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Primary cilia formation is diminished in schizophrenia and bipolar disorder: A possible marker for these psychiatric diseases

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ABSTRACT

Primary cilium (PC) is a microtubule-rich organelle that protrudes from the plasma membrane and acts as a cellular antenna sensing extracellular signals during brain development. *DISC1* (*Disrupted-in-Schizophrenia-1*) is involved in PC formation and is considered a risk factor for neuropsychiatric disorders. We have previously described altered subcellular distribution of DISC1 and an aberrant microtubule organization in olfactory neuronal precursors (ONP) obtained from schizophrenia (SCZ) and bipolar disorder (BD) patients. Herein, we analyzed *in vitro* PC formation in healthy control subjects, SCZ and BD patients. The results indicated that 66.73 ± 4.33% of ONP from control subjects showed immunostaining for the PC marker, acetylated α-tubulin. By contrast, only a small percentage of cells in culture from paranoid SCZ and BD patients showed PC staining (SCZ, 12.8 ± 4.43%; BD, 12.32 ± 5.86%). However, cells from an affected proband with disorganized SCZ and a subject with BD displayed a higher percentage of cells with cilia (SCZ, 42.20%; BD, 38.59%). Additionally, cilia elongation was observed in lithium-treated ONP derived from all groups, with a more evident response in cells from the BD group. The present study provides novel evidence that the molecular pathways involved in PC formation are defective in SCZ and BD, and impairment in these processes may be involved in the physiopathology of both diseases. Our observations also suggest that ONP is a patient-derived cell model with a potential use for diagnosis and high-throughput drug screening for brain diseases.

1. Introduction

The neurodevelopment hypothesis of the etiology of schizophrenia (SCZ) suggests that this disease originates through alterations in early brain development. These include disturbances of corticogenesis, resulting in the loss of gray matter and neuronal connectivity in the prefrontal cortex (Harrison, 1997; Selemon and Zecevic, 2015). This hypothesis has acquired new support by studies performed in olfactory mucosa cells of neuronal lineage obtained from neuropsychiatric patients by an outpatient procedure (Fan et al., 2013; Horiuchi et al., 2016; McCurdy et al., 2006; Muñoz-Estrada et al., 2015).

The human olfactory neuroepithelium has been proposed as a surrogate experimental model for the analysis of dynamic neuronal processes associated with neuropsychiatric disorders (Cascella et al., 2007; Matigian et al., 2010). This tissue is an accessible source of multipotent cells with mesenchymal characteristics that retain similar gene expression profiles as the human brain tissue (Horiuchi et al., 2013; Mor et al., 2013). Foregoing studies conducted in olfactory mucosa-derived neural cells from SCZ and BD patients have shown alterations in cell cytoskeletal components and functions, including altered ratio between free cytosolic and polymerized tubulin (Solis-Chagoyan et al., 2013), abnormal microtubule organization, as well decreased migration and cell adhesion (Fan et al., 2013; Feron et al., 1999; Muñoz-Estrada et al., 2015).

The primary cilium (PC) is a non-motile organelle composed of nine pairs of peripheral microtubules with no central pair of singlet microtubules (9 + 0), in contrast with the ciliary axoneme, which can be found in motile cilia (9 + 2) (Satir and Christensen, 2007). This organelle protrudes from the plasma membrane in almost every single cell type, including brain neurons (Fuchs and Schwark, 2004), and use the mother centriole as a cytosolic template, known as the basal body (Sorokin, 1962). Primary cilia are formed during the G0 or G1 phase of the cell cycle (Kim and Tsiokas, 2011) and its membrane is a selectively enriched center of GPCRs (Schou et al., 2015), which are carried along the axoneme by intraflagellar transport (IFT) (Kozminski et al., 1993; Rosenbaum and Witman, 2002).

Abbreviations: PC, primary cilia; ONP, olfactory neuronal precursors; SCZ, schizophrenia; BD, bipolar disorder.

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In vertebrates, PC acts as an essential center for transduction of the Sonic Hedgehog (Shh) signaling pathway and deletion of this organelle results in neural tube defects (Louvi and Grove, 2011; Murdoch and Copp, 2010). Additionally, evidence in murine models has shown that disruption of PC in cortical and hippocampal neurons is followed by dendrite outgrowth impairment, loss of synaptic integration and memory failure (Berbari et al., 2014; Guadiana et al., 2013; Kumamoto et al., 2012).

Disrupted in Schizophrenia 1 (DISC1) is a genetic risk factor for SCZ and BD, and variants of this gen have been associated with alterations in frontal and hippocampal brain regions (Chubb et al., 2008; Duff et al., 2013). Molecular and cellular biology studies have shown that DISC1 interacts with centrosome proteins and depletion of this protein in mammalian cells reduces formation of the PC significantly (Marley and von Zastrow, 2010; Porteous et al., 2011; Wang and Brandon, 2011). Furthermore, AHI1 (Abelson helper integration site 1) variants and the PCM1 (Pericentriolar Material 1) gene locus have been associated with susceptibility to SCZ and neuroanatomical abnormalities (Amann-Zalcenstein et al., 2006; Gurling et al., 2006; Ingason et al., 2010). Both, AHI1 and PCM1 are required for PC formation and knockdown analysis of these two proteins cause disorganization of the cytoplasmic microtubule network (Hsiao et al., 2009; Kim et al., 2008). Overall, these studies indicate that multiple genetic factors and proteins involved in PC formation are implicated in SCZ and BD. Despite this information, the involvement of PC in major psychiatric syndromes remains elusive.

In a previous work, we identified decreased levels of cytoplasmic DISC1, a diminished cell migration and aberrant microtubule organization at the leading edge and the nuclear cage in ONP obtained from SCZ and BD patients (Muñoz-Estrada et al., 2015). Herein we studied the time course of PC formation in ONP obtained from healthy control subjects. We also characterized this organelle in SCZ and BD patients and its response to lithium. Our results show that PC formation is significantly diminished in ONP derived from paranoid SCZ and BD patients not related to alterations in the proportion of cells recruited on quiescent stage of the cell cycle (G0/G1 phase). Nonetheless, these results and our previous findings (Muñoz-Estrada et al., 2015; Solis-Chagoyan et al., 2013) support that the deficits in ciliogenesis observed in patients' cells are related with abnormal cytoplasmic microtubule organization and altered subcellular distribution of DISC1. The data presented here using human-derived neuronal precursors support that BD and SCZ originate during the neurodevelopment and that PC may be potentially involved in the etiology of these diseases.

2. Materials and methods

2.1. Subjects

Participants were recruited from the Schizophrenia and Bipolar Disorder Clinics of the Instituto Nacional de Psiquiatría in Mexico City. Diagnoses were established independently by two general psychiatrists specially trained in the diagnosis and treatment of BD or SCZ patients, following the clinical criteria of the Diagnostic and Statistical Manual of Mental Disorders—fourth edition, revised (DSM-IV). All participants provided written informed consent prior to their involvement in the study, which was previously approved by the Institution's ethics committee and that follows the Helsinki ethical principles for medical research. Exfoliates of nasal mucosa were obtained from six patients with SCZ, five patients with BD type I, and ten from healthy control subjects with no psychiatric precedents or medication. Medical history, current medical status and absence of psychiatric disorders (Structured Clinical Interview for DSM disorders, SCID) were recorded for each control subject. Review of clinical histories found no evidence of major psychiatric or neurological illnesses.

At the time of the exfoliation procedure two of the probands with SCZ were drug naïve (n = 2) and the rest of the group was receiving atypical antipsychotics: olanzapine (n = 1), risperidone (n = 2) or amisulpride (n = 1). For the BD group, three individuals were with valproate monotherapy (n = 3) and the other two patients were taking either lithium (n = 1) or lithium with risperidone (n = 1) (for details see Table 1). All of them were free of allergies and at chronic stages of their clinical conditions. None of them were in the first psychotic episode (Table 1).

2.2. Cell culture

Olfactory Neuronal Precursors (ONP) cells obtained by exfoliation of the anterior region of the medial lateral turbinate as previously described (Benitez-King et al., 2011), were harvested in Dulbecco's modified Eagle and F-12 media (DMEM/F-12) supplemented with 10% FBS, 4 mM L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin. Cells were propagated, frozen and stored in liquid nitrogen. The experiments were carried out with cells in passages 6 to 10 cultured in Petri dishes (Nunc, Rosklide, Denmark) or in 24-well multidishes (Corning Costar, Corning, NY, USA).

2.3. Induction of primary cilia formation

ONP in culture were maintained in DMEM without FBS for 3, 6, 9 and 12 h to stimulate PC formation (Tucker et al., 1979). Then samples were processed by flow cytometry or immunofluorescence, as described below. In another experiment ONP derived from a healthy control subject, paranoid SCZ, disorganized SCZ and BD participants were cultured with either the vehicle, 50 mM NaCl (Sodium Chloride) or LiCl (Lithium Chloride) in DMEM without serum for 24 h. After the incubation time cells were processed for immunofluorescence.

2.4. Flow cytometry

FACS (Fluorescence-activated cell sorting) cell cycle was conducted as described (Darzynkiewicz et al., 2001). Briefly, cells were fixed with 70% ethanol and maintained at least for 2 h on ice. The cell suspension was centrifuged at 200 ×g 5 min and ethanol was thoroughly decanted. The pellet was rinsed two times with PBS and cells resuspended in 300 μL of the staining solution: propidium iodide (0.02 mg/mL), 0.1% Triton X-100 (v/v) and RNAse A (0.2 mg/mL) for 15 min at 37 °C. At least 10,000 cells were analyzed for each condition with a FACScalibur (Becton Dickinson) and Cell Quest ™Pro ver 5.1.1 software (BD Biosciences).

2.5. Immunofluorescence

ONP cells grown on glass coverslips (10, 000 cells/cm^2) were processed by immunofluorescence as previously described (Benitez-King et al., 2011). Briefly, cells were fixed with 3.7% formaldehyde in cytoskeletal buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2 HPO_4, $NaHCO_3$, $MgCl_2$, 2 mM EGTA, 50 mM glucose, 5 mM PIPES, pH, 6.0) and extensively washed with PBS, followed by permeabilization for 15 min with 0.1% Triton X-100. Non-specific binding was blocked with 1% BSA for 1 h at room temperature. ONP were sequentially stained with an anti-acetylated α tubulin (Sigma-Aldrich) (1:200) and either anti-PCM1 (Cell Signaling Technology) (1:400) or anti-AC3 (1:1000) antibodies, and incubated overnight at 4 °C. Secondary staining was done by incubation with a FITC and/or CY3 labeled secondary antibodies (Jackson Immunoresearch Laboratories) at 1:100 dilution for 1 h at room temperature. Nuclei were stained with 20 ng/mL DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) for 10 min. Coverslips were deposited on slides with mounting media Vectashield (Vector Labs) and examined by confocal microscopy (SP2 Leica Microsystems, Wetzlar, Germany) or with an epifluorescence Nikon microscope equipped with a Nikon OS-2Mu Digital Sight camera. Morphometric analysis of cilia was performed in 10 random chosen

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