Supporting Information

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SI Methods

Animal Husbandry and Rearing Protocols. Following delivery, infants were housed in isolettes and bottle-fed by hand in the nursery until achieving temperature regulation, typically 7–10 d from birth. Infants received standard infant formula (Enfamil Premium with iron; Enfamil). Post-isolette nursery caging contained a cloth surrogate device and formula feeder until 21 d, at which time infants were rehoused in individual cages. Animal biscuits (Purina Mills) were introduced during the first month but rarely ingested until about 3 mo of age. Additional fruit treats were provided as appropriate. Animals underwent assessments for the development of neonatal reflexes, object concept permanence (OCP), discrimination learning, and behavior during the pre-social living period, which have been reported separately (17). These assessments were based on the protocols developed at the Infant Primate Research Laboratory at the Washington National Primate Research Center and have been extensively published (41, 43–46).

Infants underwent testing as follows: from birth to 20 d, infants were assessed for the development of neonatal reflexes and perceptual and motor skills; from postnatal day 14 to ∼3.5 mo of age, infants were examined for the development of OCP; from ∼3 to 6 mo of age, animals underwent discrimination learning assessments; from ∼5 to 8 mo of age, animals were assessed for learning set development; and from 30 d to 12 mo of age, animals underwent assessments of behavior before group living. These developmentally appropriate tests are measures of neurodevelopment, learning, cognitive abilities, and social behavior in young macaques (45). At ∼13 mo of age, animals were transferred to juvenile caging where they were group housed (n = 4 males per group) with animals from within their peer group for the duration of the study. All subsequent behavioral data were collected while animals were in their home cage.

Vaccine Dosing and Administration. The concentration of EtHg in vaccine aliquots was periodically verified throughout the study using an independent testing laboratory (Quicksilver Scientific). All infants received either a vaccine or saline injection, administered i.m. or s.c. in a 0.5-mL volume, or orally, depending on recommended procedures, according to study group assignment. The vaccine dosing schedule was adjusted for all but one group (1990s Pediatric) to accommodate the approximate 4:1 developmental trajectory of infant Old World monkeys (47–50). Thus, when the human schedule called for vaccines to be administered at birth, 2 mo, 4 mo, 6 mo, 15 mo, and 48 mo, the timing of the primate vaccine schedule was accelerated fourfold and given at birth, 2 wk, 4 wk, 6 wk, 15 wk, and 52 wk. Vaccines were administered according to the schedule used in the 1990s (Table S5) or the expanded vaccine schedule from 2008 (Table S6), when fewer vaccines contained thimerosal. To follow the recommendation that pregnant women receive an influenza vaccine during gestation, pregnant dams of infants assigned to the 2008 group also received a single influenza vaccine containing 25 μg EtHg at ∼4 wk before delivery. All other dams received a saline injection.

Assessments of Behavior. Order of testing was randomized for each session. Scored behaviors included the following: Passive, Explore, Play, Sex, Aggression, Withdrawal, Fear-disturbed, Rock-huddle-self-clasp, and Stereotypy, and could be scored as either a social interaction or a nonsocial behavior (Table S1). Although negative behaviors (Withdrawal, Fear-disturbed, Rock-huddle-self-clasp, and Stereotypy) are considered part of the typical repertoire of behaviors for macaques of this age, significant increases in these behaviors could be considered "ASD-like behaviors" as the definition of ASD includes stereotyped repetitive patterns of behavior (2). Additional behaviors scored during each 5-min focal period, but not included in the analyses, included eating, drinking, grooming, and scratching. A mean duration and frequency was computed for each 30-d period for each of these nine behaviors. The first 30-d average for each animal began at initiation of social living (∼12 mo of age), and the final 30-d average was the last full 30-d period before necropsy (i.e., the final period was excluded if it contained fewer than 30 d). Animals averaged 5.78 ± 1.11 (mean ± SD) 30-d periods in social living.

Before fitting models to the behavioral data, descriptive statistics for behavior duration and frequency were examined. Means and SDs for durations and frequencies are displayed in Table 2. Since duration and frequency closely mirrored each other, only duration was used as an outcome in the analytic models. Also, because the duration of the negative behaviors was low overall, Stereotypy, Rock-huddle-self-clasp, Fear-disturbed, and Withdrawal were summed for each animal, as were durations of the positive behaviors (Aggression, Sex, and Play). Thus, for both nonsocial behavior (involving no other animal) and social behavior (involving one or more animals), there were four outcomes used in the analysis: passive, explore, positive, and negative. Duration values were natural log-transformed to reduce the possibility of disproportionate influence from extreme values.

Linear mixed models were fit to describe behaviors following recommendations for longitudinal model building (41). Linear mixed models accommodate data with multiple observations from the same subject and unequal numbers of observations per subject, both of which were characteristics of the present data. In addition, time can be treated as a continuous variable allowing it to be flexibly modeled to capture the trajectory of change in the outcome across the course of the study and interactions between experimental conditions and the trajectory of change. Before adding animal-level variables, a series of unconditional growth models were fit, which included an unconditional mean (i.e., no change), linear, and quadratic models. The best growth model for a putative outcome was determined by comparing unconditional growth models using the Akaike information criterion (51). Time was centered at the first month of social living. Among the social data, the best growth model was the unconditional means model for passive, explore, and negative behaviors, indicating that these behaviors remained constant across social living. The best growth model for positive behaviors was the quadratic model. Among nonsocial data, the best growth model for passive and explore was the linear model. The best growth model for negative behaviors was the unconditional means and the best growth model for positive behaviors was the quadratic model. After establishing the growth model for each outcome, the intervention condition main effect was added to the model and all interactions between the intervention condition, and time parameters were added to the models. In addition, age at the beginning of social living (mean = 365.66 d, SD = 35.33) was included in each model. A false discovery rate correction was applied to each parameter across the eight models. In the event of either a significant group or a group × time interaction effect, simple slope comparisons between the control group and each of the other groups were used to assess whether there were differences at initiation of social living and at the sixth month (the median number of months in

social living) (52). These models were previously used for the analysis of macaque behavior (17).

Preparation of Brain Tissues. The entire cerebellar hemisphere was cut in the sagittal plane. Every section was saved and if not used for Nissl/immunohistochemical staining, it was put into a labeled (brain # and section #) polypropylene tube and archived for future use. Some brains were not available for stereological analysis due to unsatisfactory cryoprotection.

Immunohistochemistry. Free-floating sections were blocked with 4% (wt/vol) goat serum/PBS plus 0.2% Triton X-100 and incubated overnight with antibodies against calbindin (1:20,000 dilution; Swant Laboratories), GFAP (1:4,000 dilution; Abcam), CD11b (1:4,000 dilution; Abcam), and doublecortin (1:500 dilution; Abcam). ABC reagents (Vector Laboratories) and SigmaFast DAB peroxidase substrate (Sigma-Aldrich) were also used. Subsequently appropriate secondary antibodies were used that are complementary to the primary antibodies. Sections were counterstained for Nissl (0.09% Cresyl violet or neutral red).

Immunoblot analysis. Frozen cerebellar tissue (~100 mg) was dissected from the posterior lobe. The tissue was homogenized in lysis buffer, 1% SDS, 1× PBS, and Complete Protease Inhibitor Mixture (Sigma), using a Diax 900 homogenizer (Sigma). After homogenization, the homogenate was centrifuged at 8,100 × *g* for 10 min, and the resulting fractions were collected. Protein levels were quantified using the BCA Kit (Pierce) with BSA standards and analyzed by immunoblot. The supernatant was used to separate proteins by SDS/PAGE [Tris-glycine 4–20% (wt/vol) gradient precast gels; Bio-Rad] and subjected to immunoblot analysis with the following antibodies: Iba1 (1:2,000 dilution; Abcam); GFAP (1:5,000; Abcam); calbindin (1:2,000 dilution; Waco Laboratories); GAD-67 (1:750 dilution; Abcam); and β-actin (1:10,000 dilution; Cell Signaling). Subsequently appropriate secondary HRP-conjugated antibodies (Vector Laboratories) were used that are complementary to the primary antibodies. Immunoblot signals were visualized with chemiluminescence. For densitometry analysis of the protein bands for different antibodies, the data were analyzed by using optical density measurement tools in ImageJ software (National Institutes of Health).

Stereological analysis. Three brain regions were analyzed.

i) Cerebellum: The optical fractionator method was used to estimate the number of Purkinje neurons in the cerebellum at a magnification of 20× [as described by Pakkenberg et al. (53)]. More than 400 cells were counted per brain. The optical fractionator counting frame was 425 × 275 μm, and the sampling grid was 3,000 × 3,000 μm. A Purkinje cell was defined as a large-sized soma in the Purkinje cell layer that occurred within the *z*-plane of the counting frame (10 μm, with 1-μm upper and lower guard zones).

ii) Hippocampus: Doublecortin immunostaining was used to identify newborn dentate granule cell neurons. The counting frame was 100 × 100 μm and the sampling grid was 400 × 400 μm, and cells were identified using 40× magnification. Eight sections were examined at an interval of every 10th section. More than 500 cells were counted per brain.

iii) Amygdala lateral nucleus: Neuron number was estimated using 40× magnification. The counting frame was 200 × 125 μm and the sampling grid was 800 × 800 μm. Every 10th section was examined with seven sections per brain. More than 400 cells were counted per brain, and neurons with a nucleolus in focus within the counting frame were counted.

Estimation of cell area. The cross-sectional areas of neurons were measured in the cerebellum, hippocampus, and lateral nucleus of the amygdala using the Nucleator method (54). Each time a neuron was encountered with a clearly identified nucleolus in focus within the *z*-plane, the nucleolus was marked, and a grid of two radially extended lines emanated from the nucleolus to the cell membrane. The four points at which each line intersected the neuronal cell membrane were marked, to calculate the neuronal cross-sectional area.

i) Cerebellum: To estimate the cell area of Purkinje neurons, a magnification of 40× was used, and cell area was determined in both calbindin-positive and calbindin-negative (i.e., Nissl-positive) Purkinje cells. The counting frame was 200 × 125 μm and the sampling grid was 1,250 × 1,250 μm; two sections per brain were examined at medial and lateral portions of the cerebellum. From 100 to 150 calbindin-positive and Nissl-positive cells were measured per brain.

ii) Hippocampus: Two configurations were used to measure the cell area of the CA1 neurons. Leica DMRE Microscope configuration: Brain sections were analyzed under the microscope using 40× magnification with a counting frame of 100 × 100 μm and a sampling grid of 250 × 250 μm. Two sections were examined per brain, and 100 to 200 cells were measured per brain. Hamamatsu NanoZoomer 2.0HT configuration: Brain sections were scanned in eleven 1-μm layers (*z*-planes). A digital 63× lens was used for cell soma measurements. Three sections and ~250 to 350 cells were measured per brain. Two independent researchers measured a mean neuronal cross-sectional area for each brain and were blind to animal groups. The percent difference between the independently measured means did not exceed 5.5% wt/vol.

iii) Lateral nucleus: To estimate the cell area of neurons in the lateral nucleus of the amygdala, 40× magnification was used with a counting frame of 200 × 125 μm and a sampling grid of 450 × 600 μm. More than 200 cells were measured per brain in one section located at the rostral-caudal center of the nucleus.

Estimation of volume. The volume of the cerebellum, amygdala, lateral nucleus of the amygdala, and dentate granule cell layer of the hippocampus was measured using the Cavalieri method.

i) Dentate gyrus: To estimate the volume of dentate gyrus of the hippocampus, outlines were drawn around the structure at 1.25× magnification. Eight 60-μm-thick sections were examined in each brain, spaced 10 sections apart.

ii) Amygdala: The outline of the amygdala was drawn at 1.25× magnification using the brain atlas of Paxinos et al. (37). The outlines in nine coronal sections were spaced 10 sections apart through the rostral-caudal extent of the nucleus.

iii) Amygdala lateral nucleus: The outline of the lateral nucleus of the amygdala was drawn at 1.25× magnification according to the brain atlas of Paxinos et al. (37) in seven coronal sections through the rostral-caudal extent of the nucleus.

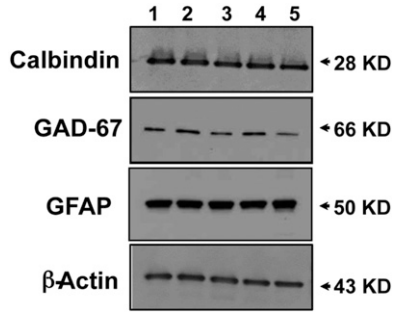

Fig. S1. Western blots of cerebellum proteins. Proteins were measured from five different regions of the cerebellum in one brain. The density of calbindin, GFAP, Iba1, and GAD-67 is similar in all cerebellar regions.

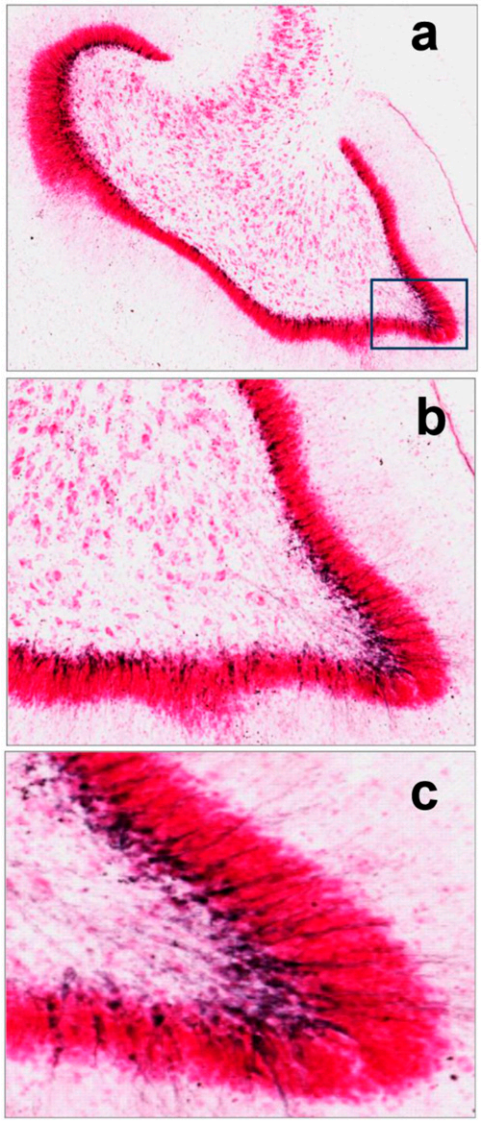

Fig. S2. Newborn cells in the granule cell layer. The new dentate gyrus neurons are illustrated in a doublecortin (black cells) immunostained section. This section was counterstained with neutral red. (*A*–*C*) Black-labeled doublecortin cells at higher magnifications.

Table S1. Description of social and nonsocial behavioral categories scored for all animals

Behavior*	Description of behaviors
Passive	No intense interaction with other animals, self, or objects. Can include a slow visual scanning component, social contact such as huddling, or proximity within one foot, and occurs without locomotion.
Explore	Visual and/or tactual inspection of other animals, self, or objects, with or without locomotion.
Play	Behaviors with greater physical intensity than explore, involving "ears back-mouth puckering" expression, open mouth without teeth exposure or ears back, chasing, wrestling, bouncing, running or jumping, rolling, biting without injury, or 'tug-of-war' with an object.
Sex	Presenting rear area, inspection of genitalia, masturbation, with thrusting toward another animal or tester, mounting and thrusting an animal or object.
Aggression	'Stiff' stance, piloerection, open-mouth threat, back, rolling and hitting with or without injury.
Withdrawal	Retreat from an animal or object creating increased distance by locomotion, but with no fear behaviors.
Fear-disturbed	Fear display involving submissive posture, retraction of lips, cooing, screeching, convulsive jerking, or three successive hoots, with or without withdrawal or locomotion.
Rock-huddle-self-clasp	Strong clasping/grasping of another monkey without play behavior, or self-clasping with arms, legs, hands, or feet, without locomotion and no active inspection of own or other's body.
Stereotypy	Repetitive body movements, with or without locomotion, requiring three or more consecutive, repetitive movements.

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*Behaviors can be either interactive with other animals (social behavior) or individual behaviors not involving any other animal (nonsocial behavior).

Table S2. Purkinje cell number (mean ± SEM) for five groups of animals

Group	Mean ± SEM
Control (n = 16)	795,754 ± 10,544
1990s Primate (n = 12)	777,423 ± 6,560
2008 (n = 8)	800,267 ± 14,811
TCV (n = 5)	824,977 ± 18,129
MMR (n = 5)	787,866 ± 8,422

ANOVA indicated that there was no difference among the groups (F = 1.68, P = 0.172).

Table S3. Purkinje cell size measured in tissue stained for both Nissl and calbindin (n = 8/group)

Control		1990s Primate	
Nissl	Calbindin	Nissl	Calbindin
273.1 ± 7.7	488.5 ± 7.9	279.2 ± 15.4	502.1 ± 9.8

Data represent mean ± SEM (μm^2).

Table S4. Vaccine source, EtHg content, and route of administration

Vaccine	Trade name (manufacturer) NDC #	Abbreviation	EtHg content (μg/0.5 mL dose)	Administration
Hepatitis B	Recombivax HB (Merck) 0006–4981-00	Hep B	1.98	i.m.
Diphtheria, tetanus, acellular pertussis	Infanrix (GlaxoSmithKline) 58160–810-46	DTaP	3.96	i.m.
Haemophilus influenza b	ActHIB (Sanofi Pasteur) 49281–545-05	Hib	3.96	i.m.
Measles mumps rubella	MMR-II (Merck) 0006–4682-00	MMR	NA	s.c.
Inactivated polio vaccine	IPOL (Sanofi Pasteur) 49281–860-10	IPV	NA	i.m. or s.c.
Rotavirus	Rotateq (Merck) 0006–4047-41	Rota	NA	Oral gavage
Pneumococcal 7-valent conjugate vaccine	Prevnar (Wyeth) 0005–1970-67	PCV	NA	i.m.
Hepatitis A	VAQTA (Merck) 0006–4831-41	Hep A	NA	i.m.
Varicellar	Varivax (Merck) 0006–4827-00	Vari	NA	s.c.
Meningococcal polysaccharide vaccine	Menomune (Sanofi Pasteur) 49281–489-05	MCV	3.96	s.c.
Influenza	Fluzone (Sanofi Pasteur) 49281–009-50	Inf	3.96	i.m.
Influenza (for pregnant dams only)	Fluzone (Sanofi Pasteur) 49281–382-15	Inf	25	i.m.

Modified with permission from ref. 17. NA, not applicable.

Table S5. Primate equivalents of EtHg dosing and timing of the US pediatric vaccine recommendations in the 1990s

Vaccine dosing	Birth	2	4	6	15	48
Human (age in mo)						
EtHg (μg) in vaccines						
Hepatitis B × 3 doses	12.5	12.5	12.5	—	—	—
DTaP × 5 doses	—	25	25	25	25	25
Hib × 4 doses	—	25	25	25	25	—
MMR × 2 doses	—	—	—	—	0	0
Total EtHg (μg) for infant boys	12.5	62.5	62.5	50	50	25
10th percentile weights for infant boys (kg)*	2.8	4.4	5.8	6.8	9	14
μg EtHg/kg bodyweight for infant boys	4.46	14.20	10.78	7.35	5.56	1.79
Primate (age in wk)						
95th percentile weights for infant primates (kg)†	0.62	0.73	0.84	0.94	1.20	2.47
Weight ratio infant boys:primates	4.52	6.03	6.90	7.23	7.50	5.67
EtHg (μg) in vaccines‡						
Hepatitis B × 3 doses	1.98	1.98	1.98	—	—	—
DTaP × 5 doses	—	3.96	3.96	3.96	3.96	3.96
Hib × 4 doses	—	3.96	3.96	3.96	3.96	—
MMR × 2 doses	—	—	—	—	0	0
Total EtHg (μg) for primates vaccines	1.98	9.9	9.9	7.92	7.92	3.96
μg EtHg/kg bodyweight for primates	3.20	13.59	11.81	8.44	6.61	1.61

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*Based on 10th percentile weights for infant boys from the weight-for-age percentiles from the National Center for Health Statistics, 4/20/01.

†Based on 95th percentile weights for infant male macaques.

‡EtHg content of primate vaccines was determined by first averaging the weight ratios for human infant boys: male infant primates across the six time points of vaccine administration. The average weight ratio was 6.3:1. The EtHg content in each pediatric vaccine was then divided by 6.3 to determine the dosing of EtHg for each primate vaccine. This method provided a similar dosing of μg EtHg/kg body weight for infant boys and primates.

Table S6. The 2008 pediatric vaccination schedule adjusted for infant primates

Prenatal*	Birth	2 wk	4 wk	6 wk	12 wk	15 wk	18 wk	26 wk	52 wk
	Hep B	Hep B Rota DTaP Hib PCV IPV	Rota DTaP Hib PCV IPV	Hep B Rota DTaP Hib PCV IPV	Hib PCV	DTaP			DTaP IPV
Inf				Inf†					
						MMR Varicella			MMR Varicella
					Hep A		Hep A		
								MCV‡	

The timing of all vaccine administration for primates was accelerated ~4:1 to account for the faster developmental trajectory of infant Old World primates. DTaP, diphtheria, tetanus, acellular pertussis vaccine; Hep A, hepatitis A vaccine; Hep B, hepatitis B vaccine; Hib, *Haemophilus* influenza B vaccine; Inf, influenza vaccine; IPV, inactivated polio vaccine; MCV, meningococcal, vaccine; MMR, measles mumps rubella vaccine; PCV, pneumococcus vaccine; Varicella, chicken pox vaccine.

*Pregnant dams giving birth to infants assigned to this study group received a single prenatal influenza vaccine containing 25 μg EtHg at ~4 wk before delivery. All other dams received a single saline injection.

†Influenza was administered to infants at 6 wk of age and then every 12 wk thereafter, mimicking the pediatric schedule of annual influenza vaccination. All vaccines administered were thimerosal free, except for the influenza and meningococcal vaccines, which were both formulated to contain 3.96 μg EtHg.

‡Meningococcal vaccine is recommended for certain high-risk groups and was therefore included to maximize EtHg exposure in animals in this group.