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

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Abstract

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 neurobiological processes are likely candidates to underlie schizophrenia by integrating genetic data with existing pathway analysis tools in a comprehensive bioinformatics pipeline. Using unbiased pathway analysis methods to weigh the role of biological processes in schizophrenia, we demonstrate enrichment of schizophrenia-associated single-nucleotide polymorphisms (SNPs) in pathways and gene sets associated with synaptic functioning. We subsequently performed targeted analyses of neurotransmitter gene sets, through which we detected enrichment of gene sets representing the dopaminergic synapse, cholinergic synapse and long-term potentiation. We furthermore highlight that enrichment is mostly located in postsynaptic membrane and postsynaptic signaling components. We thus 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 to target core symptoms 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] Antipsychotics 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 one of the major hallmarks in the field thus far being the identification of 108 independent schizophrenia-associated risk loci.[8, 9] Pathway and gene set enrichment analysis methods are widely used to provide more 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 results from the latest schizophrenia genome-wide association study (GWAS) have shown enrichment of schizophrenia-associated variants in neuronal, immune and histone pathways, and the involvement of calcium signaling processes.[13, 14] Nevertheless, several conventional pathway analysis tools have been left unused in these studies, despite their proven success for other diseases and input from comprehensively annotated databases.[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 largest GWAS in schizophrenia (Figure 1).[8] Our results elucidate the involvement of synaptic dysfunction in schizophrenia, and enable a more nuanced understanding of the several actionable classes of neurotransmitters implicated in the disease.

Methods

Input data and analysis overview

We downloaded summary statistics from the largest and most recent GWAS in schizophrenia[8] from the website of the Psychiatric Genomics Consortium (https://www.med.unc.edu/pgc/results-and-downloads; downloaded on 26 March, 2015). The summary statistics contained results from approximately 9.4 million single nucleotide polymorphisms (SNPs). As detailed below (also see Figure 1), we successively mapped SNPs to genes, applied Gene Ontology (GO) term enrichment analysis and Ingenuity Pathway Analysis (IPA) as exploratory pathway analysis methods, followed by targeted analyses using Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA) and Multi-marker Analysis of GenoMic Annotation (MAGMA).

Mapping SNPs to genes and assigning p-values to genes

Schizophrenia summary statistics were clumped using the PLINK[18] clump function (version 1.90b3z), thereby clustering SNPs in linkage disequilibrium (LD) with one another in blocks around top-associated SNPs. We used 1000 Genomes Phase 1 as a reference for calculating LD.[19] In a first round of clumping, we used a genomic window size of 250 kilobase (kb) and removed SNPs with an LD $r^2 > 0.5$. This was followed by a second round of clumping with a window size of 5 megabase (Mb) and an LD cutoff of $r^2 > 0.2$. We removed regions with a complex LD structure, including the major histocompatibility complex on chromosome 6 (Supplementary Table 1). Using MAGMA version 1.05b,[20] we annotated SNPs to corresponding genes, extending gene footprints by an additional 20 kb up- and downstream as a large proportion of regulatory elements involved in gene expression is likely to be captured by including this region.[21] We then applied a gene-based test in MAGMA to obtain a p-value for each gene to which at least one SNP was mapped. The p-value of a gene

(containing at least one SNP) was based on the mean of χ^2 statistics of SNPs contained in that gene and a known approximation of the sampling distribution.[20] Again, 1000 Genomes Phase I was used as a reference set for LD. We extracted 1,705 unique genes with an association p-value to schizophrenia $\leq 5\times10^{-5}$ for use in GO term enrichment analysis and IPA, thus aiming to also include genes containing sub-threshold SNPs that still explain part of the variance in schizophrenia and may in the future reach genome-wide significance in larger sample size studies.

Exploratory gene set enrichment analyses

Gene Ontology (GO) term enrichment analysis

We used the packages *mygene*[22] and *Gostats*[23] and the Bioconductor databases *Go.db* and *org.Hs.eg.db* within R version 3.3.2 (www.r-project.org) to perform GO term enrichment analysis on the list of genes with p-values $\leq 5\times10^{-5}$ (threshold explained in the previous section). Genes were annotated with ontological terms describing biological processes. Overrepresentation of GO terms in the gene list was tested with a conditional hypergeometric test, excluding GO terms with less than 5 and more than 1000 annotations. The conditional test was applied to correct for the hierarchical structure of the Gene Ontology database, so that information on significance of 'child terms' was taken into account when assessing significance of 'parent terms' (where 'child terms' are the more specialized terms that are hierarchically connected to broader 'parent terms'). Bonferroni-correction for the number of GO terms tested was applied to the significance threshold for enrichment ($n = 471$, $\alpha = 0.05/471 = 1.06 \times 10^{-4}$).

Ingenuity Pathway Analysis (IPA)

We analyzed the same extracted 1,705 genes with an association p-value $\leq 5\times10^{-5}$ as mentioned above for enrichment in all canonical pathways available in QIAGEN's Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, CA, USA, www.qiagen.com/ingenuity). Enrichment p-values were calculated using a right-tailed Fisher's exact test. The threshold for significant enrichment was again Bonferroni corrected for the total number of pathways tested ($n = 310$, $\alpha = 0.05/310 = 1.61 \times 10^{-4}$).

Targeted pathway analyses

Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA)

MAGENTA is a summary statistics-based gene set enrichment analysis tool that assesses enrichment of pre-defined gene sets for common variants with modest association to a trait.[24] 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 95th percentile cut-off (based on all gene p-values in the genome) compared to equally sized gene sets randomly sampled from the entire genome. Following evaluation of the results of our exploratory gene set enrichment analyses, we used seven gene sets representing synaptic signaling from the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.kegg.jp, downloaded on 3 January, 2017),[25] as input for MAGENTA: 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 again extended genes with 20 kb up- and downstream regions to capture regulatory elements. The major histocompatibility complex region on chromosome 6 was also excluded by MAGENTA. In the event we found a significant enrichment of a gene

set (Bonferroni-corrected $\alpha = 0.05/7 = 7.14\times10^{-3}$ for n = 7 gene sets tested), we visualized enrichment in pathway components using the R package *pathview*.[26]

MAGMA gene set analysis

Using the results from the gene-based test performed to obtain a gene list for input in GO term analysis and IPA, we applied a gene set test on the seven synaptic signaling gene sets derived from KEGG using MAGMA. We first applied a basic competitive gene set analysis to test whether genes in our gene sets exhibit a stronger association with schizophrenia than other genes. Gene length, gene density (the relative amount of LD in a gene) and inverse minor allele count were used as covariates in this analysis. Second, because many genes overlapped between two or more of the tested gene sets we applied a competitive gene set analysis where we conditioned the enrichment signal of each of the gene sets on that of the other gene sets to correct for the influence of shared genes on the enrichment signal. In line with all other statistical analyses outlined above, the threshold for significant enrichment was again Bonferroni corrected ($\alpha = 0.05/7 = 7.14\times10^{-3}$ for n = 7 gene sets tested).

Results

Exploratory GO term enrichment analysis and IPA

We first applied a hypothesis-generating approach to investigate top-enriched pathways for schizophrenia-associated SNPs. Using GO term enrichment analysis, we identified 76 overrepresented GO-terms, with the top overrepresented term being 'synaptic signaling' ($p = 9.57 \times 10^{-22}$, Figure 2, Supplementary Table 2). To corroborate the strength of the evidence, we used IPA and identified 13 significantly enriched pathways (after Bonferroni correction), with the strongest evidence again pointing to enrichment of synaptic processes (Figure 2, Supplementary Table 3).

Targeted follow-up analyses of synaptic signaling

To gain a more nuanced understanding of the synaptic molecules enriched for schizophrenia-associated variants, we tested enrichment of schizophrenia GWAS SNPs in specific gene sets representing synaptic signaling which we derived from KEGG. A significant enrichment was found for gene sets representing the dopaminergic synapse (18 genes with p-values above the 95th percentile cutoff, where 6 were expected, $p = 5.3 \times 10^{-5}$), synaptic long-term potentiation through glutamate (10 genes with p-values above the 95th percentile cutoff, where 3 were expected, $p = 1.9 \times 10^{-3}$) and the cholinergic synapse (13 genes with p-values above the 95th percentile cutoff, where 6 were expected, $p = 3.2 \times 10^{-3}$; Figure 3, Supplementary Table 4). We mapped SNP enrichment in MAGENTA on components within these KEGG pathways. SNP enrichment was restricted to trans-membrane and postsynaptic components in the cholinergic and dopaminergic synapses. The long-term potentiation pathway only included elements in these cellular domains. High enrichment was found 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 and the dopamine D2 receptor (DRD2) were highly enriched (Figure 4).

Replication analyses using MAGMA

Using a competitive gene set analysis in MAGMA, we replicated the enrichment of the dopaminergic synapse that we found with MAGENTA ($p = 5.41 \times 10^{-4}$; Figure 3, Supplementary Table 5). We did not replicate enrichment of schizophrenia–associated variants in long-term potentiation ($p = 9.75 \times 10^{-3}$) or the cholinergic synapse ($p = 1.21 \times 10^{-2}$) after correction for multiple gene sets tested, although these were still the most highly enriched gene sets after the dopaminergic synapse. When conditioning the enrichment signal of each of the gene sets on that of the other gene sets, the enrichment p-value of the dopaminergic synapse gene set remained Bonferroni-corrected significant ($p = 6.95 \times 10^{-3}$; Figure 3, Supplementary Table 5). Enrichment p-values for the other gene sets remained non-significant. Finally, all genes that showed significant enrichment at the 95th percentile cutoff in MAGENTA turned out to have gene p-values < 0.05 through our SNP-to-gene mapping analysis in MAGMA (data not shown), further pleading for the implication of the highlighted synaptic pathways in schizophrenia.

Discussion

By implementing existing pathway and gene set enrichment analysis tools into a comprehensive bioinformatics pipeline, we aimed to detect biological processes underlying schizophrenia. Starting with an exploratory approach, we confirmed enrichment of schizophrenia-associated SNPs in synaptic processes. We followed this up in a targeted analysis on gene sets representing synaptic systems for each major neurotransmitter, showing enrichment of schizophrenia SNPs particularly in the dopaminergic system and postsynaptic signaling cascades.

Dysfunctional synaptic transmission impacts synaptic plasticity and brain development, mediated through long-term potentiation (LTP) and long-term depression (LTD).[27] Although all five major neurotransmitter systems (dopamine, gamma-aminobutyric acid, glutamate, serotonin, and acetylcholine) have been implicated in disease mechanisms underlying schizophrenia, the extent to which each of them is involved had remained elusive.[6, 28] We here studied SNP enrichment in this 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, 29] *DRD2* genetic variants are also implicated in schizophrenia and several of its intermediate phenotypes.[30, 31] We here confirm an accumulation of genetic variants in this receptor 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.

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),[32] in whose receptor (IP_3R) we report enrichment of schizophrenia-associated SNPs. Gene deletions in PLC are reported to be associated with schizophrenia and altered expression of PLC and schizophrenia-like behavior has been reported in PLC knock-out mice.[33-35] 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.[36] 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.[37, 38] 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. [39, 40] Interestingly, phosphorylation of glycogen synthase kinase 3 beta (Gsk3β) by the antipsychotic aripiprazole is mediated by Akt.[41] 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.[42]

Enrichment of schizophrenia-associated SNPs in the cholinergic synapse primarily came from our MAGENTA analysis. Cholinergic transmission may be relevant to

symptomatology of schizophrenia, especially in light of its high rates of nicotine abuse and cognitive impairment.[43-45] 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.[46] Enrichment of the glutamate-induced LTP pathway was another finding that could only be verified using 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.[47] The lack of cross-validation of the cholinergic and LTP pathways with other techniques than MAGENTA is likely due to technical differences between these programs.[20, 24] Nevertheless, individual genes that were significantly enriched in MAGENTA were all found to be enriched in MAGMA, leading us to hypothesize that reduced power may be at the root of such lack of replication.

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, e.g. glutamatergic and GABAergic neurotransmission.[6, 48, 49] 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.[46, 50] 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. 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 underlying schizophrenia.

In conclusion, using our comprehensive pathway analysis pipeline we highlight downstream signaling cascades as the most likely part of the dopamine system to have a role in schizophrenia. Conditional analysis correcting mainly for post-synaptic signaling genes showed a decreased enrichment of all neurotransmitter gene sets, supporting the hypothesis that postsynaptic signaling in general may be a stronger underlying mechanism in schizophrenia than these neurotransmitter systems independently. Our results open avenues for follow-up research aimed at elucidating signaling pathways in schizophrenia. They also provide leads for novel drug development targeting downstream cascades to hopefully reduce the burden imposed on quality of life in patients suffering from this disabling disorder.

Acknowledgments

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Figures

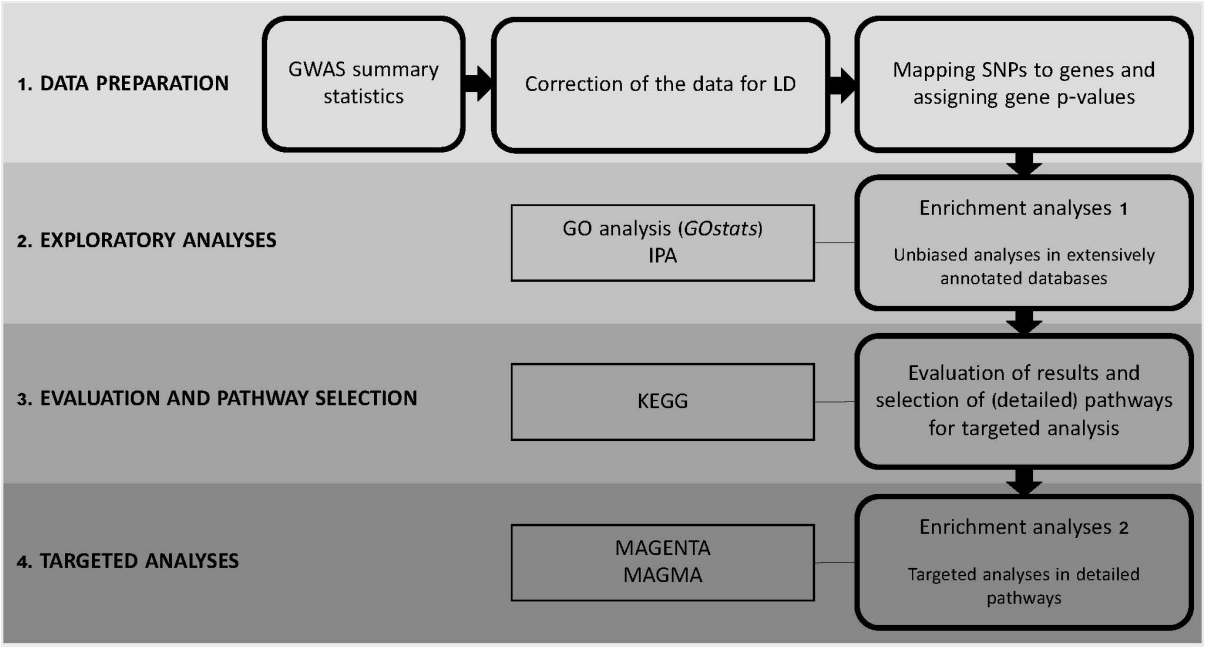

Figure 1 Overview of our pathway analysis pipeline. The analysis pipeline consists of four stages. In the first stage, linkage disequilibrium (LD) is removed from the data and SNPs are mapped to genes. Second, exploratory enrichment analyses are performed in gene sets included in the densely-annotated databases of Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO). Third, results of the exploratory analyses are evaluated and pathways representing enriched biological processes are selected from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, which contains detailed molecular pathways. In the final stage, we perform targeted enrichment analyses on the selected pathways using Meta-Analysis Gene-set Enrichment of variaNT Associations (MAGENTA) and Multi-marker Analysis of GenoMic Annotation (MAGMA).

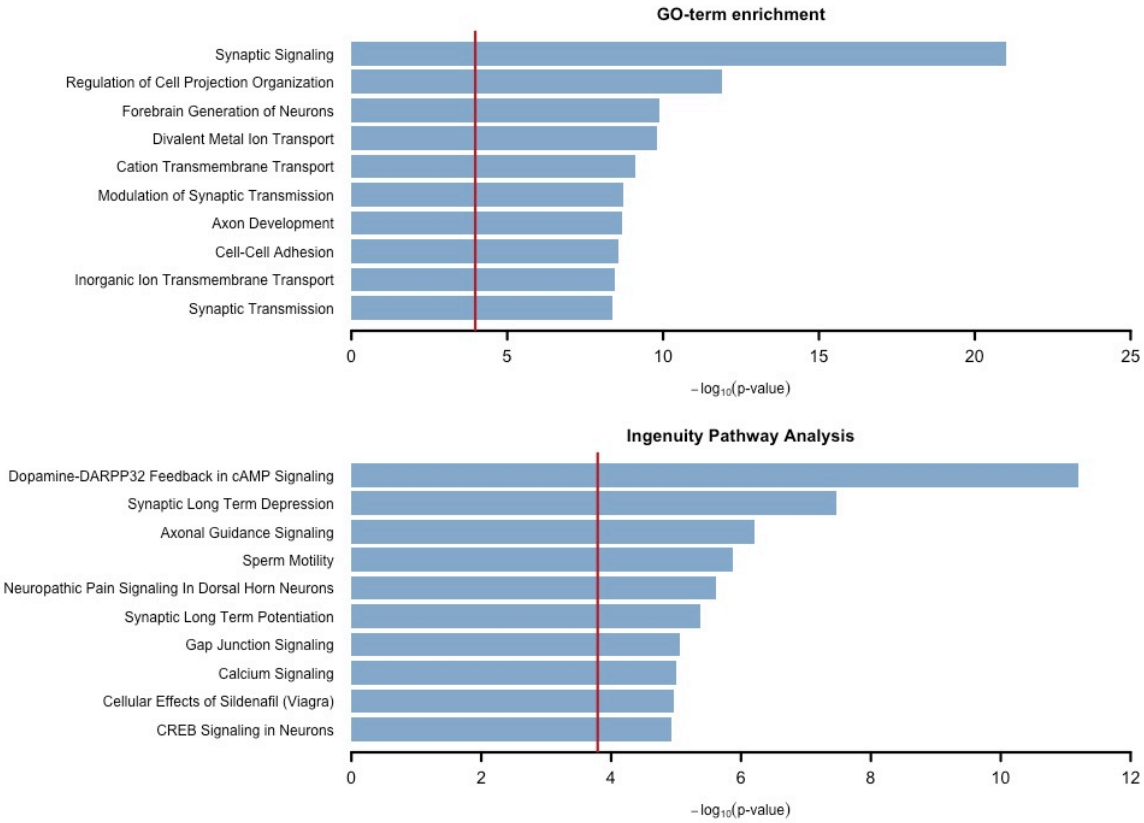

Figure 2 Results of exploratory gene set enrichment analyses. Top-10 enriched gene sets and pathways from GO term enrichment and IPA are shown. $-\log_{10}$ converted enrichment p-values are represented with blue bars. Red lines indicate the Bonferroni-corrected significance threshold for GO-term enrichment analysis ($\alpha = 1.06 \times 10^{-4}$, red line) and IPA ($\alpha = 1.61 \times 10^{-4}$).

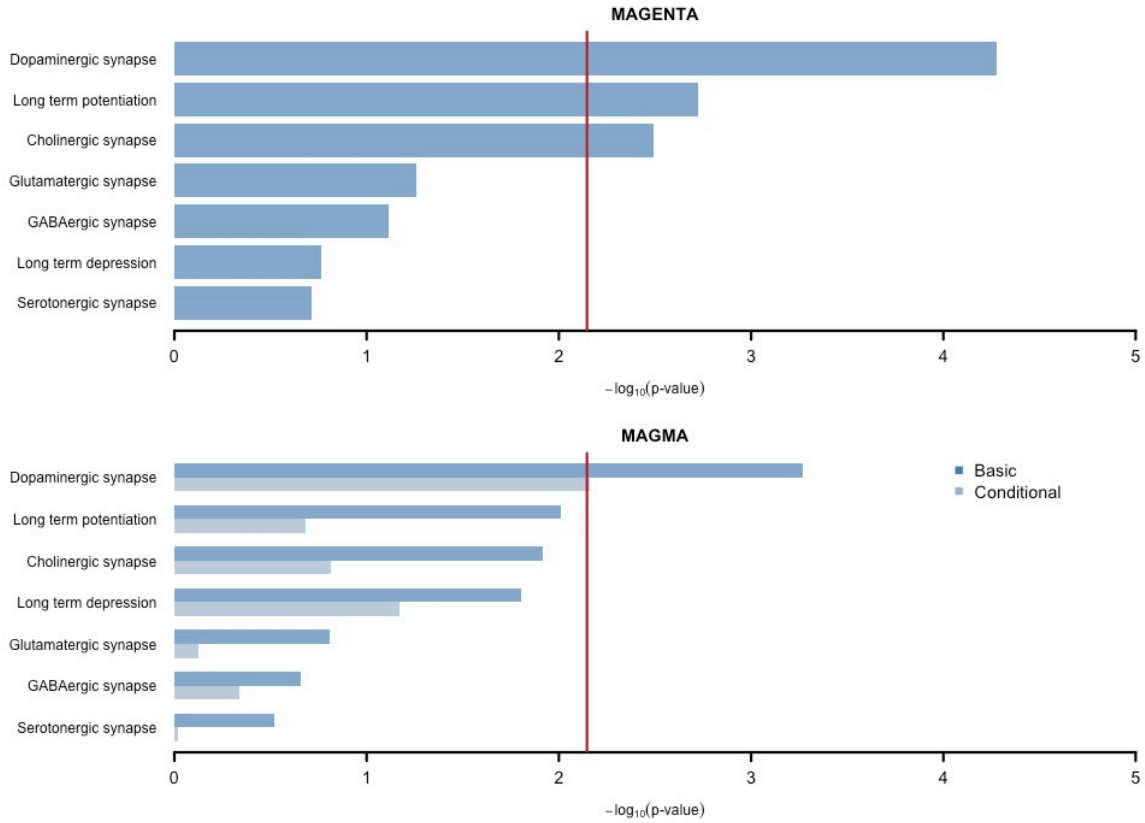

Figure 3 Results of targeted pathway enrichment analyses in KEGG nervous system gene sets. Results from MAGENTA and MAGMA for seven tested synaptic signaling gene sets are shown. Bars show -$\log_{10}$ converted enrichment p-values. For MAGMA analyses, the results from both the basic (blue) and conditional (grey) competitive gene set analyses are shown. The vertical red lines indicate the Bonferroni-corrected significance threshold ($\alpha = 7.14 \times 10^{-3}$).

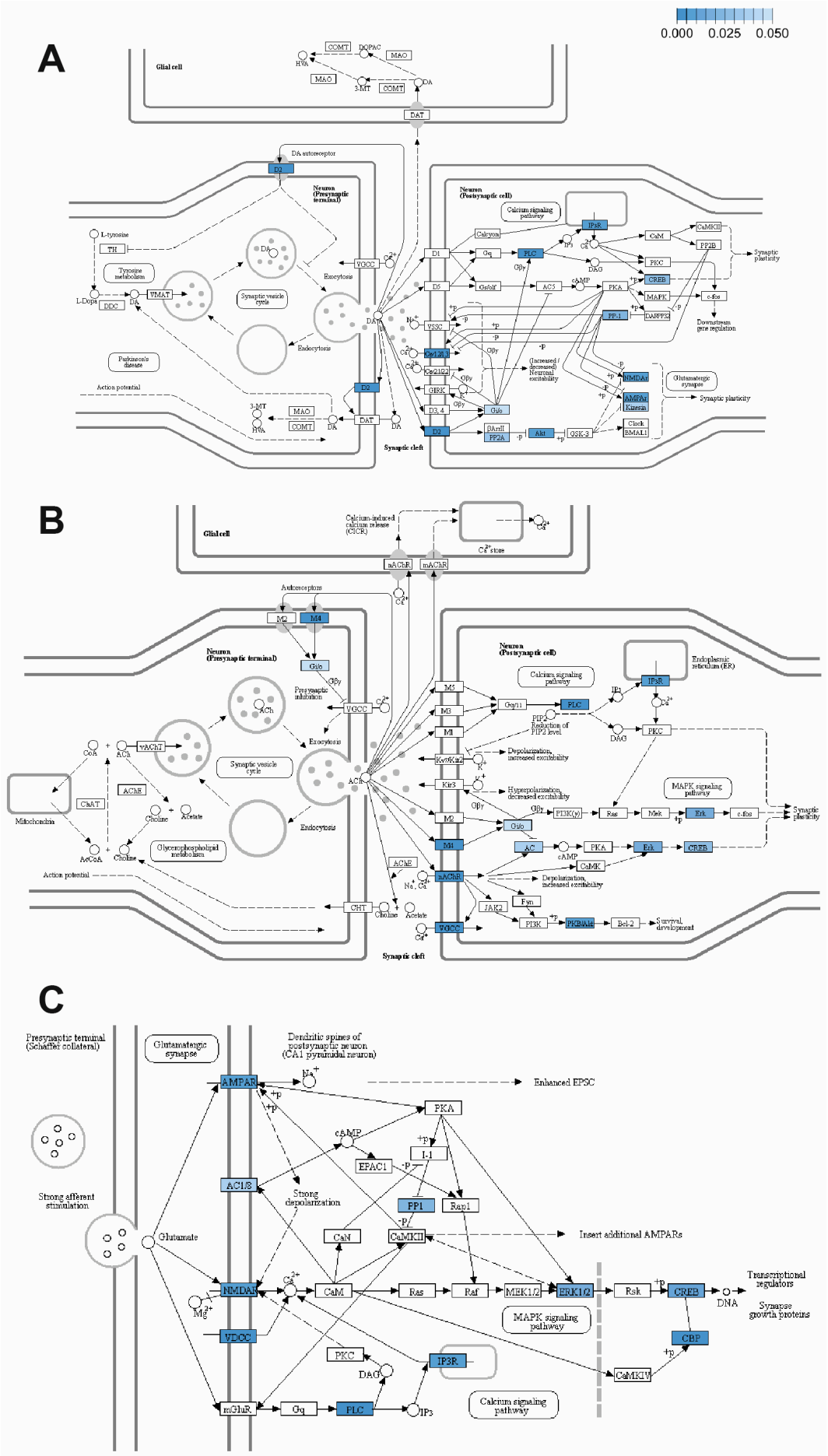

Figure 4 Localization of SNP enrichment in molecular components of synaptic signaling in A) Dopaminergic synapse, B) Cholinergic synapse and C) Long-term potentiation

Enrichment p-values < 0.05 for components are marked with blue shading, with darker blue for lower enrichment p-values. Components with enrichment p-values > 0.05 are not marked. Data represented on graphs derived from KEGG[25] using the *pathview*[26] package in R.

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