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Serotonin Transporter Genotype Affects Serotonin 5-HT$_{1A}$ Binding in Primates

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Abstract

Disruption of the serotonin system has been implicated in anxiety and depression and a related genetic variation has been identified that may predispose individuals for these illnesses. The relationship of a functional variation of the serotonin transporter promoter gene (5-HTTLPR) on serotonin transporter binding using in vivo imaging techniques have yielded inconsistent findings when comparing variants for short (s) and long (l) alleles. However, a significant 5-HTTLPR effect on receptor binding at the 5-HT$_{1A}$ receptor site has been reported in humans, suggesting the 5-HTTLPR polymorphism may play a role in 5-HT function. Rhesus monkeys possess a 5-HTTLPR length polymorphism similar to humans and serve as an excellent model for studying the effects of this orthologous genetic variation on behaviors and neurochemical functions related to the 5-HT system. In this study, PET imaging of [F-18]mefway was performed on 58 rhesus monkeys (33 *l/l*, 25 s-carriers) to examine the relation between 5-HT$_{1A}$ receptor specific binding and 5-HTTLPR genotypes. Significantly lower 5-HT$_{1A}$ binding was found in s-carrier subjects throughout both cortical brain regions and the raphe nuclei. These results demonstrate that the underlying 5-HT neurochemical system is influenced by this functional polymorphism and illustrate the strong potential for extending the nonhuman primate model into investigating the role of this genetic variant on behavior and gene-environment interactions.

Keywords

5-HTTLPR; serotonin; PET; 5-HT$_{1A}$; mefway; endophenotype; rhesus nonhuman primate

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Introduction

Disruptions in the serotonin (5-HT) system, including biosynthesis, transmission, reuptake and degradation, are implicated in a wide variety of mood and anxiety-related neuropsychiatric illnesses. The 5-HT transporter (5-HTT) clears serotonin from the synaptic cleft and thereby plays a major role in serotonergic neurotransmission. The gene encoding 5-HTT contains a functional length polymorphism in the promoter region (referred to as 5-HTTLPR) that is associated with the development of emotional traits and psychopathology. The role of allelic variation in 5-HTTLPR as a contributing factor to neuropsychiatric illness has been extensively studied. Such studies have strongly implicated 5-HTTLPR through behavioral association and neuroimaging studies of brain structure and function (see Canli and Lesch, 2007 for review). Imaging studies of neurochemical function in humans have focused on the baseline binding and expression of 5-HTT and have yielded inconsistent relations with 5-HTTLPR variants (Heinz et al.,2000;Reimold et al.,2007;Praschak-Rieder et al.,2007;Shioe et al.,2003;Parsey et al.,2006). Extending beyond the search for direct 5-HTTLPR allelic effects on 5-HTT expression, the 5-HT_{1A} system has served as a candidate for altered function due to its role in serotonin regulation and its responsivity of receptor expression to selective serotonin reuptake inhibitor (SSRI) drugs. Using PET imaging of [C-11]WAY100635 in a moderately large sample of healthy volunteers (n=35), a significant reduction in 5-HT_{1A} binding was revealed in carriers of the short 5-HTTLPR allele (David et al.,2005), thus providing intriguing evidence for 5-HTTLPR influence on serotonergic function.

The rhesus monkey (*Macacca mulatta*) serves as an excellent model for studying the influence of 5-HTTLPR allelic length on neurochemical endophenotypes. 5-HTTLPR in humans has an analogous biallelic length variation in the rhesus monkey (Lesch et al.,1996). In the same region, although not exactly same location as the 5-HTTLPR polymorphism in humans, a 21 bp insertion/deletion variant is found in the rhesus monkey. Moreover, the ability to control the rearing environment has made the rhesus monkey an ideally suited model for studying 5-HTTLPR × environment interactions. For example, adverse early life events were found to interact with 5-HTTLPR allelic length to influence stress reactivity (Bennett et al.,2002;Barr et al.,2004b), neonatal response (Champoux et al.,2002) and alcohol sensitivity (Barr et al.,2004a). Behavioral studies with rhesus monkeys reported that s-carriers experiencing environmental challenge showed reduced cognitive flexibility (Izquierdo et al.,2007), neonatal irritability (Kraemer et al.,2008) and increased activation of the orbitofrontal cortex and the amygdala (Kalin et al.,2008). Similar studies that attempt to establish coupling between genetic and environmental influences are often not suitable for human subjects, making the rhesus monkey an invaluable resource for research in 5-HTTLPR × environment interactions.

In this paper, we examine the relation between 5-HTTLPR allelic length and 5-HT_{1A} binding using a large cohort of rhesus monkeys with a close age range and similar rearing conditions. We employed a highly selective 5-HT_{1A} PET radioligand, [F-18]mefway, with high resolution imaging. The rhesus monkey model was selected to minimize the environmental variability present in the general human population due to life experiences and conditions, with the goal of increasing the sensitivity for detecting gene-endophenotype relations.

Methods

Subjects

Fifty-eight rhesus (*Macaca mullata*) monkeys (36 females, 22 males; 10.4 ± 2.2 kg) were included. The age at the time of scanning was 14.5 ± 1.9 years. Genotyping for the rhesus 5-

HTTLPR was based upon a modified protocol of Lesch and colleagues (Lesch et al.,1996) as previously reported by our group for some of the subjects in this cohort (Kraemer et al., 2008). Thirty-three subjects were *l/l* homozygous (21 females, 12 males) and 25 subjects (15 females, 10 males) were s-carriers, including *s/s* homozygous (n=3) and *l/s* heterozygous (n=23) subjects. Combining the *s/s* and *s/l* subjects within the s-carriers group is consistent with analyses methods for many human and rhesus 5HTTLPR studies due to the autosomal dominance of the 5HTTLPR s-allele (Lesch et al., 1996). The subjects were incorporated from two separate protocols involving the investigation of prenatal stress and prenatal alcohol exposure on development (see Schneider et al.,1999 and Schneider et al.,1997 for details). Briefly, prenatal stress consisted of daily exposure of the pregnant mother to three unpredictable horn blasts. Prenatal alcohol exposure consisted of the pregnant mother voluntarily consuming moderate levels of alcohol (0.6 mg/kg daily) during gestation. These offspring from the prenatal stress condition (7 *l/l*, 7 s-carriers) and prenatal alcohol condition (15 *l/l*, 11 s-carriers), along with controls (11 *l/l*, 7 s-carriers), were mother-reared for 6 months and housed in peer groups until 30 months of age and subsequently in pairs similar in age, sex and prenatal condition. All experimental procedures involving the rhesus monkeys were approved by the Institutional Animal Use and Care Committee (IACUC).

PET Measures

The assay of 5-HT_{1A} binding was performed using dynamic PET imaging of the 5-HT_{1A} specific antagonist radioligand [F-18]mefway, which we have previously validated in rhesus monkeys (Wooten et al.,2011). The radiotracer was produced using previously published methods (Saigal et al.,2006) resulting in typical end of synthesis specific activities of 150 GBq/μmol. The PET studies were conducted with an injected [F-18]mefway activity of 59 – 130 MBq administered through a catheter placed in the saphenous vein in a volume of 3 mL physiological saline. In preparation for the PET scans, the subjects were initially anesthetized with 10 mg/kg (i.m.) ketamine and subsequently maintained at approximately 1.5% isoflurane throughout the course of the experiment. Vital signs including body temperature, breathing rate, heart rate and oxygen saturation levels were monitored and recorded throughout the experiment. Atropine sulfate (0.27 mg) was given to reduce secretions. The subjects were positioned in the PET scanner using a custom built stereotaxic headholder with the head facing down. The PET scans were acquired on a Concorde Systems microPET P4 scanner (Tai et al.,2001). A 518 second transmission scan using a Co-57 rotating point source was first performed to correct for tissue attenuation and scatter of the annihilation radiation. The acquisition of the emission data was initiated with the 30 second bolus injection of [F-18]mefway and continued for a duration of 90 minutes. Upon completion of the study, anesthesia was turned off and the monkey was returned to its cage when swallowing reflex was restored, and continuously monitored until fully alert.

Data Processing

The dynamic PET data were binned into temporal frames and reconstructed using filtered back projection (0.5 cm^{-1} ramp filter) with corrections applied for deadtime, scanner normalization, radiation attenuation and scatter. The reconstructed PET time-series were 128 × 128 × 63 × 23 (x,y,z,t) voxels with spatial dimensions of 1.90 × 1.90 × 1.21 mm^3. The time-series data for each subject were converted to a parametric image of the distribution volume ratio (DVR) to serve as a metric of [F-18]mefway binding to the 5-HT_{1A} receptor site. The DVR represents an index of receptor density (B_{avail}), apparent affinity ($1/K_D$) and the ligand free fraction in the nondisplaceable tissue compartment (f_{ND}), as given by the relation: $DVR = f_{ND}B_{avail}/K_D + 1$ (Innis et al.,2007). The cerebellar cortex was used as a reference region of negligible 5-HT_{1A} specific binding and the DVR was estimated using a multi-linear reference tissue model (MRTM2) (Ichise et al.,2003). The DVR image volumes for each subject were spatially transformed into a standard space based upon the atlas of

Paxinos (Paxinos et al.,2000). A rhesus monkey MRI atlas (McLaren et al.,2009) was used to create a [F-18]mefway template and the DVR image volumes were transformed into the common space using the SPM8 software (http://www.fil.ion.ucl.ac.uk/spm/doc/). The spatially normalized DVR images were then smoothed with a $4 \times 4 \times 4$ mm^3 gaussian filter to account for anatomical variation among subjects. Brain regions of interest (ROIs) were selected based upon the 5-HTTLPR regional findings reported by David et al. (2005), with each ROI consisting of subregions outlined according to atlas specifications (Saleem and Logothetis,2007). Specifically, ROIs were extracted for the areas of the anterior cingulate gyrus (aCg: 1.4 cm^3), posterior cingulate gyrus (pCg: 1.3 cm^3), hippocampus (Hp: 1.6 cm^3), amygdala (Am: 0.4 cm^3), dorsal lateral prefrontal cortex (dlPFC: 4.2 cm^3), parietal cortex (PC: 4.9 cm^3), occipital cortex (OC: 16.1cm^3), lateral temporal cortex (lTC: 7.0 cm^3) and the agranular frontal cortex (aFC: 4.4 cm^3). The placement of all ROIs was consistent across all subjects, however, for the raphe nuclei (RN) a fixed volume ROI (3 voxel diameter circle placed on 3 consecutive transaxial planes: 0.10 cm^3) was centrally placed over the region of focal binding on the DVR images in native space to minimize variability in positioning over this small brain region. The location of the ROIs displayed on a [F-18]mefway image are shown in figure 1.

Statistical Analyses

A multivariate analysis of variance model (MANOVA) was used to determine the relationship between the 5-HTTLPR variant and the 5-HT_{1A} binding (DVR) of the brain regions while adjusting for the effects of age, sex and treatment. The multivariate response variable consisted of all of the brain ROIs under investigation and the predictors of interest were 5-HTTLPR, age, sex and treatment group. The treatment groups consisted of subjects exposed to prenatal stress (n=14), prenatal alcohol (n=26) and age-matched controls (n=18). In this paper, we report only on the 5-HTTLPR main effect results, while including the treatment groups in the model. The multivariate analysis of variance tests the hypothesis that the vector of 5-HT_{1A} DVR means in the *l/l* group is equal to the vector of means in the s-carrier group while adjusting for age, sex and treatment. Pillai's trace is reported as the test statistic in our multivariate analysis of variance (MANOVA) in our analysis because it is the most robust statistical measure when there is unequal cell size as in this case (Hair et al., 2006). Analysis was performed using SAS (version 9.2) and R (version 2.13) (Everitt,2007). Univariate analyses on specific brain regions were conducted using the same predictors as the multivariate model to follow up significant multivariate effects.

Results

Overall significant differences between the regional 5-HT_{1A} DVRs of the two 5-HTTLPR groups (*l/l* homozygotes and s-carriers) were found in the model that contained only 5-HTTLPR status, Pillai's trace=.435 (p-value= 0.0025) and these overall differences were present after adjusting for age, sex and treatment status (Pillai's trace : p-value= 0.002). As shown in Table 1, MANOVA also revealed a significant overall effect of age (Pillai's trace=0.467, p-value=0.001) as well as sex (Pillai's trace=0.334, p-value=0.039).

The reduced binding with age was not statistically significant in any of the univariate models; thus the age effect represents an overall decline in 5-HT_{1A} binding. In univariate analyses sex differences were significant in the amygdala (p=.013, females higher than males) and there was a trend toward the same sex differences in anterior cingulate (p=.051), hippocampus (p=.055), and aFC (p=0.060), but they did not reach significance.

In the univariate tests to focus on the specific brain regions responsible for the significant effect 5-HTTLPR group, the s-carriers showed significantly lower binding then the *l/l* subjects in the following brain regions: raphe nucleus (p= 0.009), occipital (p = 0.031),

parietal (p = 0.036). A trend toward lower [F-18]mefway binding in the s-carriers was also found for the anterior cingulate (p = 0.059) and posterior cingulate (p = 0.058), but these did not reach significance (see Table 2).

The assumption of multivariate normality was verified by examining residual plots of the model (i.e. chi-square plots for the multivariate normality of the error terms). No significant departures from the multivariate normal distributions were observed. One outlier was identified (female, *l/l* homozygote with elevated 5-HT_{1A} binding) and the analysis was performed both including and excluding this subject. No differences in the significance of our results were observed when this observation was removed, therefore the animal was retained for the reported analyses. Within the s-carrier group, the three *s/s* homozygous subjects displayed no detectable trend towards elevated or decreased 5-HT_{1A} binding compared to the *l/s* heterozygous subjects.

Discussion

The present findings that 5-HTTLPR allelic variations significantly alter the 5-HT_{1A} receptor system in the rhesus monkey are consistent with a similar study in humans (David et al.,2005). In that retrospective human study, PET imaging of a 5-HT_{1A} antagonist radioligand in a total of 35 healthy individuals revealed carriers of the short 5HTTLPR genotypes exhibited decreased 5-HT_{1A} binding throughout 5-HT_{1A} expressing regions of the brain. As with our results, this decrease in binding was seen in all cortical brain regions as well as the raphe midbrain region. A more recent study in humans (n=54), however, did not find an 5-HTTLPR association with 5-HT_{1A} binding (Borg et al.,2009). Both these human studies describe similar methods, using [C-11]WAY100635 in middle age subjects. However, the study with negative findings reported a much higher variation in 5-HT_{1A} binding across the subjects (~2-fold greater c.o.v.) suggesting a more heterogeneous population or some unknown source of variance. It is also possible different processing methodologies might have contributed to large variabilities across subjects.

It has been proposed that the short allele genotype yields reduced transcriptional efficiency in expression of the 5-HT transporter (Lesch et al.,1996;Collier et al.,1996). This could in turn possibly result in elevated 5-HT tone and 5-HT_{1A} receptor desensitization (David et al., 2005). Although a number of human (Shioe et al.,2003;Jacobsen et al.,2000;Van Dyck et al., 2004) and rhesus (Christian et al.,2009;Jedema et al.,2010) PET neuroimaging studies do not support the notion that 5-HTT expression is compromised in s-carriers, we cannot fully dismiss this mechanistic component from the model of 5-HT system dysregulation associated with the s-carrier 5-HTTLPR genotype.

Radioligand imaging studies of 5-HTT binding in 5-HTTLPR variants have reported a metric of “binding potential” representing an index proportional to the receptor density (B_{max}) and ligand-protein affinity ($1/K_{Dapp}$). Using this composite index, potential decreases in receptor density could be masked by matching increases in affinity, or accompanying decreases in endogenous 5-HT competition. With this limitation, the interpretation of the decreased 5-HT_{1A} binding reported in s-carriers could be due to either elevated 5-HT tone or 5-HT_{1A} downregulation. Additional studies and methodologies interrogating 5-HT function, such as measurements separating receptor density from affinity in pre- and post-synaptic receptors and 5-HT metabolism, will be required to understand the role of the 5-HTT and the mechanisms by which this decrease in 5-HT_{1A} binding was achieved.

The rhesus 5-HTTLPR length polymorphism has been used extensively to study neurobehavioral functioning. Research on resiliency to stressful events or conditions in infant and juvenile rhesus monkeys has shown that carriers of the short allele demonstrate

heightened physical aggression and HPA response, altered serotonin metabolism and regional brain metabolism (Bennett et al.,2002;Barr et al.,2004b;Champoux et al., 2002;Kalin et al.,2008;Barr et al.,2003), illustrating the strength of the rhesus monkey model for the study of gene × environment interactions (Suomi,2006). Previous work from our lab using subjects included with the research reported herein found that prenatal alcohol-exposed carriers of the s-allele exhibited increased neonatal irritability and increased stress responses compared to *l/l* homozygotes independent of prenatal alcohol exposure (Kraemer et al.,2008). The statistical model for the present research included treatment groups to account for potential prenatal condition alterations, permitting us to focus on the effect of 5-HTTLPR allelic variations over and above any prenatal treatment main effects. We regard this research as a first step in understanding the effects of 5-HTTLPR variations on 5-HT function in the rhesus monkey model. Future analysis of this cohort will examine interactions between 5HTTLPR, 5-HT1A and prenatal treatment with behavioral measures.

A recent study from another lab evaluated 5-HT_{1A} binding in rhesus monkeys as a function of 5-HTTLPR length polymorphisms, among other biomarker comparisons. Using a smaller sample size (n=8) of all male subjects, Jedema and colleagues found no statistically significant difference in 5-HT_{1A} binding between s-carriers and (*l/l*) homozygotes (Jedema et al.,2010). However, consistent with our results, reduced 5-HT_{1A} binding was observed in all reported cortical and midbrain regions ($p < 0.116$ using multivariate analysis). As suggested by the authors, their study may have been underpowered for detecting the allelic group differences. In our study, there was a 7% difference between groups when averaged over all regions of the 58 subjects. Using MRI T1 images of their cohort, Jedema et al. found statistically significant differences in cortical morphology in regional gray matter volume, with s-carriers having reduced volume in several brain regions that were implicated in measured cognitive tasks. A limitation of the present study is that we do not have MRI images available on this cohort. Therefore, it is conceivable that a thinner cortex in s-carriers would result in lower measured 5-HT_{1A} binding due to image resolution (i.e. partial volume) effects even if 5-HT_{1A} receptor density was preserved. To address this, in part, the PET DVR images were spatially smoothed after transforming to atlas space to minimize the variability of imperfect ROI placement over peak DVR values. Further, all cortical volumes consisted of large regional sampling resulting in lower [F-18]mefway DVR values when compared to our previously published values using [F-18]mefway (Wooten et al.,2011) and perhaps masking large differences in 5-HT_{1A} binding in focal brain regions.

In conclusion, carriers of the 5-HTTLPR short allele were found to have significantly reduced binding at the 5-HT_{1A} receptor sites, suggesting that 5-HTTLPR length variations play a role in the regulation of the 5-HT system. The rhesus model was used to provide a study cohort that minimizes variability potentially affecting the 5-HT system which is found in the human population, including psychosocial stress and substance abuse. These findings also confirm that 5-HT related alterations persist into adulthood, given the age of the rhesus subjects (14.5 yrs) were approximately equivalent to humans in their fourth decade of life, an age similar that of the study by David and colleagues in humans (David et al.,2005). An intriguing question is that of the developmental course of differences in 5-HT function between 5-HTTLPR variants and the possibility of developmentally vulnerable periods of 5-HT disruption.

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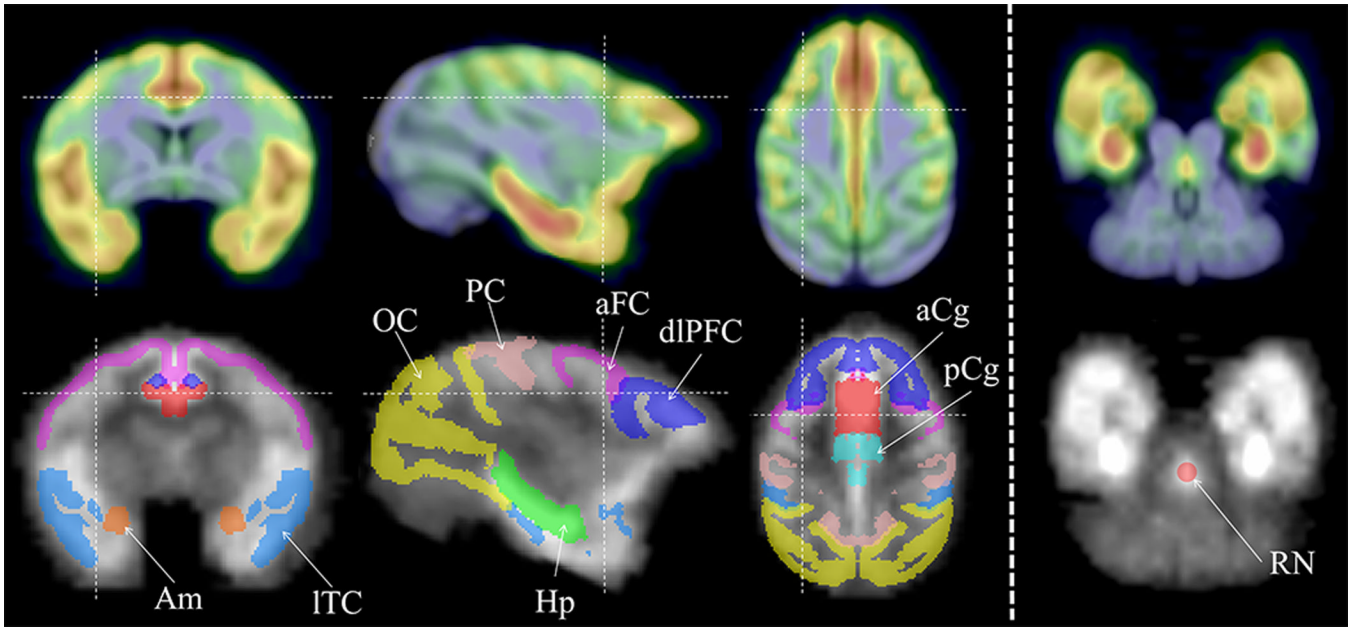

Figure 1. 5-HT$_{1A}$ Binding Distribution and Region of Interest Definitions

Top row: [F-18]Mefway binding displayed on MRI template in coronal, sagittal and two transaxial views. Bottom row: Regions of interest defined in template space for the amygdala (Am), lateral temporal cortex (lTC), occipital cortex (OC), parietal cortex (PC), hippocampus (Hp), agranular frontal cortex (aFC), dorsolateral prefrontal cortex (dlPFC), anterior cingulate gyrus (aCg), posterior cingulate gyrus (pCg) and raphe nuclei (RN). There was a slight overlap of some adjacent regions (approximately 1% of volume) due to spatial smoothing of the template masks.

Table 1

Multivariate Effects

Variable	Pillai's Trace	F	*df*	Error *df*	Pr>F
5-HTTLPR	0.445	3.46	10	43	0.002
Sex	0.334	2.16	10	43	0.039
Age	0.467	3.77	10	43	0.001
Treatment	0.414	1.15	20	88	0.317

Table 2

5-HT_{1A} binding values (DVR) for each region by 5-HTTLPR (mean±s.d.)

5-HTTLPR ROI	*l/l* (n=33)	s-carrier (n=25)	Mean difference estimate (l/l-s-carrier)	p-value*
RN	3.13 ± 0.43	2.87 ± 0.27	0.26	.009**
aCg	3.65 ± 0.80	3.28 ± 0.57	0.36	.059
pCg	2.56 ± 0.49	2.32 ± 0.34	0.23	.058
OC	1.28 ± 0.19	1.18 ± 0.14	0.10	.031**
PC	2.44 ± 0.51	2.17 ± 0.34	0.25	.036**
lTC	2.72 ± 0.47	2.60 ± 0.38	0.12	.305
dlPFC	3.35 ± 0.74	3.11 ± 0.52	0.24	.164
aFC	3.08 ± 0.65	2.84 ± 0.42	0.24	.097
Hp	3.65 ± 0.55	3.67 ± 0.50	−0.01	.914
Am	3.30 ± 0.53	3.24 ± 0.33	0.06	.585

* p-values are from the univariate analyses and test if the difference between the *l/l* and s-carrier groups was significant (models were adjusted for sex, age, treatment).

** significant p-values