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Degree of Chinese ancestry affects behavioral characteristics of infant rhesus macaques (*Macaca mulatta*)

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Abstract

Background—The use of Chinese-origin macaques in biomedical research is problematic for some scientists because of the reported behavioral and physiological differences from those of Indian origin. However, few studies have examined the effects of varying degrees of Chinese ancestry (DCA) on behavior, and they were typically based on small sample sizes and unusual rearing conditions.

Methods—Using data from a colony-wide program, we examined whether DCA was related to behavior and temperament ratings reflecting emotionality (fearfulness, aggression and anxiety) and activity. The 249 subjects, born and reared in outdoor social environment, were separated from their groups for a 25-hr indoor testing. Their DCA (range: 0.6 to 99.4%) was determined by analyzing the frequency of short tandem repeat alleles.

Results and Conclusions—The major effect of DCA was on fearfulness, with the more hybridized animals scoring lowest and the more pure Indian- and Chinese-origin macaques resembling each other.

Keywords

Fearfulness; Aggression; Anxiety; Chinese ancestry; Rhesus macaque

Introduction

Rhesus macaques (*Macaca mulatta*) are among the most widely-used nonhuman primate species in research in the US [4]. The original source of these animals during the middle 20th Century was India; however, because the Indian government suspended exportation of rhesus monkeys in 1978, the principal source of rhesus macaques has been domestic breeding colonies within the US. To meet the growing demand for rhesus macaques for research, however, many facilities in the US started to import rhesus monkeys from China. For example, the current population of rhesus macaques at the California National Primate Research Center (CNPRC) comprises animals of pure Indian-origin (most of the population), a few animals that are pure-Chinese origin, and a large number of rhesus monkeys with varying degrees of Chinese descent, owing to an early decision to interbreed Indian- and Chinese-origin animals.

Investigation of potential differences between Indian-origin and Chinese-origin rhesus macaques began in the early 1990s [5, 11]. Because rhesus monkeys are the principal animal model for human immunodeficiency virus (HIV) infection, several studies on simian immunodeficiency virus (SIV) infection of rhesus monkeys suggested that there were differences in pathogenesis between animals of Indian and Chinese origin [e.g., 11]. These studies, however, had some limitations, such as small sample size, and none examined the suitability of Chinese-Indian hybrid animals. (We note, however, that more recent research has indicated that Chinese-origin animals may be a suitable SIV disease model [eg. 16, 17].)

Other studies focused on identifying physiological, immunological, and genetic differences between the Chinese- and Indian-origin rhesus monkeys. For example, animals with 50% Chinese descent differ from Indian-origin monkeys in routine blood chemistry and hematological values [6]. In addition, differences in the major histocompatibility complex have been found between Indian- and Chinese-origin rhesus monkeys [eg. 18, 24]. Moreover, several studies have reported significant genetic divergence between Indian-derived rhesus macaques and those originating from China [eg. 1, 12, 14, 19, 20, 27].

Finally, a limited number of reports have considered behavioral differences related to ancestral background, and the results have been mixed. Anecdotal reports suggested that Chinese-origin adult animals seemed to be more aggressive towards both other rhesus monkeys and humans (described in [7]). Two studies by Champoux and colleagues compared nursery-reared infant rhesus macaques of Indian-origin with infants having 50% Chinese descent. One study found rhesus macaque neonates with 50% Chinese descent temperamentally more irritable and reactive than Indian-origin animals [5]. The other study indicated that rhesus macaque infants with half Chinese descent habituated more slowly to separation, and showed lower soothability and higher levels of anxiety [7]. Activity level was also assessed in the above studies, but no effects of Chinese descent were reported. Finally, another report based on management records of captive rhesus macaques in five U.S. non-human primate research centers did not find significant differences in aggression-related incidents between adult Chinese- and Indian-origin animals [2].

As just described, previous studies (especially the behavioral studies) focused largely on differences between animals of pure Indian-origin and those of 50% degree of Chinese ancestry (DCA). Often those studies had very small sample sizes. In addition, the two behavioral studies only examined nursery-reared animals, which have been shown to be more emotionally reactive compared to animals reared in large social groups [3].

The current study was designed to address these shortcomings. Our subjects were all born and reared in large, outdoor field cages in a rich social environment that approximates that found in the wild, and our sample size of 249 is much larger than that in previous studies. In addition, the DCA of our subjects has a wider range, from 0.6 to 99.4%, based on the frequency of short tandem repeat (STR) alleles [12]. To be consistent with previous research, we examined emotionality- and activity-related behaviors. Emotionality specifically included measures of fearfulness, aggression, and anxiety.

Materials and Methods

Subjects

Subjects were 249 rhesus macaque infants (142 females), that were born and raised with their mothers in the 0.2 hectare outdoor field cages at CNPRC from the years 2001 to 2008. Each field cage included up to 150 animals of all age/sex categories, which provides animals with a rich social environment that approximates that of troops in the wild. Each animal was

separated from its mother for 25 hr between 90 and 130 days of age (mean = 108.5 days) and tested in the Biobehavioral Assessment (BBA) project as described below.

Degrees of Chinese ancestry (DCA)

Estimation of DCA was based on allele frequencies across 14 STR loci that were typed at the Veterinary Genetics Laboratory (VGL), University of California, Davis, CA. Further information on STR markers, DNA extraction, PCR amplification of STRs, and fragment analysis of PCR products can be found in Kanthaswamy *et al.* [12, 13]. Using the STR allele frequencies, the program STRUCTURE 2.1 [9] was used probabilistically to compute each study animal's fractional membership in the Chinese ancestral population or DCA with at least 95% confidence limits. The STRUCTURE analysis was conducted at sweeps of 10^5 reiterations after a burn-in period of 10^5 and it was assumed that allele frequencies among all study animals are correlated since most of these animals descend from common founders. Genotype information from a reference population of 105 pure blood Chinese and 99 pure blood Indian animals was used to estimate the DCA of the test population which ranged from 0.006 to 0.994 with a mean of 0.513. Based on the assignment probabilities of the pre-defined pure blood Chinese (> 0.90 DCA) and Indian (< 0.10 DCA) animals in the reference population into their respective ancestral clusters (12), hybrid animals in the study population exhibited partial inferred ancestry with DCA estimates ranging from 0.11 to 0.89. Study animals with DCA estimates that fell outside the range of DCA for hybrids were categorized as either pure blood Indian ($N = 97$) or pure blood Chinese ($N = 104$).

BioBehavioral Assessment (BBA) Overview

The purpose of the BBA program is to assess individual differences in the behavioral and physiological characteristics of 3–4 month old infant rhesus macaques in a highly standardized situation; the program is described in detail elsewhere [3, 10]. Briefly, on the day of testing (designated Day 1), up to 8 infants at a time are separated from their mothers before 0900 and are brought from their field cages to individual indoor holding cages (60 cm $\times$ 65 cm $\times$ 79 cm, Lab Products, Inc., Maywood, NJ) that are out of visual and auditory contact with their mothers. During the 25-hr period, infants are tested in the same predetermined random order on a variety of behavioral tests, and have their blood sampled on four occasions (details are described in [10]). The infants are returned to their mothers at approximately 1000 on the following day (Day 2). All behavioral data collection is done by trained observers who have achieved greater than 85% agreement in tests of reliability.

In the current study, we utilized behavioral measures from three assessments in the BBA program. These are Holding Cage observations, Human Intruder test, and Temperament ratings.

Holding Cage Observations

This procedure was conducted at the beginning (0915 on Day 1) and the end (0700 on Day 2) of the 25-hr period. The purpose of the Day 1 observation is to assess immediate responses to separation and relocation; the Day 2 observation reflects adaptation to the novel situation. An experienced observer sat 2.6m from the cage and observed each animal for 5 min on both Day 1 and Day 2, in a pre-determined random order. The observer recorded behaviors using The Observer software (Noldus, 1991). Behaviors included states (such as *locomote* and *hang*) measured as frequencies and durations, and events (such as *threat* and *yawn*) measured as frequencies. Behaviors assessed as 'states' were mutually exclusive with each other. The complete ethogram and definitions can be found in Table 2 of Golub *et al* [10].

Human Intruder Test

This test was conducted in the room adjacent to that of the Holding Cage at 1400h on Day 1. Each animal was placed inside a cage of $38.7 \times 52.0 \times 39.0$ cm with a clear plastic front through which the subject animal was able to see the human intruder, who was always a seated female with height between 1.7 and 2 m. The behaviors of the animal were video recorded, with the camera 1 m from the front of the testing cage. Each animal was tested in a single session of four 1-min conditions in fixed order. In the first condition, the intruder presented her left profile 1 m from the plastic front of the testing cage (profile far); at the end of the 1 min, the intruder moved laterally towards the animal and stayed at approximately 0.3 m from the front of the cage, presenting her left profile (profile near); at the end of this condition, the intruder moved to the position of 1 m from the testing cage, and stared directly at the animal (stare far); one minute later, the intruder moved to the near condition and repeated the previous condition (stare near). Behaviors were coded later from the videotapes by a trained technician using an ethogram nearly identical to that used in the Holding Cage observation. The principal difference for the present analysis was that “active” defined as “whole body movement; step; jump,” was scored instead of locomotion.

Temperament Rating

At the end of testing, just prior to the infant’s reunion with its mother, the technician who performed the Holding Cage observations and other test procedures during the 25-hr period rated each animal on 16 broad temperament traits. The ratings used a Likert-type scale of 1–7, with 1 reflecting a total absence of the behavior and 7 reflecting an extremely large amount of the behavior. Inter-rater reliability and agreement for the ratings are described by Weinstein and Capitanio [25] and Golub *et al* [10]. For the present animals, a single rater, who was blind to DCA status, rated all animals.

Data analysis

Owing to slight differences in lengths of observation periods, duration behaviors for Holding Cage observation and Human Intruder test were converted from raw scores to proportions of the total observation period, and frequency behaviors were converted to rate per 60 sec. Data for each behavioral measure from three assessments were analyzed in separate regression models that included DCA, the square of DCA (to permit examination of quadratic effects), sex, and age. To facilitate interpretation, DCA, sex, and age were centered by subtracting the mean from each individual’s values.

Based on previous research (described in the Introduction), emotionality (comprising fearfulness, aggression, and anxiety) and activity were the behavioral characteristics of particular interest. These characteristics were inferred from the behavioral measures and temperament ratings, and are described in detail in Table 1.

For the Holding Cage and Human Intruder observations, *fear grimace* and *screech* were selected to represent fearfulness; *bark*, *threat* and *cage shake* reflected aggression; *coo*, *scratch* and *yawn* were selected as measures of anxiety; and *locomote* (for Holding Cage) or *active* (for Human Intruder) and *hang* (for both assessments) were considered to reflect activity level. Based on the definitions of the rating adjectives, ratings of *fearfulness* reflected fearfulness; ratings of *aggressive* and *gentle* were thought to reflect aggression; *nervous*, *calm* and *flexible* were considered to be related to anxiety; the rating for *active* was considered to be associated with activity levels.

All behavioral data selected above (except *gentle*) had a Poisson-like distribution. Tweedie’s compound Poisson model, able to deal with non-integer data with a Poisson-like distribution, was used for all the analyses [21, 23, 26]. Alpha was set to 0.05. A Poisson-like

distribution with a log link function was chosen to model these data (*gentle* has a normal distribution). Specifically in our analyses, $\text{Log}(Y) = \text{DCA} + \text{DCA}^2 + \text{Sex} + \text{Age}$, where Y equals behavioral or temperament outcome measures. Exploratory analyses were performed to locate the optimal variable power for each model before establishing models. For each model, variable power specifies the type of distribution of Y. Analyses were conducted using R 2.13.2, including packages Statmod [22], and Tweedie [8].

For Holding Cage data, each behavioral measure on each day was analyzed in separate models. For the Human Intruder test, each behavioral measure in each of the two most intense conditions (stare far and stare near) was tested in separate models. For Temperament ratings, each rating item was fitted into single models. Significant effects of DCA were plotted based upon the final models, using mean values (which, because of centering, were zero) for the nonsignificant effects. All figures show predicted values based upon the statistical models; we also present means (and SE) for our principal measures in Table 2.

Assurance of Compliance with Animal Codes

All procedures followed the Guidelines for Use and Care of Laboratory Animals of the National Research Council and the CNPRC Standard Operating Protocols (SOPs). The CNPRC is an accredited institution by the Association for Assessment and Accreditation of Laboratory Animal Care. Experimental protocols were approved by the University of California, Davis, Institutional Animal Care and Use Committees (IACUC) before implementation.

Results

Fearfulness

The effects of DCA on fearfulness were detected in all three assessments. A linear effect was found for fear grimace during the Holding Cage observation ($b = -3.831$, $t = -3.347$, $P < 0.001$): fear grimaces on Day 1 (but not Day 2) were more frequent among animals with greater Indian descent and declined with greater DCA (Figure 1). A quadratic effect was found for fear grimace in the stare far condition ($b = 152.748$, $t = 2.152$, $P < 0.05$; Figure 2) and for screech in the stare near condition of the Human Intruder test ($b = 17.367$, $t = 1.974$, $P < 0.05$; Figure 3), and for the temperament ratings of fearfulness ($b = 1.043$, $t = 2.273$, $P < 0.05$; Figure 4). For all quadratic effects, animals with a greater degree of a single ancestry tended to be more fearful than hybrid animals that had a more equal mix of Chinese and Indian ancestry. Table 2 displays mean values for measures based on 25% cut-offs for DCA.

There was also a significant sex effect on screech in the stare near condition, with females showing more screech than males ($b = -2.122$, $t = -2.607$, $P < 0.01$; Figure 3).

Aggression

Among all aggression-related measures, significant linear effects of DCA were only evident for *bark* in the stare far condition in the Human Intruder test ($b = -1.014$, $t = -1.991$, $P < 0.05$): animals with higher DCA showed less barking. There were no quadratic effects of DCA on any aggression-related measures.

Anxiety

DCA's effects on anxiety-related measures were only found in the Human Intruder test. There were quadratic effects of DCA on *yawn* in the stare far condition ($b = -8.409$, $t = -2.726$, $P < 0.01$; Figure 5; Table 2): rates of yawning were greatest for animals with intermediate values of DCA, and lowest for animals with more extreme values. DCA

showed linear effects on *coo* in the stare near condition ($b = -0.697$, $t = -1.990$, $P < 0.05$), indicating that animals with higher DCA tended to *coo* less.

Activity

We found no significant effects of DCA on any activity-related measures in any of the three testing conditions.

Discussion

The data presented above indicated that the most consistent effect of DCA was on fearfulness in infant rhesus macaques, and the effect was predominantly non-linear: animals with around 50% DCA were the least fearful, and animals with almost 0 or 100% DCA tended to be more fearful. The principal exception to this pattern was found in the Holding Cage Observation in which DCA had a linear effect: animals with greater DCA displayed less fear. Few effects of DCA were found for anxiety- and aggression-related measures, and those that were found were evident only in the Human Intruder test: Linear effects were found, indicating that animals with higher DCA exhibited fewer bark and *coo* vocalizations, and significant quadratic results suggest that animals with intermediate DCA were more likely to show anxiety-associated behavior (yawning) than were animals at either end of the DCA distribution. No significant effects of DCA were found on activity-related measures. Together, these results strongly suggest that differences in Chinese ancestry, among infant monkeys reared in rich, social environments, are mostly associated with fearfulness, and that the effects are predominately non-linear.

Comparisons with previous findings

The findings in the current study differ from those of previous behavioral studies, which compared pure Indian animals (0% DCA) with 50% DCA hybrids. Unlike in those studies [5, 7], we found that greater Indian descent in infant rhesus macaques predicted higher frequencies of fearfulness-related behaviors when animals were not being actively challenged (i.e., a linear effect in the Holding Cage observations); in contrast, during active challenge (the Human Intruder test), or on an integrated measure of temperament across the entire 25-hr period (Temperament ratings), infants with higher Indian descent tended to resemble the animals with higher Chinese descent in fearfulness, and it was the hybrids that differed from (and were lowest in fear, and higher in anxiety, compared to) the animals with both low and high DCA (i.e., quadratic effects). Our results also did not support anecdotal reports that Chinese animals were more aggressive than the Indian ones, inasmuch as we saw an effect on only one of our measures of aggression (bark). Differences between our results and those of others could be due to a number of reasons.

First, effects of rearing condition could have an important influence on the results. The subjects in the present study were born and raised by their mothers in an outdoor, socially rich environment. In contrast, animals in the prior behavioral studies were nursery-reared animals that were separated from their mothers between one to three days after birth and raised in an indoor laboratory by humans without conspecific social interactions in the first month of life. There is good evidence that nursery-rearing and rearing in a field cage result in broad differences in responsiveness. For example, other studies in our laboratory using the same measures showed that field-cage-reared animals exhibited significant increases in activity from Day 1 to Day 2 in the Holding Cage observations; and nursery-reared animals were generally less active, and showed more distress vocalizations than field-cage animals in the Human Intruder test, suggesting greater emotionality [3]. Thus, it is likely that the DCA effects from the different studies are overlaid on basic differences in emotionality arising from the rearing conditions themselves.

Second, the differences between the studies could reflect genotype by environment interactions. Studies have demonstrated that there are genetic differences between rhesus monkeys of Chinese and Indian origin [13, 27]. Other studies have demonstrated that nursery-rearing does interact with genetics to affect biobehavioral outcomes [15]. Therefore, it is possible that the effects of rearing (nursery versus field-cage) may affect behavioral outcomes differentially based on some genetic factor that distinguishes animals of Chinese versus Indian origin.

Finally, differences in testing conditions and data collection methods between the current study and previous ones also likely contribute to the differences in results. For example, one of the previous studies tested the animals in their living cage with their cage mates [7], while the animals in our study were tested while alone in their cages and following a maternal separation. Moreover, in another study, aggression was measured by a collection of behaviors such as bites, hairpulls, aggressive chases, threats, hitting, or slapping observed in a social setting [7]; in the current study, aggression was inferred from frequencies of bark, threat, and cageshake during a nonsocial assessment.

Implications and future work

The findings in the current study provide good evidence that the relationship between DCA and behavioral characteristics of infant rhesus macaques may be more complex than expected. Based on the behavioral differences found between pure Indian and 50% hybrid animals in previous research [5, 7], one might naturally assume a linear relationship between DCA and behaviors such that pure Chinese animals would be even more different from the Indian ones than are the 50% hybrids. However, our findings have challenged this assumption. Our results indicate that DCA may not always have linear effects on different dimensions of emotionality—both Indian-origin and Chinese-origin rhesus macaques appear to resemble each other in emotional responses (especially fear-related responses) under conditions of challenge, and it is the hybrids that appear most different from the more pure Indian- or Chinese-origin animals.

The current study has some limitations, but also provides some opportunities for future work. Additional behavioral measures in both novel and familiar environments (and especially in social settings) on animals born in field cages would be helpful to validate and extend the current findings. Moreover, it would be useful to study mother-infant interactions to better understand the extent to which infant differences, such as those seen in the present study, are a reflection of variation in maternal care, which we know can impact behavior and immune function (Vandeleest, Mendoza, and Capitanio, in prep). Future research might also examine the extent to which the behavioral differences that we have identified might persist into adulthood. For example, do adult Indian-origin monkeys remain more fearful than hybrids with intermediate DCA during challenging situations? Examination of a broader array of physiological measures in socially-reared animals ranging in DCA might provide insight into the value that such animals might have for biomedical research. Finally, better genetic characterization of animals from different locations may help us to better understand the differences between animals of different geographic ancestry.

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Figure 1.
Linear effects of DCA on Day 1 fear grimace

Figure 2.
Quadratic effects of DCA on stare far fear grimace

Figure 3.
Quadratic effects of DCA on stare near screech

Figure 4.
Quadratic effects of DCA on ratings of fearful

Figure 5.
Quadratic effects of DCA on stare far yawn

Table 1

	Assessments	Measures	Definition
Fearfulness	Holding Cage Observation & Human Intruder Test	Feargrimace	Exaggerated grin with teeth showing
		Screech	Intense, very high pitched
	Temperament Rating	Fearful	Fear grins; retreats readily from others or from outside disturbances.
Aggression	Holding Cage Observation & Human Intruder Test	Bark	Gruff, abrupt low-pitched vocalization
		Threat	Scored with at least two or more of the following: open mouth stare, head bob, ear flaps, bark vocalizations.
	Temperament Rating	Cageshake	Holding onto cage and shaking it, generating a lot of noise
		Aggressive	High frequency of displays; threats.
Anxiety	Holding Cage Observation & Human Intruder Test	Gentle	Subject responds to technicians in an easy-going, kind, and considerate manner; subject is not rough or threatening.
		Coo	Medium-pitched, moderately intense, clear call
		Scratch	Common usage.
	Temperament Rating	Yawn	Wide open mouth displaying teeth.
		Nervous	Jittery, anxious, seems to be anxious about everything.
		Calm	Reacts in an even, calm way; is not easily disturbed; not agitated; restful, peaceful.
Activity level	Holding Cage Observation	Flexible	Adapts to situations; is able to accommodate new ways of doing things.
		Locomote	Directed movement from one location to another.
	Human Intruder Test	Hang	Holding onto ceiling, sides or front of the cage; all four limbs off of floor
		Active	Whole body movement; step, jump.
	Temperament Rating	Hang	Holding onto ceiling, sides or front of the cage; all four limbs off of floor
		Active	Moves about a lot, distance traveled by walking, running, climbing or jumping; not lethargic.

Table 2

DCA				
Measures	0~25%	25.01~50%	50.01~75%	75.01~100%
Day 1 feargrimace	0.060(.03)	0.000(.00)	0.000(.00)	0.002(.00)
Stare far feargrimace	0.151(.07)	0.000(.00)	0.000(.00)	0.060(.06)
Stare near screech	0.967(.48)	0.000(.00)	0.000(.00)	0.290(.24)
Ratings of fearfulness	2.459(.12)	2.083(.29)	2.182(.30)	2.466(.10)
stare far yawn	0.053(.05)	0.165(.17)	0.347(.27)	0.017(.01)
N	109	12	11	117