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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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.22 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 test₁=35.5, P=.12), sex (χ²₁=0.09, P=.77), or postmortem interval (Mann-Whitney test₂=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 ethanol to xylene isomers, and embedded in paraffin, as previously described.32 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-
associated protein GAP43 (1:1000, Sigma Chemical, St Louis, Mo), 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+matue) 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 ×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, postmortem 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.
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 test, t = 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, 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. If the olfactory bulb is removed and axons emanating from...
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 no significant relationship between smoking status and effect size for olfactory impairments in schizophrenia.40 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.41 None of our subjects were active smokers and nonsmokers for attachment of OE biopsies 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.42 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. 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 nervous system, neurochemistry, and functioning of limbic and frontal regions as being important in schizophrenia, there are also numerous data indicating neurobiological abnormalities throughout the central nervous system. 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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