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# Serotonin transporter genotype modulates HPA axis output during stress: effect of stress, dexamethasone test and ACTH challenge

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## Abstract

**Background—**Studies show that the hypothalamic–pituitary–adrenal (HPA) axis is dysregulated in depression. Some studies suggest that variation in the serotonin transporter genotype (hereafter *5HTT*) modulates both risk for depression and psychopathological HPA axis responsiveness. Rhesus monkeys are well suited to model such relationships. Rhesus macaque models of human psychopathology have assessed the effect of the serotonin transporter (*rh5HTT*) on levels of cortisol in stressed subjects. These studies show that that under conditions of stress, heterozygous females (Ls) reared under adversity exhibit high levels of cortisol. Studies have not to our knowledge, however, assessed the potential additive effect on the cortisol response in a number of macaque subjects homozygous for the serotonin transporter short allele (ss). Moreover, little is known about the level of the central or peripheral nervous system at which the *5HTT* genotype acts to modulate the cortisol response.

**Methods—**This study assesses a relatively large number of subjects homozygous and heterozygous for the *rh5HTT* short and long alleles (a) during stress; (b) following a dexamethasone suppression test; and (c) following an adrenocorticotropic hormone (ACTH) challenge. Subjects included 190 infant rhesus macaques (*Macaca mulatta* – 84 males and 106 females; 118 LL, 60 Ls, and 12 ss subjects), obtaining two blood plasma samples during the stress of separation from their mothers. Then on the following day, we obtained a blood sample following a dexamethasone test, and later that day we obtained a blood sample after an ACTH challenge test. Subjects ranged in age between 90 and 128 days, with a mean age of 107 days.



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**Conflict of interest**

There is no conflict of interest in the present study for any of the authors.

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**Results—**Subjects homozygous for the short allele had significantly higher levels of cortisol across all test conditions, when compared to those homozygous for the long allele, or those heterozygous with Ls alleles. Subsequent analyses showed a high correlation between individual cortisol levels across the three different tests.

**Conclusions—**These data suggest that subjects homozygous for the short allele are more likely to show dysregulated cortisol levels in response to stress. Given the correlation in individual responses of the HPA axis across the different tests, our data suggest that the effect of the *5HTT* genotype shows some commonality in its regulation of stress, feedback, and ACTH-stimulated cortisol output. Our data suggest that under conditions of stress, the serotonin transporter may modulate HPA axis psychopathology.

## Keywords

Serotonin; stress; cortisol; HPA axis; serotonin transporter genotype; depression; dexamethasone; Nonhuman primate; Rhesus monkey

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A wide variety of studies have shown that hypothalamic–pituitary–adrenal (HPA) axis functioning is modulated, at least in part by central nervous system (CNS) serotonin (1–3), and some studies suggest that genetic differences in the serotonin transporter play a role in this serotonin modulation of the HPA axis (4–6). Studies about the serotonin transporter gene effects on the HPA axis in human subjects show that girls in late childhood and early adolescence with one or two copies of the serotonin transporter short allele (Ls, ss, respectively) show high waking cortisol when compared to the female subjects homozygous for the long allele (LL) (7). Most studies, however, have only found serotonin genotype differences in HPA axis output when environment, sex, hormone, or other environmental influences are considered. Alexander et al., for example, found higher cortisol in male subjects homozygous for the short allele when compared to the other genotypes, but only under conditions of a public speaking stressor and only if the subjects experienced severe prior stressful events (8). Pre- and post-menarcheal girls show higher and more prolonged cortisol output following a stressor than do girls with the Ls or LL genotypes, while showing no differences at baseline (9). Newborns homozygous for the short allele show higher cortisol than infants with the other genotypes, but only after the stressor of a heel stick for blood sampling (10). In a separate study, researchers found higher adrenocorticotropic hormone (ACTH) in subjects homozygous for the short allele when compared to the other genotypes, but only under stressful conditions (11), and in the same study, the ss genotype females exhibited a greater response to stress, showing a higher percentage change in cortisol when compared to males across all three serotonin transporter genotypes, and for females, those homozygous for the short allele also showed higher cortisol when compared to both Ls and LL genotypes (11). An opposite sex interaction was found by Wankerl and colleagues when studying an aging population. They found higher cortisol in the ss genotype subjects when compared to the other two genotypes, but only in men (12). Josephs and colleagues measured cortisol after a variety of social stressors and found that subjects homozygous for the short allele showed higher cortisol than subjects homozygous for the long allele, but only in subjects with high testosterone (13). An additional study reported higher cortisol in subjects with the ss genotype, but it is difficult to ascertain from the report

whether that was a main effect or an interaction (14). With the variety of studies showing a gene×environment or other interacting effects of the serotonin transporter genotype on cortisol levels, it seems reasonable to assume that studies with a large sample size, on average, a main effect of genotype should be detected. In a recent comprehensive meta-analysis, the authors concluded that on average, across studies investigating the effects of stress on cortisol output, subjects homozygous for the short allele exhibit high cortisol reactivity when compared to heterozygotes subjects, as well as subjects homozygous for the long allele (14, 15). Yet other researchers have concluded that few studies have convincingly demonstrated a main effect of serotonin genotype on cortisol output (9, 10), but see Alexander et al. (8) and Gotlib et al. (9) for an exception. To cloud the issue even further, some studies have failed to demonstrate any differences in cortisol levels when comparing the ss genotype to the other two genotypes. Reimold and colleagues, for example, found no differences in baseline, or dex-CRH, stress-induced cortisol levels when comparing across genotypes (15, 16).

As rhesus macaques show HPA axis responses to stress that are closely akin to humans and possess a bivariate serotonin transporter genotype that is orthologous to that found in humans, they are ideally suited to study serotonin genotype effects on HPA axis functioning. Shortly after the discovery of an orthologous serotonin transporter genotype in rhesus macaques (5, 16), Barr et al. published the first study (5) investigating the effect of the serotonin transporter genotype on plasma ACTH and cortisol levels in 208 infant rhesus with Ls or LL genotypes. In this study, both male and female Ls subjects showed significantly higher ACTH following separation stress. When comparing cortisol, the effect was different for males and females with males showing no genotypic variation in cortisol, but females with the Ls genotype exhibited blunted cortisol output, but only if they were reared without parents in peer-only conditions. While this study had a large sample size, there were too few homozygous short allele (ss) animals to compare with the heterozygous and homozygous subjects with the long allele and subsequent studies have had similar problems obtaining sufficient homozygous short allele subjects to make such comparisons. In a separate study of macaques, McCormack and colleagues assessed the effect of the serotonin transporter genotype in abused infants and their mothers. While their sample size was also small and prevented a comparison of the ss homozygotes, they found that Ls subjects exhibited significantly higher plasma cortisol, both as adult and infant macaques (17). Using a small sample, Kraemer et al. also found higher cortisol in macaques with the Ls genotype, but only if the infants had been exposed to alcohol prenatally (18). Jarrell and colleagues found no difference between the subjects homozygous for the short allele and other groups both during baseline, after a dexamethasone test, and following the stress of group reformation (19), but this was only in a group of 40 subjects and the genotypes were not randomly distributed among groups. Thus, while macaques show some evidence of HPA axis modulation by the serotonin transporter genotype, cortisol response in subjects homozygous for the short allele is still somewhat in question.

Nonhuman primates have a long tradition in psychiatry of modeling various aspects of psychiatric disease, and when parallel findings are found in both humans and nonhuman primates, this strengthens the validity of the findings supporting a relationship between the studied variables. Testing a group of nonhuman primate subjects homogeneous for the short

allele would address questions of its role in both nonhuman and human primates, providing compelling evidence for a functional direct effect of stress-mediated serotonin genotype modulation of cortisol output and facilitate the utility of the nonhuman primate model. Given the above mixed findings in humans, and the paucity of studies investigating ss genotype nonhuman primate subjects, we propose obtaining cortisol under stressful conditions from a large group of nonhuman primates with sufficient numbers to allow comparisons of the ss, Ls, and LL genotypes.

Classically, the dexamethasone test has been used to test for abnormalities in depression with depressed individuals showing a reduced suppression of cortisol after an evening dose of dexamethasone (20) and is considered a valid method to assess stress-mediated cortisol output. Kinnally and colleagues studying a small sample of rhesus macaques with the LL versus Ls/ss genotypes (*there were too few ss subjects to study separately, so they were combined with the Ls genotype subjects*) showed that dexamethasone administration decreased levels of cortisol, with higher cortisol in mother-reared subjects, but found no difference between the LL and Ls/ss subjects (21). Dexamethasone testing is often followed by ACTH stimulation, but to our knowledge, we know of no studies assessing the effect of the serotonin transporter genotype following ACTH stimulation of the adrenal cortex after dexamethasone testing. Individual differences in stress-mediated cortisol output show individual differences that are robustly stable across time (22, 23). Testing very young infant subjects following a highly stressful experience (maternal separation) allows both an assessment of serotonin genotype mediation at an age when the biological foundations of the HPA axis response are being formed and also allows an assessment of the effect of genotype on feedback and adrenal response, allowing researchers to assess serotonin transporter genotype-mediated adrenal and pituitary control of cortisol output.

## Methods

### Subjects

Subjects were 190 infant rhesus macaques (*Macaca mulatta* – 84 males and 106 females; 118 LL, 60 Ls, and 12 ss subjects) (Table 1). Subjects were born in 2007 at the California National Primate Research Center (CNPRC) and were reared by their mothers in outdoor 0.2-ha enclosures consisting of 100–150 animals of a mixture of ages, social groups, and familial relationships, a social condition that closely parallels that of the natural condition rhesus macaques (24). All subjects were removed from their mothers for 25 h when they were aged between 90 and 128 days (with a mean age of 106.5 days). The UC-Davis ACUC approved all procedures that were used.

### Infant testing

Subjects tested were removed from their mothers and housed in individual indoor housing for testing. During this time, infants experienced several novel situations designed to assess infant behavioral and physiological reactivity (details of the BioBehavioral Assessment testing are provided in Capitanio et al. (25, 26)). During testing, water was freely available and food was provided on the same schedule as a typical day. After this 25-h long test period, infants were reunited with their mothers and were returned to their home cage.

### Plasma cortisol concentrations

Over the course of testing, four blood samples were obtained via femoral venipuncture while the subjects were manually restrained and awake. The first blood sample was obtained 2 h after the infants were separated from their mothers (around 1100). The second blood sample was obtained 5 h later (around 1400), and immediately after the second blood sample, infants were injected intramuscularly with 500 μg/kg dexamethasone. A third blood sample was obtained at 0830 on the second day of testing, immediately after which 2.5 IU of ACTH was injected intramuscularly. The final blood sample (Sample 4) was obtained 30 min later at 0900 on the second day of testing. For each sample, 0.5 ml of whole blood was obtained and centrifuged for 10 min at 3,000 rpm at 4°C. After centrifuging the blood, plasma was removed and pipetted into tubes. Plasma samples were then stored at −80°C. Samples were later assayed in duplicate using commercially available kits (Diagnostic Products Corporation, Los Angeles, CA, USA). Inter-assay and intra-assay coefficients of variation were 5.8 and 7.9%, respectively.

### Genotyping

Genomic DNA was isolated from blood using a Qiagen DNeasy Tissue kit (Valencia, CA, USA) or a modified salting out method (25). Serotonin transporter promoter polymorphism (*5-HTTLPR*) genotyping was performed through multiplexing on an ABI 3730 DNA Analyzer (Applied Biosystems, ABI, Foster City, CA, USA) using fluorescence technology (26). The *rh5-HTTLPR* primers (GenBank Accession number AF285761) have been described elsewhere (4). Each 15 μl reaction contained 1.5 μl of DNA, 1.5 μl 1× polymerase chain reaction (PCR) buffer (ABgene, Rochester, NY, USA), 0.2 mM dNTP, 1.5 mM Betaine, 1.5 mM $MgCl_2$, 0.25 U Taq polymerase (Denville Scientific Inc., Metuchen, NJ, USA), 0.1 μM *5-HTTLPR* primer mix (fluorescently labeled reverse primer). PCR was performed on a GeneAmp 9700 thermal cycler (Perkins Elmer, Foster City, CA, USA) with the following profile: (a) initial denaturation for 5 min at 95°C, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and (b) a final primer extension at 72°C for 10 min. Genotypes were determined using STR and software with respect to the GeneScan 350 ROX size standard. Allelic sizes and identity were confirmed by direct sequencing on an ABI 3730 DNA Analyzer using BigDye Terminator Sequencing chemistry v3.1 (ABI) as described elsewhere (27).

### Statistical analysis

Hardy–Weinberg assessment showed that the genotype distribution did not violate expected frequencies. Preliminary analyses showed that sex did not significantly affect cortisol levels at this age and did not interact with serotonin genotype. Thus, it was not used in subsequent analyses. Preliminary analyses showed no difference in the two Day-1 Stress samples, and because of the high correlation between the two Day-1 Stress samples ($r$=0.61, $P$<0.0009), they were averaged and the mean of the two Day-1 Stress samples was used in analyses, leaving three repeated cortisol measures (Day-1 Stress, dexamethasone, and ACTH samples). A mixed design two-way repeated-measures ANOVA analysis was used to test the effect the serotonin transporter and treatment on cortisol regulation, with the between-group independent variable being genotype (LL, Ls, and ss), and within-group variable being the

three cortisol measures described above. The dependent variable was plasma cortisol levels. In those few cases where a value was missing (<5%), linear regression was used to predict the missing point using the overall cortisol mean. The repeated measures showed evidence of violations of sphericity, thus all values and their associated *df* are reported using Roy's Largest Root multivariate tests.

## Results

Significant main effects were found for both sample condition ($F$ =165.09, $df$ 2/187, $P$ <0.009) and genotype ($F$ =4.196, $df$ 2/187, $P$ <0.017). There was a significant sample×genotype interaction ($F$ =3.24, $df$ 4/372, $P$ <0.04). As expected, further analysis of the sample condition effect showed that cortisol decreased from stress levels following dexamethasone and then rose to levels significantly higher than stress following ACTH administration ($P$<0.001). Further analysis showed a linear trend for the main effect of genotype, with a dose-like effect of increasing cortisol levels in Ls genotype subjects and a further increase in cortisol in subjects homozygous for the short allele ($F$=17.30, $df$ 2/187, $P$ <0.0009). Comparisons across genotypes showed that subjects with the ss genotype had significantly higher cortisol levels than both the LL ($P$<0.02) and Ls subjects ($P$<0.01; Fig. 1).

Comparisons within the interaction showed the genotype linear trend was significant for the dexamethasone sample, with levels of cortisol higher in the Ls subjects when compared to the LL subjects and further increase for the ss subjects, showing a significant difference between the Ls and ss genotypes ($P$<0.05). A similar linear trend was seen for the ACTH-stimulated sample, although it failed to reach traditional statistical significance ($P$<0.07). The stress sample showed no such trend, with the cortisol levels significantly higher in the ss subjects than in both the Ls and LL subjects but no difference between the LL and Ls genotype subjects ($P$<0.01).

Bivariate correlation of the cortisol values across the samples also showed a modest correlation between the mean Day-1 Stress and dexamethasone and ACTH samples ($r$=0.49, $P$<0.0009; $r$=0.63, $P$<0.0009, respectively). The dexamethasone and ACTH samples were also highly correlated ($r$=0.74, $P$<0.0009).

## Discussion

Our results suggest that there is, in part, common serotonin transporter genotype regulation of cortisol output across stress, feedback, and ACTH stimulation of the adrenal cortex, as demonstrated by the correlation between the three samples, and similar serotonin genotype-modulated linear effects for the stress, dexamethasone, and ACTH samples. As in our study, Michopoulos and colleagues demonstrated that there is a high correlation between the stress sample and the dexamethasone sample (28), suggesting at least partial common regulation of stress-induced cortisol and dexamethasone-mediated cortisol levels. Our findings are consistent with a number of studies cited earlier in the introduction showing higher cortisol levels in humans who are homozygous for the short allele following a stressor. For example, human infants with the ss genotype exhibited higher cortisol levels following the stressor of

heel prick when compared to the LL genotype with the heterozygotes showing an intermediate response (10), and lend credence to the relatively more replicated finding that under conditions of stress, cortisol output is mediated in part by the serotonin genotype. Moreover, as in the Mueller and colleagues study (10), the effect was demonstrated early in life, at time when the biological foundations of the HPA axis response are being formed.

The review by Contesse and colleagues (29) present compelling evidence to show that variation in the serotonin system plays a critical role in modulating the adrenal response to ACTH and the cortisol negative feedback response. Our data suggest that this modulation may be in part due to differences in serotonin transporter genetic effect. It is likely that there are central mechanisms mediating cortisol output that are mediated by serotonin genotype. CNS serotonin activity is mediated by the serotonin transporter genotype (30), and Reimold et al. demonstrated that reduced CNS transporter binding in a wide band of the limbic system, particularly in the thalamus, is negatively associated with high cortisol levels following the dexamethasone/CRH test (16); see also (28). For the dexamethasone and ACTH tests, there was a smooth and statistically significant linear trend of genotype, with the LL genotype subjects showing the lowest values and the ss genotype subjects showing the highest cortisol values, with a dose-dependent effect of the allele. For the separation-stress values, however, the LL animals and Ls genotype animals showed similar values, although the effect was trending in the same direction. The three cortisol samples were designed to model activation of the HPA axis (Day-1 Stress), negative feedback (Dexamethasone), and adrenal output capacity or hypertrophy (ACTH stimulation) (31, 32), and while our findings as well as others show a strong correlation between stress-mediated and dexamethasone-induced cortisol levels, our findings suggest similar serotonin genotype effects on cortisol feedback (dexamethasone) and ACTH-mediated adrenal output or adrenal cortex capacity, but suggest that stress-mediated activation may be regulated somewhat differently. While speculative, it may be that centrally, the perception of the stressor is different for the LL and Ls genotype subjects than the ss genotype subjects, which may explain why the linear trend for the stress sample was attenuated, and did not parallel as strongly the linear trend for the biochemically-mediated dexamethasone and ACTH-stimulated samples. Central CRH stimulation of the HPA axis using the CRH test in subjects varying in serotonin transporter genotype would address this question.

While our overall sample size is the largest to date, it is noteworthy that there were still only a limited number of subjects homozygous for the short allele and it will be of interest to see the results replicated. We also note that while the dose-like linear effect of the three genotypes is consistent with our hypothesis, the effects are modest in size. Given the substantial heritable influences on HPA axis functioning and the relatively small effect for the serotonin transporter genotype, as well as other studies showing effect of other genes on HPA axis regulation [see for example Barr et al. (33)], it is likely that modulation of the HPA axis is polygenic. Miller and colleagues (15) also note that effect sizes for the modulation of the HPA axis 5HTT genotype effects are small and show an effect on cortisol output similar to what others studying mono-amine oxidase (MAO) genotypic effects on cortisol. Additionally, they also suggest that it is likely that the serotonin transporter gene interacts with other non-serotonin genes. It is also probable that the MAOA gene and other

non-serotonin genes interact with the serotonin gene and system to regulate and fine-tune HPA axis response, an exciting area for future research.

Studies suggest that individuals with the short allele are more prone to affective disorders (34) and other forms of psychopathology (35, 36). Several of our earlier studies show that individual differences in cortisol output are remarkably stable across time (23, 37). To the extent that our findings generalize to humans, the above studies, along with the findings in this study suggest a potential life-long risk for cortisol hypersecretion and other forms of stress-mediated psychopathology in subjects homozygous for the short allele of the serotonin genotype, particularly under conditions of stress.

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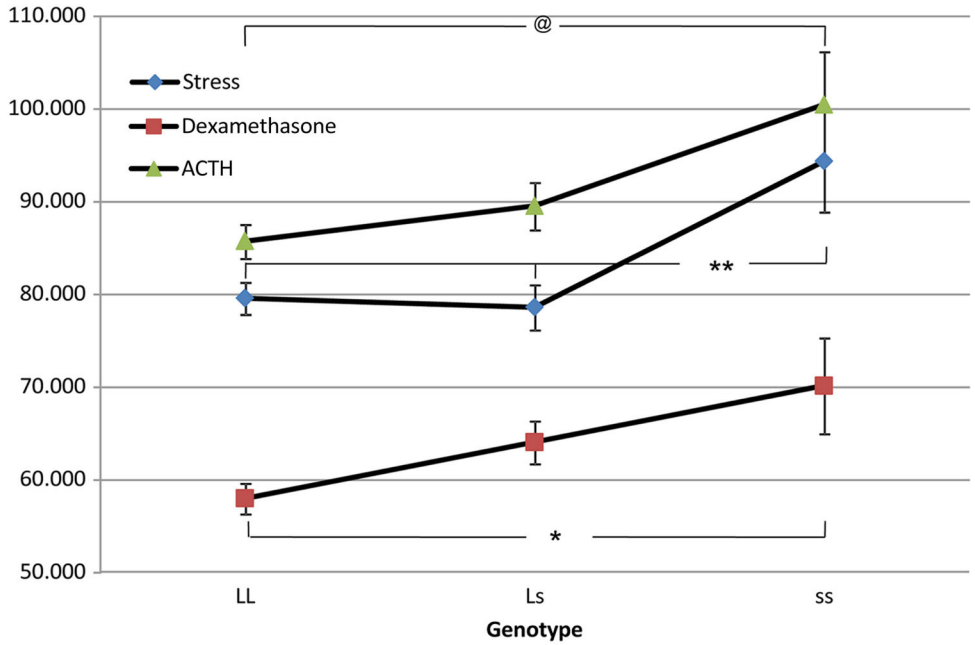


**Fig. 1.**

Plasma cortisol concentrations following the social separation stressor (diamond symbol), dexamethasone challenge (rectangle symbol), or ACTH stimulation (triangle symbol) are shown. Error bars are standard errors. There was a main effect of genotype, with a dose-like linear trend of increasing cortisol levels in Ls genotype subjects and a further increase in cortisol in subjects homozygous for the short allele ($P<0.0009$). Further analyses showed a genotype linear effect with a dose-dependent increase for the dexamethasone ($P<0.05$) and ACTH stimulation tests ($P<0.07$). There was no difference between the LL and Ls groups during the separation stressor, but there was a significant increase in cortisol for the ss genotype subjects when compared to the LL and Ls genotypes ($P<0.01$). Within the LL and Ls genotypes, there was a significant difference between each of the sampling three cortisol sampling conditions (P<0.01). For the ss genotype, there was significant difference between the Dexamethasone sample and the Stress-mediated (P<0.05) and ACTH stimulated samples (P<0.01). There was a nearly significant difference between the Stress and ACTH sample (P<0.07). @= P<0.07 linear trend; *= linear trend, and **= no linear trend but a significant increase when ss is compared to LL and Ls.

**Table 1**

Demographics and samples sizes for each of the genotypes

| HTTP | | Gender | | Weight (kg) | Age (days) |
|---|---|---|---|---|---|
| L/L | 118 | Male | 53 | 0.988 | 105.96 |
| | | Female | 65 | 0.935 | 105.62 |
| L/s | 60 | Male | 28 | 1.036 | 108.89 |
| | | Female | 32 | 0.960 | 106.53 |
| s/s | 12 | Male | 3 | 1.033 | 120.00 |
| | | Female | 9 | 0.920 | 104.33 |

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