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ORIGINAL PAPER

Metabolic and reproductive consequences of the serotonin transporter promoter polymorphism (5-HTTLPR) in adult female rhesus monkeys (*Macaca mulatta)*

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Abstract The serotonin (5HT) reuptake transporter (SERT) plays a key role in 5HT homeostasis by recycling 5HT into the presynaptic neurons. Recently, polymorphisms in the length of the promoter region of the gene that encodes SERT have been linked to functional differences in reactivity to psychosocial stress, as the short (s) promoter length allele shows reduced transcriptionally activity in vitro and is associated with reduced 5HT activity and increased vulnerability to affective disorders. Given 5HT's important role in appetite regulation, polymorphisms in the SERT gene could also affect metabolic parameters. In addition, since reduced 5HT activity may also predispose females to reproductive deficits, polymorphisms in the SERT gene may help explain individual differences in ovulatory function. The present study, using a rhesus monkey model, tested the hypothesis that the presence of the *s*-variant allele would be associated with altered metabolic regulation and impaired ovulatory cycles compared with the *l*/*l* genotype. Females homozygous for the long allele in the SERT gene (*l*/*l*, $n = 19$) were compared to those with the *s*-variant allele (*l*/*s* or *s*/*s*, $n = 20$). All females had similar social histories. Body weights ($P = 0.026$) but not heights ($P = 0.618$) were significantly lower in *s*-variant compared to *l*/*l* females. In addition, both BMI ($P = 0.032$) and sagittal abdominal diameters (SAD) ($P = 0.031$), as indices of adiposity, were significantly lower in *s*-variant females. Consistent with these differences, fasting and non-fasting levels of leptin were significantly lower in *s*-variant females ($P = 0.002$). While there were no genotype differences in non-fasting levels of insulin, *s*-variant females had significantly lower concentrations of insulin during a fast than did *l*/*l* females ($P = 0.052$). Neither glucose, T_3, T_4, nor ghrelin varied significantly between groups during either the fasted or non-fasted condition ($P > 0.05$). Analysis of a subset of females indicated that significantly fewer *s*-variant females (62.5%) exhibited ovulatory cycles than *l*/*l* females (100%, $P < 0.05$). However, there were no differences in serum estradiol or progesterone in *l*/*l* females and those *s*-variant females that did ovulate ($P > 0.05$). In addition, females with the *s*-variant genotype also had reduced 5HT activity ($P = 0.030$), assessed from the acute increase in serum prolactin following the administration of the 5HT reuptake inhibitor, citalopram. Finally, *s*-variant females were significantly less responsive to glucocorticoid negative feedback ($P = 0.030$) yet more responsive to corticotropin releasing hormone (CRH, $P = 0.016$) in terms of plasma cortisol than were *l*/*l* females. These data indicate that adult female rhesus monkeys with the *s*-variant polymorphism in the SERT gene exhibit metabolic and reproductive alterations in conjunction with reduced serotonergic responsivity and increased LHPA activity and suggest

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the possibility that this genotype may predispose females exposed to psychosocial stressors to further metabolic and reproductive deficits.

Keywords Serotonin reuptake transporter · Genetic polymorphism · Metabolism · Reproduction

Introduction

The neurotransmitter serotonin (5-hydroxytryptamine/5-HT) modulates numerous physiological and behavioral processes [1–4]. In turn, individual differences in serotonergic neurotransmission, including factors related to synthesis, release, turnover, and degradation, are believed to contribute to variation in mood, impulsivity, eating patterns, as well as to physiological indices such as blood pressure, insulin sensitivity, and plasma lipids [5]. The serotonin reuptake transporter (SERT/5-HT transporter/5-HTT) is a pivotal protein in 5HT neurotransmission because it terminates serotonin's synaptic effects and thus determines the magnitude and duration of the 5-HT synaptic signal [6, 7].

Recent attention has focused on determining the degree to which genetic factors may account for differences in serotonin neurotransmission and turnover and thus contribute to variation in physiology and behavior. Of particular interest are those polymorphisms that directly regulate transcriptional activity of the SERT gene. Two such polymorphisms containing variable-number of tandem repeat elements (VNTRs) have been identified in the human non-coding region of the SERT gene: a 44 base pair (bp) insertion/deletion in the promoter region (5-HTT gene-linked polymorphic region, 5-HTTLPR) [8], and a 17 bp VNTR in the second intron of the gene (VNTR-2) [9]. The VNTR-2 polymorphism consists of alleles composed of 9, 10, and 12 repeat units that act as strong tissue specific enhancer sequences [10]. Although a specific function for this polymorphism has not been identified, significant associations are observed between the STin2.12 allele and the incidence of schizophrenia [11] as well as generalized anxiety disorders [12].

Of particular interest is the 5-HTTLPR polymorphism. The short variant polymorphism of this bi-allelic repeat has diminished transcriptional activity in vitro compared to the long allele [13] and is associated with a higher incidence of anxiety and depression in response to life stressors [14–18]. Mice with targeted disruption of the SERT gene [19] are more anxious and have increased pituitary-adrenal responses to stressors [20, 21]. Rhesus monkeys have homologous polymorphisms in SERT gene [13], and, like humans, the presence of the short allele has reduced transcriptional activity [22]. Studies of rhesus monkeys clearly show a gene by environment interaction, as animals with the short allele exposed to adverse rearing conditions show more anxiety-like behaviors in standardized tests of emotionality [23, 24], increases the LHPA response to social separation [25], and preference for alcohol consumption [26]. Taken together, the data indicate this ''*s*'' allele in the SERT gene may predispose individuals to psychopathology when exposed to socio-environmental stressors.

As stated above, 5HT is known to affect eating behavior and, in fact, decreased 5HT function is associated with disorders such as anorexia nervosa (20). However, the role of polymorphisms in the 5HT system and metabolic state is not yet understood. There is evidence that the short variant polymorphism is associated with a greater incidence of anorexia nervosa, an observation that has been made in some [27] but not all studies [28]. Thus, in addition to greater emotional reactivity to socio-environmental stressors, individuals with the short variant allele in the SERT gene may be metabolically different than those homozygous for the long allele. Because fertility is tightly coupled with sufficient caloric intake of [29], one consequence of metabolic differences due to polymorphisms in the SERT gene may be alterations in gonadal activity as well. In the present study, we examined the metabolic and reproductive consequences of polymorphisms in the promoter region of the SERT gene in female rhesus monkeys in order to gain insight into the genetic variation in 5HT activity that may regulate metabolic and reproductive status.

Methods

Subjects

Subjects were 40 adult female rhesus monkeys (*Macaca mulatta*) that were members of one of five breeding groups located at the Yerkes National Primate Research Center Field Station. These groups contained multiple adult females, juveniles, and 2–3 adult males each. Animals were housed in outdoor compounds with attached indoor quarters as described previously [30]. Groups were fed a standard low fat, high fiber monkey diet (Ralston Purina Company, St. Louis MO) twice daily ad libitum and supplemented daily with fresh fruits and vegetables. The protocol was approved by the Emory University Animal Care and Use Committee in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services ''Guide for Care and Use of Laboratory Animals''.

Females were selected on the basis of three criteria: parity, dominance status within their respective group, and SERT genotype. All females were multiparous and were between 7 and 10 years of age. First pregnancy for females in our breeding colony typically occurs at 4 years of age [31]. Female macaques that are socially housed readily

form a dominance hierarchy that functions to control aggression but also access to resources [32]. Thus, only females that were determined to be from the middle portion of the dominance hierarchy were selected [32], as we were interested in the effects of SERT genotype on reproductive and metabolic parameters independent of dominance. Dominance status was determined from the outcome of dyadic interactions [33], based on 10 h of observation of each group over a four-week period. Initially, 179 females in nine breeding groups were identified on the basis of parity and dominance status. A single blood sample was obtained from each of these females for determination of 5HTTLPR polymorphisms as described previously for rhesus monkeys [13]. Specifically, DNA was extracted from whole blood using the Pure Gene Blood Kit (Gentra, D-4000). Polymorphisms in the promoter region of the 5-HT transporter gene were identified following amplification of the relevant gene segments by polymerase chain reaction using the oligonucleotide primers fwd (cag ggg aga tcc tgg gag gga) and rev (ggc gtt gcc gct ctg aat gc) based on the protocol described previously. The ''*s*'' amplicon (398 bps) and the ''*l*'' amplicon (419 bps) were separated on an agarose gel containing ethidium bromide and identified by direct visualization. Figure 1 is a representative gel comparing human and rhesus monkey length variants in the promoter region of the SERT gene. Results indicated that of the 179 adult females screened, 50.2% had an *l/l* genotype, 45.3% had an *l/s* genotype, and 4.5% had an *s/s* genotype. Based on these results, we chose 20 females with an *l/l* genotype and 20 with either an *l/s* or *s/s* genotype, as the heterozygous short variant (*l/s*) produces a similar phenotype as the homozygous genotype(*s/s*) on most [22, 23], but not all measures [24]. Four females of each genotype were identified in each of 5 groups, yielding four *l/l* and four *l/s or s/s* per group. However, one female identified as having an *l/l* genotype developed a gastrointestinal bacterial infection during the course of the study; her data were not included.

Females were studied under two different conditions. Differences in reproductive parameters were determined on a subset of seven *l/l* and eight *l/s* females housed in two of the five groups. The eighth *l/l* female was the female dropped from the overall analysis for clinical reasons stated above. Because rhesus monkeys housed in an outdoor environment breed seasonally, with ovulations restricted to the fall and winter months [34, 35], all data were collected between Sep 10 and Dec 23. To characterize ovulatory cycles and patterns of menstruation, blood samples for the analysis of estradiol and progesterone were collected three days per week (Mon, Wed, Fri). Ovulation was inferred from a sustained rise in serum progesterone (>1 ng/ml for >5 days). Short luteal phase cycles were identified by a blunted rise in progesterone (1–3 ng/ml) for 4–6 days followed by the appearance of menstruation [36]. Blood samples were collected without anesthesia after females were habituated to the procedures. This approach has previously been shown not to adversely affect limbic—hypothalamic—pituitary—adrenal (LHPA) arousal or reproductive performance [30, 37].

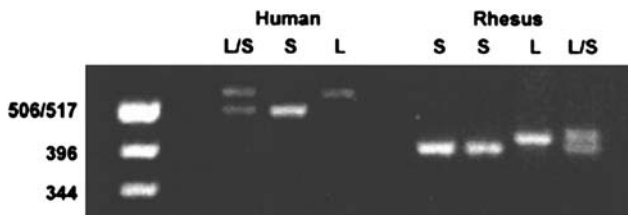

Fig. 1 Representative gel showing length variants in the promoter region of the human and rhesus monkey 5-HT transporter where S is the short allele and L is the long allele. The assays for detection of polymorphisms in the human vs. monkey 5-HT transporter are identical with the exception of primer sequence

Following the collection of the reproductive data, all 39 females were ovariectomized as part of a larger study designed to investigate hypothalamic and adrenal responses to exogenous hormone treatments. Following recovery from surgery, females were implanted with silastic capsules of estradiol that produced serum concentrations of approximately 40 pg/ml [35]. All females were then subjected to a dexamethasone suppression test. Plasma samples were obtained at 0900, 1200, and 1730 h, with the final sample followed by a dexamethasone injection (0.25 mg/kg, IM). Additional plasma samples were obtained at 0900 and 1200 h the next day to assess glucocorticoid negative feedback on both pituitary ACTH and adrenal cortisol release. The following week, females received a CRH stimulation test in which CRH (1.0 μg/kg, iv) was given following a blood sample. Subsequant samples were obtained at 30, 60 and 90 minutes. In the weeks following this assessment, serum samples were collected following a 10 h overnight fast and again within 60 min of the morning feed for metabolic hormone determination in samples during a fasted and non-fasted condition. Samples were assayed for leptin, insulin, ghrelin, glucose, T_3, and T_4. Finally, central serotonergic responsivity was determined by treatment with the highly selective 5HT reuptake inhibitor (SSRI), *s*-citalopram. The increase in 5HT by citalopram activates hypothalamic 5HT receptors, stimulating the release of prolactin from the pituitary [38, 39]. The procedure involved first dissolving *s*-Citalopram-HCl in a 1% ethanol solution in Sesame oil that was then administered intramuscularly as a dose of 0.55 mg/kg. Serum samples were collected prior to and 60 and 120 min following the injection for measurement of prolactin as a surrogate for 5HT [40].

Two anthropometric measures were obtained on all females while they were anesthetized (10 mg/kg ketamine

hydrochloride). ''Height'' was determined on the basis of a measurement made from the top of the head to the bottom of the heel while animals were positioned supine with their legs fully straightened. The measurement was taken by two individuals using MHC vernier calipers and the mean of these was used as that animal's height index [41]. Sagittal abdominal diameters (SAD) were also obtained while females were anesthetized and in a supine position. SAD is measured, using calipers, as the distance from the back to the top of the abdomen at the level of the navel [42]. Again, two individuals measured the distance and the mean was calculated for an animal's SAD. In addition, body weights were obtained monthly. Body mass index was calculated as body weight in kg divided by height in m^2.

All hormone assays were performed in the Yerkes Biomarkers Core Lab. Serum estradiol was determined by a modification of a commercially available radioimmunoassay (Diagnostics Products Corporation, Los Angeles, CA). Prior to assay, samples (250 µl) were extracted twice with 5 ml of anesthesia grade ether. Following evaporation of the solvent, samples were reconstituted with 250 µl of the zero calibrator and 100 µl aliquots were assayed in duplicate. The assay has a sensitivity of 5 pg/ml using 100 µl of extracted serum, with an inter- and intra-assay coefficient of variation (CV) of 5.4% and 12.1%, respectively. Sample values of estradiol were corrected for extraction efficiencies, which exceeded 95%. Progesterone was measured using a modification of a previously described assay that employs a commercially available kit from DPC [36]. Samples (125 µl) are extracted with 2.5 ml of anesthesia grade ether and the organic layer is evaporated to dryness under a stream of N_2. The sample is reconstituted in 125 µl of the assay buffer and replicates (50 µl) are assayed following the kit protocol. The assay has a sensitivity of 0.10 ng/ml with an inter- and intra-assay CV of 8.14% and 7.73%, respectively. Sample values of progesterone were corrected for extraction efficiencies, which exceeded 95%. Serum levels of cortisol were determined by radioimmunoassay with a kit from Diagnostic Systems laboratory (Webster TX). Using 25 µl, the assay has a range from 0.5 to 60 µg/dl with an inter- and intra-assay CV of 4.9% and 8.7%, respectively. Serum levels of prolactin were measured by radioimmunoassay with a kit from DSL. The assay has a sensitivity of 0.10 ng/ml with an inter- and intra-assay CV of 6.3% and 9.1%, respectively. Serum concentrations of leptin were measured by radioimmunoassay using a commercially available kit validated for nonhuman primates (Linco, St. Louis MO). Assaying 100 µl, the assay has a range of 0.5 to 100 ng/ml. Intra-assay CVs were 6.84% and inter-assay CVs were 7.24%. Serum insulin was assayed with a kit from DPC having a sensitivity of 3 to 372 IU/l and an inter- and intra-assay CV of 9.02% and 5.87%, respectively. Serum levels of glucose were determined by a commercially available colorimetric enzyme assay (Stanbio Laboratory, Boerne TX), having a range from 0 to 27 mmol/l and inter- and intra-assay CVs of 2.12% and 4.21%, respectively. Serum T_4 was assayed with a kit from DPC having a sensitivity of 0.5–24 µg/dl and inter- and intra-assay CVs of 10.46% and 6.96%, respectively. Serum T_3 was assayed with a kit from DPC having a range from 0 to 600 ng/ml and inter- and intra-assay CVs of 11.17% and 2.06%, respectively. Active (acylated) ghrelin concentrations were measured by an ELISA following the sample collection protocol to minimize ghrelin degradation (ALPCO, Boston MA). The assay has a range from 1.96 to 250 pg/ml with an inter- and intra-assay CV of 4.47% and 5.50%, respectively.

Data were summarized as mean ± SEM. The effect of SERT genotype on reproductive, metabolic, and morphometric parameters were determined by univariate (*t*-tests) or analyses of variance (ANOVA). In those cases where females were studied under two conditions (fasted vs. non-fasted samples) or over time, repeated measures ANOVA were performed. Differences in ovulation rates were evaluated by the χ^2 statistic. Statistical tests having a probability of $P \leq 0.05$ were considered significant.

Results

Assessment of serotonergic responsivity

Serum prolactin was used as a surrogate measure for the extent of increased 5HT neurotransmission in response to the acute administration of citalopram. As expected, citalopram induced a substantial and significant increase in prolactin over the course of the 120 min assessment (Fig. 2; $F_{1,\ 37} = 59.806$; $P < 0.001$). Notably, the response

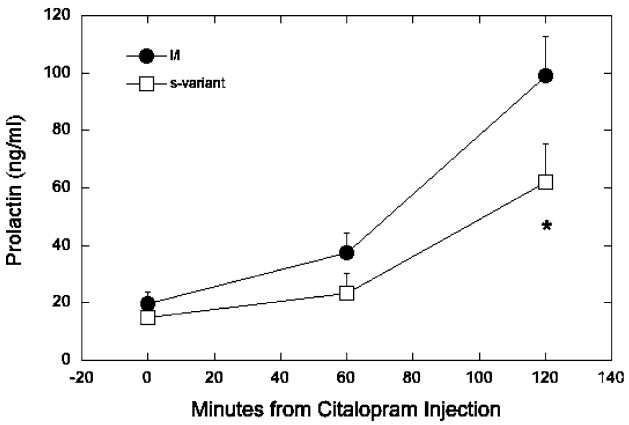

Fig. 2 Mean ± SEM serum levels of prolactin (ng/ml) in response to citalopram (0.55 mg/kg, im) as an estimate of 5HT between *l/l* and *l/s or s/s* females. The asterisk indicates a significant difference at the time point

to citalopram varied significantly by SERT genotype ($F_{1, 37}$ = 3.671; P = 0.030), with significantly lower concentrations of prolactin at 120 min following citalopram injection in females with the *s*-variant compared with *l/l* females. That is, the *s*-variant females exhibited evidence of blunted serotonin function in comparison to their *l/l* counterparts.

Assessment of glucocorticoid negative feedback

An assessment of glucocorticoid negative feedback by means of a dexamethasone suppression test revealed a significant difference in cortisol release as a function of SERT genotype, with *s*-variant females having significantly higher levels of cortisol throughout the assessment period ($F_{1,37\backslash1\ \backslash2\ \backslash3}$=5.076; P = 0.030; Figure 3). Post-hoc analyses revealed *l/s* and *s/s* females had significantly higher levels of cortisol at the time of the dexamethasone injection and at 18 h post-injection. Indeed, the suppression in plasma cortisol following dexamethasone compared to the same time of day 24 h earlier was significantly greater in *l/l* (–77.4 ± 3.0%) compared with *s*-variant females (–63.8 ± 4.6%; t_{37} = 2.49, P = 0.017). Although dexamethasone significantly suppressed plasma levels of ACTH ($F_{4, 148}$ = 34.88, P < 0.001), there was no difference in ACTH release between the *l/l* and *s*-variant females ($F_{1, 37}$ = 0.949; P = 0.438) from pre-injection values (*l/l*: 78.01 ± 11.46 pg/ml; *s*-variant: 96.96 ± 11.18 pg/ml) to 18 h post-injection (*l/l*: 42.28 ± 4.81 pg/ml; *s*-variant: 40.20 ± 4.69 pg/ml).

The increase in plasma cortisol in response to CRH was also significantly influenced by SERT genotype (Fig. 4; $F_{1, 37}$ = 3.594; P = 0.016), with females carrying the *s*-allele having significantly higher concentrations of plasma cortisol at 90 min following CRH administration. As observed with the ACTH response to dexamethasone, the increase in ACTH to CRH was not significantly affected by SERT genotype ($F_{1, 37}$ = 1.176; P = 0.322). Although plasma ACTH levels were higher in *s*-variant compared to *l/l* females throughout the assessment period (e.g., time 0: (*l/l*: 99.85 ± 15.66 pg/ml; *s*-variant: 124.02 ± 15.26 pg/ml; +90 min: *l/l*: 199.34 ± 24.80 pg/ml; *s*-variant: 257.84 ± 24.17 pg/ml), differences were not significant ($F_{1,37\backslash1\ \backslash2\ \backslash3}$=2.546; P = 0.119).

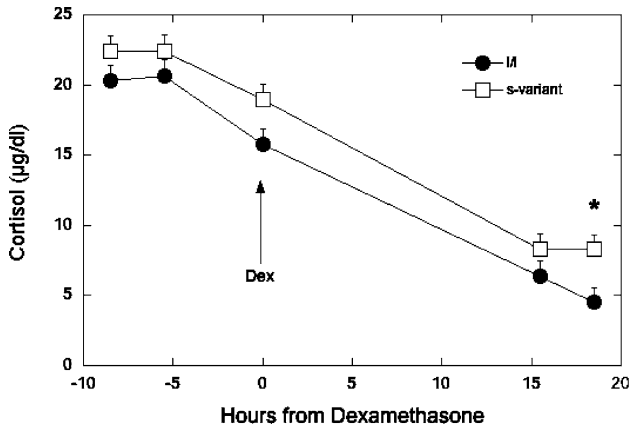

Fig. 3 Mean ± SEM concentrations of cortisol (µg/dl) prior to and following the administration of dexamethasone (Dex; 0.25 mg/kg, IM) between *l/l* and *l/s or s/s* females. Sample collection controlled for time of day as samples were obtained at 0900 h (–8.5 h) and 1200 he (–5.5 h) prior to Dex and 0900 h (+15.5 h) and 1200 h (+18.5 h) following Dex. The asterisk indicates a significant difference at the time point

Morphometric parameters

As illustrated in Table 1, an analysis of body weights taken at two different time points (May and July) indicated that females with an *l/l* genotype were significantly heavier than those with the *s*-allele ($F_{1, 37\backslash1\ \backslash2\ \backslash3}$=5.409; P = 0.026). The significant increase in body weights from May to July ($F_{1, 37\backslash1\ \backslash2\ \backslash3}$=138.71; P < 0.001) was not different as a function of genotype ($F_{1, 37\backslash1\ \backslash2\ \backslash3}$=0.487; P = 0.489]. In contrast, heights were not significantly different between *l/l* (76.24 ± 0.66 cm) and *l/s or s/s* females (75.59 cm ± 0.58 cm; t_{37} = 0.507; P = 0.615]. The significant differences in body weight also translated to similar differences in BMI as a function of genotype (Table 1; $F_{1, 37}$ = 4.942; P = 0.032). BMI scores also increased significantly from May to July ($F_{1, 37}$ = 137.908; P < 0.001) but again changes were not influenced by genotype ($F_{1, 37}$ = 0.344; P = 0.561). Finally, SAD as a direct index of adiposity was significantly greater in *l/l* compared with *l/s* or *s* females (Table 1; t_{37} = 2.241; P = 0.031).

Metabolic parameters

Fasted and non-fasted serum concentrations for insulin, glucose, T_4, T_3, leptin, and ghrelin were compared between

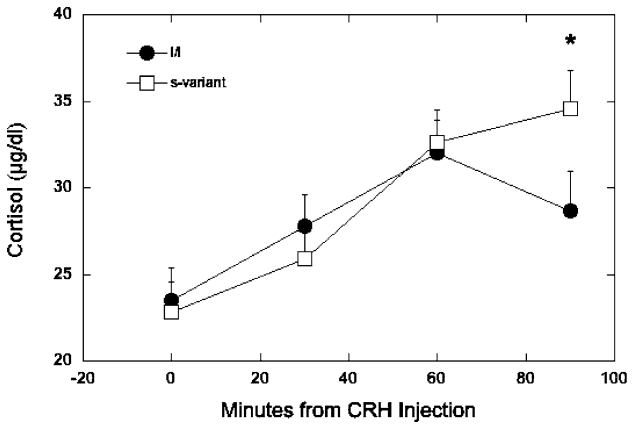

Fig. 4 Mean ± SEM concentrations of cortisol (µg/dl) prior to and following the administration of CRH (0.5 µg/kg, iv) between *l/l* and *l/s or s/s* females. The asterisk indicates a significant difference at the time point

Table 1 Mean ± SEM body weights, height, body mass index (BMI) scores, and sagittal abdominal diameters (SAD) between females with an *l/l* or *l/s—s/s* (*s*-variant) females. Probabilities shown reflect the results of the ANOVA for repeated measures for each hormone. Heights and SAD were only obtained at the May assessment; thus, no data (ND) are shown for the July assessment

Parameter	*l/l*		*s*-variant		Statistics		
	May	July	May	July	*l/l* > *s*	May vs. Jul	SERT × Month
Body weight (kg)	7.82 ± 0.25	8.53 ± 0.28	7.04 ± 0.22	7.68 ± 0.24	$P = 0.026$	$P < 0.001$	$P = 0.489$
Height (cm)	77.08 ± 0.59	ND	76.68 ± 0.49	ND	$P = 0.618$	ND	ND
BMI	13.16 ± 0.40	14.35 ± 0.43	11.98 ± 0.35	13.05 ± 0.39	$P = 0.032$	$P < 0.001$	$P = 0.561$
SAD (cm)	9.69 ± 0.25	ND	8.92 ± 0.23	ND	$P = 0.031$	ND	ND

Table 2 Metabolic hormone values (mean ± SEM) between females with an *l/l* or *l/s—s/s* (*s*-variant) SERT genotype following a 12-h·overnight fast (''*F*'') or following the consumption of a morning meal (''NF''). Probabilities shown reflect the results of the ANOVA for repeated measures for each hormone

Parameter	*l/l*		*s*-variant		Statistics		
	NF	*F*	NF	*F*	*l/l* > *s*	NF > *F*	SERT × *F*
Leptin (ng/ml)	5.88 ± 0.60	2.50 ± 0.26	3.59 ± 0.59	1.41 ± 0.26	$P = 0.002$	$P < 0.001$	$P = 0.142$
Glucose (mmol/l)	5.35 ± 0.38	5.42 ± 0.34	4.49 ± 0.37	4.60 ± 0.33	$P = 0.083$	$P = 0.616$	0.888
T_4 (μg/dl)	5.44 ± 0.27	5.48 ± 0.28	4.94 ± 0.26	5.21 ± 0.28	$P = 0.305$	$P = 0.150$	$P = 0.280$
T_3 (ng/dl)	155.14 ± 6.71	160.71 ± 5.38	156.76 ± 6.55	155.20 ± 5.24	$P = 0.808$	$P = 0.506$	$P = 0.240$
Insulin (μU/ml)	57.27 ± 8.26	30.50 ± 7.64	42.10 ± 8.05	29.62 ± 7.45	$P = 0.451$	$P < 0.001$	$P = 0.052$
Ghrelin (pg/ml)	17.5 ± 3.8	26.6 ± 7.9	21.7 ± 3.1	29.5 ± 6.6	$P = 0.606$	$P = 0.039$	$P = 0.824$

l/l and *s*-variant females (Table 2). Analyses revealed that serum leptin was significantly higher in *l/l* versus *s*-variant females ($F_{1,37}$ 11.02, $P = 0.002$). Fasting significantly reduced leptin concentrations ($F_{1,37\backslash 1\ \backslash 2\ \backslash 3}$=48.032; $P < 0.001$) in a similar fashion for both genotype ($F_{1,37} = 2.247$; $P = 0.142$). Fasting also significantly reduced serum insulin ($F_{1,37} = 30.356$; $P < 0.001$). There was one important interaction ($F_{1,37} = 4.024$; $P = 0.052$), as the higher non-fasting insulin of the *l/l* versus *s*-variant animals were reduced significantly more by fasting than those of the *s*-variant animals. No other metabolic hormones varied significantly by genotype or fasting condition with the exception of ghrelin. Concentrations of ghrelin were significantly elevated during the fasting condition ($F_{1,37} = 4.60$, $P = 0.039$), independent of SERT genotype ($F_{1,37} = 0.27$, $P = 0.606$).

Reproductive parameters

Prior to the ovariectomies, a number of reproductive parameters were assessed in a subset of l/l and *s*-variant females. Analysis of ovulation rates on a subset of seven *l/l* and eight *l/s* females revealed that a significantly greater number of *l/l* females (100%) ovulated in comparison to *l/s* females (62.5%), ($\chi^2 = 8.28$; $P < 0.05$). However, for those females that did ovulate, follicular (63 ± 7 vs. 59.5 ± 4 pg/ml) and luteal phase levels of estradiol (28.2 ± 3 vs. 32.8 ± 6 pg/ml) were not significantly different between *l/l* and *s*-variant females ($F_{1,\ 10} = 0.860$; $P = 0.376$). Nor did follicular (0.56 ± 0.07 vs. 0.70 ± 0.08 ng/ml) or luteal phase progesterone secretion (2.70 ± 0.32 vs. 3.09 ± 0.27 ng/ml) differ significantly between animals of each genotype that ovulated ($F_{1,10} = 1.43$; $P = 0.258$). In addition, for those females that ovulated, there was no difference in the length of the luteal phase between the two SERT genotypes *l/l* vs. *l/s* respectively (10.3 ± 0.5 days) vs. (11.2 ± 0.5 days; $P = 0.24$). Serum sampling frequency was not sufficient to fully characterize the ovulatory surge in estradiol.

A post-hoc multiple regression analysis was done to identify what variables found to vary between the two SERT genotypes accounted for the anovulatory condition in these three *l/s* females. Results indicated that the statistical combination of non-fasting leptin and cortisol concentrations 18 h after dexamethasone administration significantly accounted for 62.4% of the variance ($F_{2,\ 12} = 9.66$, $P = 0.003$). Variables not added into the forward regression equation were non-fasting insulin, body weight, SAD, and the response to citalopram. Comparing the non-ovulating females to the subset of *l/s* who did ovulate, the data indicate the non-ovulating females had significantly lower serum levels of leptin (1.7 ± 0.6 vs. 5.8 ± 1.9 ng/ml;

$t_6 = -2.33$; $P = 0.024$, one-tailed) and higher levels of cortisol at 18 h following Dex (14.74 ± 4.32 vs. 6.46 ± 0.92, $P = 0.040$, one-tailed).

Discussion

The association between the presence of a short allele variant in the promoter region of the gene that encodes SERT and an increased incidence of affective disorders in humans is well documented [14–17]. Similarly, studies in rhesus monkeys indicate that the presence of the short allele increases the incidence of anxiety-like behaviors in standardized tests of emotionality in infants and juveniles [23, 24] and increases the LHPA response to social separation [25] and the preference for alcohol consumption [26] in animals exposed to adverse rearing conditions. These findings suggest that individuals with the *s*-variant polymorphism may be more vulnerable to the consequences of psychosocial stressors on emotionality. However, little work has been done to understand the non-behavioral, physiological consequences associated with this polymorphism. For the first time, the present study shows that adult female rhesus monkeys with the *s*-variant 5HTTLPR exhibit substantial metabolic and reproductive alterations in conjunction with reduced serotonergic responsivity and increased LHPA activity.

Our data support the notion that 5HT responsivity is compromised in females with the *s*-variant allele in the SERT gene. Indeed, females with the *s*-allele had overall lower levels of prolactin throughout the assessment but values at 120 min post-injection were significantly different than *l/l* females. Studies in people indicate the prolactin response to clomipramine [43], a 5HT reuptake inhibitor, or fenfluramine [44] is reduced in individuals with the short promoter variant. Other studies show that this reduced serotonergic response in *s*-variant individuals is significantly associated with lower socio-economic status [45]. Previous assessment of 5HT tone did not find a 5HTTLRP polymorphic differences using fenfluramine in juvenile monkeys [24]. However, unlike the group housed animals in the present study, these animals were pair housed, underscoring the notion that exposure to a complex social environment interacts with SERT polymorphisms to influence 5HT responsivity in this test. Given the present animals had similar social histories, these data suggest that the 5HTTLPR genotype differences observed in response to citalopram may be exacerbated when individuals are exposed to adverse social environments, which in the case of macaque monkeys is social subordination [46, 47]. SERT is important for maintaining 5HT homeostasis and the temporal interaction of 5HT with its receptor by removing 5HT from the extracellular space to the presynaptic terminal where it is recycled to vesicles for re-release [48]. SERT gene knockouts show a gene-dose increase in 5HT in the extracellular space [49] and reduced cellular levels of 5HT [50] in limbic, hypothalamic, and brain stem regions. Furthermore, many of the specific phenotypes observed in SERT knockouts are seen in individuals with the *s*-variant allele in the gene [51]. Thus, the decreased transcriptional activity caused by the *s*-allele [13, 52] likely disrupts the reuptake and re-cycling of 5HT, reducing the amount of 5HT available for release and affecting central and peripheral systems dependent on 5HT.

This study also shows that females with the short promoter length variant have reduced glucocorticoid negative feedback, as assessed from the dexamethasone suppression test, and increased response to CRH. Overall, cortisol levels were significantly higher in females with the *s*-variant allele and, while dexamethasone significantly suppressed cortisol in all females, the degree of suppression 18 h following treatment was significantly greater in *l/l* females. Furthermore, *s*-variant females responded with a significantly greater increase in plasma cortisol to exogenous CRH. Despite these differences in cortisol, the pattern of ACTH in response to dexamethasone and CRH was not significantly different between females of each SERT genotype, suggesting a difference in adrenal sensitivity to ACTH [53]. Further evaluation, including ACTH stimulation tests, can address this possibility. Nevertheless, the data from the present study suggest the LHPA axis is differentially regulated in *s*-variant females independent of socio-environmental influences and that reduced glucocorticoid negative feedback is indicative of generalized LHPA dysregulation [54]. Since 5HT input to the hippocampus limits the behavioral and neuroendocrine responses to stress [55–57] it follows that disruptions in the SERT gene [19] or the presence of the short variant [21], results in increased LHPA responses to every day stressors. Studies in monkeys that have evaluated environmental—SERT polymorphic interactions indeed show greater neuroendocrine responses to adverse experiences [58].

The morphometric and metabolic analyses indicate robust differences between *s*-variant and *l/l* females. The *s*-variant females had consistently lower body weights, even as females gained weight during the long-days of summer. Although heights were similar, these differences in weight translated to higher BMI scores and a greater sagittal abdominal diameter or SAD in *l/l* females. In addition to BMI, high SAD is a predictor of adiposity in people [42] and monkeys [59]. While glucose levels were similar between the two genotypes, *l/l* females had significantly higher serum concentrations of insulin during the fed but not the fasted condition compared with *s*-variant females. Furthermore, serum leptin concentrations were significantly higher in *l/l* females during both conditions, despite the significant fall in leptin during the fast. These higher levels of insulin and

leptin in *l/l* females map directly to higher indices of body fat and are consistent with studies in humans [60].

We assessed plasma levels of ghrelin to determine if differences matched those seen with body weight. Although biologically active ghrelin showed the characteristic increase during a fast [61], this orexigenic peptide was not different between *l/l* and *s*-variant females. Given that all animals had ad libitum access to food, the lower body weights and reduced indices of fat suggest the *s*-variant females ate less; however, we did not measure food intake. Since ghrelin is elevated more following a longer fast [62] one might expect ghrelin levels to be higher in *s*-variant females if indeed their food intake is reduced and the interval between meals lengthened. However, since meal size is controlled by a number of satiety signals from the gut, including CCK [63], it is possible these signals differ in *s*-variant females. In addition, given the results of the LHPA assessment, it is possible that a reduction in feeding, leading to the lower body weight and adiposity in *s*-variant females, is due to the anorexic effects of CRH [64] secondary to the reduced glucocorticoid negative feedback evident in these females. On the other hand, one might expect *l/l* females to have lower body weights given the anorexic action of 5HT [65]. Indeed, metabolic syndrome is characterized by low 5HT responsivity in humans [39] and SSRIs reduce body weight [66] while formulations of SSRI combined with norepinephrine re-uptake inhibitors are now prescribed for some forms of obesity [67]. On the other hand, some [27, 68] but not all studies [69] show an association between anorexia nervosa and the presence of the *s*-variant. Obviously, the influence of 5Ht on food intake is complex. However, our data indicate that the contribution of the SERT genotype to body weight regulation in female rhesus monkeys is significant and suggest that this model can be used to determine how socio-environmental as well as nutritional variables interact with 5HT responsivity to affect metabolism.

The higher incidence of anovulation characteristic of the *s*-variant females is potentially significant from a life-history perspective. At this point it is not possible to determine the mechanism responsible for this reproductive deficit in *s*-variant females, but it could be due a combination of factors delineated in this study that relate to inherent differences in 5HT neurotransmission. This notion is supported by data from cynomolgus monkeys who become anovulatory in response to a series of mild stressors, including a socially stressful situation and metabolic insult [70, 71]. These ''stress sensitive'' females show diminished 5HT regulation, including reduced response to fenfluramine and reduced expression of SERT mRNA in the brain stem and this ''5HT phenotype'' is evident absent any exposure to experimental stressors. However, this phenotype is not due to polymorphic differences in SERT, as in contrast to humans and rhesus monkeys, cynomolgus monkeys do not show the 5HTTLPR [71]. Nevertheless, the similarities speak to the importance of the 5HT system for regulating reproduction in females. Although underpowered, our post-hoc analyses indicate that anovulation in the *s*-variant females is associated with reduced circulating levels of leptin and increased cortisol following escape from negative feedback. These data must be interpreted cautiously until confirmed by prospective studies. However, this result is supported by data showing anovulation is associated with hypoleptinemia [72, 73] as well as activation of the LHPA axis [74, 75]. The stimulatory effect of leptin on the neuroendocrine axis of monkeys is well-documented [76] and 5HT neurons in the raphe are a target of [77]. Indeed, there is evidence that 5HT may mediate, in part, leptin's effect on reproduction, as estrous cyclicity is rescued in leptin deficient mice with an SSRI [78]. Nevertheless, the data suggest that a global dysregulation of metabolic and neuroendocrine systems that may emerge in *s*-variant females may be responsible for these reproductive deficits.

It is important to note that a major assumption in the design of our study was that by selecting females from similar (i.e., middle) social status positions we had controlled for possible socio-environmental effects. Although it is not possible to rule out random effects, the main effect of SERT genotype was significant for many of the analyses performed. Since both human [17, 45] and previous non-human primate studies [25, 26, 79] focus on the importance of how the 5HTTLPR interacts with psychosocial stressors, it is likely that neuroendocrine, reproductive, and metabolic phenotypes of individuals with the different SERT polymorphisms will be even more prominent when psychosocial factors are considered. Specifically, future studies might focus on the effect of dominance status (a feature of the psychosocial environment that has been linked to variability in ovulatory function and adrenocortical activity (e.g., [80]) on the physiological responses of l-l and *s*-variant individuals. In summary, because the 5HT system is integral to both stress responsivity and metabolic regulation and metabolic dysregulation is a consequence of stressor exposure, the consideration of the 5HTTLPR may provide a better understanding of how psychosocial stress impacts reproduction in women.

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