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Glutamate hypothesis in schizophrenia

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Schizophrenia is a chronic and severe psychiatric disorder that has profound impact on an individual's life and on society. Thus, developing more effective therapeutic interventions is essential. Over the past quarter-century, an abundance of evidence from pharmacologic challenges, post-mortem studies, brain imaging, and genetic studies supports the role of glutamatergic dysregulation in the pathophysiology of schizophrenia, and the results of recent randomized clinical trials based on this evidence have yielded promising results. In this article, we review the evidence that alterations in glutamatergic neurotransmission, especially focusing on the *N*-methyl-D-aspartate receptor (NMDAR) function, may be a critical causative feature of schizophrenia, how this contributes to pathologic circuit function in the brain, and how these insights are revealing whole new avenues for treatment development that could reduce treatment-resistant symptoms, which account for persistent disability.

Keywords: *D-serine, glutamate, N-methyl-D-aspartate receptor, schizophrenia, serine racemase.*

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Schizophrenia is a chronic and severe psychiatric disorder that has a profound impact on both an individual's life and on society. It is characterized clinically by positive symptoms (i.e., hallucinations, delusions, disorganized thinking, and grossly disorganized or abnormal motor behavior), negative symptoms (i.e., diminished emotional expression, avolition, alogia, anhedonia, and asociality), and cognitive deficits (i.e., deficits in attention, working memory, and executive function). The cognitive deficits are known to appear before clinical diagnosis[1] and are strongly linked to functional outcomes, such as academic and occupational function and independent living.

The prevalence of schizophrenia has been estimated to be approximately 1% in the general population, and it exhibits high heritability. According to a recent twin study with a large, adequately powered sample and improved statistical methods,[2] the heritability was calculated to be 79%, consistent with prior results. However, the pattern of inheritance is non-Mendelian, supporting the role of complex genetics. Thus, genetic risk factors strongly contribute to the cause of schizophrenia, but at the same time environmental factors also play a significant role. Schizophrenia is considered to be a neurodevelopmental disorder because environmental insults, such as infection, are concentrated in the perinatal period.[3]

In this article, we will review the mounting evidence that dysfunction of the *N*-methyl-D-aspartate receptor (NMDAR) may be a critical causative feature of schizophrenia, how this contributes to pathologic circuit function in the corticolimbic system, and how these insights are revealing whole new avenues for treatment development that could ameliorate the current treatment-resistant negative and cognitive symptoms, which account for persistent disability.

The history of pathological hypotheses in schizophrenia

Hypotheses before the discovery of antipsychotic drugs

Existence of people with psychiatric symptoms, such as hallucinations and delusions (phrenitis), was already known in the era of Hippocrates (460–375 BC), the so-called father of medicine. His contemporaries believed that these disorders were due to abnormal conditions of the body caused by physical factors. They tried to treat these disorders in several ways believed to have potential therapeutic effects, including eating the root of *Rauvolfia serpentina*, which contains reserpine that inhibits the storage and use of biogenic amines.

In 1899, Emil Kraepelin, a German psychiatrist, integrated several psychiatric conditions, such as hebephrenia, catatonia, and dementia paranoides, which are now known to be subtypes of schizophrenia, and developed the concept of dementia praecox.[4] Since his assumptions were that those conditions led to irreversible loss of cognitive functions and were resistant to treatment, he classified them as a dementia.

The term 'schizophrenia,' which is used currently, was coined in 1908 by Eugen Bleuler, a Swiss psychiatrist.[5] He revised the idea of dementia praecox and renamed it 'schizophrenia.' Notably, he identified a disconnect between affect and thought processes (splitting, e.g., 'schiz') as the most fundamental feature of schizophrenia and ambivalence and autism as basic symptoms, whereas positive symptoms, such as hallucinations and delusions, were considered as accessory symptoms.[6,7] He also speculated that the core symptoms may respond to different treatments than the ancillary symptoms. This hypothesis proved prescient when antipsychotics were found to be effective against positive symptoms but ineffective against negative and cognitive symptoms.[8] Later, Kurt Schneider, a German psychiatrist, proposed a list of highly disorder-specific forms of delusions and hallucinations based on a disturbance of self-identity, which he termed 'first-rank symptoms,' whereas he deemed negative and cognitive symptoms as secondary symptoms.[4,9] However, the specificity of first-rank symptoms for schizophrenia has been questioned.[10]

Dopamine hypothesis

The first antipsychotic, chlorpromazine, which was originally developed as an antihistaminergic drug for use in a range of conditions, including nausea and allergies, and reserpine, a Rauvolfia alkaloid, were demonstrated to be effective in treating the psychosis of

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schizophrenia[11–13] in the early 1950s. Shortly afterward, more potent phenothiazines and butyrophenones drugs were developed and proved to be highly efficacious in alleviating symptoms of schizophrenia.[14–16] However, they were prone to inducing Parkinsonian-like extra-pyramidal side-effects.[15] Moreover, reserpine was found to cause depletion of monoamine, such as dopamine, in synaptic vesicles via inhibiting vesicular monoamine transporter. Alpha-methyl-para-tyrosine, which was known as a specific inhibitor of tyrosine hydroxylase, the initial enzyme in the synthesis pathway for catecholamines, was also shown to reduce symptoms of schizophrenia.[17] Carlsson synthesized these observations and proposed that antipsychotics exerted their therapeutic effects by blocking dopamine receptors, for which he received the Nobel Prize.[18]

'Stimulants,' such as amphetamine, which inhibits the presynaptic vesicular monoamine transporter 2 and the dopamine transporter, increase dopamine concentrations in the synaptic cleft. Consistent with the antipsychotic effects of depleting dopamine, high doses of stimulants induce a psychosis that clinically resembles the acute phase of paranoid schizophrenia.[19] Animal data indicated that both phenothiazines and butyrophenones reduced dopamine-mediated behaviors induced by amphetamine or apomorphine.[20–22] Synthesizing the opposing effects of stimulants and antipsychotics on dopaminergic neurotransmission, Snyder proposed the 'dopamine hypothesis' that schizophrenia was due to excessive stimulation of dopamine receptors.[23]

Glutamate hypothesis

The dopamine hypothesis can account for certain aspects of the psychopathology of schizophrenia, especially positive symptoms.[24] However, with the possible exception of clozapine, antipsychotics have negligible effects on negative and cognitive symptoms, the most robust predictors of disability in schizophrenia.[25] Furthermore, cortical atrophy correlates with negative and cognitive symptoms in chronic schizophrenia but not with the severity of psychosis.[26,27] Thus, the core features of schizophrenia, which are primarily responsible for persistent disability, are linked to pervasive cortical pathology and are unlikely the consequence of simply dopamine dysfunction.[28]

Ketamine and phencyclidine (PCP), which were developed as dissociative anesthetics in 1970s, have been known to induce schizophrenic-like symptoms, including not only psychotic symptoms and thought disorders but also negative and cognitive symptoms in healthy humans.[29,30] Moreover, psychosis induced by ketamine or PCP is clinically difficult to distinguish from the primary psychosis of schizophrenia.[31,32]

The existence of binding sites for ketamine and PCP were described in 1979 followed by reports in the early 1980s that they are noncompetitive antagonists of the NMDA subtype of glutamate receptor.[33] Based on clinical observations of patients intoxicated with PCP and ketamine and careful laboratory studies of the effects of infusion of subanesthetic doses of ketamine in normal volunteers. It was proposed that schizophrenia results from hypofunction of NMDAR.[30,32,34]

What is the NMDA Receptor?

Glutamate is recognized as the most abundant excitatory amino acid neurotransmitter in the brain. It activates G protein-coupled metabotropic (mGlu) receptors and ionotropic receptors. The ionotropic receptors are divided into three subtypes based on their sensitivity to high-affinity selective ligands: NMDAR, α-amino-3-hydroxy-5-methylisoxaole-4-propionate receptors, and kainite receptors. NMDAR show relatively slow and incomplete desensitization compared to non-NMDA ionotropic glutamate receptors.

Structure and function (see Fig. 1)

NMDAR comprise a heterotetrameric complex of two obligatory GluN1 subunits with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits. The GluN1 subunit is encoded by a single gene (*GRIN1*) but has eight different isoforms owing to alternative splicing. GluN2 subunits and GluN3 subunits also have four (GluN2A - D) and two variants (GluN3A - B), which are encoded by separate genes, *GRIN2A-D and GRIN3A-B*, respectively. Each GluN subunit has a typical modular architecture with two large clamshell-like extracellular domains (an amino-terminal domain involved in assembly and channel modulation, and a ligand-binding domain), a transmembrane domain, and a carboxy-terminal domain involved in receptor trafficking and signaling. The amino-terminal domain and carboxy-terminal domain regions are the most divergent and account for much of the functional diversity of NMDAR.[35] D-serine and glycine bind to GluN1 and GluN3 subunits, which is designated the glycine modulatory site (GMS), and glutamate binds to GluN2 subunits at the glutamine binding site.[36] This receptor is modulated by the GluN2A and GluN2B allosteric antagonists divalent zinc and the phenylethanolamines ifenprodil or Ro25-6981, respectively.[37] Moreover, there are binding sites for adjusting ion channel activation, such as the redox modulatory site where glutathione binds on GluN1 and 2A,[38] spermine binding site on GluN2B and Mg^{2+}, MK-801, and the ketamine and PCP binding site on the transmembrane domain.[35]

Activation of the NMDAR uniquely requires three simultaneous events: (i) postsynaptic depolarization, typically by activation of α-amino-3-hydroxy-5-methylisoxaole-4-propionate receptors, to remove the magnesium block of the GluN1 cation channel; (ii) occupancy of the GMS on GluN1 by either glycine or D-serine; and (iii) binding of the neurotransmitter glutamate to its receptor on GluN2 to open the channel and permit calcium entry. Calcium induces a cascade of intracellular events that mediate local, acute functional synaptic plasticity and changes in gene expression that promote long-term neural structural plasticity.[39]

Pathophysiological Role of Serine Racemase and D-Serine

Vertebrates were thought not to utilize D-amino acids, although bacteria were known to synthesize several D-amino acids. Serine racemase (SR) and D-serine were discovered in eukaryotic insects in 1966.[40] In 1992, the Nishikawa laboratory was the first to report the presence of free D-serine in the mammalian brain and overthrew the shibboleth that vertebrates do not synthesize D-amino acids.[41] This discovery also resolved a quarter-of-a-century conundrum about the reason for the expression of D-amino acid oxidase (DAAO), an enzyme that de-aminates D-amino acid to imino acid in the brain.[42]

The Snyder laboratory developed the evidence that functional activity of NMDAR required endogenous D-serine as a co-agonist by showing loss of NMDAR activity by perfusing acute hippocampal slices with purified DAAO, which degraded synaptic D-serine, but not glycine.[43] Furthermore, they demonstrated that there was a close correspondence in the expression of SR, NMDAR, especially glutamate, glycine, and PCP binding sites, and D-serine levels in the forebrain[44,45] and an inverse association in DAAO expression and D-serine levels in the cerebellum and hindbrain where glycine was found in high concentrations.[28] Thus, they concluded that D-serine is the primary co-agonist at synaptic NMDAR in the forebrain.[28,46,47] They succeeded in the purification and characterization of SR from the rat brain in 1999.[48] This enzyme is classified as a member of the family of pyridoxal-5'-phosphate-dependent enzymes and catalyzes the formation of D-serine from L-serine.

Cellular localization

D-serine is enriched in corticolimbic regions of the brain and localized to the same areas as NMDAR.[44] The cellular source of D-serine has been hotly disputed. In initial *in vitro* studies,[49] D-serine was believed to be enriched in astrocytes and mainly synthesized in astrocytes, and therefore, SR was considered an astrocytic enzyme.[44,48] While there is still a widely held belief that D-serine and SR are

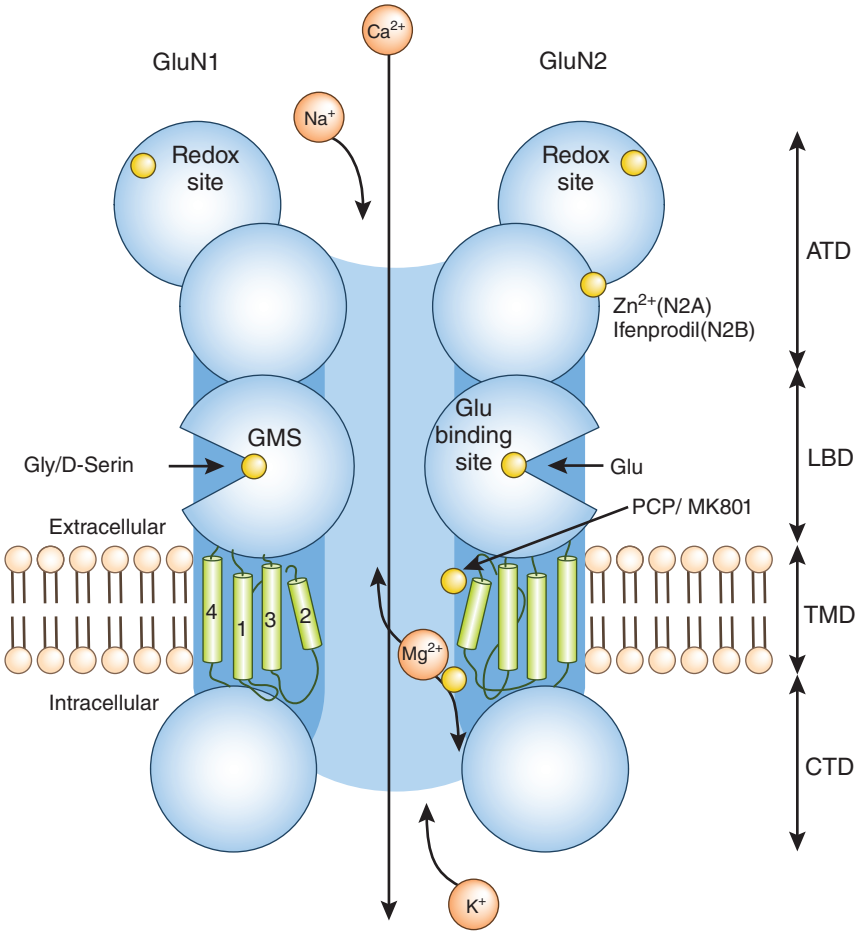

Fig.1 Structure of the *N*-methyl-d-aspartate receptor (NMDAR; GluN1/GluN2). The conventional NMDAR comprises a heterotetrameric complex of two GluN1 and two GluN2 subunits. ATD, amino-terminal domain; CTD, carboxy-terminal domain; GMS, glycine modulatory site; LBD, ligand-binding domain; PCP, phencyclidine; TMD, transmembrane domain.

components of a glial transmitter system,[50] immunohistochemical studies show that the majority of SR is expressed in neurons, rather than in astrocytes. For example, the Mori laboratory called into question the glial synthesis of D-serine a decade ago.[51] In their study, they reported that SR immunoreactivity was predominantly localized to neurons: pyramidal neurons in the cerebral cortex and hippocampal CA1 region, medium-spiny neurons in the striatum and weakly gamma-aminobutyric acid (GABA)ergic Purkinje cells in the cerebellum by using novel SR knockout mice as a control for immunospecificity of their SR antibodies. Furthermore, using mice with conditional deletions of SR either in excitatory forebrain glutamatergic neurons or glial fibrillary acid protein-expressing astrocytes, Benneyworth *et al*. reported that the majority of SR was expressed in glutamatergic neurons and that, by contrast, less than 15% was in astrocytes in the hippocampus and none in the cerebral cortex.[52] Moreover, in human post-mortem neocortex, SR was also found in both excitatory and inhibitory neurons, but not in astrocytes.[53]

Recently, inflammatory A1 reactive astrocytes were found to express SR and synthesize and release D-serine. Reactive astrocytes typically proliferate after brain insults, such as traumatic brain injury (TBI). Hippocampal TBI was shown to cause proliferation of SR-expressing reactive astrocytes over 7 days while neuronal SR in injured neurons declined.[54] Notably, silencing the expression of SR in astrocytes by conditional *srr* inactivation only in astrocytes prevented the electrophysiologic and cognitive deficits after TBI. D-Serine released from reactive astrocytes would preferentially act at extrasynaptic neuronal NMDAR, which cause excitotoxic neuronal damage and death. Consistent with the TBI findings, primary cultures of astrocytes obtained from neonatal mouse brain express markers associated with reactive astrocytes and also express SR and release D-serine.[55,56]

D-Serine homeostasis: The serine shuttle (see Fig. 2)

Recent studies indicate that resting adult astrocytes *in vivo* are the primary source of brain L-serine and express all the necessary enzymes to convert glucose into L-serine.[57,58] SR and D-serine are co-localized with postsynaptic density protein 95 (PSD-95) at postsynaptic densities in glutamatergic synapses on both excitatory and inhibitory neurons, but not within the presynaptic vesicular glutamate transporter-1.[46] Deletion of Phgdh in mouse brain astrocytes dramatically reduces brain L- and D-serine by about 80%,[58] with D-serine deficits localized to neurons.[59] Thus, L-serine is considered to be synthesized from glucose via a 3-phosphoglycerate dehydrogenase-dependent pathway in adult astrocytes and then transported into postsynaptic neurons to be converted to D-serine by neuronal SR.[49] This process has been designated the 'serine shuttle,' which highlights the role of glia–neuron cross-talk for optimal NMDAR function.[60] As D-serine is released from postsynaptic spines at glutamatergic synapses, D-serine appears to function in an autocrine fashion and not as a presynaptically localized co-transmitter. This autocrine property of D-serine ensures that the postsynaptic NMDAR's GMS are occupied with D-serine to respond immediately to released glutamate.[28] Termination of D-serine signaling is attained by reuptake into astrocytes where it is catabolized by DAAO. On the other hand, under pathologic conditions, A1 reactive astrocytes express SR and release D-serine, which may contribute to their neurotoxic effects by acting at extrasynaptic NMDAR.[28,56]

Evidence Supporting the NMDAR Hypothesis

Clinical findings

The most impressive clinical evidence that PCP psychosis resembles schizophrenia is the fact that PCP users have been mistaken by experienced psychiatrists for having schizophrenia before obtaining the

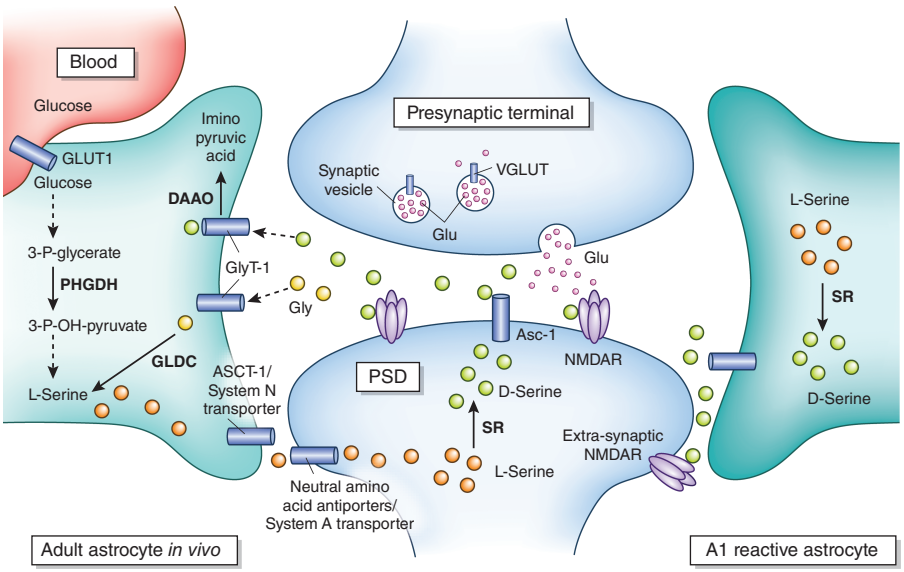

Fig.2 Schematic representation of serine shuttle. L-serine is synthesized from glucose, which diffuses from blood vessels and is transported into astrocytes by glucose transporter 1 (GLUT1). L-serine is then transported into postsynaptic neurons via neutral amino acid exchangers (such as ASCT1, neutral amino acid antiporters, or system A transporter) to be converted to D-serine by serine racemase (SR). This process is known as the 'serine shuttle.' SR and D-serine are concentrated at the postsynaptic densities in glutamatergic and SR-expressing GABAergic neurons. D-serine is subsequently released from neurons by Asc-1 or other transporters and binds to the glycine modulatory sites on synaptic *N*-methyl-d-aspartate receptor (NMDAR). Termination of D-serine signaling is attained by reuptake into astrocytes where it is catabolized by D-amino acid oxidase (DAAO). A1 reactive astrocytes express SR, and synthesize and release D-serine, which may contribute to their neurotoxic effects by acting at extrasynaptic NMDAR. Asc-1, alanine-serine-cysteine-1 (Slc7a10; solute carrier family 7 member 10); ASCT-1, alanine/serine/cysteine/threonine transporter 1 (Slc1a4; solute carrier Family 1 member 4); GLDC, glycine decarboxylase; GlyT-1, glycine transporter 1; PHGDH, phosphoglycerate 3-dehydrogenase; PSD-95, postsynaptic density protein 95; VGLUT, vesicular glutamate transporter. Adapted from Coyle and Balu[28] and Wolosker *et al*.[49] with permission.

history of drug use. A particularly dramatic instance occurred in Washington, DC, during the fall of 1973. The admission rate for what appeared to be unusually long, severe, and treatment-resistant initial schizophrenic psychoses suddenly tripled in a community mental health center. These patients had all smoked PCP before becoming psychotic, and their presenting picture was at first indistinguishable from a florid schizophrenic episode.[61,62] With regard to psychotic symptoms, lysergic acid diethylamide (known as 'LSD') generally produces a psychosis in healthy volunteers characterized by distortions in visual perception lasting for only 8–16 h whereas NMDAR antagonists, such as PCP and ketamine, produce not only schizophrenic-like psychosis (including auditory hallucinations), but also negative and cognitive symptoms lasting for periods up to 2 weeks.[62] Furthermore, LSD administration induces no more severe effects in patients with schizophrenia than in healthy volunteers. By contrast, administration of glutamate antagonists to stabilize patients with schizophrenia exacerbates their symptoms.[63,64]

Anti-NMDAR encephalitis is now well established as an autoimmune disorder having schizophrenia-like symptoms caused by autoantibodies against an extracellular domain of NMDAR.[65] The antibodies are considered to lead the internalization of postsynaptic NMDAR clusters to causing NMDAR downregulation. The disorder most commonly presents with abnormal behavior, such as agitation and aggression, abnormal speech, catatonia, seizures, psychosis (including delusions and hallucinations), and cognitive impairments.[66] It was reported that 6.5% of patients at first presentation of psychosis fulfilled the diagnostic criteria for schizophrenia and were shown to be NMDAR-antibody positive.[67]

Genetic findings

Twin and family studies over the last half-century have provided compelling evidence of the high heritability of schizophrenia, approaching a ratio of 0.8.[2] However, the pattern of familial risk declined strikingly from identical to fraternal twins, consistent with complex genetics wherein multiple risk genes of modest effect interacting with environmental risk factors cause the phenotype. Early in the 21st century, many studies were carried out to determine if candidate genes, typically linked to the dopamine hypothesis, were significantly associated with the risk for schizophrenia. A number of positive, significant associations were reported, often reinforced by meta-analyses. Unfortunately, experience with studies of common disorders involving complex genetics indicated that these studies were statistically underpowered, leading to false positive results, further complicated by publication bias (non-publication of negatives results).

Sufficiently powered studies to agnostically search the genome for linkage between sites and risk for schizophrenia requires thousands of subjects to satisfy the high bar of a significance threshold of 5×10^{-8} required for multiple comparisons. Obviously, such large cohorts of patients cannot be acquired at a single clinic but rather require extensive collaborations and sharing of data among many sites throughout the world. A recent genome-wide association study (GWAS) was carried out involving over 100 000 controls and nearly 40 000 subjects with schizophrenia, which identified 108 sites on the genome that met the strict statistical threshold of 5×10^{-8}. Although one site was linked to the dopamine receptor (*DRD2*), several were associated with glutamatergic neurotransmission or downstream mediators: for example, the $mGlu_3$ (*GRM3*), the GluN2A (*GRIN2A*), SR (*SRR*), and AMPA receptor 1 (*GRIA1*).[68–70]

Furthermore, other studies have revealed that an increased burden of rare gene variants, taking the form of both large copy-number variants (CNVs) and single-nucleotide variants, which often occur as *de novo* mutations, exert significantly larger effects than common single-nucleotide polymorphisms.[71–74] According to large-scale CNV studies, *de novo* CNV encode the NMDAR and proteins associated with the postsynaptic density with increased risk of schizophrenia.[75–79] Especially, 11 rare CNV (deletions at 1q21.1, *NRXN1*, 3q29, 15q11.2, 15q13.3, and 22q11.2, and duplications at 1q21.1, 7q11.23, 15q11.2-q13.1, 16p13.1, and 16p11.2) showed highly significant evidence (odds ratio: 2–60) for association with schizophrenia. About 2.5% of patients and 0.9% of controls carry a CNV at one of these loci.[80] Moreover, nearly all of them are associated with a range of other neurodevelopmental disorders, such as autism spectrum disorder and intellectual disabilities, and some of them are also associated with particular physical disease phenotypes, such as epilepsy, congenital heart disease, microcephaly, and obesity.

Recently, there has been accumulating evidence also supporting a role for rare variants in schizophrenia causation, especially rare loss of function (LoF; nonsense, splice site or frame shift) variants.[81] For example, in the recent mutation screening study for the exonic regions of the NMDAR subunits in schizophrenia and autism spectrum disorder, 40 rare variants were identified and two of them in *GRIN2C* and *GRIN2D* in schizophrenia were rare LoF mutations.[81] These findings support the view that ultra-rare variants with LoF in

glutamatergic pathways, especially NMDAR-related genes, increase the risk of schizophrenia.

Neurophysiological findings

The development of neuroimaging technology has provided a way forward for non-invasive visualization of the brain structure and activity *in vivo*.[82] Especially, proton magnetic resonance spectroscopy (^{1}H-MRS), positron-emission tomography (PET), and single-photon emission computed tomography (SPECT) afford researchers the ability to measure brain parameters, such as dopamine and glutamate function in the brain, in living human subjects.

^{1}H-MRS

^{1}H-MRS studies have shown that acute administration of ketamine to healthy subjects leads to a significant increase in anterior cingulate glutamine, a putative marker of glutamatergic neurotransmission, and this increase was marginally related to cognitive function.[83,84] On the other hand, in schizophrenia, a recent meta-analytic study reported that there was a significant elevation in glutamatergic transmission in the limbic system, in glutamate indices in the basal ganglia, glutamine levels in the thalamus, and Glx (glutamate + glutamine) levels in the basal ganglia and medial temporal lobe.[85] Poels also showed that there was a positive correlation between elevated Glx in the hippocampus and worse executive functioning, global clinical state, and decreased hippocampal volume in unmedicated patients.[86,87]

Some ^{1}H-MRS studies have investigated glutathione (γ-L-glutamyl-L-cysteinyl-glycine) levels in the brains of patients with schizophrenia. Glutathione is a tripeptide synthesized from glutamate, cysteine, and glycine. It has an important role in protecting the brain against oxidative stress as an intracellular antioxidant. Furthermore, it modulates the redox site on the NMDAR. Thus, increasing extracellular glutathione levels promotes glutamate-induced depolarization via NMDAR activation. Some studies indicated that glutathione levels in the medial prefrontal cortex (PFC) were lower in schizophrenia than in healthy controls.[88] On the other hand, higher levels were found in the medial temporal lobe.[89] However, significant differences were not observed between schizophrenia and healthy subjects in the other studies.[90,91] Thus, the results are mixed.

One main limitation of glutamate ^{1}H-MRS studies is that ^{1}H-MRS provides a total tissue measure and does not distinguish between intracellular or extracellular compartments, or between intra- or extra-neuronal compartments, further limiting the specificity of such measurements. PET and SPECT allow for a more selective measurement of brain neurochemistry than does ^{1}H-MRS.[87]

PET/SPECT

One ketamine administration study, which used PET, supports the notion of hypersensitivity to NMDAR antagonists in schizophrenia. In this study, ketamine administration caused regional cerebral blood flow elevations reflecting hypermetabolism in frontal and cingulate regions in both the healthy control group and the schizophrenia group. However, in comparisons of the two groups, the schizophrenia group had greater relative blood flow increases in the anterior cingulate, a region suspected of abnormal glutamatergic function in many ^{1}H-MRS studies.[92] On the other hand, PET/SPECT ligands for the glutamate binding site, the GMS, or the redox site on the NMDAR are being developed and are still in the subclinical stage. Regarding the PET ligands, [^{11}C] ABP688 and [^{18}F] FPEB, which bind to an allosteric site on $mGlu_5$, which co-localizes with NMDAR functionally and has physically close interactions with NMDAR activation, have demonstrated potential as a marker for glutamatergic transmission.[87] The translocation protein 18 kDa (TSPO) expression, which is elevated with microglial activation, is starting to be used as another target for PET studies in psychiatric disorders suspected to involve microglial activation, including schizophrenia.[93] It is known that activated microglia induce reactive astrocytes by cytokines,[55,94] which could contribute to glutamatergic dysfunction in schizophrenia via the activation of extrasynaptic NMDAR by releasing D-serine.

Mismatch negativity/P300/gamma band oscillations

Electroencephalography (EEG) is one of the least invasive and easy-to-use methods to monitor neurophysiological brain function with high temporal resolution in people with psychiatric disorders. Magnetoencephalography is also a neuroimaging technique to visualize neural activity with higher spatial resolution than EEG. Mismatch negativity (MMN), P300 (or P3), and gamma band oscillations are electrical phenomena that appear to be informative markers for schizophrenia.

MMN is a negative-going wave in the event-related potential component and is evoked by a deviant stimulus, which is occasionally inserted in a repetitive train of standard stimuli. The onset of the auditory MMN response occurs within 50 ms of the deviant stimulus, and peaks after an additional 100–150 ms.[95] MMN is thought to reflect auditory sensory memory or pre-attentive processing.[96] P300 is a positive-going wave in the event-related potential and is elicited during oddball paradigm with a latency of roughly 250–300 ms after deviant stimulus. Its (P3a) component is thought to reflect a re-orienting or covert shifting of attention.[95,97]

Administration of the non-competitive NMDAR antagonist, ketamine, is known to attenuate MMN and P300 amplitude in healthy subjects.[98,99] Furthermore, in individuals with high risk, first-episode, or chronic schizophrenia, attenuation of the MMN amplitude has been reported.[100,101] Moreover, some studies have provided more direct evidence, showing the linkage of these attenuations and glutamate hypothesis in schizophrenia. For example, the plasma levels of glutamatergic amino acids were correlated with MMN amplitude attenuation in subjects in the early stages of psychosis.[96] Another EEG and MRS study suggested that there were positive correlations between P300 amplitude and both the glutamine/glutamate ratio and the glutamine concentration in the anterior cingulate.[102] Therefore, MMN and P300 amplitude attenuation has been hypothesized to reflect NMDAR hypofunction and has attracted much attention as a biomarker for schizophrenia. During a continuous performance test, there is a highly significant reduction of no-go anteriorization, which was also observed in both first-episode and chronic schizophrenia.[103,104]

Synchronous neuronal oscillations in the 30–100 Hz range, known as 'gamma oscillations,' are normally correlated with performance of a variety of cognitive tasks, including the allocation of attention and working memory.[105] The gamma rhythms are generated by fast-spiking parvalbumin-positive GABAergic interneurons, which are regulated by NMDA-dependent excitatory input. The NMDAR has also been directly implicated in the emergence of the rhythms.[105] In other words, spontaneous gamma rhythms reflect the excitatory/inhibitory balance between excitatory neuron and inhibitory interneurons. The NMDAR antagonists induce schizophrenic cognitive behaviors and increase spontaneous gamma rhythms.[106–109] Converging evidence from many studies suggests that abnormalities in the synchronized gamma oscillatory activity elicited by a variety of sensory stimuli and cognitive tasks may be an important biomarker for glutamatergic dysfunction in schizophrenia.[110,111]

Post-mortem neurochemical findings

Studies of cortical NMDAR expression in schizophrenia using human post-mortem brain tissue have revealed variable changes in transcript and protein expression depending on the brain region and receptor subunit examined.[112] A recent meta-analysis indicated that the GluN1 subunit (mRNA and protein) in the PFC of the post-mortem brains of subjects with schizophrenia was significantly decreased relative to controls. In the same study, they also conducted a qualitative review about the GluN2 (A, B, and D) and GluN3A subunits and demonstrated no consistent statistically significant changes in cortical mRNA expression or protein levels of these subunits in schizophrenia

compared to controls, with decreasing GluN2C mRNA expression in the PFC.[113] Some studies also suggested a decreased expression of GluN1 mRNA selectively in the dentate gyrus of subjects with schizophrenia compared to controls[114,115] and decreased expressions in GluN1 and GluN2B subunits (mRNA and protein) in the left hippocampus of schizophrenia compared to the right.[115–118]

In addition to direct quantification of the individual NMDAR subunits, increasing postsynaptic density of NMDAR in the post-mortem dorsolateral PFC (dlPFC) in schizophrenia was also reported despite decreased NMDAR signaling at the post-receptor level.[119] Furthermore, in a quantitative autoradiograph study, NMDAR binding in the anterior cingulate cortex was suggested to be increased in schizophrenia compared to controls due to a postsynaptic compensation for impaired glutamatergic neurotransmission.[120]

There are also numerous abnormalities of NMDA GMS modulators described not only in the brain but also in the periphery of subjects with schizophrenia. In fact, reductions of brain SR and D-serine have been reported in schizophrenia.[121–123] The level of kynurenic acid, an endogenous GMS antagonist, is elevated in the cerebral spinal fluid and post-mortem brain tissue in schizophrenia.[124–126]

Moreover, in addition to the NMDAR itself, altered expression of several NMDAR-associated post-synaptic density proteins have been reported in the post-mortem brains of subjects with schizophrenia. Increased mRNA but decreased protein expression has been reported for both PSD-93 and PSD-95 in the anterior cingulate cortex.[112,127] The expression of PSD-95 mRNA was significantly decreased in the dlPFC, in spite of the fact that it was increased in the occipital cortex.[128,129] Similarly, the expression of NF-L mRNA was significantly increased in the dlPFC, even though the protein was decreased.[127] Furthermore, the kainate subtype of glutamate receptor was first measured in the post-mortem brains of subjects with schizophrenia by Nishikawa *et al.*, who reported a 25–50% increase in [3H] kainic acid binding in the PFC. The increased kainate receptor may also reflect a reduced activity at certain glutamatergic synapses and impaired cognitive functions in the PFC.[130,131] Some studies also reported that the levels of N-acetylaspartylglutamate (NAAG) and glutamate, as well as the activity of glutamate carboxypeptidase II (GCP-II) were altered in schizophrenia. NAAG, which is catabolized by the enzyme GCP-II, has dual roles as an endogenous NMDAR antagonist and mGlu3 agonist. The elevation of NAAG levels and the reduction of glutamate levels, as well as reduced GCP-II activity in schizophrenia support the hypothesis that NAAG-mediated signaling contributes to NMDAR hypofunction in schizophrenia.[28,39,132–134]

Animal models

Pharmacologic, developmental, and genetic animal models have the potential to provide a platform for advancing the mechanistic understanding of alterations that can lead to schizophrenia and the possibility to compensate for limitations of human post-mortem studies. There is an abundance of data from these animal models that support the hypothesis of NMDAR hypofunction contributing to the pathophysiology of schizophrenia.[39] In pharmacological models, administration of NMDAR antagonists or kynurenic acid to animals led to neurochemical, morphological, and cognitive and/or behavioral features similar to what is observed in schizophrenia. Developmental models, given potential environmental risk factors of schizophrenia during the perinatal and/or early postnatal period, lead to abnormal-brain-development-related NMDAR activity and show neuronal alterations and cognitive and/or behavioral features similar to what is observed in schizophrenia. Genetic animal models have also provided a wealth of data suggesting that reduced NMDAR activity can lead to changes in the brain and behavior that is similar to what is observed in schizophrenia.[39]

For example, mice lacking the enzyme SR (SR−/−), which is encoded in the schizophrenia risk gene SRR, identified in the large genome-wide association study[69] showed >85% reduction of endogenous D-serine in the cortex and hippocampus and cognitive impairments associated with schizophrenia.[135] SR−/− mice also demonstrate a phenotype that closely replicates many aspects of schizophrenia, including enlarged lateral ventricles, cortical atrophy, reduced dendritic length, reduced spine density, downregulation of the cortical fast-spiking parvalbumin-positive GABAergic interneurons, reduced brain-derived neurotrophic factor expression, reduced Akt signaling, and reduced microRNA-132 levels.[28,39,82,135,136] Mice lacking the synaptic protein dysbindin encoded by *DTNBP1,* which is reduced in *dl*PFC and the hippocampus of schizophrenia,[137,138] show a schizophrenia-like phenotype, including NMDAR hypofunction, disrupted inhibitory transmission, hyperexcitability in the PFC, as well as cognitive impairments, such as working memory.[39] Other genetic models, such as insertion of *G72* mice[139] and inactivation of *DAO* mice,[140] are also known to display NMDAR hypofunction and neurochemical, morphological, and cognitive and/or behavioral phenotypes, such as schizophrenia.

Treatment based on Glutamate Hypothesis

Pharmacotherapy based on the dopamine D_2 receptor (D2R) blocking activity of all antipsychotics developed since the discovery of chlorpromazine over 50 years ago has universally been the mainstay of treatment for patients with schizophrenia. The introduction of antipsychotics was associated with dramatic reduction in the number of patients with schizophrenia and related psychotic disorders in chronic mental hospitals. By introducing 5-hydroxytryptamine$_2$ (5-HT$_2$)-receptor blocking activity into the structure of antipsychotics, a second generation of antipsychotics (SGA) was developed that had markedly reduced propensity for causing extrapyramidal neurologic side-effects. Clinical studies indicate that delays in treatment with antipsychotics are associated with poorer outcomes. Thus, D2R blocking approach has provided substantial benefits to patients with schizophrenia. However, negative and cognitive symptoms are refractory and are associated with persistent disability.[141] Furthermore, risks of troublesome side-effects, such as weight-gain, hyperlipidemia, glucose intolerance, type 2 diabetes, and metabolic syndrome, further detract from current treatments, especially with SGA.

Meta-analyses indicate that clozapine, a weak D2R antagonist, is associated with consistently better outcomes than all other antipsychotics and appears to affect negative symptoms.[142] The mechanism whereby clozapine is significantly more effective remains unclear as clozapine interacts with several receptors aside from the D2R, including muscarinic, histamine, and serotonin receptors. However, recent studies indicate that clozapine may enhance NMDAR function through different mechanisms, including blocking glycine uptake, enhancing GMS occupancy, and interacting with the mGlu$_5$.[143–146]

The glycine modulatory site

The GMS of the NMDAR is the first glutamatergic strategy pursued as a novel mechanism to treat schizophrenia, especially the negative and cognitive symptoms. This modulatory approach trumped direct NMDAR agonists because it is associated with a reduced risk of excitotoxicity and neuronal death associated with direct NMDAR agonists.[147] There are several strategies by which the availability or concentration of GMS co-agonists and antagonists can be altered as a means to augment NMDAR function.[39]

Glycine is an α-amino acid, which also has a role as an inhibitory neurotransmitter via binding to strychnine-sensitive glycine receptors. Furthermore, glycine enhances NMDA channel opening via the GMS of the NMDAR. Some randomized controlled trials (RCT) with oral glycine suggested improvement of negative symptoms.[148–151] D-serine is known to be an endogenous co-agonist at the NMDAR and induces more activation of NMDAR than glycine. Some RCT with D-serine or D-alanine also reported efficacy for the treatment of negative symptoms.[152–154]

D-cycloserine (DCS) is an anti-tubercular drug that inhibits bacterial cell wall synthesis. Neuropsychiatric symptoms, such as depression, sedation, psychosis, and seizures, are side-effects that may occur

with high-dose DCS treatment. DCS is also a partial agonist at the GMS with about 50% efficacy. DCS is a less efficient ligand for NMDAR function than the endogenous full agonists, such as glycine and D-serine. At high doses, DCS acts as an antagonist by displacing more efficacious endogenous full agonists, but at moderate doses, DCS facilitates glutamatergic neurotransmission via the NMDAR.[155]

RCT of DCS added to antipsychotics produced mixed results. In the DCS added on first-generation antipsychotics trial, negative symptoms were significantly improved but positive and cognitive symptoms were not.[156] On the other hand, when DCS was added to clozapine, negative symptoms worsened by contrast to the addition glycine[157] or D-serine.[158] In the trial of addition of DCS to risperidone, which is an SGA, DCS was associated with reduction in negative symptoms. However, the degree of improvement appeared to be intermediate between improvement of negative symptoms observed with combination of DCS with first-generation antipsychotics and worsening of negative symptoms observed with combination of DCS with clozapine.[159] This pattern of response suggests that, as a partial agonist at some NMDAR, DCS may attenuate clozapine effects via the activation of the GMS of the NMDAR.[160]

A large multi-site RCT of glycine and DCS added to antipsychotics suggested that they were ineffective. But it was a 'failed' trial as there were significant differences in the outcomes among sites.[160] Post-hoc analysis indicated that at inpatient sites where compliance was assured, glycine and DCS significantly reduced negative symptoms. Along this line, Iwata *et al.*[161] recently published a meta-analysis of the results of placebo-controlled add-on trials of NMDAR-positive modulators and concluded that they had no effects on cognitive deficits in schizophrenia but did not address negative symptoms, which has been more consistently improved by GMS agonists.[162] Furthermore, they neglected certain confounds, such as desensitization with continuous DCS treatment[163] or negative interactions of DCS with clozapine because of its effects on endogenous GMS occupancy.[143–146] Finally, they neglected to address how DCS has been shown to augment cognitive remediation in schizophrenia, a strategy that employs 'real-world' learning.[164]

Increasing GMS occupancy would permit greater NMDAR activation by glutamate. One strategy that has been explored is to inhibit the uptake of the co-agonist, glycine. Previous studies in the acute hippocampal slice at the glutamatergic synapse on the CA1 pyramidal neuron revealed that the GMS is not saturated with endogenous co-agonists and that inhibiting the glycine transporter (GlyT-1) potentiates NMDAR currents at a synapse where D-serine is the dominant co-agonist.[165] Thus, blocking GlyT-1 appeared to be a plausible way to enhance NMDAR function in schizophrenia.

Sarcosine, an endogenous GlyT-1 inhibitor, is generated in the process of glycine synthesis. Three RCT of sarcosine added to the stable antipsychotic drug regimen lasting 6 weeks demonstrated that sarcosine was associated with greater reductions in Positive and Negative Syndrome Scale (PANSS) total scores than the placebo and the D-serine group in patients with stable chronic schizophrenia, as well as in drug-naïve patients in the acute phase of schizophrenia. Moreover, a meta-analysis reported sarcosine was effective in total psychopathology, negative symptoms, and general psychopathology. However, several drugs that exploited sarcosine as the 'back-bone' for high-affinity GlyT-1 inhibitors produced undesirable side-effects, such as hypoactivity and ataxia, and have therefore prompted the development of non-sarcosine-based GlyT-1 inhibitors. Unfortunately, the noncompetitive GlyT-1 antagonist, bitopertin, failed to reach its endpoints to improve PANSS total score and negative symptoms in Phase II/III and III trials for schizophrenia,[166–168] even though it significantly reduced negative symptoms in a Phase IIb study in patients with stable, medicated schizophrenia.[39,169] Moreover, in an RCT to investigate adjunctive treatment with Org25935, a selective inhibitor of GlyT-1, Org25935 did not differ significantly from placebo in reducing negative symptoms or improving cognitive functioning when administered as adjunctive treatment to SGA.[170] Other types of GlyT-1 inhibitors, such as PF-03463275[171,172] and ASP2535,[173] have shown promise in preclinical studies of cognitive remediation for schizophrenia.

DAAO, the primary enzyme responsible for catabolizing D-serine, is sensitive to inhibition by benzoic acid. Inhibition of DAAO would presumably increase the availability of D-serine. In an RCT to investigate the effects of add-on treatment with sodium benzoate, benzoate significantly improved a variety of symptom domains and neurocognition in patients with chronic schizophrenia.[174] Benzoate is also known to induce the expression of brain-derived neurotrophic factor in primary human neurons and astrocytes,[175] which might be another pathway whereby benzoate could reduce schizophrenic symptoms.

Stimulating the redox modulatory site on the NMDAR also enhances NMDAR function. Therefore, the redox modulatory site is considered to be another potential target for treatment of schizophrenia. Glutathione can protect a brain from reactive oxidative stress and harmful xenobiotics as a nucleophilic scavenger and an enzyme-catalyzed antioxidant. It also induces NMDAR activation via binding to the redox modulatory site. Glutathione is synthesized from three amino acids, L-glutamate, glycine, and L-cysteine, which is regulated by extracellular *N*-acetyl-L-cysteine (NAC) concentration. NAC increases extra-cellular L-glutamate levels and intracellular glutathione levels via cystine-glutamate anti-porter. Moreover, NAC promotes activation of the Group II mGlu receptors. As a result of these complementary mechanisms, NAC can enhance NMDAR function and reduce excitotoxicity.

The results of several clinical trials with NAC in schizophrenic subjects stabilized on antipsychotic drugs have been reported. In one of the earliest RCT, NAC added to SGA improved the PANSS scores (Negative, General, and total scores) and the Clinical Global Impression scales (Severity and Improvement scales) in chronic schizophrenia.[176] In other trials, in which NAC was added to risperidone in chronic schizophrenia[177] or was added onto clozapine in clozapine-resistant schizophrenia,[178] NAC was also reported to be efficacious for reducing negative and cognitive symptoms. Furthermore, one RCT reported efficacy not only with negative and cognitive symptoms but also with positive symptoms in chronic schizophrenia.[179] Moreover, a recent RCT demonstrated continuous effects of adjunctive NAC approach in long-term treatment[179] and effects in early psychosis.[180] A recent meta-analysis from 6 RCT showed that adjunctive NAC appears to have efficacy for schizophrenia.[181]

Another strategy to reduce negative and cognitive symptoms in schizophrenia would be to treat for excitotoxicity-related neuropathology with adjunctive memantine. Memantine is a moderate-affinity noncompetitive NMDAR antagonist, which binds preferentially to the same site as MK-801 and PCP within the NMDAR channel. Like Mg^{2+}, memantine shows a strong voltage dependency. Memantine can enter the channel and block current flow only if the channel is open. Thus, it is defined as an 'open-channel blocker' or a 'trapping-channel blocker' of NMDAR.[182] Memantine is thought to block pathologically activated NMDAR when synaptic glutamate concentrations are abnormally high whereas it does not influence the normal functioning of physiologically activated receptors. In other words, memantine blocks the effects of sustained, pathologically elevated levels of glutamate that would lead to neuronal dysfunction.[183] A meta-analysis (including eight RCT[183]) and a systematic review article (including 10 studies[182] in which memantine was add onto antipsychotic drugs in schizophrenic patients) were recently published. Both studies concluded that memantine selectively improves negative symptoms while cognitive and positive symptoms were not significantly affected. Moreover, the meta-analysis also demonstrated that the most robust effects on negative symptoms were associated with young adult schizophrenic patients.[183] However, long-term effects and tolerance of the approach have been unclear.

Metabotropic glutamate receptors

The mGlu receptors are classified into three groups (Group I: $mGlu_{1 \text{ and } 5}$; Group II: $mGlu_{2 \text{ and } 3}$; and Group III: $mGlu_{4,\ 6,\ 7 \text{ and } 8}$) differentiated by their amino acid sequence, ligand selectivity, and signaling

cascades. Subtypes 2, 3, and 5 have been investigated as potential therapeutic targets for schizophrenia.

Group II mGlu receptors are widely expressed throughout the brain, particularly in those regions implicated in schizophrenia, including the hippocampus, cortex, nucleus accumbens, striatum, and amygdala.[39,184] These receptors are expressed presynaptically as autoreceptors, activated by astrocytic glutamate release or glutamate overflow from the synapse during excessive glutamate release. $mGlu_3$ is also found postsynaptically as well as on astrocytes where it mediates neuroprotective effects and participates in astrocytic-neuronal communication.[185] Group II mGlu receptors also have a reciprocal relationship with $5\text{-}HT_{2A}$ receptors. Activation of the $5\text{-}HT_{2A}$ receptor enhances thalamocortical neurotransmission in rodents and this effect is antagonized by activation of Group II mGlu receptors. On the other hand, orthosteric agonists of $mGlu_{2/3}$ functionally antagonize $5\text{-}HT_{2A}$ receptor signaling.[185]

The $mGlu_5$ is primarily enriched postsynaptically in both GABAergic interneurons and pyramidal neurons in the hippocampus, cortex, striatum, caudate nucleus, nucleus accumbens, septum, and olfactory bulb. The receptor has close interaction with the NMDAR activation via intracellular signaling pathways and scaffolding proteins, such as HOMOR, SHANK, and GKAP-PSD95.[185] Therefore, it would be of great interest to study whether positive allosteric modulators (PAM) of $mGlu_5$ can treat schizophrenia.

One of the $mGlu_{2/3}$ agonists, LY2140023, showed effects for both positive and negative symptoms compared to placebo without prolactin elevation, extrapyramidal symptoms, or weight gain in a Phase II trial.[186] However, in a follow-up multicenter Phase II study, LY2140023 did not significantly separate from placebo due to the large placebo effect.[187] The active comparator in this trial did not differ from placebo, indicating that this was a failed trial. Another $mGlu_2$ PAM, AZD8529, also failed to show efficacy in a Phase II trial.[188] As for $mGlu_5$ agonists, a PAM of $mGlu_5$, VU0409551, has shown robust cognitive and behavioral effects in several animal models of schizophrenia.[189,190] Another Phase II trial has recently been initiated.[191]

The failed clinical trials or highly variable outcomes of drugs directed at the NMDAR hypothesis of schizophrenia indicate that our current approach to drug development that relies on categorical diagnoses will subvert meaningful advances. First, the GWAS results indicate that schizophrenia is a disorder of complex genetics involving more than 100 genes, each of which has modest effects. Thus, some patients may be genetically biased much more towards glutamatergic dysfunction than others. Second, sub-grouping to enrich for patients with likely glutamatergic pathology could be accomplished through genotyping (polygenic scores) and assessment of other informative biomarkers, such as cortical glutamate measured by magnetic resonance.[192] Third, additional strategies to reduce variance would be to: focus on clinics that have 'real' patients and not volunteers recruited by advertisements, reduce the number of sites with larger numbers of patients, and focus on the early stages of schizophrenia as chronic patients likely have a different pathology.[193]

Conclusion

In this review, we have marshaled the abundant evidence from pharmacologic challenges, post-mortem studies, brain imaging, and genetics supporting the role of dysregulation of glutamatergic neurotransmission in the pathophysiology of schizophrenia. Of course, this pathology must be understood in terms of its disruption in the function of critical circuits, such as downregulation of the corticolimbic PV + GABAergic, disinhibition of pyramidal neurons, and increased release of striatal dopamine.[194] Furthermore, reduced NMDAR function attenuates corticolimbic spine development on pyramidal neurons resulting in an approximately 30% reduction in glutamatergic synapses.[82,195] Taken together, this cortical pathology accounts for cognitive and negative symptoms of schizophrenia as well as positive symptoms, a downstream consequence of the cortical pathology.

Elucidating the pathologic circuitry in schizophrenia provides novel targets for therapeutic interventions. First, given that the disinhibition of striatal dopaminergic input, which correlates with psychosis, is a consequence of increased glutamatergic output from the cerebral cortex, it is not surprising that D2R blocking antipsychotics have little impact on cortically determined negative and cognitive symptoms. Secondly, pre-clinical studies with a genetic model of schizophrenia indicate that restoring NMDAR function by correcting D-serine deficits or by augmenting NMDAR responsiveness with an $mGlu_5$ PAM reverses the schizophrenic-like pathology. Although the results have been inconsistent due to a variety of factors, properly executed placebo-controlled clinical trials with NMDAR GMS agonists added to stable doses of antipsychotics suggest improved negative symptoms and enhanced cognitive remediation. Unfortunately, drugs that addressed alternative sites, such as an $mGlu_{2/3}$ agonist to downregulate glutamate release or inhibition of GlyT-1, showed promise in Phase IIb trials only to falter in the Phase III trials. These failures reflected high placebo responses and the reliance on the categorical diagnosis untethered from any biomarkers that could identify likely responders with glutamatergic risk genes or biomarkers.

John F. Nash, Jr., who was awarded the 1994 Nobel Prize in Economics for his path-breaking thesis research on game theory, developed schizophrenia in his late twenties. Years after its onset, he gave an invited lecture to the World Congress of Psychiatry, stating: 'I would not treat myself as recovered if I could not produce good things in my work. A remission might not be worthwhile in the end.' Thus, he reminds us that the ultimate goal is not to develop drugs that suppress certain symptoms, like antipsychotics, but rather drugs that correct the fundamental pathology to restore the sense of pleasure and liveliness of thought that are stolen from the individual with schizophrenia. Mounting evidence suggests that understanding the glutamatergic dysregulation in schizophrenia may provide a way forward in developing 'curative' treatments.

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Disclosure statement

Y.U. declares no competing interests. J.T.C. holds a patent on the use of D-serine to treat serious psychiatric disorders that is owned by Massachusetts General Hospital but could yield royalties to J.T.C.

Author contributions

Both Y.U. and J.T.C. wrote the manuscript.

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