

Dysregulation of Olfactory Receptor Neuron Lineage in Schizophrenia

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Background: Growing evidence implicates abnormal neurodevelopment in schizophrenia. While neuron birth and differentiation is largely completed by the end of gestation, the olfactory epithelium (OE) is a unique part of the central nervous system that undergoes regeneration throughout life, thus offering an opportunity to investigate cellular and molecular events of neurogenesis and development postmortem. We hypothesized that OE neurons exhibit deviant progress through neurodevelopment in schizophrenia characterized by an increase in immature neurons.

Methods: Olfactory epithelium was removed at autopsy from 13 prospectively assessed elderly subjects who had schizophrenia and 10 nonpsychiatric control subjects. Sections were immunolabeled with antibodies that distinguish OE neurons in different stages of development, including basal cells (low-affinity nerve growth factor receptor, p75NGFR), postmitotic immature neurons (growth-associated protein 43 [GAP43]), and mature olfactory receptor neurons (olfactory marker protein). Absolute and relative densities of each cell type were determined.

Results: We observed a significantly lower density of p75NGFR basal cells (37%) in schizophrenia and increases in GAP43+ postmitotic immature neurons (316%) and ratios of GAP43+ postmitotic immature neurons to p75NGFR+ cells (665%) and olfactory marker protein+ mature neurons to p75NGFR+ basal cells (328%). Neuroleptic-free schizophrenia subjects exhibited the highest GAP43+ postmitotic immature neuron values.

Conclusions: Abnormal densities and ratios of OE neurons at different stages of development indicate dysregulation of OE neuronal lineage in schizophrenia. This could be because of intrinsic factors controlling differentiation or an inability to gain trophic support from axonal targets in the olfactory bulb. While caution is necessary in extrapolating developmental findings in mature OE to early brain development, similarities in molecular events suggest that such studies may be instructive.

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GROWING evidence from clinical and postmortem research implicates abnormal neurodevelopment in the pathogenesis of schizophrenia. One of the field's major challenges is to delineate abnormal neurodevelopmental processes that could culminate in the disease at the cellular and molecular level. For the neuropathologist, this would require either examination of brain tissue from fetuses destined for schizophrenia (impossible to know) or study of neural tissue in which there is ongoing neurogenesis from known patients with schizophrenia. Neurons in the nonhuman primate and human brain are born, migrate, and assume their mature phenotype in a complex and highly orchestrated process during fetal development and the immediate postnatal period.¹⁻³ By the time the

clinical expression of schizophrenia is apparent, neurogenesis and development is mostly complete. While there has recently been great interest in several small populations of proliferating cerebral neurons in adulthood,⁴ their functional destiny is unknown. By and large, the brain's neurons are morphologically static and molecularly homeostatic. Thus, it would seem as if the opportunity to examine developing neurons in schizophrenia is unavailable. However, components of the olfactory system, that is, the olfactory epithelium (OE) and its synaptic targets in the olfactory bulb provide an opportunity for a snapshot of morphologic and molecular neurodevelopmental processes that are ongoing even in late life.

The olfactory system is a unique part of the central nervous system where neurodevelopment robustly occurs throughout life.⁵ The OE continuously regenerates, with

SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Autopsies were conducted on 13 elderly patients who had schizophrenia and 10 age- and sex-compatible nonpsychiatric controls. All schizophrenia subjects were prospectively accrued from 8 state hospitals in Pennsylvania and were clinically assessed and diagnosed according to DSM-IV criteria by research psychiatrists (S.E.A., P.J.M., and R.E.G.) of the University of Pennsylvania's Schizophrenia Mental Health Clinical Research Center, Philadelphia, as previously described.²⁹ This involved a standardized medical record review with recording of demographic variables, presenting and subsequent symptoms, treatment history, medical history, and laboratory and neuroimaging findings. Caregivers were interviewed regarding clinical status and patients were examined with a systematic focus on issues pertinent to confirming the diagnosis of schizophrenia and establishing the presence of other psychiatric or medical disorders that would warrant exclusion. Based on all information, diagnoses and inclusion were established by research team consensus. Nonpsychiatric controls were obtained through the University of Pennsylvania's Alzheimer Disease Center Core. While none of these controls had antemortem assessments, review of clinical histories found no evidence of prior major psychiatric illness. Presence or absence of smoking history was documented by medical record review. If there was no mention of smoking in the medical record but autopsy found lung emphysematous changes, we considered that subject to have been a smoker.

The mean (SD) age of the schizophrenia sample was 78.2 (9.0) years and included 6 males and 7 females. The mean age of the control sample was 70.0 (11.9) years, including 4 males and 6 females. Postmortem interval was identical at 10.9 hours for schizophrenic (3.5) and control (5.9) samples. There were no significant differences

between groups for age (Mann-Whitney test₂₁=35.5, $P=.12$), sex ($\chi^2_1=0.09$, $P=.77$), or postmortem interval (Mann-Whitney test₂₁=56.5, $P=.60$). The mean age of onset for the schizophrenic sample was 23.5 (4.4) years, and mean (SD) cumulative duration of hospitalization was 54.8 (9.35) years. All schizophrenic cases had been treated with antipsychotic medications; however, 7 had not been receiving antipsychotic medication for at least 1 month and 4 had not been receiving antipsychotic medication for at least 1 year. Of the 6 who were receiving antipsychotic medication 1 month prior to death, the mean (SD) daily dosage, expressed as chlorpromazine milligram equivalents, was 375 (206) mg. While none of the subjects had smoked within at least 2 years before death, 7 of the 13 schizophrenic cases and 3 of the 10 controls had smoked previously.

All cases had gross and microscopic diagnostic neuropathologic examinations at the time of death that included examination of multiple cortical and subcortical regions. No neuropathologic abnormalities relevant to mental status were found in the schizophrenic and nonpsychiatric groups, although minor abnormalities were noted in 3 schizophrenic cases (2 with lacunar infarcts, 1 with a posterior fossa meningioma); 1 control had Parkinson disease. Data analyses including and excluding these cases were conducted with identical results.

IMMUNOHISTOCHEMISTRY FINDINGS

At autopsy, the OE, bony septae, and contiguous cribriform plate were removed en bloc and fixed for 24 to 36 hours in 10% neutral-buffered formalin, decalcified for 14 to 16 days in distilled water with sodium EDTA, sodium hydroxide, and glycerol at pH 7.1 to 7.4.^{30,31} Tissue blocks were then cut into coronal blocks, dehydrated in graded ethanols to xylene isomers, and embedded in paraffin, as previously described.³⁰ Ten-millimeter-thick serial sections were cut from a central block of olfactory tissue in each case. Three double immunohistochemical labeling procedures were conducted for each case, in duplicate, using antibodies: (1) Me20.4 (1:10 for p75NGFR³²), (2) growth-

dividing stem and basal precursor cells giving rise to immature neurons that migrate through the epithelium, differentiate into mature olfactory receptor neurons (ORNs), send axons to reinnervate the glomeruli of the olfactory bulb, and form new synapses with target neurons there. As ORNs pass through different stages of development, they express different proteins that can serve as molecular markers of the ORN's particular stage of development.

The importance of examining the olfactory system in schizophrenia is underscored by the marked impairments in olfaction that are present in the disorder. Deficits have been reported in odor detection threshold sensitivity,^{6,7} odor identification,⁸⁻¹⁴ and odor memory.^{13,15} The pathoetiologies of these deficits are unknown and could be caused by abnormalities in the OE, the olfactory bulb, or central olfactory regions. In a recent meta-analysis of olfaction research in schizophrenia, Moberg et al¹⁶ assessed the presence and relative levels of impairment in the 4 main olfactory domains (identification, detection threshold sensitivity, discrimination, and

memory), and found "very large" effects of diagnosis for all 4. They also examined possible influences of medication treatment, sex, and smoking on effect sizes and found that these were not significant moderator variables. Additionally, Moberg et al^{17,18} have observed that olfactory impairments worsen with duration of illness, independent of normal aging effects or other potential confounds. Most recently, Turetsky et al¹⁹ documented a significant reduction in olfactory bulb volumes with magnetic resonance imaging in subjects with schizophrenia relative to healthy controls.

Given the many known cellular and molecular similarities in neurogenesis, differentiation, and maturation between the OE and other regions of the central nervous system,²⁰⁻²⁸ we propose that study of these events in the OE is instructive for understanding putatively anomalous neurodevelopment in schizophrenia. We hypothesized that OE exhibits deviant progress through neurodevelopmental events in schizophrenia. In particular, we hypothesized an increase in immature neuronal forms that are trying to establish synaptic connections with tar-

associated protein GAP43 (1:1000, Sigma Chemical, St Louis, Mo³³), and (3) olfactory marker protein (OMP; 1:2000³⁴). The p75NGFR antibody recognizes a receptor to trophic molecules and is selectively expressed in the dividing precursor basal cells of the OE.^{30,31} As basal cells commit to neuronal maturation, expression of p75NGFR ceases and the postmitotic immature neurons begin to express GAP43. As these attain a mature phenotype, they begin to express OMP and GAP43 diminishes.^{35,36} Slides were double labeled for p75NGFR/GAP43, p75NGFR/OMP, and GAP43/OMP. Immunohistochemistry studies were performed by the peroxidase-antiperoxidase method, and used 3',3'-diaminobenzidine as previously described.^{30,37} In each instance, the 3',3'-diaminobenzidine chromogen solution for the first antibody procedure included 2.5% nickel sulfate, while this was excluded for the second antibody. This yielded a black reaction product identifying the first antibody and brown for the second. All cases (schizophrenic and control samples), as well as positive and negative control slides (with and without primary antibody), were run simultaneously with precise timing of reactions for each double immunohistochemical run.

QUANTITATIVE MICROSCOPY

We determined the densities of distinctly labeled cells at each of the 3 stages of development (p75NGFR+ progenitor, GAP43+ immature, and OMP+ mature) as well as the relative proportions of cells to each other within defined segments of the OE. In the adult olfactory mucosa, portions of olfactory neuroepithelium (distinguished on the basis of their immunolabeling for neuron-specific antigens) are irregularly interspersed with metaplastic respiratory epithelia along superior portions of the nasal septum and turbinates.

Manual cell counting was performed on a personal computer (Macintosh; Apple Computers, Cupertino, Calif) using the public-domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at: <http://rsb.info.nih.gov/nih-image>) after codification

for blind analysis. Two closely adjacent double-labeled sections were used for density determinations of each cell type (p75 NGFR basal cells, GAP43 immature ORNs, OMP+ mature ORNs). Three to five 1-mm horizontal segments of OE adjacent to the apex of the nasal cavity were delimited at low magnification (**Figure 2A**). After raising the magnification to $\times 400$, all cells with visible nuclei of each immunolabeled cell type within the delimited segment were manually counted, yielding a density measurement (ie, the number of cells per millimeter in length). Because we counted all cells within each segment, we did not need a random systematic sampling grid or the optical fractionator. Similarly, because we counted only nucleated cells, split cell artifact was minimized. The average densities of each cell type in each case were calculated.

Relative densities of each cell type for each case were expressed as a ratio, that is, [(GAP43+cells)/(p75NGFR+cells)], [(OMP+cells)/(p75NGFR+cells)], and [(OMP+cells)/(GAP43+cells)]. By using ratios of the densities of different cell types, we create dimensionless indices of ORN lineage stage representation in the OE within each case. This eliminates the potential methodological problems associated with comparing cases that may have had differing degrees of tissue shrinkage during autolysis, fixation, processing, or other nonspecific factors, as well as variability in the thickness of the OE between and within cases.

STATISTICAL ANALYSES

Between group differences were assessed using the Mann-Whitney test for independent samples with an α level of .05 used to determine statistical significance. This test was deemed most appropriate because of the sample size and distribution of data. All tests were 2-tailed. In addition, we assessed possible effects of age, history of smoking, post-mortem interval, and antipsychotic drug dose (expressed in daily chlorpromazine milligram equivalents 1 month prior to death) on the neuropathologic indices using Mann-Whitney tests and/or correlation analysis.

gets in the olfactory bulb. In this study, we quantitatively characterized the cellular-molecular phenotypic development within the ORN lineage in human OE tissues obtained at autopsy from prospectively accrued and diagnosed individuals with schizophrenia and matched control subjects. Cell type-specific antibodies directed at the low-affinity nerve growth factor receptor (p75NGFR), postmitotic immature neurons (growth-associated protein 43 [GAP43]), and olfactory marker protein (OMP) were used to label ORN precursor cells, immature ORNs, and mature ORNs, respectively (**Figure 1**). Examining the densities and relative proportions of neurons at different stages of differentiation within the ORN lineage provides insights into neuron turnover, birth, and capacity for attaining a mature phenotype in the OE.

RESULTS

Double immunolabeling for p75NGFR/GAP43 and p75NGFR/OMP identified distinct neuron populations at different stages of development (Figure 2). There was

coexpression of GAP43 and OMP in many ORNs, thus making a determination of a ratio between mature and immature ORNs difficult in the GAP43/OMP slide series. The **Table** summarizes ORN subpopulation densities and ratios.

The density of p75NGFR+basal cells was significantly lower in schizophrenic cases compared with controls (**Figure 3**). In contrast, the density of GAP43+ORNs was significantly increased by more than 3-fold in the schizophrenic group. Olfactory marker protein+ORN density was also somewhat increased in schizophrenic cases, but the difference was not statistically significant.

In comparing the relative proportion of different developmental subpopulations in the OE, we found that the ratio of GAP43+immature neurons to p75NGFR+basal cells was significantly increased compared with the control group by more than 6-fold. The mean [(OMP+neurons)/p75NGFR+] ratio was also significantly increased. Given the substantial coexpression of GAP43 and OMP in postmitotic neurons, a ratio of these

cell types could not be directly determined in the same section. Instead, the mean densities of each cell type in adjacent sections were used to calculate a ratio of [OMP+/(GAP43+ORNs)]. No between group difference was observed.

There were no significant effects of age, postmortem interval, or smoking history on the cellular composition of the OE in the total sample or the schizophrenic or control subsamples examined separately. However, the ratio of GAP43+ORNs to p75NGFR+basal cells was significantly elevated in those patients who had not been receiving antipsychotic medication for at least 1 month prior to death (ratio=3.99, SD=1.07) compared with those

who were receiving antipsychotic medication (ratio=1.08, SD=0.92; Mann-Whitney test₁₁=1.0, P=.01) in the setting of comparable densities of p75NGFR+basal cells and modestly elevated GAP43+ORNs and OMP+ORNs.

COMMENT

In a previous study of the OE in schizophrenia,³¹ we examined the expression of cytoskeletal proteins, synaptophysin, glial fibrillary acidic protein, protein gene product 9.5, and p75NGFR and found the molecular phenotype of OE to be similar to that of controls. However, that study was not quantitative and aside from p75NGFR, it did not assess proteins that are selectively expressed at different stages of differentiation. Here, our focus was the neurodevelopmental composition of the OE using antibodies that were specific for neurons at different stages of maturation. We found significant differences in the densities and ratios of immature neuronal forms within the ORN lineage. The density and relative proportion of the immature GAP43+neurons is increased in the OE in schizophrenic cases while there is a decrease in p75NGFR+basal cells and no difference in the density of mature OMP+neurons. The finding that these effects were most notable in patients who had not been receiving antipsychotic medication for at least 1 month prior to death, and some for longer than 1 year, suggests it is not a confound of treatment.

Alterations in ORN lineage have been identified in various experimental conditions where the OE was challenged with injury or synaptic targets in the olfactory bulb were disrupted. For instance, in rodents, temporary destruction of the OE (eg, with detergent) results in an increase in GAP43 as the OE reconstitutes itself and reestablishes synaptic connections with the olfactory bulb.³⁵ If the olfactory bulb is removed and axons emanating from

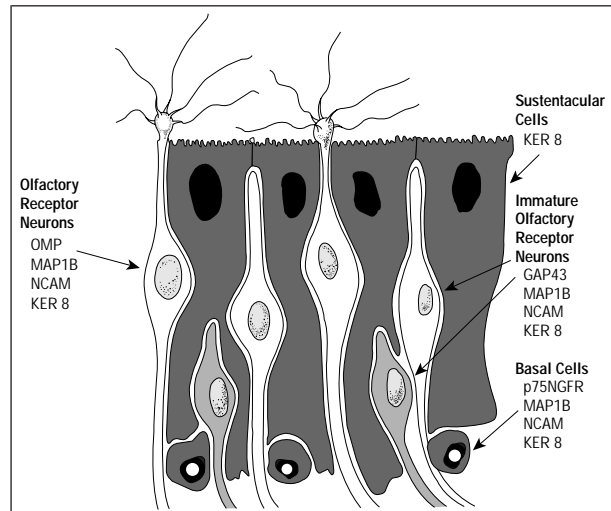


Figure 1. Schematic illustration of adult olfactory epithelium showing 4 cell types and their immunocytochemical profile. Note that low-affinity p75 nerve growth factor receptor (p75NGFR), growth-associated protein 43 (GAP43), and olfactory marker protein (OMP) are proteins that distinguish different stages of the olfactory receptor neuron lineage. KER 8 indicates keratin 8; MAP1B, microtubule-associated protein 1B (also called MAP5); and NCAM, neural cell adhesion molecule.

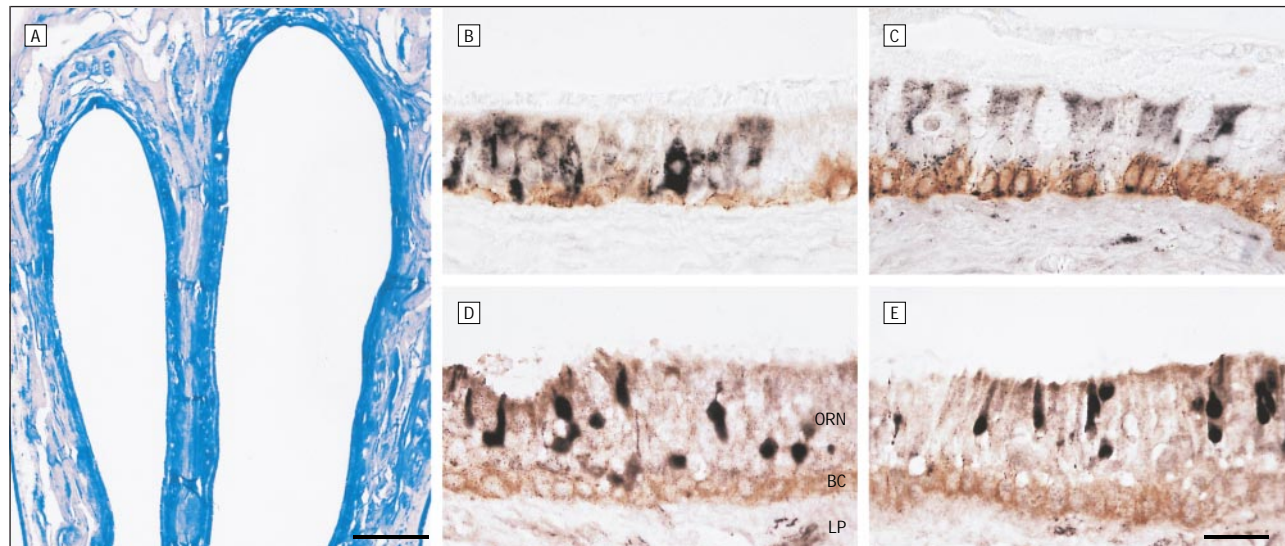


Figure 2. Olfactory epithelium (OE). A, Coronal section of nasal cavities (toluidine blue). Olfactory epithelium lines the walls of left and right nasal cavities along turbinates (lateral walls) and septum (medial walls). Segments for analysis were from the OE adjacent to the apices of nasal cavities. Bar indicates 1 mm in part A. Nonpsychiatric control (B) and schizophrenic case (C) OE was double-labeled for low-affinity p75 nerve growth factor receptor (p75NGFR)+basal cells (brown) and immature olfactory receptor neurons (ORN) expressing growth-associated protein 43 (GAP43) (black or dark gray). Nonpsychiatric control (D) and schizophrenic case (E) OE was double-labeled for p75NGFR+basal cells (brown) and olfactory marker protein (black) (immunohistochemistry). BC indicates basal cell layer; LP, lamina propria. Bar indicates 10 μ m in part E.

Cellular Composition of Olfactory Epithelium*

Type of Cell†	Total Sample			Nonsmokers Only			Smokers Only‡	
	Schizophrenic Case (n = 13)	Control (n = 10)	Mann-Whitney Test ₂₁ P Value	Schizophrenic Case (n = 6)	Control (n = 7)	Mann-Whitney Test ₁₁ P Value	Schizophrenic Case (n = 7)	Control (n = 3)
p75NGFR basal cell density, cells/mm	54.2 (15.5)	86.5 (30.3)	21.0, .01	59.9 (16.4)	79.3 (29.1)	9.0, .16	50.1 (14.6)	103.2 (31.2)
GAP43 ORN density, cells/mm	83.0 (58.4)	26.2 (13.9)	23.0, .009	105.7 (50.6)	25.1 (13.1)	1.0, .004	63.6 (61.1)	28.8 (18.5)
OMP ORN density, cells/mm	76.4 (35.7)	57.6 (46.8)	33.0, .12	84.8 (38.6)	65.5 (55.2)	12.0, .37	69.5 (35.1)	39.0 (2.6)
GAP43/p75NGFR ratio	2.26 (1.68)	0.34 (0.21)	16.0, .002	2.35 (1.53)	0.37 (0.22)	0.0, .003	2.17 (1.92)	0.29 (0.23)
OMP/p75NGFR ratio	5.09 (3.93)	1.55 (2.90)	11.0, .002	5.93 (4.95)	2.07 (3.40)	4.0, .03	4.39 (3.17)	0.32 (0.04)
OMP/GAP43 ratio§	6.86 (12.6)	4.02 (4.27)	53.0, .89	2.74 (1.80)	4.87 (4.84)	15.0, .68	10.31 (16.8)	2.04 (1.86)

*Data are given as mean (SD) unless otherwise stated.

†p75NGFR indicates low-affinity p75 nerve growth factor receptor; GAP43, growth-associated protein 43; ORN, olfactory receptor neuron; and OMP, olfactory marker protein.

‡Sample sizes preclude statistical analysis.

§Given the frequent overlap in GAP43 and OMP expression, this ratio was calculated using raw density values obtained in adjacent sections, not the same section.

normal OE are unable to establish connections with their targets, there is an arrest of the OE in an immature state as evidenced by a chronic increase in GAP43+ neurons and a decrease in mature ORNs. Presumably this is because of the ORN's inability to obtain trophic support from the olfactory bulb. Our findings of an increase in the number and proportion of GAP43+ neurons and a decrease in p75NGFR+basal cells in the OE could be because of an intrinsic dysregulation of the differentiation of ORNs (ie, accelerated differentiation). Alternatively, there may be an as yet undefined difficulty in establishing healthy synaptic connections with the olfactory bulb that accelerates OE turnover. Preliminary findings suggest this latter possibility.³⁸

Caution must be heeded in interpreting our findings because of the small sample size. Another consideration is that the advanced age of our subjects and the chronicity of their illness may affect our findings and their generalization to schizophrenia-at-large. However, the fact that we identify all stages of the ORN lineage, even in later life, is supportive of the OE as a useful model for neurodevelopmental studies. Furthermore, quantitative studies of the OE in aged rodents have shown that the rate of cell proliferation decreases with advancing age,³⁹ thus, making our findings of more immature ORNs in schizophrenia even more noteworthy.

Concerning our methods of quantitation, the sections and segments used for counting were systematically chosen to be from a block in the center of the rostral-caudal extent of the OE. As this was not strictly a random selection nor a random systematic sampling throughout the extent of the OE, potential bias may have been introduced.

Given the prevalence of smoking among schizophrenic subjects, we considered this as another potential confound. Our own previous clinical psychophysical studies, current analysis of postmortem data, and extensive review of the literature indicate that this issue is of minimal concern for interpretation of our data. In a recent meta-analysis of olfactory functioning in schizophrenia, there was

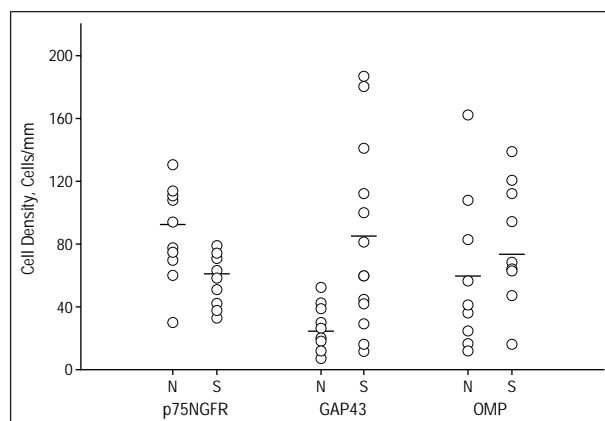


Figure 3. Scatterplots of cell density data (number of cells per millimeter linear length of olfactory epithelium). N indicates nonschizophrenic control subject (n=10); S, schizophrenic case (n=13); p75NGFR, basal cells expressing the low-affinity p75 nerve growth factor receptor; GAP43, immature olfactory receptor neurons expressing growth-associated protein 43; and OMP, mature olfactory receptor neurons expressing olfactory marker protein.

no significant relationship between smoking status and effect size for olfactory impairments in schizophrenia.¹⁶ Furthermore, while, nonsmokers tend to outperform active smokers in olfactory identification tasks in a dose-related manner, this effect is small and typically resolves within 1 year of smoking cessation.⁴⁰ None of our subjects were active smokers, although we cannot rule out exposure to secondhand smoke. Directly related to possible smoking effects on OE, Feron et al⁴¹ reported no differences between active smokers and nonsmokers for attachment of OE biopsy cells to culture slides, for in vitro behavior of outgrowing cells, for the mitotic ratio or cell death ratio, or for cellular response to dopamine therapy.⁴¹ Finally, in our study, 7 of the 13 schizophrenic cases and 3 of the 10 controls had a documented history of smoking (by clinical history or autopsy evidence of emphysema). While we cannot be sure of any possible permanent residual effects of a history of smoking on the OE, no differences between smokers and nonsmokers were found for any study variable.

A potential limitation of a general nature concerns the relevance of any findings in peripheral olfactory components (ie, OE) to neurobiological processes occurring in the cerebrum. It is uncertain how similar the growth, development, and behavior of ORNs are to hippocampal, frontal, or other telencephalic neurons that are presumably abnormal in schizophrenia. However, the OE is embryologically closely related to important limbic and neuroendocrine regions of the brain.^{42,43} It is derived from the olfactory placode that also generates some cells that migrate to the forebrain and that further has been proposed to have a morphogenetic and inducing effect on the forebrain. In addition, while much attention has rightfully focused on the neuroanatomy, neurochemistry, and functioning of limbic and frontal regions as being important in schizophrenia,^{44,45} there are also numerous data indicating neurobiological abnormalities throughout the central nervous system.⁴⁶⁻⁴⁹ The reason why the preponderant symptoms of schizophrenia may preferentially involve higher cognitive, emotional, and social domains could be that the cellular and molecular abnormalities of schizophrenia are most highly expressed in brain regions of high plasticity, complexity, or prolonged maturation. If this is true, then studying ongoing, highly dynamic neurodevelopmental processes in the OE and its synaptic targets in the olfactory bulb may be very instructive.

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