-treated brain was axonal guidance signaling (Fig 5a, Supplementary fig. 3 and table 7), a subfield of neural development, in which there were 96 differential genes enriched. Pathways related to the synaptic functions such as synaptic LTP and CREB signaling in neurons were also among the top affected pathways (Figs. 5a and 5b and Supplementary table 7). Differentially expressed genes also highly enriched for dopamine-DARPP32 signaling pathway (Supplementary fig. 4), which is very important for the primary neural rewarding mechanism (Arias-Carrion and Poppel, 2007). Dysregulation of dopamine-DARPP32 signaling pathway could be a cause for depression-like behavior in thimerosal-treated mice. These thimerosal-affected canonical pathways showed high overlaps with the gene ontology categories previously identified as part of the ASD human brain transcriptional analysis: synaptic function (LTP, CREB, dopamine-DARPP32, and GABA receptor

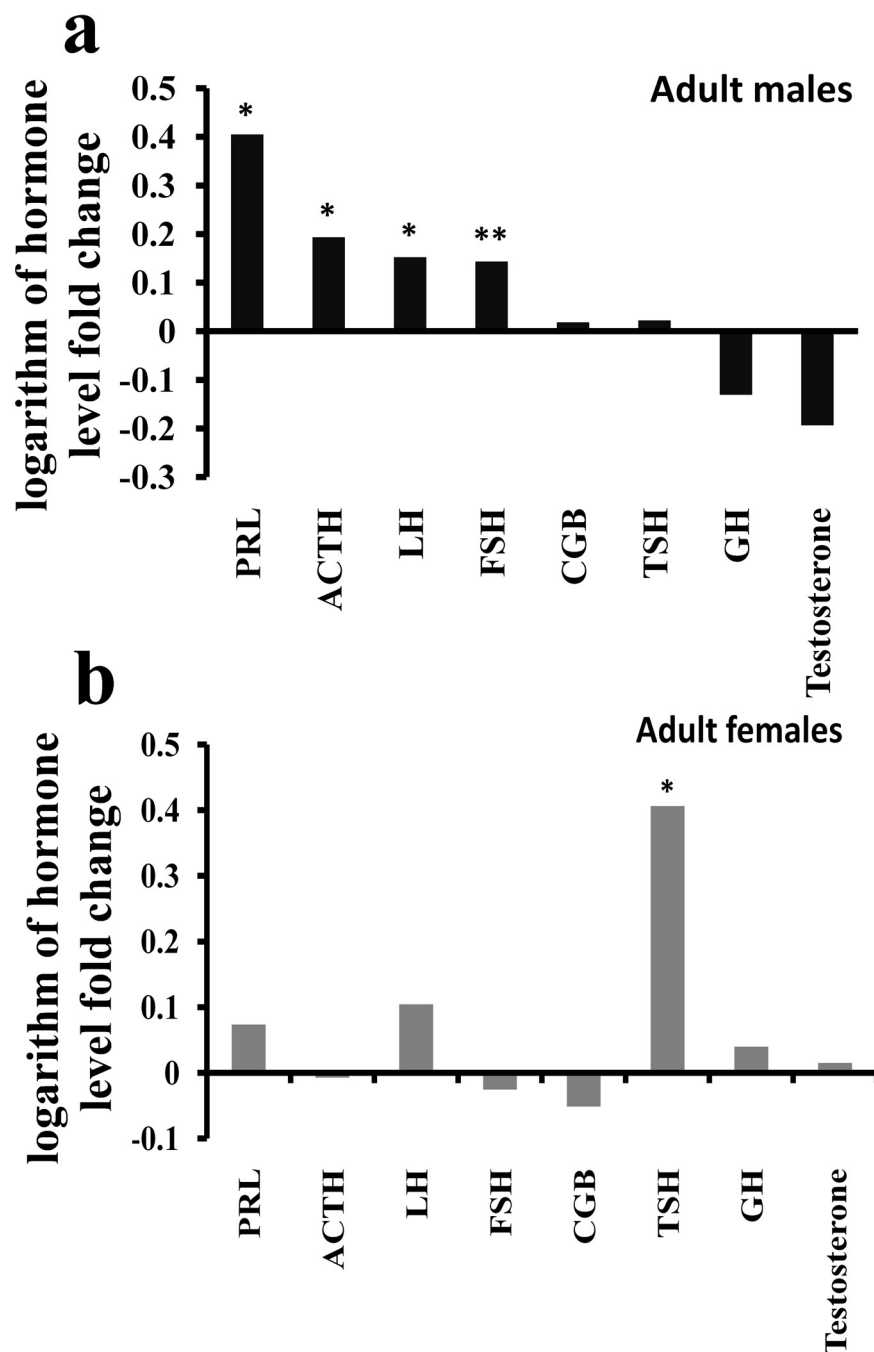
signaling pathways), and neuronal projection (axonal guidance signaling pathway).

The GnRH (gonadotropin-releasing hormone) signaling was on the top 3 in the canonical pathways list (Figs. 5A and 5C and Supplementary table 7). GnRH activates GnRH receptors that reside primarily in the pituitary and stimulates the production and release of pituitary hormones such as FSH and LH. Several important players of GnRH signaling pathway such as GnRHR, CGA, FSHB, and LH, etc. were found significantly up-regulated in thimerosal-treated male mouse brains, and the significant elevation of some female reproduction-related sex hormones (PRL, FSH, and LH) occurred exclusively in male but not in female thimerosal-treated mice, demonstrating the gender bias of thimerosal-mercury toxicity. Therefore, early neonatal exposure of thimerosal caused long lasting dysregulation of sexual endocrine system (hypothalamus-pituitary-gonadal (HPG) axis) in adult male mouse which may account for the behavioral change and the vulnerability of males to thimerosal-mercury toxicity.

## DISCUSSION

ASD, a group of heritable neurodevelopmental disorders, is characterized by deficits in social interaction and communication, impairments in language and repetitive, stereotyped behavior and interests. The ASD prevalence in California has risen by 600% from 1990 through 2006 which cannot be sufficiently explained by changes in diagnostic criteria, awareness, and inclusion of milder cases (Hertz-Picciotto and Delwiche, 2009). A study of ASD concordance in monozygotic versus dizygotic twins revealed that the susceptibility to ASD has moderate genetic heritability and a substantial environmental component (Hallmayer *et al.*, 2011), suggesting the important causal involvements of environmental factors in ASD pathogenesis. One such an environmental factor may be increased mercury burden via industrial sources and vaccine preservative thimerosal (American Academy of Pediatrics and United States Public Health Service, 1999; CDC (Centers for Disease Control and Prevention, 1999). Concerns about the use of thimerosal in infant vaccines stem primarily from the traits and neurological abnormalities of ASD are analogous to that of mercury poisoning (Bernard *et al.*, 2001).

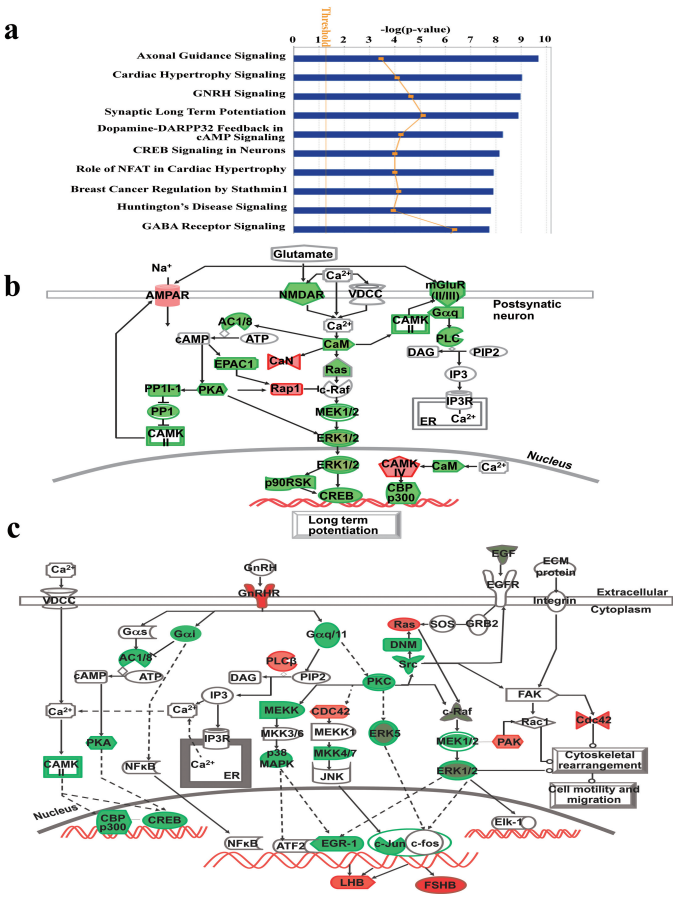
Animal models have been used to evaluate the potential neurotoxicity of vaccine thimerosal by mimicking the U.S. child inoculation schedule at 2, 4, 6, and 12 months of age at different doses. Thimerosal has been shown to cause neurological deficits in autoimmune propensity mice (Hornig *et al.*, 2004), hamsters (Laurent *et al.*, 2007), infant monkeys (Hewitson *et al.*, 2010), and rat (Olczak *et al.*, 2009, 2010, 2011), although no neurotoxic effect of thimerosal in autoimmune diathesis mice was also reported (Berman *et al.*, 2008). Immunization schedules vary depending on a country's health policy, and the thimerosal containing vaccines inoculate as early as the



**FIG. 4.** Neonatal thimerosal-induced changes of pituitary hormone levels in mouse brains. Radioimmunoassay was carried out to detect the serum hormone levels of PRL, ACTH, LH, FSH, CGB, TSH, GH, and testosterone. The *t*-test was used to analyze the difference of each hormone level between the thimerosal-treated samples and the placebo-treated samples. *p*-values (*t*-test) < 0.05 are considered significant difference. The fold change (Bultynck *et al.*, 2004) for each hormone: FC = thimerosal mean/placebo mean. In order to be more intuitive, we plotted the hormone level changes as the logarithm of FC. (a)  $\log_2$  (Bultynck *et al.*, 2004) for each hormone in 3 weeks old male mice (*n* = 3). (b)  $\log_2$  (Bultynck *et al.*, 2004) for each hormone in 3 weeks old female mice (*n* = 3). \**p* < 0.05; \*\**p* < 0.01.

first 12–24 h after birth in some developing countries such as Brazil and China. In this study, we modeled the possible neurotoxicity of this 1-day-old immunization schedule by injecting 20× thimerosal on postnatal day 1, 3, 5, and 9 (accumulative

EtHg 914 µg/kg). We found that the early neonatal thimerosal exposure caused neurodevelopment delay, deficits in sociability and depression-like behavior. Impairment in social interaction is the key feature of ASD. Consistent with previous report



**FIG. 5.** Pathway analyses of the neonatally-thimerosal-dysregulated genes in adult male mouse brains. The pathway data represent the logarithm of *p*-values calculated by Fisher's exact test, with a threshold for statistical significance, *p* < 0.05. Canonical pathways with *p* < 0.05 were defined as significant. Genes involved in each pathway were provided in Supplementary table 7. (a) Top 10 canonical pathways affected by up-/down-regulated genes/isoforms in thimerosal-treated male mice brain samples. The pathway analysis of genes that affected by thimerosal was identified by Ingenuity Pathway Analysis, and the expanded list of the top 10 canonical pathways were presented in the supplementary data (Supplementary table 7). (b and c) Synaptic long-term potentiation pathway and GNRH signaling pathway. Dysregulated genes in neonatally thimerosal-treated male mice brains are enriched in synaptic long-term potentiation pathway (b) and GNRH signaling pathway (c). Color indicates the strength of fold change of the indicated gene expression. Red represents the gene expression increase in thimerosal-treated mouse brains; Green represents gene expression decrease in thimerosal-treated mice brains. Each gene involved in the signaling pathways is listed in Supplementary table 7.

of thimerosal-induced neuropathology in developing rat brains (Olczak *et al.*, 2010), our neuro-histology results showed that ~70% thimerosal-treated mouse brains manifested more dying “dark” neurons in prefrontal cortex, temporal cortex, and hippocampus. Increased propensity to cell death (increases in proapoptotic and decreases in antiapoptotic molecules) has also been observed in frontal cortex in adult human autistic cases (Araghi-Niknam and Fatemi, 2003), suggesting that the neurodegeneration induced by thimerosal exposure could be one

of the mechanisms for behavior abnormalities. We should point out here that 20× higher dosage of thimerosal than that used in humans does not represent the real vaccination situation in humans. Further studies are needed to evaluate the potential toxicity of thimerosal with similar dosage as human conditions.

High-throughput RNA sequencing allowed us to obtain a comprehensive transcriptomic comparison between normal and thimerosal-treated autistic-like mouse brains. Alternations of a number of important functional categories/pathways affected by neonatally thimerosal exposure are identified in this study. Pathways involving neuronal development (axonal guidance signaling) and neuronal synaptic function (synaptic long term potentiation, glutamate receptor, and CREB signaling, dopamine-DARPP32 feedback signaling, etc.) are mostly affected by neonatal thimerosal administration. Axonal guidance signaling is the process by which neurons accurately send out axons to reach the correct targets. It has recently been proposed that ASD results from a developmental disconnection of brain regions that are involved in higher order associations (Geschwind and Levitt, 2007). A large-scale genomic studies implicate numerous gene candidates of autism are known or suspected to mediate neuritic outgrowth and axonal guidance in fetal and perinatal life (reviewed by McFadden and Minshew, 2013). Our RNA-sequencing and IPA analysis reveal that axon guidance is the most affected pathway, suggesting that dysregulation of neurodevelopment may account for the behavior abnormalities in thimerosal-treated mice. However, we need to point out that the RNA sequencing results, behavior, and neuropathology results are only applicable to male mice. The transcriptional and pathological effects of thimerosal on neonatal females require more studies.

Long-term synaptic potentiation (LTP) is a key mechanism in learning and memory, and its alteration is associated with mental disorders like fragile X syndrome. Loss key proteins in LTP, like NMDAR, AMPAR, mGluR, and CaMK2 could induce autistic-like behavior in animal models. LTP appears as the top 4 thimerosal-affected functional pathway (Fig. 5b, Supplementary table 7).

One of the most striking functional dysregulations is endocrine system. To our knowledge, this is the first report that thimerosal mercury affects endocrine system. Our RNA sequencing and qRT-PCR data revealed that both HPG axis and hypothalamic-pituitary-adrenal (HPA) axis are affected by neonatally thimerosal administration. GnRH pathway controls a complex process of follicular growth, ovulation, and corpus luteum maintenance in the female, and spermatogenesis in the male. GnRH is also a potent stimulator of PRL secretion. Though some reports suggested the elevation of FSH, LH, and PRL levels in male epileptic patients (Gerra *et al.*, 2000; Rodin *et al.*, 1984; Tripodianakis *et al.*, 2007), how this up-regulation of HPG axis related to neurological deficits in thimerosal-treated mice is not clear. The relationship between HPG axis over-activation and thimerosal-induced neurological deficits in mice requires further studies.

HPA axis, the stress-responsive endocrine system, is also affected by neonatal thimerosal exposure. ACTH is an important component of the HPA axis and is often produced in response to biological stress (along with its precursor corticotrophin-releasing hormone from the hypothalamus). Its main function is increased production and release of adrenal corticosteroids. Serum levels of ACTH and cortisol in subjects with autism were found significantly higher than those in healthy controls (Iwata *et al.*, 2011). HPA axis is influenced by the limbic system (Dallman *et al.*, 2004), which is the neural basis of emotions and social interactions, and is involved in the neuropathology of autism (Sweeten *et al.*, 2002). The abnormal levels of ACTH and cortisol may be due to alterations in limbic system function. Therefore, significant elevation of ACTH in thimerosal-treated mouse brains may represent the functional alternations in limbic system which affects emotions and social interactions. The neurotoxic effects of neonatal thimerosal on limbic system remain to be determined.

A very interesting aspect of thimerosal's toxicity to endocrine system is the sexual dimorphism. The elevation of anterior pituitary secreting hormones (gonadotropin-FSH, LH; PRL; adrenocorticotropin-ACTH) occurred exclusively in male but not in female thimerosal-treated mice, demonstrating the gender bias of thimerosal-mercury toxicity. From the first published description of autism, it has been a male-biased neurological disorder. Prevalence surveys have reported a range of male biases from 1.33:1 (male:female, M:F) to 15.7:1 (Black *et al.*, 2002), and a commonly referenced consensus ratio is 4:1 (Chakrabarti and Fombonne, 2001). The mechanism underlying this male-biased neurological disorder is not clear. Though the SNPs within RYR2, UPP2 and microdeletion of SHANK1 suggest a sex-differential ASD risk (Sato *et al.*, 2012), they cannot fully explain the male bias in ASD. Sex chromosomal genes have been proposed to be key player in molecular mechanisms driving females' protection from ASD, however, except fragile X syndrome, ASD transmission in most families does not follow an X-linked pattern (Werling and Geschwind, 2013). Thus, genetic mutation could partially rather than fully explain the sex bias in ASD. Our discovery of thimerosal-induced male-specific elevation in anterior pituitary secreting hormones provides an explanation how environmental factors may relate to the gender bias of ASD.

Complex human disorders such as ASDs involve a mixture of genetic and environmental risk factors. In this study, early neonatal exposure of thimerosal produced two of the hallmark characteristics of autism—sociability deficits and a male-oriented bias, suggesting that thimerosal mercury exposure is potentially a causal environmental risk factor for ASD. Moreover, our study provides the first comprehensive insight into the transcriptome of thimerosal-treated autistic-behaved brain tissues in animal model. Using a whole transcriptome RNA sequencing technique, we were able to identify the differentially expressed genes, and to reveal functional categories and pathways affected by thimerosal exposure which may relate to the

neurological deficits. To our knowledge, this is the first report to systematically elucidate pathways possibly involved in thimerosal-induced autistic-like behavior. Besides the alternations of neuronal development and synaptic function pathways, up-regulation of pituitary secreting hormones (gonadotropin-FSH, LH; PRL; adrenocorticotropin-ACTH) reveals a possible mechanistic link between thimerosal-induced autistic like behavior and gender bias. How thimerosal exposure causes long-lasting transcriptional/splicing dysregulation and potentially leads to neurological deficits require further study?

## SUPPLEMENTARY DATA

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

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