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# Increased neuroinflammatory and arachidonic acid cascade markers, and reduced synaptic proteins, in the postmortem frontal cortex from schizophrenia patients

Jagadeesh Sridhara Rao [a,*], Hyung-Wook Kim [a], Gaylia Jean Harry [b], Stanley Isaac Rapoport [a], Edmund Arthur Reese [a]

[a] Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA
[b] Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

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ABSTRACT

Schizophrenia (SZ) is a progressive, neuropsychiatric disorder associated with cognitive impairment. A number of brain alterations have been linked to cognitive impairment, including neuroinflammation, excitotoxicity, increased arachidonic acid (AA) signaling and reduced synaptic protein. On this basis, we tested the hypothesis that SZ pathology is associated with these pathological brain changes. To do this, we examined postmortem frontal cortex from 10 SZ patients and 10 controls and measured protein and mRNA levels of cytokines, and astroglial, microglial, neuroinflammatory, excitotoxic, AA cascade, apoptotic and synaptic markers. Mean protein and mRNA levels of interleukin-1β, tumor necrosis factor-α, glial acidic fibrillary protein (GFAP), a microglial marker CD11b, and nuclear factor kappa B subunits were significantly increased in SZ compared with control brain. Protein and mRNA levels of cytosolic and secretory phospholipase $A_2$ and cyclooxygenase also were significantly elevated. N-methyl-D-aspartate receptor subunits 1 and 2B, inducible nitric oxide synthase and c-Fos were not significantly different. In addition, reduced protein and mRNA levels of brain-derived neurotrophic factor, synaptophysin and drebrin were found in SZ compared with control frontal cortex. Increased neuroinflammation and AA cascade enzyme markers with synaptic protein loss could promote disease progression and cognitive defects in SZ patients. Drugs that downregulate these changes might be considered for new therapies in SZ.

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## 1. Introduction

Schizophrenia (SZ) is a progressive neuropsychiatric illness with widespread impairment of behavior and cognitive functioning (Harvey et al., 1995; Pantelis and Brewer, 1995). The cognitive deficits are present regardless of illness stage, and are particularly on tasks related to frontal and temporal lobe functioning, including attention, processing speed, executive functioning, verbal memory, and learning (Censits et al., 1997; Townsend et al., 2001). A number of neurobiological alterations have been linked to the cognitive impairment, such as loss of white and gray matter and frontal lobe hypofunction (Weinberger et al., 1988; de Castro-Manglano et al., 2011; Price et al., 2010). Additionally, several molecular changes also have been linked, including loss of brain synaptic proteins and increased markers of neuroinflammation, excitotoxicity and arachidonic acid (AA) metabolism (Chen et al., 2011; Rao et al., 2011a, 2011b, 2012a). Although changes in these markers also have been recognized in Alzheimer's disease (AD) (Hatanpaa et al., 1999; Esposito et al., 2008), HIV-1 associated dementia (Everall et al., 1999; Aoki et al., 2005) and bipolar disorder (Aoki et al., 2005; Kim et al., 2010), it is not clear whether they are found in SZ. Additionally, there is no currently established linkage of cognitive phenotypes in SZ to genetic variations (Bilder et al., 2011).

Neuroinflammation is implicated in progressive cognitive impairment in several neurodegenerative conditions (Hatanpaa et al., 1999; Esposito et al., 2008). Studies have reported activated microglia (Steiner et al., 2008; van Berckel et al., 2008) and elevated levels of pro-inflammatory cytokines in postmortem brain tissue of SZ patients, and elevated cytokine levels in plasma (Licinio et al., 1993; Drexhage et al., 2008; Muller and Schwarz, 2008). Microglial and astrocytic activation can induce release of the pro-inflammatory cytokines (interleukin-1 beta (IL-1β)) and tumor necrosis factor alpha (TNFα) as well as activation of numerous signal transduction pathways including the AA cascade *via* a nuclear factor kappa B (NF-κB) pathway (Hernandez et al., 1999; Laflamme et al., 1999; Blais and Rivest, 2001; Moolwaney and Igwe, 2005). Chronic activation of N-methyl-D-aspartate (NMDA) receptors selectively upregulates mRNA, protein and activity of AA-selective

*Abbreviations:* AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase $A_2$; $iPLA_2$, calcium-independent $PLA_2$; COX, cyclooxygenase; LOX, lipoxygenase; DHA, docosahexaenoic acid; IL-1β, interleukin 1beta; TNFα, tumor necrosis factor alpha; $sPLA_2$, secretory phospholipase $A_2$; NMDA, N-methyl-D-aspartate; $PGE_2$, prostaglandin $E_2$; SZ, schizophrenia; iNOS, inducible nitric oxide synthetase; TXS, thromboxane synthase; PGES, prostaglandin E synthase; TX, thromboxane; NR, NMDA receptor; BDNF, brain derived neurotrophic factor; RIN, RNA integrity number; GFAP, glial fibrillary acid protein.

* Corresponding author at: 9000 Rockville Pike, Bldg. 9, 1S-126, BPMS, National Institute on Aging, NIH, Bethesda MD-20892, USA. Tel.: +1 301 594 3134; fax: +1 301 402 0074.
*E-mail address:* jrao@mail.nih.gov (J.S. Rao).

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cytosolic phospholipase $A_2$ ($cPLA_2$) in the rat brain (Rao et al., 2007). During excitotoxic insults, specific biomarkers of excitotoxicity such as inducible nitric oxide synthetase (iNOS) (Acarin et al., 2002) and c-Fos, are expressed in the brain (Rogers et al., 2005).

AA is found mainly in the stereospecifically numbered (*sn*)-2 position of membrane phospholipids, from which it can be hydrolyzed by $cPLA_2$ or secretory $sPLA_2$. The released AA can be converted into pro-inflammatory lipid mediators, such as prostaglandin (PG) $H_2$, leukotrienes, by the action of cyclooxygenase (COX), lipoxygenase (LOX) and thromboxane synthase (TXS) enzymes. Increased brain AA markers have been reported in an experimental model of neuroinflammation in the rat (Rosenberger et al., 2004; Basselin et al., 2011).

An association of AA and its pro-inflammatory metabolites with neuronal apoptosis and synapse loss has been demonstrated *in vivo* and *in vitro* (Okuda et al., 1994; Williams et al., 1998; Farooqui et al., 2001; Yagami et al., 2002; Fang et al., 2008). Further, reduced dendritic spine density and complexity have been associated with deficits in learning, memory, and general cognitive function (Masliah et al., 1997). Pre- and post-synaptic proteins such as synaptophysin and drebrin, respectively, are expressed in synaptic vesicles and at dendrites (Kojima et al., 1988; Huang et al., 1992; Terry-Lorenzo et al., 2000; Aoki et al., 2005) and changes in their levels have been used as an indicator of neuronal damage (Harigaya et al., 1996; McCarthy et al., 2006). In the SZ brain, associations between synapse loss, elevated markers of the AA cascade, neuroinflammation and synaptic protein loss have not been identified as a feature of the disease pathology. However, previous studies indicated structural, metabolic, and signaling abnormalities in the frontal cortex of SZ patients (Weinberger et al., 1988; Beasley et al., 2009; de Castro-Manglano et al., 2011; Price et al., 2010).

We hypothesized that the progression and reported cognitive impairments in SZ are associated with neuroinflammation and activation of the AA cascade. To test this hypothesis, we determined protein and mRNA levels of specific markers for neuroinflammation, excitotoxicity, AA cascade, and synapses in the postmortem frontal cortex region from SZ patients and matched control subjects.

## 2. Materials and methods

### 2.1. Postmortem brain samples

This study was approved by the Institutional Review Boards of McLean Hospital and the National Institutes Health, Office of Human Subjects Research (OHSR). Frozen postmortem human frontal cortex (Brodmann area 10), from 10 SZ patients and 10 age-matched controls, was provided by the Harvard Brain Tissue Resource Center (McLean Hospital, Belmont, MA) under PHS grant number R24MH068855 to J. S. Rao. Characteristics of the subjects with regard to age, sex, cause of death, and drug usage have been reported in detail elsewhere (Rao et al., 2012b) (Table 1).

### 2.2. Preparation of membrane and cytoplasmic extracts

From each tissue sample, 200 mg was used to prepare membrane, cytoplasmic and nuclear fractions as previously described (Rao et al., 2007). Protein concentrations were determined with Lowry's protein reagent (Bio-Rad, Hercules, CA). Membrane and cytosolic fractions were distinguished using cadherin and tubulin antibodies.

### 2.3. Western blot analysis

Proteins (50 μg) from the membrane, cytoplasmic and nuclear extracts were separated on 4–20% SDS-polyacrylamide gels (PAGE) (Bio-Rad). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane. Membranes were incubated overnight in tris-buffered-saline solution, containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies (1:200 dilution) for NMDA receptors NR-1, NR-2B, or IL-1R and cadherin (Cell Signaling, Beverly, MA). Individual cytosolic protein blots were incubated with primary antibodies (1:500 dilution) for IL-1β, TNFα, CD11b, GFAP, iNOS, $cPLA_2$-IVA, $sPLA_2$-IIA, $iPLA_2$-VIA, COX-1, COX-2, 5-, 12-, and 15-LOX, m-PGES-1 (1:200), cPGES, synaptophysin, drebrin (Abcam, Cambridge, MA) or tubulin (Cell Signaling). Nuclear blots were incubated with primary antibodies for NF-κBp50 and NF-κBp65 (1:200) (Abcam). Membrane, cytoplasmic and nuclear blots were incubated with appropriate HRP-conjugated secondary antibodies (Bio-Rad) and visualized using a chemiluminescence reaction (Amersham, Piscataway, NJ) detected by X-ray autoradiography (Biomax Chemiluminescence Film, Kodak, Rochester, NY). Optical densities of individual bands were measured using Alpha Innotech Software (Alpha Innotech, San Leandro, CA) and were normalized to β-actin (Sigma, St. Louis).

### 2.4. Immunohistochemistry

The tissue samples were cryosectioned while maintained on a dry ice bed. For immunohistochemistry, frozen sections were warmed to −20 °C from −80 °C storage conditions. Each section was gently raised to room temperature and immersion-fixed with 4% paraformaldehyde for 18 h and cryoprotected in 30% sucrose. The cryostat sections were air dried for 60 min, rinsed in 1× automation buffer (Biomedia Corp, Foster City, CA), treated with 0.3% hydrogen peroxide, and then incubated for 1 h at room temperature (RT) with a monoclonal human leukocyte antigen-D related (HLA-DR) antibody (1:750; MBL, Woburn, MA) or anti-GFAP (1:3500; Dako) for 32 min at RT as previously described (48). Images were collected using an Aperio Scanscope T2 Scanner (Aperio Technologies, Vista, CA) and viewed using an Aperio Imagescope v. 6.25.0.1117. Images were rated by two independent scorers blind to subject classification. Samples were rank-ordered based upon the level of the glial responses as identified by staining density of the cell body of astrocytes and process ramification of microglia as previously described (Funk and Kumar, 2011), then clustered based upon subject classification.

### 2.5. BDNF protein levels

BDNF protein levels were measured in brain cytosolic extracts using an ELISA kit according to the manufacturer's instructions (Chemicon International, Temecula, CA). Values are expressed in pmol/mg protein.

### 2.6. Total RNA isolation and real time RT-PCR

Total RNA was isolated from each frontal cortex sample and RT-PCR conducted as previously described (Rao et al., 2010). RNA integrity number (RIN) was measured using a Bioanalyzer (Agilent 2100 Bioanalyzer, Santa Clara, CA). RIN values were SZ 6.8 ± 0.26 and control 6.9 ± 0.18 (mean ± SEM), respectively. mRNA levels (NR-1, NR-2B, IL-1R, IL-1β, TNFα, GFAP, CD11b, $cPLA_2$-IVA, $sPLA_2$-IIA, $iPLA_2$-VIA, COX-1, COX-2, mPGES, cPGES, 5-, 12-, 15-LOX, NF-κbp50, NF-κBp65, BDNF, synaptophysin and drebrin) were measured by quantitative RT-PCR, using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Carlsbad, CA). The fold-change in gene expression was determined by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Data are expressed as the relative level of the target gene in the SZ brain normalized to the endogenous control (β-globulin) and relative to the control (calibrator). Experiments were carried out in duplicate.