Archival Report

Biological Psychiatry

# Altered Glutamate Protein Co-Expression Network Topology Linked to Spine Loss in the Auditory Cortex of Schizophrenia

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## ABSTRACT

**BACKGROUND:** Impaired glutamatergic signaling is believed to underlie auditory cortex pyramidal neuron dendritic spine loss and auditory symptoms in schizophrenia. Many schizophrenia risk loci converge on the synaptic glutamate signaling network. We therefore hypothesized that alterations in glutamate signaling protein expression and co-expression network features are present in schizophrenia.

**METHODS:** Gray matter homogenates were prepared from auditory cortex gray matter of 22 schizophrenia and 23 matched control subjects, a subset of whom had been previously assessed for dendritic spine density. One hundred fifty-five selected synaptic proteins were quantified by targeted mass spectrometry. Protein co-expression networks were constructed using weighted gene co-expression network analysis.

**RESULTS:** Proteins with evidence for altered expression in schizophrenia were significantly enriched for glutamate signaling pathway proteins (GRIA4, GRIA3, ATP1A3, and GNAQ). Synaptic protein co-expression was significantly decreased in schizophrenia with the exception of a small group of postsynaptic density proteins, whose co-expression increased and inversely correlated with spine density in schizophrenia subjects.

**CONCLUSIONS:** We observed alterations in the expression of glutamate signaling pathway proteins. Among these, the novel observation of reduced ATP1A3 expression is supported by strong genetic evidence indicating it may contribute to psychosis and cognitive impairment phenotypes. The observations of altered protein network topology further highlight the complexity of glutamate signaling network pathology in schizophrenia and provide a framework for evaluating future experiments to model the contribution of genetic risk to disease pathology.

*Keywords:* Auditory cortex, Glutamate, Postmortem, Proteomics, Schizophrenia, Spine

http://dx.doi.org/10.1016/j.biopsych.2014.09.006

Individuals with schizophrenia (SCZ) display auditory symptoms associated with altered activation of the primary auditory cortex (AI) including auditory hallucinations (1–5). Additionally, they manifest impairments in the processing of sensory information within the AI that contribute to disease burden. For example, a reduction in the ability to discriminate tones (6–9) impacts interpretation of spoken emotion (prosody) (10,11), impairing social cognition (12,13). Likewise, deficits in auditory event-related potentials, such as reduced amplitude of mismatch negativity, are present in SCZ (6,7,14,15). Impairments in tone discrimination and mismatch negativity are correlated and likely involve the same intracortical circuits (8) in AI layer 3 (16,17).

Several lines of evidence suggest that disrupting glutamate signaling in the AI can induce or exacerbate these auditory impairments. *N*-methyl-D-aspartate (NMDA) receptor antagonists produce reductions in mismatch negativity amplitude in normal human subjects, nonhuman primates(17), and mice (18–20). Similarly, NMDA receptor antagonists exacerbate psychotic symptoms in individuals with schizophrenia, including recurrence (or intensification) of auditory hallucinations (21). The role of glutamate signaling network impairments in SCZ is supported by findings that postsynaptic genes involved in NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor signaling are significantly enriched for mutations in disease (22–25). However, these SCZ risk genes have not yet been linked specifically to auditory impairments in SCZ, despite evidence for heritability of some of these impairments (26).

Structural impairments of glutamatergic layer 3 pyramidal neurons, such as reduced dendritic spine density, are among the most consistently observed findings in postmortem studies of individuals with SCZ (27) and have been reported in multiple brain regions, including AI (28–36). As dendritic structural features are critical for signal processing (37–40), it has been postulated that this spine loss underlies the AI processing deficits observed in SCZ. Not surprisingly then, numerous studies have demonstrated that attenuating presynaptic or postsynaptic glutamate signaling can decrease dendritic spine density (41–45). Surgical deafferentation of glutamatergic projections (43–45) or knockout of glutamate release proteins results in decreased density of spines (46,47).

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Postsynaptically, pharmacologic blockade or knockout of glutamate receptors (AMPA or NMDA) results in decreased spine density (41–45), as does decreasing levels of the NMDA receptor co-agonist d-serine (48).

We therefore hypothesized that alterations in glutamate signaling protein expression are present in the AI of SCZ subjects. In addition, as emerging genetic data indicate that many SCZ-associated loci converge on the glutamate signaling network, we further hypothesized that alterations in network features would also be present in the AI of SCZ subjects. We tested these hypotheses using a liquid chromatography-selected reaction monitoring/mass spectrometry (LC-SRM/MS) targeted proteomics approach (49) to quantify the expression of 155 synaptic proteins in AI gray matter homogenates from 22 SCZ and 23 matched control subjects. Functional gene annotation analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (50) was used to determine if proteins with altered expression were enriched for terms related to function and cellular compartment, while weighted gene co-expression network analysis (WGCNA) was used to investigate patterns of co-regulated protein expression. Differentially expressed proteins were enriched for the Gene Ontology (GO) term glutamate signaling pathway. Analysis of protein co-expression network topology revealed a significant loss of correlated protein expression in SCZ. The exception to this loss was a small module of postsynaptic density proteins whose co-expression increased in SCZ. The averaged expression of these proteins was significantly inversely correlated with spine density in the subset of SCZ subjects for which AI layer 3 spine density had previously been determined (51).

## METHODS AND MATERIALS

### Subjects

Brain specimens from all subjects were obtained during autopsies conducted at the Allegheny County Office of the Medical Examiner after receiving consent from the next-of-kin. An independent panel of experienced clinicians made consensus DSM-IV diagnoses using a previously described method (28). Twenty-two subjects diagnosed with SCZ and 23 control subjects for which total protein extracted from AI gray matter was available were studied. Every effort was made to balance these two groups by age, sex, race, and postmortem interval (PMI) (Table 1; Table S1 in Supplement 1). Control subjects underwent identical assessments and were determined to be free of lifetime psychiatric illness. Procedures were approved by the University of Pittsburgh Institutional Review Board and Committee for Oversight of Research Involving the Dead.

### Rhesus Monkeys

The tissue utilized here has been extensively described elsewhere (52,53). Briefly, rhesus monkeys (3–8 years of age) were randomly assigned to one of three treatment groups: vehicle (5 male, 5 female animals), clozapine 5.2 mg/kg daily (5 male, 5 female animals), haloperidol .14 mg/kg daily (5 male, 5 female animals). Later, a fourth group was treated with a higher dose of haloperidol (2.0 mg/kg twice daily) (4 male, 4 female animals). Drugs were administered in either peanut butter or fruit treats and all animals were treated for 6 months.

**Table 1. Summary of Subject Characteristics**

| | Control | Schizophrenia | *p* |
|---|---|---|---|
| *n* | 23 | 22 | |
| Mean Age (SD) | 45.8 (11.3) | 47.2 (13.7) | .71 |
| Range | 19–67 | 25–71 | |
| Sex (F/M) | 5/18 | 5/17 | |
| Handedness (R/L/A/U) | 21/2 | 14/3/1/4 | |
| PMI (SD) | 17.9 (6.6) | 18.9 (8.1) | .64 |
| Storage Time, Months (SD) | 130 (43) | 126 (37) | .69 |
| pH (SD) | 6.7 (.3) | 6.5 (.3) | .045 |
| Suicide, *n* (%) | | 7 (32%) | |
| Schizoaffective, *n* (%) | | 7 (32%) | |
| Alcohol/Substance Abuse ATOD, *n* (%) | | 15 (68%) | |
| Antipsychotic ATOD, *n* (%) | | 19 (86%) | |

There were no diagnostic group differences in age, sex, postmortem interval, storage time, or in the distribution of handedness between the diagnostic groups. There was a small but significant difference in pH.

A, ambidextrous; ATOD, at time of death; F, female; L, left-handed; M, male; PMI, postmortem interval; R, right-handed; U, unknown.

### Sample Preparation and LC-SRM/MS

**Human.** Gray matter was harvested as previously described (54,55). Tissue slabs containing the superior temporal gyrus with Heschl's gyrus located medial to the planum temporal were identified, and the superior temporal gyrus was removed as single block. The samples were then distributed in a block design for preparation and analysis to evenly distribute SCZ and control samples and blind the experimenters during sample preparation, analysis, and peak integration. Gray matter was collected from Heschl's gyrus by taking 40-μm sections and frozen at −80°C (54). Total protein was extracted using sodium dodecyl sulfate extraction buffer (.125 mol/L Tris–HCl (Sigma-Aldrich, St. Louis, Missouri) [pH 7], 2% sodium dodecyl sulfate, and 10% glycerol) at 70°C, and protein concentration was measured by bicinchoninic acid assay in triplicate (BCA Protein Assay; Pierce, Rockford, Illinois). Twenty micrograms of these gray matter homogenates were mixed with 10 μg of lysine $^{13}C_6$ stable isotope labeled neuronal proteome standard and processed for LC-SRM/MS analysis by in-gel trypsin digestion as previously described (49). Variability of peptide and protein quantification was assessed as described in MacDonald *et al.* (49). Briefly, four 10-μg aliquots from the same AI gray matter homogenate were prepared and analyzed by LC-SRM/MS. For details on LC-SRM/MS, see Supplemental Methods in Supplement 1.

### Statistical Analysis

For each peptide light/heavy ratio, an analysis of covariance (ANCOVA) model was fitted to compare SCZ versus control groups. Age, PMI, and assay group were adjusted in the model. In addition, there was a small, albeit statistically significant, difference in pH between groups, so pH was also included in the model. The ratio (between SCZ and control groups) and the corresponding *p* value were reported from