

Research Article

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Authors for correspondence:

*Giuseppe Blasi,

E-mail: giuseppe.blasi@uniba.it

Antonio Rampino, E-mail:

antonio.rampino@uniba.it

Evidence of an interaction between *FXR1* and *GSK3 β* polymorphisms on levels of Negative Symptoms of Schizophrenia and their response to antipsychotics

Antonio Rampino^{1,2*} , Silvia Torretta¹ , Barbara Gelao¹, Federica Veneziani^{1,3}, Matteo Iacoviello¹ , Aleksandra Marakhovskaya³, Rita Masellis¹, Ileana Andriola², Leonardo Sportelli¹ , Giulio Pergola^{1,4} , Alessandra Minelli^{5,6} , Chiara Magri⁵ , Massimo Gennarelli^{5,6} , Antonio Vita^{5,7}, Jean Martin Beaulieu³, Alessandro Bertolino^{1,2} and Giuseppe Blasi^{1,2*}

¹Group of Psychiatric Neuroscience, Department of Basic Medical Sciences, Neuroscience and Sense Organs, University of Bari Aldo Moro, Bari, Italy; ²Azienda Ospedaliero-Universitaria Consorziale Policlinico, Bari, Italy; ³Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada; ⁴Lieber Institute for Brain Development, Johns Hopkins Medical Campus, Baltimore, Maryland, USA; ⁵Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy; ⁶Genetics Unit, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy and ⁷Department of Mental Health and Addiction Services, ASST Spedali Civili of Brescia, Brescia, Italy

Abstract

Background. Genome-Wide Association Studies (GWASs) have identified several genes associated with Schizophrenia (SCZ) and exponentially increased knowledge on the genetic basis of the disease. In addition, products of GWAS genes interact with neuronal factors coded by genes lacking association, such that this interaction may confer risk for specific phenotypes of this brain disorder. In this regard, fragile X mental retardation syndrome-related 1 (*FXR1*) gene has been GWAS associated with SCZ. *FXR1* protein is regulated by glycogen synthase kinase-3 β (*GSK3 β*), which has been implicated in pathophysiology of SCZ and response to antipsychotics (APs). rs496250 and rs12630592, two eQTLs (Expression Quantitative Trait Loci) of *FXR1* and *GSK3 β* , respectively, interact on emotion stability and amygdala/prefrontal cortex activity during emotion processing. These two phenotypes are associated with Negative Symptoms (NSs) of SCZ suggesting that the interaction between these SNPs may also affect NS severity and responsiveness to medication.

Methods. To test this hypothesis, in two independent samples of patients with SCZ, we investigated rs496250 by rs12630592 interaction on NS severity and response to APs. We also tested a putative link between APs administration and *FXR1* expression, as already reported for *GSK3 β* expression.

Results. We found that rs496250 and rs12630592 interact on NS severity. We also found evidence suggesting interaction of these polymorphisms also on response to APs. This interaction was not present when looking at positive and general psychopathology scores. Furthermore, chronic olanzapine administration led to a reduction of *FXR1* expression in mouse frontal cortex.

Discussion. Our findings suggest that, like *GSK3 β* , *FXR1* is affected by APs while shedding new light on the role of the *FXR1/GSK3 β* pathway for NSs of SCZ.

Introduction

Genome-Wide Association Studies (GWASs) identified hundreds of low penetrance genetic loci involved in risk for Schizophrenia (SCZ) [1,2]. GWAS alleles clustering to specific biological pathways may underlie specific illness phenotypes [3–5]. However, risk genes also interact with genes that, though not surviving statistical thresholds of Genome-Wide association, may have a role in the pathophysiology of SCZ, thus potentially impacting on the full biological manifestation of risk [6].

Among genetic loci associated with SCZ by GWAS, fragile X mental retardation syndrome-related 1 (*FXR1*) codes for fragile X mental retardation syndrome-related protein 1 (FXR1P), an RNA binding protein related to the fragile X mental retardation protein (FMRP) [1,7]. FXR1P is known to interact with FMRP [8,9], and large-scale genetic studies have consistently indicated involvement of FMRP targets in the genetic architecture of SCZ [10,11]. Furthermore, molecular studies have demonstrated that FXR1P is potentially regulated by dopamine receptor [12,13] and regulates ionotropic Glutamate Receptor [13]. Both types of receptors have robustly been

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implicated in the pathophysiology of SCZ and mechanism of action of Antipsychotic (AP) medication [14,15]. However, whether FXR1P can be modulated by APs has not been demonstrated.

In a previous study [12], we have demonstrated a functional interaction between FXR1P and the glycogen synthase kinase-3 β (GSK3 β). This kinase phosphorylates FXR1P and facilitates its degradation in neurons. Importantly, the GSK3 β gene has been consistently implicated in the modulation of SCZ-related phenotypes [16], along with response to APs [17–19]. Furthermore, analyses of *postmortem* brains have showed decreased GSK3 β phosphorylation and protein levels in frontal cortex or lower GSK3 β mRNA levels in dorsolateral prefrontal cortex (PFC) of SCZ as compared to healthy individuals [20–22]. GSK3 β is a known effector of Type 2 Dopamine Receptor (DRD2) signaling [23–25]. DRD2 has been involved in the pathophysiology of SCZ [26–29] and is the main molecular target of AP medication [30,31]. In addition, the contribution of GSK3 β to AP response has been also related to alternative molecular pathways not directly involving DRD2 and dopamine neurotransmission as a whole, such as those related to Wnt pathway, glutamate receptors, and serotonin receptors [22,32,33].

We identified two SNPs associated with *postmortem* PFC FXR1 and GSK3 β mRNA expression rs496250 and rs12630592, that have a combined effect on behavioral and brain phenotypes related to the processing of emotions [12]. More specifically, the interaction between the rs496250 and rs12630592 SNPs in healthy subjects is associated with Emotional Stability, as defined within the Big Five Personality Trait model, as well as with amygdala activity during an emotion recognition task. These variants may also affect symptom severity in bipolar disorder [34].

Emotional Stability and amygdala activity during emotion processing are linked with Negative Symptoms (NSs) of SCZ [35–39], a core clinical domain of the disorder at least partially heritable [40,41] and associated with genetic variation by both candidate gene approaches and GWASs [42–47]. This suggests that the GSK3 β –FXR1 signaling module and related genetic variation affecting GSK3 β and FXR1 expression levels may be involved in brain and clinical phenotypes related to NSs, potentially including response to AP treatment. On this basis, we investigated the interaction between rs496250 and rs12630592 functional variations within FXR1 and GSK3 β [12,16] on NS severity and response to AP in patients with SCZ.

Furthermore, we investigated putative modulation of FXR1 by AP—as already reported for GSK3 β [17–19]—by studying the effect of chronic administration of the second generation AP olanzapine on mouse frontal cortex FXR1 gene expression.

We hypothesized that rs496250 and rs12630592 interact on NS severity and response to AP in patients with SCZ and that olanzapine administration is associated with FXR1 expression in mouse frontal cortex.

Methods and Materials

Experiments in humans

Samples

Discovery Sample. We pooled data from two independent samples (Samples 1 and 2) into a single Discovery Sample (DS) in order to maximize our sample size and reduce Type I errors.

Sample 1 included 266 patients with SCZ or Schizoaffective disorder (201 males; Mean Age: 35.9 \pm SD = 10) recruited in the region of Apulia, Italy. Recruitment procedures were carried out in

accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), and approval was given by the local ethics committee (“Comitato Etico Indipendente Locale—Azienda Ospedaliero-Universitaria Consorziata Policlinico di Bari”). Diagnosis of SCZ was made using the Structured Clinical Interview for the DSM-5, Axis 1 disorders (*Diagnostic and Statistical Manual of Mental Disorders: DSM-5*, Arlington, VA: American Psychiatric Publishing, 2013), which was administered by psychiatrists. Patients were excluded if they had: a significant history of drug or alcohol abuse; active drug abuse in the previous year; experienced a head trauma with a loss of consciousness; or if they suffered from any other significant medical condition. NSs were assessed at study entry (T0) and at Day 28 (4 weeks or T1) with the PANSS. Such a scale was administered by a trained psychiatrist, who was blind to FXR1 rs496250 and GSK3 β rs12630592 genotypes.

Patients were treated for 4 weeks with an AP therapy (Mean AP stable dose = 574.9-mg Chlorpromazine Equivalents [CEs]). More in detail, the majority of patients underwent monotherapy with Olanzapine (73 out of 266). Other interventions included Risperidone, Clozapine, Quetiapine, Aripiprazole, Paliperidone, and Haloperidol. Fifty one out of 266 patients received more than one AP, and 20 out of 266 underwent concomitant medication with antidepressants, while 51 out of 266 underwent concomitant medication with mood stabilizers.

Sample 2 included a subgroup of individuals recruited within the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) Study [48]. Characteristics of the CATIE sample are described elsewhere [49]. For the purpose of the current study, 121 subjects with diagnosis of SCZ according to DSM-4 having full genetic and clinical information were studied (91 males; Mean Age: 38.9 \pm SD = 11.5). As in Sample 1, NSs were assessed at the study entrance (T0 or study baseline) and 1 month later (T1; mean AP stable dose = 479.8-mg CEs). In detail, patients underwent treatment with Olanzapine, Quetiapine, Risperidone, Perphenazine, and Ziprasidone, and overlap between different APs was permitted only for the first 4 weeks after randomization. Concomitant medications were allowed throughout the trial, except for additional AP agents. Sixty one out of 148 patients in Sample 2 underwent concomitant medications with antidepressants.

Replication Sample. The Replication Sample included 116 patients with SCZ and schizophreniform disorder (49 males; Mean Age \pm SD: 39 \pm 12.85) recruited at the University of Brescia who satisfied the criteria of DSM-5 [50]. Subjects underwent monotherapy with Olanzapine ($N = 58$) or Risperidone ($N = 58$). Changes in symptom severity were monitored by administering the PANSS scale at the study entrance (T0 or study baseline) and after 2 weeks of stable treatment (T1)

Genotyping

Sample 1

FXR1 rs496250 and GSK3 β rs12630592 genotypes in Sample 1 were ascertained using an Illumina HumanOmni2.5-8 v1 BeadChip platform (Illumina, Inc., San Diego, CA, USA). More in detail, approximately 200-ng DNA was used for genotyping analysis. DNA was concentrated at 50 ng/ml (diluted in 10-mM Tris/1-mM EDTA) with a Nanodrop Spectrophotometer (ND-1000). Each sample was whole-genome amplified, fragmented, precipitated, and resuspended in appropriate concentrations of hybridization buffer. Denatured samples were hybridized on the prepared Illumina HumanOmni2.5-8 v1 BeadChip. After hybridization, the

Table 1. Genetic data distribution of the Discovery Sample (Samples 1 and 2) and of the Replication Sample.

		Samples used to study the effect of genotypes on Negative Symptoms (NSs)			Samples used to study the effect of genotypes on NS response to antipsychotics				
		<i>GSK3β</i> rs12630592			<i>GSK3β</i> rs12630592				
Discovery Sample		GG	GT	TT	GG	GT	TT		
Sample 1	<i>FXR1</i> rs496250	GG	54	90	32	GG	8	21	6
		A-carriers	27	52	11	A-carriers	7	17	4
Sample 2	<i>FXR1</i> rs496250	GG	30	43	16	GG	30	42	16
		A-carriers	9	18	5	A-carriers	9	18	5
Replication Sample		GG	T-carriers		GG	T-carriers			
	<i>FXR1</i> rs496250	GG	32	46	GG	32	46		
		A-carriers	17	21	A-carriers	17	21		

BeadChip oligonucleotides were extended by a single labeled base, which was detected by fluorescence imaging with an Illumina Bead Array Reader. Normalized bead intensity data obtained for each sample were loaded into the Illumina GenomeStudio (Illumina, v.2010.1) with cluster position files provided by Illumina, and fluorescence intensities were converted into SNP genotypes. After genotypes were called and the pedigree file was assembled, we removed SNPs showing minor allele frequency (MAF) < 1%, genotype missing rate > 5%, or deviation from Hardy–Weinberg equilibrium ($p < 0.0001$). Individuals were also removed if their overall genotyping rate was below 97%. Sample duplications and cryptic relatedness were ruled out through identity-by-state analysis of genotype data.

Sample 2

Genotyping procedures for Sample 2 are described elsewhere [49].

Because of the low MAF (A) of rs496250, in all analyses, A-homozygote subjects (AA) were collapsed with heterozygotes (AG) as in a previous report [12]. Genotype composition of our samples is described in Table 1.

Replication Sample

Genotyping procedures for Replication Samples are described elsewhere [50].

Furthermore, since, in this cohort, the number of minor allele carrier individuals was extremely small (*FXR1* rs496250 AA/*GSK3β* rs12630592 GG = 1, *FXR1* rs496250 AA/*GSK3β* rs12630592 GT = 0, *FXR1* rs496250 AA/*GSK3β* rs12630592 TT = 0, *FXR1* rs496250 AG/*GSK3β* rs12630592 GG = 16, *FXR1* rs496250 AG/*GSK3β* rs12630592 GT = 14, and *FXR1* rs496250 AG/*GSK3β* rs12630592 TT = 7), within the following statistical analyses, we collapsed individuals with *FXR1* rs496250 AA and AG genotypes in a single “A-carriers” group, and *GSK3β* rs12630592 TT and AT genotypes in a single “T-carriers” group.

Genotype composition of the Replication Sample is provided in Table 1.

Statistical analyses

Pooling of Samples 1 and 2

Before pooling Samples 1 and 2 into the DS, the two samples were investigated for putative differences in age, gender, PANSS Negative Scores at study baseline, and dose of APs as converted to CEs [51]. One-way analysis of variances (ANOVAs) using sample as the

independent variable and either age, PANSS Negative Scores, or CE AP dose as the dependent variable were used to assess sample matching as for these variables. A Pearson’s chi-square test was used to check for gender matching between the two samples. Moreover, in order to further control for inter-sample heterogeneity, each individual was given a factor level dichotomous variable (Sample Factor [SF]), indicating the sample s/he belonged to and SF was introduced as covariate of no interest in all statistical analyses [52].

ANOVA revealed that age was lower in Sample 1 than in Sample 2 ($p < 0.003$), PANSS Negative Scores were higher in Sample 1 than in Sample 2 ($p < 0.0001$), and mean stable dose of APs expressed in CE was higher in Sample 1 than in Sample 2 ($p = 0.0005$). No statistically significant difference was observed across gender distribution in the two samples ($p > 0.05$).

Therefore, age, gender, CE, and SF were introduced as covariates of no interest in the statistical model. Furthermore, we used genome-wide genotypes to compute genomic eigenvariates, which afford a multidimensional representation of ancestry by means of singular value decomposition applied to allelic count at each polymorphic locus considered. We thus obtained, within each dataset we used for our analyses, a set of variables representative of population stratification. Both the cohorts we recruited in Bari for the current study (Sample 1) and the CATIE sample (Sample 2) included Caucasian ancestry male and female participants; hence, genomic eigenvariates in these samples indexed a relatively restricted range of population stratification.

More in detail, we computed genomic eigenvariates by performing a Principal Component Analysis separately for each of the two cohorts using SNPs with high imputation quality (INFO > 0.8), low missingness (<1%), MAF > 0.05, and in relative linkage equilibrium after two iterations of linkage disequilibrium (LD) pruning ($r^2 < 0.2$, 200 SNP windows). We removed long-range-LD areas (MHC and chr8 inversion).

Effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes and their interaction on NSs

We performed a factorial ANOVA to investigate the main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes and their interaction on NSs, with the genotypes of interest as independent variables and the PANSS NS Score after 1 month of stable dose of AP treatment (T1, or Day 28 for Sample 1, and Visit 1 for Sample 2), as the dependent one. Potential confounding effects of population stratification were corrected for by marginalizing the PANSS NS

Score for the first five principal genomic eigenvariates, separately for each cohort. Standardized residuals were computed by performing linear regression analysis with the first five principal genomic eigenvariates as independent variables, and the PANSS NS Score as the dependent variable. Site-specific standardized residuals were then used for the analysis. To provide further confirmation of results, analogous analyses were performed in Samples 1 and 2 of the DS separately with the same statistical approach described above (See the Supplementary Material).

Confirmatory analysis was performed on the Replication Sample by using the same statistical approach. Principal genomic eigenvariates were computed as described for the DS. CEs were not used as covariates in this analysis, because they were not available in this sample.

Finally, in order to assess the specificity of rs496250 and rs12630592 effects on NSs, similar analyses were performed on Positive and General Symptoms of SCZ, respectively, measured with the “Positive” and “General” subscales of the PANSS.

All post hoc analyses were performed using Fisher’s test. Based on our strong a priori hypothesis on the effects of rs496250 and rs12630592 on phenotypes of interest based on the DS results, one-tailed statistics was used in post hoc analyses on the Replication Sample.

Effect of *FXR1* rs496250 and *GSK3 β* rs12630592 genotypes and their interaction on Negative Symptom response to APs

Response to APs in terms of NSs was measured as the variation of PANSS Negative Scores from T0 to T1 that we indicated as Δ -N-PANSS. In order to establish the main effect of *FXR1* rs496250 and *GSK3 β* rs12630592 genotypes and their interaction on Δ -N-PANSS, we performed a factorial analysis of covariance (ANCOVA), with *FXR1* rs496250 and *GSK3 β* rs12630592 genotypes as independent variables and Δ -N-PANSS as the dependent one. Since response to APs could be affected by severity of NSs at the study entry (T0) and by the stable dose APs subjects were assuming, we normalized the Δ -N-PANSS to PANSS Negative Scores at T0.

Again, potential confounding effects of population stratification were corrected for by marginalizing the Δ -N-PANSS for the first five principal genomic eigenvariates, separately for each cohort. Standardized residuals were computed by performing linear regression analysis with the first five principal genomic eigenvariates as independent variables, and the Δ -N-PANSS as the dependent variable. Site-specific standardized residuals were then used for the analysis.

Because of study discontinuation, 183 out of 387 patients in the DS (males 121; Mean Age = 29.4 \pm SD = 8.2) who entered the study were assessed at T1 and were available for Δ -N-PANSS computation.

Confirmatory analysis using the same statistical approach was performed on the Replication Sample by using the same statistical approach. Principal genomic eigenvariates were computed as described for the DS. CEs were not used as covariates in this analysis, because they were not available in this sample.

Moreover, to provide further confirmation of results, analogous analyses were performed in Samples 1 and 2 separately with the same statistical approach described above (See the Supplementary Material).

Finally, in order to assess the specificity of rs496250 and rs12630592 effects on NSs, similar analyses were performed on Positive and General Symptoms of SCZ, respectively, measured with the “Positive” and “General” subscales of the PANSS.

All post hoc analyses were performed using Fisher’s test. Based on our strong a priori hypothesis on the effects of rs496250 and

rs12630592 on phenotypes of interest based on the DS results, one-tailed statistics was used in post hoc analyses on the Replication Sample.

Animal experiments

Animals

Ten-week-old C57BL/6 J mice were used for current experiment. All mice were housed individually in controlled 12-hr light/12-hr dark cycle, constant temperature, and humidity environment. No changes in corn cob layer were made during the entire experimental period. All animals in the experiment were drug naïve and were used only for a single experiment. All animal procedures were performed in accordance with the Canadian Council of Animal Care guideline and following formal approval by the University of Toronto Animal Ethics Committee.

Treatment

The activity of *GSK3 β* has been shown to be affected by APs in several experimental settings [20,53]. To verify whether *FXR1P* can also be affected by AP drugs, mice were treated with olanzapine for 30 days in chow. Mice were randomly assigned to two different arms of treatment (10 mice for each arm), one olanzapine-treated and the other one vehicle-treated. 54 mg/kg concentration pure olanzapine administered to animals in chow. Olanzapine dose was adjusted in order to reach a steady-state plasma level (21 \pm 5 ng/ml) closed to previously reported [54] clinically relevant range (10–50 ng/mL). Chow without olanzapine was used as vehicle.

Tissue dissection

Mice were sacrificed after 30 days of treatment by rapid cervical dislocation. Brains were dissected on an ice-cold surface. PFC 500-nm-thick serial coronal sections were prepared using ice-cold adult mouse brain slicer and matrix (Zivic Instruments, Pittsburgh, PA, USA), and PFC was sectioned with microsurgical knife. Finally, samples were stored at -80°C until analysis.

PFC RNA extraction and qPCR

Impact of treatment on *FXR1* expression in the PFC was evaluated using quantitative PCR. Total RNA was extracted from mouse PFC using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA, USA) and converted into cDNA using SuperScript IV VILO Master Mix synthesis system (Invitrogen #11756050; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instructions.

qPCR analysis was performed according to TaqMan Fast Advanced Master Mix protocol on a QuantStudio3 Real-Time PCR System (Thermo Fisher Scientific) using Thermo Fisher Scientific Mm00484523_m1 *FXR1* probe and Thermo Fisher Scientific Mm99999915_g1 *GAPDH* probe as internal control. Relative expression quantification analyses were carried out on biological triplicates of each sample on a QuantStudioTM Design and Analysis Software (Thermo Fisher Scientific). Mean Ct values of *FXR1* were normalized to those of *GAPDH*. These normalized values were analyzed through the comparative Ct Method for the relative quantification of targets as previously reported [55].

Statistical analysis

A one-way ANOVA with *FXR1* gene expression level as the dependent variable and treatment arm (olanzapine vs vehicle) as the independent variable was performed in order to establish the impact of olanzapine as compared to vehicle on *FXR1* expression.

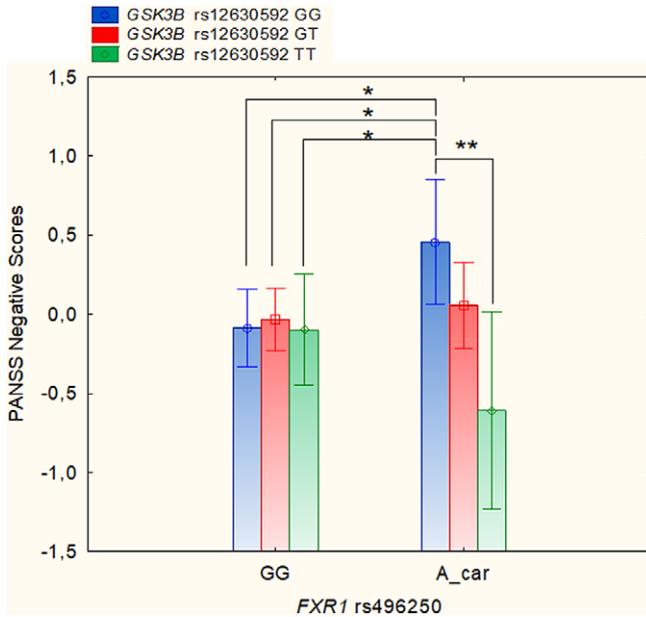


Figure 1. Interaction between *FXR1* rs496250 and *GSK3β* rs12630592 genotypes on Negative Symptom severity in the Discovery Sample. Subjects carrying *GSK3β* rs12630592 GG genotype and *FXR1* rs496250 A-carrier have higher N-PANSS compared with *GSK3β* rs12630592 GT/*FXR1* rs496250 A-carrier and with *GSK3β* rs12630592 TT/*FXR1* rs496250 A-carrier subjects. Furthermore, *GSK3β* rs12630592 GT/*FXR1* rs496250 A-carrier subjects have higher N-PANSS than *GSK3β* rs12630592 TT/*FXR1* rs496250 A-carrier subjects. Bar graphs show mean \pm SE. * indicates $0.01 < p\text{-value} < 0.05$. ** indicates $0.001 < p\text{-value} < 0.01$. See text for detailed statistics.

Results

Interaction of *FXR1* rs496250 and *GSK3β* rs12630592 on Negative Symptom severity

In the DS, factorial ANOVA on NS severity indicated no main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes (all $p\text{-values} > 0.05$), while their interaction was significant ($F = 3.11$; $p = 0.045$; Figure 1). Fisher's post hoc analyses showed that, in the context of *FXR1* rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher N-PANSS compared with rs12630592 TT ($p = 0.005$) subjects. Furthermore, *FXR1* rs496250 A-carrier/rs12630592 GG subjects have higher N-PANSS than *FXR1* rs496250 GG/rs12630592 GG subjects ($p = 0.02$), *FXR1* rs496250 GG/rs12630592 GT subjects ($p = 0.03$), and *FXR1* rs496250 GG/rs12630592 TT subjects ($p = 0.04$).

Similar analyses on the Replication Sample indicated consistent results with those obtained on the DS. In detail, we found that *FXR1* rs496250 and *GSK3β* rs12630592 genotypes interacted on NS severity ($F = 4.3$; $p = 0.04$; Figure 2). Fisher's one-tailed post hoc analyses showed that, in the context of *FXR1* rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher PANSS NS Scores compared with rs12630592 T-carrier ($p = 0.045$) subjects. Furthermore, rs12630592 GG/*FXR1* rs496250 A-carrier subjects have higher PANSS NS Scores than *GSK3β* rs12630592 GG/*FXR1* rs496250 GG subjects ($p = 0.025$).

Separate analyses on Samples 1 and 2 indicated consistent results with those obtained with the pooled Samples 1 and 2 (see the Supplementary Material).

No main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes, nor rs496250-by-rs12630592 interaction was observed on the PANSS "Positive" and "General" subscale scores (all $p\text{-values} > 0.05$).

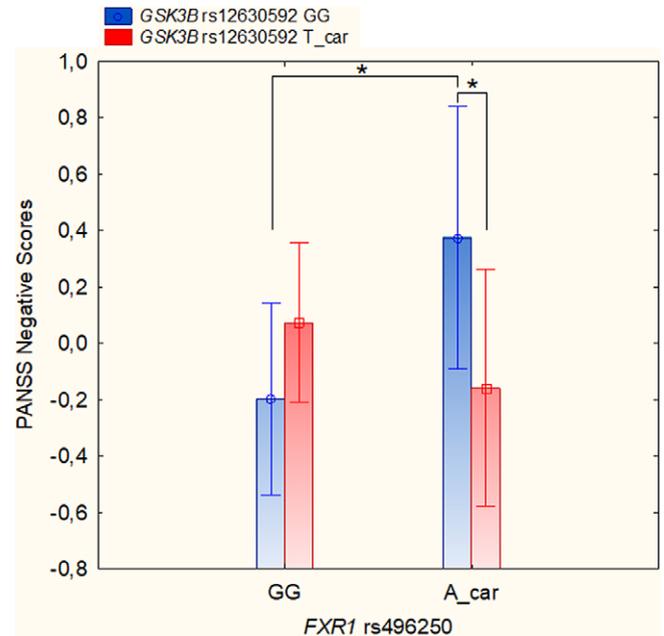


Figure 2. Interaction between *FXR1* rs496250 and *GSK3β* rs12630592 genotypes on Negative Symptom severity in the Replication Sample. In the context of *FXR1* rs496250 A-carrier genotype, subjects carrying rs12630592 GG genotype have higher PANSS NS Scores compared with rs12630592 T-carrier subjects. Furthermore, rs12630592 GG subjects have higher PANSS NS Scores than *GSK3β* rs12630592 GG/*FXR1* rs496250 GG subjects. Bar graphs show mean \pm SE. * indicates $0.01 < p\text{-value} < 0.05$. See text for detailed statistics.

Interaction of *FXR1* rs496250 and *GSK3β* rs12630592 on Negative Symptom response to APs

In the DS, factorial ANCOVA showed no main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes on Δ -N-PANSS (all $p\text{-values} > 0.05$). Nonetheless, the same analysis indicated a significant interaction between rs496250 and rs12630592 on Δ -N-PANSS ($F = 3.3$; $p = 0.05$; Figure 3). Post hoc analyses indicated that, in the context of *FXR1* rs496250 A-carrier genotype, subjects with rs12630592 TT genotype have higher Δ -N-PANSS compared with both rs12630592 GT ($p = 0.003$) and rs12630592 GG ($p = 0.006$) genotypes. Statistically significant difference in Δ -N-PANSS was also observed across rs12630592 genotypes in the context of *FXR1* rs496250 GG individuals. More specifically, in the context of *FXR1* rs496250 A-carrier genotype, subjects with rs12630592 TT genotype have higher Δ -N-PANSS compared with *FXR1* rs496250 GG/rs12630592 GG ($p = 0.01$), *FXR1* rs496250 GG/rs12630592 GT ($p = 0.007$), and with *FXR1* rs496250 GG/rs12630592 TT ($p = 0.03$) individuals. Separate analyses on Samples 1 and 2 indicated consistent results with those obtained on pooled Samples 1 and 2 (see the Supplementary Material).

Similar analyses on the Replication Sample found no main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes, nor any interaction between the two genotypes, on Δ -N-PANSS. We reasoned that a possible interpretation of such an inconsistency may be related to the T0-T1 time interval used in the replication cohort. On this basis, we explored mean values of Δ -N-PANSS as a function of the different genotypic configurations. This inspection revealed that, in the context of *FXR1*-A-carrier genotype, *GSK3β* rs12630592 TT individuals had greater mean values of Δ -N-PANSS compared to *GSK3β* rs12630592 GG and GT subjects, which is consistent with directionality of results in the DS.

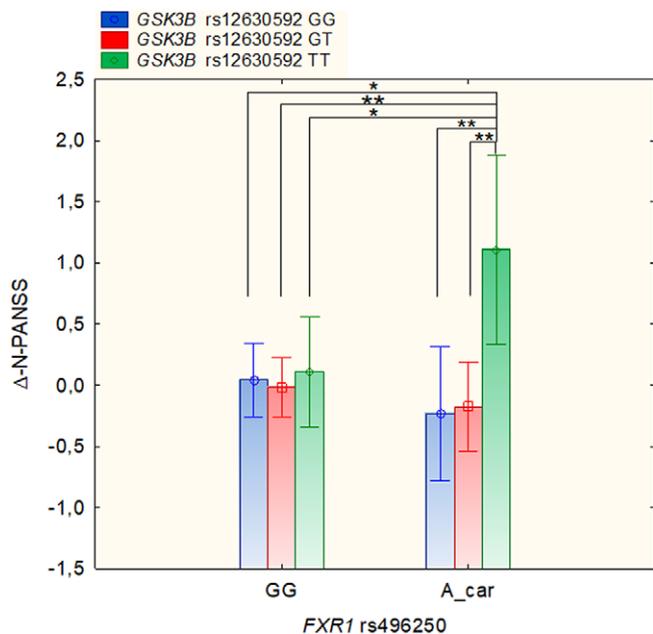


Figure 3. Interaction between *FXR1* rs496250 and *GSK3β* rs12630592 genotypes on Negative Symptom response to antipsychotics in the Discovery Sample. Subjects carrying *GSK3β* rs12630592 TT genotype and *FXR1* rs496250 A-carrier have higher Δ -N-PANSS compared with *GSK3β* rs12630592 GT/*FXR1* rs496250 A-carrier and with *GSK3β* rs12630592 GG/*FXR1* rs496250 A-carrier subjects. Bar graphs show mean \pm SE. * indicates $0.01 < p$ -value < 0.05 . ** indicates $0.001 < p$ -value < 0.01 . See text for detailed statistics.

No main effect of *FXR1* rs496250 and *GSK3β* rs12630592 genotypes, nor rs496250-by-rs12630592 interaction was observed on the variation of the PANSS “Positive” and “General” subscale scores (all p -values > 0.05).

FXR1 gene expression is affected by AP treatment in the mouse brain

ANOVA revealed PFC *FXR1* expression was reduced ($0.282 \pm .077$ -fold, $n=4$ animals per group) in mice treated with olanzapine as compared to those treated with vehicle [ANOVA: $F(3.2)$, $p < 0.05$].

Discussion

Previous evidence has suggested that the molecular interplay between *FXR1* and *GSK3β* may have a role in the pathophysiology of SCZ, in particular, in regulation of emotional phenotypes associated with NSs of the disorder [12,56]. Previous findings have also implicated *GSK3β* in the mechanism of action of APs [53]. Here, we show that *GSK3β* interacts with *FXR1* functional variation in modulating SCZ NS severity and response to APs and that, similarly to *GSK3β* [17–19], *FXR1* expression is affected by APs.

In humans, we found that rs496250 and rs12630592 interact on NS severity and response to APs in patients with SCZ, providing evidence that the *FXR1*–*GSK3β* pathway is involved in modulation of such a symptom domain. In fact, we found that subjects carrying a genotypic condition associated with lower PFC *GSK3β* expression (rs12630592 TT individuals) show lower NS severity and greater response to APs in terms of negative symptomatology compared with other rs12630592 genotypes in the context of higher predicted *FXR1* PFC expression (rs496250 A-carrier individuals). Notably, in previous work by our group [46], the same genotypic configuration

(*FXR1* rs496250 A-carrier *GSK3β* rs12630592 TT individuals) was associated with higher emotional stability and left amygdala activity during emotion processing. Therefore, it is possible that genetic regulation of brain *FXR1*–*GSK3β* expression, while modulating emotion processing and stability in healthy individuals, impacts on NS regulation in patients with SCZ. Such a possibility is in line with evidence that NSs are associated with both emotional stability [48] and amygdala activity [36] in patients with SCZ.

Indeed, results on *FXR1*-by-*GSK3β* interaction on response to APs were not replicated in the current study, even though, when exploring mean values of delta PANSS as a function of the different *FXR1*-by-*GSK3β* genotypic configurations, we observed that in the context of *FXR1*-A-carrier genotype, *GSK3β* rs12630592 TT individuals had greater mean values of delta PANSS compared to *GSK3β* rs12630592 GG and GT subjects, which is consistent with directionality of results in the DS. Lack of replication in the results might be due to the T0–T1 time interval used in the replication cohort, which is designed to investigate quite fast responses to treatment and is shorter compared to those of the DS. This aspect may have prevented statistically significant detection of subtle genetic effects on response. Furthermore, we found that chronic administration of Olanzapine at a clinically relevant dose for 30 days to a sample of C57BL/6 J mice was associated with lower *FXR1* transcription levels in mouse *postmortem* PFC compared with vehicle-treated animals. This suggests that *FXR1* is engaged in the chain of molecular events involved in the mechanism of action of APs, or at least of Olanzapine, as previously reported for *GSK3β*. Quite interestingly, a Transcriptome-Wide Association Study [57] has reported brain *FXR1* mRNA expression is upregulated in patients with SCZ, a finding that might be in line with our current result of an *FXR1* mRNA reduction following chronic administration of an AP agent in mouse brain. To the best of our knowledge, this is the first study reporting a possible link between AP treatment administration and *FXR1* expression in brain. We are aware that the animal model we here adopted is limited by the exclusive use of olanzapine as AP treatment. Nonetheless, it represents a strong indication for future replicative studies on animal models adopting different AP drugs.

At the protein level, *GSK3β* and *FXR1* are involved in a negative regulatory interaction in which *GSK3β* inactivates *FXR1* [12]. Therefore, it is possible that genetic factors modulating brain expression of the two proteins, such as rs1263590 and rs4962590 allelic variation, may amplify *FXR1*/*GSK3β* physiological signaling. Importantly, this signaling has been implicated in a number of molecular events that may play a role in the regulation of NS severity and response to treatments. For example, *FXR1*–*GSK3β* interplay has been implicated in dopamine signaling mediated by DRD2 [25], that has been consistently linked with NS pathophysiology and response to APs [12,58–60]. On this basis, it is possible that genetically determined imbalance between *GSK3β* and *FXR1* brain expression modulates the impact of DRD2-mediated dopamine signaling on NSs and their response to agents that primarily target and antagonize DRD2, that is, APs. *GSK3β* activity is known to be regulated by DRD2 signaling and can be pharmacologically inhibited by APs having a DRD2 antagonist activity [17,24,61]. Furthermore, CRISPR/Cas9-mediated selective knockout (KO) of *GSK3β* in DRD2 expressing neurons of the adult mouse PFC results in a reduction in social interaction, a prototypical proxy of human social withdrawal, which, in turn, is a typical NS of SCZ [62].

Another possible interpretation of our results is related to the relationship between *GSK3β*/*FXR1* pathway, glutamate signaling,

and phenotypes of relevance for NSs of SCZ. In this regard, a recent report indicates that, in mouse, *FXR1* and *GSK3 β* modulate glutamatergic neurotransmission via regulation of AMPA receptor subunits GluA1 and GluA2, as well as vesicular glutamate transporter VGlut1 [56]. Furthermore, other findings reveal that KO of glutamate AMPA receptor 1 in mice (GluA1-KO) is associated with impaired social behavior [63], a prototypical model of SCZ NSs [64,65]. Moreover, in humans, genetics and neurophysiology studies have consistently implicated glutamatergic signaling in NSs [40,66], and pharmacology investigations have suggested that such a signaling represents a potential target for NS treatment [67,68]. Furthermore, *GRIA2* gene, coding for the glutamate ionotropic receptor AMPA type subunit 2, is associated with improvements in NSs in patients treated with APs [69]. It is thus possible that genetically determined modulation of the *FXR1*–*GSK3 β* pathway in brain impacts on NS severity and response to APs by its intermediate tuning on glutamate neuronal signaling. Rather importantly, our results indicate that the impact of genetic variation potentially regulating *FXR1*–*GSK3 β* signaling on psychopathology of SCZ is quite specific for NSs, whereas it is not significant for positive symptoms. One possible explanation of such a finding is that, since there is evidence that SCZ negative and positive symptom domains are supported by different neurobiological and brain circuitry systems [70,71], it is possible that genetic variation impacting on one of the two domains has less pronounced effects on the other one. This seems particularly plausible in light of existing evidence that genomic variation associated with SCZ at genome-wide level of significance clusters to different biological ontologies that distinctly support either positive or NSs [46].

As a whole, our results suggest that, while individually implicated in the pathophysiology of SCZ by GWASs and molecular biology studies, *FXR1* and *GSK3 β* are players of one single molecular pathway with a potential role in modulation of NS severity and response to APs. This could be relevant to future setup of new pharmacological tools to treat NS domain of SCZ. We are aware that a major limitation of our study is that genetic variation of rs12630592 and rs4962590 is only a proxy of *GSK3 β* and *FXR1* PFC mRNA expression, respectively, thus not providing any actual measure of these genes' transcriptional levels in the whole brain. In addition, mRNA expression itself is a proxy of one gene translation into the corresponding protein. Therefore, it is possible that the genetic interaction we, here, observed is not directly due to the exclusive contribution of *GSK3 β* and *FXR1* proteins to regulation of NSs, so that other molecular pathways, possibly related with *GSK3 β* /*FXR1* signaling, may be hypothesized behind these clinical manifestations. Another possible limitation of our study is that samples we studied had quite a large internal heterogeneity in terms of AP medication used (olanzapine, risperidone, quetiapine, paliperidone, aripiprazole, clozapine, and haloperidol). In addition, part of the sample in the DS was taking antidepressant and mood stabilizer medication at the time of their recruitment in the current study. Further studies with reducing such heterogeneity in terms of pharmacological agents used are warranted.

In conclusion, findings of both our animal and human experiments highlight an involvement of the *FXR1*–*GSK3 β* signaling pathway in the pathophysiology and possibly pharmacoresponse of NSs, thus shedding light on the intricate molecular basis of these complex clinical phenomena and on possible new pharmacological treatments.

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Authorship Contributions. Antonio Rampino wrote paper draft. All authors contributed to draft revision and finalization. Antonio Rampino, Matteo Jacovello, Silvia Torretta, and Barbara Gelao performed statistical analyses on human subjects data. Federica Veneziani, Aleksandra Marakhovskaya, and Jean Martin Beaulieu performed all animal experiments and analyses. Jean Martin Beaulieu supervised all animal experiments and analyses. Antonio Rampino, Rita Masellis, Silvia Torretta, and Ileana Andriola contributed to human subject recruitment. Leonardo Sportelli contributed to data analysis. Antonio Rampino, Jean Martin Beaulieu, Giulio Pergola, Alessandro Bertolino, and Giuseppe Blasi supervised final results and the overall paper scientific quality. Massimo Genarelli, Alessandra Minelli, Chiara Magri, and Antonio Vita provided clinical and genotype data of the Replication Sample. Giuseppe Blasi substantially contributed to revise paper draft and coordinate the study.

Data Availability Statement. The data that support the findings will be available in the Open Science Framework repository at <https://osf.io/d5e9t> following a 6-month embargo from the date of publication to allow for commercialization of research findings.

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