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Increased Stability of Microtubules in Cultured Olfactory Neuroepithelial Cells from Individuals with Schizophrenia

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Abstract

Microtubules (MTs) are essential components of the cytoskeleton that play critical roles in neurodevelopment and adaptive central nervous system functioning. MTs are essential to growth cone advance and ultrastructural events integral to synaptic plasticity; these functions figure significantly into current pathophysiologic conceptualizations of schizophrenia. To date, no study has directly investigated MT dynamics in humans with schizophrenia. We therefore compared the stability of MTs in olfactory neuroepithelial (OE) cells between schizophrenia cases and matched

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nonpsychiatric comparison subjects. For this purpose, we applied nocodazole (Nz) to cultured OE cells obtained from tissue biopsies from seven living schizophrenia patients and seven matched comparison subjects; all schizophrenia cases were on antipsychotic medications. Nz allows MT depolymerization to be followed but prevents repolymerization, so that in living cells treated for varying time intervals, the MTs that are stable for a given treatment interval remain. Our readout of MT stability was the time at which fewer than 10 MTs per cell could be distinguished by anti- β -tubulin immunofluorescence. The percentage of cells with ≥ 10 intact MTs at specified intervals following Nz treatment was estimated by systematic uniform random sampling with Visiopharm software. These analyses showed that the mean percentages of OE cells with intact MTs were significantly greater for schizophrenia cases than for the matched comparison subjects at 10, 15, and 30 minutes following Nz treatment indicating increased MT stability in OE cells from schizophrenia patients ($p=.0007$ at 10 minutes; $p=.0008$ at 15 minutes; $p=.036$ at 30 minutes). In conclusion, we have demonstrated increased MT stability in nearly all cultures of OE cells from individuals with schizophrenia who received several antipsychotic treatments, versus comparison subjects matched for age and sex. While we cannot rule out a possible confounding effect of antipsychotic medications, these findings may reflect analogous neurobiological events in at least a subset of immature neurons or other cell types during gestation, or newly generated cells destined for the olfactory bulb or hippocampus, suggesting a mechanism that underlies findings of postmortem and neuroimaging investigations of schizophrenia. Future studies aimed at replicating these findings, including samples of medication-naïve subjects with schizophrenia, and reconciling the results with other studies, will be necessary. Although the observed abnormalities may suggest one of a number of putative pathophysiologic anomalies in schizophrenia, this work may ultimately have implications for an improved understanding of pathogenic processes related to this disorder.

Keywords

schizophrenia; microtubules; olfactory

1. Introduction

Microtubules (MTs) are hollow cylindrical filaments polymerized from α/β -tubulin heterodimers. As essential components of the cytoskeleton, MTs play critical roles in various neurodevelopmental processes and adaptive central nervous system functioning (Dent and Gertler, 2003; Dent et al., 2011; Hoogenraad and Akhmanova, 2010; Rodriguez et al., 2003). In developing neurons, MTs steer growth cones as they interact with actin filaments contributing to growth-cone advance and turning in filopodia, and consequently govern axon guidance (Geraldo and Gordon-Weeks, 2009).

MTs are also critical for ultrastructural changes that are integral to development of synaptic plasticity (Jaworski et al., 2009). While stable MTs do not undergo polymerization-depolymerization (Bulinski and Gundersen, 1991) and are confined to the shafts of dendrites, dynamic MTs enter dendritic spines, where they regulate levels of F-actin, maintaining the structure of individual spines and their synapses. Thus, modulation of MT dynamics can affect and remodel ultrastructural components of synapses.

During neurodevelopment, the roles of MTs figure significantly into current pathophysiologic theories of schizophrenia (Balu and Coyle, 2011; Greer and Greenberg, 2008; Rosoklija et al., 2000). Some postmortem studies have suggested deficits of neuronal migration and outgrowth of axons and dendrites, two processes that depend, in part, on MTs (Falkai et al., 2000; Balu and Coyle, 2011). The N-methyl-D-aspartate (NMDA) receptor hypofunction hypothesis of schizophrenia is supported by numerous investigations and can account for multiple symptom domains of this disorder (Greer and Greenberg, 2008; Wayman et al., 2008). NMDA receptors are located primarily on dendritic spines, whose size and shape are important determinants of the effects of activated receptors. In turn, activation of NMDA receptors increases MTs in the dendritic spines and increases their size (Merriam et al., 2011). NMDA receptor hypofunction can thus lead to altered MT dynamics as well as decreased dendritic spine densities as found in neocortex and subiculum of schizophrenia cases (Balu and Coyle, 2011; Garey et al., 1998; Glantz et al., 2000; Rosoklija et al., 2000).

Altered MT dynamics represent a potential mechanism by which a mutation in the disrupted-in-schizophrenia-1 (*DISC1*) gene has been hypothesized to contribute to schizophrenia (Kamiya et al., 2005). The product of the DISC1 gene, in which a disruption by a translocation is strongly associated with schizophrenia and other psychiatric disorders in a large Scottish pedigree, interacts with proteins that affect centrosomal and MT function, and may affect MT dynamics; these proteins include NUDEL, and the microtubule-associated proteins (MAPs), MIP-T3 and MAP1A (Morris et al., 2003). DISC1 is a component of the microtubule-associated dynein motor complex (with dynein, dynactin, NUDEL, and LIS1) and appears to be essential for maintaining the complex at the centrosome and for normal neuronal migration and formation of axons (Kamiya et al., 2005).

MAPs, which bind to MTs but not to unpolymerized tubulin, act as MT stabilizers and influence axon guidance. Among these is the stable tubule only polypeptide (STOP, or MAP6), which is proposed to have a role in regulating MT dynamics by imparting cold-stability to MTs (Andrieux et al., 2002). Interestingly, mice with a loss-of-function mutation of this gene exhibit synaptic hippocampal, neurotransmitter, and behavioral abnormalities analogous to those found in schizophrenia (Andrieux et al., 2002; Brun et al., 2005; Fradley et al., 2005). Most strikingly, the mothers fail to feed their pups, and this behavior is reversed by chronic, but not acute, treatment with antipsychotic medications (Andrieux et al., 2002; Brun et al., 2005). Davunetide (NAP), an 8-amino acid peptide derived from activity-dependent-neuroprotective protein (ADNP), interacts with MTs to promote neurite outgrowth and synaptogenesis. Interestingly, this medication diminished hyperlocomotion and improved object recognition/discrimination and spatial memory in the STOP heterozygous mouse (Merenlender-Wagner et al., 2010). Epothilone D, a MT stabilizer, was shown to reverse abnormalities in STOP-null mice, including increasing synaptic vesicle density, improving long-term potentiation, and markedly improving maternal behavior and pup survival (Andrieux et al., 2006).

In a 12-week multi-center double blind clinical trial, davunetide was related to significant improvement in the UCSD Performance Based Skills Assessment (UPSA) (Javitt et al.,

2012), which is considered to measure functionally meaningful cognition. In addition, davunetide led to a trend-level increase in the N-acetylaspartate (NAA)/creatine (Cr) ratio and a significant increase in the choline/Cr ratio in dorsolateral prefrontal cortex (DLPFC) in schizophrenia (Jarskog et al., 2013), suggesting neurotrophic or neuroprotective effects. In culture, davunetide increased the MT network area, had neurotrophic effects, and protected cells against tubulin and tau loss from assembled MT related zinc toxicity (Oz et al., 2012).

Notwithstanding the important findings in mice, studies of STOP in humans have not been consistent with this model. For example, a postmortem brain study of DLPFC showed that mRNA levels of two STOP isoforms were *increased* in schizophrenia (Shimizu et al., 2006). A proteomic analysis also revealed increased STOP levels, as well as increased MAP2 and MAP1A in the DLPFC (Martins-de Souza et al., 2009) in schizophrenia. Increased MAP2 levels have also been found in hippocampus (Cotter et al., 2000), while normal levels of MAP2 were found in cerebellum in schizophrenia cases (Mukaetova-Ladinska et al., 2002). These findings would predict increased stability of MTs in patients with schizophrenia. Notably, however, studies from other laboratories have reported decreased MAP2 expression in hippocampus and prefrontal cortex (Arnold et al., 1991; Rioux et al., 2004; Somenarain and Jones, 2010), and thus the question of MT stability in schizophrenia patients has not been resolved.

To date, no study has directly investigated MT dynamics or stability in humans with schizophrenia. Studies of the dynamics of MTs require live neurons from patients, posing daunting ethical constraints. Accordingly, we approached this problem by employing olfactory neuroepithelium (OE), which can be obtained by biopsies that are very safe and efficient. The regenerative neuroepithelial tissue contains pluripotent cells, from which *in vitro* neuroepithelial cells can be propagated with little modification of genomic or epigenomic profiles. As such, these cells are more likely to harbor *in vivo* neurobiological characteristics of donors compared to neurons derived from pluripotent stem cells induced to form neurons (Pang et al., 2011), which requires expression of appropriate transcription factors. Solís-Chagoyán et al (2013) have utilized OE to evaluate MT organization in schizophrenia cases and controls.

The application of nocodazole (Nz), a synthetic compound that prevents the polymerization of tubulin, allows for direct assessment of MT stability. For this initial study, we chose the Nz resistance assay as it would allow for measurement of MT stability both qualitatively (increase or decrease in stability) and quantitatively (a modest or more dramatic change in stability). Since Nz blocks repolymerization of tubulin, living cells can be treated for varying time intervals to assess the degree of stability of cellular MTs (Piperno et al., 1987). Here, we applied Nz to cultured cells harvested from OE of live individuals with and without schizophrenia in order to directly compare the stability of their MTs. We hypothesized that there would be increased stability of MTs in patients with schizophrenia.

2. Methods

2.1 Subjects

Individuals aged 18–55 were recruited through the Schizophrenia Research Center at the University of Pennsylvania. Consensus diagnoses of schizophrenia were determined based on interview with the Structured Clinical Interview for the *Diagnostic and Statistical Manual of Mental Disorders, 4th ed., Patient Edition* (SCID-P). Non-psychiatric comparison subjects were interviewed with the SCID, Non-Patient Edition and were determined to be free of any Axis I or Axis II cluster A (i.e. schizotypal, schizoid, or paranoid) disorder and had no first-degree relative with an Axis I psychotic disorder. Cases and comparison subjects were matched for age within 10 years and sex. Exclusion criteria for all subjects included the following: 1. Presence or history of medical and neurological disease that may affect brain function (hypertension, cardiac disease, endocrine disorders, renal disease, pulmonary disease, orthopedic circumstances interfering with prolonged testing); 2. Not proficient enough in English to be able to understand the consent and experimental procedures; 3. Mental retardation, defined as a full scale IQ less than 70; 4. Self-reported pregnancy; 5. Use of any anticoagulant; 6. Presence or history of substance related disorders by DSM-IV criteria; 7. History of significant olfactory trauma or disease that would contraindicate a biopsy of the OE in the judgment of the otorhinolaryngologist; 8. Active nasal infection, post-traumatic anosmia, or known olfactory nerve pathology.

2.2 Human OE biopsies

OE biopsies were obtained from seven individuals with schizophrenia and seven non-psychiatric comparison subjects matched as described above. OE tissues were obtained in collaboration with the Department of Otorhinolaryngology at the University of Pennsylvania. Subjects were informed of the nature and potential risks of participation in the study and provided written informed consent for participation. Work with human tissue described in this manuscript was conducted in accordance with the Declaration of Helsinki according to protocols approved by the Institutional Review Board of the University of Pennsylvania. OE biopsies were obtained as previously described (Lowry and Pribitkin, 1995). Briefly, the nasal cavity was anesthetized with tetracaine topical spray. After 15 minutes, two 1 mm³ biopsies were obtained with giraffe forceps, one from the high middle turbinate and the other from the opposed septum, and transferred to culture media for transport to the laboratory.

2.3 Culture of OE cell lines

OE biopsy tissues were washed in Hank's Balanced Salt Solution (HBSS) (Gibco), divided into 5–8 pieces and positioned on etched tissue culture plastic for development of explants in a humidified incubator (5% CO₂, 37 degrees C). Cells in complete culture medium [Dulbecco's Modified Eagle's Medium (DMEM); low glucose (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Gemcell), 2mM L-glutamine (Invitrogen) and 2000U/mL Penicillin-Streptomycin (Invitrogen)] were permitted to attain confluence. At confluence, wells were washed with Phosphate-Buffered Saline (PBS) without cations, dissociated with trypsin-EDTA (Gibco) and then diluted with an equal amount of complete media. The solution was centrifuged at 600× g and supernatant was removed. The cell pellet was gently triturated and

replated in 10 cm tissue culture plates in DMEM, 10% FBS. At confluence, cells were passaged, frozen in cell freezing medium [5% FBS and dimethyl sulfoxide (DMSO), in DMEM], and stored at -140 degrees C in a liquid nitrogen vapor phase freezer.

For experiments, cell lines at passage two were thawed and grown to confluence in 10 cm tissue culture plates, dissociated and plated in complete cell culture medium in 12 well tissue culture plates on acid-washed coverslips at a density of 40,000 cells/mL to achieve 50% confluence by 48 hours.

2.4 Nocodazole assay

Nz and Taxol were dissolved in DMSO as 10 mM stock solutions. Cells from paired cases and comparison subjects were treated in parallel. Each pair of cell lines was plated in duplicate 12-well plates as described above. When the cells were 50% confluent, culture medium was removed from all wells and coverslips were rinsed with 1 ml HBSS. Nz (10 μ M final concentration in 1mL HBSS) was added (T=0) and plates were incubated at 37 degrees C. At each point in the time course (T=10, 15, 30, 60 minutes), Nz solution was removed and coverslips were rinsed with HBSS. To remove depolymerized tubulin (whose staining by anti-tubulin antibodies would obscure the view of the remaining MTs), cells were exposed for 3 minutes to extraction buffer (0.5% Triton X-100, 0.1M PIPES, 1mM EGTA, 4% PEG 8000, 1 μ M Taxol) and then fixed in methanol at 20 degrees C for 8 minutes. The coverslips were air-dried and placed between two sheets of filter paper.

2.5 Immunohistochemistry

For this initial study, we did not seek to differentiate subpopulations of MTs since abnormalities of both neurons and glia have been reported in schizophrenia. As noted previously, abnormalities of neurons and other cell types including astrocytes, oligodendrocytes, and microglia have been implicated in schizophrenia, and all could play roles in neurodevelopmental and physiologic processes related to the disorder (Dwork et al., 2007). Hence, even if a proportion of the cells represented non-neuronal cell types, we believe this would represent an important finding. Therefore, for this initial study, we did not seek to differentiate MTs in subpopulations of cells and thus we used the anti- β tubulin antibody that recognizes all isoforms of β -tubulin. Consequently, we were able to assess MT stability regardless of the type of cell from which the MTs were derived. It is of note, however, that the majority of the OE cells express β -III-tubulin, suggesting that they do harbor molecular characteristics of neurons (Hahn et al., 2005). Some proportion of the OE cells express glial markers such as glial fibrillary acidic protein (GFAP) (Hahn et al., 2005), indicating characteristics of glial cells or the capacity to differentiate into a glial phenotype. As noted below, in future work, we shall examine the possible relationship between cell type and MT stability.

The fixed, dried samples were placed in coverslip racks and rehydrated for 24 hours in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) at room temperature. Blocking was performed for 30 minutes with 10% normal horse serum in the same buffer. Coverslips were rinsed once in TBS and then incubated for 30 minutes at room temperature in anti- β -tubulin antibody described above, diluted 1:100 in TBS, rinsed in TBS, and incubated for 30 minutes at room

temperature in fluorescently tagged anti-rabbit IgG secondary antibody (Alexa 488 F_{ab} from goat; Invitrogen), 0.02 mg/ml in TBS with 1% normal horse serum. After a final rinse in TBS, coverslips were mounted on glass slides with aqueous mounting medium (Fluoromount-G; Sigma-Aldrich) containing 0.0001% 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and sealed with acrylic after 24 hours.

2.6 Microscopy

Systematic random sampling was achieved with a fractionator probe. On an Olympus BX61 fluorescence microscope, controlled by Visiopharm software (MicroImager module), the outline of each coverslip was traced on a computer. Using a sampling grid of 1.16×1.16 mm, approximately 180 locations on each coverslip were photographed automatically through a 60X, 1.2 N.A. water-immersion objective at a linear resolution of 0.108 microns/pixel. In each location, a single 137×111 micron field was photographed, first under illumination for Alexa 468 (which was also used for auto-focusing) to visualize tubulin immunoreactivity, and then under illumination for DAPI, to display nuclei. All photographs were made automatically, and counting was performed offline, with the NewCast module. Although the goal was *not* to obtain estimates of the total OE cell population, in order to avoid subjective decisions about which cells to include, we employed a central 95×75 micron counting frame with the conventional inclusion and exclusion lines.

Each included OE cell was classified, by an observer blind to the donor's psychiatric status, as having either 10 or more visible MTs, or fewer than 10. [Some apparently solitary MTs may consist of more than one, since their diameter (~25 nm) is smaller than the optical resolution of the microscope (theoretically, 216 nm for this dye)]. The percentage of included OE cells with ≥ 10 intact MTs for each cell line at each time point of Nz exposure was calculated. Assays were run in duplicate, and the results averaged for each duplicate time point; for 14 of 140 (10%) time points only one result was available. On average, 110 ± 43 cells from each well were evaluated.

2.7 Statistical analysis

Consistently, we found that before 10 minutes post-baseline, nearly all OE cells had many intact MTs, while at 60 minutes virtually none had any (see "Results"). Therefore, in the main analyses, we compared the percentages of OE cells with intact MTs between cases and matched comparison subjects, respectively, at 10, 15, and 30 minutes. Student's independent sample t-test was used for this purpose as well as to compare the cases and controls with regard to age. Fisher's exact test was used to compare cases and controls with regard to ethnicity and smoking.

3. Results

Because of the efforts in matching subjects, age (mean, SD) of the cases (47.3, 6.3) and controls (43.1, 10.8) did not differ significantly ($p=0.40$, $t=0.87$, $DF=12$). Three of 7 (43%) cases and 5 of 7 (71%) controls were Caucasian ($p=0.59$); the remaining subjects were African-American. Five of 7 (71%) cases and 3 of 5 (60%) controls were smokers ($p=1.00$); information on smoking was missing on 2 controls (assuming both controls were

nonsmokers, $p=0.59$). All cases were treated with antipsychotics (virtually all atypical antipsychotics), most with antidepressants, and some with anxiolytics, while no controls were treated with psychotropic medications.

In nearly every matched pair, the percentages of OE cells with intact MTs at 10, 15, and 30 minutes after incubation with Nz were greater for the schizophrenia case than the nonpsychiatric comparison subject (Figure 1). The mean percentages of OE cells with intact MTs were significantly greater for schizophrenia cases than for the matched comparison subjects at each time point tested in the main analyses: 10 minutes [mean (SD) for schizophrenia cases and nonpsychiatric comparison subjects, respectively, were 60.2% (15.1) and 27.6% (11.7), $p=.0007$, $t=4.50$, $DF=12$]; 15 minutes [43.0% (15.3) and 14.2% (8.0), $p=.0008$, $t=4.42$, $DF=12$]; and 30 minutes [13.8% (10.1) and 3.7% (5.1), $p=.036$, $t=2.36$, $DF=12$]. As expected, there were no group differences between schizophrenia cases and nonpsychiatric comparison subjects at baseline [87.3% (12.5) and 85.7% (8.1), $p=0.78$, $t=0.29$, $DF=12$], while at 60 minutes virtually no OE cells had intact MTs in either group [0.99% (2.2) and 0.15% (0.25), $p=0.35$, $t=1.02$, $DF=12$]. Figure 2 depicts representative cultured olfactory neuroepithelial (OE) cells from one matched pair, fixed before adding nocodazole, and at 10, 15, and 30 minutes after adding nocodazole, demonstrating progressive depolymerization of MTs in the presence of nocodazole.

4. Discussion

These findings provide evidence that MT stability is increased in olfactory neuroepithelial (OE) cells of adult patients with schizophrenia. Specifically, we observed a significant decrease in depolymerization of MTs during a time course of nocodazole treatment in cultured OE cells derived from individuals with schizophrenia, compared with cells derived from nonpsychiatric comparison subjects.

These findings must be reconciled with those of a recent publication (Solís-Chagoyán et al., 2013). In that study, olfactory neuroepithelial cells from patients with schizophrenia exhibited wide intracellular areas without MTs by anti β -III tubulin staining. The ratio of unstained areas to total cell volume in the cells from schizophrenia cases was, on average, 5 times greater than in cells from healthy individuals. However, no differences in the amount of total β -III tubulin or either the amount of globular and polymerized tubulin were found in cells from cases as compared to comparison subjects. These findings are indicative of altered MT organization in olfactory neuronal precursors in schizophrenia patients. The study benefited from novel methods to expand and propagate neuronal precursors obtained from the OE (Liu et al., 1998; Benítez-King et al., 2011). Although the two studies may have different apparent implications, they are not necessarily contradictory, since our study assayed the rate of disassembly of MTs, while the study of Solís-Chagoyán et al (2013) measured MTs at steady state.

It will also be important to determine whether greater MT stability is associated with the usual post-translational modifications of tubulin, which would suggest increased MT stability under physiological conditions. Consequently, we plan to conduct MT staining with an antibody that recognizes N-Lys-40-acetylated tubulin, which predominates in stable MTs

(Piperno et al., 1987), in these schizophrenia cases and comparison subjects as well as evaluate whether differences in expression of MAPs, which also alter MT stability, account for the observed differences. Furthermore, in order to attempt to replicate the findings of Solís-Chagoyán et al (2013), we intend to conduct a more extensive morphometric analysis, including MT length, thickness, and number of MTs per cell. Moreover, we shall address specificity for cell type, notably whether the cells with more stable MTs represent a neuronal lineage in these subjects, by staining with β -III tubulin and other markers.

Our findings may appear to be surprising when compared to studies of STOP-deficient mice (Andrieux et al 2002), since these mice demonstrated schizophrenia-related phenotypes that were associated with diminished cold-stability of MTs. However, STOP mRNA (Shimizu et al., 2006) and protein (Martins-de Souza et al., 2009) are greater in postmortem brains from patients with schizophrenia. Second, in our view, inherent in any animal model is the question of the fidelity with which it represents human disease. Generally, we would not necessarily expect that an animal model of schizophrenia will represent all brain and behavioral phenotypes found in the disorder; likewise, patients with schizophrenia are not anticipated to exhibit all of the neurobiological manifestations found in the animal model. Finally, we did not assay specifically for cold-stability of MTs.

While these and other studies are needed clarify our findings, we offer a potential explanation for a relationship between an increase in MT stability and functional consequences relevant to schizophrenia. For this purpose, we underscore the role of growth cone guidance and neuronal migration, given evidence of MT effects on these processes and their disruption in schizophrenia (noted in Introduction and below). The growth cone is a highly dynamic, sensory-motile structure at the tip of growing axons (Geraldo and Gordon-Weeks, 2009). MTs have the capacity to explore the periphery of growth cones and MT orientation often predicts the direction of outgrowth (Sabry et al., 1991; Tanaka and Kirschner, 1995; Buck and Zheng, 2002).

One potential mechanism by which excess MT stability may contribute to neurodevelopmental abnormalities in schizophrenia involves plus-end-tracking proteins (+TIPs), some of which bind preferentially to rapidly growing plus ends of MTs (Dent et al., 2011; Jaworski et al., 2008) in a process known as “capping.” +TIPs are concentrated on MTs extending into the distal axonal growth cone regions, where the plus ends of dynamic MTs abound; this places +TIPs in close proximity to guidance cue receptors (Dent et al., 2011). +TIPs have been shown to dissociate from MTs slightly stabilized by the administration of low concentrations (200 nM) of taxol (Perez et al., 1999). This suggests that other factors that cause small or modest increases in stability of MTs may have analogous effects. One imputed function of leading edge capping of MTs is to provide directional information for motile cells (Gundersen and Bulinski, 1988). Thus, the dissociation of +TIPs from MTs and the resulting ablation of +TIP-dependent MT capping functions would render the cells less responsive to guidance cue receptors and disrupt the direction of cell migration. While this explanation is tentative, and requires confirmation in independent analyses, the study of post-translational modifications and MAPs, and their relationship with neurodevelopmental events relevant to schizophrenia, would begin to test its likelihood. It is possible that the stability changes measured here would have subtle

effects on post-translational modifications and binding of MAPs, which could be studied in future experiments.

In summary, we have shown here that cultured OE cells from individuals with schizophrenia exhibit increased MT stability compared to matched comparison subjects. If our findings in OE cells reflect similar neurobiological events among immature neurons or other cell types during fetal development, or perhaps among newly generated cells destined for the olfactory bulb or hippocampal dentate gyrus, this would suggest a mechanism to explain observations in schizophrenia, from autopsy studies or *in vivo* imaging, of altered numbers of neurons in white matter (Akbarian et al., 1996; Anderson et al., 1996; Eastwood and Harrison 2003; Rioux et al., 2003; Eastwood and Harrison 2005; Connor et al., 2011; Fung et al., 2011; Yang et al., 2011; Joshi et al., 2012; Beasley et al., 2002), altered organization of white matter tracts (Buchsbaum et al., 2006; Whitford et al., 2011; Zalesky et al., 2011; Marengo et al., 2012; Wang et al., 2012), deficits in dendritic spines in the subiculum (Rosoklija et al., 2000) and the prefrontal cortex (Garey et al., 1998; Glantz and Lewis 2000; Kalus et al., 2000), and deficits in interneurons (Guidotti et al., 2000; Volk et al., 2000; Straub et al., 2007; Fung et al., 2010; Curley et al., 2011; Lewis et al., 2012).

A potential limitation is the possibility that our results were confounded, at least in part, by antipsychotic and other medications. In previous studies, MT organization changes occurred in the presence of antipsychotics including haloperidol and trifluoperazine (Benítez-King et al., 2004). Among other findings, haloperidol and clozapine in therapeutic dose equivalents caused increased neuritogenesis evidenced by enlarged MTs in neurites, while cells incubated with high concentrations of these medications showed cytoskeletal collapse. Moreover, trifluoperazine at therapeutic concentrations prevented MT depolymerization caused by calcium-activated calmodulin.

However, OE culture cells that were born and propagated *in vitro* may not harbor immediate effects of drugs as the cells in the OE biopsy tissues. We note that OE cells examined for this study have gone through more than 15 cycles of divisions following their removal during biopsy. If we assume that the intracellular content of pharmacologic agents retained in the cytoplasm is diluted by 50% in each division, the intracellular concentration of drugs would be decreased by 2000-fold or more. Moreover, any drugs remaining in the cytoplasm could be metabolized or could leave the cells, just as they must have crossed the plasma membrane to enter the cell. Nonetheless, it is possible that the initial exposure of the OE to medications could exert effects, such as modulation of their intracellular proteins and responses, that outlast the causative agents. We further note that the case and comparison groups in this study were similar with regard to age, sex, ethnicity, and smoking status. For further assurance, however, we shall attempt to replicate these findings in medication-naïve patients with schizophrenia in future studies.

In addition, we do not know whether or not the observed alterations in cultured OE cells reflect changes in the entire brain. For example, in a previous study the correlation between ADNP and ADNP2 (an ADNP homologue which shares 33% identity and 46% similarity with ADNP) in the same schizophrenia patients was greater in postmortem frontal cortex specimens than in the hippocampus (Dresner et al., 2011). The authors suggested that this

may reflect differences in the relative expression of these proteins in the two brain regions in schizophrenia. Finally, we wish to comment on comparison subject 4, who had high levels of MTs at 30 minutes post-Nz administration compared to the matched case. We postulate that this comparison subject, while free of psychiatric disease, may have had other neurobiological characteristics that could have compensated for the high level of MTs, thereby protecting or providing resilience against schizophrenia, which is believed to be a multifactorial disorder (van Os et al., 2008).

The findings could be further reinforced by an experimental animal model of schizophrenia in which the stability of MTs is increased, for example, by treating animals with low concentrations of a microtubule-stabilizing drug such as Taxol. Such a model could investigate whether elevated MT stability disrupts phenotypic outcomes that have been suggested in schizophrenia, including neurotransmitter dysfunction (e.g. hyperdopaminergia in the limbic system, diminished NMDA receptor function), neurophysiologic alterations (disturbances of sensory gating), and behavioral anomalies including social withdrawal (Keshavan et al., 2008; Tandon et al., 2009).

5. Conclusion

We have demonstrated increased MT stability in nearly all cultures of OE cells from individuals with schizophrenia who received several antipsychotic treatments, versus comparison subjects matched on age and sex. While we cannot rule out a possible confounding effect of antipsychotic medications, these findings, in OE cells, may reflect analogous neurobiological events among at least a subset of immature neurons and other cell types during gestation, or perhaps among newly generated cells destined for the olfactory bulb or hippocampus, suggesting a mechanism that underlies findings from autopsy studies or *in vivo* imaging investigations of schizophrenia (Akbarian et al., 1996; Eastwood and Harrison 2005; Joshi et al., 2012). Future studies aimed at replicating these findings, including samples of medication-naïve subjects with schizophrenia, and reconciling the results with other studies, will be necessary. Previous evidence indicates that schizophrenia is most likely a multifactorial disorder (van Os et al., 2008), and thus the observed abnormalities may suggest one of a number of putative pathophysiologic anomalies; nonetheless, this work may ultimately have implications for an improved understanding of pathogenic processes that give rise to this disorder.

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Abbreviations

MTs	microtubules
OE	olfactory neuroepithelium
Nz	nocodazole
NMDA	N-methyl-D-aspartate

MAPs	microtubule associated proteins
DISC1	disrupted-in-schizophrenia-1
DLPFC	dorsolateral prefrontal cortex
NUDEL	nuclear distribution protein nude-like 1
NAA	N-acetylaspartate
Cr	creatine
Nz	nocodazole
SCID-P	Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, 4 th ed., Patient Edition
HBSS	Hank's Balanced Salt Solution
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
EDTA	ethylenediaminetetraacetic acid
PBS	Phosphate-Buffered Saline
DMSO	Dimethyl Sulfoxide
DAPI	4',6-diamidino-2-phenylindole
GFAP	glial fibrillary acidic protein
+TIPs	plus-end-tracking-proteins
DF	Degrees of freedom
ADNP	activity-dependent neuroprotective protein (ADNP)

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Figure 1.

Comparison of percentages of olfactory neuroepithelial (OE) cells with intact microtubules^a between schizophrenia cases and matched comparison subjects at 10, 15, and 30 minutes following administration of nocodazole. Note that scale is expanded in the 30-minute graph.

^a Defined as a cell containing 10 or more visibly preserved microtubules (see sec. 2.6)

Figure 2.

Representative cultured olfactory neuroepithelial (OE) cells, from one matched pair, fixed before adding nocodazole (A, B), or 10 (C, D), 15 (E, F), or 30 (G, H) minutes after adding nocodazole, demonstrating progressive depolymerization of microtubules in the presence of nocodazole. It is apparent that not all microtubules depolymerize at the same rate, so to obtain the data presented in Figure 1, we used systematic uniform random sampling to estimate the fraction of cells that had not yet reached the end point of depolymerization, which we chose to be fewer than 10 distinguishable microtubules within the cell. Each image here is approximately the size of one counting field. Stained, as described in text, with anti- β -tubulin (green) and DAPI (blue). A, C, E, G culture from nonpsychiatric comparison subject. B, D, F, H matched schizophrenia subject. Scale = 50 microns.