Neuropsychopharmacology (2015) 40, 1600–1608

www.neuropsychopharmacology.org

Variation in Dopamine D2 and Serotonin 5-HT2A Receptor Genes is Associated with Working Memory Processing and Response to Treatment with Antipsychotics

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Dopamine D2 and serotonin 5-HT2A receptors contribute to modulate prefrontal cortical physiology and response to treatment with antipsychotics in schizophrenia. Similarly, functional variation in the genes encoding these receptors is also associated with these phenotypes. In particular, the *DRD2* rs1076560 T allele predicts a lower ratio of expression of D2 short/long isoforms, suboptimal working memory processing, and better response to antipsychotic treatment compared with the G allele. Furthermore, the *HTR2A* T allele is associated with lower 5-HT2A expression, impaired working memory processing, and poorer response to antipsychotics compared with the C allele. Here, we investigated in healthy subjects whether these functional polymorphisms have a combined effect on prefrontal cortical physiology and related cognitive behavior linked to schizophrenia as well as on response to treatment with second-generation antipsychotics in patients with schizophrenia. In a total sample of 620 healthy subjects, we found that subjects with the rs1076560 T and rs6314 T alleles have greater fMRI prefrontal activity during working memory. Similar results were obtained within the attentional domain. Also, the concomitant presence of the rs1076560 T/rs6314 T alleles also predicted lower behavioral accuracy during working memory. Moreover, we found that rs1076560 T carrier/rs6314 CC individuals had better responses to antipsychotic treatment in two independent samples of patients with schizophrenia ($n = 63$ and $n = 54$, respectively), consistent with the previously reported separate effects of these genotypes. These results indicate that *DRD2* and *HTR2A* genetic variants together modulate physiological prefrontal efficiency during working memory and also modulate the response to antipsychotics. Therefore, these results suggest that further exploration is needed to better understand the clinical consequences of these genotype–phenotype relationships.
Neuropsychopharmacology (2015) **40,** 1600–1608; doi:10.1038/npp.2015.5; published online 28 January 2015

INTRODUCTION

Schizophrenia is a heritable brain disorder whose risk is likely increased by multiple genes adding small effect (McGuffin *et al*, 2001; Ripke *et al*, 2013). Dorsolateral prefrontal cortex dysfunction (Callicott *et al*, 2003) and working memory deficits (Egan *et al*, 2001) in schizophrenia are also heritable and modulated by interacting genetic variations (Bertolino and Blasi, 2009a). Importantly, individual genetic profiles also impact responses to pharmacological treatment (Blasi and Bertolino, 2006).

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Received 24 July 2014; revised 28 October 2014; accepted 5 November 2014; article preview online 7 January 2015

Dopamine D2 and serotonin 5-HT2A receptors are particularly relevant in this context. D2 receptors modulate cognitive processing (Seamans and Yang, 2004), and their altered signaling appears to confer liability for abnormal prefrontal response and clinical symptoms in schizophrenia (Abi-Dargham *et al*, 2000; Durstewitz and Seamans, 2008; Seamans and Yang, 2004). Also, they are involved in the action mechanism of antipsychotics. Furthermore, genetic variation within *DRD2* is associated with schizophrenia at genome-wide significance levels (Ripke *et al*, 2014).

The *DRD2* gene (11q23) encodes two isoforms with a mechanism of alternative splicing. The D2 long (D2L) isoform is located mainly postsynaptically, whereas the D2 short (D2S) is mainly a presynaptic autoreceptor (Usiello *et al*, 2000). In previous reports, we have found that the T allele of a single-nucleotide polymorphism (SNP) positioned in intron 6 (rs1076560, G > T) and affecting *DRD2* splicing is associated with a lower ratio of expression of

D2S/D2L compared with the G allele. Furthermore, the T allele is associated with altered working memory prefrontal physiology and behavior (Bertolino *et al*, 2009b; Zhang *et al*, 2007). This effect may reflect lower stability of prefrontal response networks during working memory processing (Seamans and Yang, 2004), which is driven by greater D2 postsynaptic signaling putatively associated with the T allele (Usiello *et al*, 2000; Zhang *et al*, 2007). Other findings also suggest association between the T allele and better clinical response after antipsychotic treatment in patients with schizophrenia (Blasi *et al*, 2011).

5-HT2A receptors have been implicated in prefrontal physiology and in schizophrenia pathophysiology. For example, 5-HT2A signaling improves prefrontal cognition (Harvey, 2003) and contributes to greater spatial tuning of prefrontal pyramidal neurons during working memory in animal models (Williams *et al*, 2002). Other investigations have revealed abnormal 5-HT2A expression and binding in patients with schizophrenia (Abi-Dargham, 2007; Gonzalez-Maeso *et al*, 2008) as well as a relationship between 5-HT2A binding and psychotic symptoms in antipsychotic-naive patients (Gonzalez-Maeso *et al*, 2008).

The gene coding for 5-HT2A (*HTR2A*, 13q14-21) has been weakly associated with schizophrenia (Ayalew *et al*, 2012; Collins *et al*, 2012; Sanders *et al*, 2008). Other investigations have linked genetic variation within *HTR2A* to response to antipsychotics (Arranz *et al*, 1998; Chen *et al*, 2009; Masellis *et al*, 1998; Olajossy-Hilkesberger *et al*, 2011). In this gene, a non-synonymous SNP located at exon 3 of *HTR2A* (rs6314, C>T, histidine>tyrosine) (Ozaki *et al*, 1996) has been linked with calcium signaling and function of phospholipases C and D (Hazelwood and Sanders-Bush, 2004; Ozaki *et al*, 1997), human hippocampal volume and activity (Filippini *et al*, 2006; Schott *et al*, 2011), episodic memory performance (de Quervain *et al*, 2003; Schott *et al*, 2011), response to treatment with clozapine, and diagnosis of schizophrenia (Arranz *et al*, 1996, 1998;Masellis *et al*, 1998; Olajossy-Hilkesberger *et al*, 2011). Furthermore, the rs6314 T compared with the C allele predicts lower 5-HT2A expression and inefficient cognitive processing in healthy subjects as well as attenuated improvement after antipsychotic treatment in patients with schizophrenia (Blasi *et al*, 2013a).

The 5-HT2A receptor interacts with dopamine systems (Di Giovanni *et al*, 2010; Fink and Gothert, 2007; Lieberman *et al*, 1998). In particular, some studies indicate that antagonism on 5-HT2A receptors indirectly increases DA release in the prefrontal cortex (Fink and Gothert, 2007). On the other hand, other studies report that 5-HT2A agonists increase but 5-HT2A antagonists decrease prefrontal dopamine efflux (Bortolozzi *et al*, 2005; Pehek *et al*, 2006). In general, a complex link between 5-HT2A and dopamine tone appears to modulate brain activity (Di Pietro and Seamans, 2007; Lieberman *et al*, 1998). Consistently, 5-HT2A and D2 receptors are both located on prefrontal pyramidal and nonpyramidal neurons (Fink and Gothert, 2007; Jakab and Goldman-Rakic, 1998, 2000; Seamans and Yang, 2004), and their signaling is also transmitted through the common intraneuronal pathways (Beaulieu, 2012; de Bartolomeis *et al*, 2013). Overall, these findings suggest that the relationship between D2 and 5-HT2A modulates neuronal function through multiple mechanisms, and that their balanced stimulation is crucial.

The aim of this study was to test whether the combined effect of *DRD2* rs1076560 and *HTR2A* rs6314 is relevant for phenotypes linked to schizophrenia. On the basis of previous findings, we hypothesized that these polymorphisms together modulate cognitive processing and response to treatment with antipsychotics. In earlier studies, the GT and CT genotypes of rs1076560 and rs6314, respectively, were associated with suboptimal cognitive processing in healthy subjects. On the other hand, rs1076560 GG and rs6314 CT were associated with better response to treatment with antipsychotics in patients with schizophrenia. Therefore, we hypothesized a joint effect of rs107650 GT and rs6314 CT genotypes in predicting suboptimal cognitive processing as well as a joint effect of rs1076560 GG and rs6314 CT genotypes in predicting worse response to antipsychotic treatment.

METHODS

Prefrontal Physiology

Subjects. Healthy subjects ($n=620$) from the region of Puglia, Italy were evaluated with the Structured Clinical Interview (First *et al*, 1996) for the Diagnostic and Statistical Manual of Mental Disorders to exclude any psychiatric disorder. Individuals were included if they had no history of significant drug or alcohol abuse, no active drug use in the past year, no head trauma with loss of consciousness, and no significant medical condition. All healthy subjects underwent one or more fMRI and cognitive testing procedures described below and were genotyped for *DRD2* rs1076560 and *HTR2A* rs6314.

Genotyping. *DRD2* rs1076560 was analyzed with allele-specific PCR primers as previously described (Zhang *et al*, 2007). Determination of *HTR2A* rs6314 genotype was conducted using the 5′exonuclease TaqMan assay as previously described (Blasi *et al*, 2013a). Both genotypes displayed Hardy–Weinberg equilibrium. Given the low number of homozygous subjects for the T allele for each genotype ($N=8$ for rs6314 and $N=7$ for rs1076560, respectively), they were collapsed in one group with heterozygous individuals (T carriers) for all subsequent analyses as previously done (Blasi *et al*, 2013a; Zhang *et al*, 2007).

fMRI

Subjects and cognitive tasks. Three hundred and twenty-two healthy subjects (169 females, mean ± SD age: 27.9 ± 7.7; IQ: 109.2 ± 12.2; handedness: 0.77 ± 0.37—Edinburgh Inventory (Oldfield, 1971)) underwent fMRI scanning while performing the two-back working memory task (see Supplementary Materials and Methods) (Blasi *et al*, 2013a). A part of these individuals was investigated in previous studies by our group addressing association of *DRD2* rs1076560 ($N=42$) (Zhang *et al*, 2007) and of *HTR2A* rs6314 ($N=169$) (Blasi *et al*, 2013a) with fMRI activity during working memory. There were 196 *DRD2* GG/*HTR2A* CC subjects, 43 *DRD2* GG/*HTR2A* T carriers, 70 *DRD2* T carriers/*HTR2A* CC, and 13 *DRD2* T carriers/*HTR2A* T carriers in this sample.

To extend working memory findings to another cognitive domain, 265 healthy subjects (144 females, mean ± SD age: 25.9 ± 5.1; IQ: 110.0 ± 11.7; handedness: 0.7 ± 0.4) were

scanned while performing the variable attentional control (VAC) task (Blasi *et al*, 2005). There were 178 *DRD2* GG/*HTR2A* CC subjects, 33 *DRD2* GG/*HTR2A* T carriers, 43 *DRD2* T carriers/*HTR2A* CC, and 11 *DRD2* T carriers/*HTR2A* T carriers in this sample. The VAC is an event-related design task that has been used in several previous investigations (Blasi *et al*, 2007, 2010, 2013a; Rasetti et al, 2010) to elicit brain activity during three levels of attentional control (low, intermediate, high) (see Supplementary Materials and Methods).

Data Acquisition and Analysis

The fMRI data were acquired with a 3.0 Tesla scanner (GE Healthcare) (see Supplementary Materials and Methods) and analyzed using Statistical Parametric Mapping 8 (SPM8) (www.fil.ion.ucl.ac.uk/spm) (see Supplementary Materials and Methods). Pre-determined condition effects at each voxel were calculated using a t-statistic for both the two-back and the VAC tasks. This procedure produced a statistical image for the contrast of two-back *vs* zero-back (N-back working memory task) and for BOLD responses relative to brain processing of stimuli for each level of attentional control elicited by the VAC (high level—HIGH, intermediate level—INT, and low level—LOW).

After individual processing, the contrast images were used in second-level random-effects models to determine task-specific regional responses at the group level. With regard to the two-back working memory task, analysis of variance (ANOVA) was performed on the contrast of interest, with *DRD2* rs1076560 and *HTR2A* rs6314 genotypes as predictors. We used a statistical threshold of $p<0.05$ and a minimum cluster size (k) = 20. Family-wise error correction was performed using BA46 within prefrontal cortex, which was our hypothesized region of interest based on previous findings (Blasi *et al*, 2013a; Zhang *et al*, 2007). The WFU_PickAtlas (http://fmri.wfubmc.edu/software/pickatlas) was used to build such volume of interest.

With regard to the VAC task, ANOVA was again performed on the contrasts of interest, with *DRD2* rs1076560 and *HTR2A* rs6314 genotypes as predictors and load as the repeated measure factor. Given that the aim of the VAC analysis was to replicate working memory findings in another cognitive domain, we used as a volume of interest a 10 mm sphere centered on the peak of activity of the prefrontal cluster associated with a *DRD2* rs1076560 by *HTR2A* rs6314 interaction in the N-back analysis. Thus, we used a statistical threshold of $p<0.05$, minimum cluster size (k) = 20, family-wise error corrected within this volume of interest.

ANOVA was used as appropriate to investigate genotype effects on behavioral data during fMRI.

Behavioral Performance During Working Memory

Five hundred and nineteen healthy subjects (260 females, mean ± SD age: 26.9 ± 7.6; IQ: 107.8 ± 12.2) performed the two-back working memory task outside the scanner. Some of these individuals were investigated in previous studies by our group addressing association of *DRD2* rs1076560 (N = 88) (Zhang *et al*, 2007) and of *HTR2A* rs6314 (N = 221) (Blasi *et al*, 2013a) with working memory behavior. There were 327 *DRD2* GG/*HTR2A* CC, 77 *DRD2* GG/*HTR2A* T carriers, 90 *DRD2* T carriers/*HTR2A* CC, and 25 *DRD2* T carriers/*HTR2A* T carriers in this sample. This task was identical to that performed in the fMRI setting (see above). Performance data were recorded as the number of correct responses (accuracy) and reaction time. ANOVA was used to investigate genotype effects on behavioral data.

Response to Antipsychotic Treatment

Subjects and designs. Two cohorts were included in this investigation. The first cohort included 63 Caucasian patients with SCID diagnosis of schizophrenia, and they were all from the region of Puglia, Italy (Table 1). Inclusion criteria were the same used for the healthy subjects enrolled in this study (see above). On the basis of *DRD2* rs1076560, there were 49 GG and 14 GT individuals, whereas there were 49 CC and 13 CT subjects based on *HTR2A* rs6314. As per protocol, all patients had not received any psychotropic

Table 1 Characteristics of the Patients with Schizophrenia Included in the Italian and American Samples to Investigate the Interaction between *DRD2* rs1076560 and *HTR2A* rs6314 on Response to Antipsychotic Treatment

N	Sex	Age	Premorbid IQ (WRAT)	Length of illness (months)	Mean chlorpromazine equivalents (mg/day)	PANSS scores at baseline	Drug-free period
Italian sample							
63 29 drug free 34 drug naive	15 females 48 males	28.3 ± 7.4	102.0 ± 7.7	59.0 ± 72.2	621.4 ± 217	Total: 103.7 ± 22.1 Positive: 25.3 ± 6.2 Negative: 25.8 ± 11.2 General psychopathology: 52.1 ± 11.9	11.9 + 19.8 months
N	**Sex**	**Age**	**IQ (WAIS)**	**Length of illness (months)**	**Mean chlorpromazine equivalents (mg/day)**	**PANSS scores at baseline (drug)**	**PANSS scores at baseline (placebo)**
American sample							
54	18 females 36 males	27.6 ± 6.3	91.9 ± 14.1	80.2 ± 70.2	593.4 ± 293.9	Total: 62.4 ± 14.4 Positive: 14.4 ± 4.4 Negative: 16.7 ± 5.4 General psychopathology: 31.2 ± 7.6	Total: 62.9 ± 14.8 Positive: 14.4 ± 4 Negative: 16.8 ± 6 General psychopathology: 31.3 ± 7.9

medication, including benzodiazepines, antidepressants, or mood stabilizers, for at least 1 week or 1 month if they were receiving depot medication prior to study inclusion. All patients then received olanzapine monotherapy for 8 weeks (mean olanzapine dose: 20.9 ± 7.3 mg). Titration was allowed for the first 4 weeks, and the dose was then kept constant until week 8. Symptoms were assessed at study entry (day 0) and at day 56 (8 weeks) with the positive and negative syndrome scale (PANSS) by only one trained psychiatrist (GC), who was blind to genotype.

The second cohort consisted of 54 partially treatment-resistant patients with schizophrenia who were admitted to the Clinical Brain Disorders Branch schizophrenia inpatient research unit at the National Institutes of Health Clinical Center, Bethesda, MD, USA. All these patients were self-reported Caucasians of European ancestry and were diagnosed with chronic schizophrenia using DSM-IV criteria. All individuals volunteered to participate in a double-blind, placebo-controlled cross-over study with standard doses of atypical antipsychotics, including olanzapine, quetiapine, risperidone, ziprasidone, and aripiprazole (Apud *et al*, 2012). The program typically enrolled patients who previously had adequate trials of standard antipsychotic medication interventions, which were only partially effective in decreasing their symptoms (see Supplementary Materials and Methods). There were 42 GG and 12 GT according to *DRD2* rs1076560, whereas there were 47 CC and 7 CT for *HTR2A* rs6314 (Table 1).

All patients were taking atypical antipsychotics before their admission. After the initial evaluation period, patients were maintained on a standard dosage of one atypical antipsychotic in an open-label fashion for several weeks before transitioning to the coded trial medication and being tapered from their other medication over a period of 4–7 days. All other medications were discontinued. Subgroup 1 ($N=32$) underwent a sequence of 4 weeks of coded placebo followed by 4 weeks of a coded standard atypical antipsychotic. Subgroup 2 ($N=22$) underwent the inverse sequence; that is, after a similar open medication and taper period, they received a standard atypical antipsychotic treatment for 4 weeks followed by placebo for 4 weeks. Weekly assessments with the PANSS were conducted independently by one of four trained research nurses. Two weeks before starting the double-blind protocol and up to 4 weeks after completing it, these evaluations were performed twice a week (see Supplementary Materials and Methods for further details).

Data Analysis

The sample size of the two patient groups was relatively small, and it was impossible to test for main effects and interactions as we did for fMRI and behavioral data. Therefore, we investigated the combined effect of *DRD2* rs1076560 and *HTR2A* rs6314 on response to antipsychotic treatment using groups reflecting available multilocus genotypic combinations. Thus, the Italian sample was composed of 14 *DRD2* GG/*HTR2A* CT, 35 *DRD2* GG/*HTR2A* CC, and 14 *DRD2* GT/*HTR2A* CC individuals. None of the subjects had the *DRD2* GT/*HTR2A* CC combination. Therefore, ANCOVA was performed with the genotypic combination as the predictor, and the difference between PANSS scores at 0 and 56 days of olanzapine treatment was the dependent variable. Mean olanzapine dose and PANSS baseline scores were specified as nuisance variables.

In the American sample there were 5 *DRD2* GG/*HTR2A* CT, 37 *DRD2* GG/*HTR2A* CC, 2 *DRD2* GT/*HTR2A* CC, and 10 *DRD2* GT/*HTR2A* CC individuals. Here, ANCOVA was performed with the genotypic combination as the predictor and the difference between the PANSS last measure on placebo and on antipsychotics as the dependent variable. Mean chlorpromazine equivalents during the trial, order, number of days on placebo, number of days on antipsychotics, PANSS baseline scores on placebo, and PANSS baseline scores on antipsychotics were used as nuisance variables.

RESULTS

Prefrontal Activity during Cognition in Healthy Subjects

Genotype groups were matched according to age, gender, IQ, and handedness (all $p>0.1$). Furthermore, there were no significant main effects or interaction between rs1076560 and rs6314 on two-back behavioral data in the fMRI sample when scanned. Thus, genotype effects on brain responses during working memory processing in this sample reflect how the brain processed working memory and not how individuals scored on the test. Consistent with previous studies (Blasi *et al*, 2013a; Zhang *et al*, 2007), ANOVA in SPM indicated that *DRD2* rs1076560 T carriers have greater activity in the left BA46 at two-back relative to GG individuals ($x=-48$, $y=40$, $z=14$; $K=100$; $Z=3.70$). Similarly, *HTR2A* rs6314 T carriers have a greater BA46 BOLD response compared with CC subjects ($x=-50$, $y=46$, $z=10$; $K=26$; $Z=3.26$). Notably, there was also an interaction between rs1076560 and rs6314 ($x=-56$, $y=36$, $z=14$; $K=45$; $Z=3.29$) (Figure 1a). Here, *post hoc* analysis on parameter estimates extracted from the significant cluster in BA46 revealed that individuals carrying the T allele for rs1076560 and the T allele for rs6314 have greater BOLD responses relative to all other genotype configurations (*post hoc*, Fisher's test, all $p<0.001$) (Figure 1b).

In order to extend fMRI working memory findings to another cognitive domain, we investigated the interaction between *DRD2* and *HTR2A* on prefrontal responses during attentional control. With this aim, we centered a 10-mm ROI on the local maxima in which the rs1076560 by rs6314 interaction was found at two-back ($x=-56$, $y=36$, $z=14$). Again, we found that the interaction between rs1076560 and rs6314 is associated with the BOLD response in this brain region ($x=-52$, $y=34$, $z=6$; $K=82$; $Z=3.45$) (Figure 1c). *Post hoc* analysis on parameter estimates was completely consistent with those of the working memory data in indicating greater BA46 activity in T carriers for rs1076560 and for rs6314 (all $p<0.05$) (Figure 1d).

Results of an exploratory, uncorrected analysis investigating further putative combined effects of rs1076560 and rs6314 in the working memory and attentional control networks are presented in Supplementary Table 1.

Behavior during Working Memory

Genotype groups were matched according to age, gender, IQ, and handedness (all $p>0.1$). ANOVA on accuracy data

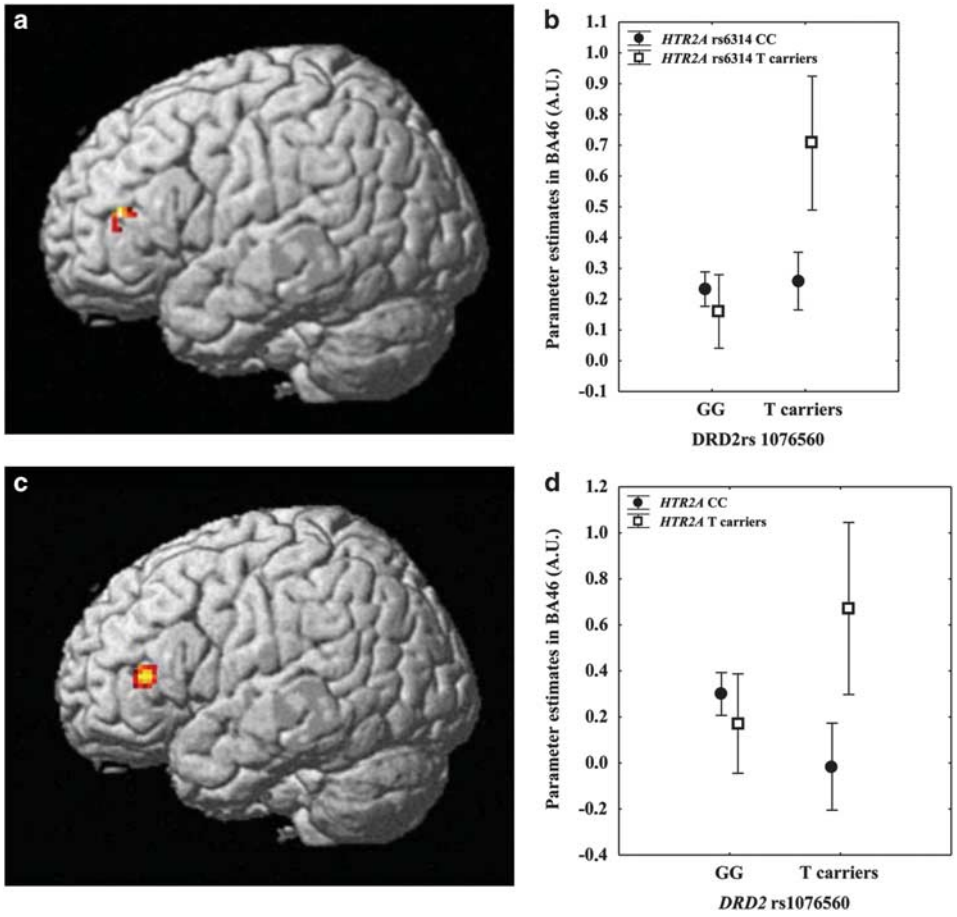

Figure 1 (a) Render of the brain showing the dorsolateral prefrontal cluster in BA46 associated with the *DRD2* rs1076560 by *HTR2A* rs1130233 interaction during performance of the N-back task, and (b) relative parameter estimates to illustrate the difference in activity between genotype groups. (c) Render of the brain showing the dorsolateral prefrontal cluster in BA46 associated with the *DRD2* rs1076560 by *HTR2A* rs1130233 interaction during performance of the VAC task, and (d) relative parameter estimates to illustrate the difference in activity between genotype groups.

indicated a main effect of rs1076560 ($F_{1, 515} = 10.1$; $p = 0.001$) with greater percent correct responses in GG individuals compared with T carriers. Furthermore, there was a main effect of rs6314 ($F_{1, 515} = 4.8$; $p = 0.02$) such that there was greater accuracy in CC compared with T-carrying subjects. Importantly, there was an rs1076560 by rs6314 interaction ($F_{1, 515} = 10.8$; $p = 0.001$). More in detail, subjects carrying the T allele for rs1076560 and for rs6314 had a lower percentage of correct responses relative to all other genotype groups (Fisher's *post hoc*: all $p \leqslant 0.001$) (Figure 2).

Response to Treatment with Antipsychotics

In the Italian sample, groups were matched according to age, gender, premorbid IQ, length of illness, drug-free period, and baseline PANSS scores (all $p > 0.15$). Mean olanzapine dose during the trial was greater for *DRD2* GG/*HTR2A* CT *vs* *DRD2* GG/*HTR2A* CC and *DRD2* GT/*HTR2A* CC subjects (all $p < 0.04$), whereas it did not differ between the latter two groups ($p > 0.3$). ANCOVA with olanzapine mean dose and baseline PANSS scores as nuisance variables indicated a main effect of genotypic combination on the difference in negative symptoms between 0 and 56 days of olanzapine treatment ($F_{2, 58} = 3,1$; $p = 0.05$) (Figure 3a). Fisher's *post hoc* test revealed that *DRD2* GT/*HTR2A* CC individuals have greater improvement in negative symptoms relative to those with *DRD2* GG/*HTR2A* CC ($p = 0.02$) and *DRD2* GG/*HTR2A* CT ($p = 0.005$) genotypic combinations. There was no difference between *DRD2* GG/*HTR2A* CT and *DRD2* GG/*HTR2A* CC patients ($p = 0.2$). Furthermore, no main effect of genetic combination was present on total, positive, and general psychopathology PANSS scores (all $p > 0.5$).

Groups of the American sample did not differ for age, gender, IQ, length of illness, day on placebo and on antipsychotics, and chlorpromazine equivalents during the active phase of the trial. Baseline PANSS scores did not differ as a function of genotypic combination in both the placebo and the antipsychotics arm (all $p > 0.2$). ANCOVA, with order, PANSS baseline scores, mean chlorpromazine equivalents during the active arm, and days on placebo and days on antipsychotics as nuisance variables indicated a statistical trend for an effect of genotypic combination on positive symptoms scores ($F_{3,44} = 2.4$; $p = 0.07$) (Figure 3b), which was significant after removing the two individuals with *DRD2* GT/*HTR2A* CT genotypes (Figure 3b) ($F_{2,43} = 3.5$; $p = 0.03$). Exploratory Fisher's *post hoc* analysis indicated that *DRD2* GT/*HTR2A* CC subjects had better

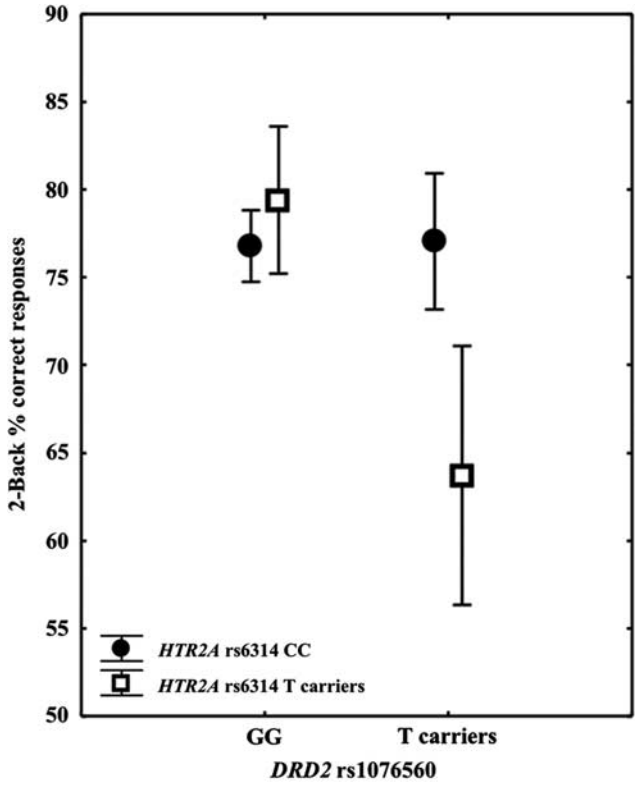

Figure 2 Graph showing the interaction between *DRD2* rs1076560 and *HTR2A* rs6314 on behavioral accuracy at the two-back working memory task.

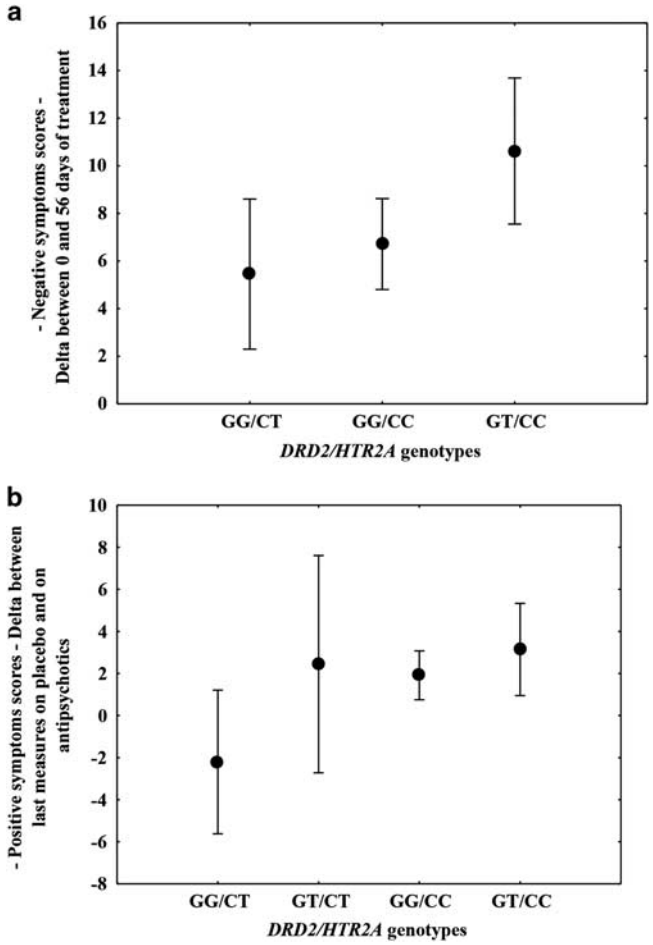

Figure 3 Association of *DRD2*/*HTR2A* genotypic combinations scores with response to treatment with antipsychotics. (a) In the Italian sample, there was an association between genotypic combination and response to olanzapine treatment in terms of improvement in negative symptoms. (b) In the American sample, there was a similar association in terms of positive symptoms. See text for statistics.

responses to antipsychotic treatment compared with *DRD2* GG/*HTR2A* CT individuals ($p<0.007$). This result is similar to those found in the Italian sample, even if in another symptom domain. No statistically significant difference was present between *DRD2* GT/*HTR2A* CC, *DRD2* GT/*HTR2A* CT, and *DRD2* GG/*HTR2A* CC groups (all $p>0.2$). Furthermore, the latter two genotypic configurations had better responses compared with the *DRD2* GG/*HTR2A* CT group (all $p\leqslant 0.04$). No statistically significant effects of the genotypic combination was present on PANSS total, negative, and general psychopathology measures (all $p>0.6$).

DISCUSSION

The present results suggest that *DRD2* rs1076560 and *HTR2A* rs6314 together modulate brain activity and behavioral accuracy during working memory in healthy subjects; therefore, these results extend in larger samples previous findings indicating their separate effects. Furthermore, these results also suggest the combined effect of these polymorphisms in the modulation of response to antipsychotic treatment in patients with schizophrenia.

The pooled impact of rs1076560 and rs6314 on working memory processing is manifested in individuals carrying the T allele in both genes, which is consistent with our earlier results (Blasi *et al*, 2013a; Zhang *et al*, 2007). Of note, this combined effect was reproduced when we investigated prefrontal activity associated with attentional processing, which is another cognitive domain involved in the pathophysiology of schizophrenia (Blasi *et al*, 2010; Weickert *et al*, 2000). Furthermore, we also found that individuals carrying the T allele in both genes also have lower behavioral accuracy during working memory in a large sample of healthy subjects. Previous studies have indicated that genetic variability in healthy subjects drives variation in prefrontal activity (Blasi *et al*, 2013b; Huffaker *et al*, 2009) and lowers behavioral performance (Goldberg *et al*, 2003). All together, these findings are reminiscent of results in patients with schizophrenia (Callicott *et al*, 2000), and they have been interpreted as the need for greater recruitment of cortical resources to perform the task with reduced or similar behavioral proficiency (Bertolino and Blasi, 2009a; Manoach, 2003). Consistent with this interpretation, our imaging and behavioral results together suggest that healthy subjects with the T allele for *DRD2* rs1076560 and the T allele for *HTR2A* rs6314 have inefficient working memory processing reflected by greater prefrontal activity and lower behavioral accuracy relative to individuals with different genotypic configurations.

The mechanisms on which these genotype/phenotype relationships rely may be based on molecular effects of *DRD2* rs1076560 and *HTR2A* rs6314 variants on D2 and 5-HT2A expression and signaling. The rs1076560 T allele is associated with relatively greater expression of D2L *vs* D2S (Zhang *et al*, 2007) compared with the G allele. This effect may increase D2 postsynaptic signaling (Usiello *et al*, 2000) and thus decrease the stability of prefrontal response networks and efficiency during working memory processing (Seamans and Yang, 2004). Also, the T allele of *HTR2A* rs6314 has been linked to less efficient activity and behavior during working memory in a previous report (Blasi *et al*, 2013a). This effect may be based on association of the T allele with reduced expression of the 5-HT2A receptor (Blasi *et al*, 2013a), whose signaling appears to facilitate mnemonic processes in prefrontal pyramidal neurons involved in working memory (Williams *et al*, 2002). Although these findings implicate a separate impact of rs1076560 and rs6314 on prefrontal processing via modulation of D2 and 5-HTA signaling, consistent evidence also indicates that 5-HT2A affects dopamine release (Di Giovanni *et al*, 2010). Based on this knowledge, a possible interpretation of the results of the present study is that the combined genetic effect of *DRD2* and *HTR2A* affects working memory processing via their modulation of D2 and 5-HT2A signaling on activity of prefrontal pyramidal neurons. For example, a genetically mediated 5-HT2A expression on prefrontal dopaminergic terminals may differentially regulate dopamine release (Fink and Gothert, 2007). Furthermore, 5-HT2A may also be differently expressed on prefrontal pyramidal neurons and interneurons as a function of rs6314 genotype. Thus, genetically induced levels of 5-HT2A receptors may in turn interact with lower or greater expression of D2L postsynaptic receptors as a function of *DRD2* variation in determining differential levels of stimulation of prefrontal pyramidal neurons or interneurons of relevance for cognitive processing. Another synergistic or alternative mechanism of the combined effect of rs1076560 and rs6314 on working memory processing is that stimulation of D2 and 5-HT2A receptors implicates intraneuronal adjustments that modulate common pathways including molecules such as AKT1 and GSK3beta (Beaulieu, 2012; de Bartolomeis *et al*, 2013), whose expression and related genetic variation have been associated with schizophrenia (Blasi *et al*, 2011, 2013b; Tan *et al*, 2008). In line with the relevance of common signaling cascades, current evidence also suggests that the cross-talk between D2 and 5-HT2A may be facilitated by the assembly of heteromers (Albizu *et al*, 2011; de Bartolomeis *et al*, 2013; Fuxe *et al*, 2014). Thus, the genetic variation in *DRD2* and *HTR2A* may affect how D2/5HT2A heteromers modulate signaling transduction of relevance for schizophrenia-related phenotypes.

We also find that *DRD2* rs1076560 and *HTR2A* rs6314 together impact the response to antipsychotic treatment in patients with schizophrenia. Specifically, we find that rs1076560 GT/rs6314 CC individuals have better responses to antipsychotics. This association, which is statistically significant in the Italian sample and at a statistical trend level in the American sample, is mainly elicited when comparing rs1076560 GT /rs6314 CC with rs1076560 GG/rs6314 CT subjects. The rs1076560 T allele and the rs6314 C allele separately predicted better response to treatment with second-generation antipsychotics in previous studies (Blasi *et al*, 2011, 2013a). The fact that genetic variation related to D2 and 5-HT2A signaling have a combined effect on clinical response to second-generation antipsychotics is in line with the antagonistic effects of these drugs on both these receptors. Furthermore, the finding that the therapeutic effect of second-generation antipsychotics is better elicited by specific *DRD2* and *HTR2A* configurations suggests that specific *DRD2* and *HTR2A* alleles may produce patterns of D2 and 5-HT2A expression that are more likely to receive benefit from treatment. The fact that the T allele of rs1076560 confers risk for suboptimal prefrontal activity in healthy subjects but that it is also associated with advantage in responding to treatment with antipsychotics requires some explanation. These results are to be interpreted in the context of a possible abnormal dopamine tone and 5-HT2A expression in patients. It is indeed possible that the reduction of dopamine tone and of 5HT2A receptors in the prefrontal cortex of patients with schizophrenia interact with the effects of genetic variation to determine the clinical effects. Regardless of the specific interpretation, these results add that the *DRD2*/*HTR2A* interaction is also relevant to modulate clinical response to treatment in patients with schizophrenia.

A limitation of the pharmacogenetic results is the limited size of the two samples used in this study; these samples did not allow us to canonically test for separate main effects of genotypes and their interaction. Larger sample sizes are needed to detect multilocus interaction, or conversely, to demonstrate additivity. Notwithstanding this limitation, we find that the genotype–genotype combination is—or tends to be—associated with response to second-generation antipsychotic treatment in different symptom domains in the two populations investigated, that is, negative symptoms in the Italian population and positive symptoms in the American sample. These results may reflect another limitation of the study. In fact, these independent samples had different characteristics and underwent different protocols of treatment. In particular, the Italian cohort included drug-free (mean drug-free period: 11.9 + 19.8 months) or drug-naive, acutely ill patients undergoing an 8-week olanzapine-treatment trial. The American sample was composed of suboptimally treated patients before their admission in a placebo-controlled, cross-over trial. Indeed, the antipsychotic dose levels were significantly tapered in this sample before the standardized psychopharmacological protocol. Thus, it is possible that different trial and population characteristics may reflect on differential detection of genotype effects as clinically elicited. However, the relationships with clinical symptoms that we find in two cohorts suggest that the combined effect of *DRD2* and *HTR2A* polymorphisms is still present even if different methodological approaches are used in limited samples of patients with non-homogeneous characteristics.

In conclusion, the imaging, behavioral, and pharmacogenetic findings of this study implicate the combined effect of *DRD2* and *HTR2A* genetic variations in phenotypes of physiological and clinical interest linked to schizophrenia. They also suggest that the investigation of interactions among multiple genetic characteristics might shed light on the aspects of the physiology of the brain and on the development of brain disorders.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Professor Bertolino is a full-time employee of Hoffman-La Roche.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)