Altered G Protein Coupling in Olfactory Neuroepithelial Cells From Patients With Schizophrenia

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Increasing evidence suggests that olfactory dysfunction is an endophenotype of schizophrenia, and thus the olfactory system can be studied both in relation to this sensory dysfunction and also as a means of examining pathophysiological mechanisms of schizophrenia. In this study, we examined human olfactory neuroepithelial (ON) biopsy tissues and their in vitro culture cells for ligand-induced guanine nucleotide-binding protein (G protein) activation and downstream signaling. We assessed the binding of a nonhydrolyzable GTP analogue [α35S]GTPγS binding to specific G protein subtypes in response to odorants, dopamine, or serotonin in ON cell membranes from matched schizophrenia-control subjects. In response to odorant mixtures, we found decreased [α35S]GTPγS binding to Gαs/olf in schizophrenia patients. These changes were not mediated by mRNA expression of key molecules of G protein coupling, including adenylate cyclase III (ACIII), protein kinase A (PKA), protein kinase Cγ (PKCγ), or Gαs or Gαolf in ON cells or ON biopsy tissues. In contrast, dopamine (DA)- and serotonin (5HT)-induced S35-GTPγS binding to Gαs/olf and Gαq/11 were significantly increased in schizophrenia cases, while these parameters were strikingly reduced by in vitro treatment with antipsychotics. Patients with schizophrenia exhibit increases in electrophysiological (EOG) recordings, suggesting enhanced odorant-induced activation. Our results of decreased odorant-induced G protein activation may point further downstream for underlying mechanisms for increased EOG measures. Increased G protein activation in response to DA and 5HT may suggest increased postreceptor DA or 5HT signaling as an additional mechanism of dopaminergic or serotonergic dysregulation in schizophrenia.

Key words: schizophrenia/olfactory/G protein/dopamine/serotonin

Introduction

G protein–coupled receptors (GPCRs) include multiple neurotransmitter/neuropeptide systems implicated in the pathophysiology of schizophrenia. Dopamine receptor type 2 (D2R) is a molecular target for virtually all antipsychotics1 and recent neuroimaging evidence indeed demonstrates evidence for alterations in DA signaling.2 Serotonin receptor type 2 (5HT2R) also plays an important role in therapeutics of psychosis as well as in various physiological alterations associated with schizophrenia.3 In addition, schizophrenia patients exhibit many neuropsychological dysregulations, such as auditory, visual, and olfactory function, for which GPCRs may play an important role. Nevertheless, study of G protein signaling in neural cells of patients with schizophrenia has been limited.

The olfactory neuroepithelial (ON) biopsy approach offers a unique opportunity to investigate neural cells derived from patients without genetic reprogramming.4 When propagated in vitro, these cells express D2R and 5HT2 receptors as well as odorant receptors5 and permit us to investigate receptor-specific activation of G protein signaling in neural cells of patients. Moreover, ON cells are derived from the olfactory system, and thus molecular signaling in these cells can also be interpreted in the context of circuit function of the olfactory system. In this study, we examined GPCR signaling in response to odorants in the context of olfactory dysfunction observed in schizophrenia patients and in response to DA or 5HT.

Increasing evidence suggests that olfactory dysfunction is an endophenotype of schizophrenia6–12 and as such, biological underpinnings for olfactory dysfunction may offer clues to pathophysiological mechanisms affecting other brain regions.4 Olfactory function reflects the activity...
of the circuitry connecting olfactory receptor neurons (ORNs), the olfactory bulb, and the primary olfactory cortex. Several research methodologies permit investigating each segment of the circuitry: ORNs are accessible via ON biopsy,\(^5\)\(^{13,14}\) and circuit activity can be measured via electrotactogram (EOG)\(^{15}\) and olfactory evoked potentials (EP).\(^{16}\) Indeed, patients with schizophrenia have demonstrated alterations in EP and EOG recordings\(^ {16,17}\) as well as in neuronal differentiation in the ON.\(^ {18}\)

Schizophrenia patients exhibit decreases in EP recordings, consistent with olfactory dysfunction as observed in psychophysical assessment.\(^ {16}\) However, EOG recordings, which represent action potentials of ORNs in the ON, are increased in schizophrenia patients compared to healthy subjects,\(^ {15}\) suggesting increases in odorant-induced activation of ORNs. Notably, individuals at high risk for schizophrenia also showed decreased amplitude of event-related potentials\(^ {19}\) and similar changes in EOG recordings to patients with schizophrenia (data not shown), suggesting that this dysregulation is integral to olfactory dysfunction in schizophrenia.

Odorant signaling is initiated by binding of odorants to odorant receptors that activate GoGα, which in turn triggers downstream signaling.\(^ {20}\) Alterations in odorant signaling might, therefore, be determined at the level of odorant receptors and/or G protein coupling and its downstream signaling.

Dopamine and 5HT receptor signaling, whose functions are also mediated by G protein–coupled receptors (GPCRs), have been implicated in the pathophysiology of schizophrenia.\(^ {21,22}\) In addition, metabotropic glutamate receptors, also signaled by G protein coupling, have been reported to be altered in postmortem studies of schizophrenia\(^ {23}\) and have been investigated as a possible therapeutic target.\(^ {23,24}\) More recently, molecular imaging studies with PET or SPECT examined 18F/11C-L-DOPA uptake and found significant increases in schizophrenia, suggesting presynaptic dysregulation of DA signaling in schizophrenia patients and individuals at risk for schizophrenia.\(^ {25-27}\) At the postsynaptic receptor level, multiple groups have examined the expression levels of receptors and binding affinities for DA and 5HT and reported inconsistent changes in postmortem brains of schizophrenia patients.\(^ {28}\) Beyond the receptor level, however, GPCR signaling has not been extensively studied.

The goal of this study was to examine G protein activation in ON cells as a mechanism for increased EOG measurements, which may reflect activation of odorant-induced and G protein activation–mediated enhancement of intracellular calcium influx. We hypothesized that the observed alterations in odorant signaling are determined downstream to receptor activation, ie, G protein activation, activation of effectors, and further downstream. Indeed, recent behavioral threshold sensitivity data in schizophrenia patients is consistent with this hypothesis.\(^ {29}\)

ON cells, derived from olfactory biopsies from living subjects with schizophrenia, have previously been examined for alterations in cellular and molecular characteristics.\(^ {14,30}\) In this study, we examined dysregulations of GPCR signaling in ON cells from patients with schizophrenia.

### Methods

**Human Olfactory Neuroepithelial Biopsies**

Olfactory epithelial biopsies were obtained from 27 individuals with schizophrenia and 27 nonpsychiatric controls (see table 1 for subgroups of subjects included in each assay) who were recruited and assessed in the Schizophrenia Research Center at the University of Pennsylvania. Olfactory 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 (supplemental methods).

Olfactory biopsies were obtained as previously described.\(^ {31}\) Briefly, the nasal cavity was anesthetized with pontocaine spray. After 15 minutes, two 1-mm\(^3\) 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.

**Culture of Olfactory Neuroepithelial Cell Lines**

ON cells were prepared as previously described.\(^ {5}\) At confluence, cells were passaged and frozen in cell freezing medium (5% FBS in DMSO) and stored at −140°C in liquid nitrogen vapor phase freezer for subsequent studies (supplemental methods).

**Odorant, Dopamine, and 5HT Receptor–Mediated G Protein Activation**

As a measure of the functionality of various G protein–coupled receptors, we examined G protein activation in ON culture cells derived from olfactory biopsies of patients with schizophrenia and age- and sex-matched control using 2 odorant mixes: Mix A (citral, hedione, geraniol, phenethylalcohol, citronella, eugenol, menthone) (17 schizophrenia-control pairs) and Mix B (lyral, lilial, triethylamine, ethyl vanillin, isovaleric acid, phenylethylamine) (10 schizophrenia-control pairs). Immunoprecipitation was performed as previously described.\(^ {32}\) Cell membranes were incubated at 4°C with anti-Gαs/olf, -Gαi, -Gαo, or -Gαq/11 followed by 1 hour incubation with 25 μL protein A–conjugated agarose. The levels of immunoprecipitated \(^ {[35]}\)GTPγS-bound Gα proteins were measured by scintillation spectrometry (supplemental methods).
ON cell lines from 13 schizophrenia-control pairs were grown to 90% confluence, homogenates were extracted in RIPA buffer and processed through 2 freeze-thaw cycles. Twenty microgram of protein extracts were loaded onto 7.5% Tris-glycine gels (Biorad), run under denaturing conditions at 200 V and transferred to PVDF membrane (Millipore). Membranes were blocked for 1 hour at RT in 5% milk/TBS with 0.05% Tween-20 (TBST) and probed overnight at 4°C with antibodies against Gαs/olf (1:100; sc-55545, Santa Cruz), ACIII (1:500; sc-588, Santa Cruz), PKCγ (1:500; sc-211, Santa Cruz), PKA (1:1000; C-α #4782, Cell Signaling), and β-actin (1:10000; A2228, Sigma Aldrich). Blots were washed 5×10 minutes in TBST, incubated in HRP-conjugated secondary antibody for 1 hour at RT, (1:5000 anti-rabbit or anti-mouse, Jackson Immunoresearch Laboratories), washed again 5×10 minutes in TBST and developed using ECL or ECL plus (GE Healthcare).

Quantitative Real Time PCR From ON Cells
Total RNA was extracted from the ON cells of 15 schizophrenia-control pairs using Trizol (Life Technologies) and
RNeasy mini kit (Qiagen) following the manufacturer’s protocol. RNA was further purified by DNase I digestion (DNase I, 30U/100mg total RNA, Qiagen). Total RNAs were reverse transcribed using High Capacity cDNA Reverse Transcriptase kit (Life Technologies). Twenty-five nanograms of total RNA equivalent cDNA from each sample was used for quantitative real-time PCR (qPCR) in duplicate wells using 2× SYBR Green PCR master mix (Life Technologies) using gene specific primers for CNGA2, PRACA, PRKCG, GNAS, GNAL (supplementary methods) using ABI 7300 Sequence Detection System. Data from each qPCR run were analyzed using ABI Prism Software version 1.2.3.

Results

Odorant-Induced G protein Activation Is Decreased in Patients With Schizophrenia

ON culture cells express odorant receptors and these receptors can be activated by odorants in vitro. Binding of odorants to their receptors activates a subset of G proteins, which can then be monitored in ON and measured by the binding of a nonhydrolyzable GTP analogue, GTPγS into specific Gα proteins. To establish this experimental paradigm in human ON cells, we isolated membrane fractions from the ON cells of 3 healthy control subjects, which were then incubated with varying concentrations of Mix A (citralva, hedione, geraniol, phenethylalcohol, citronella, eugenol, menthone) and Mix B (lyral, lilial, triethylamine, ethyl vanillin, isovaleric acid, phenylethylamine) in the presence of [35S]GTPγS. Odorant-stimulated samples were then immunoprecipitated with antibodies for specific Gα classes, Gαs/Gαolf, Gαi, Gαo, Gαq/11, Gα12, and Gα13. Mix A increased [35S]GTPγS binding to Gαs/Gαolf, Gαi, Gαo, and Gα13 by 142%–192% (Gαs/Gαolf: \( P < .00001 \); Gαi: \( P < .00001 \); Gαo: \( P < .00001 \); Gαq/11: \( P = .05903 \); Gα13: \( P < .00001 \)), whereas Mix B increased [35S]GTPγS binding to Gαs/Gαolf, Gαo, and Gα13 by 75.1%–148.2% (Gαs/Gαolf: \( P < .00001 \); Gαi: \( P = .067564 \); Gαo: \( P < .00001 \); Gαq/11: \( P = .31904 \); Gα12: \( P = .10527 \); Gα13: \( P < .00142 \)).

We then examined ON cells derived from a subset of patients with schizophrenia and their age- and sex-matched controls (table 2). Mix A-induced incorporation of [35S]GTPγS was decreased for G protein subunits, Gαs/Gαolf (student’s t test; 2 tailed \( t(16) = 2.38, \ P = .03 \)) and Gαo (\( t(16) = 2.56, \ P = .02 \)) and Gα13 (\( t(16) = 2.34, \ P = .03 \)) in the schizophrenia group (17 matched subject pairs) (Figure 1A). A subgroup of the same cohort (10 matched subject pairs) was also examined for G protein activation in response to odorant Mix B (figure 1B). Interestingly, we observed similar decreases in Mix B-induced [35S]GTPγS binding to Gαs/Gαolf (\( t(8) = 2.82, \ P = .02 \)) as well as Gαo (\( t(8) = 2.69, \ P = .03 \)).

Table 2. Assay Subgroups

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X denotes the use of the specific matched pair in the assay indicated in the column headings.
Expression Levels of Odorant Signaling Proteins Are Altered in ON Cells From Schizophrenia Patients

To assess whether the expression levels of signaling proteins in the odorant signaling cascade are altered in ON cells from schizophrenia patients, we examined expression of ACIII, PKA, PKCγ, and Gαs/olf, in ON cell lysates by western blot in a subgroup of 13 matched subject pairs (table 2). Gαs/olf is the main Gα subunit coupled with odorant receptors and Gαs/olf in turn activates ACIII, the effector of odorant signaling, producing cAMP (figure 2A). We found a significant decrease in ACIII (t(13) = 3.121, P = .009), which can lead to decreased cAMP formation (representative western blot figure 2B). No between-group differences were seen in PKA or PKCγ. We next examined mRNAs for key signaling molecules in ON cells.
mRNAs of Odorant Signaling Molecules Are Unaltered in Ex Vivo ON Cells

In vitro culture cells were derived from biopsy tissues from 10 schizophrenia patients and 10 age- and sex-matched control subjects. We assessed RNA expression of the transcripts for 5 genes which play critical roles in odorant signaling: cAMP-dependent protein kinase A (PRKACB), protein kinase Cγ (PRKCG) adenylate cyclase III (ADCY3), Guanine Nucleotide-Binding Protein (G protein), Alpha Activating Activity Polypeptide, Olfactory Type (GNAS) and Guanine Nucleotide-Binding Protein (G protein), Alpha Activating Activity Polypeptide and Olfactory Type (GNAL) or β-tubulin III (TUBB) in ON cells derived from each of the subjects. There were no between-group differences for PRKACB, ADCY3, GNAS, or GNAL transcripts between schizophrenia patients and their matched controls (figure 3).

Increased DA- and 5HT-Induced G Protein Activation in ON of Schizophrenia Patients Is Attenuated by Antipsychotics

G protein signaling transduces various receptor systems, sharing some of molecular components in common. To test whether dysregulations in G protein signaling observed in response to odorants are shared by other receptor systems, we examined DA- and 5HT-mediated G protein activation, which are of particular interest in both the pathophysiology and treatment of schizophrenia.

To that end, we incubated ON membranes derived from the same 10 matched control-schizophrenia pairs (table 2) with DA or 5HT for 5 minutes in the presence of [35S]GTPγS and assessed the level of [35S]GTPγS incorporation into Ga subunits as described previously.32 DA (1 μM) induces [35S]GTPγS binding to Gas/olf, Goi, and modestly to Gaq/11. DA-induced activation of both Gas and Gai was significantly increased in ON cells of schizophrenia patients. Similarly, 5HT increases [35S]GTPγS binding to Gas, Goi, and Gaq/11. 5HT-induced Gas, Goi, and Gaq/11 activation was heightened in ON cells of schizophrenia patients (figure 4A).

Given that antipsychotics block these receptors, we tested the effects of antipsychotics on 5HT-induced G protein activation in ON culture cells. To assess the acute effects of antipsychotics, ON cells were incubated with 10 μM haloperidol or clozapine for 30 minutes and cell membranes were incubated with [35S]GTPγS and 5HT. As predicted, activation of Gas and Gaq11 was strikingly suppressed by both antipsychotic agents. To evaluate the effects of chronic treatment, ON cells were treated with haloperidol or clozapine for 5 days and G protein activation was then measured by [35S]GTPγS incorporation induced by 1 μM 5HT. Similar to the acute effects of haloperidol and clozapine, chronic exposure to either antipsychotic decreased 5HT-induced Gas/olf and Gaq11 activation but increased basal [35S]GTPγS binding to Gas/olf and Gaq/11 (figure 4B).

Discussion

The goal of this study was to investigate G protein coupling as a locus of dysregulation in GPCR signaling in neural cells of patients with schizophrenia. Data presented here demonstrate ligand-specific dysregulations in G protein coupling in ON cells from schizophrenia patients: notably decreases in response to odorants but increases to DA and 5HT.

Increased EOG findings in schizophrenia patients predict greater neuronal excitation resulting from enhanced odorant signaling. Decreased G protein activation as we observed in schizophrenia patients, however, would reduce cAMP formation leading to decreased excitability of ORNs. In addition, we found a decrease in ACIII expression in patients, which can further lower cAMP formation. Our results together indicate that the activity of the proximal segment of the odorant signaling, ie, odorant receptors, G proteins, and ACIII, is lowered. Thus, the alterations in this segment may not be the primary cause for EOG abnormalities in schizophrenia.

It follows that increased EOG measures may result from enhanced activity of the segment of odorant signaling pathway that is downstream to G protein and ACIII. In this case, one explanation for decreased G protein coupling as observed in ON cells of patients could be that it is a compensatory mechanism. Downstream to the activation of Gas/olf are activation of CNG channels and voltage-dependent [Ca2+] and [Na+] channels, which regulate neuronal activity of ORNs. Given that voltage sensitive [Ca2+] and [Na+] channels have been implicated for the pathophysiology of schizophrenia, it will also be important to examine these channels in ON cells and in postmortem brains of schizophrenia patients.

Fig. 3. mRNAs of odorant signaling molecules are unaltered in cultured ON cells. mRNA expression was examined in ON culture cells derived from olfactory biopsies from 10 age/sex-matched schizophrenia and control pairs. Amplified transcripts from OE cells were examined for ADCY3, PRKACB, GNAS, and GNAL. Data represented as means ± SEM (bars). Statistical significance was assessed paired student’s t test, 2 tailed, P < .05.
It would be interesting to examine the extent to which the G protein dysregulation observed in ON cells might be reflected in other sensory modalities in patients. If G protein dysregulation is inherent to the illness, it is possible that similar changes could be found in other sensory modalities, such as in gustatory function. If, on the other hand, it is a compensatory response to the changes in the further downstream segment of the pathway, it is less likely to be observed in other sensory modalities.

Our results do not elucidate the molecular underpinnings for decreased odorant-induced G protein coupling in schizophrenia. However, there are several possibilities to be considered. Reduced odorant-induced G protein activation may reflect changes in the quantity of odorant receptors, odorant binding affinity, G protein expression, and/or receptor-G protein coupling efficiency. While it is possible that our findings could be due to reduced expression of odorant receptors it is of note that, humans have more than 300 different odorant receptors, each of which responds to specific odorants or combinations of odorants.\textsuperscript{33,34} If increased EOG measures are caused by altered expression of odorant receptors, schizophrenia should be associated with altered expression of hundreds of odorant receptor genes, which is not a likely scenario.

Decreased G protein activity could be due to altered expression of key molecules in the G protein signaling pathway. Alternatively, the altered G protein activation may be a result of differential levels of heteromeric GPCR complexes that are known to exhibit different signaling properties\textsuperscript{35} although currently there is no concrete evidence to support the existence of heteromeric GPCR complexes in olfactory neuroepithelium. RNA quantification in ON cells, however, fails to show any dysregulation in PRKACB, PRKCG, ADCY3, GNAS, or GNAL, demonstrating that the observed dysregulation in G protein coupling is not caused by alterations at the mRNA level. Neither did we find discernible changes in Gα expression levels in ON cells derived from schizophrenia patients. Interestingly, decreased Gαs/olf was found in a cohort of individuals who are at clinically high risk for schizophrenia (Borgmann-Winter et al, under review). Thus, it will be important to further investigate Gαs/olf protein expression levels in a larger sample size and to delineate progressive changes in G protein expression in the clinical risk period for onset of schizophrenia. DA and 5HT receptors are of particular interest as they are critical for the pathophysiology and therapeutics of schizophrenia. In contrast to odorant-induced signaling, DA- and 5HT-induced G protein activation were increased, indicating that G protein signaling dysregulation in schizophrenia is receptor specific.

Schizophrenia patients exhibit increased DA-induced Gαs/olf and Gαq but not Gαq11 activation suggesting enhancement of DR1-, D2R-, and D1R-like signaling. The dopamine hypothesis of schizophrenia has been extensively investigated by molecular brain imaging of living subjects.\textsuperscript{2} Meta-analyses have shown that DOPA uptake was overall increased, indicating a presynaptic dysregulation in the DA system.\textsuperscript{36–38} At the postsynaptic level, however, evidence has been inconsistent for the levels of D2R and D3R in postmortem or molecular imaging studies.\textsuperscript{38} Interestingly, postmortem studies of patients with schizophrenia have demonstrated a number of alterations in expression levels of dopamine pathway-mediated proteins and mRNA, such as G protein–coupled receptor kinase GRK,\textsuperscript{39} and the transcript for calcyon which mediates DA and Gq11 crosstalk in the cortex and thalamic nuclei of patients with schizophrenia.\textsuperscript{40,41} Our findings indicating heightened G protein coupling in ON cells suggest postsynaptic DA receptor...
function as a potential mechanism via which DA function is increased. It will be important to further examine such changes in postsynaptic DA function in postmortem brains of schizophrenia patients.

We considered whether altered G protein signaling could be mediated by antipsychotics. This scenario seems unlikely since in vitro antipsychotic treatment (acute or chronic) produced opposite effects on G protein coupling to those observed in patients. Furthermore, no significant correlations between antipsychotic use at the time of biopsy and the alterations in odorant-stimulated G protein coupling observed in patients were found (supplementary data). Smoking is another factor that could affect G protein coupling. Interestingly, subjects with schizophrenia who are smokers have increased Mix A-induced Gαo coupling compared to nonsmokers (supplementary data). No differences are seen between smokers and non-smokers in control subjects. Interestingly, the increased Mix A-induced Gαo coupling associated with smoking in patients with schizophrenia is in the opposite direction of the changes we saw in G protein coupling in subjects with schizophrenia in which we observed decreased Mix A-induced Gαo coupling. A paradoxical effect of smoking specifically in patients with schizophrenia has been observed in psychophysical measures of olfactory function such as olfactory identification.42,43 While subjects observed in psychophysical measures of olfactory functioning specifically in patients with schizophrenia has been increased. It will be important to further examine K. E. Borgmann-Winter et al.

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References

Supplementary Material
Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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