Epigenetic Regulation of Serotonin Transporter Expression and Behavior in Infant Rhesus Macaques

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Abstract

Epigenetic mechanisms may moderate genetic and environmental risk (G x E) for mood disorders. We used an experimental rhesus macaque model of early life stress to test whether epigenetic regulation of serotonin transporter (5-HTT) may contribute to G x E interactions that influence behavior and emotion. We hypothesized that peripheral blood mononuclear cell (PBMC) DNA methylation within an 800 bp CpG island that overlaps with the5-HTT transcription initiation start site, a hypothesized model of the same genomic region in brain tissue, would mediate or moderate the effects of early life stress and a functional5-HTT promoter polymorphism (rh5-HTTLPR) on two outcomes: PBMC5-HTT expression; and behavioral stress reactivity. Eighty-seven infant rhesus macaques (3–4 months of age) were either mother-reared in large social groups (n= 70) or nursery-reared (n= 17). During a maternal/social separation, infants’ blood was sampled and behavioral stress reactivity recorded. PBMC DNA and RNA samples were used to determine: rh5-HTTLPR genotype; 5-HTT mRNA expression using qRT-PCR; and 5-HTT CpG methylation status using sodium bisulfite pyrosequencing. Consistent with human data, carriers of the low expressing rh5-HTTLPR alleles exhibited higher mean5-HTT CpG methylation, which was associated with lower PBMC5-HTT expression. Higher5-HTT CpG methylation, but not rh5-HTTLPR genotype, exacerbated the effects of early life stress on behavioral stress reactivity in infants. 5-HTT CpG methylation may be an important regulator of 5-HTT expression early in development, and may contribute to the risk for mood disorders observed in “high-risk” rh5-HTTLPR carriers.

Keywords

Serotonin transporter; rhesus macaque; DNA methylation; rh5-HTTLPR genotype; behavior; development

Background

Early life stress is a risk factor for adult psychiatric illness and for poor stress adaptation, a hypothesized endophenotype of mood disorders (Nemeroff et al., 2004; Charney and Manji, 2004). Altered serotonin transporter (5-HTT) expression early in life may moderate the effects of early life stress on influence emotional development. For example, pharmacologic
blockade of 5-HTT in the early postnatal period in mice results in reduced 5-HTT expression in adulthood (Maciag et al., 2006) and an adult depressive phenotype (Ansorge et al., 2004), suggesting that temporary reduction of serotonin uptake during this critical period can have permanent effects on brain development and emotion. Understanding the role of 5-HTT regulation and stress adaptation in infants will inform our understanding of risk for mood disorders in adulthood.

Both genomic and environmental factors have been linked with 5-HTT expression and behavior. The “short” allele of the serotonin transporter (5-HTT) promoter polymorphism (5-HTTLPR) predicts lower 5-HTT expression (Heils et al., 1996; Hranolovic et al., 2004). Early life stress is also linked with reduced 5-HTT expression, an effect that can persist into adulthood (Lee et al., 2007; Kinnally et al., 2008; Kinnally et al., 2009; Miller et al., 2009). Intriguingly, structural genetic variation interacts with early life adversity to influence neurobehavioral outcomes. Carriers of the “short”, low-expressing 5-HTTLPR (rh5-HTTLPR) allele are at greater risk for developing mood disorders and poor stress adaptation, while carriers of the “long”, high-expressing allele are somewhat protected from this risk (Champoux et al., 2002; Caspi et al., 2003; Barr et al., 2004; Kaufman et al., 2004; Cichetti et al., 2007; Stein et al., 2008; Zalsman et al., 2006, although see Surtees et al., 2006; Munafó et al., 2008; Risch et al., 2009). The mechanisms and intermediate phenotypes that mediate such gene × environment interactions are not yet well understood. It is likely that the most immediate outcome of gene × environment interactions should be gene expression itself, although few studies have focused on this outcome.

Epigenetic modifications may play a role in 5-HTTLPR × early stress interactions influencing neurobehavioral outcomes. CpG islands are CG (nucleotides cytosine and guanine, with phosphodiester = phosphate deoxyribose bond (p)) -rich regions of the genome often located in or near promoter regions (Bird, 1987). Greater methylation of cytosines within CpG islands is associated with reduced gene transcription (Jones and Takai, 2001). An 800 bp CpG island is located approximately 200 bp downstream of the 5-HTTLPR and overlaps with the transcription initiation start site of the 5-HTT gene (UC Santa Cruz Genome Browser) in humans and rhesus macaques. This is a candidate region that may contribute directly to G × E interactions. Methylation of this CpG island regulates 5-HTT expression, as greater average DNA methylation is associated with lower 5-HTT expression in human lymphoblast cell lines (Philibert et al., 2007) and using reporter gene constructs in vitro (Ölsson et al., 2010), as well as an increased risk for depression (Philibert et al., 2008; although see Ölsson et al., 2010). The factors that influence methylation of the 5-HTT CpG island are only just beginning to be explored. Examples are carrying the low expressing 5-HTTLPR allele as well as the experience of childhood abuse: both have been associated with higher average lymphoblast DNA 5-HTT CpG methylation in humans (Philibert et al., 2007; Beach et al., 2009).

When brain tissue is unavailable for methylation analysis, which is often the case in human or animal studies with longitudinal research designs, peripheral blood 5-HTT markers may serve as a useful surrogate for brain (Uebelhack et al., 2006; Cupello et al., 2009). We have previously observed that early life stress results in lower peripheral 5-HTT expression in infant rhesus macaques, and that 5-HTT expression patterns are associated with behavioral disinhibition during a stressful separation from mothers and social partners at this stage in development in rhesus macaques (Kinnally et al., 2008; Kinnally et al., 2009). The hypotheses that guided the present study were that 5-HTT CpG methylation may mediate or, alternatively, moderate the effects of genotype and early life stress on 5-HTT expression and behavior. To test these hypotheses, we investigated the relationships among peripheral blood DNA 5-HTT CpG methylation, rh5-HTTLPR genotype, and experimental early life stress in infant rhesus macaques. If 5-HTT CpG methylation mediated the effects of genotype or early...
life stress or their interaction, we expected that 5-HTT CpG methylation would be higher in nursery reared infants or in carriers of the “high risk” short rh5-HTTLPR allele. In contrast, we hypothesized that if 5-HTT CpG methylation patterns moderated the effects of genotype and/or early life stress, that 5-HTT CpG methylation would interact with genotype or rearing to influence 5-HTT expression and behavior.

**Materials and Methods**

**Experimental Subjects**

Subjects were 87 (56 female) infant rhesus macaques aged 90–120 days. Infants were selected from two rearing conditions: mother-rearing (MR) and nursery rearing (NR; for full description of rearing protocols, see Capitanio et al., 2005). Briefly, MR animals are raised with their mothers in half-acre outdoor enclosures. Each field cage contained one large social group comprising at least 6 genetically distinct matrilines with extended kin networks. NR animals are separated from their mothers on the day of birth and were subsequently reared indoors in an incubator for the first month of life. Following this month, infants are housed in indoor individual cages (.46 × .61 × .69 m) with intermittent or continuous access to a sex- and age-matched pair-mate. Within rearing groups, all genotypes (long/long homozygotes, or l/l; long/short heterozygotes, or l/s; and short/short homozygotes, or s/s) were represented (MR l/l, n = 50; FR l/s = 18; FR s/s n = 2; NR l/l, n = 12; NR l/s = 4, NR s/s, n = 1). Because s/s homozygotes were rare, l/s and s/s carriers were combined into one “s carrier” group.

**Blood Sampling and PBMC Extraction**—Blood was sampled via femoral venipuncture four times over a 25-hour maternal/social separation as part of a standardized biobehavioral assessment at the California National Primate Research Center (CNPRC). Each sample was decanted into an EDTA-treated collection vial. The first sample was collected at 1100 h (AM sample), approximately 2.0 hrs following social separation/relocation. For the present study, 5-HTT expression analysis was considered only for these “baseline” AM samples.

Whole blood samples were centrifuged for 10 minutes at 3000 RPM at 4°C. Plasma was removed and decanted into 1.5 ml sarstedt (VWR, South Plainfield, NJ) tubes for storage at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from the remaining sample within one hour of sampling. White blood cells were aliquotted to RPMI media (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum and applied to lymphocyte separation media (MP Biomedicals, Solon, OH). Samples were centrifuged at 2000 RPM at 23°C for 30 minutes. The purified phase was washed three times with media, centrifuged, and then resuspended in Trizol RNA stabilizing reagent (Invitrogen, Inc., Carlsbad, CA). Samples were stored for no longer than one year at −80°C.

**Rh5-HTTLPR Genotyping and 5-HTT Expression Analysis**—Methods for genotyping infants in the present study were conducted as described previously (Kinnally et al., 2008). Genotyping was conducted in 25 ul reactions consisting of 1.5 mM MgCl₂ (Promega, Madison, WI), 3X enhancer buffer with betaine, 20μM primer (F1 and R1), 10 X amplification buffer (Promega, Madison, WI), 2.0 μM dNTP and 0.5 U Taq Polymerase (Promega, Madison, WI). Primers sequences were as following: STR F1 5’GGCGTTGCGCGCTCTGAATGC3’; STR R1 5’GAGGGACTGAGCTGGACAACCAC3’. PCR was performed for 35 cycles with initial 5-minute denaturation of 95°C, annealing at 52°C, extension at 74°C, denaturation at 95°C, each step for 30 seconds. A final extension step lasted for 5 minutes. Following amplification, rh5-HTTLPR products were cleaved using restriction enzyme Pst I for at least 1.5 hours at 37°C. Samples were then run on a 3% agarose gel with ethidium bromide for DNA fluorescence.
For PBMC 5-HTT mRNA quantitation, total RNA was isolated from purified PBMC’s stabilized with Trizol reagent (Invitrogen, Inc., Carlsbad, CA). Lysed cells were subjected to phenol extraction and washed with ethanol. RNA was quantified using a spectrophotometer reading at 260/280 nM. One microgram of RNA was then treated with DNAse (Ambion, Inc., Austin, TX) and incubated at 37° C for 60 minutes. Samples were then subjected to reverse transcriptase PCR to synthesize complementary DNA (cDNA). cDNA preparation entailed extension with random hexamers (GE-Amersham Biosciences, Piscataway, NJ), and reverse transcriptase using MMLV-RT (Invitrogen, Inc., Carlsbad, CA). cDNA samples were stored at −20 ° C for no more then 6 months. Real time PCR was conducted using ABI PRISM 7700 Sequence Detection System. A human Taqman quantitative gene expression assay (Applied Biosystems, Inc., Foster City, CA) targeting a region of the 5-HTT gene that we determined to be 100% homologous with rhesus macaques using the published sequence (GenBank accession number AF285761) was used for quantitative PCR. B-actin was selected as an endogenous control, as it was determined to amplify at comparable efficiency to 5-HTT (the slope of the log input amount vs. ΔCt < 0.1). B-actin probe (Applied Biosystems, Inc., Foster City, CA)/primer (Integrated DNA Technologies, Inc., Coralville, IA) sequences are as follows: probe: 5‘ ACC ACC ACG GCC GAG CGG 3’; forward primer 5’ TGA GCG CGG CTA CAG CTT 3’; reverse primer 5’ CCT TAA TGT CAC ACA CGA TT 3’. 83.5 ng cDNA was applied to the primer/probe cocktail and Universal Master Mix (Applied Biosystems, Inc., Foster City, CA) and run in duplicate. All samples were amplified as following: 2 minutes 50 ° C; 10 minutes denaturation at 95 ° C, 40 cycles each: 15 seconds at 95° C, 1 minute at 60 ° C. Human RNA (Invitrogen, Inc., Carlsbad, CA) was included on each plate as a control to establish inter-plate variability. Interplate assay coefficients of variance (calculated as the standard deviation of a human control sample/mean of the human control samples) were less than 3%. 5-HTT values were calculated using the 2 −ΔΔCt method (5-HTT Ct −B-Actin Ct). 5-HTT expression data were available for a subset of subjects (n=46; FR l/l =21, MR l/s = 8; MR s/s =0; NR l/l =12, NR l/s =4; NR s/s = 1).

5-HTT CpG Methylation Analysis—DNA was converted for methylation sequencing using a commercially available sodium bisulfite modification kit (Qiagen, Inc., Valencia, CA). Completion of sodium bisulfite conversion was confirmed by ensuring that known lone cytosines were determined to be thymines during sequencing. The target region was amplified using polymerase chain reaction. PCR amplification was conducted with three primer sets (Integrated DNA Technologies) targeting contiguous regions of the first 650 bp of the 5-HTT CpG island. Primers could not be designed for the 150 bp region of the CpG island that overlaps with the transcription initiation start site. Primers were as follows: F1 5’GGGAAGAAGGTTTTGGAAAAAGA AA3’ R1 5’CCACATATCAAATAACCCATATAAA3’; F2 5’ GGTTGTTTAA A3’; R2 5’ TTTCTTTTATATGGTTTATTTTAGATAA3’; R3 5’ CCTACCGCTACCTACCT ACTACTCC3’. Reverse primers were tagged with biotin. Biotin-labeled amplicons were captured on streptavidin beads (Roche, Inc., Basel, Switzerland), washed sequentially with 70% ethanol, denaturing buffer (10mM Sodium hydroxide) and washing buffer (10 mM Tris, pH7.6). Beads with amplicon were incubated with binding buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6) and 0.4 uM sequencing primer at 80 ° C to anneal primers to the template. Amplicons were subjected to pyrosequencing (Tost and Gut, 2007; Biotage, Inc., Uppsala, Sweden). The quantity of methylated residues was assessed using Q-CpG software (Biotage, Inc.). Each amplicon was sequenced in two to three separate reactions. Sequencing primers were as follows: SEQ1 5’AAGGAAGTCTTGGAAAGAAA A3’; SEQ2 5’TTGTAGGGTTGTTAGG3’; SEQ3 5’AAGTTATTGTAATTTAAGA GGAAT3’; SEQ4 5’GGGYTAGGGTTAGGAT3’;
SEQ5 5’ATGGTTTGATTTTTAG ATAG3’; SEQ6 5’ TGAGGYGAATAAATTTAATG 3’; SEQ7 5’TAGGAGGGCAGG GAT3’. Sequencing was conducted using the PyroMark PSQ MA instrument and PyroGold reagents (Biotage, Inc., Uppsala, Sweden). Proportion of methylated residues in each reaction at each locus was assessed using Q-CpG software (Biotage, Inc. Uppsala, Sweden). Only samples with 100% sodium bisulfite conversion were included. Six residues (CpGs 54–59) were not estimable for any subject due to assay limitations, and were removed from the analysis. Data were analyzed for subjects with at least 70% coverage (mean = 87.5%) of the total 59 residues. To facilitate comparison among subjects, all samples were run on one plate for each sequencing reaction. A subset of samples was assayed twice, and underwent repeated sodium bisulfite conversion, PCR amplification and pyrosequencing. Coefficient of variance between these samples was less than 30%, indicating adequate reliability of methylation estimates.

Biobehavioral Assessment—As part of a large-scale biobehavioral assessment project at the California National Primate Research Center, infants (90–120 days of age) were separated from mothers in the field enclosure or from pair mates in indoor housing and transported to an unfamiliar testing suit. They were housed for the next 25-hrs in individual holding cages (.81 m × .61 m × .66 m) in a temperature-controlled room under a 12:12 hr light/dark cycle. Infants experienced a variety of standardized procedures over the 25-hr period designed to assess behavioral and physiological reactivity (Capitanio et al., 2005; Golub et al., 2009; Capitanio et al., 2006). Standardized procedures were designed to ensure that each subject had experiences comparable to all other subjects who underwent assessment. A detailed description of these behavioral tests can be found in a previously published report (Capitanio et al., 2006). During the 25-hour assessment period, subjects were allowed access to water and food ad libitum.

Holding Cage Observation: Five-minute focal observations were conducted on each subject twice during the 25-hour separation, and a variety of activity states, emotional behaviors, and self-directed behaviors were recorded (see Ethogram, Table 1). Using Observer 5.0 software (Noldus), an observer who had reached a reliability criterion of 85% or better recorded all occurrences of these behaviors during the trial period. The observation period commenced approximately 15 minutes after separation/relocation. Subjects were allowed at least 10 minutes to acclimate to temporary housing conditions. The observer remained at a distance of 2.5 m for the duration of the observation, avoiding eye contact.

Data Analysis

Biobehavioral Assessment—Factor analyses, to identify the underlying factor structure in the data, were conducted using M Plus statistical software, as described elsewhere (Golub et al., 2008). Briefly, the initial exploratory factor analysis used weighted least squares with robust standard errors procedure for estimation and extraction of factors followed by promax rotation. A two-factor solution provided a satisfactory fit to the data. Based on this solution, separate confirmatory factor analyses were performed using data from subsequent years. The final scales were then converted to z-scores and labeled “activity” and “emotionality.” The activity factor included the following behaviors: proportion of time spent in locomotion, proportion of time in the hang position (which loaded negatively), rate of environmental exploration, and whether the animal displayed eating, drinking, or crouching. The emotionality factor comprised rates of cooing and barking, and dichotomous codes for scratch, threat and lip smacking.

Statistics

Univariate analysis of variance was used to determine the main effects of rearing and 5-HTTLPR genotype and the interaction between them on average 5-HTT CpG methylation.
Multiple backward regression was used to test 1.) main effects of rearing, rh5-HTTLPR genotype and 5-HTT CpG methylation and interactions between them on PBMC 5-HTT expression 2.) rearing, rh5-HTTLPR genotype, 5-HTT CpG methylation status and their interactions on behavioral reactivity to stress (activity and emotionality factor scores). One-tailed significance testing was employed when the direction of association was hypothesized a priori based on human data (Philibert et al., 2007). When significant, independent samples t-tests or Pearson’s correlations were conducted to ascertain the specific CpG sites that were associated with outcome measures.

Results

DNA Methylation, Rearing and Genotype

5-HTT methylation did not differ significantly between MR and NR infants (F(1,83) = .012, p = .912), and there was no significant rearing × genotype interaction effect on methylation (F(1,83) = .643, p = .425). However, 5-HTT CpG methylation was higher in short rh5-HTTLPR allele carriers. Across all infants, carriers of the "s" allele exhibited greater methylation (average = 4.934%) than l/l homozygotes (average = 3.805%; F (1, 83) = 4.727 p = .033; see Figure 1). Before correction for multiple comparisons, only methylation at CpG 8 was higher in s allele carriers (t = −2.51, df = 80, p = .022). 5-HTT CpG methylation did not differ between male and female infants (t = .171, df = 85, p = .864)

DNA Methylation and Expression

5-HTT CpG methylation, but no other predictor, was significantly associated with 5-HTT mRNA (t = −1.826, df = 45, p = .035, see Figures 2 and 3), such that higher methylation was associated with lower 5-HTT mRNA. 5-HTT expression was not associated with rearing (t = 1.141, df = 45, p = .072) or genotype alone (t = .475, df = 45, p = .318). Interactions among factors did not significantly predict 5-HTT mRNA: neither a gene × environment interaction (t = −1.07, df = 45, p = .458), gene × methylation interaction (t = −1.12, df = 45, p = .683), nor methylation × environment interaction (t = 1.12, df = 45, p = .458) was observed. Individual CpGs 2, 4, 9, 21, 23, 25 and 47 were negatively correlated with 5-HTT expression (all p < .05), and CpGs 3, 12, and 19 predicted 5-HTT expression at a trend level (all p < .08). 5-HTT expression did not differ between male and female infants (t = .452, df = 45, p = .654).

5-HTT CpG Methylation and Behavior

5-HTT CpG methylation × early environment interactions predicted activity but not emotionality factor scores during maternal/social separation. The effect was such that NR individuals with higher 5-HTT CpG methylation displayed higher activity factor scores (Figures 4 and 5) during maternal/social separation compared with MR infants (F (2, 86) = 2.21, p = .030). Rh5-HTTLPR × rearing interactions did not predict activity scores (t = −.272, df = 86, p = .786), nor were there direct main effects of genotype (t = .404, df = 86, p = .688) or rearing alone (t = .658, df = 86, p = .512) on activity factor scores. Sex was not associated with activity factor scores (t = .325, df = 85, p = .746) or emotionality factor scores (t = 1.603, df = 85, p = .113).

Discussion

Identifying the regulatory factors that contribute to 5-HTT expression, especially early in development, will clarify the role of 5-HTT in psychological risk following early stress. It was hypothesized that genetic, epigenetic, and environmental factors would explain variation in expression of the 5-HTT gene as well as behavioral development measured early in life. We examined the relationship of rh5-HTTLPR genotype, 5-HTT CpG island
methylation, and early life stress with peripheral blood mononuclear cell (PBMC) 5-HTT expression and behavioral response to acute maternal/social separation in infant rhesus macaques. Consistent with a study in humans (Philibert et al., 2007), we found that average 5-HTT CpG methylation was higher in carriers of the low expressing rh5-HTTLPR allele and was negatively associated with PBMC 5-HTT mRNA expression in infant rhesus macaques. These data indicate that 5-HTT CpG methylation is functionally related to 5-HTT expression in PBMCs early in life, and suggests that methylation status of the 5-HTT regulatory region may explain some of the apparent relationship of stress and 5-HTTLPR genotype with subsequent psychopathology. In support of this inference, we found that in maternally-deprived NR infants, higher 5-HTT CpG methylation, regardless of rh5-HTTLPR genotype, exhibited the greatest activity during a stressful maternal/social separation. As behavioral hyper-reactivity to stress is a hypothesized risk factor for mood disorders in humans, we suggest that 5-HTT CpG methylation may play a role in the risk conferred by the “short”, low expressing rh5-HTTLPR allele that has been previously reported by some (Champoux et al., 2002; Caspi et al., 2003; Barr et al., 2004; Kaufman et al., 2004; Cichetti et al., 2007; Stein et al., 2008; Zalsman et al., 2006) but not others (Surtees et al., 2006; Munafo et al., 2008; Risch et al., 2009). These epigenetic findings must, of course, be confirmed in brain tissue before firm conclusions can be drawn.

This is the first description of individual variation in PBMC DNA 5-HTT CpG methylation in rhesus macaques. The extent of average 5-HTT CpG methylation across the first 650 bp of the 5-HTT CpG island was relatively low in our subjects; individual averages ranged from 0.82% – 11.51%. Since individual values at a given site ranged from 0% – 100%, we do not believe that these low values are attributable to decreased sensitivity at the lower bounds of the assay. Further, this range is consistent with reports of human lymphoblast cell lines (range: 0 – 20%; Philibert et al., 2007) and human buccal cells (range: 2.9 – 22%; Olsson et al., 2010). The expression data further validate our methylation findings as even modest differences in 5-HTT CpG methylation were associated with variation in 5-HTT expression, consistent with adult humans (Philibert et al., 2007) and reporter gene assay data (Olsson et al., 2010). Unsurprisingly, these findings are also consistent with our previous findings that neither rearing nor genotype independently or interactively predicted 5-HTT expression (Kinnally et al., 2008): the samples included in the present study represented a subsample from that original study. These data suggest that PBMC 5-HTT expression is partly regulated by 5-HTT CpG methylation.

Although the observed link between greater methylation and rh5-HTTLPR genotype was consistent with human data, our failure to find a relationship between methylation and early life stress was not (Philibert et al., 2007; Beach et al., 2009). Failure to detect effects of nursery rearing on average 5-HTT methylation may indicate that our target region was too large: Beach et al. focused their investigation on 21 CpG sites that were largely restricted to the first 100 bp in the 5’ end of the island, while we included 650 bp of the CpG island. Though we were underpowered to investigate effects of nursery rearing on individual CpGs in our study, we found that, before correction for multiple comparisons, CpGs 27, 31 and 32 displayed significantly greater methylation in nursery-reared infants (data not shown). These sites are restricted to the first 250 bp of the 5-HTT CpG island, somewhat consistent with the human finding. These sites may serve as a priori regions of interest in future studies.

The discovery that carriers of the short rh5-HTTLPR allele exhibit higher methylation is consistent with several human studies which have recently demonstrated a link between low-expressing alleles of functional polymorphisms and greater average methylation of a CpG islands within the regulatory regions of these genes (5-HTT, Philibert et al., 2007; MAOA, Philibert et al., 2008; BDNF: Mill et al., 2008; 5HT1A, Lu et al., 2008). We have now demonstrated this phenomenon in non-human primates: the l/s or s/s rh5-HTTLPR
genotype was associated with greater methylation of the 5-HTT CpG island, consistent with a human study (Philibert et al., 2007). These findings must be replicated, however, as only CpG 8 was determined to differ significantly by genotype. As such, it is possible that this finding was an artifact. However, inspection of individual sites reveals that 40/59 CpGs had higher proportions of methylation in carriers of the short allele (data not shown). The mechanism for a genotype-methylation status association is unknown. It is possible that methylation status of this region is inherited in linkage disequilibrium with \textit{rh5-HTTLPR} genotype (Jones and Takai, 2007), although the stability of methylation patterns during embryogenesis has not been verified empirically. It is also possible that accessibility of transcription factors (TFs) to DNA binding sites may influence de novo methylation during development (Brandeis et al., 1994; MacLeod et al., 1994). The 5-HTTLPR insertion/deletion polymorphism is flanked by several nominal TF binding sites, including those for SP-1, AP-1 and AP-2 (Heils et al., 1996). Access of TFs to this genomic region may differ based on \textit{rh5-HTTLPR} genotype, resulting in variation in CpG methylation during development. In any case, if the mechanism(s) of variation in 5-HTT CpG methylation are developmental in nature, they clearly take place relatively early in development (fetal or postnatal), as our subjects were 3–4 months of age, comparable to one year of age in humans. The stability of these marks over the lifespan remains to be determined.

NR infants are generally more reactive to stress than MR counterparts (Champoux et al., 2002; Capitanio et al., 2006; Kinnally et al., 2010), although the difference was not statistically significant in our study. Intriguingly, 5-HTT methylation status measured in peripheral blood DNA was associated with an exacerbated effect of early experience on behavioral response to stress. We found that NR, or maternally deprived, infants with higher 5-HTT CpG methylation exhibited higher activity during stress, while MR infants’ activity factor scores were not related to methylation status. Our findings therefore parallel multiple reports in humans and non-human primates that the low expressing 5-HTTLPR allele confers risk for poorer stress adaptation and mental health in the presence of early life stress (Champoux et al., 2002; Caspi et al., 2003; Barr et al., 2004; Kaufman et al., 2004; Zalsman et al., 2006; Cichetti et al., 2007; Stein et al., 2008; although see Surtees et al., 2006; Munafò et al., 2008; Risch et al., 2009; Kinnally et al., 2010; Kinnally et al., 2010).

However, our data suggest that 5-HTT CpG methylation plays a functional role in these interactions. It is possible that observed early life stress \(\times\) low expressing 5-HTTLPR genotype interactions may have resulted, in part, from greater methylation patterns in the 5-HTT regulatory region. Notably, this epigenetic mechanism for genotype effects was first speculated upon by Heils et al. (1996) when the 5-HTTLPR polymorphism was originally characterized.

While our results may help clarify the mechanisms of susceptibility conferred by \textit{rh5-HTTLPR} genotype, the mechanism by which greater 5-HTT CpG methylation leads to risk for greater behavioral reactivity in the context of early life stress remains unknown. First, we assume that this relationship is due to parallel methylation patterns between peripheral blood and relevant brain region 5-HTT methylation patterns. This must be determined empirically, however. If so, it is possible that higher methylation itself, perhaps via effects on 5-HTT expression, puts all individuals at risk for poor behavioral adaptation to stress. If this is the case, perhaps MR infants evoke a compensatory (epigenetic or otherwise) process that abrogates the association of 5-HTT CpG methylation with lower 5-HTT expression and poor stress adaptation. This will be a critical question to address in order to understand the role of 5-HTT in risk for psychiatric dysfunction following early life stress.

Several limitations to our study must be considered. Presumably, our findings arise from the accumulation of site-specific interactions of transcriptional enhancers or repressors (or both) within the 5-HTT CpG island, leading to alterations in 5-HTT expression and related...
behavior. But the present study was underpowered to explore each individual CpG site in its interaction with early life stress. Presenting interactions for each of 65 CpG sites would therefore not be compelling. Exploratory analysis suggests, however, that CpG sites 2, 4, 9, 21, 23, 25 and 49 may be of particular note, as they are significantly associated with 5-HTT expression. Notably, sites 21–23 lies within an SP-1 binding site, making it a potential *a priori* candidate site for future studies.

Recent work in biological psychiatry has demonstrated the plasticity of the epigenome in response to early life experiences (Weaver et al., 2004; Roth et al., 2009; McGowan et al., 2009; Oberlander et al., 2008; Beach et al., 2009). Our results suggest that whatever the developmental mechanism(s) for epigenetic patterning of peripheral blood DNA 5-HTT in infant rhesus macaques, that greater methylation put at-risk individuals at even greater risk for developing poor stress coping strategies. This finding requires replication and must be extended from surrogate PBMC to brain regions of interest before we can confidently relate this phenomenon to psychobiological processes. Nevertheless, we suggest that even small differences in methylation across regulatory regions of stress pathway genes represent a new risk factor for poor outcomes following early life stress.

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Figure 1.
Direct sodium bisulfite pyrosequencing of 65 CpGs in an 800 bp CpG island that overlaps with the transcription initiation start site of the 5-HTT gene in infant rhesus macaques. Average C-methylation of CpGs 1–65 is presented for long rh5-HTTLPR homozygotes and carriers of the short rh5-HTTLPR allele. Multiple regression analysis revealed that average methylation across all 65 CpG’s was significantly higher in carriers of the “s” allele, compared with l/l homozygotes.
Figure 2.
Average C-methylation is negatively associated with peripheral blood mononuclear cell 5-HTT mRNA. Multiple regression analysis revealed that average PBMC DNA 5-HTT CpG methylation determined via direct sodium bisulfite pyrosequencing was inversely associated with PBMC 5-HTT mRNA quantified using qRT-PCR in 46 subjects, indicating that 5-HTT C-methylation may regulate 5-HTT expression. Neither Rh5-HTTLPR genotype (l/l = black circles, l/s = gray circles, s/s = white circles), nor rearing (MR = circles; NR = triangles) was associated with PBMC 5-HTT mRNA. Regression fit lines are displayed for each subgroup.
Figure 3.
Average C-methylation is negatively associated with peripheral blood mononuclear cell 5-HTT mRNA. Multiple regression analysis revealed that average PBMC DNA 5-HTT CpG methylation determined via direct sodium bisulfite pyrosequencing was inversely associated with PBMC 5-HTT mRNA quantified using qRT-PCR in 46 subjects, indicating that 5-HTT C-methylation may regulate 5-HTT expression. Neither Rh5-HTTLPR genotype (l/l = black circles, l/s = gray circles, s/s = white circles), nor rearing (MR = circles; NR = triangles) was associated with PBMC 5-HTT mRNA. Regression fit lines are displayed for each subgroup.
Figure 4.
A 5-HTT CpG methylation status × early life stress interaction predicted Activity factor scores during a maternal/social separation. Nursery reared (NR) infants (triangles) with higher average 5-HTT CpG methylation exhibited higher Activity Factor scores than NR infants with lower 5-HTT CpG methylation. Rh5-HTTLPR genotype (l/l = black circles, l/s = gray circles, s/s = white circles) × early life stress interactions were not observed to influence activity factor scores. Regression fit lines are displayed for each subgroup.
Figure 5.
A 5-HTT CpG methylation status × early life stress interaction predicted Activity factor scores during a maternal/social separation. Nursery reared (NR) infants (triangles) with higher average 5-HTT CpG methylation exhibited higher Activity Factor scores than NR infants with lower 5-HTT CpG methylation. Rh5-HTTLPR genotype (l/l = black circles, l/s = gray circles, s/s = white circles) × early life stress interactions were not observed to influence activity factor scores. Regression fit lines are displayed for each subgroup.