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Central Serotonin Transporter Availability Measured with [^{123}I]ß-CIT SPECT in Relation to Serotonin Transporter Genotype

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Running Title: Serotonin Transporter Gene and Availability

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ABSTRACT

Objective: A functional polymorphism has been described in the promoter region of the gene (SLC6A4) coding for the serotonin transporter protein (SERT). This polymorphism has two common alleles that have been designated as long (*l*) and short (*s*). Each allele has been linked with a number of human clinical phenotypes, including neuropsychiatric diseases associated with dysregulation of serotonin (5-HT) transmission. In vitro studies of non-neural cells have suggested that the *l*-allele may have higher transcriptional activity than the *s*-allele. However, the relevance of these findings for SERT levels in brain remains unclear. Method: We assessed genotype at the SLC6A4 promoter polymorphism in 96 healthy European American subjects (age range 18 to 88) who also underwent SPECT scanning with [^{123}I]2ß-carbomethoxy-3ß-(4-iodophenyl)tropane ([^{123}I]ß-CIT) for measurement of central SERT protein availability. A ratio of specific to nondisplaceable brain uptake (i.e., V_3" = [brainstem-diencephalon – occipital]/occipital), a measure proportional to the binding potential (B_{max}/K_D), was derived. Results: The results showed that the main effect of genotype was significant (F=4.48, df=2,92, p=0.014, ANCOVA, age = covariate). Post-hoc Tukey pairwise comparisons revealed that the *ss*-homozygotes had significantly greater SERT availability than the *ls*-heterozygotes (p=0.024). In addition, there was a nonsignificant trend for the *ll*-homozygotes to have greater SERT availability than the heterozygotes (p=0.092), but no

difference was observed between the *ll*-homozygotes and *ss*-homozygotes (p=0.70). The effect of age was significant in the ANCOVA model (t=–3.61, df=95, p<0.001). Conclusions: These results do not suggest higher central SERT levels in association with the *l*-allele in European American subjects but point to a more complex relationship between SLC6A4 genotype and protein availability.

INTRODUCTION

A functional polymorphism has been described in the 5' flanking regulatory (promoter) region of the gene (SLC6A4) coding for the serotonin transporter protein (SERT). This polymorphism (5-HTTLPR) has two common alleles that have been designated as long (*l*, 16 repeats) and short (*s*, 14 repeats) according to their relative size (1).

The 5-HTTLPR polymorphism has been associated with a number of clinical phenotypes and diseases (for a review, see (2)). The following are selected from numerous previous reports. Healthy subjects who carry the *s*-allele have been reported to have higher scores on measures of anxiety-related personality traits (1), although this has not been observed uniformly (e.g., (3)). Conversely, the *l*-allele has been associated with depression/suicide (4), and among patients with geriatric depression *ll*-homozygotes have been observed to have more severe depressive symptoms and higher platelet factor 4 and ß-thromboglobulin levels than *s*-carriers (5). The *l*-allele has also been reported to be in linkage disequilibrium with obsessive-compulsive disorder (6). *S*-carriers have been reported to have a higher risk of bipolar disorder (7, 8) and antidepressant-induced mania in bipolar disorder (9). Susceptibility to alcoholism has yielded contradictory associations with 5-HTTLPR, including no effect (10, 11), greater risk for *ll*-homozygotes (12), and greater risk for *s*-carriers (13).

Among alcoholic patients *s*-carriers may also have a higher risk of suicide attempts (14). Finally, two studies have suggested the *s*-allele to be a risk factor for late onset Alzheimer's disease (15, 16). The clinical associations of the 5-HTTLPR polymorphism are far from established, as reports for most psychiatric phenotypes have been inconsistent, a circumstance that may reflect the genetic complexity of these phenotypes and consequently the limited power to detect associations with them.

Emerging reports have also considered intermediate phenotypes, or "endophenotypes," which, since they are thought to reflect phenomena closer to the direct effect of the SLC6A4 variant than a complex behavioral phenotype, have the potential to increase power for observing a phenotypic effect. For example, Moreno et al (17) reported that SLC6A4 genotype is related to mood response during tryptophan depletion, and Hariri et al (18) showed that SLC6A4 genotype is related to amygdalar response to emotion based on functional magnetic resonance imaging measures.

As a possible basis for some genetic-phenotypic associations of the 5-HTTLPR polymorphism, in vitro studies of non-neural cells (1, 19) have suggested that the *l*-allele may have higher transcriptional activity than the *s*-allele. However, postmortem binding studies have thus far not supported an association between the *l*-allele and increased SERT binding in human brain tissue in mixed patient-control samples. Studies to date have reported: 1) lower

SERT binding in midbrain in *ls*-heterozygotes compared to either *ll*- or *ss*-homozygotes among cocaine-dependent subjects and healthy controls (20); 2) increased SERT binding in prefrontal cortex in *ss*-homozygotes compared to *l*-carriers among suicide victims and healthy controls (4); and 3) no relationship between 5-HTTLPR genotype and SERT binding in prefrontal cortex in individuals with (n=29) and without (n=71) a history of major depression (21).

With the advent of functional brain imaging it has become possible to study the effects of genetic polymorphisms in living subjects (18, 22). As an in vivo probe for SERT, several investigators have chosen the single photon emission computed tomography (SPECT) radioligand 2ß-carbomethoxy-3ß-(4-[^{123}I]iodophenyl)tropane ([^{123}I]ß-CIT; also referred to as [^{123}I]RTI-55). [^{123}I]ß-CIT is a potent cocaine analog with high affinity in vitro for both SERT and the dopamine transporter (DAT) (23). Previous studies in humans and nonhuman primates have shown that [^{123}I]ß-CIT accumulates in two distinct brain regions: striatum and brainstem-diencephalon. Pharmacological characterization of regional [^{123}I]ß-CIT uptake in baboons (24) has indicated that striatal activity is associated almost exclusively with DAT (i.e., displaceable by GBR-12909 but not citalopram), while binding in the brainstem-diencephalon appears to be specific for SERT (i.e., displaceable by citalopram but not GBR-12909). Thus, regional uptake of [^{123}I]ß-CIT in the brainstem-diencephalon appears to provide a pharmacologically relevant measure of SERT in humans.

Two recent neuroimaging investigations with SPECT and [^{123}I]ß-CIT have examined the effects of the SLC6A4 5-HTTLPR polymorphism on SERT availability in humans. Heinz et al (25) studied 14 abstinent alcoholics and 8 healthy control subjects and found that among healthy subjects, SERT availability was higher for *ll*-homozygotes than for *s*-carriers, whereas among alcohol-dependent subjects SERT availability was lower for *ll*-homozygotes than for *s*-carriers. Jacobsen et al (26) studied 27 healthy control subjects and reported no effect of SLC6A4 genotype on SERT availability.

The discrepant results in these two studies may be attributable to a number of factors, including small sample size, different outcome measures and image analysis techniques, and diagnostic and population heterogeneity. We therefore sought to examine a larger, diagnostically and racially homogeneous group. In the present study, the effect of SLC6A4 5-HTTLPR genotype on SERT protein availability was measured in 96 healthy European American subjects.

METHOD

Subjects

The study population consisted of 96 healthy European-American volunteers (54 male, 42 female) who ranged in age from 18 to 88 (50±19 years, with this and

subsequent averages reported as mean±S.D.). Subjects underwent a clinical examination by a research psychiatrist to exclude any neurological or psychiatric disease, alcohol or substance abuse. Screening procedures included a physical and neurological examination, EKG, serum chemistries, thyroid function studies, CBC, urinalysis, and urine toxicology screen. Female subjects of childbearing potential were required to have a negative pregnancy test (serum at screening; urine immediately prior to tracer injection). Those subjects ≥55 years of age (N=31) were also required to have no significant evidence of cognitive impairment, as indicated by a Folstein Mini-Mental State Examination (MMSE) (27) score ≥27. Those subjects ≥68 (N=25) were required to have a normal brain MRI scan. No subject was taking medication known to affect the brain 5-HT system. After complete description of the study procedures to the subjects, written informed consent was obtained. The present subject sample is a subset of that (N=126) in which we previously reported an age-related decline in DATs (28) and SERTs (29) using the same [^{123}I]ß-CIT SPECT scans. It also contains 12 subjects' scans in common with our earlier [^{123}I]ß-CIT SERT genetic report (26). All scans, including these common scans, were analyzed by a different rater from the previous report using somewhat different methodology, as discussed below.

DNA analyses

The SLC6A4 promoter VNTR polymorphism was genotyped as described previously (10). Alleles were designated according to their relative size: short (14 repeats) and long (16 repeats).

SPECT imaging

All subjects received 0.6 g potassium iodide (SSKI solution) in the 24 h prior to the SPECT scan. They then received an injection of [^{123}I]ß-CIT (6.0±0.7 mCi; specific activity >5,000 Ci/mmol) on day 1, followed 22.9±1.9 h later by a 24 min scan with a Picker (Cleveland, OH) PRISM 3000 (N=72) or 3000XP (N=24) SPECT camera equipped with a low energy, high resolution (LEHR) fanbeam collimator (128 x 128 matrix, 120˚ angular range, 3˚ angular step, 40 steps, 36 seconds per step, 15.5 cm radius of rotation). In this configuration, the PRISM 3000 acquires images at a reconstructed full-width at half-maximum resolution of 12.3 mm as determined by an ^{123}I point source in water. Comparability of the two cameras has previously been confirmed by imaging 26 subjects on both cameras from a single [^{123}I]ß-CIT injection (29). Previous studies (30, 31) have demonstrated that [^{123}I]ß-CIT reaches equilibrium binding in the brain by 18 to 24 h, yielding a simple unitless ratio of regional radioactivities (V_3" = specific/nondisplaceable binding = [brainstem-diencephalon – occipital]/occipital) which is proportional to SERT number (i.e., B_{max}), assuming that both the affinity

(K_D) and the nondisplaceable binding (V_2) do not vary significantly within the population. Prior to scanning, four or five fiducial markers filled with 5 μCi of $Na^{99m}TcO_4$ were attached to the skin along the canthomeatal plane to identify this plane during image analysis.

Images were reconstructed from photopeak counts (159±16 keV) using standard filtered backprojection methods (Butterworth, power 10, cutoff 0.24 cm^{-1}) and displayed as a 128 x 128 x 64 matrix with a voxel size of 2.07 x 2.07 x 3.56 mm (15.25 mm^3). Subsequent image analysis was performed by an operator who was unaware of subject demographics. SPECT data were reoriented to correct for deviations from the canthomeatal plane, as identified by the fiducial markers. Eight contiguous transaxial slices with the highest uptakes in brainstem-diencephalon and striatum, respectively, were identified from a reconstructed midsagittal image and digitally summed to yield two transaxial slices, each 28.5 mm thick. Attenuation correction was performed using a Chang zero order method (attenuation coefficient $\mu = 0.15$ cm^{-1}) within an ellipse drawn around the skull. Standard region of interest (ROI) templates (previously published (29)) for brainstem-diencephalon (432 voxels or 6.6 mL) and occipital cortex (7912 voxels or 120.6 mL) were positioned on their respective summed slices, with the occipital ROI placed at the striatal level. No attempt was made to correct for scatter or partial volume effects.

Statistical analysis

Chi square tests were used to compare observed genotype frequencies to those predicted by Hardy Weinberg equilibrium and also to compare sex distributions among genotypic groups. Subject age was compared across groups using analysis of variance (ANOVA). V_3" for brainstem-diencephalon was computed without conversion of SPECT cpm to absolute units of radioactivity as $[(cpm/voxel)_{brainstem\text{-}diencephalon} - (cpm/voxel)_{occipital})] / (cpm/voxel)_{occipital}$. Values of V_3" for brainstem-diencephalon were then compared across genotypic groups using analysis of covariance (ANCOVA), controlling for age which has been strongly correlated with brainstem-diencephalon V_3" in previous studies with [^{123}I]ß-CIT (29). For significant main effects by ANOVA or ANCOVA, Tukey post-hoc pairwise comparisons were performed. All statistical analyses utilized the SPSS (SPSS Inc., Chicago, IL) or SYSTAT (SYSTAT Inc., Evanston, IL) software packages and employed two-tailed tests of significance.

RESULTS

Genotype Composition of the Sample

Table 1 about here

Frequencies of SLC6A4 5-HTTLPR genotypes for the 96 subjects are displayed in Table 1. Of these, 36 were classified as 5-HTTLPR *ll*-homozygotes, 18 as *ss*-homozygotes, and 42 as *ls*-heterozygotes. Overall frequencies of the *l*- and *s*-alleles, 59% and 41% respectively, are similar to those observed by Lesch et al (1) (57% and 43%, respectively) in a predominantly Caucasian population, as well as those observed in homogeneous healthy European American samples by Gelernter et al (10) (60% and 40%, respectively). The distribution of the three common genotypes did not differ significantly from Hardy-Weinberg equilibrium expectations (Table 1).

Unexpectedly, the three genotypic groups differed significantly with respect to age ($F = 8.22$, $df = 2,93$, $P = 0.00052$, ANOVA), as the *ll*-homozygotes (59.2±19.3 years) were significantly older than both the *ss*-homozygotes (40.7±14.4 years, $P = 0.0016$, Tukey pairwise comparison), and *ls*-heterozygotes (46.2±17.7 years, $P = 0.0050$). The genotypic groups did not differ significantly with regard to sex (M/F = *ll:* 17/19, *ls:* 27/15, *ss:* 10/8, $\chi^2=2.30$, df=2, p=0.32).

Effect of genotype on Central SERT availability

Figure 1 about here

The values of V_3" for brainstem-diencephalon are displayed in Figure 1. When the contribution of age was controlled for, the main effect of genotype was significant (F=4.48, df=2,92, p =0.014, ANCOVA). Post-hoc Tukey pairwise comparisons revealed that the *ss*-homozygotes had significantly greater SERT availability than the *ls*-heterozygotes (p=0.024). In addition, there was a nonsignificant trend for the *ll*-homozygotes to have greater SERT availability than the *ls*-heterozygotes (p=0.092), but there was no difference between the *ll*-homozygotes and *ss*-homozygotes (p=0.70). The effect of age was significant in the ANCOVA model for brainstem-diencephalon (t=–3.61, df=95, p<0.001). The interaction of age*genotype was also significant (F=5.19, df=2,93, p=0.007) with the *ss*-homozygotes showing a greater decline in V_3" with age than either of the other two groups, possibly because of the small number of elderly *ss*-homozygotes (only one subject >60 years). As in our previous report (29), the addition of gender (with or without the interaction of age and gender) did not significantly improve the prediction of V_3" after controlling for the contribution of age.

DISCUSSION

We examined the effect of SLC6A4 5-HTTLPR genotype on SERT availability in the largest and most homogeneous subject sample reported to date—a sample of 96 healthy European Americans. When the contribution of age was controlled for, the main effect of genotype was significant, with the *ss*-homozygotes demonstrating significantly greater SERT availability than the *ls*-heterozygotes, and the *ll*-homozygotes showing a nonsignificant trend for greater SERT availability than the heterozygotes. No difference was observed between the *ll*-homozygotes and *ss*-homozygotes.

The highly significant difference in age distributions of the 3 genotypic groups (*ll* > *ls* and *ss*) is difficult to explain. The *l*-allele could conceivably possess a survival or sampling advantage in older healthy subject populations, given the association of the *s*-allele with several conditions (depression/suicide (4, 14), bipolar disorder (7, 8), and late-onset Alzheimer's disease (15, 16)) that might eliminate older individuals from the healthy subject pool. However, any advantage of the *l*-allele would likely be insufficient to account for these large age differences, and another report found no difference in genotype frequencies between young and older healthy subject groups (16). A random sampling effect seems more likely. In any case, the age differences among genotypic subgroups dictate caution in interpreting the present results. Similarly, the significant interaction of genotype*age in the ANCOVA results (with *ss*-homozygotes evidencing a steeper age-related decline than the other two groups) must be taken

with caution, given the skewed age distribution of the *ss*-subjects (only one *ss*-subject >60 years).

Comparison with other neuroimaging studies

These results differ from those of two previous neuroimaging investigations (also using [^{123}I]ß-CIT SPECT) of SLC6A4 genotype and protein availability in smaller samples. Heinz et al (25) found that among 8 healthy control subjects, SERT availability was increased in *ll*-homozygotes compared to *s*-carriers, whereas among 14 abstinent alcoholic subjects, SERT availability was lower for *ll*-homozygotes than for *s*-carriers. Jacobsen et al (26) reported no effect of SLC6A4 genotype on SERT availability in 27 healthy control subjects.

The divergent results among these three studies are potentially attributable to several methodological differences. Heinz et al (25) employed the outcome measure BP'—a ratio of the specific tracer uptake to the total plasma [^{123}I]ß-CIT concentration at equilibrium, whereas Jacobsen et al (26) and the present study both used V_3"—a ratio of specific to nondisplaceable binding at equilibrium. V_3" has the theoretical disadvantage of assuming equivalent nondisplaceable tracer uptake between subjects (or at least study groups) (30, 31) but may be more reproducible, as it does not depend on plasma measurements. Our results are unlikely to be confounded by a systematic difference in nondisplaceable [^{123}I]ß-CIT uptake among *ll*-homozygotes, *ss*-homozygotes, and *ls*-heterozygotes. Heinz

et al (25) and Jacobsen et al (26) both used MRI coregistration for ROI placement, permitting the use of cerebellum as reference region, whereas the present study utilized occipital cortex as reference region. Although a previous primate study (24) demonstrated no displaceable component of cortical [^{123}I]ß-CIT uptake, human post-mortem studies have found low but measurable levels of SERT in occipital cortex (32, 33) including by [^{123}I]ß-CIT binding studies (34). However, this difference is again unlikely to yield systematic differences among genotypic groups. Only Jacobsen et al (26) performed measured attenuation correction, whereas Heinz et al (25) and the present study both assumed uniform attenuation within an ellipse drawn around the skull. However, the increased accuracy in [^{123}I]ß-CIT quantitation by measured compared to uniform attenuation correction appears to be small (35) and, in any case, would not introduce systematic biases among genotypic groups.

Other important differences among these three studies include varying diagnostic and racial composition of subject groups. Whereas the present study and that of Jacobsen et al (26) included only healthy subjects, the subjects of Heinz et al (25) comprised 14 abstinent alcoholics and 8 control subjects. SLC6A4 5-HTTLPR allele frequency is known to vary across population groups (10, 36), although no effect on SERT availability has been determined for race or the interaction of race and genotype. As we lacked sufficient racial subsamples to undertake a systematic investigation of racial effects, we opted to restrict our

analysis to our largest population subgroup–-of European Americans. The other two studies (25, 26) both included small numbers of subjects of non-European ancestry, although neither study possessed the statistical power to address the effect of race on SERT availability rigorously. Future studies using larger samples of different racial groups will be necessary to address this question.

All three studies are limited by [^{123}I]ß-CIT's dual affinity for SERT and DAT. Although displacement binding studies in primates have demonstrated that [^{123}I]ß-CIT uptake in brainstem-diencephalon is primarily associated with SERT (24) (i.e., displaceable by citalopram but not GBR-12909), a small component of this uptake is likely attributable to DAT. Ex-vivo autoradiography studies in 3 monkeys from our group (unpublished data) suggest that [^{123}I]ß-CIT uptake in brainstem-diencephalon is distributed (starting from the region with highest uptake) in: 1) superior colliculus, 2) dorsal and median raphe nuclei, 3) substantia nigra, and 4) several hypothalamic and thalamic nuclei. Although this distribution closely mirrors that of SERT binding in homogenate binding and autoradiographic studies in human and nonhuman primates (32, 33, 37), the substantia nigra also contains significant levels of DAT. This relatively small DAT component of brainstem uptake may account for the less than complete displacement of [^{123}I]ß-CIT binding in human brainstem-diencephalon by citalopram (38). Therefore, replication of the present findings using a more selective PET or SPECT tracer will be valuable.

Comparison with previous postmortem and in vitro studies

The present results are somewhat paradoxical in suggesting the lowest SERT availability for the *ls*-heterozygotes, with relatively higher values for either homozygote condition (in the case of the *ll*-homozygotes, by a nonsignificant trend). However, they are remarkably similar to the postmortem results of Little et al (20) in 17 human cocaine users and 21 healthy control subjects, in which reduced SERT binding (by [^{125}I]ß-CIT in dorsal and median raphe and substantia nigra) was observed in *ls*-heterozygotes compared to *ll*-homozygotes or *ss*-homozygotes. They also bear similarity to the postmortem findings in 8 suicide victims and 15 healthy controls of Du et al (4) who reported increased SERT binding (by [^{3}H]paroxetine in prefrontal cortex) in *ss*-homozygotes compared to *l*-carriers. Comparison of the present results with those obtained in mixed healthy-patient samples is clearly limited by the possibility that disease states may alter the relationship between SLC6A4 genotype and SERT binding.

The emerging brain binding and imaging studies of the SERT genotype-phenotype relationship are thus somewhat at odds with the previous in vitro data from non-neural cells. In their original landmark report, Lesch et al (1) used cultured human lymphoblastoid cell lines and observed reductions in SERT mRNA concentrations, SERT expression, and 5-HT reuptake in lymphoblasts carrying the *s*-allele compared to *ll*-homozygotes. Heils et al (19) also performed

functional studies of the native 5-HTTLPR in a human placental choriocarcinoma cell line (JAR) and found that transcriptional activity (both basal and induced by cyclic AMP or protein kinase C) was increased by the *l*-allele compared to the *s*-allele. Greenberg et al (39) investigated the expression and function of 5-HTTLPR in human platelets and found no difference in SERT expression but more rapid initial 5-HT uptake in *ll*-homozygotes compared to *s*-carriers. In the aforementioned postmortem brain study of Little et al (20), the authors also measured SERT mRNA levels and observed the same pattern as Lesch et al (1), with lower SERT mRNA levels in the *s*-carriers than the *ll*-homozygotes. Overall, the available postmortem/imaging and in vitro studies are in substantial agreement with regard to the comparison between *ll*-homozygotes and *ls*-heterozygotes but diverge strikingly with regard to the *ss*-homozygote condition.

These differences between in vitro results in non-neural cells and brain binding and imaging studies are not readily explained. However, the 5-HTTLPR polymorphism may play a more complex role in neural tissue than in lymphoblasts or placental choriocarcinoma cells, and SERT binding sites may also differ in these tissues. Alternatively, SERT protein levels in brain may not strictly reflect transcriptional activity, and putative associations between the 5-HTTLPR polymorphism and clinical phenotypes and diseases may result from a mechanism other than adult basal levels (e.g., SERT levels during development, under conditions of stress, etc.). The differences could also reflect interactions

with other loci, i.e., epistatic effects. Finally, associations between this polymorphism and protein levels (as well as disease) may simply be population-dependent.

In conclusion, these results do not suggest higher central SERT levels in association with the *l*-allele in European American subjects but point to a more complex relationship between SLC6A4 genotype and protein availability. SLC6A4 5-HTTLPR *ss*-homozygotes (and possibly *ll*-homozygotes) may have increased SERT availability compared to heterozygotes. These results may enhance understanding of previously observed associations between SLC6A4 genotype and neuropsychiatric diseases and phenotypes.

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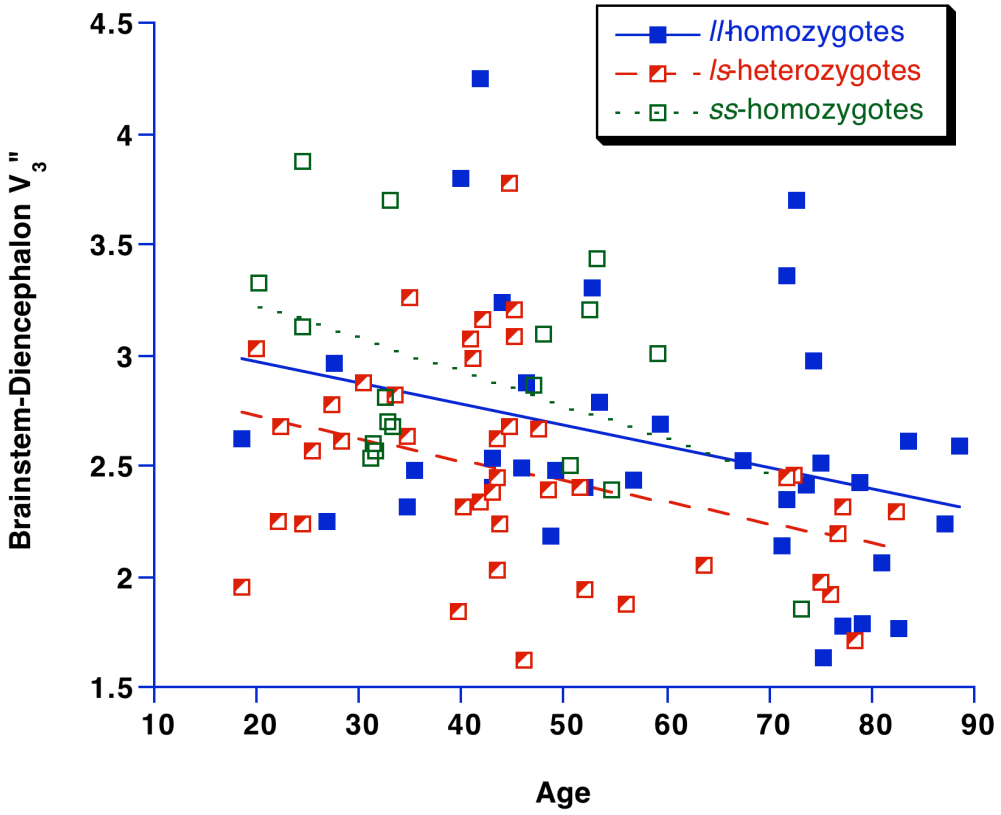
Table 1. Frequencies of 5-HTTLPR Polymorphism of the Serotonin Transporter Gene (SLC6A4) in Healthy European American Subjects

	Genotype (5-HTTLPR)	*N*	%
Observed	*l–l*	36	38%
	l–s	42	44%
	s–s	18	19%
Expected	*l–l*	33.8	35%
	l–s	46.3	48%
	s–s	15.8	17%
	χ^2	0.83	
	df	2	
	p	0.66	

FIGURE LEGENDS

FIGURE 1. Serotonin transporter (SERT) availability (V_3") in brainstem-diencephalon, as measured by [^{123}I]ß-CIT and SPECT, versus age in 96 healthy European American subjects, grouped according to SLC6A4 5-HTTLPR genotype. The main effect of genotype was significant, controlling for age (F=4.48, df=2,92, p=0.014, ANCOVA). Post-hoc Tukey pairwise comparisons revealed that the *ss*-homozygotes (N=18) had significantly greater SERT availability than the *ls*-heterozygotes (N=42) (p =0.024). In addition, there was as a nonsignificant trend for the *ll*-homozygotes to have greater SERT availability than the *ls*-heterozygotes (N=36) (p=0.092), but there was no difference between the *ll*-homozygotes and *ss*-homozygotes (p=0.70).


l/l-homozygotes
l/s-heterozygotes
s/s-homozygotes
Brainstem-Diencephalon V3"
Age
4.5
4
3.5
3
2.5
2
1.5
10
20
30
40
50
60
70
80
90