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**Title:** Insula serotonin 2A receptor binding and gene expression contribute to serotonin transporter polymorphism anxious phenotype in primates

**Short title:** Insula serotonin 2A receptor and anxiety

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**Abstract**

Genetic variation in the serotonin transporter gene (*SLC6A4*) is associated with vulnerability to affective disorders and alterations in the efficacy of pharmacological treatment. We recently identified sequence polymorphisms in the common marmoset *SLC6A4* repeat region (AC/C/G and CT/T/C) associated with individual differences in trait anxiety, gene expression and response to antidepressants. The mechanisms underlying the effects of these polymorphisms are unknown, but a key mediator of serotonin action is the serotonin 2A receptor (5HT2A). Thus, we correlated 5HT2A binding potential (BP) and *post mortem* RNA gene expression in 16 *SLC6A4* genotyped marmosets with responsivity to 5HT2A antagonism during the human intruder test of anxiety.

Voxel-based analysis and RNA measurements showed a reduction in 5HT2A BP and gene expression specifically in the right posterior insula of individuals homozygous for the anxiety-related variant AC/C/G. These same marmosets displayed an enhanced anxiety-related, dose-dependent response to the human intruder after 5HT2A pharmacological antagonism, whilst CT/T/C individuals showed no effect. A voxel-based correlation analysis, independent of *SLC6A4* genotype, revealed that 5HT2A BP in the adjacent right anterior insula and insula proisocortex was negatively correlated with trait anxiety scores. Moreover, 5HT2A BP in both regions were good predictors of the size and direction of the acute emotional response to the human intruder threat after 5HT2A antagonism.

Our findings suggest that genetic variation in the *SLC6A4* repeat region may contribute to the trait anxious phenotype via neurochemical changes in brain areas implicated in interoceptive and emotional processing, with a critical role for the right insula 5HT2A in the regulation of affective responses to threat.

**Significance statement**

Serotonin transporter polymorphisms within the primate-specific promoter repeat region are associated with altered emotion regulation, vulnerability to psychiatric disorders and differences in antidepressant responsiveness. However, the underlying neurobiological mechanisms are poorly understood. Using PET imaging, a behavioral anxiety test and *post mortem* expression measurements, we show that marmoset monkeys carrying a high anxiety-related serotonin transporter variant have reduced serotonin 2A receptor (5HT2A) binding and RNA expression in right posterior insula and differential sensitivity to anxiogenic effects of acute 5HT2A antagonism, the latter predicted by 5HT2A binding in adjacent right anterior insula and insula proisocortex. Our findings highlight the value of marmosets in studying gene-brain-behavior relationships and may contribute to genetic and imaging screens development in personalized medicine for treating anxiety.

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**Introduction**

Genetic variation in the serotonin transporter gene (*SLC6A4*) has been associated with early life stress reactivity, vulnerability to affective disorders and altered social cognition (1). Specifically, a variable number of tandem-repeats (VNTR) located upstream of the promoter region has been identified in human and non-human primates, with *short* alleles being linked to reduced gene expression and emotionally vulnerable phenotypes (2, 3). Neuroimaging studies have provided strong evidence supporting these gene-behavioral associations and suggest neurodevelopmental mechanisms underlying the *SLC6A4* genetic variation. Individuals carrying the *short* alleles show hyperactivity to threat related stimuli (such as fearful faces or Pavlovian conditioned stimuli in humans or a human intruder in macaques) or during negative self-reflection, in a network of structures that consistently include the amygdala, insula and dorsomedial prefrontal/anterior cingulate cortex (4–11). Altered connectivity has also been reported between structures in this network and related areas, including medial, lateral and orbital prefrontal cortex in *short* allele carriers (8, 12–14), suggesting impaired regulation of the emotional response. In particular, , the right anterior insula has been implicated in altered emotion regulation in *short* allele carriers (9, 17, 18). This key region integrates the interoceptive information from the posterior insula with cognitive processing from prefrontal areas and it is hypothesized to form the subjective feeling of emotion and self-awareness, specifically with respect to negative affect (15, 16). In addition to these functional changes, reduced grey matter volume in areas involved in emotional processing, including amygdala, medial prefrontal cortex and hippocampus, have also been described in *short* allele carriers (12, 19). Finally, although serotonin transporter binding studies have not provided consistent results (19–22), reduced serotonin 1A receptor binding in cortical areas has been reported in *short* allele carriers, both in humans and macaques (23, 24).

Polymorphisms in the *SLC6A4* have also been associated with treatment efficacy of selective serotonin reuptake inhibitors (SSRIs), with *short* allele carriers showing a slower remission rate and more severe side effects (25). Whilst SSRIs are the most widely used drugs for the treatment of anxiety and depression, one third of the patients show a poor response (26), and it has been proposed that the anxiety experienced by some patients at the early stages of treatment may contribute to this reduced treatment efficacy (27). In some SSRI resistant patients, a therapy combining SSRIs with serotonin 2 receptor (5HT2) antagonists has been shown to improve the efficacy of the antidepressant treatment (27–29). However, the mechanisms by which 5HT2 regulates the emotional response are still unknown and vary according to the specific brain areas and the type of behaviors measured (28). Reports studying brain serotonin receptors alterations have provided conflicting results with respect to depression (30) and little is known about anxiety disorders. However, the density of medial prefrontal cortex serotonin receptor 2A (5HT2A) does correlate negatively with right amygdala reactivity to threat stimuli (fearful faces) (31), but only when 5HT1A binding is relatively low (32). In addition, a positron emission tomography (PET) imaging study in monozygotic twins suggests that approximately 40-50% of interindividual variability in cortical 5HT2A density is genetically driven (33). However, whether the *SLC6A4* polymorphisms contribute to alterations in cortical 5HT2A density and subsequently to the anxious phenotype has not yet been explored.

Studies in animals can provide a detailed mechanistic understanding of the brain-behavior interactions related to this polymorphism but other than humans the *SLC6A4* repeat region has only been found in primates including apes and monkeys, but not prosimians or rodents (34). Moreover, VNTR within the *SLC6A4* repeat region has only been reported in humans and old world monkeys but not in marmosets (35). However, we recently identified sequence variation within the common marmoset *SLC6A4* repeat region that revealed a similar difference in *SLC6A4* RNA expression patterns and sensitivity to threat stimuli to that of the human and macaque *SLC6A4* VNTR. Thus, marmosets carrying the low-expressing haplotype (AC/C/G) show an enhanced anxiety-like behavioral repertoire towards a human intruder threat compared to marmosets carrying the high-expressing variant (CT/T/C) (36). Moreover, these two haplotypes are associated with, respectively, opposing anxiogenic and anxiolytic effects of acute administration of the SSRI citalopram during the human intruder threat, effects that were specifically associated with the average distance maintained from, and reflecting avoidance of, the human intruder, without changing the overall behavioral repertoire. Such genotype-dependent differential sensitivity to the acute effects of an SSRI may underlie, at least in part, differential sensitivity to pharmacotherapy in people with anxiety and depression (36).

The marmoset’s small size, short gestation period and accelerated development compared to old world monkeys, alongside its sophisticated social and emotional behavior (37, 38) and primate brain with expanded associated neocortex compared to rodents, make it an ideal species for laboratory studies of gene-brain-behavior interactions during development and adulthood (39). Moreover, the use of marmosets that are bred ‘in house’ affords considerable control/restriction over the environmental influences during development thereby helping to expose the influence of genetic variation. Thus, taking advantage of this newly discovered polymorphism in marmosets the relationship between cortical 5-HT2A density and the anxious phenotype in the two *SLC6A4* homozygous haplotypes was determined. We employed a unique combination of PET imaging of the 5HT2A specific radioligand [18F]-altanserin (40), and psycho-pharmacological challenge with a 5-HT2A antagonist *in vivo*, followed by measurements of *post mortem* 5-HT2A RNA expression in those brain regions showing differential altanserin binding. Based on the neurobiological changes reported in *short* allele carriers mentioned above, and the relationship between cortical 5-HT2A density and amygdala reactivity, we hypothesized that marmosets homozygous for the AC/C/G anxiety-related haplotype may show reduced 5-HT2A binding in brain areas implicated in emotional processing compared to the low anxious CT/T/C homozygous. To define the relationship between genotype, brain 5-HT2A receptors and sensitivity to the behavioral effects of a 5HT2A pharmacological challenge, we measured each marmosets’ anxiety response to an unknown human (uncertain threat) after an acute dose of a specific 5-HT2A antagonist (M100907) and investigated whether the response was related to 5-HT2A binding potential.

**Results**

**Selective reductions in 5HT2A BP and *post mortem* RNA levels in the right posterior insula of marmosets homozygous for the high anxiety-related *SLC6A4* haplotype AC/C/G**

PET imaging of the 5HT2A specific radioligand [18F]-altanserin (40) in a cohort of 16 marmosets, balanced by *SLC6A4* genotype and sex, revealed that whole brain 5HT2A BP did not differ significantly between genotypes (global signal AC/C/G vs CT/T/C, 95% CI -0.06-0.15; two-tailed t-test p=0.36). However, a voxel-based analysis identified one specific cluster within the right posterior insula (Table 1 and Fig. 1a) that showed a 27.3% (95% CI 21-34%) reduction of 5HT2A BP in those animals carrying the anxiety-related AC/C/G haplotype compared to those carrying the CT/T/C haplotype (one-way ANOVA F(1,15)= 78.72, p=4.02E-07, Fig. 1b). Consequently, real time PCR was performed on the right posterior insula at *post mortem* and this revealed paralleled these findings. 5HT2A RNA expression not only correlated positively with 5HT2A BP (Pearson correlation r=.722, p=.005, Fig. S1a) but also showed a similar reduction in the animals homozygous for the anxiety-related AC/C/G haplotype (20.1%, 95% CI 10.4- 29.9%, one-way ANOVA F(1,12)= 20.96, p=7.9E-04, Fig. 1c). To confirm the laterality of this effect, the right posterior insula cluster identified with PET was reflected about the midline to extract homologous values of 5-HT2A BP from the left hemisphere. Whilst the left posterior insula similarly showed a significant reduction in 5HT2A BP in the AC/C/G group (one-way ANOVA F(1,15)=4.69, p=.048, Fig. S2g), it did not survive corrections for multiple comparisons. Likewise, RNA expression in the left posterior insula, which correlated positively with 5HT2A BP (Pearson correlation r=.633, p=.020, Fig. S1d), only showed a trend towards a reduction in the AC/C/G group (one-way ANOVA F(1,12)=4.26, p=.063, Fig. S3g).

***SLC6A4* genotype-dependent, anxiogenic response to threat after acute 5HT2A antagonism in marmosets homozygous for the anxiety-related haplotype AC/C/G**

A previous study from our group has shown that the *SLC6A4* polymorphisms in marmosets are associated with trait anxiety, as measured by the human intruder test (36). The human intruder test measures an array of behavioral responses directed towards an unfamiliar person staring at the marmoset for two minutes while the animal is in its home cage (Fig. 2a). The uncertain nature of this stimulus induces a pattern of anxiety-like behavior. A principal component analysis (PCA) of the array of behavioral variables including vocalizations, locomotion, head bobbing and distance from the intruder, revealed two components: PC1 (anxiety) and PC2 (coping strategy), that together explained the variability observed within the behavioral repertoire. Specifically, the AC/C/G homozygous marmosets presented increased trait anxiety (high PC1 scores), characterized by larger average distance from the human intruder, reduced locomotion and jumps to the front and high numbers of head bobbing, Egg and Tse-like vigilant calls. In addition, they showed a passive coping strategy (low PC2 scores), driven mainly by low numbers of Tsik and Tsik-Egg aggression related calls. Remarkably, the CT/T/C group showed the opposite pattern (36). Comparable to this large cohort (N=52) from which the PCA was derived, the cohort in our current study (N=16) showed a similar differential pattern for PC1 and PC2. However, as expected for polymorphisms presenting high frequency and low penetrance, such as the ones in the *SLC6A4* repeat region, the effects of genetic variation on phenotype in a small sample (N=16 vs N=52) became weaker due to increased variation and reduced power, with only PC2 reaching statistical significance (*A priori* hypothesis: for PC1 AC/C/G>CT/T/C and for PC2 AC/C/G<CT/T/C, 1-tailed t test PC1: p=.07, PC2: p=.03, Table S1 and Fig. S4).

Given that the present study revealed specific reductions in 5-HT2A in the right posterior insula of the anxiety-related AC/C/G group, we hypothesized that the anxiety response of the animals homozygous for this haplotype would be more sensitive to 5-HT2A antagonism. To test this hypothesis, we administered a 5-HT2A antagonist (M100907) peripherally and measured the responsivity of marmosets to a human intruder using a repeated-measure block design (see Materials and Methods).

Previous studies have shown that acute administration of anxiolytics (41) have a marked impact on average distance from the human threat (avoidance response) and recently we identified a *SLC6A4* genotype-dependent response to acute administration of the SSRI citalopram on this measure too, without altering the overall anxiety trait, i.e. PC1 or PC2 (36). Thus, we focused the subsequent analysis of the acute effects of M100907 on *average distance* (see Materials and Methods) although all other behavioral measures and PCA scores calculated for this pharmacological study are summarized in Table S2. As predicted, after acute 5-HT2A pharmacological antagonism with the higher of the two doses administered, marmosets homozygous for the anxiety related haplotype AC/C/G displayed an enhanced anxiogenic response to the human intruder with a significant increase in *average distance* from the human intruder compared to vehicle (planned comparisons: vehicle vs M100907 0.3 mg/kg dose: paired two-tailed t test p=.038, Fig. 2b). There was no effect of either dose in the CT/T/C group and no effects on any other measure (Table S2).

Together these results suggest a functional role for the observed genotype differences in 5HT2A BP and RNA expression in the posterior insula, in the regulation of anxiety-like behavior that may underlie their differences in trait anxiety.

**5-HT2A BP and RNA levels in the right anterior insula and insula proisocortex predict trait anxiety**

In order to establish whether 5HT2A binding in the posterior insula or anywhere else in the brain was related to trait anxiety *per se*, we performed a voxel-based analysis correlating 5-HT2A BP with trait anxiety (PC1) and coping strategy (PC2) scores of the present cohort of marmosets, independently of *SLC6A4* genotype.

At the same level of significance used previously, corresponding to a maximum of *p* value of 0.001 for cluster-generating threshold as recommended by Eklund et al. (42) there were no behavioral correlations. However, as an exploratory approach, we relaxed the stringency of the correction for multiple comparisons with a cluster-generating threshold of p<.005 as a less stringent but still widely used value to conduct an exploratory analysis, and a smaller extent threshold of k=50, which still excluded clusters too small to be of interest (see Materials and Methods for details). This analysis revealed two specific clusters for PC1 (Table 1 and Fig. 3). One cluster located in the right anterior insula (Fig. 3a-c), and another one located in the right insula proisocortex (Fig. 3d-f). Both areas showed a negative relationship, with low 5-HT2A BP corresponding to high anxiety scores (Pearson correlation right anterior insula: r=-.753, p=.001, right insula proisocortex: r=-.746, p=.001, Fig. 3b and 3e, respectively). Direct measurement of 5-HT2A RNA levels within *post mortem* tissue of the right anterior insula region assessed using real time PCR, mirrored this negative relationship (Pearson correlation r=-.656, p=.015, Fig. 3c). There was no such relationship though between 5-HT2A RNA expression and anxiety scores in the right insula proisocortex (Pearson correlation r=-.173, p=.571, Fig. 3f). Whilst the right anterior insula 5-HT2A RNA expression levels correlated positively with 5-HT2A BP (Pearson correlation r=.661, p=.014; Fig. S1b), this was not the case for the right insula proisocortex (Pearson correlation r=-.466, p=.108, Fig. S1c). We used the same approach as described above to confirm the laterality of this effect, with no significant relationship between anxiety and either 5-HT2A BP or RNA expression within these two regions in the left side (Fig. S2 and S3). No significant findings were detected for PC2 at either threshold.

To determine whether these variables were also predictors of the trait anxiety scores, we performed linear regression analyses with the significant correlating variables. We included 5-HT2A BP and RNA expression in right anterior insula and 5-HT2A BP in right insula proisocortex as predictors, and anxiety scores (PC1) as the dependent variable. All three variables were good predictors of anxiety scores (Table 2).

**5-HT2A BP in the right anterior insula or insula proisocortex predict the behavioral response to threat after 5-HT2A antagonism in the human intruder test**

Since both PET and RNA expression provided strong evidence that 5-HT2A in the right anterior insula and insula proisocortex contributed to overall trait anxiety scores, we next determined whether 5-HT2A in these regions also contributed to a marmoset’s responsivity to the human intruder after acute 5-HT2A antagonism with M100907, as described above. To test this, we analyzed the relationship between 5-HT2A BP and RNA levels, and the pharmacologically-induced anxiety response after 5-HT2A antagonism. We calculated the drug-induced effect on *average distance* from the human threat as the percentage of change of the *average distance* variable following vehicle (see Materials and Methods). This measure correlated negatively with 5-HT2A BP in both right anterior insula (Pearson correlation: r=-.660, p=.014) and insula proisocortex (Pearson correlation: r=-.645, p=.017), (Fig. 4). In contrast, right anterior insula RNA expression did not correlate significantly with the response to threat. A regression analysis on the significant correlating measures revealed that 5-HT2A BP in both right anterior insula and insula proisocortex were good predictors of the increased anxiety response after 5-HT2A antagonism (Table 3). 5-HT2A BP in both insula regions also highly correlated with each other (Pearson correlation: r=.857, p=.0002) thus, they were not significantly different as predictors of the threat response.

**Discussion**

The neurobiological mechanisms that contribute to the development of emotionally vulnerable phenotypes with increased risk for psychiatric disorders are still poorly understood. One of the most studied genetic variants within the psychiatric field is the *SLC6A4* repeat region polymorphisms, in which low expressing variants have been associated with vulnerability to psychiatric disorders and low treatment efficacy. Here weshow that marmosets homozygous for the low-expressing, high anxiety-related *SLC6A4* variant, AC/C/G, show a significantly reduced 5HT2A BP and RNA expression specifically in the right *posterior* insula, and display an enhanced anxiety-like response to a human threat after acute, systemic 5HT2A pharmacological antagonism. In addition, we reveal that 5HT2A BP and RNA levels in the adjacent right *anterior* insula and 5HT2A BP in the right insula proisocortex, have a negative relationship with trait anxiety regardless of genotype, with lower 5HT2A levels corresponding to higher anxiety scores. Moreover, 5HT2A BP in the right *anterior* insula, or the right insula proisocortex, were both good predictors of the size and direction of the emotional response to threat after 5HT2A acute pharmacological antagonism.

To our knowledge, this is the first study to investigate not only the contribution of the *SLC6A4* polymorphisms to 5HT2A levels and its relationship with trait anxiety, but also, critically, to directly compare *in vivo* BP and *post mortem* RNA expression in the same cohort of monkeys and demonstrate correspondence across measures. Our findings of reduced 5HT2A BP and associated RNA expression in the right posterior insula of marmosets carrying the low-expressing, emotionally vulnerable *SLC6A4* variant extend previous studies, one in humans (24) and one in macaque (23), reporting reduced 5HT1A binding in individuals homozygous for the *short* allele in cortical areas including the insula. It is also consistent with a study showing reduced 5HT2A cortical receptor binding in *SLC6A4* KO mice (43). Besides the relationship between the *SLC6A4* variant and 5-HT2A levels in the posterior insula, we also revealed a negative relationship between cortical 5HT2A and overall anxiety scores in the adjacent right anterior insula and the more posterior insula proisocortex. Whilst the negative relationship between BP and anxiety scores was mirrored by RNA levels in the right anterior insula, this was not the case for the insula proisocortex. Given that BP and RNA expression correlated positively in right anterior insula but not in insula proisocortex, a likely reason for this non-correspondence may be due to the presence of presynaptic 5HT2A specifically within this region. Presynaptic 5HT2A would contribute to BP but not necessarily to RNA levels, since the RNA coding for presynaptic receptors would normally reside in cell bodies in a distal part of the brain, such as for example the 5HT2A expressing thalamocortical inputs to the insula (44). Thus, depending on the overall balance between pre and post synaptic 5HT2A there will be more or less correspondence between BP and RNA in different brain areas. Nonetheless, the negative relationship found between 5HT2A BP within these two insula regions and anxiety scores is supported by our results from the pharmacological study in which the magnitude and direction of the behavioral response to the human intruder threat after 5HT2A antagonism was directly associated with 5HT2A BP within both regions (Fig. 4). A similar negative correlation between 5HT2A and in this case, amygdala reactivity to fearful faces, has been found in medial prefrontal cortex (31), suggesting a role for PFC 5HT2A in the regulation of amygdala reactivity during anxiety (45). In contrast, a study in healthy volunteers using questionnaire measures of neuroticism showed a positive relationship with 5HT2A BP in frontolimbic areas, including the left but not the right insula (46). Thus, while these studies support the role of cortical 5HT2A in the regulation of emotion, different brain regions selectively involved in the regulation of distinct aspects of emotional behavior, may show specific 5HT2A functional patterns (47).

In the case of patients with depression, imaging and *post mortem* studies show increased cortical 5HT2A levels (30). Consistent with this, imaging studies have detected a downregulation of 5-HT2A receptor in the brain of such patients in response to SSRI treatment (48), and the therapeutic benefit gained by some patients when treated with a combination of both a SSRI and 5HT2A antagonist, seems to go in line with these observations (49). However, little is known about 5HT2A levels in patients with anxiety disorders. While 5HT2A antagonists have been used for the treatment of anxiety disorders (47), 5HT2A agonists (serotonin hallucinogens) have provided improvement of anxiety and depressive symptoms in patients suffering from life-threatening diseases (50). Thus, the role of cortical 5HT2A in the regulation of anxiety may not only depend on the specific brain area and type of behavior but also on the emotional context.

In the present study, the insula was the brain region that consistently showed alterations in 5-HT2A BP and RNA levels associated with the *SLC6A4* polymorphisms, trait anxiety scores and the anxiety-related response after 5-HT2A antagonism. The insula has been implicated in interoceptive processing and emotional decision-making (51, 52), subjective feelings (53), emotion and self-awareness (54). In primates, the insula cortex presents a complex structural and functional architecture, transitioning from granular neocortex in the posterior-dorsal insula to agranular neocortex in the anterior-ventral insula with an intermediate zone of dysgranularity (55). While the posterior insula receives sensory information from the body, it is proposed that the anterior region integrates this somatic representation with cognitive information from prefrontal areas to process subjective feelings and self-awareness (15, 16). Thus, together the posterior and anterior insula cortex play an integrated role in emotion regulation. Consistent with this is the increased anterior insula activity during emotional processing in patients with anxiety disorders (56). In addition, in depressed patients, higher neuroticism was related to increased activity within the right anterior insula cortex to incongruent compared to congruent emotional stimuli (57). Moreover, anterior insula hyper-reactivity was associated with over-interpretation of interoceptive states as threatening or negative by patients with anxiety and depression, respectively (58). Perhaps not surprisingly then, imaging studies have also shown neurophysiological changes in the insula cortex associated with *SLC6A4* polymorphism. In humans, enhanced right anterior insula activation in *short* allele carriers has been reported in response to an unpredictable laboratory stressor (17), fear conditioning (18) and negative self-reflection (9). Moreover, in patients with SAD, *short* allele carriers showed greater anterior insula activation to fearful faces (10). In macaques, increased anterior insula activity in *short* allele carriers has also been shown using [18F] fluoro-2-deoxy-D-glucose (FDG) PET scanning immediately after the human intruder test (59). The same group have also identified a positive relationship between freezing behavior in the human intruder test and anterior insula activity (11), which was highly heritable (60).

Here, we report a significant reduction of 5HT2A levels specifically in the *posterior* insula and an anxiogenic response to threat after 5HT2A antagonism in marmosets homozygous for the high anxiety-related *SLC6A4* variant. However, it was the 5HT2A binding in the *anterior* insula, where no significant differences in 5HT2A levels could be detected between *SLC6A4* genotype, that predicted the anxiety response to threat. Thus, the *SLC6A4* polymorphism may be contributing to this differential anxiety response to threat after 5HT2A antagonisms through altered 5HT2A-mediated functions in *posterior* insula, e.g. altered processing of interoceptive information. This altered information is subsequently transferred to the *anterior* insula, as predicted by the model of information flow in this area (16), where it is integrated with cognitive information from prefrontal cortex under 5HT2A-mediated regulation, ultimately contributing to the emotional response. Although the present study clearly reveals the importance of 5HT2A function within the insula in relation to anxiety and the *SLC6A4* polymorphism, the extent to which this genetic association between insula activity and *SLC6A4* polymorphism is determined by 5HT2A density, requires further investigation.

The *SLC6A4* haplotype-dependency of the heightened sensitivity to acute 5HT2A antagonism was anticipated since high anxious marmosets presented with reduced right insula 5HT2A BP and RNA levels, suggesting that further functional reduction of these receptors would have a greater behavioral impact on this haplotype. We have previously shown that these same high anxious marmosets displayed an anxiogenic response to the human intruder after an acute dose of the SSRI citalopram (36). Consistent with this, studies increasing serotonin output with acute SSRIs have shown a differential effect on right insula activation depending on the *SLC6A4* polymorphism. Smith et al. (2004) found enhanced reductions in right anterior insula activity of *long* allele homozygotes compared to subjects homozygous for the *short* allele, during FDG PET after i.v. infusion of citalopram (61). In addition, oral citalopram increased amygdala and posterior insula activity in *long* allele homozygotes but not *short* allele ones during perception of fearful faces (62). Together, these findings demonstrate genetically driven differential insula activation in response to drugs that target the 5-HT system. Their therapeutic relevance is highlighted by recent work identifying right anterior insula metabolism as the best predictor of escitalopram treatment success (63). Based on our findings, we propose that insula 5HT2A levels may contribute to the mechanism by which *SLC6A4* polymorphisms modulate insula activity and subsequent anxiety responses.

In conclusion, the present study implicates 5HT2A receptors in the right posterior insula as the neurochemical mechanism by which genetic variation in the *SLC6A4* gene contributes to the anxious, vulnerable phenotype. We reveal a differential sensitivity to acute 5HT2A antagonists depending on *SLC6A4* genotype, with individuals who carry the high anxiety-related variant and present reduced right posterior insula 5HT2A BP and RNA levels, displaying an anxiogenic response to threat. Moreover, we show that 5HT2A BP in the right anterior insula and insula proisocortex were both good predictors of the anxious response. Altogether, these findings highlight the specificity of the neurobiological changes associated with *SLC6A4* polymorphisms that are highly relevant to our understanding of the development and treatment of mood and anxiety disorders. Dissecting the neurobiological mechanisms underlying genetic variation that links selective brain areas and receptors to specific emotional behaviors, will increase our understanding of individual differences not only in anxiety and mood disorder symptoms but also in treatment efficacy, bringing us one-step closer to the development of more effective personalized therapies.

**Materials and Methods**

**Animals and housing.** Sixteen naïve common marmosets *Callithrix jacchus* (26±2 months, 413±17g) balanced for sex and genotype were used in this study. All animals had MRI and [18F]-altanserin PET scans, HIT and snake test [procedures described previously in (64)] before entering a pharmacological study, which consisted of repeated HIT with acute i.m. doses of citalopram [behavioral data reported elsewhere (36)] and after two months, with the 5HT2A antagonist M100907 (present study). Marmosets were bred on site at the Innes Marmoset Colony (Behavioral and Clinical Neuroscience Institute) and housed in pairs. Temperature (24ºC) and humidity (55%) conditions were controlled and a dawn/dusk-like 12h-period was maintained. They were provided with a balanced diet and water ad libitum. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

**Genotyping**. Marmoset were genotyped for the *SLC6A4* polymorphisms using methods previously described (36). Briefly, hair samples were taken from the animal’s back. Samples were processed using the QIAamp DNA Micro kit (Qiagen Ltd., UK). The primers used flanked the *SLC6A4* polymorphic repeat promoter region: RPRF (CAGACAACCGTGTTCATCTG) and RPRR (GATTCTAGTGCCACCTAGAC). HotStarTaq Plus DNA Polymerase (Qiagen Ltd., UK) was used in a BioRad C1000 thermal cycler (conditions: activation 15 min at 94ºC; 44 cycles of 30 sec at 94ºC, 30 sec at 55ºC and 1 min at 72ºC; and termination 5 min at 72ºC). The PCR product was visualized in an agarose gel, purified using QIAquick gel extraction kit (Qiagen Ltd., UK) and sent for sequencing (Genservice Ltd, Cambridge, UK) using the primers SeqF1 (agcagcacctaaccctccta) and SeqF2 (tccccactaggcattgctac).

**Human Intruder Test (HIT).** We have previously characterized the human intruder test of anxiety in marmosets using a large population (N=52) from our colony (36). Briefly, marmosets were isolated in the upper right-hand quadrant of their home cage (*separated phase*). After 8 minutes, an unfamiliar person entered the room (*intruder phase*). The intruder stood in front of the cage and stared at the marmoset’s eyes for 2 minutes. Marmoset performance was recorded with a HD video camera and a shotgun condenser microphone. Several measures were scored off line by an experimenter. A principal component analysis (PCA) was performed on all variables with a large cohort of marmosets, including the ones used in this study. Two principal components (PC1 and PC2) explained over 63% of the variance. According to variable loadings, PC1 corresponded to ‘Anxiety’ and PC2 corresponded to ‘Coping strategy’ in the emotional response to threat. The scores for each individual included in this study were derived from this population PCA and used for the subsequent imaging correlational analysis (see below).

**PET scan protocol.** Animals were imaged for 3 hours using a microPET Focus-220 scanner (Concorde Microsystems, Knoxville, TN, USA). Anaesthesia and body temperature was maintained. In addition, oxygen saturation, heart rate and respiratory rate were measured and maintained within physiological limits throughout.Prior to injection, singles-mode transmission data was acquired for 515 seconds using a rotating 68Ge point source (~20 MBq). An attenuation correction sinogram was produced from this scan and a blank scan of the same duration using the reconstruction and segmentation software on the Focus-220. [18F]-altanserin (3-[2-[4-(4-[18F]fluorobenzoyl)piperidin-1-yl]ethyl]-2-sulfanylidene-1H-quinazolin-4-one) (0.5±0.04 nmol/kg) was injected intravenously over 10 seconds, followed by a 5 second heparinised saline flush. For all scans the injected amount of altanserin was ~ 0.5nmol/kg. Dynamic data was acquired in list-mode for a 350-650 keV energy window and a 6ns timing window. Data were subsequently histogrammed into sinograms for the following time frames: 12 × 5s, 6 × 10s, 3 × 20s, 4 × 30s, 5 × 60s, 10 × 120s, 30 × 5min. Corrections were applied for random, dead time, normalization, attenuation and decay. Fourier rebinning (65) was used to compress the 4D sinograms to 3D prior to reconstruction with 2D filtered backprojection with a Hann window cut-off at the Nyquist frequency. The image voxel size was 0.95 × 0.95 × 0.80mm, with an array size of 128 × 128 × 95. The reconstructed images were converted to kBq/ml using global and slice factors determined from imaging a uniform phantom filled with a [18F] fluoride solution.

**PET data analysis**. Following affine and non-linear registration, binding potential maps were smoothed using an adaptive Gaussian kernel to exclude voxels outside the brain mask (the full width half maximum was 1mm isotropic). To assess the effect of *SLC6A4* genotype, voxel-based comparisons were made with SPM12 using a general linear model. Factors were included for genotype, sex and weight. Two-tailed t-tests were used to assess the main effect of genotype. A cluster-based correction for multiple comparisons was used to control the family-wise error at p < 0.05. The cluster-generating threshold was p < 0.001 (42). To investigate correlations with behavior, separate analyses were conducted with further covariates for PC1 and PC2 from the HIT. A p < 0.005 was used as a less stringent but still widely used value to conduct an exploratory analysis (the most prevalent in the AFNI software, for example from NIMH, <https://afni.nimh.nih.gov>). An extent threshold of 50 was chosen to avoid clusters too small to be of interest. We did not calculate a random-field theory threshold for this cluster-size (as we did for the p<0.001 threshold) to control the family-wise error directly as Ecklund et al. point out that the assumptions of the calculations used do not hold for more liberal thresholds. Clusters found were followed up by RNA expression measurements within these areas.

**Pharmacological manipulation on HIT.** Due to health-related issues, only 13 out of the 16 marmosets (6 AC/C/G: F=3, M=3; 7 CT/T/C: F=4, M=3) were available for inclusion in this next stage of the study. Animals were injected i.m. with the selective 5-HT2A antagonist M100907 (Sigma-Aldrich, UK) (low dose 0.1 mg/kg or high dose 0.3 mg/kg) or vehicle (0.01 M PBS-HCl) 25 minutes before the human intruder entered. M100907 doses were selected based on their reported effects on macaques (66) and squirrel monkeys (67). Repeated HIT procedures were the same as described above. To avoid habituation to the human intruder across sessions, we used different realistic rubber human masks each time (Greyland Film spol. s.r.o., Czech Republic). The experimental design was a Latin square randomized by sex, genotype and masks. Treatment order was the same for all individuals (vehicle, low dose, high dose, vehicle) with two weeks between each session. To calculate the PCA scores for each treatment, each variable was standardized using the mean and standard deviation of the control condition (average of the two injections with vehicle) of the experimental subpopulation used in this study (N=13). These standardized values were then used in the PCA function derived from the population. *Average distance* from the human intruder was calculated by dividing the testing quadrant into regions and scoring the time spent in each location, as previously described (36). Percentage of change from vehicle in *average distance* was calculated as follows: [(*average distance* after low or high dose of M100907/*average distance* after vehicle)x100]-100. Planned comparisons were performed using paired two-tailed t tests.

**Real time PCR.** At the end of the study, animals were pre-medicated with ketamine hydrochloride before being euthanized with pentobarbital sodium (Dolethal; 1ml of a 200mg/ml solution, i.e.; Merial Animal Health, Essex, U.K.). Brain were dissected, frozen using liquid nitrogen and kept at -80ºC until use. The brains were then sliced in a cryostat at -20 ºC to 200µm thick sections. Tissue samples for each target region were excised using punches of 1.5 and 2 mm radio length (Fig. S5). Total RNA was extracted with the RNeasy Plus Universal Mini Kit (Qiagen Ltd., UK) in accordance with the manufacturer’s protocol. Samples were quantified using NanoDrop (Thermo Fisher Scientific) with an average concentration of 9.40±0.24 ng/µl. RNA integrity, analyzed using Agilent RNA 6000 Pico kit with Agilent 2100 Bioanalyzer instrument, had an average RIN value of 8.60±0.04. Samples were stored at -80 ºC until use. cDNA synthesis and real time qPCR were performed using Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step (Agilent Technologies, UK) adding 2 µl of samples into each primer combination reaction. Primers for the 5HT2A gene were designed using Primer-BLAST, a software tool from the National Centre for Biotechnology Information (NCBI) (68). The predicted 5HT2A mRNA sequence used to design the primers were obtained from the Ensembl database (69). Four marmoset-specific reference genes were selected: ACTB (β-Actin), TBP (TATA-box binding protein), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (70); and SDHA (succinate dehydrogenase complex, subunit A) (70, 71). All primer combinations were designed to span exon-exon boundaries (see Table S3 for primers sequences). All reactions were performed in duplicates for each individual and controls (conditions: cDNA synthesis 30 min at 50 ºC, activation step 10 min at 95ºC, 40 two-step cycles of denaturation 30 sec at 95ºC and combined annealing/extension 1 min at 60ºC, final melting curves to check specificity of the product) using the Bio-RAD CFX96 Touch Real-Time PCR Detection System. Results were obtained using BioRadCFXManager software. Using specific efficiencies for each primer set previously calculated, and an inter-run calibrator, we ran a gene study where the relative expression levels of the gene of interest (5HT2A) were normalized to the four reference genes (ACTB, TBP, GAPDH and SDHA) and calculated using the method ΔΔCq.

**Statistical analysis.** Analyses were performed usingSPSS Statistics (version 24; IBM Corp., Armonk, NY). Data are presented as Mean ±SEM, *p* ≤ .05 was considered statistically significant. After confirming normal distribution and homogeneity of variance, one-way ANOVA and Pearson correlations were performed.

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**Figure legends**

**Figure 1**: 5HT2A related to *SLC6A4* variants. Voxel-based analysis of 5HT2A BP comparing marmosetshomozygous for the *SLC6A4* variants AC/C/G vs CT/T/C. **a)** Specific cluster in right *posterior* insula (AP=8.7) sagittal (left) and coronal (right) sections centered at the highest significant peak (t=7.14). **b)** Mean±SEM 5HT2A BP in the right posterior insula cluster for each homozygous group (AC/C/G < CT/T/C one-way ANOVA F(1,15)= 78.72, p=4.02E-07). **c)** Mean±SEM 5HT2A RNA expression in the right posterior insula region obtained at *post mortem* for each homozygous group (AC/C/G < CT/T/C one-way ANOVA F(1,12)= 20.96, p=7.9E-04).

**Figure 2**: *SLC6A4* genotype-dependent differential sensitivity to a 5HT2A antagonist during the human intruder test. **a)** Schematic of the right top quadrant of the home cage where the human intruder test takes place. An unfamiliar human stands in front of the cage and stares at the marmoset in the eyes for two minutes. Video and audio were recorded and behaviors scored post hoc. In blue, regions closer to the human intruder (corresponding to a less anxious state) and in red regions further away from the human intruder (corresponding to a more anxious state). **b)** Response to the human intruder threat after treatment with either vehicle or 5HT2A specific antagonist M100907 (low 0.1 and high 0.3mg/kg doses). Mean±SEM of *average distance* from the unfamiliar human is presented for each group in each condition (AC/C/G: vehicle vs high dose, planned comparison paired two-tailed t test p=.038).

**Figure 3**: 5HT2A related to trait anxiety scores. Voxel-based analysis correlating 5HT2A BP with anxiety scores (PC1) derived from the population PCA of the human intruder test. **a)** Specific cluster in right *anterior* insula (AP=12.2) sagittal (left) and coronal (right) sections centered in the highest significant peak (t=4.01). Below, correlations between right anterior insula **b)** 5HT2A BP (Pearson correlation r=-.753, p=.001) and **c)** 5HT2A RNA (Pearson correlation r=-.656, p=.015) with anxiety scores (PC1). **d)** Specific cluster in right insula proisocortex (AP=8.1) sagittal (left) and coronal (right) sections centered in the highest significant peak (t=3.73). Below, correlation between right insula proisocortex **e)** 5HT2A BP (Pearson correlation r=-.746, p=.001) and **f)** 5HT2A RNA (Pearson correlation r=-.173, p=.571) with anxiety scores (PC1).

**Figure 4**: Response to the human intruder threat after treatment with 5HT2A specific antagonist M100907 (high dose 0.3mg/kg) in relation to 5HT2A BP in **(a)** right *anterior* insula (Pearson correlation r=-.660, p=.014) and **b)** right insula proisocortex (Pearson correlation r=-.645, p=.017). Response to threat was calculated as percentage of change of *average distance* of the response after 0.3mg/kg relative to the response after vehicle. Values close to 0 correspond to no change from vehicle, values above and below 0 correspond to increased (enhanced anxiety), and decreased (reduced anxiety) distance, respectively.