Maternal Immune Activation in Nonhuman Primates Alters Social Attention in Juvenile Offspring

Supplement 1

Supplemental Methods

Subjects and Living Conditions

Twenty-four pregnant rhesus macaques were selected from the California National Primate Research Center (CNPRC) timed-mating program. Candidate females were between six and eighteen years of age (mean age = 11 years), had been reared in a naturalistic social group, demonstrated species-typical behaviors, and had a successful history of raising offspring. Pregnancy was confirmed at approximately 20 days of gestation and was followed by blood assays to detect fetal DNA for sex determination. Willingness to present an arm for intravenous injection while being temporality restrained (less than 1 min) was assessed at gestational day 30. To minimize stress, only animals that readily complied were included in the study. Pregnancies were monitored via ultrasound on gestational days 40, 100 and 150. Rhesus monkey gestation is approximately 165 days, and maternal immune activation (MIA) was targeted at the end of the first trimester (MIA^1 injections on gestational days 43, 44, 46) or the end of the second trimester (MIA^2 injections on gestational day 100, 101, 103). These animals were assigned to one of three experimental groups: 1) First trimester MIA (MIA^1; *n* = 5 male offspring and 1 female offspring), 2) Second trimester MIA (MIA^2; *n* = 4 male offspring and 3 female offspring) or 3) Controls (CON; *n* = 4 male offspring and 7 female offspring). Pregnant animals in the MIA groups were injected with 0.25 mg/kg synthetic double-stranded RNA (polyinosinic:polycytidylic acid [poly IC] stabilized with poly-L-lysine [poly ICLC]) (Oncovir, Inc.) via intravenous injection while briefly restrained by trained technicians on gestational days 43, 44, 46 (MIA^1) or 100, 101, 103 (MIA^2). Pregnant dams in the MIA and saline-treated CON

groups received three injections over a 72 hour period: Day 1 – 8 AM injection, Day 2 – 8 AM injection, Day 3 – no injection, Day 4 – 8 AM injection. Sickness behavior, temperature and cytokine profiles of the pregnant monkeys confirmed a strong inflammatory response to polyICLC (1).

All infants were born at term, raised with their mothers and provided three hours daily access to a social group consisting of four mother-infant pairs and one adult male to facilitate species-typical social development. Infants were weaned at 6 months of age, but continued daily peer group interactions through approximately 2 years of age. Prior to the present study, these animals were assessed in several probes of social and repetitive behaviors described elsewhere (1). At the time of the current study, all animals were housed indoors in social pairs 24 hours per day, 7 days per week. These pairs occupied two adjacent, age-appropriate laboratory cages.

All CON (n = 4) and MIA^1 males (n = 5) were selected to participate in the current series of experiments to follow-up on the social behavior differences described in our earlier report (1) (see Table S1 for description of original experimental groups). One of the MIA^1 males did not habituate to the testing procedures described below, despite 3 months of daily training (see below for details). This animal was therefore dropped from the study, yielding a final sample size of n = 4 for the MIA^1 group. Preliminary analyses revealed that the behavioral profiles of the saline-treated control monkeys and the untreated control monkeys were very similar. Control animals were selected based on availability to participate in the eye-tracking studies and pooled to form a single control group.

The housing room for all CON and MIA^1 animals was maintained on a 12-hour light/dark cycle. All animals were maintained on a diet of fresh fruit, vegetables and monkey chow (Lab Diet #5047, PMI Nutrition International Inc., Brentwood, MO), with water available *ad libitum*. All animals were also permitted unrestrained social interaction with a behaviorally-compatible, age-matched peer in a neighboring cage on weekdays. At two years of age, just prior to the eye-

tracking studies, the 8 animals were pair housed in MIA/CON pairs and weighed 3.0-4.3 kg (CON average = 4.0 kg, MIA average = 3.7 kg). The animals ranged from 24-34 months at the time of the eye tracking experiments.

Table S1. Original Experimental Groups

Experimental Group	Group Size (males, females)	Mean Age During Eye Tracking (males only)
1st Trimester MIA (MIA^1)	*n* = 6 (5m, 1f)	31 months
2nd Trimester MIA (MIA^2)	*n* = 7 (4m, 3f)	---
Saline Controls	*n* = 8 1st Trimester (1m, 3f) 2nd Trimester (2m, 2f)	26 months*
Untreated Controls	*n* = 3 (1m, 2f)	26 months*

*Mean age for control group (*n* = 4).

Training Prior to Eye-Tracking Procedures

Training methods and noninvasive head restraint strategies have been described in detail elsewhere (2). Briefly, all training and subsequent data collection occurred while the animals sat in a modified primate chair with a slanted top (Crist Instrument Co., Inc., Damascus, MD). Head restraint was accomplished noninvasively using individualized thermoplastic helmets that could be affixed to the primate chair. Each animal was habituated to sitting in the primate chair with its helmet on for successively longer periods of time up to 60 minutes. In these training sessions the experimenter would use food (dried cranberries, white grapes, banana chips, or dried mango) or juice rewords to positively reinforce the chair acclimation. Next, the animal's chair was rolled into a sound-attenuating testing chamber (Acoustic Systems, Austin, TX; 2.1 m wide x 2.4 m tall x 1.1 m deep) for habituation to this testing context and the video eye-tracker

(Applied Science Laboratories, Bedford, MA; model R-HS-S6). A wide-screen, color video monitor (60.96 cm diagonal; Gateway Inc., Irvine, CA; model LP2424) was positioned at the monkey's eye level. The video monitor was positioned 127 cm from the animals' eyes, while the eye-tracking camera was positioned on a tripod 53.34 cm from the animals' eyes. A white noise generator (60 dB) inside of the chamber was used to minimize outside auditory distractions.

Once reliably calibrated (see main text), animals were habituated to viewing photographs (color or black and white; 5-second duration) on a black background. Each photograph was separated by four black screens: 1) blank, 5-second duration, 2) a pulsating, yellow and orange star target (2.9^0 visual angle) at center, 3) same star target positioned randomly at 1 of 8 points around the screen periphery, and 4) blank, 5-second duration. Animals were required to fixate each star target for at least 250 ms to obtain a small juice reward and move on to the next picture trial, thus ensuring accuracy of the point-of-gaze data throughout a prolonged testing session and eliminating the chance that animals would sleep through one or more trials. The animals completed this phase of training once they finished 40 trials in less than 30 minutes on 3 consecutive days. Arriving at this point required an average of 29.25 days of training for CON (range = 26 – 35 days) and 23 days of training for MIA[1] (range 15 – 29 days). By the time animals reached this level of training, they no longer showed any overt behavioral signs of stress during any of the body/head restraint procedures.

Noninvasive Eye-Tracking

The animal's chair was rolled into a sound-attenuating testing chamber (Acoustic Systems, Austin, TX; 2.1 m wide x 2.4 m tall x 1.1 m deep) for testing with a video eye-tracker (Applied Science Laboratories, Bedford, MA; model R-HS-S6). A curved mouthpiece (Crist Instrument Co., Inc.; model # 5-RLD-00A) was attached to the top-left of the chair and connected to an automatic juice dispenser (Crist Instrument Co., Inc.; model # 5-RLD-E3) so that fluid reward could be dispensed throughout the testing session. A wide-screen, color video monitor (60.96

cm diagonal; Gateway Inc., Irvine, CA; model LP2424) was positioned at the monkey's eye level. All testing was conducted with the chamber lights off, so the only stimulus that the animal could see during data collection was this video monitor and test stimuli that were always presented on a black background. Infrared luminance level, pupil threshold and corneal reflection threshold were set individually for each animal at the start of each session. Sampling rate for the eye-tracking camera was set to 120 Hz. A standard nine-point calibration (3 x 3 matrix of calibration stimuli) was conducted prior to testing with each animal to ensure accuracy of gaze data collection. Calibration stimuli were videos presented in small portions of the screen (8.9 x 5.7 cm on screen, 4^0 visual angle) of rhesus monkeys from the outdoor housing enclosures at the CNPRC.

Experiment 1: Facial Expressions

Data for this experiment were gathered over 5 days. On each test day, the animal was transported to the eye-tracking room, and placed into the testing chair as described above. The animal's chair was then moved into the testing chamber, the mouthpiece for juice delivery was attached to the chair, and the eye-tracker was calibrated as described above. Face stimuli were separated from their original background using Adobe Photoshop CS5 software and placed onto a black background. The faces spanned 8.3 – 11.4^0 visual angle in the vertical direction and 9.1 – 11.4^0 visual angle in the horizontal plane. The final stimuli (face + background) were all 737 x 983 pixels (14.5^0 x 19.3^0 visual angle) and displayed in a standard 3:4 aspect ratio. Two white crosses (0.28^0 visual angle, positioned in the top left and bottom right corners) were placed on each image so that point-of-gaze coordinates could be mapped back onto each image during analysis. The animal was also required to fixate on both a center and a peripheral pulsating star target for 500 ms before moving to the next face stimulus and received a juice reward after each successful fixation (180 ms juice for the center target, 360 ms juice for the peripheral targets).

All 40 face stimuli were adjusted in terms of brightness and contrast to be balanced for luminance.

Experiment 2: Facial Expressions Embedded in Complex Scenes

Data for this experiment were also gathered over 5 days. Each day, the animal was transported to the eye-tracking room and prepared for eye-tracking in the same way as for Experiment 1. The final stimuli (faces from Experiment 1 + background) were all 960 x 1280 pixels (14.5^0 x 19.3^0 visual angle) and displayed in a standard 3:4 aspect ratio. The faces spanned 2.3^0 visual angle in the vertical direction and 2.5^0 visual angle in the horizontal plane. All 50 stimuli for this experiment were adjusted in terms of brightness and contrast to be balanced for luminance.

Data Analysis

Default parameters were used to define fixations (Applied Science Laboratories), meaning that a fixation was recorded if gaze coordinates remained within 1^0 x 1^0 visual angle for at least 100 ms. The duration of a given fixation ended when gaze coordinates deviated by more than 1^0 x 1^0 visual angle for more than 360 ms. Total fixation frequency represented the cumulative number of discrete fixations that fell within an area of interest (AOI) during each 5-second trial. Total fixation duration was the cumulative time (maximum = 5 seconds) that the animal spent fixating on a particular AOI. These two metrics are both presented as a percentage of all fixations recorded during image presentation. The average fixation duration was derived by dividing the total fixation duration by the total fixation frequency. The average dwell duration measures the average amount of time that gaze remained within a given AOI without leaving. The conditional probability measures the likelihood that fixation in one AOI will be followed by fixation immediately on another (e.g., the probability that the next fixation will be on the mouth when fixating on the eyes). Average fixation duration, average dwell duration and conditional

probability metrics were not normalized. Finally, we also measured the animals' dark-adapted pupil diameter during the presentation of each image as an index of cognitive processing load (3, 4) and physiological arousal (5, 6) (especially reflecting sympathetic nervous system activation (4, 7, 8)). Since all stimuli used in this experiment were balanced in terms of luminance, pupil diameter measurements were averaged across the entire 5 second presentation of a given stimulus and analyzed without any additional normalization.

Supplemental References

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