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### Title

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### Permalink

<https://escholarship.org/uc/item/9ns3g71r>

### Journal

Molecular Psychiatry, 22(6)

### ISSN

1359-4184

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### Publication Date

2017-06-01

### DOI

10.1038/mp.2017.33

Peer reviewed

## PERSPECTIVE

## Genetic evidence for role of integration of fast and slow neurotransmission in schizophrenia

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The most recent genome-wide association studies (GWAS) of schizophrenia (SCZ) identified hundreds of risk variants potentially implicated in the disease. Further, novel statistical methodology designed for polygenic architecture revealed more potential risk variants. This can provide a link between individual genetic factors and the mechanistic underpinnings of SCZ. Intriguingly, a large number of genes coding for ionotropic and metabotropic receptors for various neurotransmitters—glutamate,  $\gamma$ -aminobutyric acid (GABA), dopamine, serotonin, acetylcholine and opioids—and numerous ion channels were associated with SCZ. Here, we review these findings from the standpoint of classical neurobiological knowledge of neuronal synaptic transmission and regulation of electrical excitability. We show that a substantial proportion of the identified genes are involved in intracellular cascades known to integrate ‘slow’ (G-protein-coupled receptors) and ‘fast’ (ionotropic receptors) neurotransmission converging on the protein DARPP-32. Inspection of the Human Brain Transcriptome Project database confirms that that these genes are indeed expressed in the brain, with the expression profile following specific developmental trajectories, underscoring their relevance to brain organization and function. These findings extend the existing pathophysiology hypothesis by suggesting a unifying role of dysregulation in neuronal excitability and synaptic integration in SCZ. This emergent model supports the concept of SCZ as an ‘associative’ disorder—a breakdown in the communication across different slow and fast neurotransmitter systems through intracellular signaling pathways—and may unify a number of currently competing hypotheses of SCZ pathophysiology.

*Molecular Psychiatry* (2017) **22**, 792–801; doi:10.1038/mp.2017.33; published online 28 March 2017

## INTRODUCTION

Schizophrenia (SCZ) is a complex human disease, with heritability (or disease risk due to genetic factors) estimates ranging from 0.6 to 0.8.<sup>1</sup> Until recently, genome-wide association studies (GWAS) have identified only a small number of associated genes or loci accounting for a miniscule fraction of the heritability.<sup>2</sup> The turning point was the establishment of the Psychiatric Genomic Consortium,<sup>3</sup> which enabled the pooling of large numbers of independent studies, greatly increasing the power for identifying genes affecting disease risk, and confirming the polygenic nature of psychiatric disorders.<sup>2,4–6</sup> In addition to increasing the sample size, a key to improved yield from SCZ GWAS was refined statistical methods.<sup>7</sup> Recent application of this approach in SCZ<sup>6</sup> indicated an increase in power for gene discovery over standard methods,<sup>8</sup> and extended the list of putative risk genes for SCZ.<sup>8</sup> While these findings underscore the

polygenic nature of SCZ, it is a challenge to provide a comprehensive explanation linking the gene associations with underlying pathophysiology.

The recently discovered loci include genes that may provide partial evidence for several previously proposed, competing hypotheses for the pathophysiological basis of SCZ. Historically, mechanistic investigations of SCZ have been driven by a number of seemingly mutually exclusive mechanistic hypotheses—dysregulation of dopaminergic,<sup>9</sup> glutamatergic<sup>10</sup> or GABAergic<sup>11</sup> neurotransmission, disruption of calcium homeostasis or second messenger signaling,<sup>12,13</sup> and growth factor deficiency.<sup>14</sup> The latest GWAS findings<sup>6</sup> have not narrowed the set of possible mechanisms to a specific neurotransmission system or physiological process (e.g., synaptic release). Rather, they further revealed the wide spectrum of molecules involved in different aspects of brain structure and function including hits across neurotransmitter

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Received 19 October 2016; revised 5 January 2017; accepted 17 January 2017; published online 28 March 2017

systems and intracellular machinery, seemingly supporting all the competing hypotheses at once.

These findings may indicate a number of different pathological mechanisms underlying the heterogeneous clinical manifestations indiscriminately diagnosed as SCZ. Alternatively, these seemingly independent hits may be tied together through a common, unifying neurobiological framework. Here, we examine this possibility from the standpoint of classical neurobiological knowledge. Specifically, we inspect the independently discovered genes within the identified loci and show that a substantial proportion of these genes are mapped onto a common network known to be involved in integration of signaling across different neurotransmitter systems. In this network, the signaling cascades triggered by activation of G-protein-coupled receptors (GPCRs) modulate ion channels and ionotropic receptors.

## APPROACH

We examined the list of SCZ-associated single-nucleotide polymorphisms (SNPs) for the presence of genes related to neuronal electrical excitability and neurotransmission falling into the following categories or 'gene sets' (Table 1):

1. ' $Ca^{2+}$  signaling toolkit' gene set was derived from a published expert review unrelated to GWAS.<sup>15</sup> This set included  $Ca^{2+}$  channels, pumps, exchangers and transporters located on the plasma membrane as well as internal membranes of  $Ca^{2+}$  stores (endoplasmic reticulum and mitochondria),  $Ca^{2+}$ -permeable receptors for neurotransmitters and an array of  $Ca^{2+}$ -binding proteins.
2. 'Ion channels' gene set included all plasma membrane  $Ca^{2+}$  channels from the ' $Ca^{2+}$  signaling toolkit' gene set and the following additional channels: voltage-gated  $Na^+$ , voltage-gated  $K^+$ , voltage-gated  $Cl^-$ ,  $Ca^{2+}$ -dependent  $K^+$ ,  $Ca^{2+}$ -dependent  $Cl^-$ , inward-rectifying  $K^+$ ,  $K^+$  leak, cation leak and the hyperpolarization-activated current  $I_h$ . This set was compiled relying on gene naming conventions.
3. 'Ionotropic receptors for neurotransmitters' gene set included AMPA, Kainate, Delta and *N*-methyl-D-aspartate (NMDA) receptors for glutamate,  $\gamma$ -aminobutyric acid A (GABA) receptors, ionotropic serotonin (5HT) receptor 3A, nicotinic acetylcholine (ACh) receptors, and purinergic receptors P2X. This set was compiled relying on gene naming conventions.
4. 'Metabotropic receptors for neurotransmitters' gene set included mGluR receptors for glutamate, GABAB receptors, dopamine receptors, 5HT receptors (excluding ionotropic receptor 3A), muscarinic ACh receptors, noradrenalin (NA) receptors, opioid receptors, adenosine A1 and A2A receptors and purinergic P2Y receptors.
5. We derived two gene sets—'DARPP-32' and 'Dopamine pathway'—from two review articles, unrelated to GWAS, focused on integration of fast and slow neurotransmission. The first article provides an overview of molecular players from the standpoint of the critical integrative role played by DARPP-32.<sup>16</sup> The second is focused on the intracellular cascades downstream dopamine receptors and interaction with glutamate signaling.<sup>17</sup>

Finally, to confirm the relevance of the identified genes to brain function, we used data from the Human Brain Transcriptome Project<sup>18</sup> to examine mRNA expression for each of the genes in Table 1.

## GENES CODING FOR A WIDE RANGE OF IONO- AND METABOTROPIC RECEPTORS, NEUROTRANSMITTERS AND ION CHANNELS

We sought to provide a biological interpretation for the most recently published SCZ GWAS results,<sup>6</sup> reanalyzed using a

covariate modulated mixture model (CM3) statistical method.<sup>8</sup> While the original GWAS report identified 108 gene loci implicating 348 genes in SCZ, based on standard GWAS methods,<sup>6</sup> the CM3 methodology yielded 414 independent gene loci and 1285 potential gene targets with a replication rate of 80%<sup>8</sup> (Supplementary Table 1). The recent pathway analysis of the same GWAS sample<sup>19</sup> as well as studies of *de novo* mutations<sup>20–22</sup> have implicated dysregulation of synaptic function and the balance between neuronal excitation and inhibition. Therefore, we prioritized genes within a general theme of synaptic communication and electrical neuronal excitability. We hypothesized that the increase in gene discovery offered by the CM3 method would provide further support for selective involvement of glutamatergic and GABAergic neurotransmission and voltage-gated calcium channels (VGCCs)—the players that have been implicated by prior studies.<sup>19–22</sup>

In contrast to this expectation, our analyses revealed the presence of genes coding for a wide range of iono- and metabotropic receptors for neurotransmitters as well as various ion channels (Table 1). Given that SCZ is characterized by disabling and often chronic behavioral and cognitive abnormalities,<sup>23</sup> the discovery of genes within a general theme of synaptic communication and electrical neuronal excitability is not unexpected (for a discussion of this topic, see McCarroll and Hyman<sup>24</sup>). More surprising, perhaps, is the presence of hits across many neurotransmitter systems—glutamatergic, dopaminergic, serotonergic, GABAergic, cholinergic and opioid—and ion transport machinery (channels, transporters and pumps) for all four major ions determining the membrane potential:  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$  and  $Cl^-$ .

This begs the question of whether these hits reflect independent mechanisms, or are tied together through common biological pathways and functions. To address this question, we turn to classical neuroscience knowledge of how neurons integrate signaling across different transmitter systems. The original evidence for coupling across neurotransmitter systems was documented by Greengard and co-workers<sup>16,25</sup> in the neostriatum, following the discovery of the protein named DARPP-32 and its role as a convergence node for integration of 'slow' (GPCRs) and 'fast' (ionotropic receptors) neurotransmission.<sup>26</sup> The integration network described in these studies consists of GPCRs and the associated intracellular signaling cascades that—usually via a second messenger (cAMP, cGMP,  $Ca^{2+}$ , diacylglycerol or inositol trisphosphate ( $IP_3$ ))—regulate a set of kinases and phosphatases. The kinases and phosphatases, in turn, control the sensitivity/permeability of ionotropic neurotransmitter receptors and ion channels, ensuring the balance across neurotransmission of different modalities (e.g., dopaminergic and glutamatergic, reviewed in Beaulieu and Gainetdinov<sup>17</sup>).

Motivated by this framework, we evaluated whether the genes identified by our analysis comprise putative elements of this neurotransmitter integration network: (1)  $Ca^{2+}$  signaling toolkit;<sup>15</sup> (2) machinery for ion transport across plasma membrane including ion channels, transporters, pumps and ionotropic receptors; (3) typical GPCR signaling cascades involving GPCRs, G proteins and regulators of their activity, either adenylate cyclase (AC) or phospholipase C (PLC), and the downstream kinases and phosphatases. Table 1 lists the risk genes sorted into each of these categories. Functional interactions between the same genes are illustrated schematically in Figure 1. Below we describe each category; note that each gene has been discovered individually and independently of its function.

## $Ca^{2+}$ SIGNALING TOOLKIT

In excitable cells,  $Ca^{2+}$  is a main second messenger transmitting depolarization to the biochemical machinery of the cell. Therefore, intracellular concentration of free  $Ca^{2+}$  is tightly regulated by an array of channels,  $Ca^{2+}$ -permeable ligand-operated receptors,

**Table 1.** Description of the 92 genome-wide significant loci coding for the expanded version of Paul Greengard's integration of slow and fast neurotransmission (35 loci identified in the original PGC publication<sup>6</sup> are indicated by an asterisk)

Candidate gene name	Genomic location	cmrepl	Description of the candidate gene
<i>Calcium signaling kit</i>			
CACNA1C*	12p13.3	0.9999	VG Ca <sup>2+</sup> channel, L type, $\alpha$ 1C
CACNA1D	3p14.3	0.913	VG Ca <sup>2+</sup> channel, L type, $\alpha$ 1C
CACNA1I*	22q13.1	0.9956	VG Ca <sup>2+</sup> channel, T type, $\alpha$ 1I
CACNB2*	10p12	0.988	VG Ca <sup>2+</sup> channel, $\beta$ 2
ATP2A2*	12q24.11	0.9888	ER Ca <sup>2+</sup> -ATPase (SERCA)
ATP2B2	3p25.3	0.8899	PMCA
ITPR3	6p21.31	0.9559	IP <sub>3</sub> receptor
ITPRIP	10q25.1	0.8421	IP <sub>3</sub> receptor-interacting protein
SCGN	6p22.2	0.8813	Secretagoin, Ca <sup>2+</sup> -binding protein
CALN1	7q11	0.9243	Calneuron, Ca <sup>2+</sup> -binding protein
NRGN*	11q24	0.9927	Neurogranin, Ca <sup>2+</sup> -binding protein
NUCB2	11p15.1	0.8833	Nucleobindin, Ca <sup>2+</sup> -binding protein
CATSPER2	15q15.3	0.8638	Cation channel, sperm-associated
CATSPER2P1	15q15	0.8638	Cation channel, sperm-associated
TRPC4	13q13.3	0.8067	TRP cation channel, C4
TRPM6	9q21.13	0.8966	TRP cation channel, M6
<i>Ion channels (except Ca<sup>2+</sup> channels), pumps and transporters for ions and neurotransmitters</i>			
KCNB1*	20q13.2	0.9277	VG K <sup>+</sup> channel, $\alpha$ 2.1
KCNG2	18q23	0.9145	VG K <sup>+</sup> channel, $\alpha$ 6.2
KCNJ3	2q24.1	0.8246	Kir, subfamily J, member 3
KCNN3	1q21.3	0.8601	SK K <sup>+</sup> channel, Ca <sup>2+</sup> -dependent, $\alpha$ 2.3
KCNV1*	8q23.2	0.9737	VG K <sup>+</sup> channel, $\alpha$ 8.1
CLCN3*	4q33	0.9778	VG Cl <sup>-</sup> channel
HCN1*	5p12	0.9641	I <sub>h</sub> channel
NKAIN1	1p35.2	0.8901	Na <sup>+</sup> /K <sup>+</sup> ATPase (ATP1B1) interacting 1
SLC12A4*	6q22.1	0.9771	K <sup>+</sup> /Cl <sup>-</sup> cotransporter
SLC32A1*	20q11	0.9871	GABA vesicular transporter
SLC44A4	6p21.3	1.0	Choline transporter in neurons
<i>Ionotropic receptors and associated proteins</i>			
GRIA1*	5q33	0.9751	Glutamate receptor, AMPA, GluA1
GRID1	10q22	0.8385	Glutamate receptor, ionotropic, $\delta$ 1
GRIN2A*	16p13.2	0.9737	Glutamate receptor, NMDA, NR2A
SYNGAP1	6p21.3	0.9976	Synaptic Ras-GAP, NMDAR-associated
DLG2	11q21	0.9504	MAGUK, NMDAR-associated
DLG4	17p13.1	0.805	MAGUK, NMDAR-associated
SRR*	17p13.3	0.9888	Serine racemase, NMDA modulator
GABRA2	4p12	0.8194	GABAA receptor, $\alpha$ 2
CHRNA2	8p21	0.9363	Cholinergic receptor, nicotinic, $\alpha$ 2
CHRNA3*	15q24	0.9972	Cholinergic receptor, nicotinic, $\alpha$ 3
CHRNA5*	15q24	0.9972	Cholinergic receptor, nicotinic, $\alpha$ 5
CHRNB4*	15q24	0.9972	Cholinergic receptor, nicotinic, $\beta$ 4
<i>GPCR signaling</i>			
GPCRs			
DRD2*	11q23.2	0.9797	Dopamine receptor D2
GRM3*	7q21.11	0.9742	Glut receptor, metabotropic type II
OPRD1	1p35.3	0.8891	Opioid receptor, $\delta$ 1
OPCML	11q25	0.9031	Opioid-binding protein
GABBR1	6p22.1	1.0	GABAB receptor, 1
GABBR2	9q22.33	0.8618	GABAB receptor, 2
CHRM3	1q43	0.8767	Cholinergic receptor, muscarinic 3
CHRM4*	11p11.2	0.992	Cholinergic receptor, muscarinic 4
HTR1A	5q12.3	0.8956	Serotonin receptor 1A
G proteins			
GNG7	19p13.3	0.9087	G protein, $\gamma$ 7
GNB2	7q22.1	0.8654	G protein, $\beta$ 2
G-protein regulators			
RGS6*	14q24.3	0.9836	Regulator of G-protein signaling 6
RGS7BP	5q12.3	0.8956	Regulator of G-protein signaling 7 BP
GPSM3	6p21.32	1.0	G-protein signaling modulator 3
PLC → DAG pathway			
PLCB2*	15q15.1	0.9293	Phospholipase C, $\beta$ 2
PLCH2*	1p36.32	0.9668	Phospholipase C, $\eta$ 2 (eta2)
PLCL1*	2q33	0.9969	Phospholipase C-like 1
PITPNM2*	12q24.31	0.9996	Necessary for PIP2 synthesis
PLA2G15*	16q22.1	0.9771	Phospholipase A2, group XV

**Table 1.** (Continued)

Candidate gene name	Genomic location	cmrepl	Description of the candidate gene
<i>PRKCB</i>	16p12.2	0.8467	Protein kinase C, $\beta$
<i>PRKD1*</i>	14q11	0.9571	PKD
<i>PRKD3</i>	2p21	0.898	PKD
<i>DGKI*</i>	7q33	0.9772	DAG kinase, iota
<i>DGKZ*</i>	11p11.2	0.992	DAG kinase, zeta
AC $\rightarrow$ PKA pathway			
<i>ADCY1</i>	7p12.3	0.77	Adenylate cyclase 1
<i>PDE9A</i>	21q22.3	0.803	Catalyzes hydrolysis of cAMP/cGMP
<i>PDE4B</i>	1p31.3	0.9298	Catalyzes hydrolysis of cAMP/cGMP
<i>CREB1</i>	2q33.3	0.8803	CREB protein 1
<i>SPHKAP</i>	2q36.3	0.9373	A-kinase anchor protein SPHKAP
<i>AKAP6</i>	14q12	0.895	A-kinase anchor protein 6
<i>NBEA</i>	19q13.3	0.9051	A-kinase anchor protein neurobeachin
GPCR internalization			
<i>CLTA</i>	9p13.3	0.857	Clathrin, light-chain A
<i>AKT3*</i>	1q44	0.9595	PKB, $\gamma$
<i>AKTIP</i>	16q12.2	0.834	AKT (PKB)-interacting protein
DARPP-32/PP1/PP2A			
<i>PPP1R1B</i> (DARPP-32)	17q12	0.8378	PP1, regulatory subunit 1B
<i>PPP1R3B</i>	8p23.1	0.9488	PP1 regulatory subunit 3B
<i>PPP1R10</i>	6p21.33	1.0	PP1 regulatory subunit 10
<i>PPP1R13B*</i>	14q32.33	0.998	PP1 regulatory subunit 13B
<i>PPP1R16B*</i>	20q11.23	0.9871	PP1 regulatory subunit 16B
<i>PPP1CC</i>	12q24.11	0.9888	PP1 catalytic subunit $\gamma$
<i>PPP2R3A*</i>	3q22.2	0.9968	PP2A regulatory subunit B
<i>PPP2R2A</i>	8p21.2	0.8778	PP2A regulatory subunit B, $\alpha$ isoform
<i>PPP2R2B</i>	5q32	0.8242	PP2A regulatory subunit B, $\beta$ isoform
<i>PPP2R3C</i>	14q13.2	0.9437	PP2A regulatory subunit B, $\gamma$ isoform
<i>PPP2R5B</i>	11q13.1	0.8421	PP2A regulatory subunit B', $\beta$ isoform
<i>PHACTR3</i>	20q13.32	0.8345	Phosphatase and actin regulator 3
<i>ANP32E*</i>	1q22	0.9848	LANP-L, inhibits activity of PP2A
<i>CSNK2B</i>	6p21.33	1.0	Casein kinase 2, $\beta$ ; enhances PKA $\rightarrow$ DARPP-32
<i>CLK2</i>	1q21	0.8668	CDC kinase; phosphorylates PP2A
<i>CDK5R1</i>	17q11.2	0.78	CDK5, regulatory subunit 1 (p35)
<i>PRKG1</i>	10q11.2	0.9109	Protein kinase cGMP-dependent (PKG)
<i>NOS1</i>	12q24.22	0.8927	nNOS, part of NO $\rightarrow$ cGMP $\rightarrow$ PKG pathway
<i>NOSIP*</i>	19q13.3	0.9355	nNOS-interacting protein

Abbreviations: AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DAG, diacylglycerol; GABA,  $\gamma$ -aminobutyric acid; IP<sub>3</sub>, inositol trisphosphate; MAGUK, membrane-associated guanylate kinase; NMDA, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; PGC, Psychiatric Genomic Consortium; PIP2, phospholipid phosphatidylinositol-4, 5-bisphosphate; PKA, protein kinase A; PKB, protein kinase B; PKD, protein kinase D; PLC, phospholipase C; PMCA, plasma membrane Ca<sup>2+</sup> ATPase; SERCA, sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase; TRP, transient receptor potential.

pumps and exchangers. Taken together, these proteins are commonly referred to as the 'Ca<sup>2+</sup> signaling toolkit'.<sup>15,27</sup> Within the most significant detected loci (replication rate > 0.8), we found 21 genes coding for the elements of this toolkit (Table 1 and Figure 1). These included VGCC subunits (*CACNA1C*, *CACNA1D*, *CACNA1I* and *CACNB2*), plasma membrane Ca<sup>2+</sup> pump (*ATP2B2*), the endoplasmic reticulum (ER) Ca<sup>2+</sup> pump SERCA (sarco/ER Ca<sup>2+</sup>-ATPase) (*ATP2A2*), Ca<sup>2+</sup>-permeable NMDA receptor (NMDAR, *GRIN2A*), IP<sub>3</sub>-regulated ER Ca<sup>2+</sup> channel (*ITPR3*, *ITPRIP*), Ca<sup>2+</sup>-permeable transient receptor potential cation channel (*TRPC4*, *TRPM6*), Ca<sup>2+</sup>-binding proteins calneuron, secretogin, neurogranin and nucleobindin (*CALN1*, *SCGN*, *NRGN* and *NUCB2*), 'sperm-associated' VGCC expressed in the brain (*CATSPER2* and *CATSPER1*) and Ca<sup>2+</sup>-permeable nicotinic acetylcholine receptor (*CHRNA2*, *CHRNA3*, *CHRNA5* and *CHRNB4*). These results extend the primary Psychiatric Genomic Consortium findings, based on the same data, that identified 9 out of these 21 genes (indicated by an asterisk in Table 1).<sup>6</sup> In addition, we found  $\alpha$ -centractin (*ACTR1A*) and three genes coding for the divalent metal cation transporter (*CNNM2*, *CNNM3* and *CNNM4*). *ACTR1A* and *CNNM2* were reported as Ca<sup>2+</sup> homeostasis-related SCZ risk factors in an earlier GWAS.<sup>2,6</sup>

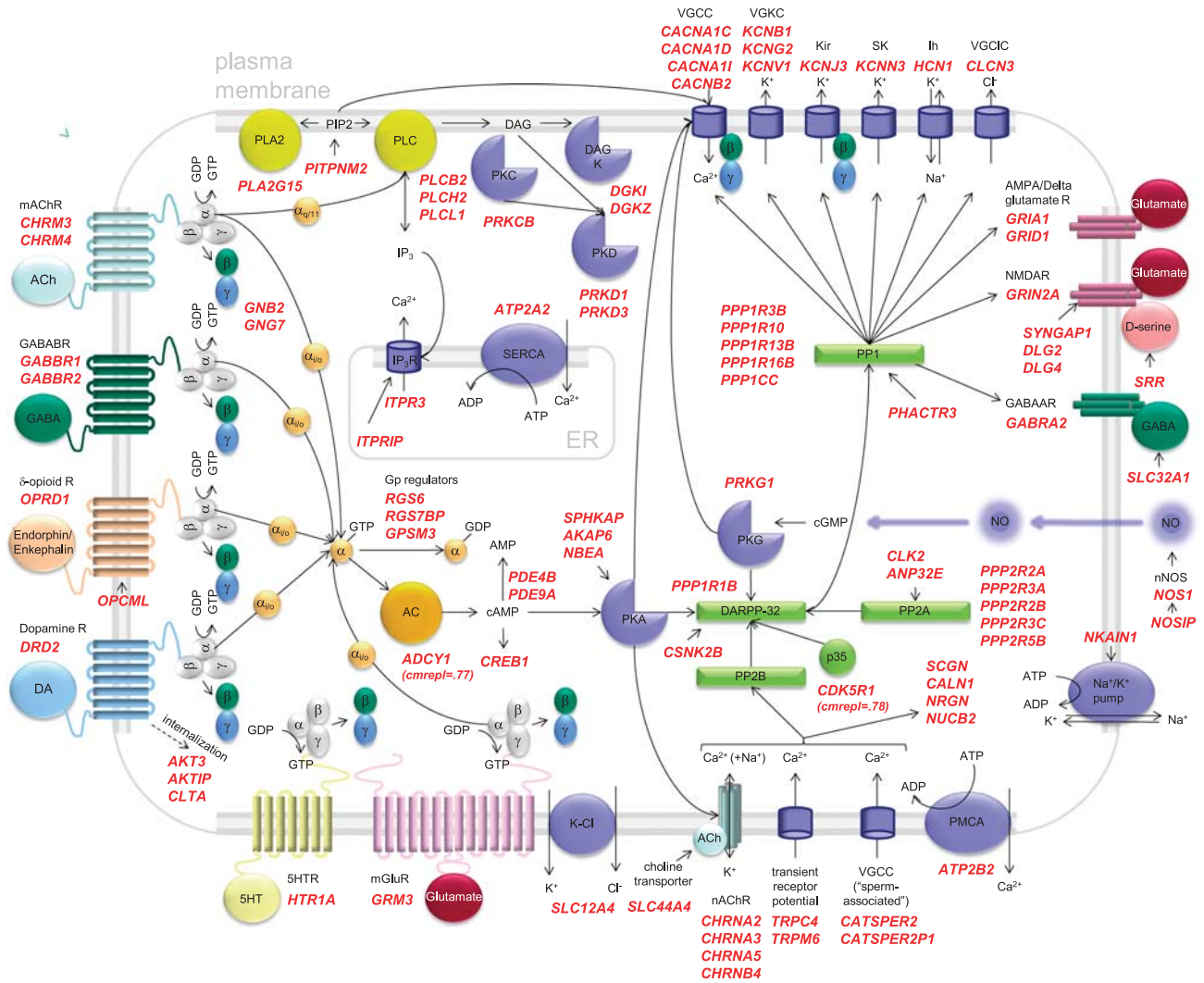
To test statistically for enrichment of SNPs within 'Ca<sup>2+</sup> signaling toolkit' genes, we derived a list of genes from a published expert review unrelated to GWAS<sup>15</sup> (Supplementary Table 2).

Ca<sup>2+</sup> signaling toolkit genes were highly enriched compared with all genes, shown in the fold-enrichment plot (Figure 2).

### ION TRANSPORT ACROSS PLASMA MEMBRANE

VGCCs constitute a hub for integration of the membrane potential, which reflects spiking and synaptic activity, with activation of GPCRs for neurotransmitters and neuromodulators. Indeed, VGCC properties (conductance, activation/inactivation, voltage dependence) are known to be modulated by the direct binding of G-protein G <sub>$\beta\gamma$</sub>  subunits.<sup>28</sup> Therefore, alteration in other ion channels, ionotropic receptors, GPCRs or G proteins may lead to similar endophenotypes as functional deficits in VGCC themselves. Below, we evaluate this hypothesis, starting with mechanisms of ion transport followed by GPCR signaling cascades.

We considered known mechanisms of ion transport across the plasma membrane for the four major ions determining the membrane potential and neuronal electrical excitability: Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Cl<sup>-</sup>. These include ion channels and ionotropic receptors as well as pumps and transporters. In addition to VGCCs (*CACNA1C*, *CACNA1D*, *CACNA1I* and *CACNB2*), we found genes for K<sup>+</sup>-permeable channels of different kinds: voltage-gated *KCNB1*, *KCNG2*, *KCNV1* and *HCN1* (*I<sub>h</sub>* current), inward-rectifying *KCNJ3* and small conductance *KCNN3*. We also found voltage-gated



**Figure 1.** Expanded version of Greengard's neurotransmission integration scheme and its relation to 92 schizophrenia (SCZ) risk genes shown in red (see Table 1 for gene description).

Cl<sup>-</sup> channel (*CLCN3*), K<sup>+</sup>/Cl<sup>-</sup> cotransporter *SLC12A4* and Na<sup>+</sup>/K<sup>+</sup> ATPase-interacting *NKAIN1*.

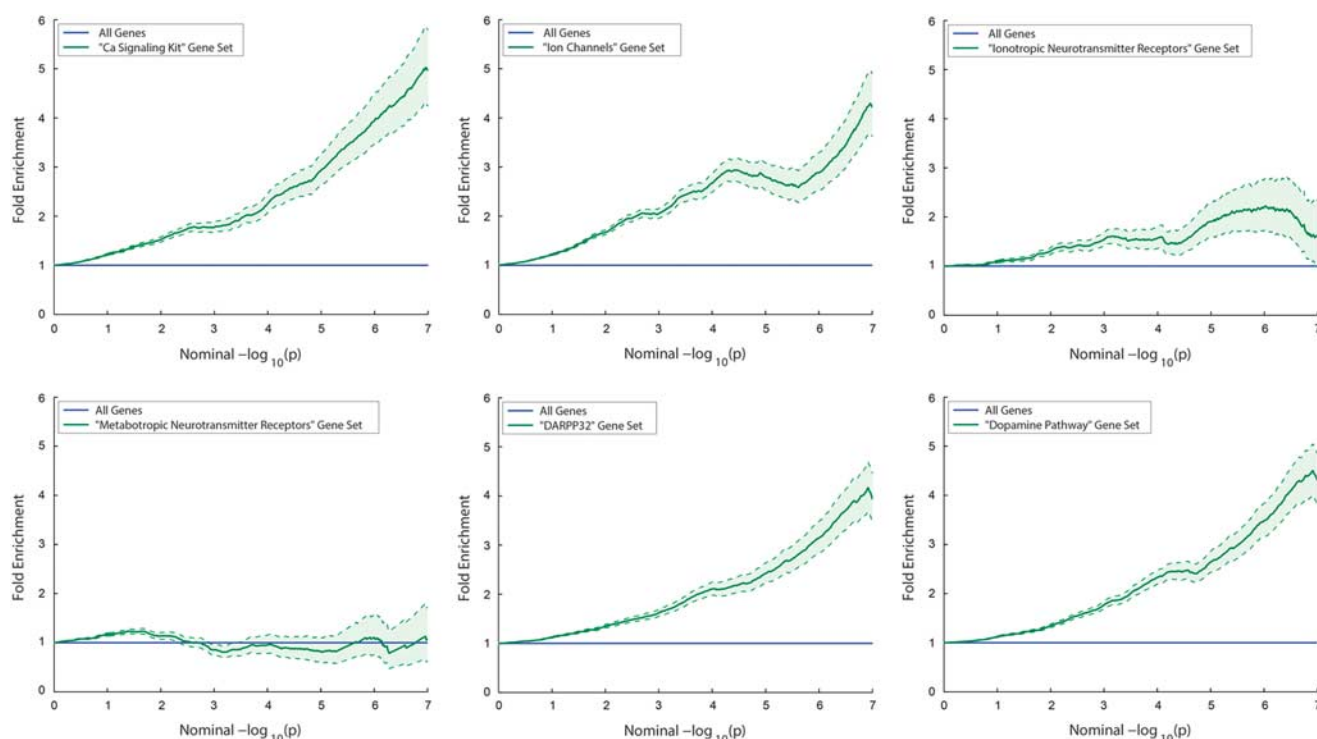
For ionotropic receptors, in addition to the NMDAR (*GRIN2A*), and nAChRs (*CHRNA2*, *CHRNA3*, *CHRNA5*, *CHRNB4*) mentioned above, we found ionotropic glutamate receptors (AMPA subunit *GRIA1* and Delta subunit *GRID1*) and GABAA receptor subunit *GABRA2*. Further, we identified a number of genes that have known direct functional interaction with NMDAR: *SRR* coding for astrocytic enzyme serine racemase known to produce a potent NMDAR modulator D-serine,<sup>29</sup> MAGUK kinases *DLG2* and *DLG4* and synaptic Ras GTPase-activating protein 1 (*SYNGAP1*). Two additional genes coded for neurotransmitter transporters: GABA vesicular transporter *SLC32A1* and choline transporter *SLC44A4*.

It is recognized that cellular membrane permeability to ions is collectively determined by channels, transporters, pumps and ionotropic receptors. However, a single list of all the molecular players is not available. To test for enrichment, we compiled two separate subsets of genes coding for ion channels and ionotropic receptors relying on gene naming conventions (Supplementary Table 2). The ion channels list included all plasma membrane Ca<sup>2+</sup> channels from the 'Ca<sup>2+</sup> signaling toolkit' gene set and the following additional channels: voltage-gated Na<sup>+</sup>, voltage-gated K<sup>+</sup>, voltage-gated Cl<sup>-</sup>, Ca<sup>2+</sup>-dependent K<sup>+</sup>, Ca<sup>2+</sup>-dependent Cl<sup>-</sup>, inward-rectifying K<sup>+</sup>, K<sup>+</sup> leak, cation leak and the hyperpolarization-

activated current *I<sub>h</sub>*. The ionotropic receptors list included AMPA, Kainate, Delta and NMDA receptors for glutamate, GABAA receptors, ionotropic 5HT receptor 3A, nicotinic ACh receptors and purinergic receptors P2X. The fold-enrichment plot in Figure 2 shows clear enrichment for the *Ion channels* and *Ionotropic receptors for neurotransmitters* gene sets.

### GPCR SIGNALING

Properties of ion channels and ionotropic receptors are regulated by downstream signaling cascade activation of GPCRs. For this reason, we examined genes within the identified loci for their association with the typical G-protein signaling cascades involving AC, PLC and the downstream kinases and phosphatases. We found eight hits on GPCRs (*DRD2*, *GRM3*, *OPRD1*, *GABABR1*, *GABABR2*, *CHRM3*, *CHRM4* and *HTR1A*), opioid-binding protein (*OPCML*) having a role in opioid receptor function, G-protein β- and γ-subunits (*GNB2*, *GNG7*), two members of the family of regulators of G-protein signaling (*RGS6*, *RGS7BP*), G-protein signaling modulator *GPSM3* and three genes related to the β-arrestins/GPCR kinase pathway of GPCR regulation/internalization: *AKT3*, *AKTIP* and *CLTA*.



**Figure 2.** Fold-enrichment plots for the following gene sets: ‘Ca<sup>2+</sup> signaling toolkit’, ‘Ion channels’, ‘Ionotropic receptors for neurotransmitters’, ‘Metabotropic receptors for neurotransmitters’, ‘DARPP-32’ and ‘Dopamine pathway’. Shaded areas indicate confidence intervals.

In addition, we found numerous genes coding for the classical AC- and PLC-mediated downstream signaling cascade of GPCRs. The AC cascade included phosphodiesterases that limit the duration of cAMP (and cGMP) signaling (*PDE9A*, *PDE4B*), *SPHKAP*, *AKA6P* and *NBEA* known for their role in anchoring of protein kinase A and cAMP-dependent *CREB1*. A hit on AC itself (*ADCY1*) was just below the chosen 0.8 replication threshold (Table 1).

PLC featured three hits: *PLCB2*, *PLCH2* and *PLCL1*. G proteins that mediate activation of PLC result in the local depletion of a membrane phospholipid phosphatidylinositol-4, 5-bisphosphate (PIP<sub>2</sub>), the presence of which is necessary for maintenance of the high-threshold VGCC currents.<sup>30</sup> Indeed, a number of additional genes were specifically associated with the PIP<sub>2</sub> metabolism: phospholipase A2 (*PLA2G15*), diacylglycerol kinase (*DGKI*, *DGKZ*), protein kinase C (*PRKCB*), protein kinase D (*PRKD1*, *PRKD3*) and a phosphatidylinositol transfer protein required for the synthesis of PIP<sub>2</sub> (*PITNM2*).

We also found numerous hits on kinases and phosphatases that are known to have a central role in the integration of fast and slow neurotransmission:<sup>16,26</sup> the main integration hub DARPP-32 (*PPP1R1B*), other PP1 subunits (*PPP1R3B*, *PPP1R10*, *PPP1R13B* and *PPP1R16B*), PP2A (*PPP2R2A*, *PPP2R3A*, *PPP2R2B*, *PPP2R3C* and *PPP2R5B*), PKG (*PRKG1*), CLK2-stimulating PP2A assembly, *PHACTR3*-inhibiting PP1 and *ANP32E*-inhibiting PP2A. Other genes deserving specific comments are neuronal nitric oxide synthase (*NOS1*) and its interacting protein (*NOSIP*), which is an enzyme producing NO necessary for the NO → cGMP → PKG cascade featured in Greengard<sup>26</sup> (see Figure 3). Finally, a neuron-specific activator of cyclin-dependent kinase 5 p35 (*CDK5R1*) acting on DARPP-32 was present just below the 0.8 replication rate threshold (Table 1).

To test for enrichment, we compiled a separate set of genes coding for metabotropic receptors (for glutamate, GABA, dopamine, 5HT, ACh, NA, opioids, adenosine and ATP), relying on gene naming conventions (Supplementary Table 2). In addition, we

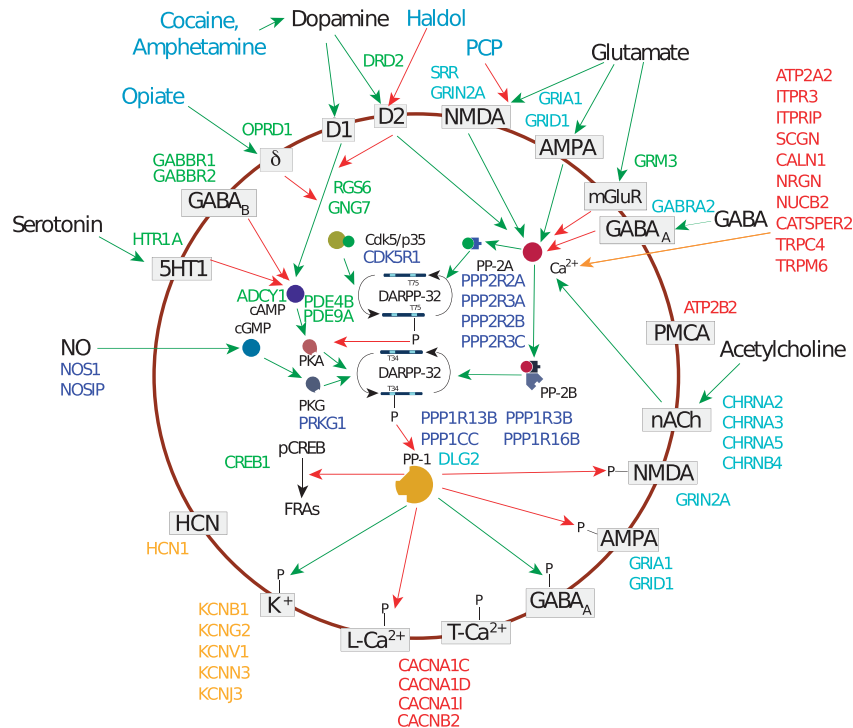
compiled two separate curated gene lists based on two review articles: The first article provides an overview of molecular players from the standpoint of the critical integrative role played by DARPP-32.<sup>16</sup> The second one focused on the intracellular cascades downstream dopamine receptors and interaction with glutamate signaling.<sup>17</sup> Although metabotropic receptors were not enriched, we found clear and strong enrichment for both the *DARPP-32* gene list, and the *Dopamine pathway* gene list (Figure 2).

#### GENE EXPRESSION PROFILE

To play the proposed role in dysregulation of neuronal excitability and synaptic integration, the genes identified by our analysis (Table 1) should be, at the very least, expressed in the brain tissue. To this end, we used data from the Human Brain Transcriptome Project<sup>18</sup> to examine mRNA expression for each of these genes in six brain regions of the developing and adult human brain. With the exception of *CATSPER2*, *RGS7BP* and *SPHKAP*, for which expression data were not available, our analysis confirmed that these genes are indeed expressed in the brain in a region-specific manner and with the expression profile following specific developmental trajectories, underscoring their relevance to brain organization and function (Supplementary Figure 1).

#### COMPREHENSIVE MODEL OF SCZ PATHOPHYSIOLOGY

SCZ is known as a complex polygenic disorder and is sometimes discussed as a heterogeneous clinical diagnosis of what could be unrelated dysfunctions on the level of cell biology.<sup>31</sup> In isolation, individual genes identified in our study can be viewed as supportive of the dopaminergic,<sup>17</sup> glutamatergic<sup>32</sup> or calcium homeostasis<sup>13</sup> hypotheses of the disease. However, considered together, the present results suggest an alternative model in which the existence of the SCZ phenotype with multiple genetic risk factors can be explained by the convergence and tight



**Figure 3.** Overlap of the present results with Greengard's neurotransmission integration scheme. DARPP-32 integrates information via a variety of neurotransmitters (black) and neuroactive drugs (light blue). Only a subset of the identified schizophrenia risk genes is shown. Gene names are color coded according to the categories in Table 1: calcium signaling kit (red), ion channels except Ca<sup>2+</sup> channels (yellow), ionotropic receptors and associated proteins (cyan), GPCR signaling proteins (green) and DARPP-32 integration pathway (blue). Red arrows indicate inhibition, green arrows indicate stimulation, and yellow arrow represents varied functions of the calcium signaling kit genes. Adapted from Greengard.<sup>26</sup>

communication between seemingly independent neurotransmitter pathways controlling neuronal excitability and, ultimately, neuronal network function/dysfunction. The overlap between the SCZ risk genes discovered using our analysis and the previously described neurotransmitter integration network is illustrated in Figure 3, which was generated by populating a figure from Greengard's 2001 review<sup>26</sup> with our identified genes (only a subset of those from Table 1/Figure 1 is shown).

The idea that multiple factors of a polygenic disease are not distributed randomly across the genome but involve SNPs that share a common biological function has been discussed previously for brain diseases,<sup>33–35</sup> and has been demonstrated for non-neuropsychiatric disorders.<sup>36–39</sup> In SCZ, the evaluation of functional relationships between individual risk variants finally became possible because of the availability of large data sets and powerful methods for statistical analysis that enable identification of a sufficient number of genome-wide molecular risk factors. Our results corroborate earlier reports on the association of SCZ with polymorphisms in genes coding for VGCCs,<sup>4</sup> and genes related to synaptic function and neuronal excitability.<sup>4,5,19–21,33,40–44</sup> Importantly, we show that many of these genes are mapped onto a common network, where the signaling cascades triggered by activation of GPCRs modulate ion channels and ionotropic receptors. This is achieved by activation of the intracellular kinases and phosphatases ensuring the appropriate level of excitability, and thus neuronal output, given the intensity and modality of the input. This is in line with the notion that specific therapeutic effects of antipsychotic agents involve mechanisms converging on normalizing synaptic transmission.<sup>45</sup> An effect on DARPP-32 of antipsychotics has also been reported in neuronal cell cultures.<sup>46</sup>

While each of the SNPs involved have only minor effects by themselves on neuronal function, a critical number of synergistic variants may result in impaired neuronal function and thus SCZ

symptoms. For example, alteration in Ca<sup>2+</sup> signaling would affect such fundamental cellular level mechanisms as communication between the apical dendrite and the soma in cortical pyramidal cells leading to altered association of inputs at the cellular level and disturbed synaptic communication in neuronal networks.<sup>47</sup> This could be collectively driven by the structural variation in the subunits of VGCCs, their post-translational modification and change in the electrical membrane excitability because of the up/downregulation of K<sup>+</sup> conductances.

There is compelling evidence that SCZ is a neurodevelopmental disorder involving abnormal synaptic formation and connectivity (for review see Insel<sup>23</sup> and Marengo and Weinberger<sup>48</sup>). With this in mind, it is tempting to speculate that any of the factors that alter the integration network depicted in Figure 1 are likely to evoke a series of compensatory mechanisms guided by neuronal plasticity rules, such as achieving the desirable end-point level of synaptic transmission.<sup>49</sup> These mechanisms of adjustment have been documented on the single-neuron and neuronal network level in the valproic acid rat model of autism.<sup>50,51</sup> In this model, a decrease in the intrinsic (single-cell) excitability was accompanied by a compensatory increase both in NMDA receptor expression and synaptic connectivity.<sup>52</sup> Thus, compensatory mechanisms set off by a deviation in the developmental course may attempt but not achieve the normal phenotype capable of supporting typical brain function. As such, these regulatory mechanisms may push the neuronal network to a new 'state', with dysfunctional information processing that it is associated with the SCZ behavioral phenotype.<sup>53</sup> Thus, the common denominator among the genetic risk factors for SCZ might be the neurodevelopmental convergence upon a similar end-point functional network organization. This convergence is not unexpected taking into account the tight interactions across neurotransmitter systems.<sup>16</sup>



While the present analysis is focused on neuronal signal transduction and membrane excitability, one can imagine that similar compensation/plasticity can be triggered by deficits in the establishment, function, and modification of synaptic connections related to abnormal surface recognition, axonal guidance, vesicular transport or exocytosis machinery. Indeed, regulated cytoskeleton-associated protein complexes, important for synaptic structure, function and plasticity, have been implicated in SCZ by recent *de novo* mutation studies.<sup>20,22</sup> In addition, impairment of cellular energetics may compromise neuronal repolarization, Ca<sup>2+</sup> clearance and ionic homeostasis contributing to the deficit in excitability/intracellular signaling. Careful consideration of these mechanisms is beyond the scope of the current perspective article.

Our current approach has a number of limitations. As with all GWASs, identification of disease-associated genes in the current study relies on the presence of common variants in the sample population. Therefore, the absence of known risk genes, such as *DISC1*, among our identified genes does not contradict their established relationship to SCZ,<sup>54</sup> but rather suggest that common inherited variants in these genes are not significant contributors to disease risk. Moreover, a lack of association for a particular gene does not imply that the molecule does not have a significant role in the relevant signaling cascades, but rather that the specific common variants or SNPs of the gene do not alter the relevant functional properties. In some cases significant functional variants may not be tolerated.<sup>55</sup> Another limitation shared between all GWASs is that each of the identified gene loci may contain multiple genes in linkage disequilibrium (i.e., inherited together) where individual contributions cannot be disentangled with current methods. Therefore, variation in some of the identified genes may not, in fact, contribute to SCZ pathophysiology.

Further investigation will be required to address the significance of specific SNP variants for the function of the respective genes (e.g., conductance, gating or voltage sensitivity of VGCC), using an approach that takes into account the polygenic context. Similarly, dedicated mechanistic studies will be needed to test the hypothesis of SCZ etiology as a dysfunction of integration across neurotransmitter systems. These studies would leverage methods and technologies beyond genetic association, such as measurement, manipulation and genetic engineering in neurons derived from human-induced pluripotent stem cells (hiPSCs). With small effects of individual risk variants, the difference in function due to, for example, alteration in a single type of ion channel may be too small to detect. Therefore, we propose using functional readouts at the level of convergence, such as neuronal excitability of single-neuron or/and neuronal network, while choosing 'cases' and 'controls' based on a predefined set of high-risk variants. Specifically, we envision the following lines of inquiry:

1. Establishing the cellular and small network-level functional endophenotype characteristic of SCZ using hiPSC-derived neurons. These experiments would take advantage of novel gene-editing methods,<sup>56</sup> which now allow replacement of large DNA segments, as well as the growing arsenal of optogenetic and chemogenetic manipulation tools<sup>57–59</sup> and optical reporters,<sup>60</sup> being developed under the BRAIN Initiative.<sup>61</sup> In this approach, we would rank patient-derived hiPSCs according to the number of high-risk variants, weighted by their strength of disease association, within a predefined set of genes, such as that comprising Table 1. Next, we would choose hiPSCs with the highest risk scores, and generate the corresponding negative controls by substituting high-risk variants with those carrying low risk. Conversely, we would choose hiPSCs with the lowest risk scores, and generate the corresponding positive controls by substituting low-risk variants with those carrying high risk. Then, we would subject the resulting cell lines to a series of well-controlled opto- and/or chemogenetic stimuli

using specific optical reporters for high-throughput phenotyping of induced responses.<sup>62</sup> These experiments would establish a mapping between groups of risk variants and the cellular/network behavior, and answer the question of whether accumulation of risk variants along seemingly independent intracellular signaling pathways can result in a similar functional endophenotype.

2. Direct opto- or chemogenetic manipulation of the key integration hubs such as DARPP-32. The question is whether directly interfering with the activity of converging nodes integrating fast and slow neurotransmission in control neurons (and networks) can evoke functional endophenotype characteristic for neurons bearing a significant load of SCZ risk variants.
3. Extension of (1) and (2) to organized cell cultures, such as 'corticoids',<sup>63,64</sup> and 'chimeric' mice.<sup>65</sup> These model systems promote neuronal maturation and may better approximate a natural neuronal circuit environment.
4. Investigation in transgenic mouse models of SCZ.<sup>66</sup> Although the validity of these models remains controversial,<sup>67</sup> some of them, such as *DISC1*<sub>L100P</sub>,<sup>54,68</sup> exhibit neuroanatomical and behavioral changes seen in SCZ patients and respond to antipsychotic drugs.<sup>69</sup> The question is whether *DISC1* dysfunction results in the same functional endophenotype as in (1).
5. Investigation of the effects of antipsychotic drugs (e.g., haloperidol and clozapine) on normalizing the SCZ functional endophenotype in hiPSC-derived neurons and in responsive mouse models.
6. Combination of the above-mentioned experimental approaches (1–5) with 'computational psychiatry'.<sup>70,71</sup> The basic idea is to use experimentally obtained mapping between SCZ risk variants and the cellular/network behavior to constrain computation models of single neurons and neuronal networks providing mechanistic biophysical basis for the SCZ functional endophenotype. A detailed 'realistic' model of cortical column<sup>72,73</sup> has recently become available because of a decade-long effort by the Blue Brain Project currently continuing under the umbrella of the Human Brain Project. These models of synaptically connected spiking neurons with realistic morphologies can be combined with the physical principles governing generation of the extracellular potentials<sup>74,75</sup> for prediction of macroscopic electro- or magnetoencephalography signals resulting from complex circuit phenomena. These models may help bridge the cellular level biophysics to well-established neuronal deficits measured noninvasively in SCZ patients such as the lack of prepulse inhibition.<sup>76</sup>

Although the focus of the current analysis is limited to SCZ, it is quite possible that the biological mechanisms are more closely associated with specific symptom dimensions, such as psychosis, across psychiatric diseases. In fact, recent cross-disorder GWAS analyses, leveraging novel statistical methodologies,<sup>77</sup> suggest extensive genetic pleiotropy among psychiatric diseases,<sup>78–81</sup> and other phenotypes.<sup>82–84</sup> The relationship between specific symptom dimensions and biological mechanisms can be further investigated by focusing on substudies with more comprehensive assessments of symptom dimensions, rather than case-control information alone.

To summarize, the spectrum of molecular risk factors identified in our study supports the concept of SCZ as an 'associative' disorder: a breakdown in the communication across different slow and fast neurotransmitter systems through intracellular signaling pathways. This emergent model may unify a number of currently competing hypotheses, for example, dopaminergic, glutamatergic, calcium, second Messenger (reviewed in Lidow<sup>13</sup>), and the recently put forward Balance between Excitation and Inhibition.<sup>20</sup> Future investigations, leveraging novel technologies

for measurement, manipulation and gene editing, as well as novel computational tools, will be required to bridge the gap between the correlational results of GWAS and the underlying biological mechanisms, enabling 'brain-based' diagnoses and, ultimately, treatment of mental disease.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

We gratefully acknowledge support from the NIH (NS057198, EB00790, R01MH111359), the Research Council of Norway (229129, 213837, 223273), the South-East Norway Regional Health Authority (2017-112, 2016-064) and KG Jebsen Stiftelsen (SKGJ-MED-008).

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)