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The Effect of Rearing Experience and TPH2 Genotype on HPA Axis Function and Aggression in Rhesus Monkeys: a Retrospective Analysis

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Abstract

Gene-environment (G×E) interactions contribute to the development of many neuro-psychiatric disorders. Tryptophan hydroxylase-2 (TPH2) synthesizes neuronal serotonin and is closely related to the hypothalamic-pituitary-adrenal (HPA) axis, while early-life experience is a critical environmental factor programming the HPA axis response to stress. This retrospective study investigated G×E interaction at the *TPH2* locus in rhesus monkeys. 28 adult, male rhesus monkeys of Indian origin, either mother-reared or peer-reared as infants, were involved in this study. These monkeys have been previously genotyped for the functional A2051C polymorphism in *rhTPH2*, and had been physiologically and behaviorally characterized. *rhTPH2* A2051C exerted a significant main effect (CC>AA&AC) on the cerebrospinal fluid (CSF) level of 5-hydroxyindole-3-acetic acid (5-HIAA) ($F_{(1,14)}=6.42, p=0.024$), plasma cortisol level in the morning ($F_{(1,18)}=14.63, p=0.002$) and cortisol response to ACTH challenge ($F_{(1,17)}=6.87, p=0.018$), while the rearing experience showed a significant main effect (PR>MR) on CSF CRH ($F_{(1,20)}=11.66, p=0.003$) and cage shaking behavior ($F_{(1,27)}=4.45, p=0.045$). The effects of *rhTPH2* A2051C on the afternoon cortisol level, plasma ACTH level, dexamethasone suppression of urinary cortisol excretion, and aggression were dependent upon the rearing experience. These results were not confounded by the functional C77G polymorphism in the mu-opioid receptor (MOR). The present study supports the hypothesis that rearing experience and *rhTPH2* A2051C interact to influence central 5-HT metabolism, HPA axis function, and aggressive behaviors. Our findings strengthen the involvement of G×E interactions at the loci of serotonergic genes and the utility of the nonhuman primate to model G×E interactions in the development of human neuropsychiatric diseases.

Keywords

Tryptophan hydroxylase-2; genotype; rearing experience; gene-environment interaction; serotonin; HPA axis; aggression; mu-opioid receptor; C77G polymorphism; rhesus monkey

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Introduction

It is now recognized that genetic and environmental factors interact to account for variation in psychopathology across the life span. Gene-environment (G×E) interaction is a common phenomenon which occurs when environmental influences on a trait differ according to a person's genetic predispositions, or when a person's genes are differentially expressed in different environments. Research aimed at identifying risk and protective factors and etiological pathways for psychiatric disorders needs to consider G×E interactions in order to reveal true genetic and environmental effects and avoid false negative results that can lead to inconsistent findings in the literature (Tsuang et al., 2004; Caspi and Moffitt, 2006; Thapar et al., 2007).

Tryptophan hydroxylase-2 (TPH2) is the second, brain-specific isoform of TPH which catalyzes the first and rate-limiting step for the synthesis of serotonin (5-HT) (Cote et al., 2003; Walther and Bader, 2003; Walther et al., 2003; Zhang et al., 2004), a major neurotransmitter implicated in many brain functions. Like other components of the 5-HT system, such as the 5-HT transporter (5-HTT) and monoamine oxidase A (MAOA), TPH2 contributes to the regulation of 5-HT neurotransmission and therefore represents a potential therapeutic target for the treatment of neuropsychiatric disorders related to 5-HT dysfunction. TPH2 gene expression exhibits a circadian rhythm and is regulated by a number of hormones and stressors (Clark et al., 2005; Malek et al., 2005; Sanchez et al., 2005; Brown et al., 2006; Hiroi et al., 2006; Malek et al., 2007; Rahman and Thomas, 2009). Particularly, the rhythmic expression of TPH2 is induced by the daily surge of corticoids released upon the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Malek et al., 2007), a critical neuroendocrine system that responds to stress, supporting the notion of reciprocal interactions between 5-HT and the HPA axis (Dinan, 1996; Lowry, 2002). In addition, TPH2 gene expression is modulated by adverse experience during both early life and adulthood (Mueller and Bale, 2008; Gardner et al., 2009). Thus, TPH2 gene expression is frequently modulated under specific physiological and stress conditions, presumably representing a mechanism underlying the activation and/or feedback of HPA axis. Accordingly, genetic or epigenetic factors affecting TPH2 expression or enzymatic activity may alter 5-HT neurotransmission and HPA axis function, and thereby lead to variation in behavioral traits and susceptibility to stress-related neuropsychiatric disorders. In support of this presumption, HPA axis reactivity has been shown to be a potential mechanism underlying the associations between the 5-HTT promoter region polymorphism (5-HTTLPR), stress and depression (Gotlib et al., 2008).

The human *TPH2* (*hTPH2*) gene is localized to chromosome 12 and an increasing number of studies have linked genetic variance in *hTPH2* to a wide variety of endophenotypes and behavioral traits or neuropsychiatric disorders, such as the amygdala responsiveness (Brown et al., 2005; Canli et al., 2005; Furmark et al., 2008; Furmark et al., 2009), personality traits (Gutknecht et al., 2007; Reuter et al., 2007b), cognitive control and emotional processing (Herrmann et al., 2007; Strobel et al., 2007; Canli et al., 2008; Osinsky et al., 2009), major depression (Zill et al., 2004a; Zhou et al., 2005; Van den Bogaert et al., 2006), suicidality (Zill et al., 2004b; Ke et al., 2006; de Lara et al., 2007; Jollant et al., 2007; Lopez et al., 2007), attention deficit hyperactivity disorder (ADHD) (Sheehan et al., 2005; Walitza et al., 2005), and drug addiction (Reuter et al., 2007a; Nielsen et al., 2008). Likewise, *hTPH2* is associated with the antidepressant response to the selective serotonin reuptake inhibitors (SSRIs) (Peters et al., 2004; Tzvetkov et al., 2008; Tsai et al., 2009). However, it is noteworthy that there are some discrepancies between studies on the association of *hTPH2* with neuropsychiatric disorders (i.e., significant associations were reported in some but not all populations). For example, despite the replicated association with major depression and suicidality (Zill et al., 2004a; Zill et al., 2004b; Zhou et al., 2005; Ke et al., 2006; Van den Bogaert et al., 2006; de Lara et al., 2007; Jollant et al., 2007; Lopez et al., 2007), *hTPH2* genetic variance was not

associated with either completed suicide in Estonian males (Must et al., 2009) or stress-induced depression (Gizatullin et al., 2008; Must et al., 2009). Thus, the behavioral translation of *hTPH2* genetic variance may vary with environmental factors.

The study of how genes and environment interact in humans is potentially difficult, as human environments cannot be easily manipulated experimentally; thus animal models can prove to be useful tools for studying G×E interactions. As the most widely used nonhuman primate in biomedical research, rhesus monkeys share genetic, physiological and behavioral similarity to humans so that they take advantage over rodents as an appropriate model for human diseases. In addition, rhesus monkeys harbor functionally parallel, though often non-identical, polymorphisms that mimic human genetic variants *in effect*, and accordingly, have emerged as a highly translational model for studying G×E interactions relevant to neuropsychiatric disorders. Notably, while a recent meta-analysis showed that G×E interactions at the 5-*HTT* locus are relatively unimportant and possibly not real in humans (Risch et al., 2009), G×E interactions at the 5-*HTT* and *MAOA* loci have been nicely replicated in monkeys (Bennett et al., 2002; Barr et al., 2004a; Barr et al., 2004b; Newman et al., 2005; Karere et al., 2009), most likely because the environment can be closely controlled in monkeys but not in humans. Thus, nonhuman primates provide unique opportunities to study the G×E interactions on physiology and complex behavior, and to help us understand the non-homogeneous effect of genes across environments.

As a novel component of the 5-HT system, TPH2 has received much attention since its identification in 2003. We have previously identified a constellation of polymorphisms in rhesus monkey *TPH2* (*rhTPH2*), among which the A2051C SNP in the 3'-untranslated region (UTR) differentiates gene expression and HPA axis function, with the CC homozygotes showing significantly higher morning cortisol level than AA homozygotes and AC heterozygotes (Chen et al., 2006). This SNP was further demonstrated to exert comprehensive effects on physiological and behavioral traits, independent of other polymorphisms in the 5' regulatory region of *rhTPH2* (in submission). Given that *TPH2* is highly polymorphic and its gene expression is frequently modulated by various environmental factors, and that inconsistent findings have been reported in *TPH2* association studies in humans, we speculate that G×E interactions also exist at the *TPH2* locus in rhesus monkeys. To test this, we re-examined data derived from a cohort of previously phenotyped and genotyped monkeys including a novel variable in our analyses, early-life experience (i.e., rearing experience), which has been shown to induce enduring neuroplasticity of the HPA axis (Liu et al., 1997; Mirescu et al., 2004; Plotsky et al., 2005; Sánchez et al., 2005; Fenoglio et al., 2006), as well to modulate *TPH2* and 5-*HTT* gene expression (Mueller and Bale, 2008; Gardner et al., 2009; Kinnally et al., 2008). The re-examination of *in vivo* functionality of *rhTPH2* genotype in the context of rearing experience makes it possible for us to further define the effects of genes, environments, and their interaction, on physiology and behavioral traits.

Methods

Animals

Amongst data derived from a cohort of 32 unrelated, adult male rhesus monkeys (*Macaca mulatta*, Indian origin) that were housed at the New England Primate Research Center, data from 28 subjects with available early rearing history as infants were included for analysis. Sixteen of the 28 monkeys were mother-reared (MR) in social groups, while the other 12 animals were reared without adults, instead with constant access to monkeys of similar age in a "peer-only reared" (PR) condition.

The general procedure for peer-rearing a monkey was to separate the monkey from its mother immediately at birth and place the infant in the nursery with 1–3 other similarly aged monkeys

until weaned, and monkeys were then placed in a much larger group of peers. All the PR monkeys were reared for at least 11 months with a group of peers (of varying sizes). The age at which peer-reared animals were placed into the large peer group ranged from 4–14 months (average 7.2 ± 1.2 months). Nine of the twelve PR monkeys were then transferred to individual housing between 12 and 55 months (average 32.8 ± 5.4 months), while the other three PR monkeys were placed directly into individual cages from their small peer groups at an average of 11.7 ± 0.3 months. Of the 16 MR monkeys, six were reared first with their mothers and other peers before being transferred into a large peer-group. These monkeys remained with their mother for 1–7 months before being separated from their mothers and transferred into the larger peer group (average 4.1 ± 0.9 months). All six of these monkeys entered individual housing at 24 months. The remaining MR monkey for which we have information were first housed with only their mother before being transferred to a peer group at 5 months of age with subsequent individual housing at 12 months. Unfortunately, specific details of the mother-rearing condition are unknown for 11 MR monkeys, although we do know that they were reared with their mothers before being transferred to individual housing. With regard to the equitability of treatment of the different groups, there was only a marginally significant difference in the age at which monkeys from either group were transferred to large peer groups (from which point experiences would have been relatively equivalent), with MR monkeys being transferred marginally earlier than were PR animals ($t=2.00, p=0.065$). Given the large amount of unknown detail for the remaining monkeys, we do not believe we have ample evidence to conclude that monkeys from either rearing experience were treated significantly differently during the first few months of rearing with the exception that PR animals were reared apart from their mothers and had much greater contact with human caretakers (both key components of peer-rearing). We believe, then, that the differences in physiological measures presented in this paper between rearing groups (or as interactions between genetics and rearing conditions) accurately reflect differences in early life experience.

All monkeys were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the *Guide for Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, publication No. (NIH) 85–23, revised 1985. The Harvard Medical School animal management program is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and meets National Institutes of Health standards as set forth in the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. (NIH) 85–23 Revised 1985). The institution also accepts as mandatory the *PHS Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions* and *NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training*.

Measurement of the cerebrospinal fluid (CSF) level of 5-hydroxyindole-3-acetic acid (5-HIAA) and HPA axis function

The CSF level of 5-HIAA (a major metabolite of 5-HT) and HPA axis function were measured between 1995 and 2000, with all the procedures except for the CSF corticotropin-releasing hormone (CRH) assay having been described elsewhere (Tiefenbacher et al., 2000; Tiefenbacher et al., 2004). Briefly, CSF samples were collected between 1000h and noon in 1995 and 1997, following an overnight fast and Telazol (5 mg/kg, IM) anesthetization. The CSF level of 5-HIAA was measured by the isocratic high-performance liquid chromatography with electrochemical detection (HPLC–EC). An AM-PM study was carried out in December 1997 to measure the plasma cortisol and adrenocorticotrophic hormone (ACTH) levels in both the early morning (0830–0900h) and mid-to-late afternoon (1530–1600h). The plasma levels of cortisol and ACTH were measured using commercial RIA kits (Tiefenbacher et al., 2000). In addition, the dexamethasone (DEX) suppression of urinary free cortisol excretion during the night (DEX-N, urine fraction collected at 0800h) and day (DEX-D, urine fraction collected

at 1600h), as well as the plasma cortisol response to combined DEX/ACTH challenge at 15 and 30 min, were performed in 1999 and 2000, respectively (Tiefenbacher et al., 2004).

Additional CSF samples were collected in 2000 for the measurement of CRH. Following an overnight fast and ketamine (10 mg/kg, IM) anesthetization, as well as the prophylactic treatment with buprenorphine (0.004 mg/kg, IM) to avoid any potential side-effects of the procedure such as headaches, 1–2 ml of CSF was withdrawn from the cisterna magna at 0900h. CRH levels were determined in C18 Sep-Pak extracted CSF samples using a commercially-available RIA kit (Advanced ChemTech, Louisville, KY).

Assessment of aggressive behavior

As described previously (Novak et al., 1992; Novak et al., 1998; Miller et al., 2004), individually housed monkeys were observed systematically between 1994 and 2002 using a modified frequency scoring system in which a 5-min sampling period was subdivided into 20, 15-second intervals and the presence/absence of 36 behavioral categories was noted for each interval. Modified frequencies (the total number of intervals in which the behavior occurred) were summed for all behavioral categories. A total of 334.3 ± 6.9 (mean $\pm$ SEM), 5-min samples were obtained from each monkey. Several indices of aggression were assessed that included threat, the early communicative aspect of aggression involving a staring, open-mouthed, teeth-baring, ear-flapping facial display and other more physical actions including cage shaking, environment-directed and self-directed aggression. Actual contact aggression was not assessed because the monkeys were housed individually. Half the observations occurred in the morning between 0900 and 1030 h, with the remainder in the afternoon between 1300 and 1430 h. Observations were carried out by observers trained to a >90% inter-observer reliability criterion using a percent agreement score. All behavioral observations were completed prior to any genetic assessments.

Genotyping for the rhTPH2 A2051C polymorphism

The A2051C polymorphism, along with another two linked polymorphisms (A1053G and S2128L) in the 3'-UTR of rhTPH2 were genotyped for all the monkeys in our previous study (Chen et al., 2006). Briefly, the segment of rhTPH2 3'-UTR spanning the A2051C and S2128L loci were amplified using the primer set TPH2-U3F5 (5'-tgtagaaacttctcatcaca-3') and TPH2-U3R5 (5'-cagcataaaattcatagtcccaag-3'), followed by *PsiI* digestion and subsequent electrophoresis for genotype interpretation. Because the alternate PCR amplicon yielded by the two alleles of S2128L may confound the A2051C genotype interpretation, we also used DNA sequencing to validate the genotype for specific subjects. Since 2051C-allele homozygotes (CC genotype) showed significantly higher morning cortisol level and cortisol response to ACTH challenge than 2051A-allele carriers (AA and AC genotypes, AA&AC for abbreviation) (Chen et al. 2006), monkeys are classified into two genotype groups: CC and AA&AC.

Data analysis

Statistics were performed using the SAS Software Version 9.1 (SAS Institute Inc., USA). The main effects of rearing condition (environmental factor with two levels: MR and PR) and rhTPH2 A2051C genotype (genetic factor with two levels: CC and AA&AC), as well as their interaction on phenotypic variables, were evaluated by two-way analysis of variance (ANOVA2) using the GLM procedure and Type III Sums-of-Squares (SS), followed by multiple comparisons of Tukey-Kramer least-squares means. Repeated measures ANOVA of one-way (RANOVA1) or two-way (RANOVA2) were carried out for phenotypic variables measured on two or more occasions, with RANOVA2 being used for two factors (rearing condition and A2051C) and RANOVA1 being used for only one factor (either rearing condition or A2051C) when the other factor was fixed. Pair-wise comparisons between individual groups

were additionally performed by t test using the TTEST procedure. For some analyses (for which we had specific predictions), post hoc tests were conducted in the absence of significant findings in the overall ANOVA. Since the directions of specific differences might be predicted, and small sample size has a negative effect on the statistical significance, we also give statistics for one-tailed t test in some cases. These analyses were intended to reveal potential interactions that may have been obscured by increased variability and small sample size.

Results

The effects of rearing experience and *rhTPH2* A2051C on CSF levels of 5-HIAA and CRH are shown in Figure 1A and 1B, respectively. Overall, while there was no effect of rearing experience (RANOVA2: $F_{(1, 14)}=1.70$, $p=0.214$), the A2051C genotype exerted a significant main effect on CSF 5-HIAA concentrations, with the 2051C-allele homozygotes (CC) showing higher 5-HIAA level than 2051A-allele carriers (AA and AC genotypes, AA&AC for abbreviation) (RANOVA2: $F_{(1, 14)}=6.42$, $p=0.024$); this effect of A2051C trended towards significance in PR monkeys (RANOVA1: $F_{(1, 6)}=4.59$, $p=0.076$) but was not significant in MR monkeys (RANOVA1: $F_{(1, 8)}=1.29$, $p=0.290$). As shown in Figure 1B, the rearing experience showed a significant main effect on CSF CRH (ANOVA2: $F_{(1, 20)}=11.66$, $p=0.003$), irrespective of the *rhTPH2* A2051C genotype (Mean±SD: 60.6±15.5 for MR vs 85.2±16.3 for PR; $t=3.03$, $p=0.039$ in CC group and $t=2.61$, $p=0.022$ in AA&AC group). In contrast, the A2051C genotype showed no significant main effect on CSF CRH levels (ANOVA2: $F_{(1, 20)}=1.23$, $p=0.283$).

The effects of rearing experience and *rhTPH2* A2051C genotype on plasma cortisol and ACTH levels, plasma cortisol response to ACTH challenge and DEX suppression of urinary free cortisol excretion are shown in Figure 2A–D, respectively. The A2051C genotype exerted a significant main effect on AM cortisol levels (CC>AA&AC; ANOVA2: $F_{(1, 18)}=14.63$, $p=0.002$), but not on PM cortisol levels (ANOVA2: $F_{(1, 18)}=0.09$, $p=0.772$). Rearing experience showed no significant effect on either AM or PM cortisol levels; however, there was a marginal time by rearing by genotype interaction (RANOVA2: $F_{(1, 15)}=3.12$, $p=0.098$). Exploration of this potential interaction revealed a marginally significant interaction of rearing experience and A2051C genotype on PM cortisol level (ANOVA2: $F_{(1, 18)}=3.80$, $p=0.070$), such that PR/AA&AC monkeys had slightly higher cortisol levels than MR/AA&AC monkeys at that time point (20.4±7.7 vs 13.7±4.5; $t=1.91$, $p=0.085$). As shown in Figure 2B, neither rearing experience nor A2051C genotype exhibited a significant main effect on ACTH levels; however, in CC group the PR monkeys showed a significant lower PM ACTH level than MR monkeys (41.9±4.8 vs 67.4±15.0; $t=3.33$, $p=0.021$), while in PR group the CC monkeys tended to have lower PM ACTH level than AA&AC (41.9±4.8 vs 64.7±20.4; $t=2.18$, one-tailed $p=0.054$). Accordingly, CC/PR monkeys showed the lowest PM cortisol level. It is noteworthy that such a tendency of ACTH pattern was also observed for the AM sample, as well as for another two sampling occasions in the late morning (data not shown). As shown in Figure 2C, A2051C showed a significant main effect on plasma cortisol response to ACTH challenge (CC>AA&AC; RANOVA2: $F_{(1, 17)}=6.87$, $p=0.018$) while the rearing experience did not, but there was a tendency for MR monkeys to show a higher response than PR monkeys (16.4±4.1 vs 12.3±4.0 at 15 min; $t=1.51$, one-tailed $p=0.075$). As shown in Figure 2D, for both DEX-N and DEX-D samples, CC monkeys exhibited lower DEX suppression of urinary cortisol excretion than AA&AC (RANOVA1: $F_{(1, 9)}=7.02$, $p=0.027$) in the MR group, while the MR subjects showed higher suppression (RANOVA1: $F_{(1, 10)}=7.43$, $p=0.021$) in the AA&AC group. There was a marginally significant main effect of A2051C genotype on DEX-D (CC<AA&AC; ANOVA2: $F_{(1, 16)}=4.18$, $p=0.062$).

The effects of rearing experience and *rhTPH2* A2051C genotype on aggressive behavior (threat and cage shaking) are shown in Figure 3A and 3B. Neither rearing experience nor *rhTPH2*

A2051C had a significant main effect on aggressive threat; however, in the MR group CC monkeys exhibited significantly lower threat behavior than AA&AC (0.091 ± 0.023 vs 0.226 ± 0.111 ; $t=4.03$, $p=0.001$), while in the 2051CC group the MR monkeys tended to have lower aggressive threat than PR monkeys (0.091 ± 0.023 vs 0.190 ± 0.084 ; $t=1.93$, one-tailed $p=0.056$). The rearing experience, but not *rhTPH2* A2051C, exerted a significant main effect on cage shaking (MR<PR; ANOVA2: $F_{(1, 27)}=4.45$, $p=0.045$), especially in AA&AC group (0.092 ± 0.066 for MR vs 0.229 ± 0.143 for PR; $t=2.55$, $p=0.031$).

We have previously demonstrated in this cohort of monkeys that a functional SNP (C77G) in the mu-opioid receptor (MOR) is also linked to HPA axis function and aggression (Miller et al., 2004). To ensure that our findings on *rhTPH2* A2051C and *rhMOR* C77G polymorphisms were not confounded by each other, as well as to identify the potential interaction between them, we re-analyzed the data by taking *rhMOR* C77G into account. Due to few subjects in MR subgroups, only PR monkeys were included for the analysis of HPA axis function. We found that the significant main effect of *rhTPH2* A2051C on AM cortisol level was independent of the *rhMOR* C77G; however, as shown in Figure S1A, with the inclusion of *rhMOR* C77G for data analysis, both the *rhTPH2* and *rhMOR* genotypes exhibited a significant main effect on PM cortisol level (ANOVA2: $F_{(1, 7)}=9.19$, $p=0.039$ for *rhTPH2* and $F_{(1, 7)}=16.03$, $p=0.016$ for *rhMOR*), and there was a marginally significant interaction between them (ANOVA2: $F_{(1, 7)}=5.05$, $p=0.088$). The *rhMOR* C77G tended to affect the cortisol response to ACTH challenge, especially at 15 min post the challenge (CC>CG&GG; ANOVA2: $F_{(1, 7)}=7.47$, $p=0.052$), and both *rhTPH2* and *rhMOR* genotype exerted their effects independently (shown in Figure S1B). We found no significant effect of *rhMOR* C77G, as well as no interaction of *rhTPH2* and *rhMOR* genotypes on CSF 5-HIAA and CRH, plasma ACTH, and DEX suppression of cortisol excretion (data not shown). As shown in Figure S2A, *rhMOR* C77G showed a significant main effect on aggressive threat in 2051AA&AC monkeys (CC<CG&GG; ANOVA2: $F_{(1, 17)}=5.96$, $p=0.026$), in consistence with our previous findings (Miller et al., 2004). Just like the case shown in Figure 3, in *rhMOR* 77CC monkeys there was a marginally significant interaction between *rhTPH2* A2051C and rearing experience on aggressive threat (ANOVA2: $F_{(1, 12)}=4.99$, $p=0.052$). In addition, *rhTPH2* and *rhMOR* genotypes tended to interact to influence the aggressive threat (ANOVA2: $F_{(1, 11)}=4.05$, $p=0.079$). In contrast, the effects of rearing experience on cage shaking were not confounded by *rhMOR* C77G (Figure S2B). Moreover, the effects of rearing experience and *rhTPH2* A2051C as well as their interactions on HPA axis function and aggressive behaviors were not essentially influenced by *rh5-HTTLPR* genotype (long vs short alleles; data not shown).

Discussion

Both genetic predisposition and environmental exposure contribute to the development of psychological traits, and G×E interaction in psychopathology has recently been a focus of investigation. As a critical neuroendocrine system that serves to maintain homeostasis in response to environmental changes, HPA axis function represents an excellent example of a complex endophenotype for which G×E interaction is important. It has been well-documented that reciprocal interactions exist between 5-HT and the HPA axis (Dinan, 1996; Lowry, 2002). Among components of the 5-HT system, 5-HTT has been extensively investigated and the *rh5-HTTLPR* polymorphism interacts with rearing experience to influence CSF 5-HIAA, HPA axis reactivity, and alcohol addiction (Bennett et al., 2002; Barr et al., 2004a; Barr et al., 2004b). Similarly, *rhMAOALPR* polymorphism and rearing experience interact to influence behavioral traits including aggression (Newman et al., 2005; Karere et al., 2009). As a novel component of the 5-HT system, *TPH2* gene expression is influenced by both genetics (Chen et al., 2006; Chen and Miller, 2008; Chen et al., 2008) and early-life stress (Mueller and Bale, 2008; Gardner et al., 2009), making it very likely that G×E interaction also exists at the

TPH2 locus. To test this assumption, this retrospective study examined the potential interaction of rearing experience and *rhTPH2* genotype on HPA axis function and aggressive behavior.

This study revealed that *rhTPH2* A2051C exerts a significant effect (CC>AA&AC) on CSF 5-HIAA, with the effect being pronounced in PR monkeys. Similarly, it has been previously reported that the *rh5-HTTLPR* polymorphism differentiates CSF 5-HIAA in PR but not MR rhesus monkeys (Bennett et al., 2002). However, while a number of studies have reported alteration in CSF 5-HIAA concentration by rearing experience (PR<MR in most cases) (Coplan et al., 1998; Higley et al., 1991; Shannon et al., 2005), we found no main effect of rearing experience on CSF 5-HIAA, except that in 2051CC homozygotes there was an apparent but non-significant elevation of CSF 5-HIAA by peer-rearing (Figure 1A). This discrepancy might be caused by differences in demographics (age for instance) of the subjects, maternal deprivation procedure, and sampling time during the day. As for the CSF CRH, the rearing experience exhibited a significant main effect (PR>MR) regardless of *rhTPH2* genotype, while *rhTPH2* A2051C showed no significant main effect. In agreement with our findings, bonnet macaques exposed to the early-life stressor - variable foraging demand rearing (VFDR) showed persistent elevation of CSF CRH (Coplan et al., 1996; Coplan et al., 2001), and maternal care was associated with low CSF CRH expression in rat, presumably resulting from thyroid hormone- and 5-HT-mediated increase in hippocampal glucocorticoid receptor (GR) expression and consequent up-regulated HPA negative feedback (Liu et al., 1997; Sapolsky, 1997; Meaney et al., 2000). Consistently, this study demonstrated that MR monkeys had significantly higher DEX suppression of cortisol excretion than PR monkeys in the 2051AA&AC group, supporting the notion that rearing experience influences regulation of GR and HPA negative feedback (Liu et al., 1997). In addition, we found that PR monkeys showed a lower plasma ACTH level than MR monkeys in 2051CC group, while such a difference has been previously reported without considering any genetic influence (Barr et al., 2004b; Cirulli et al., 2009). Nevertheless, despite the consistence with previous findings, our present analysis has revealed for the first time that rearing experience and *rhTPH2* genotype interact to influence plasma ACTH levels and HPA negative feedback. Since 5-HT is involved in the early-life programming of HPA axis reactivity by maternal care, and *TPH2* gene expression is regulated by both genetics and early-life stress, it is not surprising that rearing experience and *rhTPH2* genotype may interact to influence HPA axis function and associated behavioral traits.

While the *rhTPH2* A2051C genotype exerted a significant main effect on the morning cortisol level and cortisol response to ACTH challenge, the rearing experience only interacted with *rhTPH2* genotype to influence the PM cortisol level and probably had a minor modification effect on the cortisol response to ACTH challenge. These findings, along with the findings on CSF 5-HIAA and CRH, strongly suggest that 5-HT neurotransmission variation conferred by *rhTPH2* A2051C may primarily exert its effect on HPA axis activation (in the early morning or in response to ACTH stimulation) by mechanisms other than stimulation of CRH release, while CRH variation is influenced to a greater extent by rearing experience and may primarily contribute to the basal HPA axis activity. In support of this presumption, CSF CRH in primates peaks in the evening and is inversely related to the cortisol circadian rhythm (Garlick et al., 1987), and there was an apparent inverse correlation between CSF 5-HIAA and CRH in our study (Figure 1A and 1B).

In accordance with the well-documented disassociation of ACTH and cortisol (Bornstein *et al.* 2008), the plasma ACTH and cortisol levels were not parallel in the cohort of monkeys, especially for the AM sample (Figure 2A and 2B). It has been recognized that the pituitary and adrenal gland activation are differentially regulated. For example, the rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock, which determines the adrenal response to ACTH stimulation (Oster *et al.* 2006; Torres-Farfan *et al.*

2009). In addition, increasing evidence indicates that ACTH-independent mechanisms may have an important role in fine-tuning and modulating the highly sensitive adrenal stress system to adapt its response appropriately to physiological needs (Bornstein *et al.* 2008; Dickmeis 2009). In our present study, while the plasma cortisol differed strikingly between AM and PM (especially in 2051CC group), the plasma ACTH showed no remarkable AM-PM fluctuation, implying that the cortisol response to ACTH stimulation is sensitive in the morning but not in the afternoon. In agreement with our findings, the rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock (Oster *et al.*, 2006), and ACTH has no effect on cortisol production if the adrenal clock is not ticking as it should be (Torres-Farfan *et al.*, 2009). In addition, while 2051CC showed a significantly higher morning cortisol level and cortisol response to ACTH challenge than 2051AA&AC, the morning ACTH level of 2051CC was similar to 2051AA&AC (in MR monkeys) or even lower than that of 2051AA&AC (in PR monkeys), suggesting it is the HPA axis reactivity to ACTH stimulation, but not the ACTH level, that contributes to the morning cortisol difference between the *rhTPH2* A2051C genotypes. Thus, it can be inferred that the HPA axis reactivity to ACTH stimulation is time-dependent and can be modified by genetic factors.

It has been well established that 5-HT is involved in aggression (Olivier, 2004; Miczek *et al.*, 2007; Siever, 2008). In particular, it has been reported that both *MAOA* and *5-HTT* polymorphisms interact with rearing experience to influence aggressive behavior (Barr *et al.*, 2003; Newman *et al.*, 2005). Likewise, our present analysis demonstrates that rearing experience and *rhTPH2* A2051C polymorphism interact to influence aggressive threat, with the 2051CC associated high morning cortisol level showing significantly lower aggressive threat than 2051AA&AC in the MR but not the PR group, while the PR monkeys tended to exhibit higher aggressive threat than the MR monkeys in the 2051CC group. In contrast, the rearing experience exerted a significant main effect on cage shaking while *rhTPH2* A2051C did not, with the PR monkeys showing significant higher cage shaking than the MR subjects, especially in the 2051AA&AC group. Like the case for *rhTPH2* A2051C, *rhMOR* C77G also showed a significant effect on the aggressive threat but not cage shaking, suggesting that the cage shaking behavior might be preferentially determined by CSF CRH and basal HPA axis activity. These findings suggest that distinct aspects of the HPA axis may underlie distinct behaviors of some relatedness. In support of this assumption, it has been reported in rhesus monkeys that the AM and PM cortisol levels are differentially associated with the personality traits of confidence and excitability, respectively (Capitanio *et al.* 2004). Similarly, we found that the two forms of self-injurious behavior, self-wounding and self-biting, are likely linked to distinct aspects of the HPA axis (in submission).

It should be noted that due to the limitation of small sample size, our findings are preliminary and require further verification (especially by prospective studies); however, the validity of some specific findings might to some extent be reinforced by the repeated measurements for some variables, as well as by the compliance with findings of previous studies. For example, the effects of *rhTPH2* A2051C polymorphism on the morning cortisol level and cortisol response to ACTH challenge, as well as the effect of rearing experience on CSF level of CRH, are remarkable and independent of the other factor. In particular, the effect of *rhTPH2* A2051C polymorphism on the morning cortisol level was replicated by another sampling occasion between 1000 and 1200 h (data not shown). Thus, it is evident that *rhTPH2* A2051C exerts a significant effect on plasma cortisol level upon activation of the HPA axis by circadian or ACTH stimulation. It seems that this effect is much larger than one would expect given the typical 10–20% variance accounted for in most studies of a single gene. However, as mentioned in the Introduction section, there is strong evidence supporting that *TPH2* gene expression regulation is accompanied by the activation and/or negative feedback control of the HPA axis. In other words, *TPH2* gene expression is adapted to comply with the status of the HPA axis. Thus, it is not strange that polymorphism differentiating *TPH2* gene expression may have a

remarkable effect on the cortisol production of HPA axis. Accordingly, our findings suggest that genetic polymorphism of a single gene may exert an unexpectedly large effect on physiology and behavioral traits under specific circumstance. In addition, it is notable that for both ACTH level and DEX suppression test, a similar pattern of G×E interaction was observed for the two time points across the day.

In summary, our present study supports the hypothesis that rearing experience and *rhTPH2* A2051C polymorphism interact to influence the central 5-HT metabolism, HPA axis function, and aggressive behaviors. Our findings strengthen the involvement of G×E interactions at the loci of the major serotonergic genes, and support the utilization of the non-human primate to model the gene-environment interplay in the development of human neuropsychiatric diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The effect of rearing experience and *rhTPH2* A2051C genotype on the CSF levels of 5-HIAA (A) and CRH (B). Data are shown as Mean $\pm$ SEM, and sample size (n) for each group is indicated. The *rhTPH2* A2051C exerted a significant main effect on CSF 5-HIAA levels (CC>AA&AC; RANOVA2: $F_{(1, 14)}=6.42$, $p=0.024$), while the rearing experience showed a significant main effect on CSF CRH (ANOVA2: $F_{(1, 20)}=11.66$, $p=0.003$). * $p<0.05$ by two-tailed t test.

Figure 2.

The effect of rearing experience and *rhTPH2* A2051C genotype on the plasma levels of cortisol (A) and ACTH (B), cortisol response to ACTH challenge (C) and DEX suppression of urinary cortisol excretion (D). Data are shown as Mean±SEM, and sample size (n) for each group is indicated. The *rhTPH2* A2051C genotype exerted a significant main effect on the AM cortisol level (CC>AA&AC; ANOVA2: $F_{(1, 18)}=14.63$, $p=0.002$) and cortisol response to ACTH challenge (CC>AA&AC; RANOVA2: $F_{(1, 17)}=6.87$, $p=0.018$). The effects of rearing experience on the PM cortisol level, ACTH level and DEX suppression of urinary cortisol excretion were dependent on the *rhTPH2* A2051C genotype. * $p<0.05$ by two-tailed t test, # $p<0.05$, ## $0.05\leq p<0.10$ by one-tailed t test.

Figure 3.

The effect of rearing experience and *rhTPH2* A2051C genotype on aggressive threat (A) and cage shaking (B). Data are shown as Mean $\pm$ SEM, and sample size (n) for each group is indicated. In the MR group, the 2051CC monkeys exhibited significant lower aggressive threat than 2051AA&AC monkeys ($t=4.03$, $p=0.001$), while in 2051CC group the MR monkeys tended to have lower aggressive threat than PR monkeys ($t=1.93$, one-tailed $p=0.056$). The rearing experience exerted a significant main effect on cage shaking (MR<PR; ANOVA2: $F_{(1, 27)}=4.45$, $p=0.045$), especially in 2051AA&AC group. * $p<0.05$, ** $p<0.01$ by two-tailed t test; ## $0.05\leq p<0.05$ by one-tailed t test.