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ORIGINAL INVESTIGATION

Association and interaction analyses of 5-HT3 receptor and serotonin transporter genes with alcohol, cocaine, and nicotine dependence using the SAGE data

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Abstract Previous studies have implicated genes encoding the 5-HT3$_{AB}$ receptors (*HTR3A* and *HTR3B*) and the serotonin transporter (*SLC6A4*), both independently and interactively, in alcohol (AD), cocaine (CD), and nicotine dependence (ND). However, whether these genetic effects also exist in subjects with comorbidities remains largely unknown. We used 1,136 African-American (AA) and 2,428 European-American (EA) subjects from the Study of Addiction: Genetics and Environment (SAGE) to determine associations between 88 genotyped or imputed variants within *HTR3A*, *HTR3B,* and *SLC6A4* and three types of addictions, which were measured by DSM-IV diagnoses of AD, CD, and ND and the Fagerström Test for Nicotine Dependence (FTND), an independent measure of ND commonly used in tobacco research. Individual SNP-based association analysis revealed a significant association of rs2066713 in *SLC6A4* with FTND in AA ($\beta = -1.39$; $P = 1.6E-04$). Haplotype-based association analysis found one major haplotype formed by SNPs rs3891484 and rs3758987 in *HTR3B* that was significantly associated with AD in the AA sample, and another major haplotype T–T-G, formed by SNPs rs7118530, rs12221649, and rs2085421 in *HTR3A*, which showed significant association with FTND in the EA sample. Considering the biologic roles of the three genes and their functional relations, we used the GPU-based Generalized Multifactor Dimensionality Reduction (GMDR-GPU) program to test SNP-by-SNP interactions within the three genes and discovered two- to five-variant models that have significant impacts on AD, CD, ND, or FTND. Interestingly, most of the SNPs included in the genetic interaction model(s) for each addictive phenotype are either overlapped or in high linkage disequilibrium for both AA and EA samples, suggesting these detected variants in *HTR3A*, *HTR3B*, and *SLC6A4* are interactively contributing to etiology of the three addictive phenotypes examined in this study.

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter that mediates rapid excitatory responses through ligand-gated channels (5-HT3 receptors). The 5-HT3 receptors, unlike other serotonergic receptor classes, which are G protein coupled (Barnes et al. 2009; Boess and Martin 1994; Cravchik and Goldman 2000), belong to the superfamily of nicotinic acetylcholine (nACh), subtype A of the γ-aminobutyric acid ($GABA_A$) and glycine receptors (Maricq et al. 1991). The serotonin-gated ion channel conducts primarily Na^+ and K^+, resulting in rapid neuronal depolarization followed by a rapid desensitization and the release of stored neurotransmitter, which suggests a potentially important role for this receptor system in neuronal circuitry involved in drug abuse (Grant 1995). Further, 5-HT3 receptors are co-localized with nACh receptors on nerve terminals in several brain pathways of reward processing, including dopaminergic terminals in the striatum (Nayak et al. 2000). Although there is no evidence that they interact physically, cross-regulation may take place at a downstream molecular level (Dougherty and Nichols 2009; Nayak et al. 2000; Yamauchi et al. 2011). Besides the

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potentially important role of 5-HT3 receptors in the development of nicotine dependence (ND), they can be potentiated through acute exposure to alcohol at concentrations that produce intoxication (Narahashi et al. 2001; Sung et al. 2000).

Whereas 5-HT3 receptors assembled by 5-$HT3_A$ subunits are uniformly located in various parts of the central and peripheral nervous systems, transcripts of the 5-$HT3_A$ and 5-$HT3_B$ subunits are coexpressed in the amygdala, caudate, and hippocampus, areas implicated in alcohol, nicotine, and other drug addictions, and form pharmacologically more potent heteropentameric receptors compared with the 5-$HT3_A$ homomeric structures (Davies et al. 1999; Dubin et al. 1999; Enoch et al. 2011). The genes encoding the 5-$HT3_A$ and 5-$HT3_B$ receptor subunits (namely, *HTR3A* and *HTR3B*) lie in a 90-kb region on chromosome 11q23.1 (Miyake et al. 1995).

Serotonin transporters (SERTs), one major class of monoamine transporters, which regulate the availability of 5-HT in the synaptic cleft through re-uptake, is encoded by the *SLC6A4* gene on chromosome 17q11.2 (Ramamoorthy et al. 1993). *SLC6A4* spans 37.8 kb and is composed of fourteen exons encoding a protein of 630 amino acids (Lesch et al. 1994). Alternate promoters in combination with differential splicing involving exon 1A, B, and C in specific tissues, and alternate polyadenylation site usage resulting in multiple mRNA species are likely participants in the regulation of SERT expression in humans (Bradley and Blakely 1997; Ozsarac et al. 2002). SERTs mediate antidepressant action and behavioral effects of cocaine and amphetamines (Ramamoorthy et al. 1993). Sequence variations in *SLC6A4* have been associated with several neuropsychiatric conditions, including major depressive disorders, anxiety-related personality traits, and antidepressant response (Dong et al. 2009; Lopez-Leon et al. 2008; McCauley et al. 2004).

In addition, previous association studies have posited a significant role for *HTR3A*, *HTR3B,* and *SLC6A4* in AD (Enoch et al. 2011; Seneviratne et al. 2013), cocaine dependence (CD) (Enoch et al. 2011), and ND (Yang et al. 2013), both independently and through gene-by-gene interactions. Importantly, both studies reported by Seneviratne et al. (2013) and Yang et al. (2013) indicated significant interactive effects of genetic variations in *HTR3A*, *HTR3B*, and *SLC6A4* by influencing the etiology of AD and ND, even though both individual SNP- and haplotype-based association analyses revealed only weak association of variants in the three genes with AD and ND. Our group has also reported that a combined five-marker genotype panel in *HTR3A*, *HTR3B* and *SLC6A4* can be used to predict the outcome of treatment of alcohol dependence with the 5-HT3 antagonist ondansetron (Johnson et al. 2011, 2013). Thus, the objective of this study was to determine whether there exist significant independent and interactive effects of the three genes associated with different addictive phenotypes in the samples of both African- and European-American origin.

Subjects and methods

Subjects

SAGE is a population-based study with 4,032 subjects of either European- (EA) or African-American (AA) descent. Participants were selected from three large complementary datasets: the Collaborative Study on the Genetics of Alcoholism (COGA) (Edenberg et al. 2005), the Collaborative Genetic Study of Nicotine Dependence (COGEND) (Bierut et al. 2007), and the Family Study of Cocaine Dependence (FSCD) (Grucza et al. 2008). All subjects included in these studies include comprehensive demographic information such as age, sex, and ethnicity. Genotyping was performed on the Illumina Human 1 M platform with 1,040,107 SNPs available for each DNA sample. For a detailed description of this GWAS dataset, please see the paper by Bierut et al. (2010).

According to the quality control (QC) report of the GENEVA alcohol-dependence project accompanying the dataset, stringent QC criteria were applied to all the samples. After removal of subjects with abnormal chromosomes 11 or 17 (such as aneuploidy and mosaic cell populations), related individuals, Hispanics, 3,564 (54.8 % females) samples were retained for all analyses in this study. Among these samples, 2,428 (56.1 % females) were EA and 1,136 (52.1 % females) were AA. According to the principal component (PC) analysis results from the original study, PC1 separates the self-identified black and white subjects very well, while PC2 separates the Asian HapMap samples and the self-identified Hispanic subjects from the others; meanwhile, similar results were seen with analyses using two principal components indexing continuous variation and self-reported race as categorical variables (Bierut et al. 2010). Since Hispanic subjects were removed, self-identified racial groups were used to distinguish AA from EA in all analyses.

The dependence status of each subject for nicotine, alcohol, and cocaine was assessed by the DSM-IV criteria, which were obtained from the original dataset. In addition, the Fagerström Test for Nicotine Dependence (FTND) score of each subject was chosen as an independent measure of ND, because it is one of the commonly used measures in ND research, thus providing a means of comparing results from different studies (Yang et al. 2013). The detailed characteristics of the AA and EA samples are summarized in Table 1 and Fig. 1.

Imputation and SNP selection

In the SAGE data, there were 27 genotyped SNPs across the *HTR3B* gene region, which included the functional SNP rs1176744 (Tyr129Ser) and the missense variant rs17116138 (Val183Ile). Of the 37 SNPs within the *HTR3A* gene region, there was a coding synonymous variant, rs1176713 (Leu465Leu). For the *SLC6A4* gene, 17 SNPs were genotyped, including rs6352 (Lys605Asn), which changes an amino acid. All these SNPs follow the Hardy–Weinberg Equilibrium.

Although the 81 genotyped SNPs in SAGE well covered the three genes, in order to include more important SNPs reported by others (Enoch et al. 2011; Seneviratne et al. 2013; Yang et al. 2013), we performed imputation for four SNPs in *HTR3B* and three SNPs in *HTR3A* using the 1,000 Genomes AFR and EUR data as references for the AA and EA samples, respectively, with the MaCH program (Li et al. 2009b, 2010). Both reference panels were accessed through the 1,000 Genomes Browser (http://browser.1000genomes.org/index.html). The r^2 values, which measure the imputation quality, for six out of the seven imputed SNPs (rs33940208 was excluded from further analysis because of low imputation quality) are >0.8 for the EA sample. There are two SNPs (rs3758987 and rs4938056) with r^2 values between 0.7 and 0.8 for the AA sample; however, their minor allele frequencies are more than 35 %, which guarantees their imputation qualities with comparatively low r^2 values (Li et al. 2009b). A detailed list of genotyped and imputed SNPs is provided in Supplementary Table.

Table 1 Characteristics of the SAGE AA and EA samples used in the study

Characteristic	African-American	European-American
Sample size	1,136	2,428
Age, years (SD)	40.2 (7.4)	38.4 (9.7)
Female (%)	592 (52.1)	1,362 (56.1)
DSM-IV alcohol dependence (%)	567 (49.9)	1,084 (44.6)
DSM-IV cocaine dependence (%)	458 (40.3)	454 (18.7)
DSM-IV nicotine dependence (%)	535 (47.1)	1,066 (43.9)
FTND score (SD)	4.93 (2.32)	5.06 (2.76)
No addiction (%)	397 (34.9)	1,018 (41.9)
One type of addiction (%)	198 (17.4)	540 (22.2)
Two types of addiction (%)	260 (22.9)	539 (22.2)
Three types of addiction (%)	280 (24.6)	327 (13.5)

SD standard deviation

Statistical analysis

Individual SNP- and haplotype-based association analyses

Individual SNP-based association analyses with AD, CD, and ND were performed using logistic regression models, while FTND was analyzed using linear regression models implemented in PLINK (Purcell et al. 2007). Additive, dominant, and recessive models were all tested for each SNP, adjusted for sex, age, study (whether the subjects were from COGEND, COGA, or FSCD), and two other dependence statuses that are not used as the response variable in the AA and EA samples. For example, if ND/FTND was used as the dependent variable, sex, age, study, AD, and CD were included as covariates in the logistic/linear regression model. Pair-wise linkage disequilibrium (LD) and haplotype blocks were assessed by Haploview (v. 4.2) (Barrett et al. 2005; Gabriel et al. 2002), and their associations with the four phenotypic measures were analyzed using Haplo Stats (v.1.6.3) through computing score statistics with the same covariates and genetic models used as

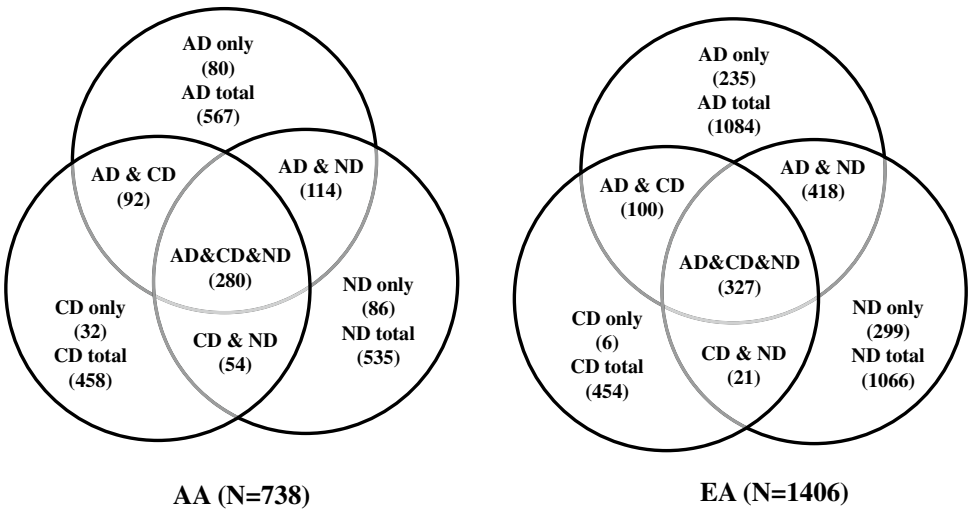

Fig. 1 *Venn diagrams* showing numbers of subjects with either sole or multiple addictions in the SAGE AA and EA samples. Numbers in parentheses stand for sample sizes of either sole or multiple addictions. Numbers at the bottom of the figure are the total sample size for AAs and EAs, respectively. *AD* alcohol dependence; *CD* cocaine dependence; *ND* nicotine dependence. There are one AA and four EAs with CD missing

the individual SNP-based association analysis (Schaid et al. 2002).

Statistically significant results for individual SNPs and major haplotypes (frequency ≥5 %) were selected after controlling for family wise error rate (FWER) using Bonferroni correction. The three genetic models and the four phenotypic measures are highly related, with the correlation coefficient between AD and CD being 0.487, AD and ND 0.453, and CD and ND 0.352. To reduce the probability of producing false-negative results and at the same time to increase statistical power, less stringent Bonferroni-corrected P values were used to select significant associations, which were corrected for the number of SNPs or haplotypes, but not phenotypes or genetic models (as they are highly correlated to each other). Uncorrected P values are presented throughout the manuscript.

SNP-by-SNP interaction analysis of HTR3A, HTR3B, and SLC6A4 variants

For the SNP-by-SNP interaction analysis of *HTR3A*, *HTR3B*, and *SLC6A4*, we performed exhaustive searches for two- to five-way interactions using the GMDR-GPU program (Zhu et al. 2013), which not only scales genetic and/or environmental factor numbers up to the GWAS level but also runs much faster than the earlier version of the GMDR program (Lou et al. 2007) by employing more efficient computational implementation (Zhu et al. 2013). Similar to the association analysis described above, by taking sex, age, and two-dependence status as covariates, and one other dependence status as phenotype for the AA and EA samples, GMDR-GPU calculates a "score" statistic for each subject based on a generalized linear model under different distributions (Lou et al. 2007). Specifically, we assumed that binary traits (AD, CD, and ND) follow a Bernoulli distribution and FTND follows a normal distribution in our gene-by-gene interaction analysis using GMDR-GPU (Zhu et al. 2013).

The best statistical SNP-by-SNP interaction model for a given order of interaction was determined by three factors: (1) the cross-validation consistency (CVC) statistics for the selected SNP combinations; (2) the prediction accuracies and the significance level or *P* value, which is determined by 10^7 permutation tests based on the observed testing accuracies; and (3) interaction analysis results of the SNP combination with all four phenotypes examined (Zhu et al. 2013). Please see the supplementary note for a detailed description of the GMDR-GPU program.

Results

Individual SNP-based association analysis

One SNP among the 88 variants tested for the three genes remained significant after Bonferroni correction ($P < 5.68E-04$), which is rs2066713 in *SLC6A4* with a *P* value of $1.6E-04$ and β value of -1.39 for FTND under the recessive model in the AA sample. The other seven SNPs presented in Table 2 showed marginal associations ($P < 0.01$) with AD, CD, ND, or FTND in either the AA or the EA sample. Within *HTR3B*, three SNPs were marginally associated with AD or FTND under the recessive model: rs12276717 showed marginal association (OR = 0.2; $P = 0.005$) with AD in the AA sample; and both rs1672717 and rs720396 were associated ($\beta = -0.58$;

Table 2 SNPs with *P* values <0.01 in individual SNP association analyses with AD, CD, ND and FTND in AA and EA samples

Sample	Gene	dbSNP ID	Minor Allele (MAF)	DSM-IV						FTND	
				AD		CD		ND			
				OR	*P*	OR	*P*	OR	*P*	BETA	*P*
AA	***HTR3B***	rs12276717	C (0.148)	0.2	0.005^{r}	0.8	0.273^{d}	0.8	0.195^{d}	0.49	0.419^{r}
	HTR3A	rs11214796	T (0.403)	0.8	0.132^{d}	0.8	0.304^{r}	0.9	0.648^{r}	0.34	0.010^{a}
		rs1563533	A (0.157)	1.7	0.004^{d}	0.8	0.274^{d}	0.7	0.065^{d}	1.13	0.041^{r}
	SLC6A4	rs2066713	T (0.262)	1.2	0.128^{a}	1.3	0.480^{r}	0.8	0.353^{r}	**−1.39**	**1.6E−04^{r}**
EA	***HTR3B***	rs1672717	C (0.389)	0.9	0.447^{d}	0.9	0.455^{r}	1.0	0.659^{r}	−0.58	0.004^{r}
		rs720396	C (0.431)	1.2	0.261^{r}	0.7	0.109^{r}	1.0	0.906^{d}	−0.53	0.005^{r}
	HTR3A	rs1020715	T (0.002)	0.5	0.490^{a}	16	0.004^{a}	0.6	0.583^{a}	−0.40	0.768^{a}
		rs2364857	C (0.112)	1.2	0.184^{a}	0.8	0.144^{d}	0.7	0.005^{d}	−0.21	0.181^{a}

MAF minor allele frequency; *AD* DSM-IV alcohol dependence; *CD* DSM-IV cocaine dependence; *ND* DSM-IV nicotine dependence; *OR* odds ratio; *BETA* beta coefficient. Significant associations are given in bold. Superscripts following *P* values indicate genetic models used for analysis: *a* additive; *d* dominant; and *r* recessive. For AD, CD and ND, logistic regression models were implemented; for FTND, linear regression models were used. Sex, age, study and two out of three addiction status were used as covariates while the other one was used as dependent variable in all statistical models. See "Subjects and methods" for details

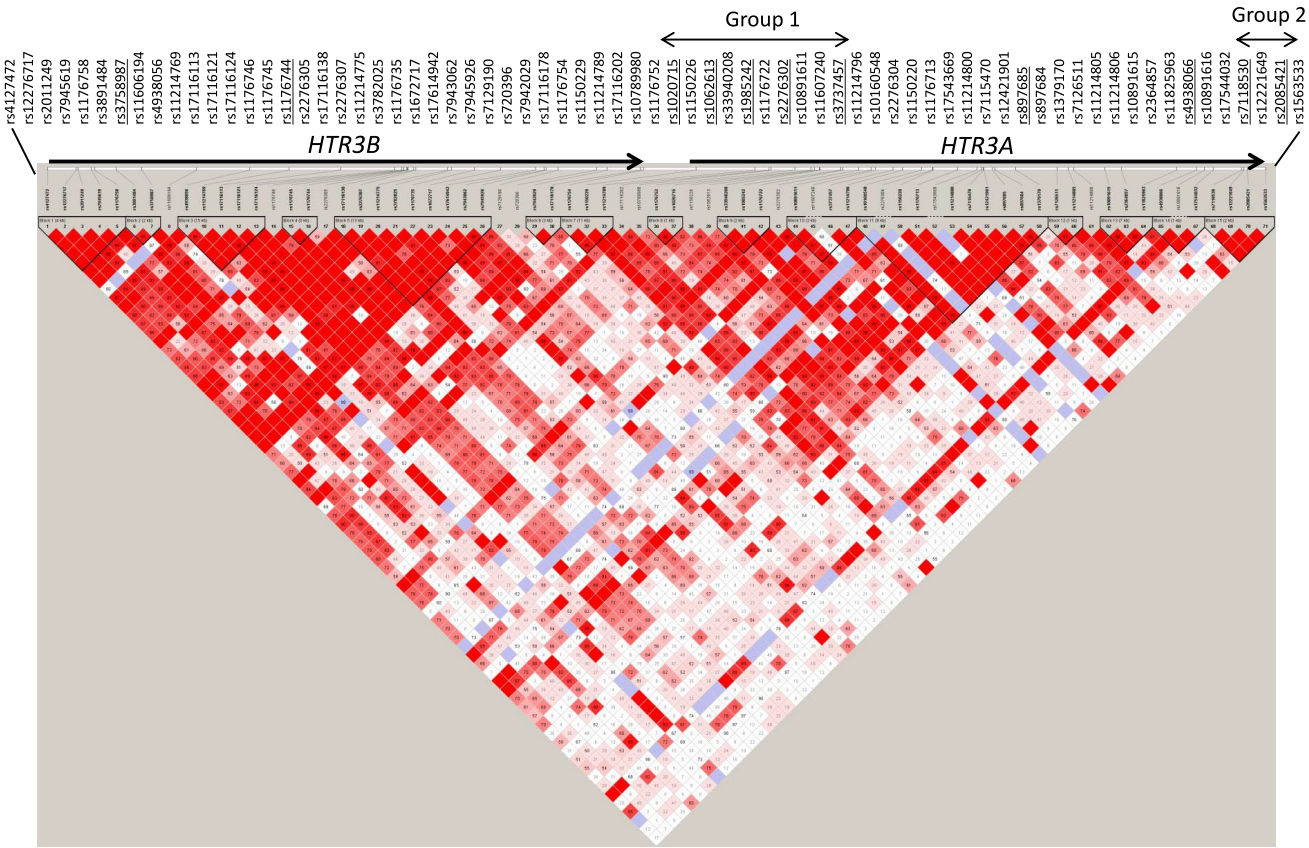

Fig. 2 LD structures for *HTR3B* and *HTR3A* SNPs in the SAGE AA sample. Haploview (v. 4.2) (Barrett et al. 2005) was used to calculate all D' values, and haplotype blocks were defined according to Gabriel et al. (2002). The number in *each box* represents the D' value for each SNP pair surrounding that box. The *arrow* on top of the figure represents the gene transcription direction from 5′- to 3′-end. SNPs involved in later interactive models are *underlined* and grouped according to D' values >0.7. Please refer to Fig. 6 for more information about SNP groups

$P = 0.004$ and $\beta = -0.53$; $P = 0.005$, respectively) with FTND in the EA sample. Of the *HTR3A* SNPs, rs11214796 was marginally associated ($\beta = 0.34$; $P = 0.01$) with FTND in the AA sample under the additive model; rs1563533 showed marginal association (OR = 1.7; $P = 0.004$) with AD in AAs under the dominant model. For EAs, rs1020715 and rs2364857 were associated with CD and ND, respectively, with P values of 0.004 (OR = 16) under the additive model and 0.005 (OR = 0.7) under the dominant model. Among these seven marginal associations, rs1020715 is questionable given its low minor allele frequency (0.002).

Haplotype-based association analysis

According to the haplotype block definition of Gabriel et al. (2002), there are 15 and 13 LD blocks in the AA and EA samples, respectively, within *HTR3A* and *HTR3B*, whereas 2 blocks were found in the *SLC6A4* region for both AA and EA samples. We used Haplo Stats to perform haplotype-based association analyses for all major haplotypes (frequency ≥5 %) in each above-mentioned LD block with the four phenotypic measures in AA and EA samples.

In AAs, there was one major haplotype C–C, formed by SNPs rs3891484 and rs3758987, located in the 5′ region of *HTR3B* (LD block 2 in Fig. 2) that was significantly associated with AD (frequency = 11.9 %; $P = 0.002$) under the dominant model. This association remained significant after Bonferroni correction among the 17 major haplotypes in AAs ($P < 0.003$). Besides this haplotype, there were two haplotypes with P values <0.01: (1) G–G–G-T-G-T-C-G-C, formed by SNPs rs17116138, rs2276307, rs11214775, rs3782025, rs1176735, rs1672717, rs17614942, rs7943062, and rs7945926 (LD block 5 within *HTR3B* in Fig. 2), with a frequency of 12.6 %, that was marginally associated with AD under the dominant model ($P = 0.004$) and ND under the additive model ($P = 0.009$); and (2) C–C–C-T-A, formed by SNPs rs6354, rs25528, rs2066713, rs8071667, and rs16965623 (LD block 2 of *SLC6A4* in Fig. 3), with a frequency of 23.7 %, that showed a marginal association with AD under the additive model ($P = 0.005$).

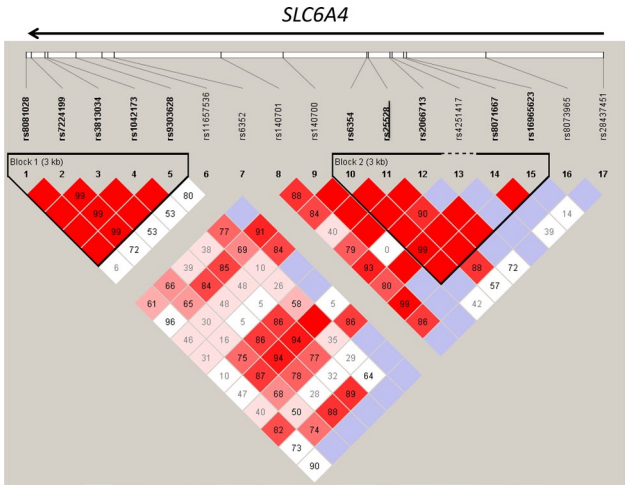

Fig. 3 LD structure for *SLC6A4* SNPs in the SAGE AA sample. Haploview (v. 4.2) (Barrett et al. 2005) was used to calculate all D' values, and haplotype blocks were defined according to Gabriel et al. (2002). The number in *each box* represents the D' value for each SNP pair surrounding that box. The *arrow* on top of the figure represents the gene transcription direction from 5′- to 3′-end. SNPs involved in later interactive models are *underlined*

For the EA sample, we found one haplotype, T–T-G, formed by SNPs rs7118530, rs12221649, and rs2085421 (LD block 13 within *HTR3A* in Fig. 4) significantly associated with FTND under the additive model (frequency = 60.5 %; $P = 0.002$), which remained significant after Bonferroni correction for 15 major haplotypes ($P < 0.003$). The global P value of this haplotype was 0.008 under the recessive model, suggesting marginal association with FTND. There are three other haplotypes showing marginal significance in EAs: (1) rs11214769 and rs1176744 (LD block 2 within *HTR3B* in Fig. 4) with ND (P global = 0.007) under the additive model; (2) A-G, formed by SNPs rs7942029 and rs17116178 (LD block 6 within *HTR3B* in Fig. 4), with CD under the dominant model (frequency = 73 %; $P = 0.007$); and (3) A–A-T-G-C, formed by SNPs rs6354, rs25528, rs2066713, rs425147, and rs8071667 (LD block 2 of *SLC6A4* in Fig. 5), with FTND under the recessive model (frequency = 39.5 %; $P = 0.007$). The detailed results of the haplotype-based association analyses in AAs and EAs are presented in Tables 3 and 4, respectively.

SNP-by-SNP interaction analysis of HTR3A, HTR3B, and SLC6A4

Two previous studies reported by our group indicated that there exist significant epistatic effects among *HTR3A*, *HTR3B*, and *SLC6A4* in both AAs and EAs in either AD or ND (Seneviratne et al. 2013; Yang et al. 2013). As shown in Table 5, we determined the best interaction models for AD, CD, ND, and FTND based on CVC >7 of 10, prediction accuracy >55 % and empirical P value <0.005 for each model based on 10^7 permutation tests. Although the SNPs involved in different interaction models were not exactly the same, Figs. 6 and 7 show great overlaps and correlations among SNPs based on the LD structure of those SNPs included in each model.

Discussion

Through individual SNP- and haplotype-based association analyses and SNP-by-SNP interaction analysis on AD, CD, ND, and FTND, we identified significant independent and interactive effects among 88 genotyped and imputed variants within *HTR3A*, *HTR3B*, and *SLC6A4* in the AA and EA samples. These findings confirm our hypothesis that interactive effects exist between 5-HT3 receptors and transporters in governing trans-synaptic serotonergic signaling underlying the pathophysiology of multiple addictions in two ethnic groups.

On the individual polymorphic level, rs2066713 was significantly associated with FTND in the AA sample. As a tag SNP located in the alternative splicing region of *SLC6A4* involving noncoding exons 1A and 1B, it is likely to regulate expression of the gene in humans, because exon 1B is surrounded by several consensus sites for transcription factors AP-1, AP-2, CREB/ATF, and NF-κB (Bradley and Blakely 1997). Rs2066713 was reported to be associated with schizophrenia in a South Indian population (Vijayan et al. 2009) and with autism in Caucasian samples (Ma et al. 2010). On the haplotypic level, two SNPs located in the 5′-region of *HTR3B* (i.e., rs3891484 and rs3758987) and three SNPs located in the 3′-region of *HTR3A* (i.e., rs7118530, rs12221649, and rs2085421) are associated with AD in the AA sample and FTND in the EA sample, respectively. However, these association signals are not as strong as the SNP-by-SNP interaction results we obtained.

In the AA sample, there are 12 SNPs included in the four interaction models of AD, CD, ND, and FTND, which can be treated as seven groups based on D′ values. Rs25528 is the only SNP located in the 5′-region of *SLC6A4* in the AD, CD, and ND interaction models, which is in strong LD with rs2066713 and also locates in the alternative splicing region of *SLC6A4*. It has been reported to be significantly associated with the Beck Depression Inventory (Su et al. 2009). The two distinct SNPs within *HTR3B* are rs3758987 and rs1176744. Rs3758987 locates in the 5′-UTR region of *HTR3B*, whereas the non-synonymous SNP rs1176744 results in a tyrosine-to-serine change at the 129th amino acid residue of 5-HT3$_B$. This amino acid substitution significantly increases the maximum response of 5-HT3$_{AB}$ to serotonin, slows its deactivation and desensitization kinetics twentyfold and tenfold,

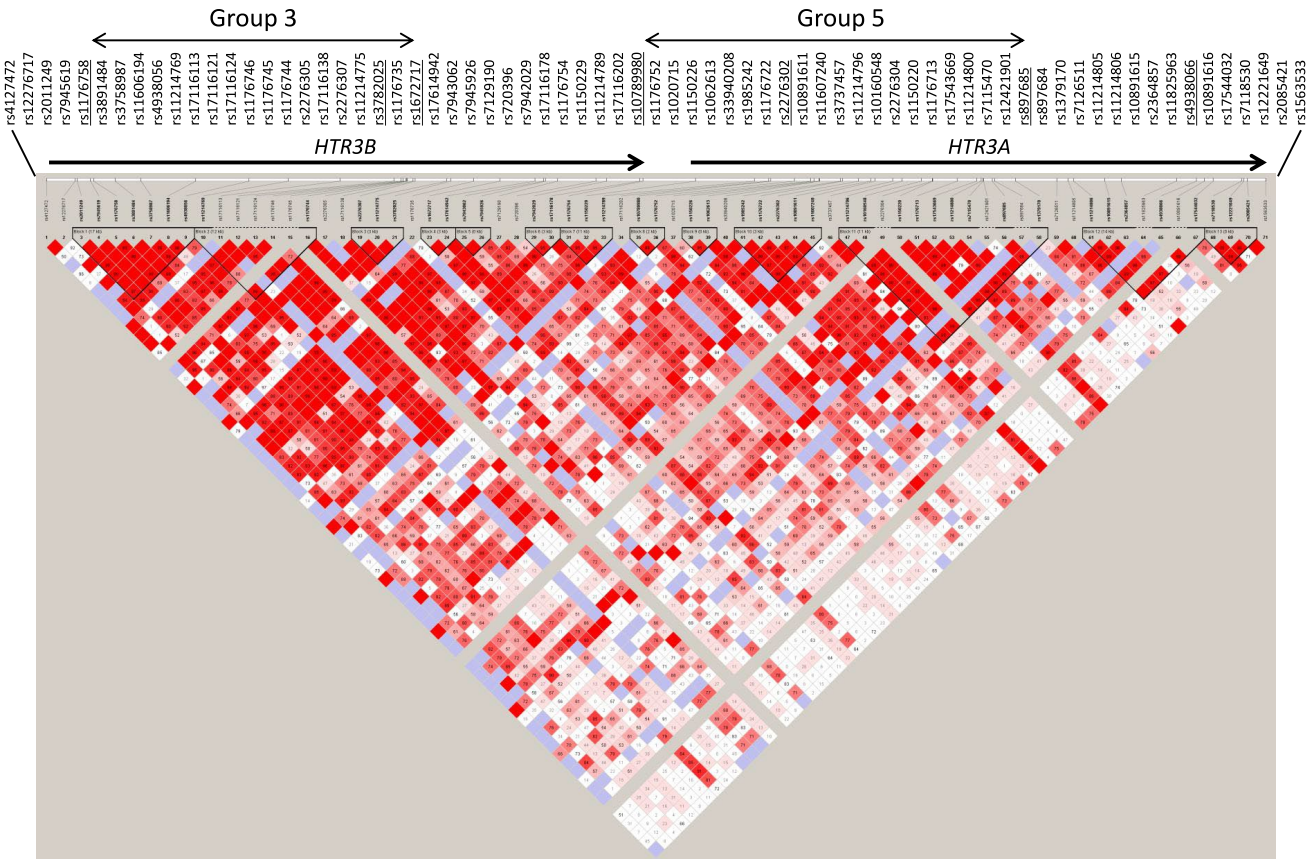

Fig. 4 LD structure for *HTR3B* and *HTR3A* SNPs in the SAGE EA sample. Haploview (v. 4.2) (Barrett et al. 2005) was used to calculate all D' values, and haplotype blocks were defined according to Gabriel et al. (2002). The number in *each box* represents the D' value for each SNP pair surrounding that box. The *arrow* on top of the figure represents the gene transcription direction from 5′- to 3′-end. SNPs involved in later interactive models are *underlined* and grouped according to D' values >0.7. Please refer to Fig. 7 for more information about SNP groups

respectively, and confers a sevenfold increase in the receptors' mean open time (Krzywkowski et al. 2008). There are two SNP groups and two individual SNPs in *HTR3A* involved in the AA interaction models, as shown in Fig. 6. Group 1 spans from the 5′-UTR to the intron region of *HTR3A*, which covers five SNPs (rs1020715, rs1062613, rs1985242, rs2276302, and rs3737457). Rs1020715 and rs1062613 are translation regulatory variants located in an open reading frame upstream of the translation initiation site of *HTR3A* mRNA (Niesler et al. 2001). Rs2276302, together with rs3737457, is part of a haplotype reported to be associated with heroin addiction in AAs (Levran et al. 2009). Rs897685, rs4938066, and Group 2 are all located in the 3′-UTR region of *HTR3A*.

In the EA sample, as shown in Fig. 7, three SNP groups and one individual SNP are included in the four interaction models for AD, CD, ND, and FTND. Of the three SNPs included in Group 3, rs1176758 is located in the 5′-UTR region of *HTR3B*; Ducci et al. (2009) reported that the intronic SNP rs3782025 was associated with alcohol use disorders + co-morbid antisocial personality disorder in Finns; rs1672717 was significantly associated with the intensity of nausea and vomiting among cancer patients treated with opioids (Laugsand et al. 2011). Although the functionality of the intronic SNPs rs3782025 and rs1672717 is not clear, the strength of interactive effects between SNPs within the gene and addictions is likely to be similar, as *HTR3B* is covered by only one LD block in Caucasians according to the HapMap data. Group 4 includes three correlated SNPs: rs9303628, rs140701, and rs2066713. The first two SNPs are located in the intron regions of *SLC6A4*, which may represent new regulatory variants or indicate that they reside in LD with such a variant. Rs2066713 has shown an independent effect on FTND, and rs25528, a SNP in strong LD with rs2066713, is the major interactive signal of *SLC6A4* in the AA sample. The variants of *HTR3A* involved in the interaction models are Group 5 (rs10789980, rs2276302, and rs3737457) and rs4938066. Three of the four SNPs are overlapped in the AA sample, whereas rs10789980 locates within the

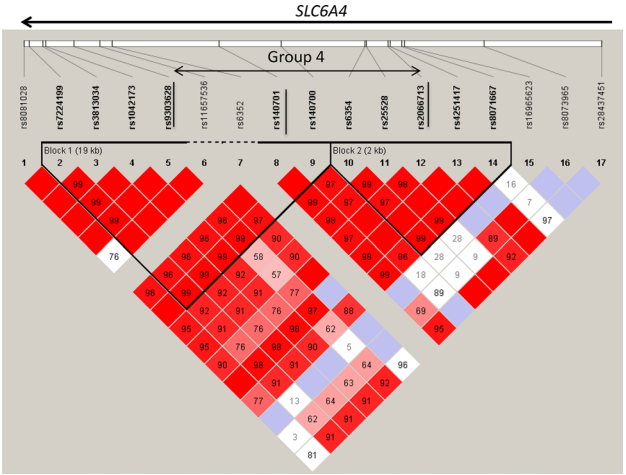

Fig. 5 LD structures for *SLC6A4* SNPs in the EA sample. Haploview (v. 4.2) (Barrett et al. 2005) was used to calculate all D' values, and haplotype blocks were defined according to Gabriel et al. (2002). The number in *each box* represents the D' value for each SNP pair surrounding that box. The *arrow* on top of the figure represents the gene transcription direction from 5′- to 3′-end. SNPs involved in later interactive models are *underlined* and grouped according to D' values <0.7. Please refer to Fig. 7 for more information about SNP groups

same open reading frame of *HTR3A* as rs1020715 and rs1062613.

By further examination of SNPs detected in these interactive models for the AA and EA samples, we found that there is one locus in *HTR3B*, one locus in *SLC6A4* and two separate loci in *HTR3A* that collaboratively contribute to AD, CD, ND and FTND in the EA sample. One locus (rs4938066 in Fig. 7) in *HTR3A* specifically influences AD, while the other locus (Group 5 in Fig. 7) affects CD, ND and FTND, which may suggest different receptor variations in AD subjects compared with CD and ND participants that couple with transporter changes in order to take effect. However, relationship among the four interactive models in the AA sample is not as obvious as it is in the EA sample. Also, Fig. 6 shows the trend that more loci in *HTR3A* are involved in the interactive models of the four phenotypes.

Previous studies by our group have shown interaction effects among *HTR3A*, *HTR3B*, and *SLC6A4* in AD and ND samples (Seneviratne et al. 2013; Yang et al. 2013). However, most case subjects included in the studies reported by Yang et al. (2013) and Seneviratne et al. (2013) have primarily only one type of addiction. Thus, this study has extended such an interaction effect among the three genes to subjects with multiple addictive phenotypes (AD, CD, and ND). This strongly implies that variants in the three genes have significant epistatic effects influencing, not only in one type of addiction but also in multiple addictions, although limited SNPs with major effects on the three genes were revealed by our previous studies (Seneviratne et al. 2013; Yang et al. 2013) and this one. Result consistency among the three studies were even revealed at the SNP level. Seneviratne et al. (2013) showed two four-variant models carried a risk for AD, which include rs10160548 in *HTR3A*, rs1176744 and rs3782025 in *HTR3B*, and 5′-HTTLPR and rs1042173 in *SLC6A4*. Yang et al. (2013) found significant interactions among rs1062613 and rs10160548 in *HTR3A*, rs1176744 in *HTR3B*, and 5′-HTTLPR and rs1042173 in *SLC6A4* in affecting ND.Rs1176744 in *HTR3B* overlaps among the three studies, which makes a residue change from Tyrosine to Serine. Besides rs1176744, this study has rs3782025 of *HTR3B* in common with Seneviratne et al. (2013) and rs1062613 of *HTR3A* with Yang et al. (2013). These three SNPs may be important serotonin-receptor- and transporter-function-modifying gene variants or in strong linkage with such variants.

One possible explanation for all these findings is that increased synaptic 5-HT, caused by limited SERT re-uptake abilities, coupled with increased 5-HT3_{AB} receptor responsiveness to 5-HT results in enhanced dopamine transmission in the reward pathway that is associated with a greater risk of multiple addictions. To take it further, cocaine inhibits SERT re-uptake (Torres et al. 2003); alcohols increase the maximal efficacy of dopamine activation of 5-HT3 receptors (Lovinger et al. 2000); both nicotine and cocaine compete with serotonin for the 5-HT3 receptor site that controls channel opening (Breitinger et al. 2001).

This hypothesis is supported by the study results of SERT-deficient mice. Researchers found that 5-HT3 receptors are upregulated in frontal cortex (+46 %), parietal cortex (+42 %), and in stratum oriens of the CA3 region of the hippocampus (+18 %) of SERT knockout mice (Mossner et al. 2004). Mutations that result in reduced or absent SERT function in mice have led to increased anxiety and stress-related behaviors. Although the effects are not as robust as those in the experimental mice, SERT-function-modifying gene variants in humans influence many of the same phenotypes (Murphy and Lesch 2008).

Considering other studies using the SAGE dataset, Bierut et al. (2010) published the first and major genome-wide association study of alcohol dependence, within which they found fifteen SNPs yielded $P < 10^{-5}$ among 948,658 SNPs analyzed. Although the best P value of our single SNP- and haplotype-based association analyses is at 10^{-4} level, on the one hand, we only analyzed 88 SNPs applying candidate gene approach; on the other hand, the significant interactive effect among *HTR3A*, *HTR3B* and *SLC6A4* may represent a way to disentangle the influence of co-morbid substance-use disorders.

In a study in AA males, Enoch et al. (2011) showed that rs1176744 in *HTR3B* influenced alcohol dependence.

Table 3 Major haplotypes (frequency ≥5 %) associated with AD, CD, ND or FTND at $P < 0.01$ level in AA sample

rs3891484-rs3758987 (*HTR3B*)			DSM-IV									FTND		
			AD			CD			ND					
1	2	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
T	T	0.633	−1.028	0.304[r]	0.012[d]	1.795	0.073[a]	0.083[r]	−0.668	0.504[d]	0.130[d]	−1.310	0.190[a]	0.064[r]
T	C	0.245	−1.501	0.133[r]		−2.360	0.018[r]		2.111	0.035[r]		1.885	0.059[r]	
C	C	0.119	3.025	**0.002[d]**		−0.341	0.733[r]		−1.978	0.048[a]		−1.817	0.069[r]	

rs17116138-rs2276307-rs11214775-rs3782025-rs1176735-rs1672717-rs17614942-rs7943062-rs7945926 (*HTR3B*)										DSM-IV									FTND		
										AD			CD			ND					
1	2	3	4	5	6	7	8	9	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
G	A	A	T	G	T	C	A	T	0.141	−1.460	0.144[a]	0.067[d]	1.832	0.067[a]	0.346[r]	−1.309	0.191[d]	0.162[a]	−0.697	0.486[r]	0.512[d]
G	A	G	T	G	T	C	G	C	0.129	0.663	0.507[r]		−0.622	0.534[d]		0.659	0.510[d]		0.457	0.647[d]	
G	A	A	T	G	T	C	G	C	0.128	–	0.264[d]		−1.346	0.178[d]		1.429	0.153[d]		−0.642	0.521[a]	
G	G	G	T	G	T	C	G	C	0.126	2.917	0.004[d]		1.131	0.258[r]		−2.618	0.009[a]		1.131	0.258[d]	
G	A	G	C	A	T	C	G	C	0.114	1.583	0.113[a]		−0.587	0.557[d]		0.309	0.757[a]		−1.477	0.140[r]	
G	A	G	C	G	T	C	G	C	0.091	0.775	0.439[r]		0.343	0.731[a]		−0.965	0.335[a]		−0.734	0.463[r]	
G	A	G	C	G	T	A	G	C	0.090	−0.576	0.564[r]		−1.213	0.225[r]		0.474	0.635[d]		1.784	0.074[d]	
A	A	G	C	A	T	C	G	C	0.083	−1.717	0.086[a]		1.143	0.253[r]		1.737	0.082[d]		−0.769	0.442[r]	
G	A	G	C	G	C	C	G	C	0.080	−0.785	0.433[d]		1.195	0.232[r]		0.784	0.433[r]		1.253	0.210[r]	

rs6354-rs25528-rs2066713, rs8071667-rs16965623 (*SLC6A4*)						DSM-IV									FTND		
						AD			CD			ND					
1	2	3	4	5	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
A	A	T	C	A	0.262	−1.631	0.103[r]	0.019[d]	−0.556	0.578[r]	0.897[d]	−0.812	0.417[d]	0.474[r]	−0.827	0.408[d]	0.215[d]
C	C	C	T	A	0.237	−2.795	0.005[a]		0.910	0.363[d]		1.442	0.149[a]		0.608	0.543[d]	
A	C	C	C	A	0.212	−1.134	0.257[r]		0.362	0.717[r]		0.307	0.759[r]		−0.992	0.321[r]	
A	A	C	C	A	0.118	1.637	0.102[r]		0.437	0.662[r]		−1.783	0.075[r]		−1.704	0.088[r]	
C	C	C	C	A	0.089	1.802	0.072[d]		−0.644	0.520[d]		−0.747	0.455[a]		−1.301	0.193[r]	
A	A	C	C	G	0.081	−0.313	0.754[d]		−1.029	0.304[d]		1.095	0.273[d]		2.119	0.034[d]	

Significant associations are given in bold. Superscripts following *P* values indicate genetic models used for analysis: *a* additive; *d* dominant; and *r* recessive. Sex, age, study and two out of three addiction status were adjusted while the other one was used as dependent variable in all statistical models

Table 4 Major haplotypes (frequency ≥5 %) associated with AD, CD, ND or FTND at $P < 0.01$ level in EA sample

rs11214769-rs1176744 (*HTR3B*)			DSM-IV									FTND		
			AD			CD			ND					
1	2	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
A	T	0.674	2.365	0.018[r]	0.058[r]	−1.159	0.246[r]	0.492[r]	−1.698	0.090[r]	0.007[a]	0.880	0.379[d]	0.625[d]
G	G	0.288	−1.608	0.108[d]		1.150	0.250[a]		−0.696	0.486[r]		−0.312	0.755[r]	
rs7942029-rs17116178 (*HTR3B*)			DSM-IV									FTND		
			AD			CD			ND					
1	2	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
A	G	0.730	−1.582	0.114[r]	0.190[r]	2.717	0.007[d]	0.055[d]	−1.317	0.188[d]	0.512[r]	0.343	0.731[d]	0.389[a]
G	G	0.169	1.036	0.300[d]		−1.356	0.175[a]		−0.665	0.506[r]		1.000	0.317[a]	
A	T	0.100	1.670	0.095[r]		−2.211	0.027[r]		1.353	0.176[r]		−1.132	0.258[r]	
rs7118530-rs12221649-rs2085421 (*HTR3A*)				DSM-IV									FTND	
				AD			CD			ND				
1	2	3	Freq	Hap Score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
T	T	G	0.605	1.252	0.211[r]	0.361[d]	1.728	0.084[d]	0.339[d]	−2.254	0.024[r]	0.101[r]	−3.119	**0.002[a]**	0.008[r]
C	T	A	0.228	−1.733	0.083[d]		−1.434	0.152[r]		0.635	0.526[d]		2.530	0.011[a]	
C	C	A	0.138	0.768	0.442[a]		−0.780	0.435[r]		1.016	0.310[r]		2.052	0.040[r]	
rs6354-rs25528-rs2066713-rs425147-rs8071667 (*SLC6A4*)					DSM-IV								FTND		
					AD			CD			ND				
1	2	3	4	5	Freq	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global	Hap score	*P* hap	*P* global
A	A	T	G	C	0.395	−1.241	0.215[d]	0.735[r]	−1.626	0.104[a]	0.082[a]	−1.296	0.195[r]	0.720[r]	−2.719	0.007[r]	0.015[r]
A	A	C	G	C	0.324	0.848	0.396[a]		0.266	0.790[a]		0.429	0.668[a]		0.807	0.420[d]	
C	C	C	G	T	0.179	0.736	0.462[d]		0.231	0.817[a]		0.388	0.698[r]		2.230	0.026[r]	
A	A	C	A	C	0.093	1.273	0.203[r]		1.418	0.156[a]		−1.177	0.239[a]		−1.180	0.238[a]	

Significant associations are given in bold. Superscripts following *P* values indicate genetic models used for analysis: *a* additive; *d* dominant; and *r* recessive. Sex, age, study and two out of three addiction status were adjusted while the other one was used as dependent variable in all statistical models

Table 5 Detected best SNP combinations in *HTR3A, HTR3B* and *SLC6A4* associated with AD, CD, ND or FTND based on cross-validation consistency (CVC), prediction accuracy and empirical *P* value from 10^7 permutations in AA and EA samples

Sample	SNP combination	Phenotype	CVC	Prediction accuracy	*P* value based on 10^7 permutations
AA	*HTR3A*: rs1020715, rs2276302, rs2085421 *HTR3B*: rs3758987 *SLC6A4*: rs25528	DSM-IV AD	8/10	57.63 %	**0.00014**
	HTR3A: rs1985242, rs4938066 *SLC6A4*: rs25528	DSM-IV CD	7/10	58.74 %	**0.0017**
	HTR3A: rs897685, rs7118530 *HTR3B*: rs1176744 *SLC6A4*: rs25528	DSM-IV ND	7/10	59.66 %	**0.000057**
	HTR3A: rs1062613, rs3737457	FTND	10/10	60.34 %	**0.000017**
EA	*HTR3A*: rs4938066 *HTR3B*: rs3782025 *SLC6A4*: rs2066713	DSM-IV AD	9/10	56.05 %	**0.00074**
	HTR3A: rs2276302 *HTR3B*: rs1672717 *SLC6A4*: rs140701	DSM-IV CD	7/10	58.27 %	**0.00029**
	HTR3A: rs897685 *HTR3B*: rs1176758 *SLC6A4*: rs9303628	DSM-IV ND	8/10	55.18 %	**0.00075**
	HTR3A: rs10789980 *SLC6A4*: rs9303628	FTND	7/10	55.25 %	**0.0039**

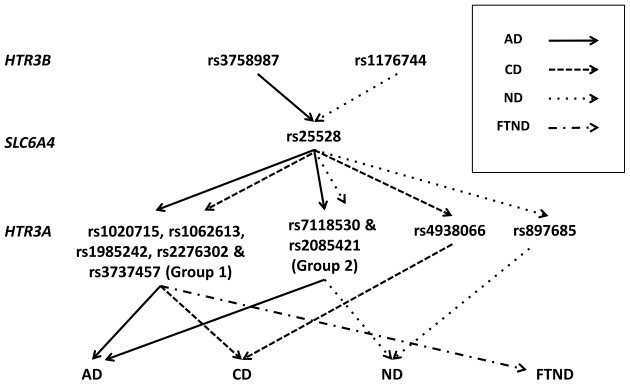

Fig. 6 Summary of detected interaction models in the SAGE AA sample. GMDR-GPU (Zhu et al. 2013) was used to perform exhaustive searches for two- to five-way interaction models. The best interaction models for AD, CD, ND, and FTND shown in the figure were determined based on CVC >7 of 10 and prediction accuracy >55 %. The *P* value associated for each model shown here was <0.005 based on 10^7 permutation tests. Interaction models with different phenotypes involved overlapped and highly correlated SNPs, which were grouped together. Group 1 includes rs1020715, rs1062613, rs1985242, rs2276302 and rs3737457; Group 2 includes rs7118530 and rs2085421. Pair-wise D' values of adjacent SNPs within each group are >0.7. SNP combinations for different phenotypes are represented by different types of *arrows*

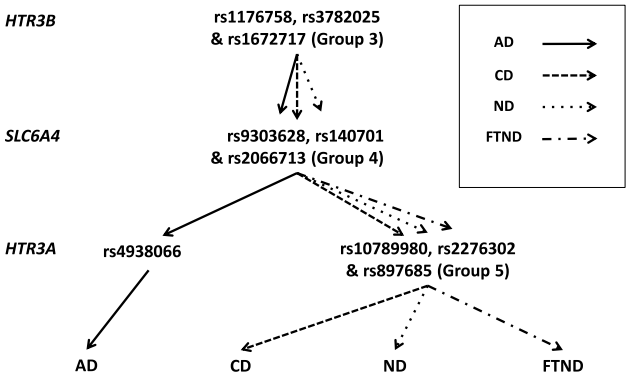

Fig. 7 Summary of detected interaction models in the EA sample. GMDR-GPU (Zhu et al. 2013) was used to perform exhaustive searches for two- to five-way interaction models. The best interaction models for AD, CD, ND, and FTND shown in the figure were determined based on CVC >7 of 10 and prediction accuracy >55 %. The *P* value associated for each model shown here was <0.005 based on 10^7 permutation tests. Interaction models with different phenotypes involved overlapped and highly correlated SNPs, which were grouped together. Group 3 includes rs1176758, rs3782025 and rs1672717; Group 4 includes rs9303628, rs140701 and rs2066713; Group 5 includes rs10789980, rs2276302 and rs897685. Pair-wise D' values of adjacent SNPs within each group are >0.7. SNP combinations for different phenotypes are represented by different types of *arrows*

In our analyses, however, we did not detect an independent effect of rs1176744; instead, we found that rs1176744 together with rs11214769 formed a major haplotype, which was significantly associated with ND in the EA sample, and together with rs897685 and rs7118530 in *HTR3A* and rs25528 in *SLC6A4* showed a significant interactive effect on ND in the AA sample. The explanation may lie in sex differences and multiple addictions.

The primary reason for us not pooling the AA and EA samples is that minor allele frequencies of most SNPs are very different for the two ethnic groups, and we wondered such pooling might yield false-positive results (Cardon and Palmer 2003). Considering the genetic heterogeneity of AA and EA populations, analyzing them separately may also reduce uncertainty and confidence interval width. Another reason is that genetic association findings in two diverse samples are providing independent replication.

This study should be considered in the context of its limitations. A functional promoter polymorphism, 5′-HTTLPR, in *SLC6A4* has been reported to have mixed associations with alcohol, cocaine, heroin, or nicotine dependence (Feinn et al. 2005; Gerra et al. 2004; Mannelli et al. 2005; Patkar et al. 2001; Saiz et al. 2009; Seneviratne et al. 2013; Yang et al. 2013). However, limited by the original GWAS data of SAGE, we do not have this polymorphism available and are not able to test its associations with the four phenotypes and interactions with other variants. This was also one of the reasons that we chose to replicate our previous findings in alcohol and nicotine dependence at gene level instead of single SNP level, since the previous interactive signals were mainly driven by 5′-HTTLPR from analyzing fewer variants compared with this study (Seneviratne et al. 2013; Yang et al. 2013). At the same time, by following this approach, we detected a new variant group (rs2066713 and rs25528) in *SLC6A4* that contributes both independently and interactively with variants in *HTR3A* and *HTR3B* to the four addictive phenotypes. These two variants are in strong LD with each other and reside in the alternative splicing region involving noncoding exons 1A, 1B and 1C, which may account for another major interactive signal in *SLC6A4*.

We also acknowledge that gene-by-gene interaction detected by this study through genetic epidemiological approach remains to be further tested experimentally in future (Cordell 2009). Even though further improvement of the GMDR-GPU is still needed, the GMDR has been successful in identifying the significant interaction of *CHRNA4* with *CHRNB2* (Li et al. 2008), *NTRK2* with *BDNF* (Li et al. 2008), and *GABBR1* with *GABBR2* (Li et al. 2009a) in ND, of *LEPR* and *ADRB2* in obesity (Angeli et al. 2011), and of *HNF4A* and *KCNJ11* in type 2 diabetes (T2D) (Neuman et al. 2010), to name a few. Although this program can theoretically handle unlimited number of SNP combinations from any GWAS data by assuming sufficient computer memory and infinite computing time, this is not the case in practice, where we are always limited by availability of hardware and computing time allowed for data analysis. Thus, one needs to keep such limitation in mind when applying this program to the dataset of their interest. Please refer to Zhu et al. (2013) for detailed descriptions of these limitations.

In summary, we showed significant interactive effects among *HTR3A*, *HTR3B*, and *SLC6A4* in AA and EA subjects with multiple addictions. Such findings not only corroborate the findings from our previous studies on single-agent addictions but also conform with the increasingly appreciated epistatic effects of variants in complex trait studies, which may account for the mysterious missing heritability (Li et al. 2008; Zuk et al. 2012).

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Conflict of interest Although not directly relevant to the work presented here, MDL has served as a consultant and board member of ADial Pharmaceuticals, LLC. JY reports no competing interests.

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