ASSOCIATION OF THE 5-HTTLPR WITH PROLACTIN RESPONSE TO CITALOPRAM IN A COMMUNITY POPULATION

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The serotonin transporter (5-HTT) is a key mechanism regulating magnitude and duration of serotonergic transmission in the central nervous system, and is the site of action of selective serotonin reuptake inhibitors (SSRIs) used for treating psychiatric conditions. Variation in treatment response to SSRIs has been correlated with a common bi-allelic length polymorphism in the 5-HTT-promoter region (5-HTTLPR), known to modulate transcriptional efficiency of the 5-HTT gene *in vitro.* The alleles, designated long (*l*) or short (*s*), result in one of three possible genotypes: *l/l*, *l/s*, or *s/s*. The (*s*) allele has been hypothesized to have a dominant functional effect, and has been associated with decreased transporter transcription efficiency and poorer therapeutic response to antidepressants. Acute transporter blockade with SSRIs rapidly increases central nervous system serotonin levels, leading to hypothalamic receptor stimulation and the release of several hormones, including prolactin. The specific aim of this study is to characterize the prolactin response to acute 5-HTT-reuptake blockade according to 5-HTTLPR genotype, to further elucidate the effect of this polymorphism on serotonin transporter function *in vivo*. This study has been designed to test the hypothesis that, when compared to subjects with the *l/l* genotype, subjects with either the *s/l* or *s/s* genotype will experience a blunted prolactin response following acute administration of the highly selective reuptake inhibitor Citalopram. To

accomplish this goal, a cohort of 206 community volunteers were intravenously administered a weight-adjusted dose of Citalopram. Each subject was genotyped for the 5-HTTLPR, and blood samples were obtained for prolactin and Citalopram levels immediately before and at regular intervals for 2.5 hours after the Citalopram injection. *Results:* Citalopram-induced prolactin response, reported as prolactin area under the curve (PRL AUC), was significantly associated with 5-HTTLPR ($F = 3.08$, $p = 0.048$). Among individuals with the s/s genotype, PRL AUC response ($\underline{M}\pm\underline{SD}$: 84.2 ± 51.8 ng/ml * 150min) was significantly lower ($p = 0.014$) than the l/l group (246.0 ± 40.2 ng/ml * 150 min). The difference in PRL AUC between subjects with the l/l genotype and the l/s group (172.5 ± 41.3 ng/ml * 150 min) was not significant ($p = 0.21$); the difference in PRL AUC between subjects with the l/s genotype and the s/s group also was not significant ($p = 0.23$). When results were analyzed as the maximum change in prolactin, the Citalopram-induced PRL MAX was similarly associated with 5-HTTLPR ($p = 0.034$). *Conclusions*: Subjects with the 5-HTTLPR s/s genotype exhibit significantly lower prolactin response in response to the SSRI Citalopram than subjects carrying two copies of the l allele. The s allele does not have a dominant effect on the prolactin response to Citalopram in a non-patient population.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1. SEROTONIN IN THE CENTRAL NERVOUS SYSTEM

Central nervous system serotonin (5-HT) is synthesized in neurons whose cell bodies lie in the brainstem raphe nuclei. Raphe 5-HT neurons form complex pathways in the human brain, consisting of at least five main 5-HT pathways into the forebrain as well as myriad branches to other neuronal pathways, which form global and local networks. Raphe nuclei fibers project to virtually every part of the brain, including limbic structures, hypothalamus, hippocampus, substantia nigra, and all cortical areas. Raphe neurons send collateral inputs to the limbic system, believed to modulate mood and behavior, and the hypothalamus, which regulates hormone secretion. Therefore, serotonin dysregulation, which has been implicated in a variety of mood and behavioral disorders, may also be reflected in plasma hormone concentrations. The serotonin transporter (5-HTT) is a key mechanism in the regulation of serotonergic activity, and is the site of action of serotonin reuptake inhibitors (SSRIs). SSRIs have proven to be clinically effective in treating many, but not all individuals suffering from mood and behavioral disorders. Consequently, much attention has been focused on discovering sources of variation in 5-HTT function to elucidate inter-individual variation in treatment response to SSRIs and future treatment strategies.

1.2. PREVIOUS RESEARCH

1.2.1. Differential biological responses to SSRIs suggest 5-HTT functional variation

Biological indices of post-synaptic serotonergic activity in individuals undergoing treatment with SSRIs were originally utilized to help clarify the differences in treatment responses between individuals. For instance, variations in biological indices of central 5-HT function following administration of SSRI's, such as levels of cerebrospinal fluid 5-hydroxyindoleacetic acid (CSF 5-HIAA) (Asberg et al., 1981; Insel et al., 1985; Thoren et al., 1980), platelet [^{3}H] imipramine binding (Castrogiovanni et al., 1995; Tollefson et al., 1996), and platelet or plasma serotonin levels (Blardi et al., 2002; Figueras et al., 1999), have been found to correlate with individual differences in therapeutic response to SSRIs, suggesting possible inter-individual differences in transporter function and/or availability. A recent study using SPECT imaging to quantify 5-HTT availability reports better treatment response to SSRI's in subjects with greater pre-treatment transporter availability (Kugaya et al., 2004).

Researchers investigated variation in the serotonin transporter genetic sequence, in hopes of discovering a functional polymorphism to explain a functional effect on the protein itself, but none have been identified to date. However, recent investigations focusing on a functional polymorphism in the 5-HTT gene linked promoter region (5-HTTLPR) have provided insight into variability in post-synaptic serotonergic activity.

1.2.2. Isolating 5-HTT functional variation

Functional variation in the promoter region of the 5-HTT contains a common 44 base pair insertion/deletion, giving rise to a bi-allelic polymorphism designated long (*l*) and short (*s*). In human platelet studies, this sequence variation has been shown to have functional consequences that modulate the gene's transcriptional efficiency and affect the rate of 5-HT transport from the extracellular space into the cell (reuptake). The (*l*) and (*s*) variants of the 5-HTTLPR have different transcriptional efficiencies when fused to a reporter gene and transfected into human placental choriocarcinoma (JAR) cells. The deletion, or (*s*) allele reduces transcriptional efficiency roughly twofold (Lesch et al., 1996; Heils et al., 1996). In cultured lymphoblasts, the (*s*) allele is associated with lower 5-HTT-gene promoter and 5-HTT gene expression, and decreased serotonin uptake (Lesch et al., 1996).

In vivo studies have shown that, compared to the (*l*) variant of the 5-HTTLPR, the (*s*) allele is associated with poorer therapeutic response to antidepressants (Arias et al., 2003; Zanardi et al., 2001; Pollock et al., 2000; Zanardi et al., 2000; Smeraldi et al., 1998), although the results of one study, utilizing a Korean population (Kim et al., 2000), associated the (*s*) allele with a significantly better response.

Other *in vivo* studies addressing functional properties of the 5-HTTLPR are informative, although not always consistent. For instance, the (*s)* allele may be associated with lower midbrain 5-HTT mRNA expression and transporter availability (Little et al., 1998; Heinz et al., 2000), reduced uptake of serotonin into platelets (Greenberg et al., 1999; Nobile et al., 1999), and lower whole blood serotonin content (Hanna et al., 1998), but these findings are not reported in all studies. For instance, Willeit et al. (2001) found no significant difference in transporter availability between 5-HTTLPR genotype groups, assessing SPECT imaging of [123]I-beta-CIT

binding *in vivo*. Stoltenberg et al. (2002) reported no association between 5-HTTLPR and whole blood serotonin content in their study population. With respect to characterizing the effects of the 5-HTTLPR allelic variation on serotonin transporter function, these studies are not ideal due to a number of factors: 1) small sample size – none of which had greater than 70 subjects, with the exception of the Stoltenberg study which included 150 subjects; 2) current subject pathology such as obsessive-compulsive disorder (Hanna et al., 1998), major depression (Nobile et al., 1999), and alcoholism (Heinz et al., 2000) – psychiatric illness and long-term alcohol dependence have been shown to alter transporter availability, although it is not clear to what degree; and 3) use of homogenized postmortem brain tissue (Little et al., 1998), analysis of which is likely to be confounded by post-mortem delay, storage time, differences in brain region, and ante mortem treatment with antidepressants.

In two large studies involving healthy volunteers, cerebrospinal fluid (CSF) concentrations of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), (an indirect estimate of 5-HT turnover in the brain) were compared with individual 5-HTTLPR genotype. Jönsson et al., (1998) reported no association of CSF 5-HIAA and 5-HTTLPR genotype in 66 healthy subjects. However, in a more recent study, CSF 5-HIAA was found to be 50% higher in persons with either one or two *l* alleles than those of persons with the *s/s* genotype (Williams et al., 2001). Although CSF 5-HIAA has been reported as an index of central serotonergic activity, the conflicting results from these studies suggested the need to measure a more direct biological correlate of transporter function.

1.2.3. Neuroendocrine Response to Transporter Blockade

Another method to measure variation in 5-HT function *in vivo* is to assess neuroendocrine response to drugs that alter serotonergic neurotransmission. In what are referred to as neuroendocrine challenge tests, plasma levels of pituitary hormones such as prolactin are measured to quantify the change in hypothalamic 5-HT receptor stimulation following administration of serotonergic agents that have known effects on enzymatic degradation, pre or post synaptic receptors, or reuptake (Raap and Van de Kar, 1999 Review).

By utilizing a serotonergic agent with an action specific to a particular mechanism, such as that of an SSRI on the serotonin transporter, functional effects of allelic variation can be compared as variation in rapid increase of synaptic 5-HT availability indexed by prolactin response. 5-HTTLPR allelic variation associated with variation in prolactin response to administration of SSRIs provides insight into the effect of the polymorphism on transporter availability and variation in treatment response.

The effect of 5-HTTLPR allelic variants in *in vivo* CNS serotonergic activity has been assessed using prolactin response to acute administration of various antidepressants. Two recent studies suggest that neuroendocrine response to pharmacological alteration of the serotonin transporter mechanism varies by 5-HTTLPR genotype. Women homozygous for the short allele exhibited smaller elevations in plasma prolactin concentration following acute administration of the tri-cyclic antidepressant clomipramine, relative to women carrying two copies of the long allele (Whale et al., 2000). In the second study, males having at least one short variant of the promoter polymorphism also showed a blunted prolactin response to the 5-HT releasing agent, fenfluramine, compared to men homozygous for the long allele (Riest et al., 2001).

Although the blunted prolactin responses reported in these two studies among participants with either the *s/s* or *s/l* genotype are consistent with one another, the serotonergic agents used limit the interpretation of the effect of genotype on transporter function. For instance, Whale et al. (2000) utilized clomipramine as the neuroendocrine challenge agent, which has mixed serotonergic and noradrenergic effects, making it difficult to attribute the rise in plasma prolactin exclusively to alteration in serotonin transporter function. In the Reist study, fenfluramine was the challenge agent used, which acts as a releasing agent as well as a reuptake inhibitor. Although fenfluramine produces a measurable prolactin response, the effect of the compound on transporter directionality complicates interpretation of the effect the genotype may have on transporter availability and normal transporter function. Additionally, the Reist study (2001) had the added complication of the study subjects having been pooled from individuals with alcohol dependence, which may itself compromise transporter availability.

1.2.4. Present Study Rationale and Purpose

To more accurately characterize the effect of the 5-HTTLPR on transporter-mediated variation in serotonergic activity, the design of this study rectifies some limitations of prior work, such as sample size, psychopathology, and pharmacological selectivity. The large sample size (n = 206) affords statistical power missing in previous studies. In addition, this community sample is screened to identify current major psychiatric disorders, alcohol dependence, and recent antidepressant exposure, all of which may be correlated with altered transporter function and availability. Lastly, the pharmacological agent used, Citalopram, is the most highly selective SSRI currently available, does not affect 5-HT release, and does not directly act at any 5-HT

receptors (Milne et al. 1991). Citalopram's profile isolates the pharmacological manipulation to the transporter reuptake mechanism, which is essential in order to associate changes in plasma prolactin with allelic variation in 5-HTTLPR.

The purpose of this study is to test possible associations between 5-HTTLPR allelic variation and variability in central nervous system serotonergic responsivity, as measured by neuropharmacological challenge, in a large community sample of healthy adults. In this experiment, I will test the hypothesis that, when compared to subjects with the l/l genotype, those subjects with one or two copies of the s allele (the s/l and s/s genotypes) will experience blunted prolactin responses following acute administration of the highly selective SSRI Citalopram.

2. RESEARCH DESIGN

2.1. SUBJECT CRITERIA

Subjects were recruited from the local population in the Pittsburgh area, and met the following criteria: good general health without reported clinical history of atherosclerotic disease; angina or peripheral arterial disease; severe and chronic diseases affecting general health (e.g., cancer [diagnosed or treated within the past year], chronic kidney or liver disease, multiple sclerosis). Subjects also were free of current major depression, and not taking any of the following disallowed medications: antidepressants; narcotics daily; anxiolytics daily; antimanics; antipsychotics; anticovulsants; antiparkinson; glucocorticoids; or weight loss medications (i.e., orlistat, sibutramine hydrochloride, or phenteramine hydrochloride).

2.2. CITALOPRAM CHALLENGE PROTOCOL

Following informed consent, participants reported to the General Clinical Research Center at UPMC-Montefiore Hospital and were administered a single, weight-adjusted dose of Citalopram, with subsequent sampling of blood for prolactin concentrations under the following conditions:

The session began between 13:30 and 15:00 hours, during the time of the day when plasma prolactin levels are relatively stable. Studies (Katznelson et al. 1998; Sassin, et al. 1972) have shown that prolactin levels are highest during sleep and just after waking, and lowest in the early to late afternoon hours. Citalopram dose is calculated by multiplying each subject's lean body mass, in kilograms, by 0.33 mg (subjects stand on an automated body composition analyzer that calculates fat free mass based on measured electrical impedance). To minimize the effects of caloric intake on circulating drug levels, Citalopram was administered following a 2-hour fast (water only). Because alcohol may affect serotonergic function (LeMarquand et al., 1994) subjects were instructed to refrain from drinking alcohol for 24 hours prior to their session. Because menstrual phase can affect prolactin response (O'Keefe et al. 1991; Wada et al. 1991), pre-menopausal women completed the challenge protocol during the early follicular phase (e.g., days 3-9) of the menstrual cycle. In addition, screening for cocaine and heroin was completed prior to the administration of Citalopram, with a positive result for either substance immediately ending the subject's participation in the study. Upon verification of negative drug screen results, an IV catheter was inserted into each forearm, one for drug administration and the other for blood sampling. After a 30-minute rest period, subjects received Citalopram intravenously over 30 minutes, which was controlled by standard infusion pump. Ten blood samples (between 4.5 – 6.5 ml each) were obtained for prolactin beginning -5 minutes and -1 minute before medication administration, and +30, +45, +60, +75, +90, +105, +120, and +150 minutes after initiation of the infusion.

3. RESEARCH METHODS

3.1. MOLECULAR GENETIC STUDIES

Genomic DNA was isolated from EDTA anticoagulated whole blood by standard procedures. The 5-HTTLPR site was assayed by DNA amplification of the sequence by the polymerase chain reaction (PCR) method using 0.3 μM of sequence flanking primers 5'-GAG GGA CTG AGC TGG ACA ACC AC-3' and 5'-GGC GTT GCC GCT CTG AAT GC-3'. The PCR reaction was carried out in a total volume of 30μl, with 1.5 μl DNA, 200 μM each dATP, dCTP, dTTP, and 100 μM dGTP, 1.83 mM $MgCl_2$, and 1.2 μl Taq polymerase. The PCR product was analyzed following electrophoresis on an ethidium bromide stained 2% agarose gel.

3.2. BIOLOGICAL ASSAYS

3.2.1. Serum Prolactin

Serum prolactin was measured using an enzyme-linked immunoabsorbent assay kit obtained from Diagnostic Systems Labs (DSL, Webster, TX USA). Standards, controls, and unknown serum samples are incubated in 96-well plates that have been coated with anti-prolactin antibody. After incubation and washing, the wells are treated with another anti-prolactin detection antibody labeled with the enzyme horseradish peroxidase. After a second incubation and washing step,

the wells are incubated with the substrate tetramethylbenzidene. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured at 450 nm is directly proportional to the concentration of prolactin present. The assay is linear from 1.5 to 90 ng/ml, with a between-assay coefficient of variation from 2.5-8.2%.

3.2.2. Plasma Citalopram Levels

Citalopram is extracted from plasma using liquid-liquid extraction along with high-performance liquid chromatography and ultraviolet detection, at wavelength 210 nm (Pollock et al. 1997). One ml of plasma is alkayzed using carbonate buffer, and then extracted using ethyl acetate in heptane (20:80, v/v) and back-extracted into 0.025M potassium phosphate buffer. The HPLC column for separation is a C18 5μm Nucelosil column, 120 x 4.6 mm i.d. from Knauer, Germany. The flow rate is 1.0 ml/minute. The assay is linear from 5 to 500 ng/ml for all compounds. Citalopram elutes at a retention time of 6.78 minutes for Citalopram and 10.00 minutes for the internal standard, Paroxetine. Inter-assay variation of spiked plasma ranges from 1.76-7.32 % C.V. for Citalopram.

3.3. STATISTICAL METHODS

Data was analyzed using SPSS (Statistical Package for the Social Sciences) Software® (version 12.1, SPSS, Inc. Chicago, IL USA), with the exception of stated manual computations. SPSS is a comprehensive software package designed for statistical data analysis. For the present analyses, the following SPSS modules were utilized: 1) descriptive statistics, to determine means, standard deviations, standard errors, variance, minimum and maximum values, and confirm normal distribution for all variables of interest; 2) t-tests to compare means of independent samples when appropriate; 3) one-way analysis of variance, to test for significant differences in group means; 4) Pearson correlations; and 5) linear regression analysis. All tests were two two-tailed, with the probability value $p \leq 0.05$ constituting statistical significance.

3.3.1. Population Stratification Test

A Chi-square test statistic was used to analyze potential differences in genotype frequencies between African American and Caucasian subjects.

3.3.2. Hardy Weinberg Equilibrium Test

A Chi-square test statistic comparing the cohort's observed vs. expected genotype distribution under the Hardy-Weinberg Equilibrium Law was manually calculated.

3.3.3. Calculation of the Dependent Measure

The dependent measure of interest, change in plasma prolactin concentration (ng/ml) following Citalopram administration, was calculated for each subject using two methods: 1) change in prolactin area under the curve (PRL AUC); and 2) maximum change in prolactin from baseline (PRL MAX). Results are reported separately for each method.

PRL AUC provides a value that includes the total prolactin response over all time points (Pruessner et al., 2003) for each subject, minus their baseline prolactin concentration. It should be noted that two baseline prolactin measurements were taken, one at t = -1 and one at t = -5. The correlation between the t-1 and t-5 measurements was highly significant (r = 0.98), so the average of the two time points was used as the baseline value. PRL AUC was calculated using the trapezoid rule, which sums the area between each successive blood sample [(PRL baseline + PRL t_1)/2 *time)+((PRL t_2 + PRL t_3)/2 *time)…((PRL t_8 +PRL t_9) – (PRL baseline * (total time)). Thus, PRL_AUC = ((PRL baseline + prl30)/2 * 15) + ((PRL30 + PRL45) * 7.5) + ((PRL45 + PRL60) * 7.5) + ((PRL60 + PRL75) * 7.5) + ((PRL75 + PRL90) * 7.5) + ((PRL90 + PRL105) * 7.5) + ((PRL105 + PRL120) * 7.5) + ((PRL120 + PRL150) * 15) – (Baseline * 150).

PRL MAX is arrived at using each subject's maximum prolactin value over the 150-minute protocol. The baseline average is then subtracted out of the peak value, using linear regression analysis, which removed any correlation of PRL MAX with baseline prolactin values. The linear regression analysis estimate the coefficient value (in this case baseline prolactin) and subtracts that value from the dependent measure (PRL MAX), creating an new value that is 'residual', or the value that remains after the baseline average is removed. The residualized value becomes the dependent measure, PRL MAX, which has a zero correlation with baseline prolactin.

3.3.4. Preliminary Data Analysis – Assessing Potential Covariates

Factors that have been known to correlate with prolactin response to serotonergic stimulation are age (McBride et al., 1990), plasma drug levels (Judson et al., 2003; Lotrich et al., 2004), and body mass index (Muldoon et al., 1996, 2004). Pearson correlations or t-tests were performed on each of these variables with PRL AUC, in order to assess their appropriate inclusion into the model as covariates.

Additionally, baseline prolactin levels may exhibit seasonal variability (Brewerton, 1989). Season was compared with baseline PRL using two separate measurements. First, season was dichotomized by: 1= March 21st through September 20th, and 2= September 21st through March 20th, and seasonal means were compared for significant differences. Season was also compared using minutes of daylight. Because women have higher baseline PRL values, groups 1 and 2 were analyzed by gender composition, and found to have an equal distribution of men and women within each season.

Other factors that may affect neuroendocrine function are reproductive status in women (van Amelsvoort et al., 2001) and sleep deprivation (Seifritz, et al., 1997). Individuals with alcohol dependence have been shown to exhibit reduced midbrain transporter availability (Heinz et al., 1998, 2000) and blunted neuroendocrine responses to serotonergic stimulation (Anathenelli et al., 2000). Thus, t-tests were performed, comparing PRL AUC and PRL MAX with women's reproductive status (dichotomized as premenstrual and postmenstrual), sleep deprivation (defined as working past 12:00 a.m. within 2 days of the session), and alcohol dependence (determined according to criteria established in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition).*

3.3.5. Primary Data Analysis

Univariate ANOVA was performed with 5-HTTLPR genotype and gender as independent measures and PRL AUC as the dependent measure. The same analysis was undertaken using PRL MAX as the dependent measure. Results are reported for both methods of calculating the dependent measure.

4. RESULTS

4.1. POPULATION STRATIFICATION

There was a highly significant difference in allele frequency between the African American (n = 73 - 77% l allele/23% s allele) and Caucasians (n = 277 - 56% l allele/44% s allele) groups (Chi-square = 15.3, $p < 0.001$), precluding analysis of the two ethnic groups together (Gelertner et al., 1997). Consequently, in this sample, only 3 of the 73 African American subjects had the s/s genotype, which was not enough to test the hypothesis under separate analysis, and so this population was not further considered.

4.2. STUDY POPULATION

The original sample included 346 subjects. From those 346 initial participants, the following subjects were removed from the dataset due to the following: the 73 African American subjects were removed due to population stratification (see section 4.1), 26 subjects voluntarily withdrew; 11 subjects experienced emesis (an independent source of prolactin increase) and their prolactin data were unusable; 18 subjects had incomplete prolactin or Citalopram data; 1 subject had baseline prolactin of greater than 50 ng/ml (normal physiological range is between 4 and 35 ng/ml); 3 subjects had PRL AUC values that were greater than 4 standard deviations from the mean and were removed as outliers; 2 subjects had Citalopram drug levels that were greater than

4 standard deviations from the mean and were removed as outliers; 6 women were taking oral contraceptives (not considered exclusion medications at the onset of the study, but found to have significantly higher mean prolactin levels (refer to Section 4.4), so women taking oral contraceptives had their data removed from the dataset. The final study population sample is comprised of 206 unrelated healthy Caucasian volunteers, 127 men and 79 women. The age range of the study population is 30-55 years (45.1 ± 0.45), with no significant difference in mean age between men and women ($t_{.05, 204} = -0.21$, $p = 0.84$).

4.3. 5-HTTLPR GENOTYPE FREQUENCIES

The study sample, n=206, had the following genotype distribution: 74 l/l; 85 l/s; 47 s/s; resulting in a Chi-square = 5.4, $0.05 < p < 00.10$, which conforms to the Hardy Weinberg Equilibrium theory. Genotype frequency was not significantly different between gender groups (Chi-square = 2.27, $p > 0.32$).

4.4. INCLUSION/EXCLUSION OF COVARIATES

Although age has been found to correlate significantly with prolactin response in other challenge studies, there was no significant correlation of age with PRL AUC ($r = -0.01$, $p = 0.90$) or PRL MAX ($r = -0.020$, $p = 0.77$) and therefore age was excluded as a covariate. This cohort encompassed a relatively narrow age range, from 30-55, likely accounting for the lack of correlation in this sample (See Table 1).

No significant association of seasonal variation with Baseline PRL was found using either by dichotomizing season ($t_{.05,\ 204}$ = 1.17, p = 0.24) or when taken as total minutes of daylight (r = -0.12, p = 0.94). There was no difference in participant's gender distribution within seasons ($X^2_{.05,1}$ = 0.61, p = 0.43).

BMI was found to significantly correlate with PRL AUC and PRL MAX responses (r = -0.19, p = 0.006; r = -0.21, p = 0.002) and, following square root transformation to normalize the distribution of BMI, was included as a covariate (see Table 1).

Individual differences in plasma Citalopram levels may partially account for variation in prolactin response between subjects. In order to minimize drug concentration differences between subjects, variation in body size was accounted for by calculating the Citalopram dose according to fat free mass (0.33 mg/kg), but there remained significant variation in post-infusion plasma Citalopram levels between subjects, particularly at +30 minutes ($\underline{M}\pm\underline{SD}$ 54.2 ± 19.0 ng/ml). Therefore, both plasma Citalopram at +30 minutes (CTL 30) and plasma Citalopram area under the curve (CTL AUC) values were tested for significant correlation with PRL AUC and PRL MAX. Although CTL AUC and CTL30 were both significantly associated with PRL AUC and PRL MAX, CTL AUC showed a slightly higher correlation and was the plasma citalopram measurement included as a covariate in the analysis of variance (see Table 1). CTL AUC was not significantly different according to genotype group (see Table 2).

PRL AUC was not found to be significantly correlated with menopausal status, alcohol dependence, or sleep deprivation (see Table 1). However, the use of oral contraceptives did significantly correlate with mean PRL AUC ($t_{.05,84}$ = -2.7, p = 0.01), and data from the 6 subjects who were currently taking oral contraceptives were excluded from the analysis (see Table 1).

Plasma Citalopram level (CTL AUC) and body mass index (BMI) were identified as covariates and were included in the model. Additionally, because baseline PRL was still significantly associated with PRL AUC ($r = -0.245$, $p = 0.001$), baseline PRL was included as a covariate in the PRL AUC analysis. There was no significant difference in age, gender, or BMI between genotype groups (see Table 2).

TABLE 1: Relations of Potential Covariates with PRL Measures

	PRL AUC	PRL MAX
Age	$r = -0.017$ $p = 0.81$	$r = -0.02$ $p = 0.77$
BMI	$r = -0.19$ $p = 0.006$*	$r = -0.21$ $p = 0.002$*
Pre/post menopause (n = 53/26)	$t_{.05,77} = -1.8$ $p = 0.07$	$t_{.05,77} = -1.3$ $p = 0.20$
Oral Contraceptive Use (n = 6)	$t_{.05,84} = -2.7$ $p = 0.01$*	$t_{.05,84} = 1.5$ $p = 0.15$
Alcohol Dependence (n = 12)	$t_{.05,204} = -0.55$ $p = 0.59$	$t_{.05,204} = -0.42$ $p = 0.68$
Sleep Deprivation (n = 11)	$t_{.05,204} = 0.58$ $p = 0.56$	$t_{.05,204} = 0.57$ $p = 0.57$
Citalopram AUC	$r = -0.14$ $p = 0.04$*	$r = -0.17$ $p = 0.02$*
Citalopram +30 min	$r = -0.12$ $p = 0.08$	$r = -0.16$ $p = 0.025$*

TABLE 2: Sample Characteristics by Genotype

	L/L	L/S	S/S	Sig.
Age	44.9 ± 0.75	45.2 ± 0.69	45.4 ± 0.94	0.92
BMI	27.1 ± 0.59	27.2 ± 0.55	27.3 ± 0.74	0.97
Baseline PRL (ng/ml)	9.4 ± 0.54	110.03 ± 0.51	9.7 ± 0.68	0.08
CTL AUC (ng/ml *150)	2633.5 ± 65.5	2595.0 ± 61.1	2581.3 ± 82.2	0.86

4.5. PRIMARY RESULTS

The 5-HTTLPR is significantly associated with area under the curve prolactin response to acute administration of Citalopram (F=3.1, p=0.048) (See Table 3). Compared to the l/l carriers (246.0 ± 40.2 ng/ml * 150 min), subjects with the s/s (84.2 ± 51.8 ng/ml * 150 min) genotype exhibited lesser increases in plasma prolactin. Pair wise comparison by genotype revealed a significantly lower prolactin response in the s/s group relative to the l/l group (p = 0.014). No significant difference was noted when comparing either the s/s group to the l/s group (p = 0.17), or the l/s group to the l/l group (p = 0.23) (See Table 4).

When the data are analyzed as maximum prolactin rather than area under the curve then results are essentially the same. The 5-HTTLPR is significantly associated with maximum prolactin response to acute administration of Citalopram (F= 3.5, p= 0.034) (See Table 3). Compared to the l/l carriers (3.69 ± 0.054 ng/ml), subjects with the s/s (3.46 ± 0.07 ng/ml) genotype exhibited lesser increases in plasma prolactin. Pair wise comparison by genotype revealed a significantly lower prolactin response in the s/s group relative to the l/l group (p = 0.01), with the s/s and l/s groups approaching but not reaching statistical significance (p = 0.056). No significant difference was noted when comparing the l/s group to the l/l group (p = 0.43) (See Table 4).

TABLE 3: Univariate Analysis of Variance

	PRL AUC				PRL MAX		
Source	Df	F	P value		Df	F	P value
CTL AUC	1, 197	4.7	0.031		1, 198	4.8	0.03
BMI	1, 197	9.7	0.002		1, 198	9.8	0.002
BASELINE PRL	1, 197	15.5	0.001				
GENDER	1, 197	0.12	0.73		1, 198	0.12	0.73
5-HTTLPR	2, 197	3.1	0.048		2, 198	3.5	0.034
GENDER*GENO	2, 197	1.06	0.35		2, 198	0.05	0.95

TABLE 4: Pairwise Comparisons by Genotype

	PRL AUC		PRL MAX	
	Mean Difference	Sig.	Mean Difference	Sig.
l/l x s/s	147.8	0.02 (*)	0.23	0.01 (*)
l/l x l/s	74.6	0.17	0.06	0.43
l/s x s/s	-73.3	0.23	-0.17	0.056

5. DISCUSSION

The major finding in this study is that Citalopram-induced prolactin response is associated with the 5-HTTLPR genotype (See Figures 1 & 2). Individuals with the s/s genotype have significantly lower prolactin responses than individuals with the l/l genotype. The s allele does not have a dominant effect on the prolactin response to Citalopram, as there is no significant difference in prolactin response between individuals with the l/s genotype and l/l genotype or the l/s and s/s genotypes.

The results of this study replicate previous associations of variation in prolactin response to SSRIs with the 5-HTTLPR. Importantly, this study was carried out using Citalopram, a highly selective serotonin reuptake inhibitor that does not act directly at 5-HT receptors, which is essential in order to associate individual differences in plasma prolactin response with proposed genetically-driven 5-HTT functional variation. This is the first study to characterize the association of the 5-HTTLPR on prolactin response to Citalopram in a relatively large (n=206) non-patient sample, including women and men.

5.1. PROLACTIN RESPONSE

The mean prolactin response reported in this study replicates the results of previous neuroendocrine studies utilizing Citalopram. Seifrietz et al., (1996) reported prolactin maximum change (M±SD: 17.80 ± 4.65 ng/ml) to 20 mg. IV Citalopram. The results reported herein (M±SD: 13.34 ± 5.83 ng/ml), are quite comparable, although by using a weight-adjusted dose (M±SD: 18.98 ± 3.78 mg) our prolactin response was slightly smaller (Lotrich et al. 2004). Attenburrow et al. (2001) used 10 mg IV Citalopram and placebo to compare within-subject differences in change in prolactin area under the curve. They found a significant difference ($p = 0.01$) in prolactin responses between the placebo (-2.2 ± 21.3 ng/ml *150 min) and 10mg. Citalopram (67.1 ± 26.7 ng/ml *150 min) treatments. The PRL AUC response in the current study (180.8 ± 25.7 ng/ml *150 min) was more pronounced than in the Attenborrow study (67.1 ± 26.7 ng/ml *150 min), presumably due to the larger Citalopram dose used in the present study (0.33 mg/kg vs. 10 mg).

For practical reasons, it is not standard to use a placebo control in studies of this size. However, in a smaller (n=75) study, Lotrich et al. (2004) compared prolactin response to placebo with a range of Citalopram doses (10mg, 0.33 mg/kg, 20 mg, and 40 mg). Lotrich's results indicated a linear dose-dependent increases in prolactin and, at the 0.33 mg/kg dose the increase was ~3.5 ng/ml (max change), which compares to the findings in this study (3.6 ± 0.26 ng/ml). Lotrich found no within-subject correlation of prolactin response between citalopram and placebo, indicating the variability in response is not related to individual physiological responses to the procedure or to temporal changes in prolactin.

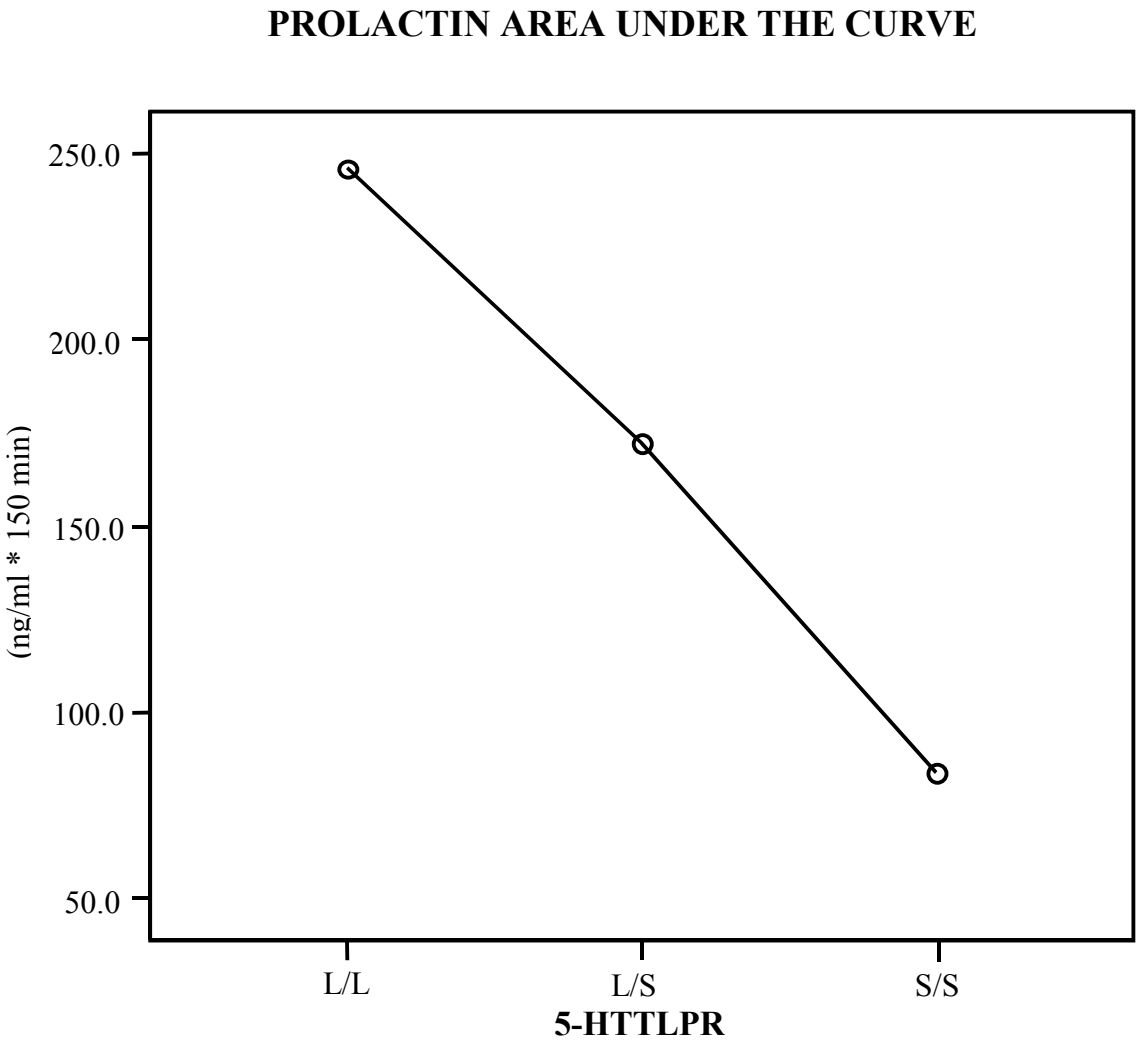

FIGURE 1: Prolactin area under the curve group means during the Citalopram Challenge Test by 5-HTTLPR genotype.

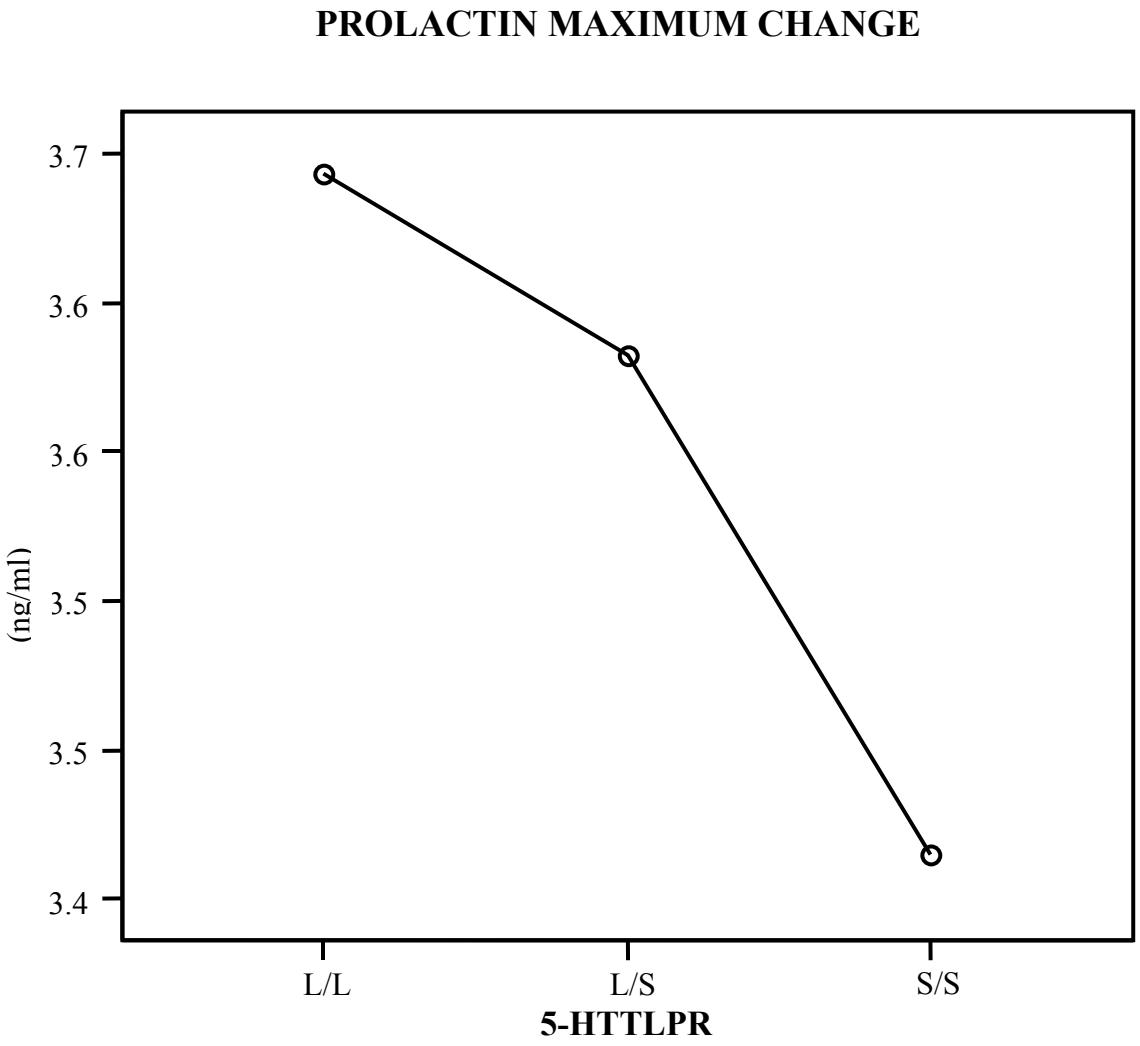

FIGURE 2: Prolactin maximum (max) change group means during the Citalopram Challenge Test by 5-HTTLPR genotype.

5.2. PROPOSED MOLECULAR MECHANISMS: TRANSPORTER VARIATION

The results of this study confirm the findings of Whale et al. (2000) and Reist et al. (2001) that prolactin response to antidepressants varies by 5-HTTLPR genotype. In contrast to the challenge agents used in the Whale & Reist studies, citalopram results in pharmacological manipulation to the transporter reuptake mechanism only, providing evidence that differences in prolactin response are related to transporter function. A growing body of evidence indicates that the s/s genotype is associated with reduced transporter availability, suggesting two possible molecular explanations for reduced prolactin response in the s/s subjects following acute 5-HT reuptake inhibition with citalopram: 1) less hypothalamic serotonergic neurotransmission under steady state conditions in individuals with the s/s genotype, and 2) relative hyper-sensitivity of the raphe nuclei somatodendritic autoreceptors in individuals with the s/s genotype.

Evidence exists for relatively less serotonergic activity in individuals with the s/s genotype. Williams et al. (2001) reported 50% more 5-HIAA in cerebrospinal fluid of subjects with either one or two copies of the l alleles, relative to subjects with the s/s genotype. Similarly, Hanna et al., (1998) found lower whole blood serotonin in subjects with the s/s genotype compared to the l/s or l/l individuals. While lower CSF 5-HIAA and blood serotonin levels are not conclusive evidence of reduced hypothalamic serotonergic activity in s/s individuals, they may reveal a tendency towards less CNS 5-HT neurotransmission, which may be reflected in prolactin response to neuroendocrine challenge and treatment response to SSRIs.

Alternately, inter-individual variability in the change in plasma prolactin following acute 5-HTT blockade is not a direct reflection of hypothalamic post-synaptic 5-HT levels, but may reflect variability in the dynamics of pre-synaptic molecular mechanisms that has developed under chronically inefficient transporter transcription. The somatodendritic autoreceptor (5-

HT1a) feedback mechanism may be one regulatory mechanism that would undergo a developmental change. For instance, if reduced transcriptional efficiency results in less 5-HTT (Little et al., 1998; Heinz et al., 2000) to remove 5-HT from raphe nuclei extracellular fluid, 5-HT1a receptors may be intrinsically up regulated. Thus, under conditions where postsynaptic (hypothalamic) 5-HT are equivalent between s/s and l/l individuals prior to the challenge, relatively greater 5-HT1a activation may inhibit firing rates in subjects with the s/s genotype immediately following transporter blockade. Relatively greater inhibition of firing rates via 5-HT1a activation would then lead to reduced hypothalamic stimulation and relatively less prolactin release in s/s individuals. A similar, but far more complex scenario could also be constructed involving differences in postsynaptic mechanisms that may develop according to 5-HTTLPR genotype.

It is likely that functional imaging techniques will be employed in future genetic studies to further quantify the associations between 5-HTTLPR genotype, transporter function, and neuroendocrine response to SSRIs. Citalopram is indicated in imaging studies, as these data show clearly that it's selective action on the serotonin transporter elicits a centrally-mediated response that varies depending on the 5-HTTLPR s and l alleles.

5.3. OTHER DETERMINANTS OF PROLACTIN RESPONSE

Data was collected on factors that have been associated with between-subjects differences in prolactin response: season, women's productive status, age, gender, sleep deprivation, and alcohol dependence. Although no correlations were found between any of these variables and variation in prolactin response in this population, it was important to rule these variables out as confounders. Likewise, study subjects were screened to rule out recent anti-depressant exposure and current major depressive disorder diagnosis, as either of these conditions could affect serotonergic function and prolactin results.

There are other possible genetic determinants of prolactin response to reuptake inhibitors that should, in future studies, be evaluated separately and in interaction with the 5-HTTLPR. A case in point is the CYP219 gene – a genetic polymorphism in this enzyme is known to effect citalopram metabolism. Dopamine receptor polymorphisms are also candidate genes for this area of research as rat studies have demonstrated dopamine's role in tonic inhibition of prolactin release. Estrogen and cortisol receptor polymorphisms are also of interest, as they may exert significant influences on both gene transcription and hypothalamic regulation of hormone secretion.

5.4. CONCLUDING STATEMENT

The purpose of this investigation was to help clarify effects of genetic variation occurring in the promoter region of the serotonin transporter gene that may, by exerting effects on transporter transcription efficiency, reflect individual differences in serotonergic neurotransmission after treatment with SSRIs. Gene transcription is a highly complex molecular process - transcription factors and variation within enhancer, silencer, and promoter sequences downstream of the 5-HTT gene may also exert influences on 5-HTT transcription.

Acknowledging these additional sources of variability in 5-HTT transcription and other genes involved with 5-HT regulation, hypothalamic stimuli magnitude, and Citalopram metabolism does not diminish the validity of the association of the 5-HTTLPR with prolactin response reported in this community sample. The results of this study provide additional evidence of a significant, genetically driven source of inter-individual variation in 5-HT regulation, further supporting the study of pharmacological-genetics in the treatment of psychiatric disease.

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