

Can serotonin transporter genotype predict serotonergic function, chronicity, and severity of drinking?

Bankole A. Johnson^{a,*}, Martin A. Javors^b, John D. Roache^b, Chamindi Seneviratne^a,
Susan E. Bergeson^c, Nassima Ait-Daoud^a, Michael A. Dawes^d, Jennie Z. Ma^e

^a Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, Charlottesville, VA, USA

^b Department of Psychiatry, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

^c Waggoner Center for Alcohol and Addiction Research, School of Biological Sciences, University of Texas at Austin, Austin, TX, USA

^d Department of Psychiatry and Behavioral Medicine, Wake Forest University School of Medicine, Winston-Salem, NC, USA

^e Department of Public Health Sciences, University of Virginia, Charlottesville, VA, USA

Received 22 May 2007; received in revised form 24 July 2007; accepted 25 July 2007

Available online 19 August 2007

Abstract

Serotonin transporter (5-HTT) activity is greater in carriers of the long (L) vs. short (S) alleles of the 5-HTT-linked polymorphic region (5'-HTTLPR) among healthy control subjects but not alcohol-dependent adults. In 198 alcoholics, we determined the relationship between current or lifetime drinking and platelet 5-HTT function and density among allelic variants of the 5'-HTTLPR. SS subjects were younger than L-carriers (LL and LS) ($p < 0.0085$) and had fewer years of lifetime drinking. For L-carriers, the mean of B_{\max} for paroxetine binding, but not V_{\max} for serotonin (5-HT) uptake, was lower than that for SS subjects ($p < 0.05$). More L-carriers than their SS counterparts had V_{\max} for 5-HT uptake below 200 nmol/10⁷ platelets-min ($p < 0.05$) and B_{\max} for paroxetine binding below 600 nmol/mg protein ($p < 0.06$). Current drinking (drinks per day during the past 14 days) correlated positively with K_m and V_{\max} of platelet 5-HT uptake ($p < 0.05$) and negatively with B_{\max} , but not K_d , of paroxetine binding ($p < 0.05$) for L-carriers alone. Years of lifetime drinking correlated negatively with K_m and V_{\max} of platelet 5-HT uptake ($p < 0.05$) and B_{\max} , but not K_d , of paroxetine binding ($p < 0.05$) for L-carriers alone. Among L-carriers alone, there were higher levels of platelet 5-HT uptake and lower levels of platelet paroxetine binding with increased drinking, and more lifetime drinking was associated with modestly lower levels of 5-HT uptake and paroxetine binding. Thus, 5-HTT expression varies with current and lifetime drinking in L-carriers alone. © 2007 Elsevier Inc. All rights reserved.

Keywords: Alcohol dependence; Genotype; Humans; Paroxetine; Platelet; Serotonin transporter

1. Introduction

Serotonergic function is an important mediator of mood, impulsivity, and appetitive behaviors, including alcohol consumption. Serotonergic pathways originating from raphe nuclei and projecting to the cortex, hippocampus, and subcortical structures can influence drinking behavior in alcohol-dependent individuals (Baumgarten and Grozdanovic, 1997) either directly by modulating alcohol's reinforcing effects or indirectly by mediating impulse control and affective state (Stoltenberg, 2003). Animal studies have documented an inhibitory role for serotonin (5-HT) in regulating alcohol intake (McBride and Li, 1998), and 5-HT reuptake inhibitors reduce ethanol consumption in rats (Gill and Amit, 1989). However, the effectiveness of

Abbreviations: 5-HT, serotonin; 5-HTT, serotonin transporter; 5'-HTTLPR, serotonin transporter-linked polymorphic region; β -CIT, [¹²⁵I]2 beta-carboxy-methoxy-3 beta-(4-iodophenyl)tropane; B_{\max} , maximum specific paroxetine binding density; DNA, deoxyribonucleic acid; DSM-IV, *Diagnostic and Statistical Manual of Mental Disorders, 4th edition*; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; K_d , affinity constant; K_m , equilibrium constant; L, long allelic variant; PCR, polymerase chain reaction; PRP, platelet-rich plasma; S, short allelic variant; SSRI, selective serotonin reuptake inhibitor; V_{\max} , maximum serotonin uptake velocity.

* Corresponding author. Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, P.O. Box 800623, Charlottesville, VA 22908-0623, USA. Tel.: +1 434 924 5457; fax: +1 434 244 7565.

E-mail address: bankolejohnson@virginia.edu (B.A. Johnson).

5-HT reuptake inhibitors at reducing drinking in humans is not clear. Naranjo and Knoke (2001) showed that selective 5-HT reuptake inhibitors (SSRIs) reduce drinking in some, but not all, subtypes of alcoholics and problem drinkers. In fact, only one study of alcohol-dependent individuals showed beneficial effects of an SSRI, and that effect was restricted to non-depressed alcoholics (Pettinati et al., 2001) or a subgroup classified as Type A alcoholics (Pettinati et al., 2000). In Type B alcoholics (Dundon et al., 2004; Kranzler et al., 1996) or Type II alcoholics (Chick et al., 2004), SSRIs may increase drinking relative to placebo treatment. In humans, low 5-HT turnover has been associated with increased alcohol-seeking behavior, alcoholism (Virkkunen and Linnoila, 1997), and impulsive violence (Virkkunen et al., 1995). Thus, alterations in 5-HT availability and function can affect drinking behavior. For a further review, see Johnson and Ait-Daoud (2000).

Of the mechanisms that control synaptic 5-HT concentration, perhaps the most compelling relates to the functional state of the pre-synaptic 5-HT transporter (5-HTT). The 5-HTT is responsible for removing 5-HT from the synaptic cleft (Lesch et al., 2002). Indeed, up to 60% of neuronal 5-HT function is gated by the 5-HTT. Synaptic clearance of 5-HT is determined by the number of 5-HTTs expressed at the pre-synaptic surface and the affinity of 5-HTTs to 5-HT (K_d) (Beckman and Quick, 1998). Platelets express 5-HTTs where they play an important role in removing 5-HT released peripherally in the bloodstream. Knowing the relationships among 5-HTT number, affinity, and transport rates is, therefore, critical to understanding 5-HT function in a given state.

The 5-HTT, wherever it is expressed, has the same amino acid sequence and pharmacological sensitivity in all tissues including neuronal cells (Da Prada et al., 1988; Lesch et al., 1993; Villinger et al., 1994). The gene responsible for encoding the 5-HTT is common to all tissues including platelets and neurons (Esterling et al., 1998; Ramamoorthy et al., 1993). It is, therefore, reasonable to propose that alterations in platelet 5-HTT state associated with genetic variation can be a proxy for similar changes in neuronal cells.

The 5-HTT gene is found at the SLC6A4 locus on chromosome 17q11.1-q12, and its 5'-regulatory promoter region contains a functional polymorphism known as the 5-HTT-linked polymorphic region (5'-HTTLPR) (Heils et al., 1996, 1997). The polymorphism is an insertion/deletion mutation in which the long (L) variant has 44 base pairs that are absent in the short (S) variant. The L-allelic variant of the 5'-HTTLPR is associated with increased transcription rates in lymphoblasts and in cell culture. In the general population, the LL genotype, compared with the SS and heterozygous (LS) genotypes, is associated with greater 5-HT uptake into human platelets (Greenberg et al., 1999) and lymphoblasts (Lesch et al., 1996) and greater [123 I]2 beta-carboxymethoxy-3 beta-(4-iodophenyl)tropane (β -CIT) binding in human raphe nuclei (Heinz et al., 2000). Hence, individuals with the LL genotype have greater uptake and, presumably, reduced intrasynaptic 5-HT levels and 5-HT neurotransmission (Heils et al., 1996; Lesch et al., 1996), rendering them more vulnerable to early-onset problem drinking, impulsivity, and antisocial behavior (Johnson, 2000; LeMarquand et al., 1994b).

However, the LL genotype has been reported to confer a greater propensity toward alcohol-induced neurotoxic damage to the 5-HTT than the SS genotype (Heinz et al., 2000, 2004; Little et al., 1998). Consequently, among alcohol-dependent individuals, those with the LL genotype might have reduced 5-HTT density and uptake. This proposal is supported by two independent lines of evidence. First, while healthy subjects with the LL genotype showed greater β -CIT neuronal binding in the raphe nucleus than did subjects with SS alleles, alcoholics with the LL genotype had reduced β -CIT neuronal binding compared with controls (Heinz et al., 2000). Second, our laboratory recently found no mean difference among the LL, LS, and SS genotypes in the platelet 5-HT uptake measures of alcohol-dependent outpatients, but compared with SS homozygotes, a higher percentage of LL alcoholics had unusually low platelet 5-HT uptake (<200 nmol/ 10^{-7} platelets-min) (Javors et al., 2005). Though these reduced measures of functional 5-HT uptake in platelets are consistent with Heinz et al.'s (2000) finding that the 5-HTT may be decreased in LL alcoholics, it is important to determine the basis for this finding. Indeed, the similarity in platelets and neuronal cells, of this effect of chronic alcohol consumption to reduce 5-HTT binding and/or function among alcoholics with the LL genotype, underscores the need to study binding and function in the two tissues.

Thus, the current study extended the findings of our previous report by examining both platelet 5-HT uptake and paroxetine binding in a larger sample of alcohol-dependent outpatients. Furthermore, we sought evidence to support the hypothesis proposed by Heinz et al. (2000), that reduced binding or functional activity of the 5-HTT in L-carriers may be related to alcohol drinking. Building upon our proposed conceptual framework, we tested, in a cohort of alcohol-dependent individuals, whether the history of drinking (i.e., lifetime drinking), the current amount of drinking, or both determine how 5-HTT genotype alters 5-HTT function.

2. Subjects and methods

2.1. Subjects

Subjects were 198 men and women, 18 to 66 years of age, who met *Diagnostic and Statistical Manual of Mental Disorders, 4th edition* (DSM-IV) criteria (American Psychiatric Association, 1994) for alcohol dependence. All participants currently were drinking ≥ 21 standard drinks/week for women and ≥ 30 standard drinks/week for men during the 90 days before enrollment and were seeking treatment to help them stop drinking through their enrollment in a pharmacotherapy trial. Racial mix was 151 White, 38 Hispanic, and 9 other. Other inclusion criteria were: 1) score of ≥ 8 on the Alcohol Use Disorders Identification Test (AUDIT) (Bohn et al., 1995), which assesses personal and social harm after alcohol consumption, and 2) negative urine toxicological screen for narcotics, amphetamines, or sedative hypnotics at enrollment. Exclusion criteria included: 1) current axis I DSM-IV psychiatric diagnoses other than alcohol or nicotine dependence; 2) significant alcohol withdrawal symptoms; 3) clinically significant physical abnormalities based on clinical examination, electrocardiogram

recording, hematological assessment, or biochemistry including serum bilirubin concentration; 4) pregnant or lactating, and 5) treatment for alcohol dependence in the 30 days before enrollment. All subjects gave written informed consent at study intake and then were screened for study eligibility. The 198 enrollees provided a blood sample for the platelet studies before randomized treatment with medication.

2.2. Current alcohol consumption

Current alcohol consumption was determined using the timeline follow-back (TLFB) procedure (Sobell and Sobell, 1992) to quantify the number of standard drinks consumed each day for the 90 days before enrollment. One standard drink was defined as 0.35 L of beer, 0.15 L of wine, or 0.04 L of 80-proof liquor. After enrollment, TLFB monitoring of drinking patterns was continued through the time of blood sample collection for the current study analysis. As platelets have an estimated life span of 8 to 14 days (Javors et al., 2000; Penington, 1981), “current drinking” was defined as drinking occurring over the 14 calendar days immediately prior to the blood draw for the measurement of platelet parameters, while “baseline drinking” was defined as drinking in the 90 days before enrollment. “Years of lifetime drinking” was calculated by subtracting the age at which subjects began experiencing symptoms of dependence from their current age.

2.3. Collection of blood samples

All blood samples for the determination of 5-HTT genotype and platelet 5-HTT function were drawn either at enrollment or within 14 days of enrollment but always before any randomized study medication treatment was begun. Each subject had 50 ml of blood drawn to obtain platelets for the measurement of 5-HT uptake into intact platelets and paroxetine binding to platelet membranes. Additionally, a 10-ml blood sample was drawn for the determination of 5-HTT genotype. Blood samples for the determination of 5-HTT genotype and platelet 5-HTT function were always drawn between 8:00 and 9:00 a.m. from fasting patients after study enrollment but before randomization to pharmacotherapy treatment. For the 198 subjects, this occurred over a 3-year study period (2002–2004), which would average circadian and seasonal variability (Modai et al., 1986, 1992).

2.4. Platelet suspension and platelet membrane preparation

Collected blood was deposited into 60-ml polypropylene syringes containing 10 ml of acid–citrate–dextrose buffer. Then the blood was centrifuged at 150 g at 23 °C for 20 min in a Beckman TJ-6 centrifuge to obtain platelet-rich plasma (PRP). To prepare adjusted PRP for the 5-HT uptake experiments only, platelet count in PRP was determined with a Coulter counter model S-plus VI and adjusted to 3×10^8 platelets/ml with the addition of platelet buffer (137 mM KCl, 1 mM MgCl₂, 5.5 mM glucose, 5 mM HEPES, pH 7.4). Adjusted PRP (3 ml) was used for platelet 5-HT uptake experiments performed on the day of the blood draw. To prepare platelet membranes for paroxetine

binding experiments, the remainder of the PRP was used. One microliter of prostaglandin I₂ solution (300 ng/ml) per ml of PRP was added to prevent platelet loss during centrifugation; then the sample was centrifuged at 550 g. The resulting platelet pellet was re-suspended in platelet buffer and then centrifuged at 35,000 g. The platelet membrane pellet was re-suspended in 1 ml of platelet buffer and then stored at –80 °C until the day of the assay to measure paroxetine binding.

2.5. Genotyping

Blood collected at enrollment was used to determine 5-HTT genotype. To accomplish this, white blood cells were separated from plasma and re-suspended, and DNA was isolated using PUREGENE® (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s protocol. The 5′-HTTLPR 44-base-pair promoter-region repeat polymorphism was amplified by polymerase chain reaction (PCR) from ~ 50 ng of DNA using two primers: 5′-CGT TGC CGC TCT GAA TGC CAG-3′ and 5′-GGA TTC TGG TGC CAC CTA GAC GCC-3′ and in a 25-μl final volume consisting of 0.5 U of Tfi DNA polymerase (EPICENTRE® Biotechnologies, Madison, WI, USA), 1X PCR buffer, 1.5 ml MgCl₂, 200 μM deoxynucleotide triphosphates, 1X enhancer, and 0.6 μM of each primer. The PCR conditions were as follows: 94 °C for 30 s; 70 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 7 min and terminal hold at 4 °C. Separation by gel electrophoresis using 4% MetaPhor® agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) allowed visualization by ethidium bromide/ultraviolet detection of the L and S variants (fragment sizes=464 and 420 bp, respectively) of the promoter region of the SCL4A gene (–1415 to –951) (Heils et al., 1996).

2.6. Serotonin uptake into intact platelets

Platelet 5-HT uptake experiments were performed successfully in 162 of the 198 subjects. The adjusted PRP suspension was used to determine platelet 5-HT uptake. Assay tubes were prepared in duplicate and contained [³H]5-HT at six different concentrations (62.5–2000 nM) and 100 μM pargyline with or without 50 μM fluoxetine. These tubes were incubated at 37 °C for 5 min; then the reaction was started by the addition of 100 μl of adjusted PRP that contained 10⁷ platelets. The assay tubes were incubated at 37 °C for an additional 5 min; then the reaction was quenched by rapid filtering through Whatman GF/B filters using a Brandel cell harvester. The filters were washed three times with 5 ml of ice-cold wash buffer (50 mM Tris–HCl, 150 mM NaCl, and 20 mM ethylenediaminetetraacetic acid (EDTA)). Filters were placed in scintillation vials containing 5 ml of Beckman Ready Protein+ scintillation counting fluid (Beckman Coulter, Inc., Fullerton, CA, USA) and immediately counted. Specific uptake was calculated by subtracting the uptake occurring in tubes containing fluoxetine from that occurring in tubes without fluoxetine. Maximum 5-HT uptake rate (V_{max}) in platelets was expressed as fmol 5-HT/min–10⁷ platelets, and the equilibrium constant (K_m) was expressed as nM. K_m and V_{max} were calculated using

the one-site hyperbolic function in Prism 4[®] software (GraphPad Software, Inc., San Diego, CA, USA).

2.7. Paroxetine binding to platelet membranes

Platelet membranes were successfully used to determine platelet paroxetine binding in 182 of the 198 subjects. Assay tubes were prepared in duplicate containing incubation buffer (50 mM Tris–HCl, 5 mM KCl, and 120 mM NaCl) and [³H]paroxetine at six different concentrations (0–2 nM) with and without 150 μM fluoxetine. The actual concentration of paroxetine in each tube was determined using a 40-μl aliquot taken from each tube before the addition of platelet membranes. The experiment was started by the addition of 80 μg of platelet membrane protein. Then the assay tubes were incubated for 1 h at 23 °C. The reaction was quenched by the addition of ice-cold wash buffer (50 mM Tris–HCl, 150 mM NaCl, 20 mM EDTA) and rapid filtering through Whatman GF/B filters treated with 0.3% polyethylenamine using a Brandel cell harvester. Filters were washed three times with ice-cold wash buffer, dried overnight, placed in scintillation vials containing 5 ml of Beckman Ready Protein+ scintillation counting fluid, and counted in a Beckman LS-6500 liquid scintillation counter. Disintegrations per minute from the 40-μl aliquots were converted into nM of paroxetine to obtain the actual concentrations in each tube. Total and non-specific binding of paroxetine was plotted against each actual concentration. Specific binding was calculated by subtracting non-specific binding from total binding. K_d and V_{max} of paroxetine binding were calculated using Prism 4[®] software. Paroxetine binding was expressed as fmol/mg of platelet membrane protein, and K_d was expressed as nM. Protein concentrations were measured using the Bio-Rad method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a SpectraMax[®] Plus³⁸⁴ microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.8. Ethical approval

The current study was approved by the local institutional review board and was conducted in full compliance with all regulatory requirements at The University of Texas Health Science Center at San Antonio. Participants were recruited between March 2001 and September 2004 by newspaper or radio advertisements.

2.9. Statistical analysis

Prism 4[®] software was used to calculate maximum specific paroxetine binding density (B_{max}) and its affinity constant K_d , as well as maximum 5-HT uptake velocity (V_{max}) and its affinity constant K_m . All of these platelet 5-HT parameters were transformed into their natural log values, which enhanced their normality for statistical analysis. The numbers of standard drinks per day (average consumption across all days), drinks per drinking day (average consumption on all non-abstinent days), percentage of heavy drinking days (average percentage of days on which men and women consumed ≥ 5 and ≥ 4 standard drinks, respectively), and percentage of days abstinent from

drinking (average percentage of abstinent days) were calculated from the TLFB calendars.

All statistical data analyses were done using SAS[®] 9.2 (SAS Institute Inc., Cary, NC, USA). The descriptive statistics for demographic characteristics, 5-HT genotype, alcohol drinking behavior measures, and platelet 5-HT uptake and paroxetine binding parameters were summarized in frequencies or percentages for categorical variables and expressed as mean \pm S.E.M. for continuous variables. During interim examination of the data, we considered using three levels of genotype (i.e., LL vs. LS vs. SS), but findings generally indicated that the SS group differed from one or the other L-carrier group (i.e., LL or LS), which generally looked similar to each other in their mean tendencies and differences from the SS homozygotes. Thus, final analyses were only carried out on the SS vs. L-carrier groups. Chi-square test and two-sample *t*-test were used to compare the differences between the SS and L-carrier groups for categorical and continuous variables, respectively. The effects of genotype and drinking on the log-transformed platelet 5-HT parameters were examined further by general linear models. By including the interactions of genotype and alcohol drinking measures in the model, we were able to examine the differential effect of alcohol drinking on the platelet 5-HT parameters for SS and L-carriers. We also examined sex and current age as possible predictors of 5-HT biology and found that these variables were never significant predictors of the platelet 5-HT measures.

3. Results

Of the 198 alcohol-dependent individuals included in this analysis, 58 (29.3%) were LL, 102 (51.5%) were LS, and 38

Table 1
Demographics, alcohol drinking, and platelet parameters for L-carrier and SS alcoholic participants

	L-carrier	SS	<i>p</i> value*
Number of subjects	160	38	–
Gender (% male)	72%	79%	0.34
Age (yr)	42.0 \pm 0.9	36.9 \pm 1.4	0.009
Age of onset (yr)	29.9 \pm 0.9	27.5 \pm 1.6	0.23
Baseline drinks per day	7.43 \pm 0.39	8.30 \pm 0.70	NS
Current drinks per day	4.89 \pm 0.31	5.49 \pm 0.65	NS
Baseline drinks per drinking day	9.20 \pm 0.40	11.0 \pm 0.85	0.05
Current drinks per drinking day	7.31 \pm 0.39	8.56 \pm 0.74	0.16
Baseline percentage of days abstinent	18.8 \pm 1.64	22.4 \pm 3.4	NS
Current percentage of days abstinent	37.3 \pm 2.66	38.1 \pm 4.8	NS
Baseline percentage of heavy drinking days	69.9 \pm 2.2	68.7 \pm 4.4	NS
Current percentage of heavy drinking days	47.1 \pm 2.6	47.6 \pm 5.1	NS
Years of lifetime drinking	12.1 \pm 0.73	9.34 \pm 1.2	0.09
Platelet serotonin uptake			
K_m (μM)	624 \pm 39	597 \pm 98	NS
V_{max} (fmol/min \times 10 ⁷ platelets)	257 \pm 20	315 \pm 40	NS
Platelet paroxetine binding			
K_d (nM)	0.256 \pm 0.07	0.229 \pm 0.037	NS
B_{max} (fmol/mg protein)	661 \pm 38	854 \pm 72	0.01

Values are means \pm S.E.M. NS = not significant.

**p* values for comparison between L-carrier and SS alcoholics.

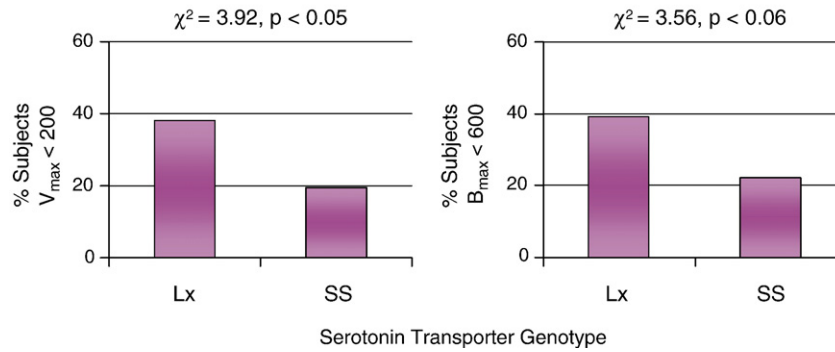


Fig. 1. Proportion of subjects within the L-carrier (Lx) and SS genotype groups with V_{max} for serotonin uptake less than $200 \text{ nmol}/\text{min} \cdot 10^7$ platelets and B_{max} less than $600 \text{ nmol}/\text{mg}$ protein. V_{max} for platelet serotonin uptake, B_{max} for platelet paroxetine binding, and genotype (LL, LS, SS) were determined as described in the Subjects and methods section. Data of participants with LL and LS were combined (L-carriers) for comparison with SS participants. A greater percentage of L-carrier than SS alcoholics had V_{max} for 5-HT uptake below $200 \text{ nmol}/10^7$ platelets-min ($p < 0.05$) and B_{max} for paroxetine binding below $600 \text{ nmol}/\text{mg}$ protein ($p < 0.06$).

(19.2%) were of the SS genotype for the 5-HTT. These genotype frequencies are distributed in a manner consistent with the Hardy–Weinberg equilibrium and are similar to those observed in previous studies by Lesch et al. (1996) (LL—33%, LS—49%, SS—18%) and Preuss et al. (2000) (LL—28%, LS—58%, SS—14%). Ages of participants ranged from 18 to 66 years, with a mean age of 41.0 years and an average duration of 11.6 years of problem drinking. Since interim analyses indicated that SS homozygotes were different from L-carriers (either LL or LS), all subsequent data analyses combined the two L-carrier groups. There were a few differences between the two genotype groups on some demographic and drinking parameters (Table 1). Briefly, alcohol-dependent individuals with the SS genotype, compared with their L-carrier counterparts, were about 5 years younger ($p = 0.009$), drank more severely on drinking days ($p = 0.05$), and had a marginally significant trend of about 3 years less lifetime drinking ($p = 0.09$). Subjects of the SS genotype did not differ significantly from the L-carriers on any other

drinking characteristic, including age of onset of problem drinking, drinks per day, percentage of heavy drinking days, or percentage of days abstinent.

Table 1 also shows the mean platelet 5-HT parameters for the two genotype groups. Compared with the SS homozygotes, the L-carrier group had a significantly lower B_{max} for paroxetine binding ($p < 0.05$). The tendency for L-carriers also to have a lower mean V_{max} for 5-HT uptake was not significant ($p < 0.2$), but the proportion of L-carriers with a low V_{max} for 5-HT uptake ($< 200 \text{ nmol}/10^7$ platelets-min) was significantly greater than for the SS homozygotes (Fig. 1) ($p < 0.05$). L-carriers also tended to be more likely than SS homozygotes to have a low B_{max} for paroxetine binding ($< 600 \text{ nmol}/\text{mg}$ protein) (Fig. 1) ($p < 0.06$). Fig. 2 shows no correlation between the 5-HT uptake (V_{max}) and paroxetine binding (B_{max}) parameters and that the subjects with unusually low uptake ($V_{max} < 200 \text{ nmol}/10^7$ platelets-min) were not the same subjects who had low binding ($B_{max} < 600 \text{ nmol}/\text{mg}$ protein).

The platelet 5-HT uptake and binding parameters were significantly related to both current drinking (Table 2) and years of lifetime drinking (Table 3) for the L-carriers but not the SS genotype group. Specifically, in the L-carriers, heavier current drinking was associated with greater uptake (V_{max}),

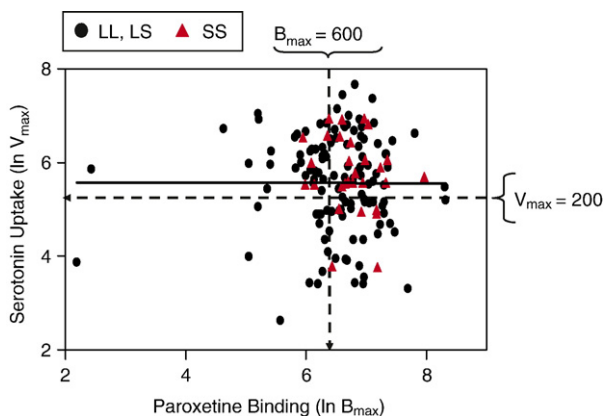


Fig. 2. Correlation between V_{max} for platelet serotonin uptake and B_{max} for platelet paroxetine binding. V_{max} for platelet serotonin uptake and B_{max} for platelet paroxetine binding were determined as described in the Subjects and methods section. The correlation was determined using natural logs (ln) of the platelet parameters. Dashed vertical and horizontal reference lines on the graph indicate positions on the x-axis of $600 \text{ nmol}/\text{mg}$ protein and positions on the y-axis of $200 \text{ fmol}/\text{min} \cdot 10^7$ platelets. The solid horizontal line is the regression line for the correlation ($p > 0.05$).

Table 2

Regression coefficients (slopes) of platelet serotonin parameters ($\ln K_m$, $\ln V_{max}$, $\ln K_d$, and $\ln B_{max}$) on current drinking

Genotype	Platelet 5-HT uptake		Platelet paroxetine binding	
	K_m vs. current drinking	V_{max} vs. current drinking	K_d vs. current drinking	B_{max} vs. current drinking
L-carriers	0.0364 $p = 0.0269$	0.0466 $p = 0.0351$	-0.0351 $p = 0.100$	-0.0383 $p = 0.0185$
SS	0.0095 $p = 0.73$	-0.0317 $p = 0.376$	-0.0259 $p = 0.415$	0.0116 $p = 0.565$
<i>b</i>		$p = 0.07$		$p = 0.06$

Values are slope estimates for the regression between each platelet serotonin (5-HT) parameter and current drinking after accounting for genotype; p values = significance of a non-zero slope. K_m is in μM ; V_{max} is in $\text{fmol} \cdot 5\text{-HT}/\text{min} \cdot 10^7$ platelets; K_d is in nM ; B_{max} is in fmol/mg protein. “Current drinking” is the number of standard drinks per day for the 14 days preceding the blood draw. “*b*” designates the significance (p value) of differences between the slopes for the two genotype groups.

Table 3
Regression coefficients (slopes) of platelet serotonin parameters ($\ln K_m$, $\ln V_{max}$, $\ln K_d$, and $\ln B_{max}$) on lifetime drinking

Genotype	Platelet 5-HT uptake		Platelet paroxetine binding	
	K_m vs. years of lifetime drinking	V_{max} vs. years of lifetime drinking	K_d vs. years of lifetime drinking	B_{max} vs. years of lifetime drinking
L-carriers	-0.0160 $p=0.0408$	-0.0214 $p<0.0412$	-0.0006 $p=0.952$	-0.0184 $p=0.0076$
SS	0.0057 $p=0.683$	0.0019 $p=0.920$	-0.0067 $p=0.695$	0.0170 $p=0.110$
<i>b</i>				$p=0.01$

Values are slope estimates for the regression between each platelet serotonin (5-HT) parameter and years of lifetime drinking after accounting for genotype; p values = significance of a non-zero slope. K_m is in μM ; V_{max} is in $\text{fmol 5-HT}/\text{min} \cdot 10^7$ platelets; K_d is in nM ; B_{max} is in fmol/mg protein. “Years of lifetime drinking” is a subject’s current age minus the age at which he/she began experiencing symptoms of alcohol dependence. “*b*” designates the significance (p value) of differences between the slopes for the two genotype groups.

lower binding (B_{max}), and reduced affinity (i.e., increased K_m) for 5-HT uptake. Also for the L-carriers, a greater number of years of lifetime drinking was associated with lower uptake and binding and higher affinity for paroxetine binding (i.e., lower K_d). There were no significant relationships between any drinking measure and any platelet 5-HT parameter for the SS homozygotes. Other measures of drinking amount (i.e., drinks per drinking day and percentage of heavy drinking days) showed similar directional trends in their relationship with the platelet 5-HT measures among the L-carrier subjects only (data not shown). Though the two genotype groups differed in current age at baseline, current age did not significantly predict the platelet 5-HT biological parameters in any of these regression analyses. The vast majority of subjects were White — i.e., of European ancestry. When race (European vs. non-European) was entered into the model, it was not a significant predictor of platelet 5-HT parameters.

4. Discussion

Our principal finding was that allelic variation in the 5'-HTTLPR was associated with differences in phenomenological characteristics of treatment-seeking alcoholics and in the biological parameters of paroxetine binding and 5-HT uptake by the 5-HTT of the platelets in these alcoholics. At the time of enrollment, SS homozygotes were younger in chronological age and had tended to drink more heavily for fewer years of lifetime drinking than their L-carrier (i.e., LL and LS) counterparts. Even more importantly, we found evidence that patterns of drinking among these alcoholics may differentially affect 5-HTT expression and function depending upon the subjects’ allelic genotype. First, we found that the 5-HTT polymorphism was functional in the platelets of alcoholics in that compared with the SS homozygotes, subjects with an L-allele (i.e., the L-carriers) actually showed reduced paroxetine binding capacity and reduced functional 5-HT uptake capacity. Second, we frequently found relationships between drinking and platelet 5-HT

parameters only within the L-carriers and never within the SS homozygotes. Third, we found that for the majority of the population who were L-carriers, heavier current drinking was associated with increased 5-HT uptake but reduced paroxetine binding, but that a greater number of years of problem drinking was associated with reductions in both of these parameters.

The data showing reduced 5-HT binding and uptake among the L-carriers contradict common expectations that L-carriers should have higher expression and/or function than should SS homozygotes (Johnson, 2000). Though we have studied the platelets and not the brains of our subject population, our findings are entirely consistent with similar observations from a neuroimaging study showing that, compared with controls, alcoholics with the LL genotype have reduced β -CIT neuronal binding to 5-HTT in raphe brain areas (Heinz et al., 2000). Although that study showed similar levels of binding in LL vs. SS homozygotes, our L-carriers actually had less 5-HT binding and uptake than the SS subjects. Heinz et al. (2003) further reported that in rhesus monkeys exposed to alcohol, this differential reduction in raphe binding potential for L-carriers is related to chronic alcohol drinking, suggesting an alcohol “toxicity” to the 5-HTT. The current study is the first to show that the reduced activity of the 5-HTT in human alcoholics is related to drinking patterns and that this can be demonstrated in platelet preparations. We previously reported reduced 5-HT uptake in the platelets of a smaller sample of LL alcoholics (Javors et al., 2005). The current study substantially expands those findings, clearly showing reduced functional 5-HTT uptake in L-carriers, and adds the findings of reduced paroxetine binding as well. Heinz et al. (2004) hypothesized that neurotoxic reductions in 5-HTT protein expression were limited to homozygous L-carriers and correlated with negative mood states as opposed to the SS genotype. Also, Sander et al. (1997) observed a behavioral dichotomy between L-carriers and SS genotype on the basis of severity of withdrawal symptoms of alcoholics. Overall, these data clearly support the hypothesis that alcohol’s toxic effect on 5-HTT gene expression affects selectively those who are L-carriers but not those of the SS genotype.

The finding of relationships between drinking and 5-HTT function among L-carriers but not SS homozygotes needs to be characterized more fully. Somewhat paradoxically, L-carriers who are currently drinking more heavily exhibit higher 5-HTT uptake and lower affinity while exhibiting less paroxetine binding. We think that a plausible explanation for this finding is that current drinking increases 5-HT release markedly (LeMarquand et al., 1994a), and there may be an attempt to compensate physiologically by increased uptake. Nevertheless, binding levels remain low because of the toxic damage to those of L-carrier status.

In the current study, SS subjects tended to drink more and to be of a younger current age than LL subjects, which is consistent with the finding of Herman et al. (2005) that binge drinking may be greater in young college-aged adults with the SS genotype. However, the current study found no significant relationships between 5-HTT genotype and age of onset of alcoholism, which is consistent with family-based studies reporting no differential association of the L or S allele with early onset of alcoholism in

offspring (Samochowiec et al., 2006). Previous reports have suggested that the SS genotype is associated with increased anxiety and affective liability (Olsson et al., 2005) and may be associated with subtypes of alcoholism complicated by a comorbid psychiatric condition (Feinn et al., 2005). Though the current study included only patients without psychiatric comorbidity, we have reported, in another study of this population, that anxiety reduction is associated with a favorable treatment outcome (Sloan et al., 2003). Since S-linkages to alcohol dependence severity have not been consistently supported (Köhnke et al., 2006; Kweon et al., 2005), these findings suggest the possibility that the SS genotype may be associated with greater tendencies to seek treatment at a younger age.

Paroxetine binding is a widely used marker for the expression rate of the 5-HTT. Previous studies have shown that the principal binding site for paroxetine in platelets is the 5-HTT but that paroxetine also binds to mitochondrial and alpha granule proteins in addition to the 5-HTT (Cesura et al., 1990). The observed lack of correlation between V_{\max} and B_{\max} in our study probably is due in part to this lack of specificity of paroxetine binding. However, our unpublished results have indicated that only about 20% of the 5-HTTs are located at the surface of platelets, which may further account for the observed lack of correlation between V_{\max} and B_{\max} (Javors et al., 2005). Although the density of paroxetine binding (B_{\max}) is certainly an indicator of the expression rate of the 5-HTT, the mechanism by which the majority of transporters are internalized is unknown, but it is likely to include phosphorylation, glycosylation, and other cellular signaling mechanisms involved with 5-HTT uptake and turnover. It is unknown whether these internalization processes may differ among the L-carriers as opposed to the SS homozygotes, and additional research is required to understand the effects of alcohol on transporter internalization processes. Research comprising such detailed analyses of alcohol's cellular effects on the 5-HTT is ongoing in our laboratory.

If our observation of the effects of drinking status and allelic variation at the 5-HTT in platelets parallels changes that occur in the brain, it would provide added support for our hypothesis that ondansetron treatment response might be greater among L-carriers than in those with the SS genotype (Johnson, 2000). This is because in the brain, 5-HTTs in raphe nuclei are somatodendritic. Decreased somatodendritic function in alcoholics of L-carriers can, therefore, be expected to reduce 5-HT firing rates by increasing self-inhibition (Little et al., 1998), resulting in reduced intrasynaptic neurotransmission and post-synaptic up-regulation of 5-HT receptors. Blockade of up-regulated post-synaptic 5-HT₃ receptors might underlie ondansetron's therapeutic efficacy. Indeed, formal study of the effect of ondansetron on drinking among alcoholics with allelic variation at the 5-HTT is ongoing.

5. Conclusion

Among treatment-seeking alcoholics, 5-HTT expression varies with current and lifetime drinking in L-carriers alone. Allelic variation in the 5'-HTTLPR is associated with differences in phenomenological characteristics and in the biological

parameters of paroxetine binding and 5-HT uptake by the 5-HTT of the platelets, resulting in a more chronic and severe pattern of drinking for L-carriers. Future studies are needed to understand the neurobehavioral components of how allelic variation at the 5-HTT affects other alcohol-mediated processes associated with the alcohol dependence syndrome, including craving, reinforcement, tolerance, and sensitization.

Acknowledgments

We are grateful to the National Institute on Alcohol Abuse and Alcoholism for their generous support through grants 7 U10 AA011776-10, 1 N01 AA001016-000, 7 R01 AA010522-12, 5 R01 AA013964-03, 5 R01 AA014628-03, and 5 R01 AA012964-06 awarded to Prof. Johnson, grant 5 K23 AA000329-06 awarded to Dr. Ait-Daoud, and grant 5 R21 AA013435-03 awarded to Prof. Roache. We acknowledge and appreciate the exceptional technical expertise of Pei Yu Tian, at The University of Texas Health Science Center at San Antonio, who performed the serotonin uptake and paroxetine binding experiments. We also thank Robert H. Cormier, Jr. for his assistance with the manuscript preparation.

References

- American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 4th ed. Washington, D.C.: American Psychiatric Association; 1994.
- Baumgarten HG, Grozdanovic Z. Anatomy of central serotonergic projection systems. In: Baumgarten HG, Gothert M, editors. Handbook of experimental pharmacology, vol. 129. Serotonergic neurons and 5-HT receptors in the CNS. New York: Springer; 1997. p. 41–89.
- Beckman ML, Quick MW. Neurotransmitter transporters: regulators of function and functional regulation. *J Membr Biol* 1998;164:1–10.
- Bohn MJ, Babor TF, Kranzler HR. The Alcohol Use Disorders Identification Test (AUDIT): validation of a screening instrument for use in medical settings. *J Stud Alcohol* 1995;56:423–32.
- Cesura AM, Bertocci B, Da Prada M. Binding of [3H]dihydrotrabenazine and [125I]azidoiodoketanserin photoaffinity labeling of the monoamine transporter of platelet 5-HT organelles. *Eur J Pharmacol* 1990;186:95–104.
- Chick J, Aschauer H, Hornik K, Investigators' Group. Efficacy of fluvoxamine in preventing relapse in alcohol dependence: a one-year, double-blind, placebo-controlled multicentre study with analysis by typology. *Drug Alcohol Depend* 2004;74:61–70.
- Da Prada M, Cesura AM, Launay JM, Richards JG. Platelets as a model for neurones? *Experientia* 1988;44:115–26.
- Dundon W, Lynch KG, Pettinati HM, Lipkin C. Treatment outcomes in type A and B alcohol dependence 6 months after serotonergic pharmacotherapy. *Alcohol Clin Exp Res* 2004;28:1065–73.
- Esterling LE, Yoshikawa T, Turner G, Badner JA, Bengel D, Gershon ES, et al. Serotonin transporter (5-HTT) gene and bipolar affective disorder. *Am J Med Genet* 1998;81:37–40.
- Feinn R, Nellissery M, Kranzler HR. Meta-analysis of the association of a functional serotonin transporter promoter polymorphism with alcohol dependence. *Am J Med Genet B Neuropsychiatr Genet* 2005;133:79–84.
- Gill K, Amit Z. Serotonin uptake blockers and voluntary alcohol consumption. A review of recent studies. *Recent Dev Alcohol* 1989;7:225–48.
- Greenberg BD, Tolliver TJ, Huang SJ, Li Q, Bengel D, Murphy DL. Genetic variation in the serotonin transporter promoter region affects serotonin uptake in human blood platelets. *Am J Med Genet* 1999;88:83–7.
- Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D, et al. Allelic variation of human serotonin transporter gene expression. *J Neurochem* 1996;66:2621–4.

- Heils A, Mossner R, Lesch KP. The human serotonin transporter gene polymorphism—basic research and clinical implications. *J Neural Transm* 1997;104:1005–14.
- Heinz A, Jones DW, Mazzanti C, Goldman D, Ragan P, Hommer D, et al. A relationship between serotonin transporter genotype and in vivo protein expression and alcohol neurotoxicity. *Biol Psychiatry* 2000;47:643–9.
- Heinz A, Jones DW, Gorey JG, Bennet A, Suomi SJ, Weinberger DR, et al. Serotonin transporter availability correlates with alcohol intake in non-human primates. *Mol Psychiatry* 2003;8:231–4.
- Heinz A, Goldman D, Gallinat J, Schumann G, Puls I. Pharmacogenetic insights to monoaminergic dysfunction in alcohol dependence. *Psychopharmacology* 2004;174:561–70.
- Herman AI, Kaiss KM, Ma R, Philbeck JW, Hasan A, Dasti H, et al. Serotonin transporter promoter polymorphism and monoamine oxidase type A VNTR allelic variants together influence alcohol binge drinking risk in young women. *Am J Med Genet B Neuropsychiatr Genet* 2005;133:74–8.
- Javors MA, Houston JP, Tekell JL, Brannan SK, Frazer A. Reduction of platelet serotonin content in depressed patients treated with either paroxetine or desipramine. *Int J Neuropsychopharmacol* 2000;3:229–35.
- Javors MA, Seneviratne C, Roache JD, Ait-Daoud N, Bergeson SE, Walss-Bass MC, et al. Platelet serotonin uptake and paroxetine binding among allelic genotypes of the serotonin transporter in alcoholics. *Prog Neuropsychopharmacol Biol Psychiatry* 2005;29:7–13.
- Johnson BA. Serotonergic agents and alcoholism treatment: rebirth of the subtype concept—an hypothesis. *Alcohol Clin Exp Res* 2000;24:1597–601.
- Johnson BA, Ait-Daoud N. Neuropharmacological treatments for alcoholism: scientific basis and clinical findings. *Psychopharmacology* 2000;149:327–44.
- Köhnke MD, Kolb W, Lutz U, Maurer S, Batra A. The serotonin transporter promoter polymorphism 5-HTTLPR is not associated with alcoholism or severe forms of alcohol withdrawal in a German sample. *Psychiatr Genet* 2006;16:227–8.
- Kranzler HR, Burslen JA, Brown J, Babor TF. Fluoxetine treatment seems to reduce the beneficial effects of cognitive-behavioral therapy in type B alcoholics. *Alcohol Clin Exp Res* 1996;20:1534–41.
- Kweon YS, Lee HK, Lee CT, Lee KU, Pae CU. Association of the serotonin transporter gene polymorphism with Korean male alcoholics. *J Psychiatr Res* 2005;39:371–6.
- LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and dependence: findings of animal studies. *Biol Psychiatry* 1994a;36:395–421.
- LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and dependence: clinical evidence. *Biol Psychiatry* 1994b;36:326–37.
- Lesch KP, Wolozin BL, Murphy DL, Reiderer P. Primary structure of the human platelet serotonin uptake site: identity with the brain serotonin transporter. *J Neurochem* 1993;60:2319–22.
- Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996;274:1527–31.
- Lesch KP, Greenberg BD, Higley JD. Serotonin transporter, personality, and behavior: toward dissection of gene–gene and gene–environment interaction. In: Benjamin J, Ebstein RP, Belmaker RH, editors. *Molecular genetics and the human personality*. Washington, D.C.: American Psychiatric Publishing; 2002. p. 109–36.
- Little KY, McLaughlin DP, Zhang L, Livermore CS, Dalack GW, McFinton PR, et al. Cocaine, ethanol, and genotype effects on human midbrain serotonin transporter binding sites and mRNA levels. *Am J Psychiatry* 1998;155:207–13.
- McBride WJ, Li TK. Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents. *Crit Rev Neurobiol* 1998;12:339–69.
- Modai I, Malmgren R, Asberg M, Beving H. Circadian rhythm of serotonin transport in human platelets. *Psychopharmacology* 1986;88:493–5.
- Modai I, Malmgren R, Wetterberg L, Eneroth P, Valevski A, Asberg M. Blood levels of melatonin, serotonin, cortisol, and prolactin in relation to the circadian rhythm of platelet serotonin uptake. *Psychiatry Res* 1992;43:161–6.
- Naranjo CA, Knoke DM. The role of selective serotonin reuptake inhibitors in reducing alcohol consumption. *J Clin Psychiatry* 2001;62:18–25.
- Olsson CA, Byrnes GB, Lotfi-Miri M, Collins V, Williamson R, Patton C, et al. Association between 5-HTTLPR genotypes and persisting patterns of anxiety and alcohol use: results from a 10-year longitudinal study of adolescent mental health. *Mol Psychiatry* 2005;10:868–76.
- Penington DG. Formation of platelets. In: Gordon JL, editor. *Platelets in biology and pathology 2*. New York: Elsevier/North-Holland Biomedical Press; 1981. p. 19–41.
- Pettinati HM, Volpicelli JR, Kranzler HR, Luck G, Rukstalis MR, Cnaan A. Sertraline treatment for alcohol dependence: interactive effects of medication and alcoholic subtype. *Alcohol Clin Exp Res* 2000;24:1041–9.
- Pettinati HM, Volpicelli JR, Luck G, Kranzler HR, Rukstalis MR, Cnaan A. Double-blind clinical trial of sertraline treatment for alcohol dependence. *J Clin Psychopharmacol* 2001;21:143–53.
- Preuss UW, Soyka M, Bahlmann M, Wenzel K, Behrens S, de Jonge S, et al. Serotonin transporter gene regulatory region polymorphism (5-HTTLPR), [3H]paroxetine binding in healthy control subjects and alcohol-dependent patients and their relationships to impulsivity. *Psychiatry Res* 2000;96:51–61.
- Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T, Chang AS, et al. Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc Natl Acad Sci U S A* 1993;90:2542–6.
- Samochowiec J, Kucharska-Mazur J, Grzywacz A, Jablonski M, Rommelspacher H, Samochowiec A, et al. Family-based and case-control study of DRD2, DAT, 5HTT, COMT genes polymorphisms in alcohol dependence. *Neurosci Lett* 2006;410:1–5.
- Sander T, Harms H, Lesch KP, Dufeu P, Kuhn S, Hoche M, et al. Association analysis of a regulatory variation of the serotonin transporter gene with severe alcohol dependence. *Alcohol Clin Exp Res* 1997;21:1356–9.
- Sloan TB, Roache JD, Johnson BA. The role of anxiety in predicting drinking behaviour. *Alcohol Alcohol* 2003;38:360–3.
- Sobell LC, Sobell MB. Timeline follow-back: a technique for assessing self-reported alcohol consumption. In: Litten RZ, Allen JP, editors. *Measuring alcohol consumption: psychosocial and biochemical methods*. Totowa, NJ: Humana Press Inc.; 1992. p. 41–72.
- Stoltenberg SF. Serotonergic agents and alcoholism treatment: a simulation. *Alcohol Clin Exp Res* 2003;27:1853–9.
- Villinger F, Faraj BA, Olkowski ZL, Jackson RT, Ansari AA. Functional expression of the serotonin transporter in human peripheral lymphocytes. *FASEB J* 1994;8:A108.
- Virkkunen M, Linnoila M. Serotonin in early-onset alcoholism. In: Galanter M, editor. *Recent developments in alcoholism, alcohol and violence: epidemiology, neurobiology, psychology, family issues*. New York: Plenum Press; 1997. p. 173–89.
- Virkkunen M, Goldman D, Nielsen DA, Linnoila M. Low brain serotonin turnover rate (low CSF 5-HIAA) and impulsive violence. *J Psychiatry Neurosci* 1995;20:271–5.