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ORIGINAL ARTICLE

Variation within the serotonin (5-HT) 5-HT_{2C} receptor system aligns with vulnerability to cocaine cue reactivity

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Cocaine dependence remains a challenging public health problem with relapse cited as a major determinant in its chronicity and severity. Environmental contexts and stimuli become reliably associated with its use leading to durable conditioned responses ('cue reactivity') that can predict relapse as well as treatment success. Individual variation in the magnitude and influence of cue reactivity over behavior in humans and animals suggest that cue-reactive individuals may be at greater risk for the progression to addiction and/or relapse. In the present translational study, we investigated the contribution of variation in the serotonin (5-HT) 5-HT_{2C} receptor (5-HT_{2C}R) system in individual differences in cocaine cue reactivity in humans and rodents. We found that cocaine-dependent subjects carrying a single nucleotide polymorphism (SNP) in the *HTR2C* gene that encodes for the conversion of cysteine to serine at codon 23 (Ser23 variant) exhibited significantly higher attentional bias to cocaine cues in the cocaine-word Stroop task than those carrying the Cys23 variant. In a model of individual differences in cocaine cue reactivity in rats, we identified that high cocaine cue reactivity measured as appetitive approach behavior (lever presses reinforced by the discrete cue complex) correlated with lower 5-HT_{2C}R protein expression in the medial prefrontal cortex and blunted sensitivity to the suppressive effects of the selective 5-HT_{2C}R agonist WAY163909. Our translational findings suggest that the functional status of the 5-HT_{2C}R system is a mechanistic factor in the generation of vulnerability to cocaine-associated cues, an observation that opens new avenues for future development of biomarker and therapeutic approaches to suppress relapse in cocaine dependence.

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INTRODUCTION

Cocaine dependence remains a challenging public health problem with relapse cited as a major determinant in its chronicity and severity.[1] With a history of cocaine use, environmental contexts and stimuli (for example, paraphernalia) become reliably associated with its use leading to durable conditioned responses ('cue reactivity') that can predict relapse as well as treatment success.[2–5] Drug cue reactivity is the attentional orientation toward such drug-associated cues that are measurable as conditioned physiological effects (for example, heart rate), subjective properties (for example, craving), appetitive approach behaviors (for example, drug-seeking) and activation of specific corticostriatal subcircuits.[1,5,6] Individual variation in the magnitude and influence of cue reactivity over behavior in humans[7,8] and animals[9,10] suggest that cue-reactive individuals may be at greater risk for the progression to addiction and/or relapse.[8,11,12] A greater understanding of the neural underpinnings of cocaine cue reactivity promises to shed light on therapeutic approaches to effectively intervene in cocaine dependence and improve recovery outcomes.

The distributed corticostriatal circuitry that controls the incentive-motivational properties of drug-associated cues involves a key modulatory role for dopamine neurotransmission.[13] Serotonin (5-HT) innervation of these interlooping pathways is also prominent[14,15] and evidence suggests a modulatory role for 5-HT neurotransmission in cue reactivity processes (for review[16]). The 5-HT_{2C} receptor (5-HT_{2C}R) is one of fourteen 5-HT-receptive proteins in brain and is prominently localized to corticostriatal subregions including the medial prefrontal cortex (mPFC) in rodents,[17] a homolog of the orbitofrontal cortex in humans.[18] This cortical region is a critical component within the circuit responsive to cocaine-associated cues in humans[19] and animals.[20,21] Stimulation of the 5-HT_{2C}R localized to the mPFC suppressed cocaine-seeking in rats,[22] an observation that recapitulates the efficacy of systemic administration of selective 5-HT_{2C}R agonists (RO 60-0175, WAY163909) to consistently reduce cue- and cocaine-primed drug-seeking.[23–27] This 5-HT_{2C}R agonist-induced functional antagonism of cocaine cue reactivity is reversed by the selective 5-HT_{2C}R antagonist SB242084 and occurs at doses of the 5-HT_{2C}R agonists that do not significantly alter general behaviors (for example, locomotor activity).[23–27] Consistent with this behavioral profile, SB242084 also increased cocaine-seeking although inter-individual variability in its efficacy was observed.[25,28–30] Finally, we recently demonstrated that cocaine cue reactivity was significantly elevated in rats following virally mediated loss of the 5-HT_{2C}R in mPFC,[31] establishing that reduced mPFC 5-HT_{2C}R function is a neurobiological mediator of cocaine cue reactivity.

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Natural variation within the 5-HT$_{2C}$R system through single nucleotide polymorphisms (SNPs) could contribute to individual differences in sensitivity to reward-associated cues in humans. The single nucleotide variant Cys23Ser (rs6318) in the human 5-HT$_{2C}$R gene (*HTR2C*) results in the substitution of a serine for a cysteine in the extracellular N-terminus of the receptor.[32] This SNP is predicted to alter protein structure and/or stability[32,33] which would be expected to alter the ability of a ligand to bind to the receptor and initiate downstream signal transduction.[34,35] In support of this concept, there is evidence that the Ser23 variant has been associated with lower sensitivity to the effects of 5-HT$_{2C}$R agonists in human studies.[36–38] As a putative reduced-function SNP in the *HTR2C* in humans, we tested the hypothesis that the Ser23 variant may associate with higher cocaine cue reactivity[31] measured as attentional bias (attentional orienting response in a computerized cocaine-word Stroop task).[6] Alignment of the human and rat 5-HT$_{2C}$R gene shows no sequence homology at the rs6318 position.[39,40] However, given our recent finding that knockdown of the 5-HT$_{2C}$R in the mPFC resulted in vulnerability to the expression of cocaine cue reactivity in rats,[31] we tested the hypothesis that individual differences in cocaine cue reactivity as measured as appetitive approach behavior [lever presses reinforced by the discrete cue complex (for example, stimulus light, pump)][6] would associate with reduced 5-HT$_{2C}$R protein expression and sensitivity to a selective 5-HT$_{2C}$R agonist.

MATERIALS AND METHODS

Assessment of 5-HT$_{2C}$R genotype and cue reactivity in cocaine-dependent subjects

Subjects. Subjects ($n = 114$) who met DSM-IV criteria for current cocaine dependence were recruited within three ongoing studies measuring cue reactivity using the same diagnostic, psychometric and advertising procedures. Subjects were recruited via newspaper advertisements, screened for psychiatric disorders using the structured clinical interview for DSM-IV (SCID-I),[41] and completed a medical history and physical examination. All subjects were tested for urine cocaine (benzoylecgonine), tetrahydrocannabinol, opioids, amphetamine, methamphetamine and benzodiazepines using the integrated E-Z split key cup II (Innovacon Company, San Diego, CA, USA) on each visit. All subjects had at least one cocaine-positive urine during screening; did not meet DSM-IV current dependence criteria for abused drugs other than cocaine, marijuana, nicotine or alcohol; did not have current or past medical disorders affecting the central nervous system; and did not have axis I disorders other than substance abuse or dependence. The subjects included non-treatment-seekers ($n = 21$) and treatment-seekers ($n = 93$). All subjects were tested during the baseline period of the studies. All subjects were free of alcohol at the time of testing as determined by a breathalyzer (Intoximeters, St Louis, MO, USA). Female subjects with a positive urine pregnancy test were excluded from the study. All data were collected in the Center for Neurobehavioral Research on Addictions at the University of Texas Health Science Center at Houston. All subjects were provided with written informed consent after being fully informed of the nature of the research in accordance with the Declaration of Helsinki. The consent form included agreement to participate in the genetic study. The study was approved by the Committees for the Protection of Human Subjects, which are the Internal Review Board of the University of Texas Health Science Center at Houston and the Baylor College of Medicine.

Cocaine-word Stroop task. The cocaine-word Stroop task was designed to measure attentional bias to cocaine-related stimuli.[42–44] It is a widely used implicit task[45] in which the participant is presented with words printed in color, and asked to discriminate the color of each stimulus; the participant is instructed to ignore the meaning of the words and concentrate only on responding to the color in which the word is written. The stimuli presented include neutral words and words that are related to the concerns or pathology under study, in this case, cocaine dependence. Slowness in responding to a color suggests distraction from color discrimination due to attention being 'captured' by the meaning of the stimulus (that is, cocaine) word.[46] Each analyzed session began with a block of 60 practice trials, followed by 30-trial blocks of test trials.[43,44] The test trials included two blocks of 30 trials with cocaine-related words, and two blocks of 30 trials with neutral words. Within each block type, each word was randomly presented three times in three different colors. Block type was alternated within each session (for example, cocaine, neutral, cocaine, neutral), and the order of block type was counterbalanced across subjects. Trials with correct responses and reaction times larger than 200 msec were used to calculate mean reaction times.[43,44] Attentional bias was operationalized as the difference between the reaction times (in msec) observed in trials with cocaine-related words and trials with neutral words, calculated for each subject and averaged across subjects. This calculation corrects for any difference in overall reaction times between cocaine-dependent and control subjects.[46] A correct response was defined as responding to the word color on an appropriately colored response button. Accuracy was assessed as the ratio of correct trials to total trials within each block type.

DNA preparation. Venous blood (10 ml) from each subject was centrifuged at 2000 r.p.m. for 30 min (Eppendorf North America, New York, NY, USA). The buffy coat was removed and stored in 2.0 ml cryogenic vials at −80 °C. DNA was isolated from the buffy coat using the Puregene Kit (Qiagen, CA, USA) according to manufacturer's recommendation. Purified DNA for each subject was dissolved in 0.25 ml DNA hydration solution.

HTR2C genotyping. The *HTR2C* has been localized to chromosome X, band q24 (female genotypes: CC, CG or GG; male genotypes: C or G).[32] All samples were assayed in duplicate on an Applied Biosystems ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Genotyping was performed with 10 ng of DNA, 1.5 µl of Taqman Genotyping Master Mix, 0.03 µl of pre-designed TaqMan primer-probe set (Applied Biosystems; Assay ID C_2270166_10) and 2.47 µl of water. PCR amplification consisted of 10 min at 95 °C, 40 cycles of 15 s at 92 °C and 1 min at 60 °C.

Statistical analyses. Reaction times and accuracy on the cocaine-word Stroop task were analyzed with a paired Student's *t*-test. Differences in age, sex, race, years of cocaine use, percent treatment-seekers, percent positive urine cocaine screens, percent alcohol abuse and percent cannabis abuse among subjects with different *HTR2C* genotypes were analyzed using one-way analysis of variance (ANOVA; age and years of cocaine use) or Fisher's exact test (sex, race, percent treatment-seekers, percent positive urine cocaine screens, percent alcohol abuse, percent cannabis abuse). Differences in attentional bias among subjects with different *HTR2C* genotypes were analyzed using one-way analysis of covariance (ANCOVA) with gene polymorphism as the independent variable and sex or race as the covariate in a general linear model. All reported *P* values for *post hoc* comparisons were Tukey–Kramer adjusted for multiple comparisons. To determine the population structure, genotypic data for ancestral informative markers for our cohort was compared against Centre d'Etude du Polymorphisme Humain–Human Genome Diversity Panel (CEPH–HGDP) samples (1035 subjects of 51 populations).[47,48] The obtained values were similar to those calculated without correction for these covariates. The alpha level for all analyses was set at $P = 0.05$.

Assessment of the 5-HT$_{2C}$R system and associated cocaine cue reactivity in rodents

Animals. Experimentally naive, male, Sprague–Dawley rats ($n = 105$) weighing 225–250 g at arrival were housed two per cage under a 12-h light-dark cycle at constant temperature (21–23 °C) and humidity (40–-50%). Food and water were available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) and with the approval of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Drugs. (–)-Cocaine (National Institute on Drug Abuse) was dissolved in 0.9% NaCl. WAY163909 ((7bR, 10aR)-1,2,3,4,8,9,10,10a-octahydro-7bH-cyclopenta-[b][1,4]diazepino [6,7,1hi]indole; a gift from Pfizer, New York, NY, USA) was dissolved in 0.9% NaCl.

Cocaine self-administration and cue reactivity assessments. Implantations of intravenous catheters with back mounts were performed under anesthesia with a cocktail containing xylazine (8.6 mg kg^{-1}), acepromazine (1.5 mg kg^{-1}) and ketamine (43 mg kg^{-1}) in bacteriostatic saline. Self-administration took place in standard operant chambers equipped with two retractable levers, a stimulus light above each lever, and a houselight housed within ventilated and sound-attenuating chambers (Med-

Associates, St Albans, VT, USA). Cocaine infusions were delivered by a syringe attached to an infusion pump located outside the chamber. Daily flushes with a solution of bacteriostatic saline containing heparin sodium (10 U ml^{-1}), streptokinase (0.67 mg ml^{-1}) and ticarcillin disodium (66.67 mg ml^{-1}) were performed to maintain catheter patency.

Self-administration consisted of 14 days of 180-min sessions, during which rats were trained to lever press to obtain a cocaine infusion (0.75 mg kg^{-1} per 0.1 ml infusion) on a fixed ratio (FR) 1 schedule before progressing to an FR5.[23,27,31,49] Schedule completions on the active lever resulted in delivery of cocaine over a 6-s period along with simultaneous illumination of the house and stimulus lights and activation of the infusion pump (discrete cue complex paired with delivery of cocaine); responses on the inactive lever were recorded but had no scheduled consequences. After cocaine delivery, the pump and stimulus light were inactivated simultaneously. The house light remained illuminated for a 20-s timeout period, during which lever presses had no scheduled consequences. Following stable self-administration on an FR5 (seven infusions per hour for at least three sessions with $<10\%$ variation in the number of infusions received for three consecutive sessions), cocaine-trained rats were subjected to a probe trial on self-administration day 12 to stratify individual rats as high cue reactive (HCR) or low cue reactive (LCR). During this 60-min probe trial, responses on the active lever were reinforced by presentation of the discrete cue complex (stimulus light, pump) previously associated with cocaine delivery. Self-administration was reinstated immediately following the end of the probe trial followed by an additional two self-administration sessions. The number of previously active lever presses during the probe trial was used to stratify rats within the HCR or LCR phenotype; a median split was used. The probe session did not interfere with the stability of self-administration as performance on the post-probe sessions was identical to the stable baseline established before the probe trial (data not shown).

Rats were returned to their home cage after 14 days of cocaine self-administration. In Experiment 1, rats ($n=12$ rats per phenotype) were reintroduced to the self-administration chambers 24 h later and assayed in a test session comprised of two sequential components. The first component evaluated whether HCR and LCR rats would exhibit differential levels of lever presses when placed in the context in the absence of the discrete cue complex. To this end, responses on both levers on an FR1 schedule were recorded but no discrete cues (for example, stimulus light, pump) were present nor delivered during the initial 10 min of the session. The second component was signaled by a single, non-response contingent delivery of the discrete cue complex presented at the termination of the first 10-min component. To assess cocaine cue reactivity, presses on the previously-active lever in the 60-min (second component) were reinforced by the discrete cue complex on an FR1; inactive lever presses were recorded but produced no scheduled consequences.[31]

In Experiment 2, rats were stratified as HCR or LCR on the basis of their performance on the probe trial (above) and returned to the self-administration chambers at 24 h of withdrawal. To assess cocaine cue reactivity, presses on the previously-active lever were reinforced by the discrete cue complex on an FR1 during a 60-min session;[31] inactive lever presses were recorded but produced no scheduled consequences. *For ex vivo* neurochemical studies, rats were sacrificed immediately after the cue reactivity test session [HCR ($n=5$), LCR ($n=6$)] or upon removal from their home cage at the expected time of that test session without re-exposure to the self-administration chambers [HCR ($n=6$), LCR ($n=6$)]; this second group of rats served as control for the behavioral experience during the cue reactivity session. For pharmacological analyses, a cohort of HCR ($n=16$ per treatment) and LCR rats ($n=16$ per treatment) were administered vehicle (saline, 1 ml kg^{-1}, intraperitoneal) or WAY163909 (0.5 mg kg^{-1}, intraperitoneal) 15 min before the start of the cue reactivity test session.

Immunoblotting. The HCR or LCR rats stratified on the probe test in Experiment 2 were evaluated for cue reactivity at 24 h of withdrawal and sacrificed immediately following the cue reactivity test session or remained in their home cages and sacrificed at 24 h of withdrawal. Rats were anesthetized [chloral hydrate solution (400 mg kg^{-1})] and decapitated; the mPFC was microdissected and flash frozen in liquid nitrogen and stored at −80 °C for subsequent crude synaptosomal protein extraction and immunoblotting.[50,51] Equal amounts of protein were separated by SDS–PAGE using 4–12% Bis-Tris gels and transferred to a PVDF membrane for immunoblotting with 5-HT$_{2C}$R antibody (D-12, sc-17797, Santa Cruz, Dallas, TX, USA; 1:100) or pan-cadherin antibody (AB6528, Abcam, Cambridge, MA, USA; 1:10 000). Membranes were incubated with mouse IgG IRDye (1:10 000) for detection by Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The integrated intensity of each band (arbitrary units) was analyzed with the Odyssey Software. The ratio of the intensity of the 5-HT$_{2C}$R-immunoreactive band to the cadherin-immunoreactive band was determined for normalization.

Statistical analyses. A one-way ANOVA (SAS 9.3) for repeated measures was used to analyze the dependent measures of the total number of active and inactive lever presses per session over the last three sessions of the self-administration phase. Student's *t*-test was employed to compare HCR and LCR rats on the total number of responses (previously active and inactive levers) and the latency to respond on the previously-active lever during the probe trial, the context-associated and cue reactivity test sessions as well as the density of 5-HT$_{2C}$R protein expression. For pharmacological analyses, a two-way ANOVA for the factors of phenotype and treatment was conducted; *a priori* comparisons between the total number of responses on the previously-active and inactive levers as well as the latency to respond on the previously active lever during the test session were made using Student's *t*-test. The experimentwise alpha level was set at $P=0.05$.

RESULTS

Assessment of 5-HT$_{2C}$R genotype and cue reactivity in cocaine-dependent subjects

The demographics of the study population are presented in Table 1. Cocaine-dependent subjects were stratified into three groups, those homozygous (CC; females) and hemizygous (C; males) for the C allele which encodes for the Ser23 variant, those homozygous (GG; females) and hemizygous (G; males) for the G allele or heterozygous (CG; females only) which encodes for the Cys variant. Genotype was not associated with the distribution of age ($F_{2,111}=0.78$; NS) or race (Fisher's exact test, NS), but was associated with sex (Fisher's exact test, $P<0.01$) (Table 1). Genotype was not associated with years of cocaine use ($F_{2,111}=0.15$, NS), percent positive cocaine urine screens (Fisher's exact test, NS), percent alcohol abuse (Fisher's exact test, NS), percent cannabis abuse (Fisher's exact test, NS), or percent treatment-seekers (Fisher's exact test, NS) (Table 1).

The response to presentation of cocaine-associated cues ('cue reactivity') was measured as attentional bias in the cocaine-word Stroop task in cocaine-dependent subjects. Several studies have reported that cocaine-dependent subjects show attentional bias to cocaine-related words whereas healthy control subjects do not.[42,44] Here, cocaine-dependent subjects had significantly longer reaction times to indicate the word color in trials with cocaine-related words than in trials with neutral words (Figure 1a; $t=6.96$;

Table 1. Demographic and clinical characteristics of cocaine-dependent subjects by *HTR2C* genotype

	Genotype		
	C/CC	*CG*	*G/GG*
Subjects (*n*)	27	6	81
Age (years ± s.e.m.)	45.37 ± 1.50	41.33 ± 2.32	43.64 ± 0.93
Sex[a]	26 M, 1 F	6 F	71 M, 10 F
Race	24 AA, 3 Cau	6 AA	54 AA, 18 Cau, 9 O
Cocaine (years ± s.e.m.)	15.56 ± 1.5	17.50 ± 2.2	15.25 ± 0.9
%Positive cocaine	78	75	74
%Alcohol abuse	31	17	25
%Cannabis abuse	22	33	10
%Treatment-seekers	70	67	86

Abbreviations: F, female; M, male; AA, African-American; Cau, Caucasian; O, other races (Hispanic, Asian). [a]Fisher's exact test, $P<0.01$.

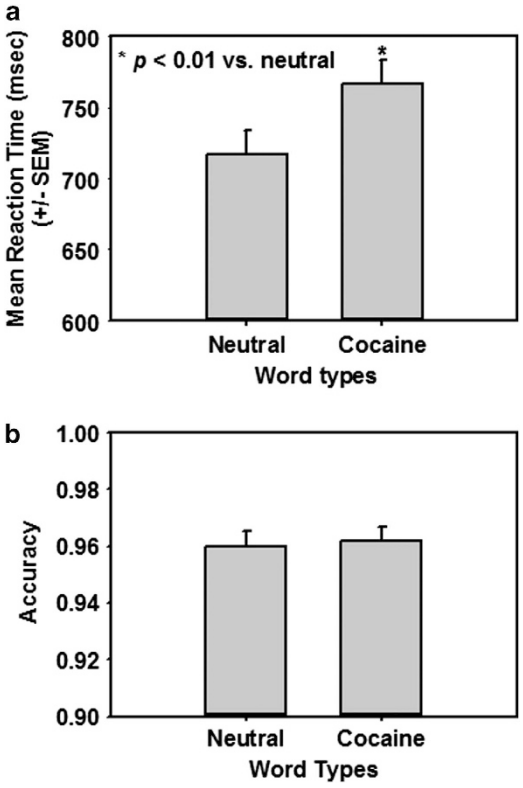

Figure 1. Cocaine-dependent subjects exhibit attentional bias toward cocaine-related words on the cocaine-word Stroop task. The difference in mean reaction times between trials with cocaine-related words and those with neutral words was used as a measure of attentional bias toward cocaine-related words. (**a**) Cocaine-dependent subjects displayed longer reaction times (msec; mean ± s.e.m.) to indicate the word color in trials with cocaine-related words vs trials with neutral words ($P < 0.01$ vs neutral words). (**b**) Accuracy (ratio of correct responses to total trials on either neutral or cocaine-related word trials) did not differ between cocaine-related and neutral word trials in cocaine-dependent subjects.

$P < 0.01$). Accuracy did not differ between cocaine-related and neutral word trials (Figure 1b; $t = -0.49$; NS). Attentional bias did not differ between cocaine-dependent treatment-seekers (48.18 ± 8.2 msec) and non-treatment-seekers (52.97 ± 14.2 msec; $t = 0.29$; NS). Our effect size is consistent with other published studies of attentional bias conducted in drug-dependent subjects.[52]

Cue reactivity was evaluated in subjects stratified for the *HTR2C* genotype. An ANCOVA demonstrated a main effect of genotype on attentional bias after adjusting for sex (Table 2; $F_{2,108} = 3.79$; $P < 0.05$). *Post hoc* comparisons revealed that attentional bias for both sexes with the C or CC genotype was significantly greater than that for subjects with the G or GG genotype (Table 2; $P < 0.05$). As 97 of 114 subjects were male and the *HTR2C* gene is X-linked, an ANOVA for male subjects only was performed. A main effect of genotype on attentional bias for male subjects was detected (Table 2; $F_{1,93} = 4.91$; $P < 0.05$); male subjects with the C genotype displayed significantly higher attentional bias than male subjects with G genotype (Table 2; $P < 0.05$). *Post hoc* comparisons indicated that attentional bias for females with the CG genotype was not different from those with the CC or GG genotype in this small sample of female subjects (Table 2, NS). An ANCOVA for all African-American (AA) subjects was also performed as 84 of 114 subjects were AA; a main effect of genotype on attentional bias was observed (Figure 2; $F_{2,81} = 4.43$; $P < 0.05$). *Post hoc* comparisons demonstrated that attentional bias for AA subjects with the C/CC genotype was significantly higher than AA subjects with the G/GG genotype (Figure 2; $P < 0.05$).

Table 2. Genotype, sex and attentional bias in cocaine-dependent subjects

Genotype		*Male*[a]	*Female*	*Both*
Male	*Female*			
C	CC	75.88 ± 14.13 (26)[b]	152.83 (1)	78.73 ± 13.89 (27)[c]
	CG	–	26.03 ± 11.04 (6)	26.03 ± 11.04 (6)
G	GG	37.80 ± 9.02 (71)	68.95 ± 27.94 (10)	41.65 ± 8.64 (81)

The numbers in the parenthesis indicate the subject number. [a]Data are presented as mean (± s.e.m.) attentional bias in msec calculated for each subject and averaged across subjects. [b]$P < 0.05$ vs G (males only). [c]$P < 0.05$ vs G/GG (both males and females).

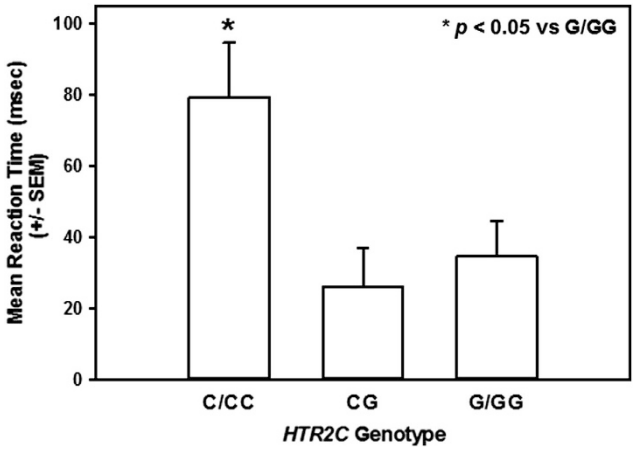

Figure 2. The highest attentional bias is seen in African-American cocaine-dependent subjects with the Ser23 protein variant. Mean reaction times (msec ± s.e.m.) for African-American subjects with the C/CC genotype which encodes for the Ser23 protein variant were significantly greater than that for African-American subjects with the G/GG genotype (*$P < 0.05$ vs G/GG genotype).

Assessment of cocaine cue reactivity in rodents

We tested the hypothesis that individual differences in HCR vs LCR rats would be observable within the context (self-administration chambers) or in the levels of cocaine cue reactivity (lever presses reinforced by the discrete cue complex). Rats in Experiment 1 readily acquired cocaine self-administration to stability; across the last three sessions (data not shown), there was no main effect of session for the number of active lever presses ($F_{2,29} = 0.14$; NS), inactive lever presses ($F_{2,29} = 1.07$; NS) or the number of infusions received ($F_{2,29} = 0.05$; NS). Rats were stratified (median split) as HCR or LCR on the basis of the number of lever presses for cocaine-associated cues during the probe session (data not shown; see Methods). Total cocaine intake did not differ between HCR rats (373.9 ± 18.3 mg kg^{-1}) and LCR rats (395.4 ± 16.6 mg kg^{-1}; NS). There was a positive correlation between previously active lever presses on the probe session with that seen on the cue reactivity test session for individual animals ($r = 0.304$; $P < 0.05$).

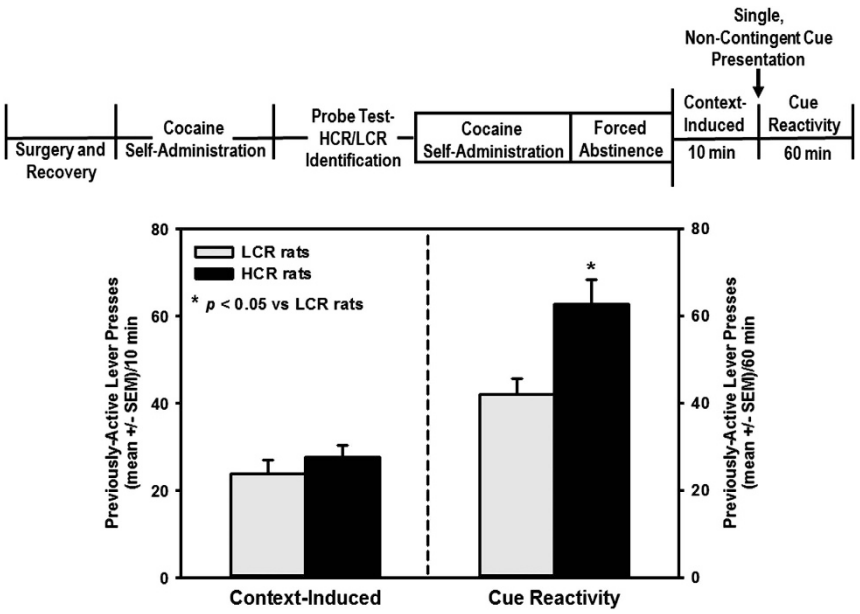

Figure 3. Individual differences in appetitive approach behavior in rats are driven by discrete cocaine-associated stimuli. The levels of operant behavior within the cocaine-associated context (left) or reinforced by the discrete cue complex (right) at 24 h of withdrawal were assessed in rats stratified as high (HCR) vs low cue reactive (LCR). Previously-active lever presses did not differ between HCR ($n = 12$) and LCR ($n = 12$) rats upon exposure to the context in the absence of the discrete cue complex (first 10 min of session; NS). HCR rats displayed significantly higher cue-reinforced lever presses (second 60 min of session) vs LCR rats (*$P < 0.05$ vs LCR rats).

The levels of operant behavior within the cocaine-taking context only or reinforced by the discrete cue complex were assessed in HCR vs LCR rats at 24 h of withdrawal. Previously active lever presses did not differ between HCR and LCR rats upon exposure to the cocaine-taking context in the absence of the discrete cue complex (Figure 3, left; $t = 0.77$; NS). HCR rats displayed significantly higher previously active lever presses that were reinforced by the discrete cue complex vs LCR rats (Figure 3, right; $t = 2.81$; $P < 0.05$). Inactive lever presses did not differ between HCR and LCR rats during the context only component (HCR = 2.5 ± 0.4; LCR = 2 ± 0.9; $t = 0.77$; NS) or the cue reactivity component (HCR = 8.4 ± 1.5; LCR = 6 ± 1.3; $t = 1.17$; NS). These data suggest that HCR and LCR rats exhibit distinct appetitive approach behavior when provided with the opportunity to deliver the discrete cue complex. The propensity to engage in appetitive behavior to deliver the discrete cue complex may represent a useful construct within which to investigate individual differences in cocaine cue reactivity.

Assessment of the 5-HT$_{2C}$R system and associated cue reactivity in rodents

We tested the hypothesis in Experiment 2 that individual differences in levels of cue reactivity would correlate with the expression of 5-HT$_{2C}$R *ex vivo*. HCR rats displayed significantly higher previously active lever presses for the discrete cue complex vs LCR rats (Figure 4a; $t = 3.65$; $P < 0.01$). Inactive lever presses (Figure 4a; $t = 0.5$; NS) and the latency to the first lever press (data not shown; $t = 0.97$; NS) were not different between HCR and LCR rats. A two-way, repeated measures ANOVA on the last three sessions of stable self-administration indicated no main effect of phenotype ($F_{1,29} = 0.02$; NS), day ($F_{2,29} = 1.43$; NS), and no phenotype × day interaction ($F_{2,29} = 1.02$; NS) for active lever presses, indicating that individual differences in cue reactivity in rats are unrelated to previous cocaine-taking history.

Figure 4b depicts representative immunoblots for mPFC synaptosomal protein from HCR and LCR rats sacrificed immediately following the cue reactivity test session. HCR rats displayed significantly lower 5-HT$_{2C}$R synaptosomal protein levels in the mPFC vs LCR rats (Figure 4c; $t = -3.75$; $P < 0.01$); an inverse correlation was observed between mPFC 5-HT$_{2C}$R synaptosomal protein and responses on the previously-active lever for the discrete cue complex in individual rats (Figure 4d; $r = 0.69$; $P < 0.05$). Because these rats underwent the cue reactivity session, such exposure could account in part for the observed changes in mPFC 5-HT$_{2C}$R protein levels. Thus, 5-HT$_{2C}$R protein levels were assessed in a cohort of cocaine-trained rats that were retained in their home cage and sacrificed 24 h after termination of cocaine self-administration sessions (that is, not tested for cue reactivity). The differential protein expression observed in HCR (0.051 ± 0.002 arbitrary units) and LCR (0.076 ± 0.008 arbitrary units) (Figure 4) rats (stratified on the probe session) is not related to the cue reactivity test itself as comparable 5-HT$_{2C}$R mPFC protein levels were observed in HCR (0.049 ± 0.01 arbitrary units) and LCR rats (0.087 ± 0.02 arbitrary units) that were not exposed to the cue reactivity test session. These data suggest that high levels of cue reactivity are associated with lower 5-HT$_{2C}$R expression in the mPFC supporting our hypothesis that differential 5-HT$_{2C}$R neurobiology may contribute to individual differences in cocaine cue reactivity.

We then tested the hypothesis that HCR and LCR rats during early withdrawal would exhibit differential pharmacological sensitivity to the suppressive effects of the selective 5-HT$_{2C}$R agonist WAY163909 over cocaine cue reactivity (Figure 5). A main effect of phenotype ($F_{1,41} = 30.93$; $P < 0.0001$), treatment ($F_{1,41} = 11.34$; $P < 0.01$), and a phenotype × treatment interaction ($F_{1,41} = 4.23$; $P < 0.05$) for previously active lever presses was observed. Saline-treated HCR rats displayed higher previously-active lever presses vs saline-treated LCR rats (Figure 5; $P < 0.05$). LCR rats treated with WAY163909 exhibited lower previously-active lever presses vs saline-treated LCR rats (Figure 5; $P < 0.05$); WAY163909 did not significantly alter previously-active lever presses vs saline in HCR rats (Figure 5; NS). WAY163909 (0.5 mg kg^{-1}) suppressed previously-active lever presses ~48% in LCR rats and ~12% in HCR rats. No main effect of phenotype ($F_{1,41} = 1.1$;

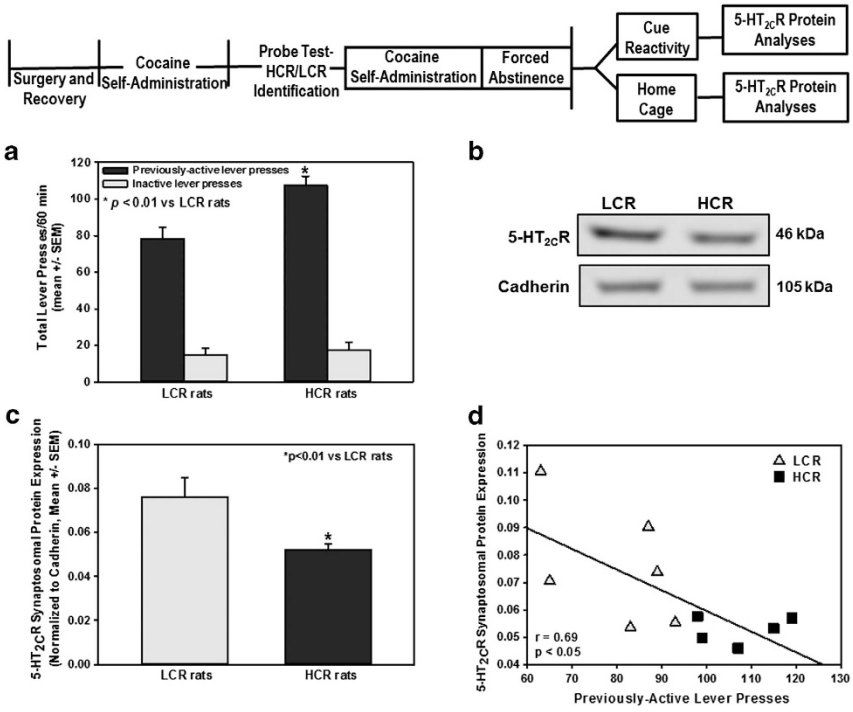

Figure 4. High cue reactive (HCR) rats exhibit lower 5-HT$_{2C}$R protein expression in medial prefrontal cortex (mPFC) relative to low cue reactive (LCR) rats. (**a**) Mean total lever presses (± s.e.m.) on the previously-active and inactive levers are presented for the cue reactivity test session. Each previously-active lever press resulted in the presentation of the discrete cue complex in the absence of cocaine delivery on an FR1. Rats identified as HCR ($n = 5$) displayed significantly higher lever presses for cocaine-associated cues vs LCR rats ($n = 6$; $^*P < 0.01$). Inactive lever presses did not differ between HCR and LCR rats. (**b**) Qualitative and (**c**) quantitative data demonstrate phenotypic differences in mPFC 5-HT$_{2C}$R synaptosomal protein expression. HCR rats displayed lower cortical synaptosomal 5-HT$_{2C}$R protein levels relative to LCR rats ($^*P < 0.05$). (**d**) An inverse correlation was observed between mPFC 5-HT$_{2C}$R synaptosomal protein and responses on the previously-active lever for cocaine-associated cues in individual rats ($r = 0.815$; $P < 0.01$). The differential protein expression observed in HCR (0.051 ±0.002 arbitrary units) and LCR (0.076 ± 0.008 arbitrary units) rats was not related to the cue reactivity test itself as comparable 5-HT$_{2C}$R mPFC protein levels were observed in HCR ($n = 6$; 0.049 ± 0.01 arbitrary units) and LCR rats ($n = 6$; 0.087 ± 0.02 arbitrary units) that remained in their home cage until sacrifice.

NS), treatment ($F_{1,41} = 0.01$; NS), and no phenotype × treatment interaction ($F_{1,41} = 1.37$; NS) for inactive lever presses during the cue reactivity test session was observed. Mean (± s.e.m.) inactive lever presses did not differ between saline-treated HCR (14.9 ±4.3; NS) and LCR rats (10.8 ± 4.6), between saline- and WAY163909-treated HCR rats (13.9 ± 6.9; NS), or saline- and WAY163909-treated LCR rats (13.2 ± 4.7; NS). No main effect of phenotype ($F_{1,41} = 3.11$; NS), treatment ($F_{1,41} = 0.25$; NS), and no phenotype × treatment interaction ($F_{1,41} = 1.34$; NS) for latency to the first lever press during the cue reactivity test session was observed. Mean (± s.e.m.) latency (sec) did not differ between saline-treated LCR (29.4 ±8.1) and HCR rats (24.5 ± 9.6; NS), saline- and WAY163909-treated LCR rats (42.9 ± 7.1; NS), or saline-and WAY163909-treated HCR rats (19.1 ± 6.4; NS). Taken together, these data demonstrate that HCR rats are less sensitive than LCR rats to the suppressive effects of WAY163909 on cue reactivity at 24 h of withdrawal.

DISCUSSION

The present study demonstrated that cocaine-dependent subjects who carry the less-common Ser23 variant of the *HTR2C* exhibit significantly higher cocaine cue reactivity than did those who carry the Cys23 variant, adding the *HTR2C* to handful of genes potentially identified as candidates involved in cocaine cue reactivity.[53,54] Likewise, in a model of individual differences in cocaine cue reactivity in rats, we identified that high cocaine cue reactivity correlated with lower levels of mPFC 5-HT$_{2C}$R protein expression and a blunted sensitivity to the suppressive effects of the selective 5-HT$_{2C}$R agonist WAY163909. Interestingly, we discovered that individual differences in drug-seeking were evident when rats were given the opportunity to deliver the discrete cue complex but not when given the opportunity to simply press levers in the cocaine-taking context, supporting the incentive-motivational value of the discrete cue complex as a key defining characteristic in provoking cocaine-seeking.[55] Together with our previous observation that knockdown of the mPFC 5-HT$_{2C}$R resulted in vulnerability to the expression of cocaine cue reactivity in rats,[31] we propose that the functional status of the 5-HT$_{2C}$R system is a mechanistic driver in the generation of vulnerability to cocaine-associated cues.

Our new finding that the Cys23Ser SNP aligns with cue reactivity in cocaine-dependent subjects supports the concept that inherent variability in 5-HT$_{2C}$R neurobiology may contribute to the liability of individuals to cocaine cues and cue-related relapse phenomena. The manner in which the Ser23 variant impacts baseline 5-HT$_{2C}$R function is not yet fully defined. The replacement of the cysteine in the extracellular N-terminus of the 5-HT$_{2C}$R is predicted to eliminate the formation of a disulfide bond, which would be expected to destabilize the receptor structure.[32,33] The impact of the Ser23 SNP on the structural integrity of the 5-HT$_{2C}$R protein could include alterations in ligand binding and downstream signaling responsivity. In COS-7 cells, the Ser23 variant exhibited lower high-affinity, but not low-affinity, binding to the 5-HT$_{2C}$R and the agonist response in these cells was more markedly desensitized relative to the Cys23 variant.[34] The 5-HT$_{2C}$R encoded by the Ser23 variant localized predominantly to the cell surface in HEK293 cells and was aligned with faster recovery of 5-HT-evoked cellular signaling following prolonged

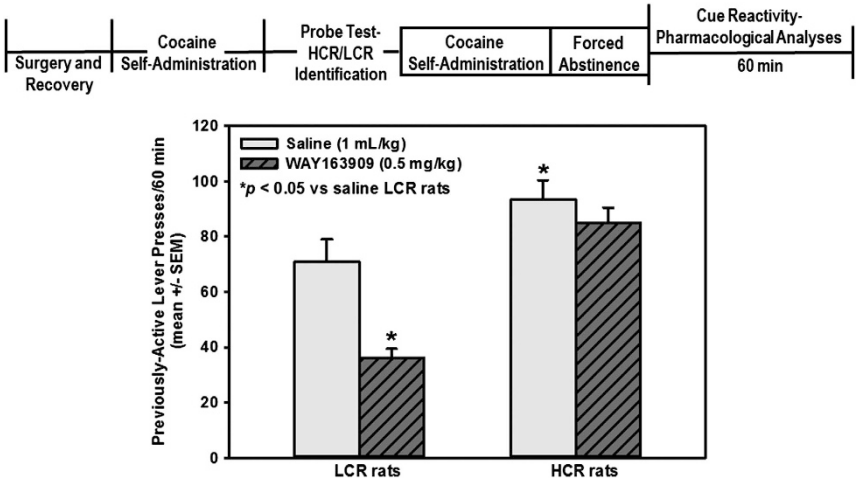

Figure 5. High cue reactive (HCR) rats exhibit lower sensitivity to the suppressive effects of the selective 5-HT$_{2C}$R agonist WAY163909 relative to low cue reactive (LCR) rats. Mean active lever presses (± s.e.m.) are presented for the cue reactivity test session. Each previously active lever press resulted in the presentation of the discrete cue complex in the absence of cocaine delivery on an FR1. Rats identified as HCR ($n = 16$ rats per treatment) and LCR rats ($n = 16$ rats per treatment) were injected with saline (15 min; 1 ml kg^{-1}, intraperitoneal) or WAY163909 (15 min; 0.5 mg kg^{-1}; intraperitoneal) prior to a cocaine cue reactivity test session on FA day 1. WAY163909 significantly attenuated lever presses for cocaine-associated cues in LCR, but not HCR, rats (*$P < 0.05$ vs saline-treated LCR rats). FA, forced abstinence.

exposure to an inverse agonist.[35] It is possible that aberrant 5-HT$_{2C}$R-mediated functions in Ser23 carriers may exhibit differential responsivity to stress[56,57] or pharmacological triggers, including 5-HT$_{2C}$R agonists.[36–38] However, there have been no experimental evaluations in animal models *in vivo* which would be valuable to tease apart the mechanisms by which the Cys23Ser SNP may drive 5-HT$_{2C}$R neurobiology and its impact on cocaine cue reactivity. Such studies are vital as a recent publication found that the Ser23 and Cys23 variants behaved indistinguishably in HEK293 and NIH-3T3 cells.[58] Thus, although there is *in vitro* evidence that the Ser23 variant leads to altered cellular responses to stimuli, definitive information remains to be collected to best understand the association reported here between expression of the Ser23 variant and enhanced cocaine cue reactivity, as well as in the clinical course of some psychiatric disorders (for review[59,60]).

There are reports of altered 5-HT$_{2C}$R responsivity after cocaine exposure in humans[61,62] and experimenter-delivered cocaine in animals.[63] Our observations that mPFC 5-HT$_{2C}$R expression and pharmacological sensitivity to a selective 5-HT$_{2C}$R agonist associate with individual variations in levels of cue reactivity in rodents are consistent with the possibility that reduced mPFC 5-HT$_{2C}$R function is a neurobiological mediator of cocaine cue reactivity.[31] These findings may be related to pre-existing neurochemical vulnerabilities specific for reward-predicting cues[31,64] or to the cyclical variations in 5-HT efflux consequent to cocaine self-administration.[65] It is currently unknown whether the difference in cortical 5-HT$_{2C}$R expression observed here translates directly to differential functional output of the receptor to manifest cue reactivity, however, high cue reactive rats were less sensitive to the suppressive effects of WAY163909. The composition of the cellular microenvironment (that is, protein-binding partners) also contributes to 5-HT$_{2C}$R-mediated signaling and agonist responsiveness.[66] We have reported that the 5-HT$_{2C}$R is localized to the postsynaptic density in PFC[50] and thus positioned to directly modulate synaptic plasticity in cortical neurons; the 5-HT$_{2C}$R agonist MK212 is reported to enhance long-term potentiation in forebrain.[67] Taken together, these biochemical and behavioral data suggest that high cocaine cue reactivity (but not sucrose cue reactivity) (Swinford-Jackson and Cunningham, unpublished)[27] may be governed by a blunted response capacity of the 5-HT$_{2C}$R. The discovery that individual differences in cue reactivity coexist concomitantly with distinct 5-HT$_{2C}$R expression patterns in the synaptosomal compartment indicates that balance in the cortical 5-HT$_{2C}$R functional status may be the key to shaping the neural state that contributes to cocaine-associated cue reactivity during abstinence.

Some limitations of this study should be noted. With the small number of female subjects in the human data set in this study and the exclusion of females in the rodent data set, the findings of this study cannot be extrapolated to women. As the *HTR2C* is X-linked, future studies should investigate the role of 5-HT$_{2C}$R neurotransmission in sex differences observed in cocaine cue reactivity as sex may be a factor that contributes to cocaine cue-related neurobiology.[68] The direct translatability of the studies presented herein is somewhat limited as there are key discrepancies in cocaine exposure patterns and cocaine use history between humans and rodents. The human data set included subjects with extensive cocaine histories, whereas the rodent data set included animals with shorter exposures to cocaine self-administration. Further, the Cys23Ser SNP has not been identified in rodents nor has the Cys23Ser SNP been tied directly to frontocortical activation patterns in response to drug-associated cues or the cortical 5-HT$_{2C}$R functional status in cocaine-dependent subjects. Nonetheless, the inclusion of the rodent study allowed for the experimental test of the hypothesis that individual differences in cocaine cue reactivity during early abstinence are associated with differential measures of cortical 5-HT$_{2C}$R neural integrity.

Our translational findings cumulatively suggest that susceptibility to cocaine cue reactivity may be related to inter-individual variation within the 5-HT$_{2C}$R system. Although other studies have examined the association of genotype with cue reactivity in cocaine users,[53,54,69] our study employed the largest sample size to date, and we are the first to have examined the association of the *HTR2C* genotype in experimentally measured cue reactivity. The rodent studies suggest that a differential 5-HT$_{2C}$R functional status, marked by lower cortical 5-HT$_{2C}$R synaptosomal protein expression and reduced pharmacological sensitivity, associates with greater reactivity to cocaine-associated cues. Future studies are required to expand on our observations to consider the 5-HT$_{2C}$R system as a risk factor or predictor of cocaine cue reactivity, and perhaps explore as a biological marker of propensity toward craving and relapse in cocaine dependence.

CONFLICT OF INTEREST

Dr Moeller is a consultant for Boehringer-Ingelheim. Dr Cunningham is a consultant for Arena Pharmaceuticals and an editor of *Neuropsychopharmacology Reviews* for which she receives compensation from the American College of Neuropsychopharmacology. The remaining authors declare no conflict of interest.

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