Administration of thimerosal-containing vaccines to infant rhesus macaques does not result in autism-like behavior or neuropathology

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Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder. Some anecdotal reports suggest that ASD is related to exposure to ethyl mercury, in the form of the vaccine preservative, thimerosal, and/or receiving the measles, mumps, rubella (MMR) vaccine. Using infant rhesus macaques receiving thimerosal-containing vaccines (TCVs) following the recommended pediatric vaccine schedules from the 1990s and 2008, we examined behavior, and neuropathology in three brain regions found to exhibit neuropathology in postmortem ASD brains. No neuronal cellular or protein changes in the cerebellum, hippocampus, or amygdala were observed in animals following the 1990s or 2008 vaccine schedules. Analysis of social behavior in juvenile animals indicated that there were no significant differences in negative behaviors between animals in the control and experimental groups. These data indicate that administration of TCVs and/or the MMR vaccine to rhesus macaques does not result in neuropathological abnormalities, or aberrant behaviors, like those observed in ASD.

pediatric vaccines | autism | rhesus macaque | thimerosal | neuropathology

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder presenting in early childhood with a current prevalence ranging from 0.7% to 2.64% in the United States (1). ASD is defined by the presence of marked social deficits, specific language abnormalities, and stereotyped repetitive patterns of behavior (2). Genetic and environmental factors have been found to play a role in the disorder (3, 4). The neuropathology of autism is now beginning to be understood; however, there is still much to be learned. Thus far, the major neuropathological changes observed in autism are changes in neuronal size in the limbic system; decreased numbers of Purkinje cells in the cerebellum; abnormalities in the brainstem, neocortex, amygdala, and hippocampus; features of cortical dysgenesis or migration disturbances; and alterations in GABAergic and cholinergic systems [see Gadad et al. (3) and Amaral (5) for reviews]. In many autism studies, comorbid conditions such as seizure disorders or intellectual disabilities contribute to the heterogeneity of the neuropathology.

An association between exposure to thimerosal-containing vaccines (TCVs) and developmental abnormalities has been debated since 1999 when the US Food and Drug Administration determined that children receiving multiple TCVs at a young age were at risk for exceeding the Environmental Protection Agency's safe exposure limits for methylmercury (MeHg). Results from an Institute of Medicine (IOM) review on the safety of childhood vaccines found that there was not sufficient evidence to render an opinion on the relationship between exposure to TCVs or the measles, mumps, rubella (MMR) vaccine and developmental disorders in children (IOM 2001) (6). The IOM review did, however, note the possibility of such a relationship and recommended further studies be conducted. A more recent second review of TCVs and autism (IOM 2004) (7) came to the same conclusion reached earlier: that there was no epidemiological data to support a relationship between TCVs and childhood developmental disorders. Several epidemiological studies sought to determine whether TCVs resulted in neurodevelopmental disorders including autism; however, both nonsignificant and significant associations have been reported (8–12). Significant associations have been reported by Thompson et al. (11), who investigated the association between TCVs and immune globulins early in life and neuropsychological outcomes in children at 7–10 y of age. The data included the evaluation of 1,047 children and their biological mothers and 24 neuropsychological tests. The only variable that was statistically significant was tics; children who were exposed to higher doses of thimerosal were more likely to exhibit tics. In a follow-up study by Barile et al. (12) examining a subset of the data from Thompson et al. (11), they found a significant association between thimerosal dosage and tics, but only in boys. They found no statistically significant associations between thimerosal exposure from vaccines early in life and six of the seven neuropsychological constructs examined.

Concern regarding the safety of childhood vaccines has had a major impact on immunization rates (13–16). It is of great importance to determine whether TCVs play a significant role in altering brain development and/or behaviors that mimic changes observed in autism. The present study provides a comprehensive

Significance

Autism is a childhood neurodevelopmental disorder affecting approximately 1 in 70 children in the United States. Some parents believe that thimerosal-containing vaccines and/or the measles, mumps, rubella (MMR) vaccine are involved in the etiology of autism. Here we gave nonhuman primate infants similar vaccines given to human infants to determine whether the animals exhibited behavioral and/or neuropathological changes characteristic of autism. No behavioral changes were observed in the vaccinated animals, nor were there neuropathological changes in the cerebellum, hippocampus, or amygdala. This study does not support the hypothesis that thimerosal-containing vaccines and/or the MMR vaccine play a role in the etiology of autism.

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Table 1. Vaccination schedules used for the six groups of animals

Group	*N*	Vaccines administered
Control	16	None, all saline placebos
1990s Pediatric	12	Vaccine regimen as recommended in the 1990s
1990s Primate	12	Vaccine regimen as recommended in the 1990s accelerated fourfold
TCV's	12	All TCVs and saline placebo for MMR
MMR	15	MMR only, all others replaced with saline placebo
2008	12	Vaccine regimen recommended in 2008

analysis of the influence of TCVs on the brain and behavior in a nonhuman primate model. The study includes 79 rhesus macaques in six groups (n = 12–16 per group): (*i*) Control, a control group given saline injections; (*ii*) 1990s Pediatric, replicating the pediatric vaccination schedule used for infants in the 1990s that included several TCVs; (*iii*) 1990s Primate, replicating the pediatric vaccination schedule used in the 1990s but accelerated fourfold representing the faster development of infant macaques; (*iv*) TCVs, only TCVs and no MMR; (*v*) MMR, only the MMR vaccine; and (*vi*) 2008, the expanded pediatric schedule used in 2008 (and very similar to that used today, which also includes a prenatal influenza vaccine; Table 1). For neuropathology, only animals in the 1990s and 2008 vaccine groups were studied because the 1990s schedule had the highest thimerosal exposure, and the 2008 schedule had the greatest number of different vaccines and is very similar to the vaccine schedule currently recommended for US infants. Analyses of early learning and cognition, from birth to 12 mo of age, in the same animals used in this study was recently reported by Curtis et al. (17).

Results

Social Behavior. Overall means and SDs for duration and frequency of social and nonsocial behaviors scored for all animals are shown in Table 2. A description of the specific behaviors measured in this study is given in Table S1. The duration and frequency of negative behaviors (e.g., Stereotypy, Rock-huddle-self-clasp, Fear-disturbed, and Withdrawal) by animals in all groups across the entire study period was very low. Behaviors that had either a significant time main effect or a time × group interaction are shown in Fig. 1 (Social: Positive Behaviors; Nonsocial: Passive Behavior; and Nonsocial: Positive Behavior). The Nonsocial Explore behavior was the most frequent of the nine behaviors measured and presented the only instance of a significant effect involving group: there was a significant time × group interaction [$F(5, 393) = 4.17$, $P = 0.004$]. Follow-up contrasts indicated that the Control animals exhibited significantly more Nonsocial Explore behavior at the beginning of social living compared with the 1990s Primate [$t(393) = 3.61$, $P < 0.001$], the 1990s Pediatric [$t(393) = -7.46$, $P < .001$], the MMR [$t(393) = -2.72$, $P = 0.011$], and the TCV [$t(393) = -2.48$, $P = 0.017$] groups (Fig. 1). However, there were no significant differences in any behavior measured between the control and experimental groups after 6 mo of social living (at ∼18 mo of age).

Brain. The neuroanatomical analyses were first performed in brains from the 1990s Primate and 2008 groups, as animals in these groups received the highest amount of EtHg exposure (1990s Primate) or the most extensive vaccine exposure (2008). Because no neuronal differences were found in either of these vaccine groups compared with the control group, no additional vaccine groups were fully studied.

Cerebellum. Abnormalities in the cerebellum have been reported in postmortem ASD brains (18, 19). Both histological and neurochemical analyses were performed on the cerebellar tissues in the present study.

Cerebellar volume and Purkinje cell number. Stereological methods were used to estimate the total number of Purkinje cells (Fig. 2) in one hemisphere. There were an average of ∼800,000 cells in one hemisphere, with a density of 270 cell/mm^3 and an overall volume of ∼3,000 mm^3. No difference in cell number, density, or cerebellar hemisphere volume was observed in the 1990s Primate and 2008 groups compared with the Control group. We also examined Purkinje cell number in some of the animals in the TCV and MMR groups, and they were similar to that of the Control group (Table S2).

Purkinje cell size. Cell size (area) was measured in both Nissl-stained sections and in calbindin-immunostained sections. The calbindin-containing Purkinje cells were markedly larger than the calbindin-negative/Nissl-positive cells [Control mean ± SD (μm^2) = 488.5 ± 7.9 and 273.1 ± 7.7; n = 8], but there was no difference in cell size between the Control and the 1990s Primate group for either calbindin-positive cells or Nissl-positive cells, respectively (Table S3).

Cerebellar proteins. Western blots were run to measure the levels of Purkinje cell-related proteins—calbindin and GAD-67—and glial proteins—Iba1 (microglial marker) and GFAP (astrocyte marker) (Fig. 3). There were no differences in the protein levels in the 1990s Primate or 2008 groups compared with the Control group (n = 8/group). Because different regions of the cerebellum were used for the protein assays, it was important to ensure that the results reflect "whole cerebellum differences." Therefore, we measured levels of the four proteins in five different cerebellar regions and found that all of the regions had similar levels of these proteins (Fig. S1).

Table 2. Duration and frequency (mean ± SD) of social and nonsocial behaviors scored for all 79 animals

	Social		Nonsocial	
Behavior	Duration (SD)*	Frequency (SD)	Duration (SD)*	Frequency (SD)
Passive	7.77 (6.95)	0.46 (0.25)	1.67 (2.85)	0.02 (0.03)
Explore	3.51 (3.30)	0.45 (0.19)	164.89 (15.18)	17.29 (2.74)
Play	14.95 (5.37)	3.67 (1.16)	4.01 (2.02)	2.56 (0.97)
Sex	0.95 (1.00)	0.16 (0.14)	0.00 (0.00)	0.00 (0.00)
Aggression	0.03 (0.07)	0.01 (0.02)	0.02 (0.12)	0.00 (0.01)
Withdrawal	0.04 (0.14)	0.01 (0.03)	0.00 (0.00)	0.00 (0.00)
Fear-disturbed	0.29 (0.53)	0.06 (0.09)	0.34 (0.72)	0.06 (0.11)
Rock-huddle-self-clasp	0.02 (0.15)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)
Stereotypy	0.00 (0.00)	0.00 (0.00)	0.27 (0.72)	0.03 (0.07)

Scoring was collected during 5-min focal periods collected 5 d/wk from ∼12 to 18 mo of age. Additional behaviors scored but not included in the analyses included eating, drinking, scratching, and self-grooming.
*Duration reported in seconds. Frequency reported as number of events per session.

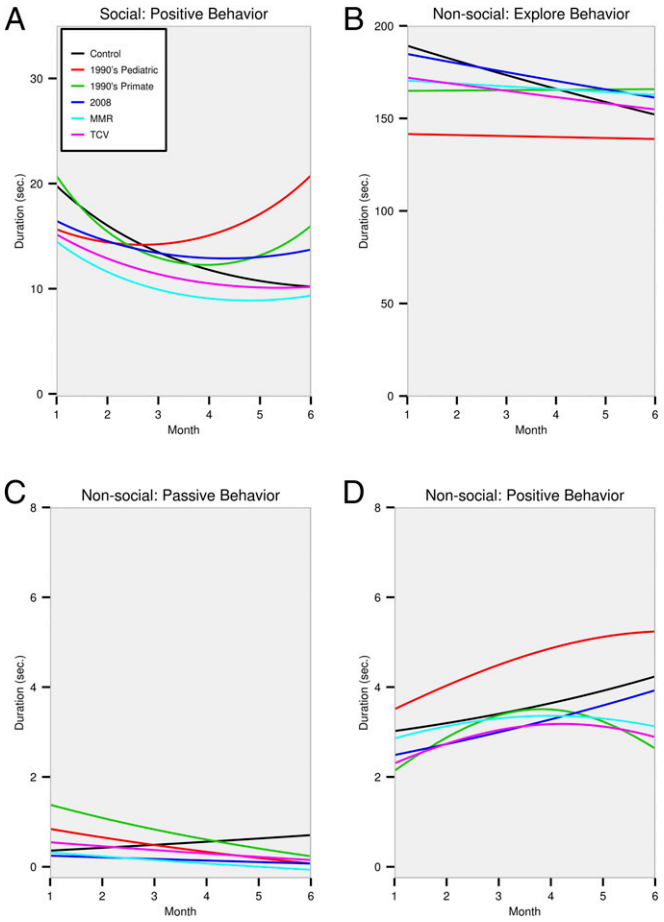

Fig. 1. Analysis of behavioral data. Fitted values from analytical models of social and nonsocial behavior for groups from age 12 to 18 mo, back-transformed with antilog. Durations of positive behaviors (play, sex, and aggression) were summed for each animal. Only behaviors that showed either a significant time main effect or a time × group interaction are shown: (*A*) Social: Positive Behavior, (*B*) Nonsocial: Explore Behavior, (*C*) Nonsocial: Passive Behavior, and (*D*) Nonsocial: Positive Behavior. Nonsocial: Explore Behavior demonstrated the only significant time × group effect, and this was only significant at the beginning of social living. Duration of behaviors is shown in seconds.

Hippocampus. The CA1 neurons in the hippocampus have been reported to be reduced in size in postmortem brains from children with autism (18).

CA1 cell size. Cell size (area) was measured in Nissl-stained sections at a rostral (section 100), middle (section 200), and a caudal (section 300) level of the CA1 region (Fig. 4). Approximately 250–450 cells were measured per animal, each with a clear nucleolus at the three levels of the nucleus. There was no significant reduction in cell area for the 1990s Primate group vs. Control group or for the 2008 group vs. Control group.

Neurogenesis. We sought to determine whether the birth of new dentate granule neurons was altered by the 1990s Primate vaccination schedule. Using doublecortin immunostaining, we counted the number of these neurons in five rostral-caudal sections/brain in the Controls and animals from the 1990s Primate group. There was no difference in the total number of cells per brain between the two groups (mean ± SEM for control brains: 4,180 ± 308 neurons, and 3,983 ± 368 neurons in the 1990s Primate group; $n = 5$/group; Fig. S2).

Dentate gyrus area. We examined the area of the granule cell layer of the dentate gyrus in the Control, 1990s Primate, and 2008 groups at eight rostral-caudal levels through the structure (Fig. 5). A two-way ANOVA was run to compare area of the dentate gyrus in these three groups across the rostral-caudal extent of the nucleus. There was no significant difference in area among the three groups ($P = 0.7565$); however, as expected, there was a significant effect for rostral-caudal level ($P < 0.0001$).

Amygdala. Abnormalities have been reported for the amygdala in ASD subjects (20). We measured the volume of the entire amygdala and of the lateral nucleus of the amygdala and the cell size and cell number for the lateral nucleus (Fig. 6). The volume of the amygdala was not significantly different in animals receiving either the 1990s Primate ($n = 12$) or 2008 ($n = 8$) vaccination schedules compared with the Controls ($n = 16$). In these same animals, we measured the volume and number of neurons in the lateral nucleus of the amygdala, and there was no difference among the three groups. Finally, the cell size in the lateral nucleus was not changed by either the 1990s Primate or 2008 vaccination schedules.

Discussion

The association between exposure to TCVs and developmental outcomes has been debated since 1999 when the US Food and Drug Administration determined that children who received multiple TCVs at a young age were at risk for exceeding the Environmental Protection Agency's safe exposure limits for MeHg. However, the safety limits for EtHg, found in TCVs, has not been extensively tested for its relationship with childhood developmental disorders.

In postmortem brains of subjects with ASD, reductions in the number of cerebellar Purkinje cells (18, 19) and amygdala lateral nucleus cells (20), and reductions in the cell size of CA1 hippocampal cell (18) have been reported. In the present study, infant male rhesus macaques received TCVs following the pediatric schedule from the 1990s (e.g., hepatitis B vaccine, diphtheria, tetanus, acellular pertussis vaccine, *Haemophilus* influenza B vaccine, measles, mumps, rubella vaccine) and the expanded 2008 schedule, and were euthanized at ∼18 mo of age. We examined cerebellar, amygdalar, and hippocampus neurons ($n = 8$–16/group), as these brain regions have been reported to be abnormal in postmortem brains from subjects with autism. There were no significant differences in Purkinje cell number or cell size, cerebellar volume, CA1 cell size, dentate gyrus volume, hippocampal neurogenesis, or lateral nucleus of the amygdala volume/cell number in animals in the 1990s Primate or 2008 groups compared with control animals.

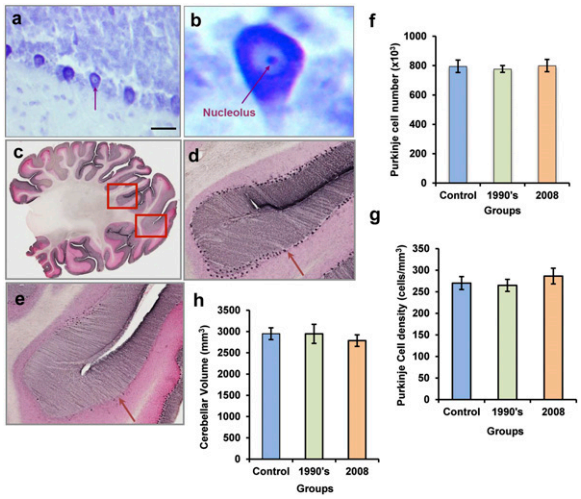

Fig. 2. Cerebellar Purkinje cells. Purkinje cells are illustrated in sections stained with Cresyl violet (*A* and *B*) and calbindin-D28k/neutral red (*C*–*E*). *C* illustrates two regions, shown at higher power in *D* and *E*, illustrating that not all Purkinje cells stain positive for calbindin (*D* vs. *E*). There was no difference in the Purkinje cell number, cell density, or cerebellar hemisphere volume among the Control, 1990s Primate and 2008 groups (*F*–*H*). Sample size: $n = 16$ for Control; $n = 12$ for 1990s Primate; $n = 8$ for 2008. [Scale bars, (*A*) 50 μm; (*B*) 10 μm; (*C*) 2.2 mm; (*D*) 200 μm; and (*E*) 200 μm.]

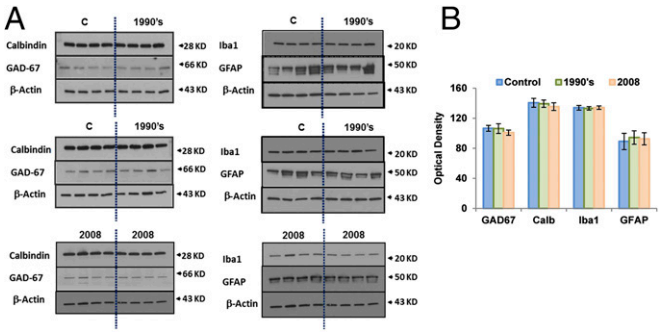

Fig. 3. Western blots of cerebellar proteins. (*A*) No differences were found in protein amounts for control, 1990s Primate, and 2008 groups. (*B*) Quantification of optical density values. Sample size: $n = 8$ for each of the three groups.

Our data do not support a role for TCVs in the neuropathology of ASD. A similar study examining the effects of TCVs on mouse neuropathology also reported normal hippocampal architecture with no changes in volume or numbers of neurons in the CA1 region or dentate gyrus (21).

There are limited studies on whether low-dose thimerosal via vaccination causes behavioral symptoms that resemble autism. Barile et al. (12) investigated the association between the receipt of TCVs and immune globulins early in life and neuropsychological outcomes in children at 7–10 y of age. The data were originally created by evaluating >1,000 children and their biological mothers. They found no statistically significant associations between thimerosal exposure from vaccines early in life on six of the seven variables, but there was a small but statistically significant association between early thimerosal exposure and the presence of tics in boys.

There is emerging evidence that autism may result from a maternal immune activation (MIA) during pregnancy. Several animal studies have examined the potential for prenatal viral exposure to induce aberrant behavioral outcomes in the offspring (22), and there are clinical reports of a maternal cytokine response to viral pathogens, suggesting a possible mechanism in the precipitation of these aberrant behaviors (23, 24). Maternal exposure to influenza and other viruses during pregnancy has been implicated in autism [reviewed by Zerbo et al. (25)]. In this study, pregnant dams whose infants were assigned to the 2008 group received a single influenza vaccine, containing thimerosal, to mimic vaccine recommendations for pregnant women. No evidence of either behavioral or neuroanatomical changes were observed in infants receiving a prenatal influenza vaccine, nor did our previous study identify any effects of prenatal influenza exposure on measures of early learning and cognition (17), suggesting that exposure to a single prenatal influenza TCV does not result in MIA.

In the present study, we examined social behavior in six groups of animals. Behaviors reported here were scored in animals from ∼13 to 18 mo of age. During this time, animals spent very little time engaged in negative behaviors such as Stereotypy, Rockhuddle-self-clasp, Fear-disturbed, and Withdrawal. In fact, there were virtually no instances of any stereotypy, a behavior characteristic of children with autism. Similar data were obtained in this same cohort of animals when examining behavior from ∼30 d to 12 mo of age (17). Overall, animals developed the normal repertoire of behaviors that is typical of animals of this age (26). Several primate studies have examined the effects of neurotoxicants on social behavior. Oral MeHg given prenatally alters the expression of social behavior in primates such that exposed offspring spend more time being passive and less time engaged in play behaviors with peers (26). Similarly, studies of postnatal lead exposure (27, 28) or prenatal TCDD exposure (29) have also produced a negative impact on social behavior in macaques. In contrast, exposure to low-dose TCVs via vaccination in our study did not significantly impact behavior.

There are several limitations to the present study. The 1990s Primate group was given an accelerated schedule of vaccinations due to the faster development of the visual system, pattern recognition, and object permanence in infant macaques (30, 31). It was therefore necessary to determine the appropriate timing for administering vaccines. In primates, there is a theoretical developmental ratio of 4:1, such that 4 wk of human development is comparable to 1 wk for a primate (32). Low-dose thimerosal exposure studies in primates have therefore used an accelerated schedule of exposure based on this developmental ratio (33, 34). It is possible that receiving multiple TCVs in an accelerated time frame could induce neurotoxicity in infant macaques, but this was not evident in tests of early learning and cognition (17). Likewise, in the present study, we did not find neuropathological or behavioral abnormalities in animals receiving TCVs. Neurobehavioral assessments followed very detailed protocols that have been used at the primate facility for more than three decades (35, 36). There were three testers involved in the scoring of social behavior data and each passed periodic reliability training to high standards. Therefore, although it is possible that primate behavioral scoring drifted over the course of this study (5 y), this should not have affected the group comparisons. Stereological analyses can result in biased data if suitable controls are not included. In the present study all cell number and cell size measurements were made with the person doing the measurements blind as to the experimental condition of the animal. In addition, at least two different people made the measurements to be certain of the validity of the data. Sometimes the neuroanatomical boundaries of nuclei are difficult to reliably define in all brain sections. To be certain of the neuroanatomical boundaries of the hippocampus CA1, the amygdala, and the lateral nucleus of the amygdala, we relied on the macaque primate brain atlas of Paxinos et al. (37), which allowed for a clear demarcation of the three brain regions. The present study focused on three brain regions found to be abnormal in postmortem ASD brains (cerebellum, amygdala, and hippocampus); however, the cerebral cortex has also been implicated in ASD neuropathology (38, 39). The cerebral cortex was not analyzed because there were no behavioral abnormalities observed in the present study nor was there neuropathology in the three regions examined.

In summary, analyses of postmortem brains from ASD subjects have often found decreases in Purkinje cell number (19) and cell size. We found no changes in Purkinje cell density or cell size in treated primates, and there was no difference in cerebellar calbindin, GAD-67, GFAP, or CD11b protein levels in the 1990s Primate or 2008 groups. Amygdala deficits have also been previously reported in autism. For instance, Schumann and Amaral (20) reported a 14% decrease in amygdala lateral

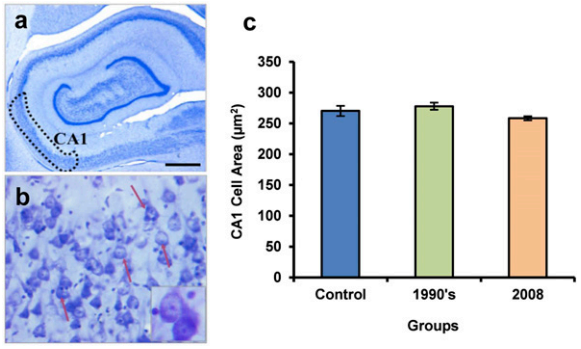

Fig. 4. CA1 cells in the hippocampus. (*A*) Location of the CA1 region. (*B*) Neurons at a higher magnification. Red arrows point to cells with a visible nucleolus. (*Inset, Right*) High magnification view of two neurons, one with a visible nucleolus. (*C*) Cell size data for Control ($n = 16$), 1990s Primate ($n = 12$), and 2008 ($n = 8$) groups. [Scale bars, (*A*) 1 mm and (*B*) 25 μm.]

nucleus cell number in postmortem ASD brains. We did not observe any changes in amygdala volume, lateral nucleus cell number, or cell volume in the 1990s Primate group. Postmortem studies (18) report smaller CA1 neurons in ASD cases, but in the present study we did not identify changes in CA1 cell size following administration of TCVs. Behaviors scored from ~13 to 18 mo of age revealed that animals spent very little time engaged in autism-related behaviors. For instance, there were virtually no instances of stereotypy, a behavior characteristic of children with autism and that can be generated by administration of various CNS toxicants during this developmental period. Overall, animals in each group developed the normal repertoire of behaviors that is typical of animals of this age. Our data strongly support the conclusion that childhood TCVs do not produce ASD-like neuropathology or behavioral changes in the nonhuman primate.

Methods

Study Design. Animal procedures followed the guidelines of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals of the National Research Council (40). All experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee. A total of 79 male infant macaques were studied in six groups: (*i*) Control (n = 16), animals received saline injections in place of vaccines; (*ii*) 1990s Pediatric (n = 12), animals received vaccines following the pediatric schedule recommended in the 1990s; (*iii*) 1990s Primate (n = 12), animals received vaccines recommended in the 1990s but on an accelerated schedule; (*iv*) TCV (n = 12), animals received all TCVs but no MMR vaccines following the accelerated schedule; (*v*) MMR (n = 15), animals only received the MMR vaccine but no TCVs following the accelerated schedule; and (*vi*) 2008 (n = 12), animals received vaccines recommended in 2008 but on an accelerated schedule. Infants were assigned to a peer group of four animals, with multiple study groups being tested each year when possible (17).

Animal Husbandry and Rearing Protocols. All infants were nursery-raised following standardized protocols (41, 42). Details are provided in *SI Methods*.

Vaccine Dosing and Administration. The vaccines used in this study are shown in Table S4. To recreate the required TCVs, thimerosal was added to the vaccines as described previously (17). Details are provided in *SI Methods* and Tables S5 and S6.

Assessments of Behavior. Social behavior was evaluated daily within the home cage for each peer group from ~12 to 18 mo of age. Each home cage contained wire mesh shelves, climbing platforms, and toys. Scoring was conducted by a blinded social tester in 5-min focal periods using a coding system of mutually exclusive and exhaustive behaviors (26). All testers were trained for 3–4 mo using the following protocol. Trainee testers score behaviors for the 5-min focal sessions along with a trained tester. This training is done for 10–15 sessions each with young infants, older infants, and young juveniles. Testers must attain a κ reliability score of 0.60 or better with their trainer with each age group being tested. All testers are retested with this procedure for reliability at 6-mo intervals. In the event of any code disagreements at or below chance levels (which was a rare occurrence), testers are retrained on the meaning of that code or codes. The three testers on this project had been social testing for 5–15 y, achieving the κ retest reliability standard at a typical value of 0.80 or better. Additional details are provided in *SI Methods*.

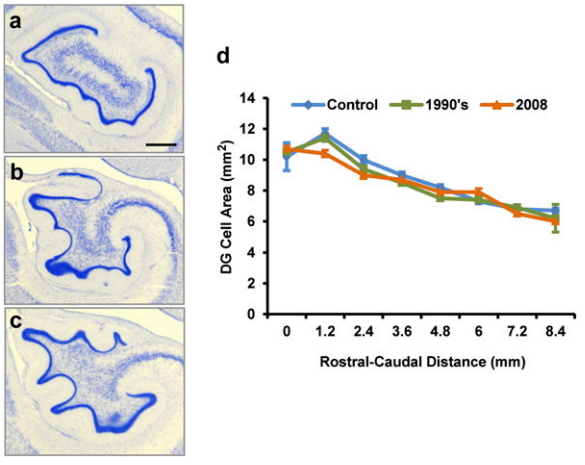

Fig. 5. The dentate gyrus. The size and shape of the dentate gyrus changes from rostral (*A*) to caudal (*C*). Illustrations were taken from sections 191, 251, and 331 (*A*–*C*). (*D*) Area of the dentate gyrus in the three groups of animals (Control, n = 12; 1990s Primate, n = 12; and 2008, n = 8). No group difference was found (ANOVA, P = 0.7565). [Scale bars, (*A*–*C*) 650 μm.]

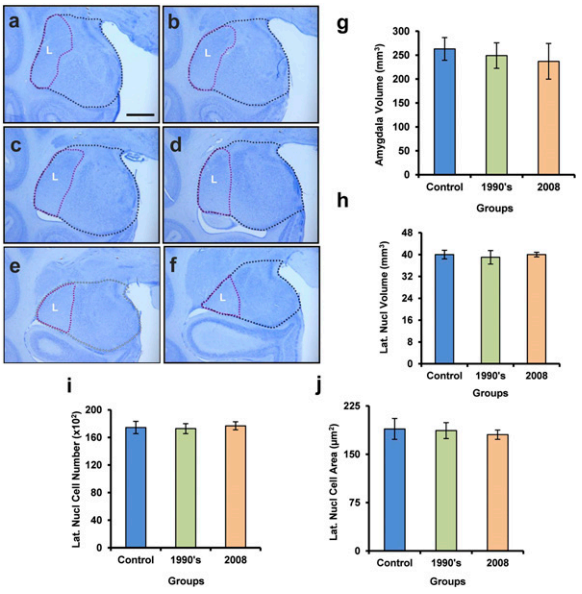

Fig. 6. The amygdala was studied in three groups of animals: Control, 1990s Primate, and 2008. (*A*–*F*) Sections through the rostral-caudal extent of the amygdala stained for Nissl substance. Outlines are provided for the amygdala borders, and the lateral nucleus of the amygdala (L). The amygdala volume (*G*), lateral nucleus of the amygdala volume (*H*), lateral nucleus of the amygdala cell area (*I*), and lateral nucleus of the amygdala cell number (*J*) did not change in the 1990s Primate, 2008, and Control groups. Sample size: n = 12 for Control; n = 12 for 1990s Primate; n = 8 for 2008. (Scale bar, 2 mm).

Preparation of Brain Tissues. Brains underwent a hemidissection with alternate hemispheres processed for immersion fixation in paraformaldehyde/PBS (pH 7.4). Tissues were postfixed in the same fixative for several weeks. After cryoprotection in 20% (wt/vol) sucrose/formalin/PBS (pH 7.4) for 2–3 d at 4 °C, the forebrain was blocked in the coronal plane, frozen, and cut at 60-μm thickness on a sliding microtome.

See *SI Methods* for details on the preparation of brain tissues, immunohistochemistry, and immunoblot analysis.

Stereological Analysis. All measurements were made using a Leica DMRE microscope attached to a Q-Imaging camera with Stereo-investigator software version 9.1, which was connected to a Dell Precision 450 workstation using Stereo Investigator software (MicroBrightField).

Cell counting. For counting Nissl-positive Purkinje neurons in the cerebellum, 10 sections were examined that were spaced 1.2 mm apart from medial to lateral through an entire cerebellar hemisphere. For counting neurons in the lateral nucleus of the amygdala, seven sections were examined that were spaced 0.6 mm apart across the entire rostral-caudal dimension.

Soma size. For measuring cell size (area), two sagittal sections (sections 100 and 300) were examined in the cerebellum, and three rostral-caudal sections were examined in the CA1 region of the hippocampus (sections 100, 200, and 300).

Volume. For volume measurements of the cerebellum, amygdala, lateral nucleus of the amygdala, and dentate gyrus of the hippocampus, 10, 7, 9, and 8 sections, respectively, that were spaced 0.6 mm apart were measured (i.e., every 10th section). Further details are provided in *SI Methods* and Tables S5 and S6.

Statistical Analysis. ANOVA was used to compare cell sizes and areas, and nuclear volumes, and multilevel modeling for analysis of behaviors among the animal groups. $P < 0.05$ was considered statistically significant.

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