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Serotonin transporter polymorphisms (SLC6A4 insertion/deletion and rs25531) do not affect the availability of 5-HTT to [^{11}C] DASB binding in the living human brain

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ABSTRACT

Studies *in vitro* suggest that the expression of the serotonin transporter (5-HTT) is regulated by polymorphic variation in the promoter region of the 5-HTT gene (5-HTTLPR); however, results from human brain imaging studies examining the relation between 5-HTT genotype and 5-HTT radioligand binding *in vivo* have been inconsistent. This inconsistency could reflect small participant numbers or the use of sub-optimal radiotracer for measuring the 5-HTT. We used positron emission tomography in conjunction with the selective 5-HTT ligand [^{11}C] DASB to examine the availability of the 5-HTT in seven brain regions in 63 healthy European caucasian volunteers who were genotyped for short (S) and long (L) variants (SLC6A4 and rs25531) of the 5-HTTLPR. [^{11}C] DASB binding potential was not influenced by the allelic status of participants whether classified on a biallelic or triallelic basis in any of the regions studied. Our PET findings, in a relatively large sample with a near optimal radiotracer, suggest that 5-HTTLPR polymorphic variation does not affect the availability of 5-HTT to [^{11}C] DASB binding in adult human brain. The reported impact of 5-HTTLPR polymorphic variation on emotional processing and vulnerability to depression are more likely therefore to be expressed through effects exerted during neurodevelopment.

Introduction

The neuronal serotonin transporter (5-HTT) plays a critical role in the regulation of serotonin (5-HT) neurotransmission and drugs targeting the 5-HTT such as selective serotonin re-uptake inhibitors (SSRIs) are used extensively in the management of mood and anxiety disorders. The gene encoding the 5-HTT has a number of polymorphic variants and one in the 5-HTT-linked promoter region (5-HTTLPR) is composed of a short (S) and long (L) version which results in differential transcription of the 5-HTT *in vitro*, with the L form being associated with higher 5-HTT expression levels (Lesch et al., 1996). More recently the 5-HTTLPR has been shown to exist in a triallelic form because the L allele itself has two functional variants (L_A and L_G) with the L_G form exhibiting similar levels of 5-HTT expression *in vitro* as the S allele (Hu et al., 2006).

It is important to determine whether 5-HTTLPR genotype affects expression of the 5-HTT in the human brain as this would have important implications for the role of individual variation of the 5-HTTLPR in the pathophysiology of emotional disorders and the effects of antidepressant treatment. It is possible to approach this question using positron or single photon emission tomography (PET or SPET) in conjunction with ligands designed to bind specifically to the 5-HTT.

The results to date of these investigations have been inconsistent probably as a consequence of relatively modest sample sizes and ligands of varying specificity for the 5-HTT. Using [^{123}I]-βCIT with SPET in 96 healthy subjects, van Dyck et al. (2004) reported higher 5-HTT binding in brainstem and striatum among carriers of the SS genotype, but this result was not confirmed in two smaller investigations also using [^{123}I]-βCIT (Jacobsen et al., 2000; Willeit et al., 2001). A limitation of studies with [^{123}I]-βCIT is that this ligand has similar affinity for the dopamine transporter and the 5-HTT and it is not possible to image the 5-HTT in cortical brain areas relevant to

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emotional regulation such as anterior cingulate cortex and limbic structures. However, two more recently developed PET ligands, [^{11}C] McN5652 and [^{11}C] DASB, allow specific quantification of the 5-HTT in these brain areas. Once again the data regarding the effect of 5-HTTLPR genotype are contradictory. Using [^{11}C] McN5652, Parsey et al. (2006) found no effect of the triallelic polymorphism on 5-HTT binding in 42 healthy subjects and 25 acutely depressed patients. However, Praschak-Rieder et al. (2007) using [^{11}C] DASB in 43 healthy subjects reported increased 5-HTT in putamen specifically in those with an L_A/L_A genotype. Reimold et al. (2007), showed a similar effect in midbrain in 19 healthy volunteers with L_A/L_A genotype (Reimold et al., 2007).

As a 5-HTT ligand, [^{11}C] DASB compares favourably to [^{11}C](+) McN 5652 in terms of the ratio of specific to non-specific binding. The aim of the present study was therefore to use [^{11}C] DASB in conjunction with PET to assess the role of 5-HTTLPR polymorphisms on the availability of brain 5-HTT to [^{11}C] DASB binding, in the largest group of healthy volunteers, using a full metabolite corrected arterial input function and graphical analysis to quantify regional activity.

Materials and methods

Participants

The study was approved by the Research Ethics Committee at Hammersmith Hospital, London, and the Administration of Radioactive Substances Advisory Committee (ARSAC), UK. All participants gave written informed consent for the study. We recruited 63 healthy European caucasian men (mean ± SD age 38.5 ± 11.1 years) with no current or past psychiatric history or substance misuse problems. Subjects with any serious medical or neurological (current or past) illness, alcohol or illicit substance dependence and other axis I or II disorders were excluded. We included only male subjects in this study because of a lack of toxicology data for [^{11}C] DASB in females.

Each subject had a PET scan with [^{11}C] DASB and a structural MRI scan for drawing accurate regions of interest. We implemented the same methods and procedures for PET and MRI scanning; image analysis; and quantification of [^{11}C] DASB binding potentials as described in detail in recent publications (Bhagwagar et al., 2007; Selvaraj et al., 2009).

The injected radioactivity dose, in this study, was between 432 MBq and 564 MBq (mean: 509 MBq, SD: 26 MBq). The radiochemical purity of the injected [^{11}C] DASB was high and ranged from 95% to 100% with a mean of 98% and a SD of 1%. The injected mass dose varied between 0.1 and 13.7 μg with a mean value of 3.1 μg and a SD of 2.3 μg. The specific activity was high, on average 100,966 MBq μmol^{-1} with a SD of 197,723 MBq μmol^{-1}.

Seven regions of interest (ROI) — amygdala, caudate, dorsal raphe, anterior cingulate cortex, hippocampus, putamen, thalamus and a reference region — cerebellum were defined on the co-registered MRI with the help of a probabilistic brain atlas template (Hammers et al., 2002). Cerebellar grey matter was used as a reference region because of the negligible density of 5-HTT in the cerebellum (Kish et al., 2005). The dorsal raphe region was manually defined as a fixed size region (900 mm^3) on the summed PET images of each individual. Dynamic PET scans were sampled by applying the individual ROI object maps. The regional volumes of distribution were determined using linear graphical analysis with metabolite corrected arterial input function and [^{11}C] DASB binding potentials estimated by using cerebellum as reference region.

Genotyping

Blood samples were collected from subjects and DNA was extracted from whole blood using the QIAamp DNA Mini Kit (Qiagen Inc, Canada). The 5-HTTLPR region of SLC6A4 was examined by amplifying with PCR using standard methods. The PCR products were visualized using gel electrophoresis performed in agarose gels prepared with ethidium bromide and 1× TBE (Tris, boric acid, EDTA). The 5-HTTLPR and rs25531 were genotyped according to the protocol by De Luca et al. (2005). To control for genotyping errors, a random subsample of 10% of the sample was re-genotyped. There were no differences between the original and the re-genotyped samples, thus we assume that the genotyping error rate was very low.

Table 1
Mean (SD) [^{11}C] DASB binding potential in different brain regions by 5-HTTLPR biallelic genotypes.

Region	SS ($N=10$)	SL ($N=35$)	LL ($N=18$)	RM ANOVA
Raphe	4.30 (1.16)	4.47 (1.59)	4.55 (1.26)	$F=0.61$,
Amygdala	2.07 (0.44)	1.87 (0.33)	1.70 (0.39)	*df* 2,59,
Ant cingulate	0.94 (0.24)	0.81 (0.16)	0.75 (0.22)	$p=0.55$
Hippocampus	1.11 (0.26)	0.99 (0.17)	0.88 (0.17)	
Caudate	1.57 (0.49)	1.46 (0.33)	1.38 (0.37)	
Putamen	2,25 (0.48)	2.02 (0.36)	2.02 (0.44)	
Thalamus	1.85 (0.51)	1.9 (0.42)	1.79 (0.39)	

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 12. Our primary hypothesis was that regional 5-HTT receptor binding potential would be lower in subjects with an SS genotype using the biallelic classification of S and L. We tested this using repeated measures analysis of variance (ANOVA) with "genotype" (LL vs SL vs SS) as a between subjects factor, and "region" (brain region of interest) as a within subjects factor with age as a covariate. We then repeated the same analysis, classifying participants on the basis of triallelic genotyping where L alleles were classified as L_A and L_G. For the purposes of this analysis, L_G and S alleles were combined as S′ and L_A was coded as L′. The three genotype groups consisted of S′S′ (SS + S L_G + L_GL_G); S′L′ (S L_A + L_AL_G); and L′L′ (L_AL_A). Age was added as a covariate to all the ANOVAs.

Results

Biallelic genotyping of the 5-HTTLPR revealed that of the 63 participants, 10 (16%) were SS, 35 (56%) were SL and 18 (28%) were LL (Table 1). The frequencies of the triallelic genotypes are shown in Table 2. These frequencies did not differ significantly from Hardy–Weinberg equilibrium (Chi squared = 1.054, and $p=0.3047$).

As reported previously the binding of [^{11}C] DASB was most concentrated in raphe with moderate densities apparent in several other brain regions including putamen, thalamus, caudate and amygdala. Lesser degrees of binding were detectable in anterior

Table 2
Mean (SD) [^{11}C] DASB binding potential in different brain regions by 5-HTTLPR gain of function triallelic genotypes.

Region	S′S′ ($N=16$)	S′L′ ($N=32$)	L′L′ ($N=15$)	RM ANOVA
Raphe	4.19 (1.02)	4.5 (1.65)	4.68 (1.31)	$F=0.09$,
Amygdala	2.06 (0.38)	1.81 (0.32)	1.71 (0.42)	*df* 2,59,
Ant cingulate	0.89 (0.21)	0.81 (0.16)	0.75 (0.24)	$p=0.91$
Hippocampus	1.06 (0.22)	0.98 (0.18)	0.88 (0.17)	
Caudate	1.47 (0.48)	1.47 (0.32)	1.41 (0.38)	
Putamen	2.14 (0.43)	2.02 (0.36)	2.05 (0.48)	
Thalamus	1.83 (0.44)	1.91 (0.42)	1.77 (0.42)	

S′S′ includes SS, SLg and LgLg genotypes; S′L′ includes SLa and LaLg genotypes; L′L′ includes LaLa genotype.

cingulate cortex and hippocampus. The RM ANOVA comparing the binding potential [^{11}C] DASB of all participants using the biallelic classification showed a significant main effect of brain region ($F=25.5$; $df=6,354$; $p=0.001$) but no main effect of genotype ($F=0.61$; $df=2$, 59; $p=0.55$) or region by genotype interaction ($F=0.28$; $df=12,354$; $p=0.83$) (Table 1; Fig. 1). There were no significant main or interactive effects of age (all values >0.5).

We then carried out the same analyses using the triallelic classification. There was no main effect of genotype ($F=0.09$; $df=2$, 59; $p=0.91$), no interaction between genotype and brain region ($F=0.63$; $df=6,330$; $p=0.48$) and no main or interactive effects of age (all p values >0.5) (Table 2, Fig. 1).

Discussion

Our cross-sectional data in a large group of healthy volunteers, suggest that polymorphic variation in the 5-HTTLPR does not alter the expression of the 5-HTT in adult human brain as measured by [^{11}C] DASB binding potential. The findings remained the same whether the 5-HTTLPR was classified on a biallelic or triallelic basis. We included among the brain regions examined, areas that previous reports have implicated as showing an effect of 5-HTTLPR genotype on 5-HTT expression, for example, putamen, caudate and midbrain/raphe. However, no effect of allelic variation on 5-HTT binding potential could be detected. Overall our findings support previous imaging studies that have not found significant effects of the 5-HTTLPR on 5-HTT availability (Parsey et al., 2006; Shioe et al., 2003).

To date, three studies have reported the effect of 5-HTTLPR triallelic genotype on 5-HTT binding using [^{11}C] DASB and PET. Praschak-Rieder et al. (2007) in a group of 43 male and female subjects reported about 12% higher 5-HTT binding only in putamen in L_A/L_A genotype carriers compared with non L_A/L_A carriers (Praschak-Rieder et al., 2007). This finding was uncorrected for multiple comparisons and the other five regions studied did not show any effect. The authors further noted that in a subset of 30 caucasians, the putamen remained significant after correction for multiple comparisons. Reimold et al. (2007), in a relatively small group ($N=19$) of male and female subjects found about 21% higher BP in midbrain in L_A/L_A carriers (Reimold et al., 2007). However, they did not find any effect in the amygdala or thalamus and they did not report putamen or other striatal regions. Kalbitzer et al. (2010) reported no significant difference in 5-HTT binding between L homozygotes and S-allele carriers (Kalbitzer et al., 2010). However they found increased BP_{ND} for [^{11}C] DASB only in the caudate nucleus ($p=0.04$, uncorrected) in the L_A/L_A genotype ($N=50$). The effect size was not reported in the manuscript. In comparison to these three studies, the current study included a larger sample size and also used metabolite corrected arterial input method to derive BP_{ND}.

While our data suggest that variation in the 5-HTTLPR does not affect the availability of 5-HTT to [^{11}C] DASB binding in the adult brain there is much evidence to indicate that 5-HTTLPR genotype influences aspects of brain function in healthy subjects. Perhaps the best described finding in this respect relates to studies using functional magnetic resonance imaging (fMRI) to measure the activity of the amygdala in response to negative emotional stimuli (Hariri et al., 2005; Munafo et al., 2008; Rhodes et al., 2007). Across a range of tasks and stimulus types, carriers of an S allele of the 5-HTTLPR consistently show greater amygdala activation. In addition, there are clinically-orientated investigations suggesting, for example, that carriers of the S allele are at greater risk of experiencing depression when exposed to stressful life events (Hariri et al., 2005; Roiser et al., 2005) and less likely when depressed to respond to treatment with SSRIs (Serretti et al., 2007). It should be noted, however, that much of this evidence has been contested (Kraft et al., 2007; Risch et al., 2009).

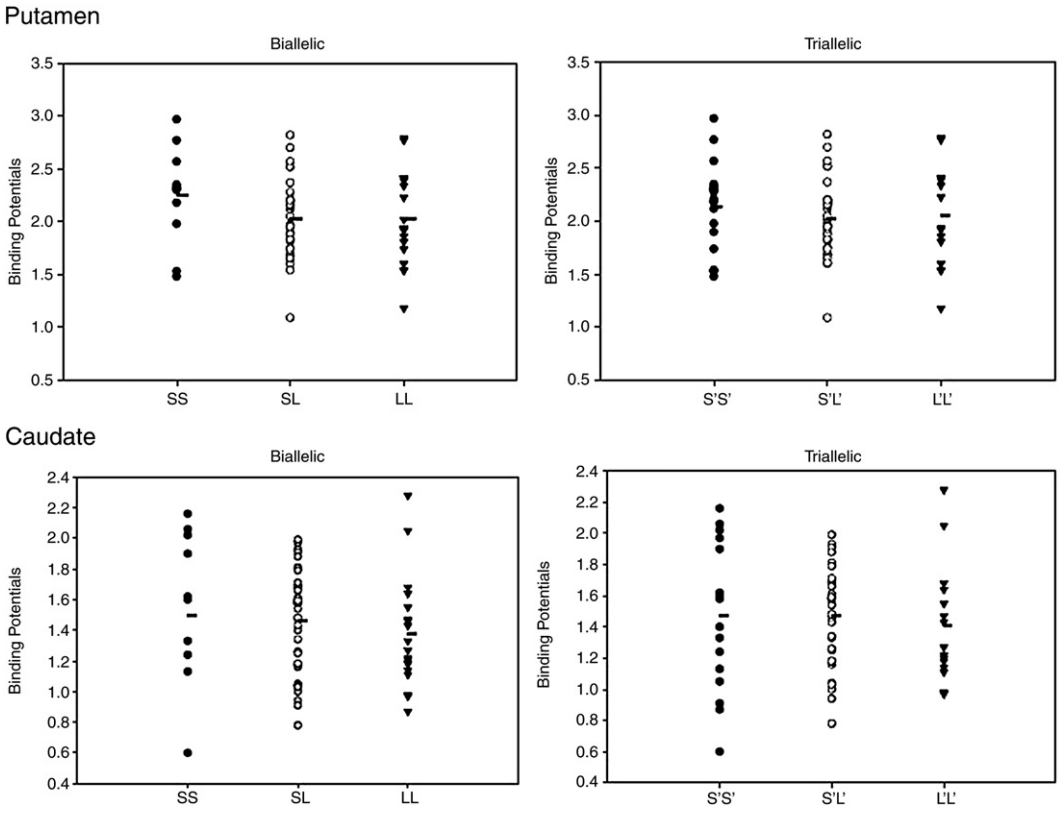

Fig. 1. Scatterplots of [^{11}C] DASB binding potentials in putamen and caudate by 5-HTTLPR biallelic and triallelic genotypes. Legend: S′S′ includes SS, SLg and LgLg genotypes; S′L′ includes SLa and LaLg genotypes; L′L′ includes LaLa genotype; horizontal lines show group mean values.

Taken together the data suggest that variation in the 5-HTTLPR does not influence brain function by changing availability of the 5-HTT to [^{11}C] DASB binding in the adult human brain but by other mechanisms. In the absence of 5-HTTLPR polymorphism related alterations in 5-HTT expression, how do we explain the reported functional associations with brain structure, function and behaviour? The current view is that the 5-HTTLPR genotype may be important in neurodevelopment so that effects of genotypes on brain function in adults are likely attributable to earlier neurodevelopmental changes. It should be kept in mind that promoter variants of gene polymorphisms play a role in not only the quantity of mRNA expression but also the timing and duration of expression, and each of these effects is tissue-specific. It could be that the L versus S promoter variants may have their main effect on the overall biological function by influencing the formation of 5-HT circuitry in fetal or childhood development, and these connectivity or functional changes might then be relatively fixed in adulthood. The 5-HTT protein available to [^{11}C] DASB binding in adult brains may be restored and maintained by homeostatic mechanisms that are independent of 5-HTTLPR genotype, and thus the PET binding potential would show no difference between S and L genetic status. This neurodevelopmental view may be supported by the observation that carriers of the S allele reportedly have reduced grey matter volume in both anterior cingulate and amygdala (Pezawas et al., 2005) and decreased functional connectivity between these brain regions (Heinz et al., 2005; Pezawas et al., 2005). Such neuroanatomical correlates may underlie abnormal functional reactions in S carriers to probes of neural circuitry regulating emotion. Furthermore, inherited differences in 5-HT transporter expression may also be compensated for by changes in tryptophan hydroxylase synthetic activity, or monoamine oxidase catabolic activity.

It is worth noting that additional variants in the 5-HTTLPR have been described and it is possible that a more complete classification of the different genotypes may allow an effect on 5-HTT availability to be described. In addition we have obtained evidence that amygdala responses to negative facial expressions measured by fMRI are influenced by the level of 5-HTT binding in left amygdala as measured by [^{11}C] DASB (Rhodes et al., 2007). This suggests that other factors involved in the regulation of 5-HTT expression might be important in explaining individual variation in emotional regulation.

It is possible that there may be gender differences in 5-HTTLPR genotype mediated 5-HTT expression, which this study could not address because we included only male volunteers. Hu et al. (2006) noted an approximately 3-fold difference in mRNA expression between L_AL_A and the SS group (Hu et al., 2006). While it is not known how such a huge *in vitro* effect translates into *in vivo* 5-HTT expression, our large PET study had adequate power to detect a between group difference of approximately 15%.

Recently, Kalbitzer et al. (2010) reported that cerebral 5-HTT binding was associated with daylight minutes at the time of the PET scan (Kalbitzer et al., 2010). Therefore, we reanalysed our data using the amount of daylight in minutes (Kalbitzer et al., 2010) as a covariate. We did not find any significant main or interaction effect of daylight minutes on either genotype or regional 5-HTT binding in biallelic or triallelic or L_AL_A vs non L_AL_A carriers (all p values >0.5). Therefore we believe that our results are unlikely to be confounded by the effect of daylight.

In summary, our cross-sectional PET study, in a large group of healthy European caucasian volunteers, shows that variations in 5-HTTLPR gene do not affect 5-HTT expression as measured by [^{11}C] DASB binding in the living human brain.

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