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ORIGINAL ARTICLE

The serotonin transporter genotype is associated with intermediate brain phenotypes that depend on the context of eliciting stressor

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A variant allele in the promoter region of the serotonin transporter gene, *SLC6A4*, the *s* allele, is associated with increased vulnerability to develop anxiety-related traits and depression. Furthermore, functional magnetic resonance imaging (fMRI) studies reveal that *s* carriers have increased amygdala reactivity in response to aversive stimuli, which is thought to be an intermediate phenotype mediating the influences of the *s* allele on emotionality. We used high-resolution microPET [^{18}F]fluoro-2-deoxy-D-glucose (FDG) scanning to assess regional brain metabolic activity in rhesus monkeys to further explore *s* allele-related intermediate phenotypes. Rhesus monkeys provide an excellent model to understand mechanisms underlying human anxiety, and FDG microPET allows for the assessment of brain activity associated with naturalistic environments outside the scanner. During FDG uptake, monkeys were exposed to different ethologically relevant stressful situations (relocation and threat) as well as to the less stressful familiar environment of their home cage. The *s* carriers displayed increased orbitofrontal cortex activity in response to both relocation and threat. However, during relocation they displayed increased amygdala reactivity and in response to threat they displayed increased reactivity of the bed nucleus of the stria terminalis. No increase in the activity of any of these regions occurred when the animals were administered FDG in their home cages. These findings demonstrate context-dependent intermediate phenotypes in *s* carriers that provide a framework for understanding the mechanisms underlying the vulnerabilities of *s*-allele carriers exposed to different types of stressors.
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Introduction

Many studies have examined the influences of a functional polymorphism (5-HTTLPR) within the promoter region of the serotonin transporter gene (*SLC6A4*) on the development of emotional traits and psychopathology. Although not entirely consistent, considerable evidence suggests that individuals with the short allele (*s*) compared to those that are homozygous for the long allele (*l*) are more likely to have anxiety-related traits.[1–9] Furthermore, gene by environment interactions involving this genetic polymorphism have been demonstrated such that *s* carriers with a history of significant stress exposure are at increased risk to develop depression.[5,6] Studies have also been performed in rhesus monkeys demonstrating that this species has a similar but not identical polymorphism in the same promoter region[10] that may also show similar gene by environment interactions.[11,12] In addition, a number of human functional magnetic resonance imaging (fMRI) studies demonstrate that *s* carriers display increased amygdala (AMYG) reactivity when exposed, in the scanner, to potentially aversive stimuli.[13–16] A few imaging studies have been performed in relation to psychopathology. A [^{15}O]-H_2O positron emission tomography (PET) blood flow study demonstrated that people with social phobia carrying the *s* allele, compared to those with the *l/l* genotype, have increased AMYG reactivity in response to an anxiety-inducing stimulus.[17] However, tryptophan depletion used as a stimulus to induce depressive symptoms in remitted patients with major depression failed to demonstrate a significant effect of the *s* allele on AMYG metabolic activity as assessed with [^{18}F]fluoro-2-deoxy-D-glucose (FDG).[18] As *s* carriers are at increased risk to develop anxiety-related traits

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and depression and in numerous studies have been shown to have increased AMYG activity, it has been proposed that the AMYG hyperactivity observed in *s* carriers is an intermediate phenotype mediating the relationship between the *s* allele and the influences of stress on the vulnerability to develop psychopathology.[19]

Imaging studies examining the relation between the *s* allele and brain activity have been performed in humans mostly using fMRI[13,14,16,17,20–25] that involve stimuli presentation within the scanner. Critical to the interpretation of findings from these studies is the recognition that the environment in which the stimuli are presented is constrained and that being in the scanner can be anxiety provoking. Although generally consistent in demonstrating that *s* carriers have altered AMYG reactivity in response to potentially aversive stimuli, the interpretation of this finding has varied in relation to the paradigms used and the conditions that were compared. One group of investigators suggests that *s* carriers are more responsive to aversive stimuli as well as to the anxiety-producing environment of the scanner[23,25] whereas another group suggests that *s* carriers have greater AMYG activity in the 'resting state'.[21,22] The use of other types of functional imaging can help clarify *s*-related intermediate phenotypes. Because of the time course of brain FDG uptake, FDG PET studies allow for the assessment of regional brain activity in ethologically relevant environments outside the scanner. Understanding the regional brain metabolic responses of *s* carriers in relation to relevant controlled stressors, outside the scanner environment as well as in the home, a nonstressful environment, would add to the current fMRI findings and help clarify their interpretation.

Rhesus monkeys are an ideal species to use to carry out such studies because they have a polymorphism within the serotonin transporter that is similar to the human *s* allele, are an excellent species to study mechanisms underlying human anxiety and high-resolution studies of regional brain metabolic activity can be performed in them. Furthermore, the impacts of different types of ethologically relevant stressors on behavioral, neurochemical, physiological and brain functional responses have been well characterized in this species.[26–28] We have been performing high-resolution FDG microPET scans in rhesus monkeys to explore the relation between patterns of brain metabolic activity and individual differences in anxiety-related traits. For example, we previously demonstrated that when threatened by a human intruder for a 30-min period, monkeys' individual differences in metabolic activity in the bed nucleus of the stria terminalis (BNST) are strongly predictive of individual differences in monkeys' anxiety-related freezing responses.[27] In the present study, we genotyped monkeys for the serotonin transporter polymorphism and performed FDG microPET studies when the monkeys remained in their home cages as well as when they were exposed to different types of stressors (alone associated with relocation and threat). The promoter repeat polymorphism in the rhesus serotonin transporter gene is analogous but not identical to the human promoter repeat polymorphism.[10,29] Among rhesus monkeys, there are two common alleles that differ by a 21 bp insertion, and this polymorphism alters levels of expression of the 5-HTT gene, with the short or 's' allele exhibiting lower levels of expression than the long allele.[29] This is remarkably similar to the effects of the short and long alleles of the human promoter repeat. The stressors used in this study, relocation and threat, were selected because they are analogous to human stressors involving exposure to novelty and potentially threatening situations.

Because the circuitry mediating emotion involves numerous components, and different stressors recruit the activity of different brain regions, we hypothesized that in addition to increased AMYG reactivity that *s* carriers would display altered function in other components of the affective circuitry. However, we predicted that these alterations would only be revealed in response to the presentation of more intense, longer-lasting, naturalistic stressors that recruit these brain regions.[27] We were particularly interested in the AMYG, BNST, nucleus accumbens (NAcc), insula and the orbitofrontal cortex (OFC) as these are interconnected components of the neural circuitry that mediate the expression and regulation of emotion,[30–33] and alterations in these structures are thought to underlie the vulnerability to develop affective and anxiety disorders.[30,34] Furthermore, different brain regions seem to be involved in mediating different types of stress-induced emotional responses. For example, preclinical studies suggest involvement of the AMYG in fear responses, whereas the BNST appears to have a prominent role in mediating anxiety.[31]

Methods

Subjects

Subjects were 30 rhesus monkeys (21 female and 9 male; average age was 30.6 ± 1.2 months) pair-housed at the Harlow Primate Laboratory and the Wisconsin National Primate Research Center. All of the monkeys were mother reared. Animal housing and experimental procedures were in accordance with institutional guidelines.

This group of experimental subjects was not closely related. The average kinship across all 30 study subjects (435 pairs of study animals) was 0.003, which is equivalent to two individuals separated by five generations (an individual would have this kinship value in common with an ancestor five generations back). Of the 435 kinship pairs in the data set, there was one pair of half-brothers both of which had the *l/l* genotype. Half-brothers share two grandparents in common. Out of the 435 kinship pairs, there were only four other pairs that were related as closely as cousins. There were only 11 pairs

out of 435 in the data set that had non-zero kinship. These eleven pairs were the one pair of half-brothers, the four pairs related as cousins and six other pairs that were more distantly related than cousins.

Genotyping of serotonin transporter polymorphism

For DNA collection, intravenous blood sampling was performed by venipuncture. We amplified the polymorphic repeat unit region of the rhesus monkey serotonin transporter promoter[10] using the following primers: forward: 5′-cagcacctaacccctaatgtccctg-3′ and reverse: 5′-gattctggtgccacctagacgccag-3′. The forward primer was fluorescently labeled. The PCR amplifications were done in ABI9700 thermal cyclers with 10 μl total reaction volumes and the following reagents: 50 ng genomic DNA, 10 μM each primer, 200 μM each dNTP, 0.5 U of HotStar Taq DNA polymerase, HotStar buffer and no additional magnesium (final $MgCl_2$ concentration of 1.5 mM). The nucleotide mix included 7′-deaza dGTP in a ratio of nine parts 7′-deaza dGTP to one part dGTP. PCR thermal cycles consisted of 15 min at 95 °C, followed by 40 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s. Amplification was completed with a 10 min extension at 72 °C and then a hold at 4 °C. The resulting PCR products were analyzed on ABI3130 Automated DNA Sequencers using ABI data collection software. Genotypes were scored using ABI GeneMapper software.

Test conditions

During the home cage baseline (BSLN) condition the animals remained in their home cage for 30 min without their cage mate. During the alone period that was associated with relocation (ALN) the subjects were separated from their cage mates and relocated to a test cage in the testing room where they remained alone for 30 min. The no eye contact (NEC) condition involved relocating the subject to the test cage in the test room. Then, a human intruded into the test room and stood completely still at a distance of 2.5 meters and presented his profile to the test subject for 10 min. The intruder then left the test room for 5 min, leaving the subject alone. The intruder then reentered the test room, and, as before, presented the profile for 5 min followed by another 5 min break and a final 5 min of the intruder for a total of 30 min. The time course of the conditions was designed to provoke a sustained response over 30 min that matched the uptake characteristics of the FDG. Test conditions were counterbalanced by condition and week of test.

FDG microPet scanning

Each animal received an intravenous injection of 10 mCi FDG prior to the 30-min behavioral test, the optimum time for FDG uptake. After the FDG injection, animals were exposed to one of the three behavioral test conditions. Subjects were then anesthetized with 15 mg/kg ketamine intramuscularly for placement of an endotracheal tube and then positioned in a custom-designed head-holder and maintained on isoflurane.

On average, imaging began 33.5 min after the end of the behavioral paradigm (ave + s.e.m. = 33.5 + 0.58 min, range = 24–60 min). The kinetics of FDG is such that over this time period it is essentially trapped into the cells in which it was taken up during the behavioral paradigm. Scanning was accomplished using a microPET scanner (Concorde Inc.) with an isotropic resolution of 2 mm^3.

Image analysis

Each FDG image was co-registered to a standard space based on the Paxinos atlas[35] using a multistep procedure. An MRI template in a standard space[35] was created by combining data from all available MRI scans ($n = 35$), including additional comparable subjects from other studies. All subjects' MRI images were manually masked to isolate the brain, then aligned to the MRI template in standard space using a nonlinear transformation computed using AIR.[35] An FDG sum image was created for each subject by registering FDG images from all conditions to a single FDG image, summing these to create an individual template, then registering each subject's FDG images to their own sum image. Next, each subject's FDG sum image was registered to their MRI image using rigid-body transformations in FSL.[36] Transform matrices were cumulated as needed to place MRI and PET data in register with the Paxinos-based template. Careful manual inspection was performed for every image to ensure valid registration. FDG images in standard space were scaled to correct for global intensity differences based on the mean FDG concentration across the whole brain.[37] Standard space MRI images were segmented using an iterated segmentation procedure in FSL.[38] Final FDG and gray-matter probability images were then smoothed using a 4 mm full-width half max Gaussian filter.[39] All data were visually inspected to ensure accurate preprocessing before statistical analyses were performed. Statistical analyses were performed on a voxelwise basis across the whole brain correcting for anatomical differences as measured by standard voxel-based morphometric techniques, as previously described in Oakes *et al*.[40] using an adapted version of Fmristat.[41] Identification of specific brain regions was accomplished with the aid of the Paxinos *et al*. atlas.[35]

Results

The genotyping revealed the following frequencies of the *s* and *l* alleles in the 30 rhesus monkeys tested: (*l*/*l* = 20; unrelated *s* carriers = 10 (*s*/*s* = 2, *s*/*l* = 8)). In comparing the *l*/*l* to the *s* individuals when administered FDG in the home cage, no significant differences in metabolic activity were found in the AMYG, BNST, NAcc, insula or OFC (using $P < 0.005$ (uncorrected) as a threshold). The only differences observed when animals were tested in the home cage were that

s carriers had increased activity bilaterally in the cerebellum ($t = 4.19$) and decreased activity bilaterally in somatosensory cortex ($t = -4.07$) and in left visual cortex (V1; $t = -3.77$).

T-tests of the interactions between genotype and stressor type: *s* (ALN-BSLN) vs *l/l* (ALN-BSLN) or *s* (NEC-BSLN) vs *l/l* (NEC-BSLN) were performed. Statistical analysis results are reported in the location (*x*, *y*, *z*) in mm relative to the anterior commissure. The ALN vs BSLN comparison revealed that the *s* carriers, compared to the *l/l* monkeys, significantly increased their metabolic activity within multiple brain regions with local maxima within the AMYG (peak = −9.98, −3.75, −8.75; $t = 4.21$), anterior insula (peak = −15.58, 7.55, 2.55; $t = 4.88$) and area 11 of OFC (peak = 7.53, 22.55, 6.25; $t = 5.24$; Figure 1a) ($P < 0.005$, two-tailed, uncorrected). Other areas of interest that demonstrated significantly increased activity in the ALN-BSLN comparison in the *s* carriers ($P < 0.005$, two-tailed, uncorrected) included lateral prefrontal cortex (areas 45, 46 and 47), superior temporal sulcus and hippocampus. Visual cortical areas (V1, V2 and V3) demonstrated significantly decreased activity in *s* carriers compared to *l/l* individuals.

The NEC vs BSLN comparison demonstrated that in response to the introduction of a threat, the *s* carriers significantly increased their metabolic activity in various brain regions including the striatum with local maxima in a region that borders the BNST and NAcc (BNST/NAcc) (peak = −13.78, 7.55, 2.55; $t = 5.11$; Figure 1b), the anterior insula (peak = −13.78, 7.55, 2.55; $t = 4.58$) and area 11 of OFC (peak = 3.73, 20.05, 3.75; $t = 3.72$) ($P < 0.005$, two-tailed, uncorrected). *S* carriers also had significantly greater activity ($P < 0.005$, two-tailed, uncorrected) in regions of medial (areas 24b and 24c) and lateral prefrontal cortex (areas 45, 46 and 47) and decreased activity in visual cortex (V1). These analyses were also performed by deleting either of the half-brothers and the results remained significant.

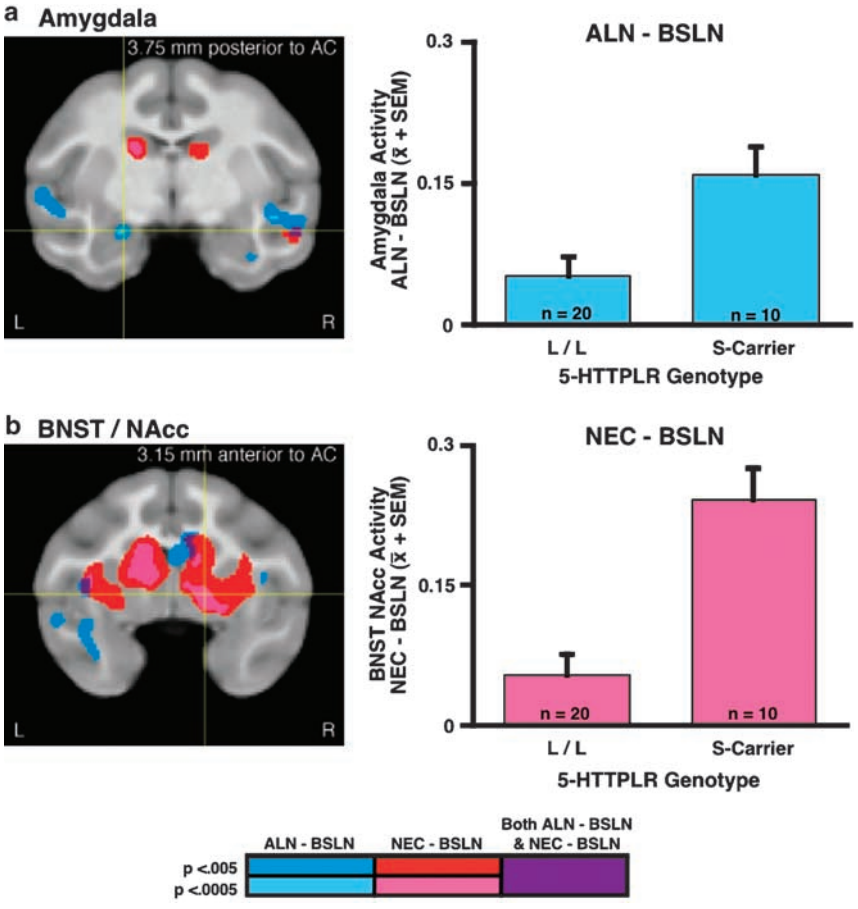

Figure 1 Serotonin transporter promoter *s* carriers have a greater increase in amygdala metabolic activity in response to the alone period (ALN) compared to baseline (BSLN) than do *l/l* individuals (**a**); and a greater increase in BNST/nucleus accumbens (NAcc) activity in response to no eye contact (NEC) compared to BSLN (**b**). Brain pictures represent the significant voxels ($P < 0.005$ and $P < 0.0005$, two-tailed, uncorrected) for the interaction between genotype and stressor type: *l/l*(ALN-BSLN) vs *s*(ALN-BSLN) in blue, and *l/l*(NEC-BSLN) vs *s*(NEC-BSLN) in red (overlap between the two tests in purple) overlaid on a rhesus monkey MRI template. Graphs represent the peak voxels from clusters within the amygdala (**a**) and BNST/NAcc (**b**) highlighted by the yellow crosses overlaid on the brain pictures.

Subsequent analyses examined the effect of genotype on AMYG and BNST activity between the two stressors; *s* (NEC-ALN) vs *l/l* (NEC-ALN). The NEC vs ALN comparison showed that during ALN the *s* carriers had significantly greater AMYG activity (peak = −8.78, −4.35, −8.75; $t = 2.8$) ($P < 0.05$, two-tailed, uncorrected), whereas during NEC the *s* carriers displayed significantly greater BNST/NAcc activity (peak = 7.53, 3.15, −3.15; $t = 2.66$) ($P < 0.05$, two-tailed, uncorrected).

Discussion

Consistent with data from human fMRI studies, these FDG findings in rhesus monkeys demonstrate that the *s* allele affects AMYG activity[13–16] as well as brain activity in numerous other brain regions.[21] Similar to the human serotonin transporter polymorphism, the rhesus short and long alleles appear to have functional effects at a molecular level, differing in their levels of gene expression.[29] Furthermore, our findings demonstrate a stressor context by genotype interaction such that the *s* allele influences different brain regions during exposure to different stressful situations. The increase in AMYG activity observed in response to the ALN condition parallels the demonstration in humans that increased AMYG reactivity, and altered AMYG-cingulate coupling occurs in *s* carriers exposed to emotion-activating stimuli.[14,18,19] However, a different pattern of genotype-dependent activation occurred in response to prolonged threat exposure occurring in the NEC condition such that a prominent response occurred in the BNST/NAcc region and not the AMYG. This pattern of brain activation involving the BNST and NAcc is consistent with previous research demonstrating these regions are closely linked anatomically and can both be involved in mediating aversive responses.[27] In contrast to these stressor-specific effects, regardless of stressor type, the same insula, OFC, and lateral prefrontal cortical regions showed greater activation in *s* carriers. It is of interest that the insula and OFC regions affected by the *s* allele have direct neuronal linkages to the AMYG and BNST/NAcc[33] and are also involved in emotion processing. The insula is of particular interest as human imaging studies demonstrate that activation in this region is associated with the integration of somatic, autonomic and emotional information related to the processing of anxiety and of fear.[42,43] The lateral prefrontal cortical regions that show increased activity in the *s* carriers are not directly linked to the AMYG and BNST but are implicated in emotion regulation via their indirect influences on limbic activity.[30,44]

When comparing the stressful conditions to the home cage BSLN state, the *s* carriers had increased activation of many of the components of the circuitry known to mediate anxiety and emotion. Differences in the metabolic activity of these regions between *s* carriers and *l/l* individuals were not apparent when the monkeys were tested in their nonstressful home cage environment. Using fMRI, Hariri *et al*.[23] originally reported that humans with the *s* allele had greater AMYG reactivity when their BOLD response to angry or fearful faces was compared with a sensorimotor matched neutral stimulus. This increased AMYG reactivity was interpreted as an enhanced response to stressful stimuli. However, two recent studies demonstrated that *s* carriers display decreased AMYG activation in response to neutral stimuli relative to a fixation cross.[21,25] Canli *et al*.[21] interpreted this finding as evidence that *s* carriers have increased AMYG reactivity at rest (viewing fixation cross) responding to neutral stimuli with a relative decrease in AMYG activation. They suggested that because in the Hariri *et al*. study responses to aversive stimuli were compared to those of neutral stimuli that the observed heightened AMYG response of *s* carriers could be due to a decreased response to the neutral stimuli, not to an increased response to the aversive stimuli. In contrast, Heinz *et al*.[25] interpreted the finding that s carriers showed decreased activation to a neutral stimulus as compared to the fixation cross as a demonstration that *s* carriers have greater AMYG reactivity to the uncertain nature of an 'affectively undefined symbol such as a fixation cross' and that this response may be heightened by the stressful nature of the scanning environment. Quantitative data from a perfusion fMRI study provided additional evidence that *s* carriers have increased resting-state AMYG activity.[22] Our FDG data from *s* carrier monkeys support the notion that increased reactivity of the AMYG, as well as other components of the emotion circuitry, is due to a heightened response to stressful stimuli.

It is important to underscore the differences in the BSLN conditions between the studies. We assessed brain activity when the monkey remained in its home cage whereas the BSLN used in the human fMRI studies was the presentation of a fixation cross while subjects were in the scanner. Furthermore, the fMRI studies examine changes on a relatively short time scale (1–10 s) related to changes in the hemodynamic response function, whereas our FDG data reflect a response averaged over a 30-min period.

Our findings demonstrate that the genetic difference between *s* carriers and *l/l* individuals leads to changes in intermediate brain phenotypes related to the circuitry of emotion following exposure to stressful challenges, but that the effect of genotype depends on the specific characteristics of a stressor. The differential involvement of the AMYG and BNST/NAcc is of particular interest as these brain regions are believed to be involved in different functions in relation to emotion, fear and anxiety.[27,31] By utilizing a variety of stressful contexts, we show for the first time a context by genotype interaction that influences anatomically separable components of the circuitry that mediate emotion. Our findings suggest that the *s* allele influences brain function in diverse environmental contexts, but that it does so through different intermediate brain phenotypes in those different

contexts. It is possible that the differential activation of the AMYG and BNST/NAcc regions in *s* carriers in response to different stressors, occurring repeatedly and over a prolonged period of time, could account for the vulnerability of *s* carriers to develop different types of anxiety-related traits and affective psychopathology.

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Author contributions: NK designed the research, analyzed data and wrote the paper. SS designed and performed the research and wrote the paper. JR performed the genotype analyses and wrote the paper. AF, TO and RD analyzed the data and wrote the paper.

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