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# Molecular evidence for decreased synaptic efficacy in the postmortem olfactory bulb of individuals with schizophrenia

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ABSTRACT

Multiple lines of evidence suggest altered synaptic plasticity/connectivity as a pathophysiologic mechanism for various symptom domains of schizophrenia. Olfactory dysfunction, an endophenotype of schizophrenia, reflects altered activity of the olfactory circuitry, which conveys signals from olfactory receptor neurons to the olfactory cortex via synaptic connections in the glomeruli of the olfactory bulb. The olfactory system begins with intranasal olfactory receptor neuron axons synapsing with mitral and tufted cells in the glomeruli of the olfactory bulb, which then convey signals directly to the olfactory cortex. We hypothesized that olfactory dysfunction in schizophrenia is associated with dysregulation of synaptic efficacy in the glomeruli of the olfactory bulb. To test this, we employed semi-quantitative immunohistochemistry to examine the olfactory bulbs of 13 postmortem samples from schizophrenia and their matched control pairs for glomerular expression of 5 pre- and postsynaptic proteins that are involved in the integrity and function of synapses. In the glomeruli of schizophrenia cases compared to their matched controls, we found significant decreases in three presynaptic proteins which play crucial roles in vesicular glutamate transport — synapsin IIa (−18.05%, $p = 0.019$), synaptophysin (−24.08% $p = 0.0016$) and SNAP-25 (−23.9%, $p = 0.046$). Two postsynaptic proteins important for spine formation and glutamatergic signaling were also decreased—spinophilin (−17.40%, $p = 0.042$) and PSD-95 (−34.06%, $p = 0.015$). These findings provide molecular evidence for decreased efficacy of synapses within the olfactory bulb, which may represent a synaptic mechanism underlying olfactory dysfunction in schizophrenia.



## 1. Introduction

Multiple lines of evidence implicate synaptic alterations in schizophrenia. A number of genes and pathways critical for synaptic function have been associated with schizophrenia in genetic studies (Fromer et al., 2014; Kirov et al., 2012; Schizophrenia Working Group of the Psychiatric Genomics, 2014). In postmortem studies, altered expression of genes and proteins involved in synaptic structure or function, such as synaptophysin, synapsin II, GAP-43, SNAP-25, Cdc42 and Duo, have been observed in the dorsolateral prefrontal cortex (DLPFC), temporal lobe, hippocampus and amygdala in schizophrenia (Eastwood et al., 1995, 2000; Eastwood and Harrison, 1995; Glantz and Lewis, 1997; Hill et al., 2006; Ide and Lewis, 2010; Karson et al., 1999; Sawada et al., 2002; Tan et al., 2014; Tcherepanov and Sokolov, 1997; Thompson et al., 1998; Varea et al., 2012; Webster et al., 2001). Furthermore, brain imaging studies indicate altered structural and functional connectivity in cortico-limbic and meso-limbic circuitry in schizophrenia (Allen et al., 2012; Alonso-Solis et al., 2015; Amad et al., 2014; Genzel et al., 2015; Whitfield-Gabrieli et al., 2009; Woodward et al., 2012), some of which may be modifiable by antipsychotic medications (Sarpal et al., 2015). As ultra-structural correlates of synaptic connectivity, decreased dendritic spine density observed in layer 3 of DLPFC (Glantz and Lewis, 2000; Rosoklija et al., 2000) and the auditory cortex Sweet et al. (2009) further supports impaired synaptic connectivity in schizophrenia.

The neural circuitry underlying olfaction may serve as a useful system for studying synaptic efficacy and connectivity in schizophrenia. The essential circuitry consists of the olfactory epithelium (OE)–olfactory bulb (OB)–pyriform cortex. In the OE, odorants bind to odorant receptors generating action potentials in glutamatergic olfactory receptor neurons (ORNs), which synapse with mitral and tufted cells in the glomeruli of the OB (Gottfried, 2010). Within the glomeruli, OB neurons are modulated by relay cells, and axons of OB neurons then travel to the forebrain via the olfactory nerve. Some of these neurons project to the olfactory tubercle, while most of the fibers terminate at the pyriform cortex, where they form synapses with pyramidal neurons that in turn terminate in other parts of the forebrain and limbic system. Neural transmission between the OE and olfactory cortex therefore relies on the relatively simple

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OE–OB synaptic connection, which is highly concentrated in the olfactory glomeruli. As such, the connectivity/strength in glomerular synapses can affect sensitivity and cognition of olfactory signals (Gottfried, 2010), which can offer clues to synaptic dysregulations in patients with schizophrenia.

Increasing evidence suggests that olfactory dysfunction is an endophenotype of schizophrenia (Turetsky et al., 2003a, 2008). Individuals with schizophrenia and those at high risk exhibit deficits in multiple domains of olfactory function including deficits in odor identification (Moberg et al., 1997), odor detection threshold sensitivity (Rupp et al., 2005), odor discrimination (Rupp et al., 2005) and odor memory and odor hedonic judgments (Moberg et al., 2014; Moberg and Turetsky, 2003; Nguyen et al., 2011; Turetsky et al., 2003b, 2009). Interestingly, a lesser degree of olfactory dysfunction also may be found among patients' family members (Moberg et al., 2014) but see (Compton and Chien, 2008; Kamath et al., 2013), suggesting a heritability of this phenotype. Together, olfactory dysfunction may be a heritable trait that co-segregates with the illness and thus meets the criteria for an endophenotype.

Structural and functional alterations in the olfactory circuitry have been reported in schizophrenia (Arnold et al., 2001; Kamath et al., 2011; Nguyen et al., 2011; Rioux et al., 2004). Olfactory event related potentials that are measured in response to odorant stimulation were found to be decreased in patients with schizophrenia compared to controls (Turetsky et al., 2003a,b,c) which indicates an overall decrease in odorant induced neural transmission. Such changes could be in part due to structural changes in key regions of the circuit, as evidenced by decreased size of the OB (Turetsky et al., 2000, 2003a) and olfactory sulci (Nguyen et al., 2011; Takahashi et al., 2013a,b, 2014) in schizophrenia patients and individuals at high risk. In addition, histologic examination of the postmortem OE showed increased ratios of immature vs. mature (GAP-43 immunoreactive vs. OMP immunoreactive) neurons (Arnold et al., 2001), suggesting that altered neuronal maturation may impact olfactory neuron function in schizophrenia patients.

The olfactory glomerulus is a specialized structure in the OB where the axons of the olfactory receptor neurons and dendrites of the olfactory nerves synapse. As such, it permits an opportunity to examine synaptic efficacy with relative ease compared to other regions of the brain. We hypothesized that there would be a decrease in key synaptic proteins in the OB of subjects with schizophrenia, which could mirror similar alterations in other areas of the brain. The results of our study indicate that key presynaptic and postsynaptic proteins are strikingly decreased in the OB of schizophrenia cases compared to their matched controls.

## 2. Method

### 2.1. Postmortem samples

Olfactory bulbs were removed at autopsy from 13 prospectively assessed elderly subjects with schizophrenia and 13 non-psychiatric control subjects carefully matched for age, postmortem interval (PMI), gender and fixative. No significant differences in age at death or PMI were found between schizophrenia and control groups (Tables 1 and 2). Subjects with schizophrenia had been diagnosed prospectively according to DSM-III-R/DSM-IV criteria based on medical history, interview with caregivers and clinical examination of the patient. Subjects with schizophrenia had been elderly participants in a prospective clinicopathological studies program with post mortem tissues stored with the University of Pennsylvania brain bank. Written informed consent for antemortem evaluation and autopsy in the event of death were obtained from next of kin according to approved institutional review board protocols. The antipsychotic exposures of individuals with schizophrenia were converted to chlorpromazine equivalent doses using established methods (Davis, 1974; Woods, 2003). Brain tissues from non-neuropsychiatric elderly controls were obtained through the University of Pennsylvania's Center for Neurodegenerative Disease Research. While none of these control subjects had undergone antemortem assessments, a review of their clinical histories found no evidence of any neurological or psychiatric illness.

### 2.2. Immunohistochemistry

Olfactory bulbs were fixed in formalin (3.7% formaldehyde in 100 mM Tris) or 70% ethanol for 24 h, paraffin embedded, and cut into 10 um thick sections, and then mounted on poly-L-lysine coated slides. Immunohistochemistry experiments were conducted according to previously described procedures (Talbot et al., 2012), to assess the expression of the synaptic proteins. Briefly, sections were deparaffinized, rehydrated and then incubated for 20 min in 3% $H_2O_2$ in methanol at room temperature to remove endogenous peroxidase activity. Antigen retrieval was performed, involving boiling for 10 min in 1 mM EDTA, 0.1 M Tris, pH 8.0. After two washes in TTB (0.1 M Tris Buffer with 0.01% Triton X-100 pH 7.6) sections were incubated in TTB containing 10% normal horse serum (NS) for 60 min at room temperature. The sections were then incubated overnight at 4 °C in primary antibodies diluted in TTB containing 10% NS. Primary antibodies used were a mouse monoclonal anti synapsin-IIa antibody (1:2000, cat #610667, BD Biosciences, San Jose, CA, USA), a rabbit polyclonal anti-synaptophysin antibody (1:2000, cat # 180130, Invitrogen, Waltham, MA, USA), a mouse monoclonal anti-SNAP-25 antibody (1:400, H-1 [sc-376713], Santa Cruz, Santa Cruz, CA, USA), a rabbit polyclonal anti-spinophilin antibody (1:1200, ABA5669, Millipore, Billerica, MA, USA), a mouse monoclonal anti-PSD-95 antibody (1:200, clone K28/43, UC Davis/NIH NeuroMab Facility, Davis, CA, USA), a mouse monoclonal anti-GFAP antibody (1:100,000, MAB360 Millipore, Billerica, MA, USA) and a mouse monoclonal anti-β-actin antibody (1:1000, cat # A2228 Sigma-Aldrich, St. Louis, MO, USA). Slides were then rinsed three times with TTB and incubated in secondary antibody (1:500, Vector Labs, Burlingame, CA, USA) in 10% NS for 1 h. After being rinsed two times with TTB, they were incubated for 1 h in Vectastain ABC reagent (1:500, Vector Labs) in TBS at room temperature. The slides were rinsed, and then incubated with the chromogen diaminobenzidine (DAB in 0.1 M Tris) and 0.03% $H_2O_2$ for 10 min. Sections were then rinsed, dehydrated and coverslipped with Cytoseal (Thermo-Fisher Scientific, Waltham, MA, USA). For PSD-95 quantification, only formaldehyde fixed samples (pairs 1–11) could be used. One section per subject was used in each run. For each protein including actin and GFAP, all cases were processed in a single, precisely timed run. Sections incubated without primary antibody or with an IgG of the same species than the primary antibody were run in parallel and served as negative controls. The concentration of the primary antibodies was based on manufacturers' recommendations and our titration studies.

### 2.3. Quantification and analysis

To determine the optical density (OD) of the DAB/AB reaction product in the glomeruli of the olfactory bulbs, we used customized Image pro 7.2 software (Media Cybernetics, Rockville, MD, USA) run on a desktop computer attached to a Leitz DMRB microscope (Leica, Wetzlar, Germany) and an Evolution OE camera (Media Cybernetics, Rockville, MD, USA). All the images were captured at the same scope, with the same illumination and settings for each antibody. For each subject a randomly selected slide section of the olfactory bulb was used, the entire section of the bulb was captured and all glomeruli contained in it were delineated. For fifteen subjects (6 cases, 9 controls), both left and right olfactory bulbs were sectioned and analyzed, while for eleven subjects (7 cases, 4 controls) a single bulb was sectioned and analyzed. Glomeruli were readily identified as areas of increased density of pre- and post-synaptic protein immunostaining within the glomerular layer of the bulb. The gray value for each glomerulus within each section was recorded and converted to an OD value, from which was subtracted the background OD (obtained from non-stained portion of the bulb),

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