Comprehensive pathway analyses of schizophrenia risk loci point to dysfunctional postsynaptic signaling

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Abstract

Background: Large-scale genome-wide association studies (GWAS) have implicated many low-penetrance loci in schizophrenia, but have yielded limited insight into disease pathophysiology. This limited understanding of the etiology of schizophrenia hampers the development of novel pharmacological treatments. Pathway and gene set analyses may provide biological context to genome-wide data and carry the potential to generate hypotheses about disease mechanisms and leads for novel drug discovery. We aimed to examine which biological processes are likely candidates to underlie schizophrenia by integrating genetic data with novel pathway analysis tools unused to date for the largest schizophrenia GWAS (N = 79,845).

Methods: Using Multi-marker Analysis of GenoMic Annotation (MAGMA), we applied a primary unbiased analysis to weigh the role of biological processes from the Gene Ontology database. We subsequently evaluated these results and performed a validation analysis in MAGMA and Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA) using detailed molecular pathways from the Kyoto Encyclopedia of Genes and Genomes

Results: We found enrichment of common genetic variants underlying schizophrenia in biological processes associated with synaptic signaling and neuronal differentiation. We validated enrichment of molecular signaling processes in the dopaminergic synapse, cholinergic synapse and long-term potentiation. Moreover, enrichment in these pathways was mostly driven by shared genes involved in post-synaptic membrane and downstream signaling components between these pathways.

Conclusions: We provide the strongest genetics-informed evidence to date that dysfunctional postsynaptic pathways are implicated in schizophrenia. Future studies in both preclinical and clinical settings may further disentangle these systems to allow the development of new treatment options in schizophrenia.

Introduction

Antipsychotics target synaptic signaling by changing neurotransmission of the dopamine D2 receptor (DRD2) and the serotonin 5-HT_2 receptor (1, 2). These drugs are the mainstay treatment modality in schizophrenia and are fairly effective at reducing positive symptoms (3). However, improving the cognitive and negative symptoms, which substantially affect quality of life, has proven challenging (4-7). Although post-mortem studies, imaging and human genetic studies have contributed to theories about pathophysiological mechanisms in schizophrenia, the underlying molecular processes have not been fully elucidated.

Genetic studies provide a valuable resource to investigate the mechanisms that are likely at play in schizophrenia. Schizophrenia is highly heritable ($h^2 \sim 80\%$) and polygenic, with genome-wide association studies (GWAS) identifying 108 independent associated risk loci (8, 9). Pathway and gene set enrichment analysis methods are widely used to provide biological context to the results of such genetic association studies by testing whether biologically relevant pathways or sets of genes are enriched for genetic variants associated with a phenotype (10-12). Pathway analyses using data derived from the latest schizophrenia GWAS (8) have shown enrichment of schizophrenia-associated variants in neuronal, immune and histone pathways, and the involvement of calcium signaling processes (13, 14), but these studies have not used the most complete and recent GWAS data (8). Additionally, several valuable and widely used pathway analysis tools have not yet been applied to these data, despite their input from comprehensively annotated databases and success in elucidating biology of other common diseases (15-17).

Aiming to comprehensively investigate the possible biological processes underlying schizophrenia, we set out to apply gene set and pathway enrichment analysis methods to the latest GWAS in schizophrenia (N = 43,241 cases and 45,604 controls) (Figure 1) (8). Our results elucidate the involvement of neuronal differentiation and synaptic plasticity in

schizophrenia, and signal an accumulation of variants in post-synaptic signaling cascades. They moreover enable a more nuanced understanding of the several actionable classes of neurotransmitters implicated in the disease.

Methods and materials

Input data and analysis overview

We used summary-level results from the largest and most recent GWAS in schizophrenia, made publically available by the Psychiatric Genomics Consortium (8) (www.med.unc.edu/pgc/results-and-downloads; downloaded on 10 May, 2017), containing results from approximately 9.4 million single nucleotide polymorphisms (SNPs) tested. As detailed below (also see Figure 1), using Multi-marker Analysis of GenoMic Annotation (MAGMA) (18) we successively (A) mapped SNPs to genes, (B) calculated gene p-values based on enrichment from GWAS data, (C) performed a primary gene set enrichment analysis using gene ontology (GO) terms, and (D) followed these findings up by a validation analysis on detailed molecular pathways derived from KEGG. Finally, we validated the results of the analysis on KEGG pathways using Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA) (19).

Mapping SNPs to genes and assigning p-values to genes

Autosomal SNPs present in the European subset of the 1000 Genomes Phase 3 dataset were extracted from the GWAS summary-level results (20). Using MAGMA v1.06, we mapped SNPs to corresponding genes, extending gene footprints by an additional 20 kilobase (kb) up- and downstream, as a large proportion of regulatory elements involved in gene expression regulation is likely to be captured by including this region (21). We then applied a gene analysis to obtain a p-value for each gene to which at least one SNP was mapped. The p-value of a gene was based on the mean χ^2 statistic of SNPs contained in that gene, obtained using a known approximation of the sampling distribution. The European subset of 1000 Genomes Phase 3 was used as a reference dataset to estimate linkage disequilibrium (LD)

between SNPs in the gene analysis, as the largest proportion of the schizophrenia GWAS meta-analysis was based on cohorts with northern and western European genetic ancestry.

Gene Ontology (GO) term enrichment analysis

We applied a competitive gene set analysis on 4,436 biological processes included in the GO database, using the genes and assigned p-values from the previous step. Gene sets were downloaded from the Molecular Signatures Database (MSigDB, release 6.0, April 2017), a regularly updated database of pathways and gene sets (22, 23). The significance threshold was adjusted for multiple GO terms tested using a permutation procedure (10,000 times) implemented in MAGMA ($p = 3.94 \times 10^{-6}$). The competitive gene set analysis in MAGMA accounts for confounders such as gene size and linkage disequilibrium between genes that are close together.

Validation using KEGG pathways

To validate the results of our primary GO term analysis, we used seven gene sets representing synaptic signaling processes from the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.kegg.jp, downloaded on 3 January, 2017) (24) as input for MAGMA: hsa04724, Glutamatergic synapse (114 genes); hsa04725 Cholinergic synapse (111 genes); hsa04726 Serotonergic synapse (113 genes); hsa04727, GABAergic synapse (88 genes); hsa04728, Dopaminergic synapse (130 genes); hsa04720, Long-term potentiation (67 genes); hsa04730, Long-term depression (60 genes). We then ran a competitive gene set analysis on these pathways. The significance threshold was again adjusted for multiple testing using a permutation procedure (10,000 times) within MAGMA ($p = 0.009$). For each pathway enriched with schizophrenia-associated SNPs, we mapped significantly enriched genes to pathway components using the *pathview* package in R version 3.3.3 (www.r-project.org)

(25). Because many genes overlapped between two or more of the tested KEGG pathways (Supplementary Figure 2), we applied a conditional competitive gene set analysis where we used gene sets different from the one tested as covariates to correct for overlapping genes present in the other gene sets in order to correct for the influence of shared genes on the enrichment signal. Finally, we tested enrichment in the same KEGG pathways using MAGENTA. MAGENTA maps SNPs onto genes that are present in the gene set, assigns a score to each gene based on the lowest SNP p-value for that gene while correcting for confounding factors (e.g., gene size). It then assesses whether a gene set is enriched with low gene p-values at the 95^{th} percentile cut-off (based on all gene p-values in the genome) compared to equally sized gene sets randomly sampled from the entire genome. The resulting enrichment p-values were corrected for multiple testing using false discovery rate (FDR < 0.05).

Results

By performing a primary enrichment analysis in MAGMA (in 4,436 biological processes described in the GO database) we identified two terms that were enriched for schizophrenia-associated SNPs (Figure 2A and Supplementary Table 1): 'Regulation of synaptic plasticity' ($p = 1.13 \times 10^{-6}$) and 'Regulation of neuron differentiation' ($p = 1.18 \times 10^{-6}$). These significant GO terms are specialized terms of synaptic signaling and neurogenesis, respectively (Supplementary Figure 1).

To gain a more nuanced understanding of molecular synaptic pathways enriched for schizophrenia-associated variants, we tested enrichment of SNPs in pathways that we derived from KEGG and that represented synaptic signaling resulting in synaptic plasticity, synaptic growth and survival (as indicated by the primary MAGMA enrichment analysis). We found a significant enrichment in pathways representing the dopaminergic synapse ($p = 1.5 \times 10^{-5}$), cholinergic synapse ($p = 2.0 \times 10^{-4}$) and long-term potentiation ($p = 3.8 \times 10^{-4}$) (Figure 2B, Supplementary Table 2). For each significant pathway, we mapped genes enriched with schizophrenia SNPs on components within these KEGG pathways (schematic in Figure 3, detailed pathways in Supplementary Figure 3). SNP enrichment was mostly restricted to trans-membrane and postsynaptic components in the cholinergic and dopaminergic synapses. The long-term potentiation pathway only included post-synaptic genes. We found high enrichment in signaling through extracellular signal-regulated kinase (ERK) and cAMP response element-binding protein (CREB), phospholipase C (PLC) and the inositol trisphosphate receptor (IP_3R), and signaling through protein kinase B (PKB/Akt). These cascades converge on mechanisms involved in synaptic growth regulation and synaptic plasticity. Furthermore, voltage-gated calcium channels, glutamatergic NMDA and AMPA receptors, the dopamine D2 receptor (DRD2) and the muscarinic acetylcholine receptor M_4 were highly enriched (Figure 3, Supplementary Figure 3).

When conditioning the competitive gene set analysis of each KEGG pathway in on the other pathways tested, thereby using overlapping genes in other pathways as a covariate in the analysis and adjusting for the effect of shared genes between pathways, the enrichment p-value of all pathways dropped (Supplementary Figure 4). None of the pathways remained significant at the established significance threshold ($p = 0.009$), indicating a contribution of shared genes to the enrichment signal.

Using MAGENTA with the same data and gene sets, we also found significant enrichment of the dopaminergic synapse (multiple test-corrected FDR = 5.0×10^{-4}), long-term potentiation (corrected FDR = 2.8×10^{-3}) and cholinergic synapse (corrected FDR = 4.8×10^{-3}) pathways (Figure 2C). We thus confirm our above-mentioned findings.

Discussion

By implementing complementary gene set enrichment analysis tools (MAGMA and MAGENTA) and annotations from biological databases (GO and KEGG) into a comprehensive analysis, we aimed to elucidate biological processes underlying schizophrenia. Thus, we first detected enrichment of schizophrenia-associated SNPs in synaptic plasticity and neuronal differentiation processes, which is in line with biological hypotheses that previous studies have made (8, 13, 14, 26, 27). We followed this up in a targeted analysis on gene sets representing synaptic signaling in all major neurotransmitter systems, and demonstrated enrichment of schizophrenia SNPs in the dopaminergic and cholinergic systems and in long-term potentiation through the glutamatergic system.

Dysfunctional synaptic transmission impacts synaptic plasticity and brain development, mediated through long-term potentiation (LTP) and long-term depression (LTD) (28). Although all five major neurotransmitter systems (dopamine, gamma-aminobutyric acid, glutamate, serotonin, and acetylcholine) have been implicated in schizophrenia, the extent to which each of them is involved had remained elusive (6, 29). We here studied SNP enrichment in these processes, using gene sets of major neurotransmitter systems from the KEGG database, in which we also visualized this enrichment. Our results strongly support the involvement of the dopaminergic system, which has been extensively examined in schizophrenia. Previous studies have reported increased dopamine synthesis and release, and increased dopamine receptor expression in schizophrenia (6, 30). *DRD2* genetic variants are also implicated in schizophrenia and several of its intermediate phenotypes (31, 32). We here confirm an accumulation of *DRD2* genetic variants in schizophrenia. Moreover, we extend this evidence for involvement of the dopaminergic system by highlighting enrichment of variants in signaling cascades downstream of this receptor. Cholinergic transmission may also be relevant to symptomatology of schizophrenia, especially in light of

the high rates of nicotine abuse and a range of cognitive symptoms (33, 34). The implication of acetylcholine in schizophrenia is further supported by a landmark study investigating chromatin interactions between enhancer regions containing schizophrenia-associated loci and promoter regions of target genes (35). Enrichment of the glutamate-induced LTP pathway was another finding that could be verified using MAGMA and MAGENTA. Mediation of LTP is, however, not limited to the glutamatergic system as post-synaptic signaling molecules such as the above mentioned CREB, IP_3R and PKB mediate synaptic plasticity in other neurotransmitter systems (e.g. the dopaminergic system). Multiple lines of evidence link LTP to cognitive deficits in schizophrenia (36).

Our detailed analysis of downstream signaling cascades in all major neurotransmitter system gene sets revealed several of these cascades to be highly enriched for schizophrenia-associated variants: the phospholipase pathway, CREB signaling and the protein kinase B signaling cascade. All of these cascades may be linked to schizophrenia by numerous lines of neurobiological evidence, as outlined below. First, the phospholipase pathway (particularly PLC) controls neuronal activity and thereby maintains synaptic functioning and development. Activation of PLCβ and PLCγ results in cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into the active form inositol 1,4,5-trisphosphate (IP_3) (37), in whose receptor (IP_3R) we report enrichment of schizophrenia-associated SNPs. Gene deletions in *PLC* are associated with schizophrenia and altered expression of *Plc* and schizophrenia-like behavior have been reported in *Plc* knock-out mice (38-40). Second, signaling through the cellular transcription factor CREB modulates synaptic plasticity. A recent study focusing on the cyclic adenosine monophosphate (cAMP)/PKA/CREB pathway shows a significant association of a SNP in this system with schizophrenia (27). Additionally, ERK is part of the CREB signaling cascade and was also found to be enriched in our analyses. Impairment of signaling through ERK is hypothesized as a disease mechanism in schizophrenia (41, 42).

Third, we found a significant enrichment of schizophrenia SNPs in postsynaptic protein kinase B (PKB or Akt). *AKT1* messenger RNA levels are higher in blood of schizophrenia patients compared to healthy controls and interactions between genetic variation in *AKT1* and cannabis use are associated with schizophrenia, possibly mediated through AKT signaling downstream of DRD2 (43, 44). Interestingly, phosphorylation of glycogen synthase kinase 3 beta (Gsk3β) by the antipsychotic aripiprazole is mediated by Akt (45). Finally, we detected an accumulation of SNPs in protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP2A is one of the mediators of sensorimotor gating, an intermediate phenotype for schizophrenia (46).

Several limitations should be considered when interpreting our results. First, no significant SNP enrichment was found in other systems hypothesized to be dysregulated in schizophrenia, such as glutamatergic and GABAergic neurotransmission (6, 47, 48). As our analyses are dependent on the power of GWAS, we cannot rule out the possibility that increased sample sizes in future studies may flag such systems. Second, we can only test for enrichment in gene sets and pathways that are annotated based on the knowledge currently available. Third, only protein-coding regions of the genome and up- and downstream regions in close proximity to genes were considered in our analyses. Recently, it has become clear that non-coding stretches of the genome account for a major part of disease heritability and transcription regulation (35, 49). As the current analyses do not allow us to probe non-coding regions, we cannot take into account the effects that such genomic areas may have on neurotransmitter systems. Despite these limitations and slight technical differences between MAGMA and MAGENTA (18, 19), such as in computation of gene p-values and calculation of gene set enrichment, we found the same pathways to be significantly enriched in results generated from each approach, suggesting our finding is robust to analytic approach. Future integration of expression quantitative trait locus (eQTL) data and genomic interactions in

pathway analysis tools has the potential to further deepen our understanding of the molecular mechanisms involved in the pathways underlying schizophrenia.

In conclusion, using two complementary enrichment analysis approaches, we highlight downstream signaling cascades as the most likely part of the dopaminergic, cholinergic and glutamatergic (mediating LTP) systems to have a role in schizophrenia. Conditional analysis correcting mainly for shared genes showed a non-significant enrichment of all neurotransmitter gene sets, supporting the hypothesis that shared signaling mechanisms across the implicated pathways are likely to be a stronger underlying factor in schizophrenia than the independent neurotransmitter systems. Genes involved in these shared signaling mechanisms might be core genes which are more important in schizophrenia pathology, a concept that has been extensively outlined in a recent publication (50). Our results open avenues for further research aimed at elucidating signaling pathways in schizophrenia, e.g. through tissue-specific manipulation of pathways in animal or cell models. Finally, our findings may aid the discovery of novel drug targets to hopefully reduce the burden imposed on quality of life in patients suffering from this disabling disorder.

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Figure legends

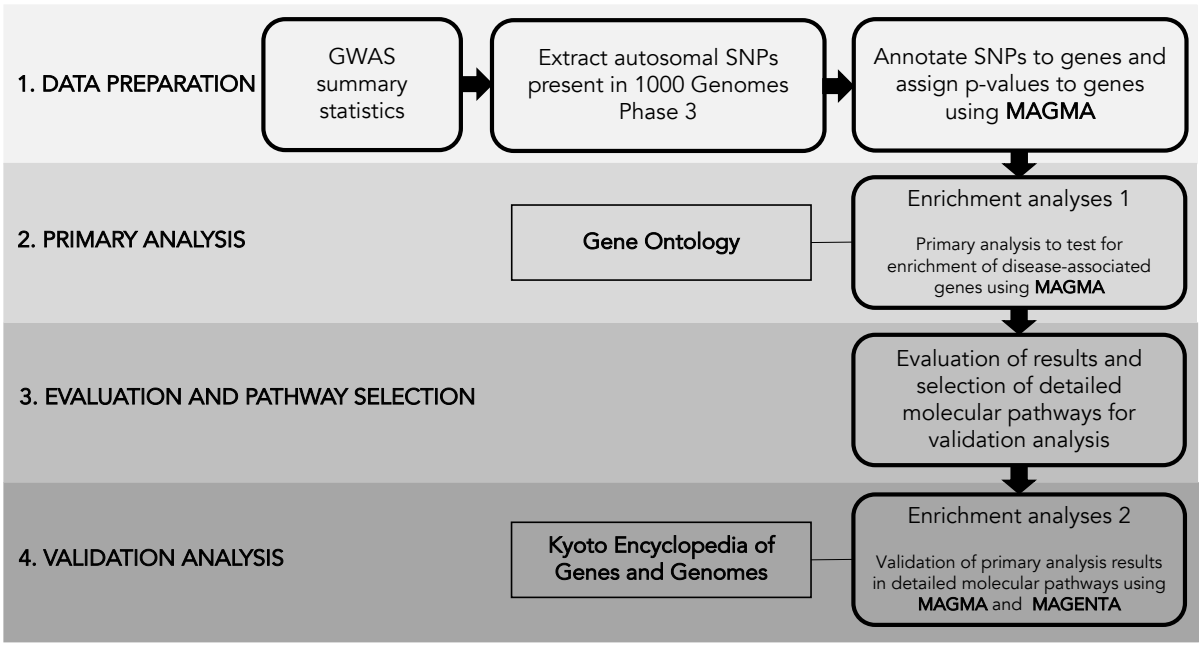

Figure 1 Overview of our pathway analysis pipeline. The analysis pipeline consists of four stages. In the first stage, data is prepared for analysis by assigning SNPs to genes and calculating gene p-values using 1000 Genomes Phase 3 as a reference for calculating linkage disequilibrium (LD). Second, a primary analysis is performed on biological processes included in the extensively annotated gene ontology database. Third, the results from the primary analysis are evaluated and biological pathways of interest are selected from detailed molecular databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) are selected. Finally, pathway analysis on these detailed molecular pathways are performed to validate involvement of biological processes found in the primary analysis and to obtain a more nuanced understanding of SNP enrichment in these processes.

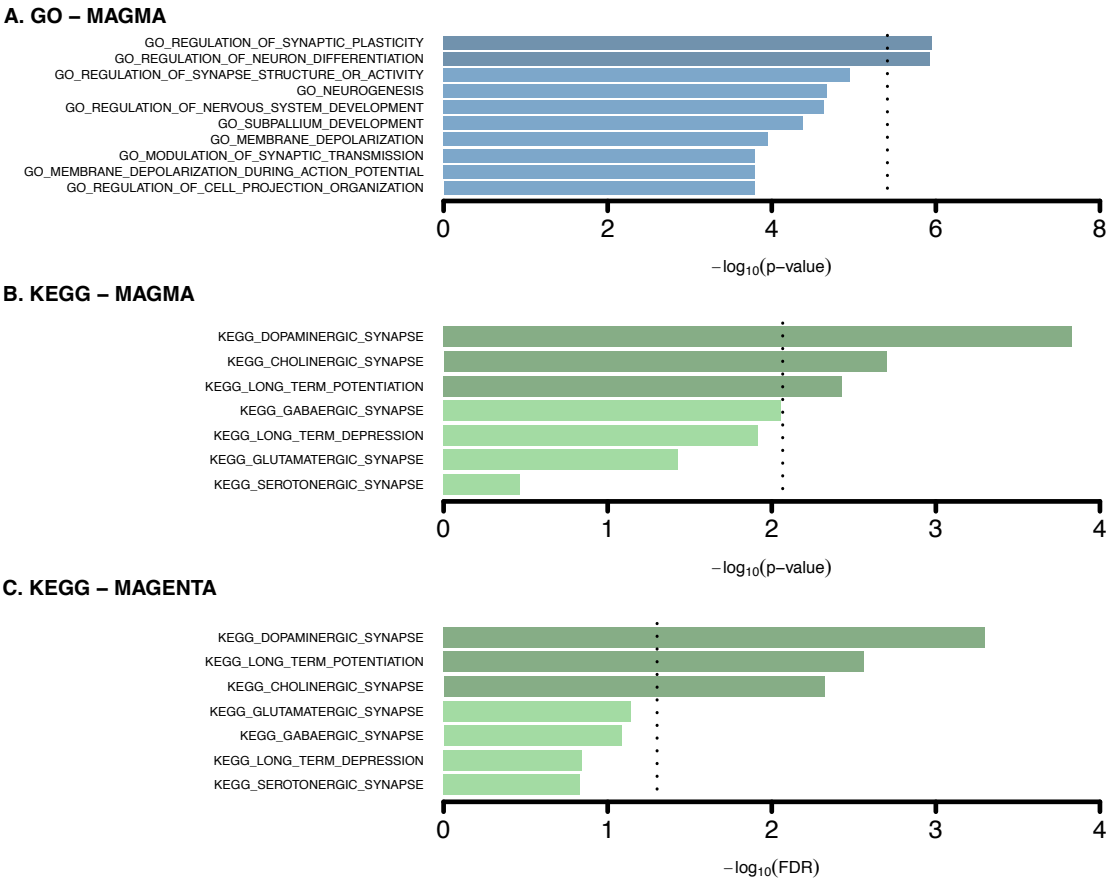

Figure 2 Results of gene set enrichment analysis on biological processes from Gene Ontology (GO) and detailed synaptic signaling pathways from KEGG. Reported p-values or FDR are $-\log_{10}$ converted. **(A)** Significantly enriched GO terms (shown in dark blue) are 'Regulation of synaptic plasticity' ($p = 1.13 \times 10^{-6}$) and 'Regulation of neuron differentiation' ($p = 1.18 \times 10^{-6}$). The significance threshold was adjusted for multiple testing using permutations ($p = 3.94 \times 10^{-6}$, dotted line). Seven KEGG pathways representing synaptic signaling processes were tested for schizophrenia SNP enrichment using MAGMA **(B)** and MAGENTA **(C)**. Significantly enriched pathways (dark green bars) were dopaminergic synapse (MAGMA $p = 1.5 \times 10^{-5}$, MAGENTA multiple-test corrected FDR $= 5.0 \times 10^{-4}$), cholinergic synapse (MAGMA $p = 2.0 \times 10^{-4}$, MAGENTA corrected FDR $= 2.8 \times 10^{-3}$) and long-term potentiation (MAGMA $p = 3.8 \times 10^{-4}$, MAGENTA corrected FDR $= 4.8 \times 10^{-3}$). MAGMA significance threshold was adjusted for multiple testing using permutations ($p = 3.94 \times 10^{-6}$, dotted line), MAGENTA significance threshold was FDR < 0.05.

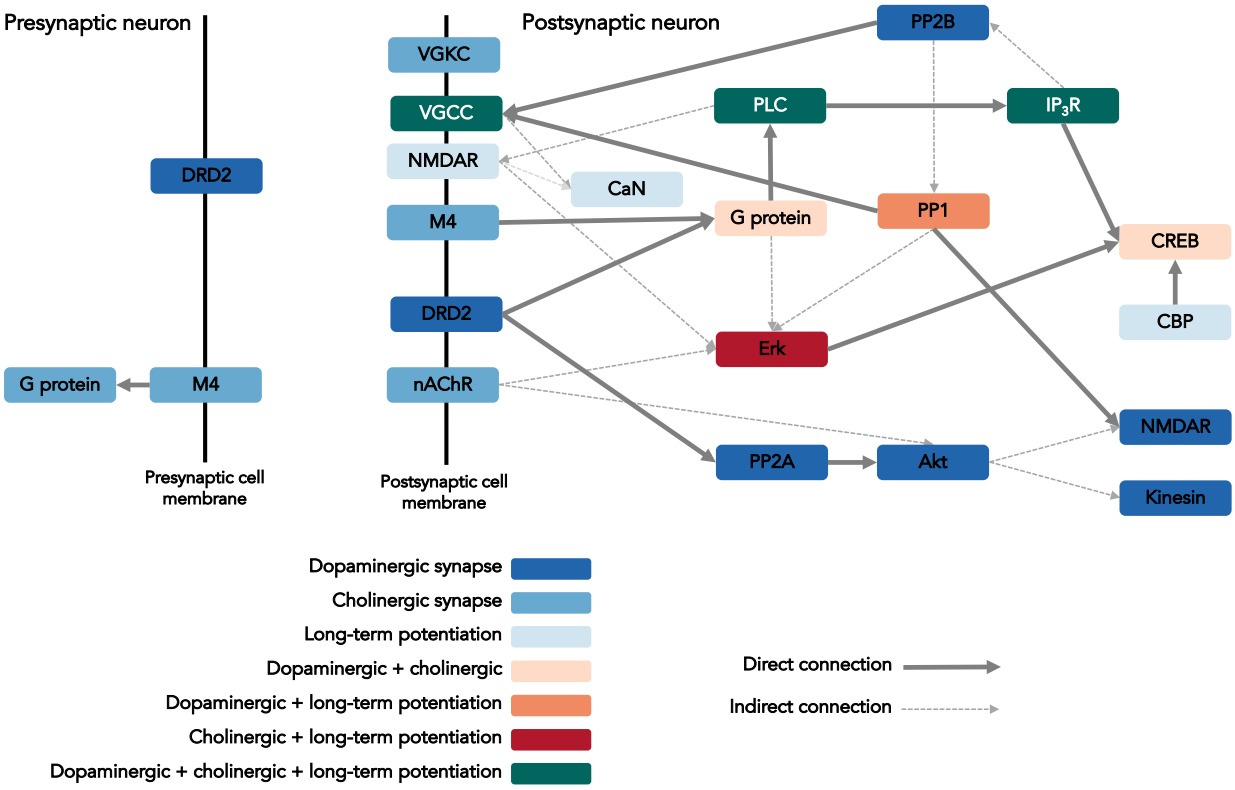

Figure 3 Overview of synaptic signaling pathway components enriched for schizophrenia SNPs. Pathway components with gene p-values below 0.05/number of genes in the corresponding gene set were included in this overview (gene significance threshold $p = 0.05/130 = 3.8 \times 10^{-4}$ for dopaminergic synapse, $p = 0.05/111 = 4.5 \times 10^{-4}$ for cholinergic synapse, and $p = 0.05/67 = 7.5 \times 10^{-4}$ for long-term potentiation). Colors indicate in which gene sets the molecular components were present and enriched. Solid arrows indicate a direct connection between signaling components, dashed lines indicate an indirect connection (meaning the connection is separated by one or more non-enriched components). Connections between components were based on KEGG (24).

Abbreviations: DRD2, dopamine receptor D2; M4, muscarinic acetylcholine receptor M4; G protein, guanine nucleotide-binding protein; VGKC, voltage-gated potassium channel; VGCC, voltage-gated calcium channel; NMDAR, N-methyl-D-aspartate receptor; nAChR, nicotinic acetylcholine receptor; CaN, calcineurin; PP2A, protein phosphatase 2A; Akt,

protein kinase B; Erk, Extracelluar signal-regulated kinase; CREB, cAMP response element binding protein; CBP, CREB-binding protein; PP1, protein-phosphatase 1; PLC, phospholipase C; IP_3R, inositol trisphosphate receptor; PP2B, protein-phosphatase 2B.

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