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Src kinase as a mediator of convergent molecular abnormalities leading to NMDAR hypoactivity in schizophrenia

Anamika Banerjee, Ph.D.[1,+], **Hoau-Yan Wang, Ph.D**[2,+], **Karin E. Borgmann-Winter, M.D.**[1], **Mathew L. MacDonald, Ph.D.**[1], **Hagop Kaprielian, B.S.**[1], **Andres Stucky, Ph.D.**[2], **Jessica Kvasic, B.S.**[2], **Chijioke Egbujo, M.D.**[1], **Rabindranath Ray, Ph.D.**[1], **Konrad Talbot, Ph.D.**[1], **Scott E Hemby, Ph.D.**[4], **Steven J. Siegel, M.D., Ph.D.**[1], **Steven E. Arnold, M.D.**[1], **Patrick Sleiman, Ph.D.**[3], **Xiao Chang, Ph.D.**[3], **Hakon Hakonarson, Ph.D.**[3], **Raquel E. Gur, M.D., Ph.D.**[1], and **Chang-Gyu Hahn, M.D., Ph.D.**[1]

[1]Department of Psychiatry, University of Pennsylvania, Philadelphia, PA 19104-3403

[2]Department of Physiology, Pharmacology and Neuroscience, City University of New York Medical School, New York, NY 10031

[3]The Center for Applied Genomics, The Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104

[4]Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, NC 27106

Abstract

Numerous investigations support decreased glutamatergic signaling as a pathogenic mechanism of schizophrenia: yet molecular underpinnings for such dysregulation are largely unknown. In the postmortem dorsolateral prefrontal cortex, we found striking decreases in tyrosine phosphorylation of N-methyl-D aspartate (NMDA) receptor subunit 2 (GluN2), which is critical for neuroplasticity. The decreased GluN2 activity in schizophrenia may not be due to downregulation of NMDA receptors since MK-801 binding and NMDA receptor complexes in the PSD were in fact increased in schizophrenia cases. At the post-receptor level, however, we found striking reductions in the protein kinase C, Pyk 2 and Src kinase activity, which in tandem can decrease GluN2 activation. Given that Src serves as a hub of various signaling mechanisms impacting GluN2 phosphorylation,

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*Corresponding Author: Chang-Gyu Hahn, M.D., Ph.D., Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania, 2216 TRL, 125 South 31st, Philadelphia, PA 19104-3403, Tel, 610-520-9131, Fax, 215-573-2041, hahnc@mail.med.upenn.edu.
+AB+ and HYW+ contributed equally to this work.

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AUTHOR CONTRIBUTIONS

A.B performed biochemical fractionations; A.B. and H-Y.W. performed all other experiments including immunoprecipitation, receptor and kinase activation assays with help from K.B.W., M.L.M., A.S., H.K J.K. C.E., R.R, and K.T.,. P.S., X.C. and H.H. conducted network analysis of GWAS data. S.E.H. and S.J.S. conducted antipsychotic treatment of rhesus monkeys and mice. The postmortem tissue and brains were collected by S.E.A., K.T. and R.E.G., who also ensured the integrity of postmortem brain tissues. P.S., X.C., and H.H conducted analyses of genetic data. A.B. and C-G.H. performed all other data analyses. C-G.H. conceived of and directed this study. The manuscript was written by A.B., K.B.W., K.T., and C-G.H. and edited by all authors.

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we postulated that Src hypoactivity may result from convergent alterations of various schizophrenia susceptibility pathways and thus mediate their impacts on NMDA receptor signaling. Indeed, the DLPFC of schizophrenia cases exhibit increased PSD-95 and erbB4 and decreased RPTPa and dysbindin-1, each of which reduces Src activity via protein interaction with Src. To test genomic underpinnings for Src hypoactivity, we examined genome wide association study results, incorporating 13,394 cases and 34,676 controls, which yielded no significant association of individual variants of Src and its direct regulators with schizophrenia. However, a wider protein-protein interaction based network centered on Src, showed significant enrichment of gene-level associations with schizophrenia compared to other psychiatric illnesses. Our results together demonstrate striking decreases in NMDA receptor signaling at the post-receptor level and propose Src as a nodal point of convergent dysregulations impacting NMDA receptor pathway via protein-protein associations.

Keywords

NMDA receptor; postsynaptic density; postmortem brain; NMDAR hypofunction; Src kinase

INTRODUCTION

Multiple lines of evidence support glutamatergic dysregulation as a key pathogenic mechanism of schizophrenia (SCZ) widely known as the NMDA receptor (NMDAR) hypofunction hypothesis [1–3]. This postulate originated from clinical observations, in which NMDAR blockade induced psychosis in healthy human subjects and worsened symptoms in patients with SCZ[4–6]. In addition, rodent studies offered further support demonstrating that NMDAR antagonists or mutation of NMDAR genes induced neurophysiological and behavioral endophenotypes of psychosis [3, 7, 8]. Furthermore, treatment studies demonstrated that clinical manifestations of SCZ in patients and SCZ like phenotypes in animal models are reduced by enhancement of glutamatergic neurotransmission [9, 10].

Despite the noted evidence for NMDAR hypofunction in SCZ, mechanistic underpinnings for this dysfunction are presently unknown. Presynaptically, decreased D-serine and glutamate carboxypeptidase II, and increased kynurenic acid were observed in the CSF or postmortem brains of patients with SCZ [11, 12]. In addition, brain imaging studies in particular, have shown alterations in gluthathione[13]. While some of the results are not fully in concert, these together suggest dysregulations in presynaptic glutamate signaling in SCZ [1, 13].

In search of post-synaptic mechanisms, multiple groups have investigated mRNA or protein levels of NMDAR subunits and their signaling partners in the postmortem brains of SCZ cases, and have reported various interesting, but inconsistent, findings [14–16]. Given that NMDAR activity arises from the receptor complexes that are targeted to specific subcellular microdomains and involves extensive intracellular signaling mechanisms [17, 18], here we examined NMDAR activity and its underlying signaling mechanisms in postmortem brains.

Glutamate binding to NMDARs activates a cascade of kinases at the post-receptor level (called “post-receptor kinases” henceforth). It enhances Ca^{2+} influx, which activates Ca^{2+}/

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calmodulin-dependent kinase II (CAMKII) and protein kinase C (PKC) [17, 19]. The latter phosphorylates proline-rich tyrosine kinase 2 (Pyk2), leading to activation of the sarcoma tyrosine kinase known as Src [20, 21]. Conversely, post-receptor kinases in turn regulate NMDAR channel activity via tyrosine phosphorylation of GluN2 subunits, which is critical for sustained enhancement of channel activity in synaptic plasticity [22]. Thus, NMDARs and its downstream post-receptor kinases are interdependent for their activation forming a loop of cascade: NMDAR → PKC → Pyk2 →Src → pYGluN2. Of the post-receptor kinases in this cascade, Src plays a primary role as it directly phosphorylates GluN2 subunits as a molecular hub of various signaling pathways, including those outside the NMDAR pathway, impacting tyrosine phosphorylation of GluN2. As such, Src serves as a conduit for various non-NMDAR signaling mechanisms that regulate tyrosine phosphorylation of GluN2 and synaptic plasticity related NMDAR function [18].

Residing in the postsynaptic density (PSD), synaptic NMDARs are associated with scaffolding proteins and many signaling molecules and comprise a macromolecular receptor complex [23, 24]_ENREF_32. Hence, the NMDAR complex can reflect alterations in diverse signaling pathways, including those implicated for SCZ. Increasing genetic evidence indeed supports this possibility. Among genetic variants found to be associated with SCZ are those of DTNBP1 [25], DISC1 [26], ERBB4 [27], neuregulin 1 (NRG1) [28, 29], and PTPRA [30, 31], whose products can impact NMDAR complexes [26, 28, 30]. Moreover, genome wide investigation of de novo CNVs as well as de novo mutations point to multiple genes within NMDAR/ARC complexes [32, 33] suggesting that NMDAR complexes may serve as molecular substrates, upon which various SCZ susceptibility factors converge to precipitate NMDAR hypofunction. A critical next step then will be to delineate molecular underpinnings for NMDAR hypofunction in SCZ and to identify the mechanisms of convergence in the brain of patients with SCZ.

Previously, we reported that NMDA/glycine-induced tyrosine phosphorylation of GluN2 subunits, critical for sustained enhancement of NMDAR activity, was decreased in the DLPFC of SCZ subjects compared to controls [28]. Tyrosine phosphorylation of GluN2 subunits is governed by Src kinase [22], which can serve as a molecular hub of alterations in various signaling pathways including the SCZ susceptibility pathways [22]. Indeed, NRG1, ErbB4, PSD-95, dysbindin-1 or RPTPα, each of which has been implicated for SCZ, can modulate Src kinase. These together led us to hypothesize that Src kinase may mediate convergent dysregulations of various SCZ susceptibility pathways to reduce tyrosine phosphorylation of GluN2 and thereby precipitate NMDAR hypoactivity.

Examination of receptor activity and intracellular signaling is a methodological challenge in postmortem studies. We developed several novel methodologies to quantify NMDAR signaling activity, protein-protein interactions in NMDAR complexes, and the enzyme activity of Src in postmortem brain tissue [28, 34, 35]. A matched pairs design was used to minimize the effects of age and sex in observed differences between SCZ and control cases. We focused on the DLPFC because disruption of this brain area is common in SCZ [36, 37], contributes to deficits in selective attention, working memory, and executive function in this disorder [36, 38, 39], and has been shown to display NMDAR hypofunction in SCZ. Here, we present evidence pointing to the post-receptor segment of the NMDAR pathway as a locus of

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dysregulation in the DLPFC of SCZ cases and demonstrate that Src hypoactivity, resulting from convergent alterations of susceptibility pathways, mediates reduction of tyrosine phosphorylation of GluN2 in the DLPFC of SCZ cases.

Materials and Methods

Postmortem brains

Flash-frozen DLPFC tissues from 17 pairs of SCZ subjects, and controls, matched for age, sex and PMI, obtained from the Penn Brain Bank at the University of Pennsylvania were used for the study. Approved by the Institutional Review Board at the University of Pennsylvania, subjects were prospectively diagnosed by DSM-IV criteria and consents for autopsy were obtained from the next-of-kin or a legal guardian. Subjects with a history of substance abuse, neurological illnesses, or the need for ventilator support near death were excluded. Neuropathological assessments were performed on PFC of all subjects to exclude cases with gross neurodegenerative process and abnormal levels of senile plaques or Lewy bodies. (Additional details in supplemental data). Detailed demographic data is shown in table S1.

Animal Studies

Rodent tissues examined in this study included PFC tissues from homozygous sdy mice (n=6) [40], from Ptpra−/− null mice (n=7) [31] (provided by Jan Sap, University of Paris and Catherine Pallen, University of British Columbia, Canada) and from antipsychotic treated rhesus monkeys (n=8/9) (provided by Scott E. Hemby, Wake Forest University, see treatment details in Figure S2). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania and all experiments on mice were conducted in accordance with University Laboratory Animal Resources (ULAR) guidelines.

MK-801 Assay

As previously described [41], 10 μgs of P2 membrane extracts were incubated with varying concentrations of [^{3}H]MK-801 (Perkin Elmer, MA), ranging from 0 to 60 nM, in the presence of 1 μM gycine, 10 μM glutamate and 10 μM spermidine in 5mM Tris-HCl (pH 7.4). The samples were incubated at 30 °C for 2 hours and the reactions were precipitated on GF/C (Whatmann) glass filters and radioactivity was measured (Fisher, PA).

Postmortem brain receptor activation

One hundred μm tissue slices from DLPFC of frozen post mortem brains were incubated with 1 μM glycine and 10 μM NMDA for 30 minutes at 37 °C and synaptosomal fractions (S) were prepared as previously described in detail [28, 42].

Src Kinase assay

Ten μgs of P2 protein extracts were incubated with 1mM of EPQ(pY)EEIPIA, which activates src, or EPQYEEIPIA (Anaspec, CA), control peptide, overnight at 4 °C. Src kinase

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activity was determined by measuring incorporation of γATP in the peptide substrate provided by the manufacturer, Millipore, CA in Liquid scintillation Counter.

Tissue fractionation

PSD fractions were isolated from flash frozen coronal slabs of the DLPFC, including Brodmann areas 9 and/or 46, by the method previously described in detail[34] (see Supplementary Text).

Immunoprecipitation, antibodies and Western Blotting

See Supplementary Text.

Src binding assay with biotinylated peptide

First, Src proteins were affinity purified from synaptic membranes of the DLPFC of SCZ and CTRL cases using Src antibodies. The EPQ(pY)EEIPIA and EPQYEEIPIA peptides (Anaspec, CA) were biotinylated following the protocol by Thermo Scientific. One μg of purified Src proteins were incubated with biotinylated peptides at 30 °C for 30 min. The reaction was terminated using ice-cold immunoprecipitation buffer and incubated with 20 μl of Nutravidin beads (Thermo) at 4C for 60 min. Pelleted beads were analyzed by western blotting with Streptavidin-HRP conjugate.

GWAS data sets—The SCZ dataset consisted of the results of a GWAS meta-analysis of 15 cohorts that included 13,394 cases, of which 11,985 were classified as schizophrenia, 377 as schizoaffective and 1,032 as bipolar, and 34,676 controls (described in Sleiman et al [43]). SNPs with missing data in more than two cohorts were excluded from further analysis. The MDD, BP and ADHD datasets were downloaded from the Psychiatric Genomics Consortium (PGC) website (http://www.med.unc.edu/pgc) and consisted of 9240 MDD cases and 9519 controls [44]; 7,481 BP cases and 9,250 controls[45]; 2,064 ADHD trios, 896 ADHD cases, and 2,455 controls [46]. The Crohn's disease dataset comprised 3685 cases and 5968 controls [47].

Gene level associations—Gene-level associations were calculated using VEGAS [48]. VEGAS performs Monte-Carlo simulations from the multivariate normal distribution based on the patterns of linkage disequilibrium (LD) in a reference population and assigns an estimated P value to each gene. SNPs located within 50kb upstream and 50kb downstream of gene boundaries were included in the analysis in order to capture regulatory regions and SNPs in LD. Gene-level significance was set at p 0.05.

Protein-protein interaction network analysis

The protein-protein interaction network was constructed based on indices of protein interactions derived from primary interaction databases including BIND, BioGRID, CORUM, DIP, HPRD, InnateDB, IntAct, MatrixDB, MINT, MPact, MPIDB, MPPI and OPHID as compiled by iRefindex [49]. To control the false positive rate, only human-human protein interactions supported by at least two publications listed in Pubmed were considered for this study. Based on the protein-protein interactions we constructed a network of the Src

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pathway genes and their interactors (Figure 5b). The resulting Src network consisted of 30 genes.

Enrichment analysis—To test if the src protein-protein interaction network was enriched for genic association with schizophrenia (SCZ), we generated gene level p-values for each of the 30 genes using VEGAS from 5 separate GWAS datasets including SCZ, major depressive disorder (MDD), bipolar disorder (BP), attention deficit hyperactivity disorder (ADHD) and a non-psychiatric disease, Crohn's disease (CD). To determine enrichment of significance we identified all SNPs within each of the 30 genes, including all SNPs 50kb upstream and downstream of the genes, as was done to generate the gene-level significance, for each of the datasets separately. Total SNP numbers varied between each of the studies depending on the genotyping platform. SNP-level significance was set at p 0.05. Enrichment of association between SCZ and each of the four other phenotypes was tested using a 2 tailed Fisher's exact test as well as between CD and the four psychiatric disorders. Due to the low cell counts in the gene-level associations, enrichment statistics were generated on individual SNVs within the genes.

RESULTS

This study employs multiple methods that were devised specifically to assess NMDAR function in postmortem brains [28, 34, 42, 50]. We conducted extensive preliminary work [28, 34, 42, 50] to verify the stability of these measures in postmortem tissues, with special attention to the range of ages, antipsychotic doses and PMIs of our cases in human (Figure S1), rodent and monkey brains (Figure S2–Figure S4).

NMDAR complexes are increased in the PSD of SCZ cases

The abundance of NMDAR complexes was assessed in synaptic membranes of the DLPFC using a binding assay with the non-competitive NMDAR antagonist MK-801 in the presence of glutamate and glycine [51, 52]. The B_{max} for MK-801 binding showed that the NMDAR density in SCZ cases was approximately 30% higher than that in controls (Figure 1a,b, $t(13)=2.837$, $p = 0.014$). To test whether such increases in NMDAR complexes occur in the PSD, PSD enriched fractions were isolated from the DLPFC and protein associations in NMDAR complexes were assessed using immunoprecipitation experiments for GluN1 or PSD-95. The results show a higher level of PSD-95 in GluN1 immunoprecipitates ($t(13)=2.279$, $p=0.0389$, Figure 1c,d), and a higher level of GluN1 in PSD-95 immunoprecipitates in SCZ brains ($t(13) = 2.405$ $p=0.0318$, Figure 1c,d). These results indicate increased number of NMDAR complexes in the PSD of SCZ cases, which may not be a likely explanation for decreased tyrosine phosphorylation of GluN2. Thus, we turned our attention to molecular events downstream to the receptors, i.e., the post-receptor level.

NMDAR signaling is hypoactive at the post-receptor level in schizophrenia

We measured NMDAR signaling responses to 10 μM NMDA + 1 μM glycine applied to 50 μm thick slices from the DLPFC of 17 SCZ cases, each matched to a normal control of the same sex, similar age (80.12 ± 1.40 y [mean ± SD] for SCZ cases vs. 79.53 ± 2.31 y for controls), equivalent pH (6.56 ± 0.039 for SCZ cases vs. 6.59 ± 0.089), and comparable, low

postmortem interval (PMI: 12.12 ± 0.98 h vs. 8.76 ± 0.08 for controls). SCZ cases showed decreases in NMDA/glycine-induced tyrosine phosphorylation of GluN2, which is critical for NMDAR channel activity (p=0.001, t(11)=6.066, Figure 2a,b). Next, we examined PKCγ, PLCγ and Src, which in tandem govern tyrosine phosphorylation of GluN2. In synaptic membranes derived from the tissues that were incubated with NMDA/Glycine, we found decreases in GluN1's association with PKCγ[53, 54] (Figure 2c,d, p= 0.027, t(13)=2.406, for PLCγ and p 0.026, t(13)=2.512 for PKCγ), indicative of decreased PKCγ activation. In addition, pY^{402}Pyk2 and pY^{419}Src, reflective of the activity of these kinases, were also decreased in GluN1 immunoprecipitates in SCZ cases compared to their matched controls (Figure 2c,d, p=0.029, t (13)=2.831 for pY^{402} Pyk2 and p<0.043. t(13)=2.701 for pY^{419} Src).

To further test Src phosphorylation, we examined GluN2A complexes in which Src directly phosphorylates tyrosine residues of this subunit. GluN2A immunoprecipitates from the DLPFC of the SCZ group showed a decrease in pY^{419}Src, which activates Src kinase, and an increase in pY^{530}Src, which suppresses this kinase (Figure 2a,b; P=0.042, t(11)=2.303 for pY^{419}Src and p=0.002 t(11)=4.171 for pY^{530} Src). These results together demonstrate that the activity of post-receptor kinases, PKC, Pyk2 and Src is attenuated in the DLPFC of SCZ cases.

All parameters of NMDAR signaling described above were derived from synaptic membrane fractions, which incorporate NMDAR complexes in synaptic as well as extrasynaptic regions. To test whether the altered NMDAR signaling occurs in the synaptic region, we examined PSD fractions of the DLPFC. In PSD fractions, the activity of post-receptor kinases is difficult assess due to the presence of detergents, but protein associations resulting from the activity of kinases can be measured. We examined PSD fractions for NMDAR's association with PLCγ and PKCγ [53, 55] as surrogate measures of GluN2 activation, which was significantly reduced in the SCZ cases compared to controls (Figure 1C; p<0.0104 t (13)=2.991 for PLCγ and p<0.004, t(13) =3.434, for PKCγ). This indicates that synaptic NMDAR complexes are hypoactive in the DLPFC of SCZ cases.

Src hypoactivity occurs independently of its upstream kinases in the NMDAR pathway

As noted above, Src serves as a molecular hub for various non-NMDAR signaling mechanisms impacting tyrosine phosphorylation of GluN2, including NRG1, erbB4 and RPTPa, SCZ susceptibility pathways. Based on this, we hypothesize that Src hypoactivity is not merely a result of hypofunction of upstream kinases within the NMDAR pathway but integrates alterations in signaling pathways that are not canonical components of the NMDAR pathway. We first tested whether Src hypoactivity is present in SCZ cases when Src activity is assessed independently of activation of NMDAR. In studies represented in Figure 2, post-receptor NMDAR signaling was assayed in response to NMDAR activation, thus the results can not resolve whether Src hypoactivity results from alterations at the receptor level or from the post-receptor segment. To distinguish between these two possibilities, we assessed Src activity with and without NMDAR activation in post-mortem tissues. First, DLPFC slices were incubated with NMDA + Glycine so that NMDAR complexes were activated and Src kinase activity was measured (Figure 3a). Second, we

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isolated synaptic membranes from the DLPFC tissues, which were then incubated with the peptide that directly activates Src, EPQ(pY)EEIPIA, instead of NMDA + Glycine [35, 56] (Figure 3b). NMDA + Glycine and EPQ(pY)EEIPIA both enhanced Src activity. EPQ(pY)EEIPIA increased Src enzyme activity on average 3.02 ± 0.37 fold in the control group, but only by 1.75 ± 0.30 fold on average in the SCZ group (Figure 3b; p=0.022, t(16)=2.859). As predicted, we found a corresponding reduction in basal levels of activated Src (pY^{419}) in the patient group compared to controls (Figure 3c,d, p= 0.015, t(13)=3.230), while Src protein levels remained unaltered. Of particular note, DLPFC tissue from the SCZ patients showed levels of Pyk2 and its activated form (pY^{402} Pyk2) that were comparable to those of the control group (Figure 3c,d). These findings indicate that Src hypoactivity in SCZ cannot be explained fully by upstream abnormalities in the NMDAR→PKC→Pyk2→Src pathway. Src is activated by association with its activated upstream kinases such as pPyk2. Since Src hypoactivity is independent of upstream kinases, we next determined the Src's binding capacity for its activators.

Binding capacity of Src for activated upstream kinases is decreased in SCZ

We isolated Src from DLPFC tissues and then assessed binding of these kinases to biotinylated EPQ(pY)EEIPIA. The SCZ group showed a significantly lower association of Src with EPQ(pY)EEIPIA at 0.1, 1.0, and 10 nM of the substrate (Figure 4 a,b, p=0.013, t(11)=2.945 for 0.1 nM; p=0.024, t(11)=2.620 for 1nM; p=0.049, t(11)=2.213 for 10 nM). Thus, the binding capacity of the SH-2 domain in Src is decreased in SCZ, which will decrease Src activity independently of the NMDAR→ PKC→Pyk2 cascade. The next step was to identify molecular alterations that may impact Src's binding capacity and/or Src's association with its activators.

Convergent molecular alterations reduce Src's binding with upstream kinases in SCZ

We found a number of molecular alterations in the DLPFC of SCZ cases that can impact Src's association with its activators. Receptor-type tyrosine-protein phosphatase α (RPTPα) increases Src's binding capacity via dephosphorylation of $SrcY^{530}$ [30]. RPTPα's association with Src, assessed in PSD-95 immunoprecipitates, was significantly decreased in SCZ cases (Figure 4c,d, p=0.006, t(11)=3.390). Given that the N-terminal of PSD-95 can interfere with Src's binding with its activators [35], increased PSD-95 in NMDAR complexes as shown in the DLPFC of SCZ cases (Figure 1 c , d) may be another molecular underpinning for Src hypoactivity activity. In addition, increased erbB4 signaling, either via altered expression [57] or cleavage of NRG1 [58] or erbB4 activity, is expected to decrease Src activity. Indeed, Src - NMDAR complexes captured by PSD-95 exhibited increased erbB4 in the SCZ group (Figure 4c).

We next further examined whether these dysregulations can indeed lead to Src hypoactivity. ErbB4 suppresses Src activity [28, 56] and is increased in PSD-95-Src complexes derived from the DLPFC of SCZ subjects (Figure 4c,d). We predicted that activation of erbB4 via NRG1 would lead to a greater reduction in Src activity of SCZ cases than of controls. Indeed, NRG1-induced suppression of Src activation was more pronounced in SCZ cases compared to controls (Figure 4e, p=0.005, t(11)=3.433). Hence, the decreased RPTPα in PSD-95-Src complexes as observed in SCZ (Figure 4c,d) should decrease Src activity. Indeed, mice

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lacking RPTPα, which were found to display several SCZ phenotypes [31], also exhibit Src hypoactivity (Figure 4e, $p=0.047$, $t(5)=2.821$). Dysbindin-1, encoded by DTNBP1, is another molecule that is decreased in the DLPFC of SCZ cases [59]. Mice lacking dysbindin-1 due to deletion mutation in Dtnbp1 (sdy –/–), which show several SCZ phenotypes [59]as well as NMDAR hypofunction [60], also exhibited Src hypoactivity (Figure 4e, $p=0.0021$, $t(7)=4.728$).

A protein – protein interaction based Src network is associated with SCZ

We next asked whether the molecular alterations in PSD-95, erbB4, RPTPa or dysbindin in the DLPFC of SCZ cases could be genetically determined. We conducted a meta-analysis of SCZ GWAS studies, consisting of 15 cohorts that included 13,394 cases and 34,676 controls. Src, PKCγ, Pyk2, PSD-95 and RPTPa (Src, PRKCG, PTK2B, DLG4, and PTPRA) as well as other direct interactors of Src showed no evidence of SNP or gene-level associations (Figure 5a). Given our results suggesting that Src hypoactivity results from altered protein association of Src with its interactors, we hypothesized that a wider protein-protein interaction (PPI) based Src network may be associated with SCZ. We generated a PPI network centered around Src, which consisted of 30 genes (Figure 5, Table S3 in supplementary information). Fourteen of these showed gene-level significant association with SCZ (Figure 5b). To determine if the observed gene-level associations in the Src network is specific to SCZ, we compared the association results of a SCZ GWAS meta-analysis against the results of 4 other datasets, MDD, BP, ADHD and CD. The enrichment of SNP associations within the Src network of 30 genes was significant in schizophrenia compared with all other phenotypes (SCZ vs MDD $p=6.921\times10^{-11}$; SCZ vs ADHD $p=3.429\times10^{-12}$; SCZ vs BP $p=9.338\times10^{-8}$; SCZ vs CD $p=7.47\times10^{-7}$); individual SNP counts for each of the 5 diseased are listed in Table S4 (See supplementary information).

Discussion

The goal of this study was to delineate the alterations in post-synaptic NMDAR signaling in SCZ and to investigate its underlying mechanisms. Our results point to the post-receptor kinases as a locus of dysregulation in SCZ and suggest that Src hypoactivity may mediate convergent abnormalities of susceptibility pathways to decrease tyrosine phosphorylation of GluN2 subunits.

NMDAR hypofunction can result from decreased NMDAR complexes, which has been extensively tested in previous studies[14–16]. Our approach was to measure NMDAR assemblies in the synaptic region by MK-801 binding assays of synaptic membranes and by immunoprecipitation of the core scaffold of NMDAR complexes, the GluN1–PSD-95 associations. Our findings of increased MK-801 binding and NMDAR complexes in the PSD suggest that NMDAR hypofunction in SCZ may not result from decreases in NMDAR complexes in synaptic regions.

At the post-receptor level, however, we found robust reductions in multiple parameters indicative of hypoactive NMDAR signaling. These include decreases in a) the activity of PKCγ, Pyk2, and Src, b) tyrosine phosphorylation of GluN2 subunits and c) NMDAR's association with its signaling partners, PLCγ and PKCγ. These three parameters are

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interconnected since the post-receptor kinases enhance tyrosine phosphorylation, which in turn recruits PLCγ into NMDAR complexes (Figure 5c). Consistent reductions in these three interconnected parameters therefore point to this specific segment of the NMDAR pathway, namely the post-receptor kinases, as a locus of dysregulation in SCZ.

The reduced post-receptor kinase activities as observed in SCZ cases could potentially result from presynaptic alterations [1], such as decreased glutamate release or increased kynurenic acid observed in SCZ cases [11, 12]. Our assessment of post-receptor kinase function, however, is based on applying relatively high concentrations of NMDA and glycine, conditions under which assay results may not capture presynaptic alterations. Thus, dysregulations in post-receptor kinases as assessed in this study are unlikely to reflect presynaptic dysregulations.

It is possible that these post-receptor kinase dysregulations are secondary to antemortem medication effects. In particular, previous studies have shown that chronic clozapine treatment decreased the activity and/or expression of PKC and PLCg [57]. We therefore examined these signaling molecules in rhesus macaques (age range: 5.9–7.9 years), who were treated with a low dose of haloperidol (0.07 mg/kg b.i.d.; n=8), high dose of haloperidol (n=8; 2.0 mg/kg b.i.d.), clozapine (n=9; 2.6 mg/kg; b.i.d.) or vehicle (n=9) for six months. GLUN1 association with PKCγ or PLCγ showed trends for increase or no changes in the three treatment conditions (Figure S2, supplementary information). Thus, decreases in GluN1 association with PLCγ and PKCγ in SCZ are unlikely to be results of antipsychotic treatment.

Among the post-receptor kinases, Src is of particular interest since it directly phosphorylates tyrosine residues of GluN2 and therefore serves as a point of convergence for various pathways impacting NMDAR signaling [22, 61]. Our results offer multiple lines of evidence supporting the notion that Src hypoactivity is a mere result of hypoactivity of upstream kinases in the NMDAR pathway. Specifically, Src activity was decreased in SCZ cases even when measured under the condition that NMDARs were not activated and Src binding capacity was also reduced, which was independent of the NMDAR → PKC→ Pyk2 cascade. These strongly suggest that Src hypoactivity may result from changes in molecular events that are outside of the canonical components of the NMDAR pathway.

Indeed, the DLPFC of SCZ cases exhibit a number of molecular alterations that can precipitate Src hypoactivity (Figure 5c). First, we find decreased RPTPa in Src-NMDAR complexes in SCZ cases. This will lead to Src hypoactivity, since RPTPα enhances Src activity by decreasing phosphorylation of pY530 of Src [30]. Second, since PSD-95 can inhibit Src activity [35], increased PSD-95 in NMDAR–Src complexes in SCZ cases will also precipitate Src hypoactivity. Third, NRG1–erbB4 signaling can reduce Src activity [56], and we find increased erbB4–PSD-95 association in SCZ cases. Finally, decreased dysbindin 1 shown in DLPFC of SCZ cases[59] may decrease Src activity as observed in the case of sdy−/− mice. Based on these we propose that Src serves as a nodal point for these molecular alterations in susceptibility pathways leading to decreased GluN2 tyrosine phosphorylation (Figure 5c).

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It will be important to ask whether Src hypoactivity arises from genomic effects. A meta-analysis of 15 cohorts demonstrate that common variants of the core members of the Src pathway, Src, PRKCG, PTK2B, DLG4, CSK, and PTPRA are not associated with SCZ. This is not unexpected since the expression of these genes in the DLPFC of SCZ cases was comparable to that of control subjects (data not shown). The results from the PPI based gene association study, however, suggest that a wider PPI based network centered on Src, consisting of 30 genes, showed significant enrichment of gene-level associations with SCZ compared to other diseases (Figure 5). Together, these genetic findings are consistent with the notion that the Src pathway as a whole is associated with SCZ; not via common variants of Src or its direct interactors but via Src's association with a wider network.

Src mediated tyrosine phosphorylation of GluN2 are critical for the synaptic plasticity (LTP) [62–65], the substrates for learning and memory. Thus, Src hypoactivity and its downstream effects observed in the DLPFC can be a basis for deficits in the neurocognitive functions modulated by the DLPFC, such as the working memory and executive function. We propose Src hypoactivity as a nodal mechanism for integrating convergent dysregulation in SCZ susceptibility pathways to reduce NMDAR activity (Figure 5c) and suggest that the Src pathway, particularly protein interactions therein, be considered as a novel therapeutic target for SCZ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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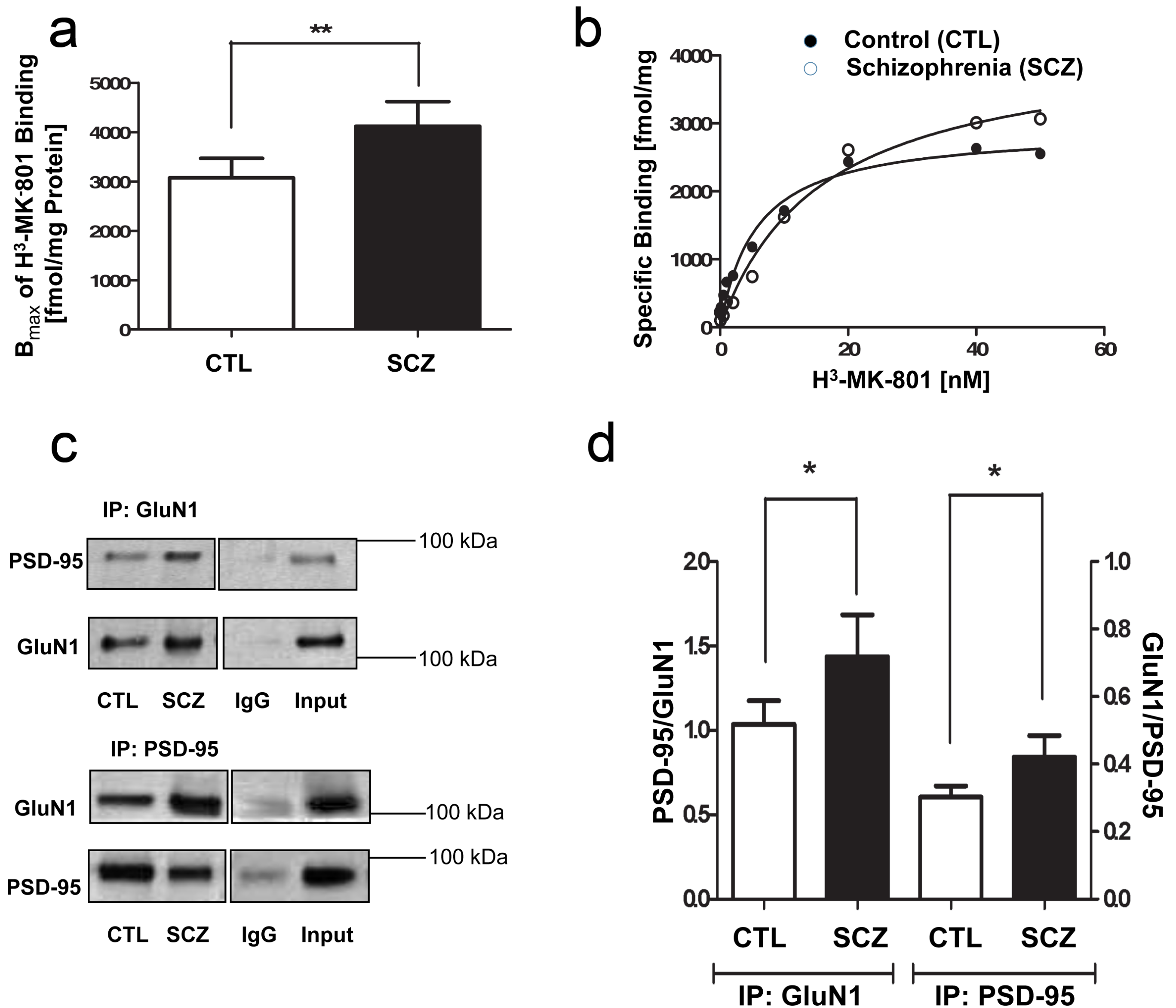

Figure 1. The abundance of NMDAR complexes is increased in the PSD of SCZ subjects

(a,b) Synaptic membranes obtained from 14 pairs of CTL and SCZ subjects were incubated with 0 to 60 nM of 3H–MK-801 in the presence of 10 μM of glutamate and 1 μM of glycine. (a) B_{max} of MK-801 binding is enhanced in the SCZ group by approximately 30%, (p=0.014). (b) Representative binding kinetics of MK-801 assay for a pair of CTL (-●-) and SCZ (-○-) subjects. (c,d) GluN1-PSD-95 complexes are increased in SCZ. PSD fractions from the PFC of 14 matched pairs were immunoprecipitated with antibodies for GluN1 or PSD-95. GluN1's association with PSD-95 was increased in the GluN1 complexes (black bar; p 0.038) and PSD-95's association with GluN1 (black bar; p 0.0318) was increased in PSD-95 complexes in the SCZ group. All data are means ± S.E.M, student's paired t-test, two tailed. *p 0.05, **p 0.01, ***p 0.001.

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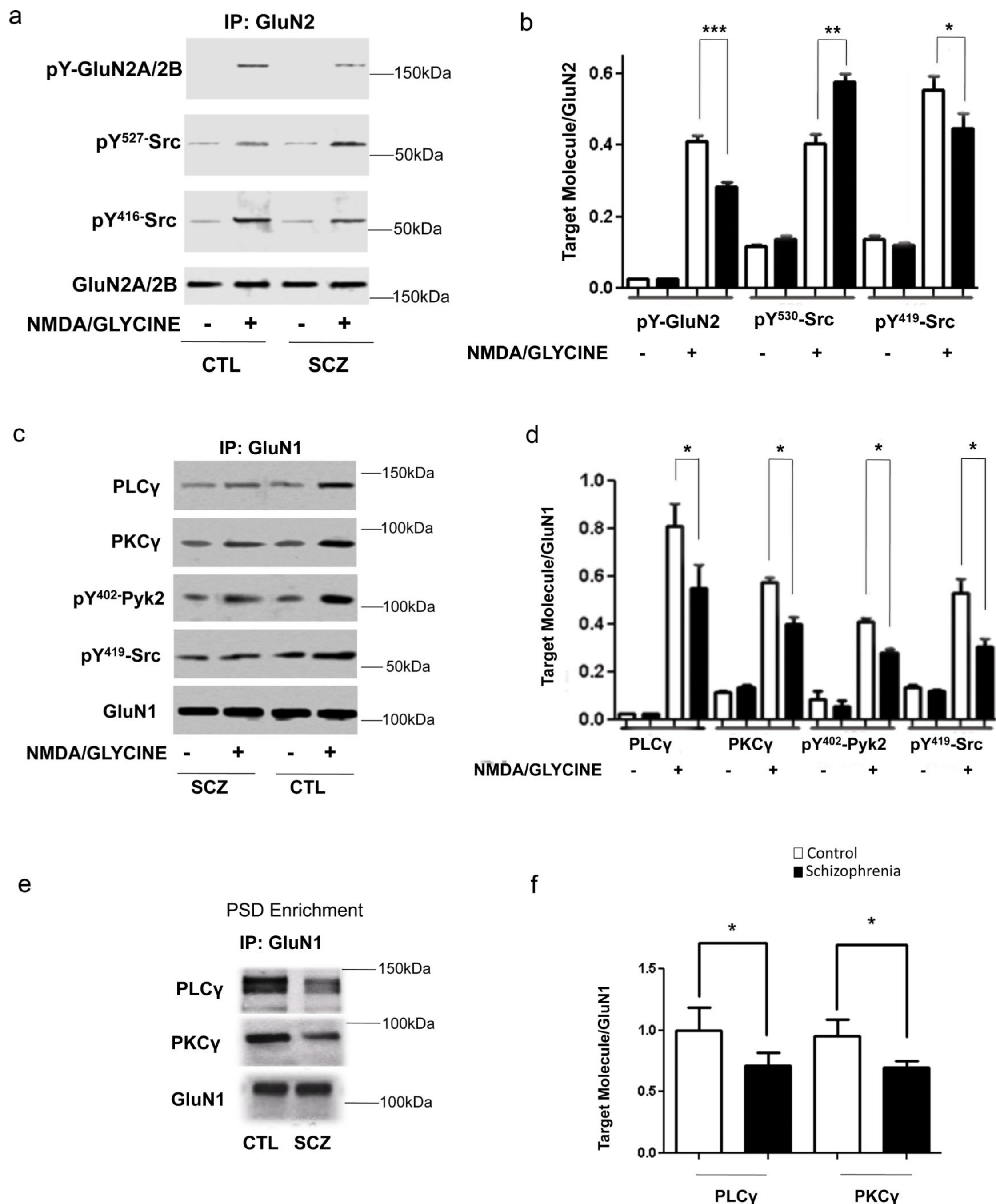

Figure 2. Post-receptor NMDAR signaling is attenuated in the postmortem DLPFC of SCZ patients

The DLPFC from 14 pairs of SCZ (black bar) and control subjects (white bar) was incubated with 10 μM of NMDA and 1 μM of glycine for 15 minutes and solubilized membrane extracts were immunoprecipitated with antibodies for GluN2 (2A/2B) (Figure 2a,b) or GluN1 (Figure 2c,d) and immunoblotted as indicated. (a,b): GluN2A/B tyrosine phosphorylation (pYGluN2) is attenuated in SCZ compared to control (p=0.001). pY419 Src is decreased and pY530 Src is increased in GluN2 complex in SCZ (p=0.042 and p=0.016 respectively). (c,d): Ligand induced enhancement of GluN1's association with its signaling

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partners, PLCγ and PKCγ is reduced in SCZ subjects compared to their controls (p=0.0278 and p=0.0380 respectively). Tyrosine phosphorylation of y402Pyk2 (p=0.029) and y419Src (p=0.0475) are also decreased in SCZ subjects in GluN1 complexes. (e,f) PSD fractions from the DLPFC of 14 matched pairs were immunoprecipitated with antibody for GluN1. Association of GluN1 with PLCγ (p=0.010) and PKCγ (p=0.0004) are decreased in the SCZ group (black bar) compared to that of the control (white bar). All data are means ± S.E.M, student's paired t-test, two-tailed. *p 0.05, **p 0.01, ***p 0.001.

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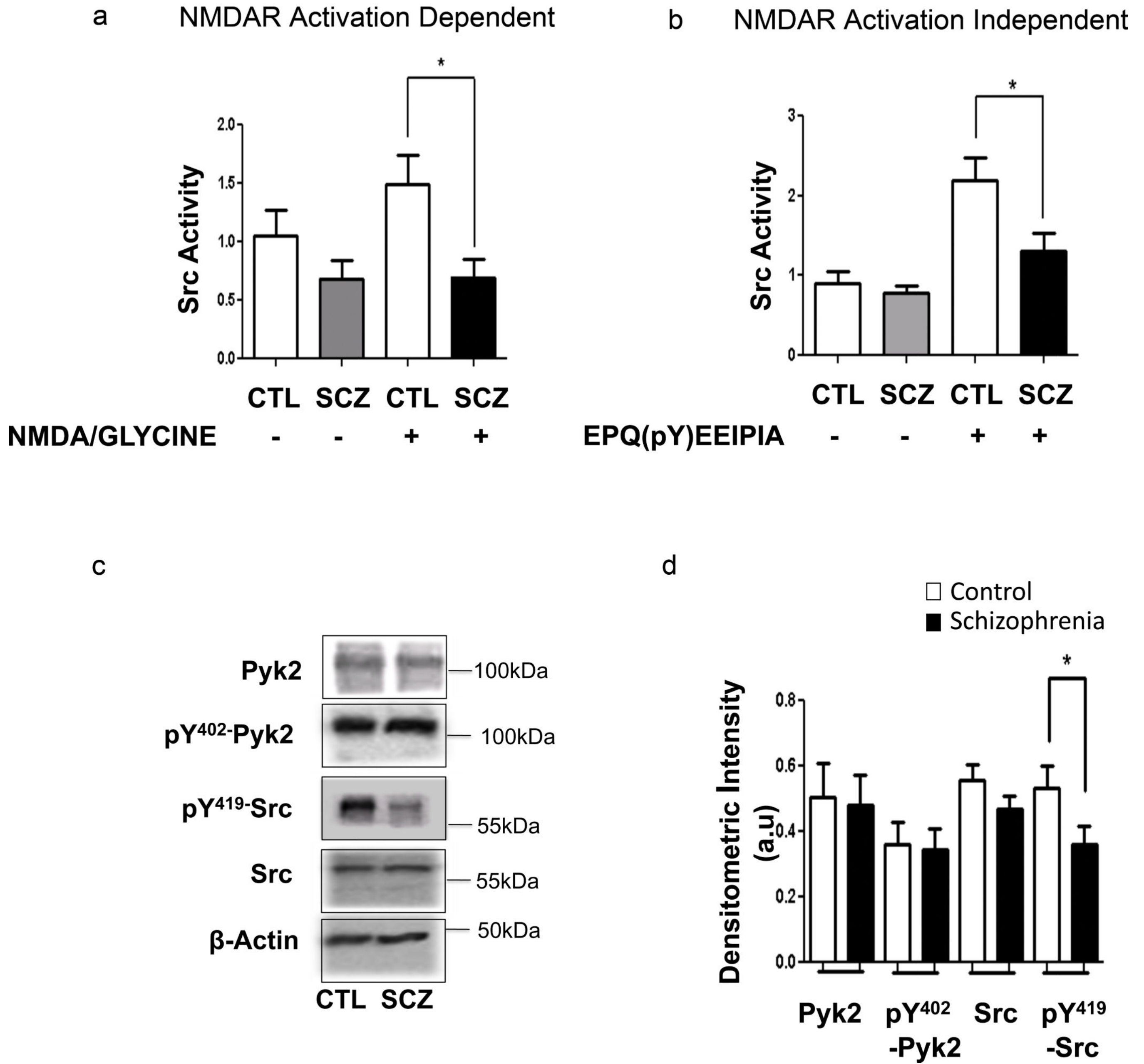

Figure 3. Src activity is attenuated in SCZ

(a) Src kinase activity was measured by P32γ-ATP incorporation into Src substrates in synaptic membranes, extracted from the tissues that were incubated with 10 μM NMDA and 1μM glycine. Ligand induced Src activation is decreased in SCZ, (black bars; p=0.003). (b) Synaptic membranes from the tissues, that were not incubated with NMDA/glycine, were reacted with the EPQ(pY)EEIPIA peptide, which binds and activates the SH-2 domain of Src. EPQ(pY)EEIPIA induced Src activity is attenuated (black bar; p=0.022) in the SCZ group compared to the control (white bar). (c,d) EPQ(pY)EEIPIA peptide induced enhancement of pY419 of Src was significantly reduced (p=0.015) but the peptide mediated pY402-Pyk2 was unaltered in SCZ subjects (black bars) compared to controls (white bars)

unlike NMDA-glycine induced condition. All data are means ± S.E.M, student's paired t-test, two tailed. *p   0.05, **p   0.01.

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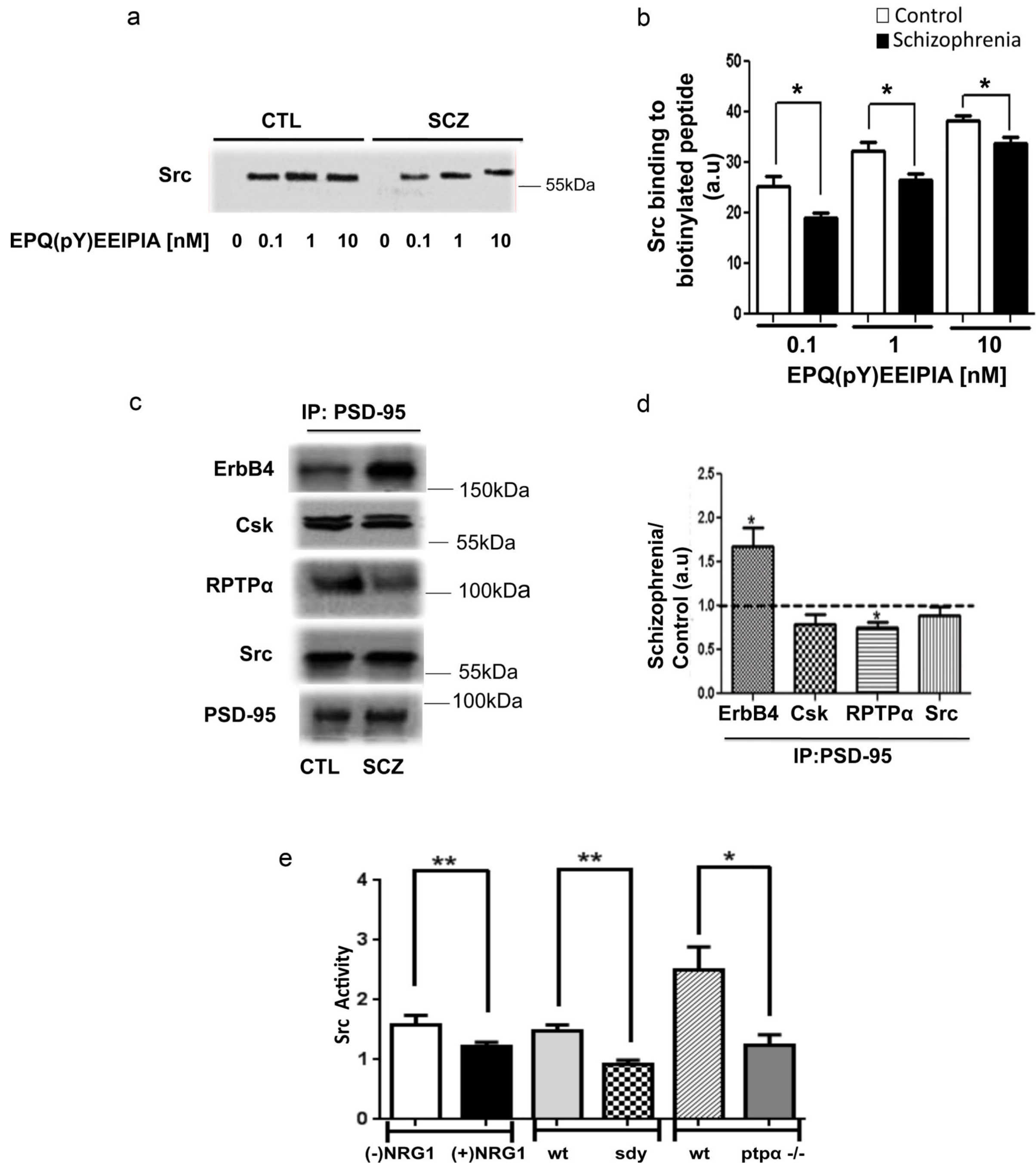

Figure 4. Underlying mechanisms for Src hypoactivity in SCZ

(a,b) Src's capacity for binding with SH-2 binding domain is decreased in SCZ. One μg of purified Src from PFC tissues was incubated with 10nM of biotinylated EPQYEEIPIA (1) or EPQ(pY)EEIPIA peptide in 0.1 (2), 1 (3), 10 (4) nM concentrations in healthy control (white bars) and schizophrenic (black bars) subjects. Binding of Src to pY peptide was significantly reduced in schizophrenia subjects at all concentrations (p=0.013 for 0.1 nM, p=0.024 at 1nM and p=0.049 at 10 nM). (c,d) **Src hypoactivity is accompanied by alterations in Src's associations with RPTPa, PSD-95 and erbB4 in SCZ**. Synaptic

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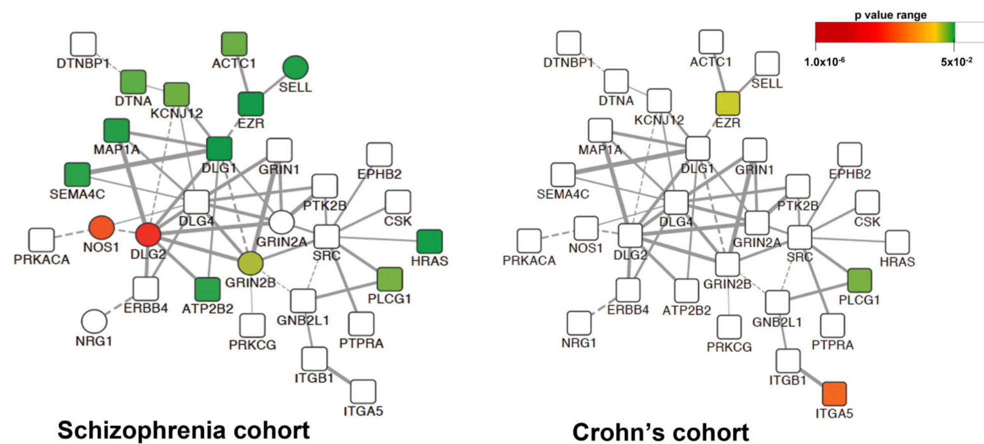
membranes of the postmortem DLPFC were immunoprecipitated with antibodies for PSD-95. RPTPα is decreased but erbB4 is increased in the PSD-95 complexes derived from SCZ (black bars) subjects (p 0.006), while Src is not altered. In addition, the ratios of PSD-95 with respect to GluN1 were increased in the NMDAR complexes derived from PSD fractions. **(e) Increased NRG1 – erbB4 signaling and decreased expression of RPTPα and dysbindin decrease Src activity**. NRG1 mediated Src activity measured by 32P-γATP incorporation in presence of NMDA/Glycine was less in the DLPFCs of human post mortem brains (n=12; p 0.005). Src activity was also measured in presence of Src-SH2 binding pY peptide, EPQ(pY)EEIPIA in dysbindin (sdy–/–) and RPTPα (Ptpra–/–) knockout mice. In both sdy–/– and Ptpra–/– mice, Src activity was attenuated compared to wild type. In sdy–/– mice src activity was decreased by ~37%, (n= 6; p 0.002) while that src activity was decreased in Ptpra–/– mice by ~47%, (n=7; p   0.015). All data are means ± S.E.M, students paired t-test, two tailed.

a Gene level associations with schizophrenia for core members of the Src pathway

Gene	Chromosome	Gene-wise p-value	# of SNP
SRC	20	0.326	37
PRKCG	19	0.438	21
PTK2B	8	0.08875	141
PTPRA	20	0.761	77
DLG4	17	0.464	29
CSK	15	0.116	58

b Protein-protein interaction derived Src network in schizophrenia and Crohn's disease



c Src mediates convergent molecular alterations leading to NMDAR hypoactivity in schizophrenia

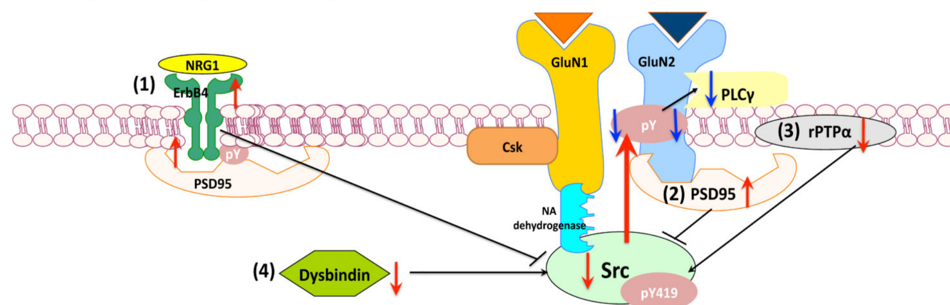


Figure 5. The Src pathway is associated with SCZ not via common variants of their own but by upstream or downstream effectors identified through protein-protein interactions

(a) Gene level associations with SCZ for core members of the Src pathway. Gene level significance was calculated based on meta-analysis of GWAS studies, which included a total of 13,394 cases and 34,676 controls. Src, PKC (PRKCG), Pyk2 (PTK2B), PSD-95 (DLG4), CSK, and RPTPα (PTPRA) failed to show the gene level significance of the p-value less than 0.05.

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(b) The protein interaction derived Src sub-network in the SCZ and Crohn's disease cohorts[47]. Based on protein - protein interactions previously reported, a sub-network of Src was constructed, which consists of 30 genes. In this illustration, nodes represent genes in the sub-network and connecting lines (or edges) show previously reported direct protein interactions. Derived from brain expression data from the Allen Brain Atlas, the information on the degree of co-expression between the proteins is incorporated into the connecting lines. The width of connecting lines corresponds to the strength of the degree of co-expression between the proteins, with solid lines signifying positive, and dotted lines negative correlations. Of 30 in the sub-work, 14 showed gene-wise significance in the schizophrenia cohort while only 3 in the Crohn's disease cohort. Gene-wise p-value significance is denoted on a green to red color scale. Circled nodes are genes with SNPs that had P-values $< 1\times10^{-3}$ for genetic association with the illness and all others are boxed.

(c) Summary diagram: Src serves as a nodal point for various molecular alterations in the DLPFC of SCZ cases, leading to reduced post-receptor NMDAR activity. (1) NRG1 –erbB4 signaling can reduce Src activity [56], therefore the increased NRG1 – erbB4 signaling as shown in SCZ cases [28] will decrease Src activity (Figure 4E). (2) PSD-95 decreases Src activity [35] and thus the increased PSD-95 in NMDAR complexes in SCZ cases (Figure 1C) will reduce Src activity. (3) RPTPα increases Src activity and thus decreased RPTPα in SCZ cases (Figure 4D) will reduce Src activity, (4) Sdy–/– mice, mutated for dysbindin, exhibit reduced Src activity. Decreased dysbindin 1, as previously seen in SCZ cases [59], can be another underlying mechanism for Src hypoactivity. Src hypoactivity precipitated by these convergent dysregulations (arrows in red) will reduce phosphorylation of its substrate, the GluN2 subunits, which is critical for the channel activity of NMDARs, as well as its downstream signaling (arrows in blue). Symbols → and ⊥ indicate positive or negative regulation of Src activity by the molecule or the pathway. Downward or upward arrows (blue) indicate alterations of the function of the molecules in the postmortem DLPFC in SCZ cases.