Comparative genomic evidence for the involvement of schizophrenia risk genes in antipsychotic effects

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Genome-wide association studies (GWAS) for schizophrenia have identified over 100 loci encoding >500 genes. It is unclear whether any of these genes, other than dopamine receptor D2, are immediately relevant to antipsychotic effects or represent novel antipsychotic targets. We applied an in vivo molecular approach to this question by performing RNA sequencing of brain tissue from mice chronically treated with the antipsychotic haloperidol or vehicle. We observed significant enrichments of haloperidol-regulated genes in schizophrenia GWAS loci and in schizophrenia-associated biological pathways. Our findings provide empirical support for overlap between genetic variation underlying the pathophysiology of schizophrenia and the molecular effects of a prototypical antipsychotic.

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INTRODUCTION

A major goal of human genome-wide association studies (GWAS) is to identify potential therapeutic targets for common, complex diseases. While genomic regions that reach genome-wide significance explain only a small fraction of disease risk, many nonetheless encode proteins that make effective drug targets.1 For example, a common genetic variant in HMGCR has a small (~5%) but significant (P = 1 x 10^-15) influence on low-density lipoprotein levels; however, its inhibition by statins effectively treats hyperlipidemia.2 Another example comes from one of the earliest GWAS, which identified a common variant in the complement factor H gene for age-related macular degeneration3-5 that led to targeting the complement cascade for the treatment of age-related macular degeneration.6

Schizophrenia is a chronic, severe and disabling brain disorder that has a median morbid lifetime risk of 0.72%.7,8 The most recent schizophrenia GWAS identified 108 genome-wide significant loci encoding over 500 genes.9 Because screening these putative schizophrenia risk genes for those that are disease- and therapeutically relevant is a substantial task, we adopted an alternative, in vivo molecular approach. We examined the overlap between schizophrenia risk genes and their orthologous mouse genes whose striatal expression was significantly altered following chronic haloperidol treatment. We improved upon prior studies of the effects of chronic antipsychotic exposure on gene expression in the rodent brain (Supplementary Tables 1 and 2) by using a better detection technology (RNA sequencing, RNA-seq) and larger sample sizes to enable the detection of more subtle effects (total N = 38 mice), and by examining several tissues including striatum, whole brain and liver for comparative controls (Supplementary Table 3). We provide evidence that schizophrenia risk genes and pathways are relevant to haloperidol effects.

MATERIALS AND METHODS

The goal of this study was to evaluate the effects of chronic administration of the antipsychotic haloperidol versus vehicle in mice. We have shown that we can reliably administer human-like steady-state concentrations of haloperidol.10-12 All experimental procedures were randomized to minimize batch artifacts13 (for example, assignment to haloperidol or vehicle, cage, order of dissection, RNA extraction and assay batch). Experimenters were blind to the treatment status.

We focused on the striatum as it is relatively dense with dopaminergic neurons and is a key site of action of the dopamine receptor antagonist haloperidol14 (confirmation of choice of tissue is described in the Discussion). We also evaluated whole brain (to measure effects outside the striatum) and liver (to identify brain-independent and hepatic alterations consequent to chronic xenobiotic administration). For striatal samples, we use RNA-seq to comprehensively identify differential gene expression resulting from chronic haloperidol exposure. For whole brain and liver, we used gene expression microarrays, which provide an inexpensive transcriptome evaluation (albeit with lesser dynamic range), and correlate well with RNA-seq (mean r = 0.87 across 88 mouse brain samples assayed with both methods).15

Mice

All animal work was conducted in compliance with the national guidelines (Institute of Laboratory Animal Resources, 1996) and was approved by the UNC Institutional Animal Care and Use Committee. The study design is summarized in Supplementary Table 3. For striatal RNA-seq we chronically treated mice with haloperidol (N = 16) or vehicle (N = 12). All striatal samples were assayed using RNA-seq. Independent mice (N = 20) were used to collect whole brain and liver (left lobe) from mice per treatment group for expression microarray analysis. To minimize the effects of the estrus cycle and other sources of heterogeneity, we evaluated male C57BL/6J mice (shipped at 6 weeks of age, Jackson Laboratory, Bar Harbor, ME, USA) for both RNA-seq and microarray experiments. Animals were maintained in standard environmental conditions (14-h light/10-h dark

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schedule, temperature 20–24 °C, and 40–50% relative humidity. Mice were housed four per cage (two haloperidol and two vehicle treated) in standard 20 cm × 30 cm ventilated polysulfone cages with laboratory grade Bed-O-Cob bedding. Water and Purina Prolab RMH3000 were available ad libitum.

Haloperidol exposure
Eight-week-old mice were implanted with slow-release haloperidol pellets (3.0 mg kg⁻¹ per day; Innovative Research of America; Sarasota, FL, USA) or vehicle and were treated for 30 days for a chronic haloperidol administration paradigm. Pellets were implanted subcutaneously, centrally above the scapulae, under isoflurane anesthesia and the incision sealed with VetBond (3M, St Paul, MN, USA). We have previously demonstrated that this procedure reliably yields human-like steady-state concentrations of haloperidol in blood plasma and brain tissue, and that this results in vacuous chewing movements (an established model of extrapyramidal symptoms) in C57BL/6J mice.10,11,12

Tissue collection
After 30 days of exposure to haloperidol or vehicle (12 weeks of age), mice were killed by cervical dislocation without anesthesia (to avoid effects on gene expression). Mice were killed between 0800 and 1200 hours, immediately after removal from the home cage. Tissues were dissected within 5 min of death, snap-frozen in liquid nitrogen and pulverized using a BioPulverizer unit (BioSpec Products, Bartlesville, OK, USA). Tissues collected were striatum, whole brain or liver (left lobe). Striatum and whole brain were collected from separate animals. The striatum dissection consisted of capturing a 2-mm-thick coronal section (Bregma coordinates +1.0 to −1.0), followed by manual isolation of the striatal region per mouse brain atlas (Figure 1A).18 Left and right striatum were pooled for each animal.

RNA-seq
Total RNA was extracted from the striatum using the Total RNA Purification 96-Well Kit (Norgen Biotek, Thorold, ON, Canada). RNA concentration was measured by fluorometry (Qubit 2.0 Fluorometer, Life Technologies, Carlsbad, CA, USA) and RNA quality was verified using microfluidics (Bioanalyser, Agilent Technologies, Santa Clara, CA, USA). We have previously demonstrated that this procedure reliably yields human-like steady-state concentrations of haloperidol in blood plasma and brain tissue, and that this results in vacuous chewing movements (an established model of extrapyramidal symptoms) in C57BL/6J mice.10,11,12

Differentially expressed genes and GWAS results
We used INRICH28 and MAGMA29 to test for enrichment of GWAS signals in transcripts showing differential gene expression in haloperidol versus vehicle. INRICH evaluates whether a given gene set or pathway has an enrichment of smaller GWAS association P-values than expected by chance (accounting for gene size, single nucleotide polymorphism density, linkage disequilibrium (LD) and pathway size). MAGMA combines the GWAS P-values for each gene (10 kb upstream to 1.5 kb downstream) into a gene-level P-value and accounts for correlations between single nucleotide polymorphisms based on the LD (using 1000 Genomes Project European reference panel Phase 3 (ref. 30)). MAGMA applies a linear regression framework to test whether gene sets are significantly associated with a trait with respect to the rest of the genome or to some subset of genes. The latter was performed, by adding a covariate consisting of all genes located at the GWAS loci. To determine whether the significant enrichment of schizophrenia GWAS was specific to schizophrenia, we performed enrichment testing with INRICH using GWAS results from other psychiatric disorders (autism, bipolar disorder, major depressive disorder and Alzheimer’s disease; URLs)31–33 and non-psychiatric traits (height, body mass index and type 2 diabetes; URLs).34,35 Because high LD and high gene density in the major histocompatibility complex (MHC) may influence these analyses, we performed enrichment tests with inclusion and exclusion of the MHC region.

Gene co-expression network analysis
To explore higher-order interactions in an unbiased manner, we used WGCNA38 (URLs). After removing transcripts with low expression levels (sum of TReC across all samples ≤ 50), resulting in 17,209 genes for analyses. TReC data were normalized using the weighted trimmed mean of M-values scale-normalization method in EdgeR.22 We tested for differential gene expression in the striatum using the negative binomial generalized linear model approach in EdgeR,22 with gene-wise dispersion applied.22,22 The drug effect was evaluated using log-likelihood statistics comparing null and alternative models. False discovery rate correction was applied to gene-based P-values to account for multiple comparisons (R package qvalue).24

Gene expression arrays
Total RNA was extracted from ~25 mg of powdered tissue from the whole brain using automated instrumentation (Maxwell 16 Tissue LEV Total RNA Purification Kit, Promega, Madison, WI, USA). RNA concentration was measured and RNA quality verified as described above. Whole-brain RNA from 20 male C57BL/6J mice (10 haloperidol, 10 vehicle) was hybridized to Affymetrix Mouse Gene 2.0 ST 96-Array Plate arrays (Santa Clara, CA, USA) using a GeneTitan instrument according to the manufacturer’s instructions. As in our prior reports,15,25 we used robust multiarray average method in the Affymetrix gene expression console to estimate normalized expression levels (default settings, median polish and sketch-quantile normalization). We excluded probes containing any known single nucleotide polymorphisms in C57BL/6J,36 resulting in 24,464 probe sets for analysis. We searched for the most intriguing principal component analysis and hierarchical clustering (R function hclust), and identified only one outlier in a liver sample. We evaluated potential confounding variables by examining the relationship between PC1–PC10 from the expression data and each variable. We found that all potential confounders had minimal impact on gene expression in the whole brain and liver. For unmeasured confounders, we performed surrogate variable analysis. Surrogate variables 1 and 2 explained the majority of variation in the residual from a model including haloperidol treatment. We used the following model to identify genes displaying differential expression: \(y = \beta_0 + \beta_{\text{drug}} + \beta_{\text{drug} \times \text{gene}} + \epsilon\), where ‘\(\beta_{\text{drug}}\)’ is an indicator of haloperidol exposure and ‘\(\epsilon\)’ and ‘\(\beta_{\text{drug} \times \text{gene}}\)’ are the first two surrogate variables. False discovery rate correction was applied to transcript-based P-values to account for multiple statistical comparisons (R package q-value).24

RESULTS
We performed RNA-seq of the striatal brain tissue from adult male C57BL/6J mice chronically treated (30 days) with implanted...
haloperidol (N = 16) or vehicle pellets (N = 12). Additional mice were examined for differential RNA expression in whole brain and liver (N = 20 mice, Supplementary Table 3). We first examined two positive control genes (Drd2 and Nts), and found that both genes showed a striatum-specific increase in expression after chronic haloperidol exposure, as expected from prior studies39,40 (Supplementary Table 5). We found that the transcriptional effects of chronic haloperidol exposure were brain-specific (Supplementary Figure 1) and most pronounced in the striatum (Supplementary Table 6). Haloperidol-regulated genes were enriched for orthologous schizophrenia GWAS risk loci (Figure 1a, INRICH $P = 0.0004$, MAGMA $P = 0.0003$, 32 loci, 39 genes, Supplementary Tables 7 and 8). This enrichment remains when the MHC region is excluded (INRICH $P = 0.0006$, MAGMA $P = 0.0001$). These effects were not seen in the whole brain (INRICH $P = 0.45$) or liver (INRICH $P = 0.95$), suggesting that these effects are anatomically specific. Although haloperidol-regulated and schizophrenia-associated genes tended to be expressed in the striatum, enrichment for schizophrenia GWAS loci remained significant when restricting the analysis to genes expressed in the mouse striatum (INRICH $P = 0.0009$).
SzGene database\(^2\) \(P = 0.05, \) Figure 1c. Notably, genetic evidence for association with schizophrenia for most of these genes is currently lacking.\(^4\) Many of these candidate genes were proposed based on pharmacological properties (Supplementary Figure 2).

**DISCUSSION**

We used an *in vivo* molecular approach to determine whether there is significant overlap between genes and pathways involved in schizophrenia risk and those regulated by chronic antipsychotic treatment. We performed RNA-seq of brain tissue from mice chronically treated with haloperidol and found that haloperidol-regulated genes are over-represented within schizophrenia GWAS loci and schizophrenia-associated biological pathways. Our findings indicate an overlap between genetic variation underlying the pathophysiology of schizophrenia and the molecular effects of a prototypical antipsychotic.

Our main analyses focused on the striatum. We confirmed our choice of this tissue using single-cell RNA-seq in the mouse brain,\(^3\)\(^4\) where we demonstrated marked enrichment of antipsychotic drug targets\(^4\) and differentially expressed genes from this experiment: both analyses pointed at the dominant cell type in ventral striatum, medium spiny neurons (particularly those expressing Drd2).\(^4\)\(^6\)

If such convergence occurs at the levels of biological pathways, it follows that some genes might be important for antipsychotic effects but do not harbor common variants that increase risk for schizophrenia. For example, the serotonin transporter and nicotinic acetylcholine receptor subunit alpha-7 are implicated in antipsychotic pharmacology,\(^4\)\(^7\)\(^8\) showing marked expression changes after chronic treatment with haloperidol, but, at present, have no genetic association with schizophrenia (additional examples include HTR2A and NTS, Supplementary Figures 3 and 4). One might also expect genes within the same pathway to show gene expression changes and association with schizophrenia. Such is the case for the dopamine receptor D2 (DRD2), the direct target of all effective antipsychotics, and for synaptosomal-associated protein 91 kDa, a novel synaptic vesicle protein (SNAP91; Supplementary Figure 3). Additional examples include CACNA1C and GRIN2A (Supplementary Figure 5).

Additional support for a role of putative schizophrenia risk genes in antipsychotic action is derived from gene co-expression network analyses (Supplementary Figures 6–8) and functional enrichment analyses (Supplementary Tables 11 and 12 and Supplementary Figures 9 and 10). Overlap between haloperidol regulation and schizophrenia risk extends to biological pathways and perhaps even to the composition of some multisubunit receptors. For example, there is enrichment of the pathway representing nicotinic acetylcholine receptor regulation of dopaminergic synapses, an active process in the striatum (\(^9\)\(^4\)\(^9\) Supplementary Figure 8a). The nicotinic receptor \(\alpha_{4}\alpha_{6}\beta_{2}\beta_{3}\) (which is critical to striatal dopamine release)\(^10\)\(^3\)\(^7\) contains subunits encoded by a gene with differential expression but no genetic association (Chrm6) and a gene with the inverse pattern of findings (Chrm4, Supplementary Figure 8b). Furthermore, the net expression changes we observe in this pathway suggest that chronic haloperidol exposure likely decreases dopamine release in the striatum.\(^4\)\(^9\)\(^5\) We also showed that the enrichment of haloperidol-regulated genes is specific to schizophrenia GWAS (Supplementary Table 9). A recent study used MAGMA to identify new drug targets using the PGC2 schizophrenia data. Interestingly, the authors found that targets of antipsychotics were enriched for association with the schizophrenia GWAS data.\(^4\) When they looked at druggable targets, they found that the PGC2 schizophrenia GWAS findings were associated with antipsychotics and anticonvulsants, as well as drugs targeting calcium channels and nicotinic acetylcholine receptors.\(^4\)

In summary, by integrating human genetic findings and mouse *in vivo* expression data, we provide evidence that some schizophrenia risk genes may be involved in chronic effects of haloperidol. Our findings suggest targets for antipsychotic drug development (for example, genes highlighted in Figure 1b and pathways in Supplementary Figure 8a). Our results support the ongoing development of \(\alpha_{7}\)-nicotinic acetylcholine receptor agonists for cognitive enhancement in schizophrenia,\(^5\)\(^3\) and suggest that \(\alpha_{6}\) is also a potential target, but this will require further experimental molecular data. We also show that the mouse can be a suitable and efficient model organism in which to use human GWAS results to learn more about drug mechanisms and to support compound development.

**URLS**

Psychiatric Genomics Consortium, http://pgc.unc.edu; human and mouse homology, ftp://ftpinformatics.jax.org/pub/reports/index.html#homology; mouse exon annotations, http://www.bios.unc.edu/~weisun/software/softfrom_files/Mus_musculus.NCB37.67.data.zip. WGCNA, http://labs.genetics.ucla.edu/horvath/CoexpressionNetwork; NIMH Psychoactive Drug Screening Program, http://pdsp.med.unc.edu/downloadKI.html. GIANT body mass index and height (http://www.broadinstitute.org/collaboration/giant), DIA-GRAM consortium type 2 diabetes results (http://diagram-consortium.org/downloads.html), Autism Spectrum Disorder Working Group of the PGC (http://www.med.unc.edu/pgc/files/reslutfiles/pgcasdeuro.gz) and a pathway regulating the effects of acetylcholine and nicotine on dopaminergic neurons (http://www.wikipathways.org/index.php/Pathway:WP16002) and MAGMA (http://ctg.cncr.nl/software/MAGMA). These data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), accession GSE67755.

**CONFLICT OF INTEREST**

PFS is a consultant to Pfizer. The remaining authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

PFS, YK, PG-R, FP-MdV and JJC designed the experiments. RJN, PG-R, AKR and CRQ performed the experiments. PFS, YK, PG-R, JJC, MGI-U, FP-MdV and PHL analyzed the data. JJC, PFS, YK and PG-R wrote the manuscript. All of the authors critically read and contributed comments to the final version of the manuscript.

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