Decreased Somal Size of Deep Layer 3 Pyramidal Neurons in the Prefrontal Cortex of Subjects With Schizophrenia

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Background: Schizophrenia is associated with deficits in working memory, a cognitive function that depends on the connections of the prefrontal cortex (PFC) with the thalamus and other cortical regions. Pyramidal neurons in PFC deep layer 3 play a central role in both thalamocortical and corticocortical circuitry. Given that somal size tends to be associated with both the dendritic and axonal architecture of a neuron, abnormalities in these circuits in schizophrenia may be associated with a change in the somal size of deep layer 3 pyramidal neurons.

Methods: We used design-based stereology to estimate the somal volume of pyramidal neurons in deep layer 3 of PFC area 9 in 28 subjects with schizophrenia, each of whom was matched to 1 normal comparison subject for sex, age, and postmortem interval.

Results: The geometric mean of the somal volume estimates in the subjects with schizophrenia was significantly ($P = .02$) decreased by 9.2%. This decrease was associated with a shift in the distribution of somal volumes toward smaller sizes. Neither antipsychotic medication treatment history nor duration of illness was associated with somal size.

Conclusions: These findings independently replicate previous reports of decreased somal size in the PFC in schizophrenia. The reduction in size of deep layer 3 pyramidal neurons is consistent with abnormalities in thalamocortical and corticocortical circuitry, suggesting that disruption of these circuits may contribute to cognitive abnormalities in schizophrenia.

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Working memory is impaired in persons with schizophrenia, and these abnormalities are associated with altered function of the dorsal prefrontal cortex (dPFC). In addition, studies in nonhuman primates indicate that working memory requires the integrity of dPFC connections with other cortical regions and the thalamus.

Recent studies have reported alterations in layer 3 of the dPFC in schizophrenia. Rajkowska et al described a decrease in the mean somal size of all layer 3 neurons and a decrease in the density of the largest neurons in deep layer 3. The authors interpreted this finding to suggest that large pyramidal neurons in deep layer 3 may be most affected in schizophrenia. Interestingly, the density of dendritic spines, a marker of the number of excitatory inputs to pyramidal neurons, has been found to be significantly decreased on layer 3 pyramidal neurons in subjects with schizophrenia, and this abnormality was reported to be most prominent on pyramidal neurons in deep layer 3 of the dPFC.

Pyramidal neurons in deep layer 3 of the dPFC play a key role in both corticocortical and thalamocortical circuitry. The principal axons of these neurons project to cortical association areas, such as the superior temporal gyrus and the inferior parietal cortex. In addition, intrinsic axon collaterals of layer 3 pyramidal neurons furnish wide-spreading, horizontal excitatory connections within the dPFC. Furthermore, deep layer 3 pyramidal neurons are located in the termination zone of axon projections from the mediodorsal thalamic nucleus, and thus likely receive excitatory input from this nucleus.

Because neuronal size is correlated with the extent of a neuron's dendritic and axonal arbor, a decrease in somal size may reflect decreased afferent and/or efferent connectivity of these neurons in schizophrenia. Consequently, we tested the hypothesis that the somal size of deep layer 3 pyramidal neurons in dPFC area 9 is decreased in individuals with schizophrenia.

The primary MANCOVA model revealed a significant ($F_{1,26} = 5.76; P = .02$) 9.2% decrease in somal size in subjects with schizophrenia compared to normal control subjects.
SUBJECTS AND METHODS

SUBJECTS

With the consent of the next of kin and the approval of the Health Sciences Institutional Review Board of the University of Pittsburgh, we obtained brain specimens from 56 subjects during autopsies conducted at the Allegheny County (Pittsburgh) Coroner’s Office (Table). Neuropathological examinations revealed no abnormalities in any of the subjects except for the following: thioflavin-S staining revealed a few neuritic plaques in 3 normal comparison subjects (pairs 3, 4, and 24) and in 1 subject with schizophrenia (pair 6), but they did not meet either clinical or neuropathological criteria for Alzheimer disease. Two subjects with schizophrenia died of cerebrovascular events (pair 6, left parietal subdural hematoma; pair 12, intracerebral hemorrhage in the right temporal lobe), but the left dPFC was not affected. An independent panel of experienced clinicians made consensus DSM-III-R diagnoses as described previously.

Twenty-eight subjects diagnosed as having schizophrenia or schizoaffective disorder (Table) were each matched to 1 comparison subject for sex, age, and postmortem interval (PMI). Individual pairs were completely matched for sex, and the mean ± SD differences in age and PMI within pairs were 3.8 ± 3.0 years and 2.6 ± 2.1 hours, respectively. Mean values for these variables (Table) and percentage of out-of-hospital deaths (comparison group, 96.4%; schizophrenia group, 89.3%) did not differ between the 2 groups. For subjects with schizophrenia, the mean ± SD age of onset was 27.3 ± 9.0 years and the duration of illness was 25.8 ± 11.8 years. Five of these subjects died by suicide, and 13 had a history of an alcohol or substance use–related disorder; these diagnoses were current at the time of death for 8 subjects. Toxicology examinations were positive for plasma alcohol in 3 subjects with schizophrenia (pairs 4 [0.13%], 7 [0.12%], and 27 [0.09%]) and in 2 comparison subjects (pairs 11 [0.03%] and 20 [0.01%]). Six subjects with schizophrenia had not been taking antipsychotic medication for at least 1 month prior to death, and 1 (pair 16) never received treatment (Table).

TISSUE PREPARATION

The left hemisphere of each brain was cut into 1.0-cm-thick coronal blocks, immersed in ice-cold 4% paraformaldehyde in phosphate buffer for 48 hours, washed in a graded series of sucrose solutions, and stored in an antifreeze solution at -30°C. Tissue storage time did not differ between the subject groups (Table). From blocks located 2 to 4 cm from the frontal pole, 40-µm coronal sections were cut on a cryostat. Every tenth section was mounted on slides and stained for Nissl substance with thionin. From a series of sections determined by cytoarchitectonic criteria to contain dPFC area 9 (Figure 1), we selected 4 sections, each separated by 400 µm, for somal size estimation. These slides were placed in random order and coded for blinded quantification.

MEASUREMENT OF SOMAL SIZE

Quantification was performed without knowledge of diagnosis by one rater (C. L. E. V.). Using a Zeiss Axiosplan microscope equipped with Stereo Investigator software and a Microvid Monitor (MicroBrightField, Inc, Colchester, VT), area 9 was identified at low magnification (×50), the border between layers 3 and 4 was located (Figure 1), and a contour outlining the lower third (determined by measuring the width of layer 3) of layer 3 was drawn (Figure 2A). The mean ± SD contour area per section was 1.93 ± 0.33 × 10^3 µm^2 for the comparison subjects and 1.85 ± 0.36 × 10^3 µm^2 for the subjects with schizophrenia. Magnification was then changed to ×1000, using a 1.4 numerical aperture, ×100, oil immersion objective, for cell measurements. To randomly sample cells, we used the optical fractionator probe of the Stereo Investigator software, which systematically and randomly placed 18 to 22 sampling boxes throughout the region of interest (Figure 2B). Each box was 110 × 75 × 8 µm in the x, y, and z directions, respectively (Figure 2C). At each sampling site, sampling was begun 2 µm below the section surface. Sections had a mean ± SD thickness of 14.0 ± 1.0 µm for both the comparison and schizophrenia groups. To estimate somal volume, we used the nucleator probe of the Stereo

Continued on next page
Investigator software, in a local vertical section design. That is, we only measured neurons in regions of area 9 where the long axis of the neurons was judged to be parallel to the plane of the section and perpendicular to the pial surface (Figures 1 and 2D). This design assumes that pyramidal neurons in deep layer 3 are isotropically oriented about the vertical axis of the tissue.

For each neuron, the nucleolus was used as the cell’s uniquely associated reference point. To estimate soma size, the operator clicked on the nucleolus, which brought up a set of 5 two-dimensional isotropic random rays (Figure 2D). The operator clicked on each ray where it intersected the boundaries of the soma. The formula used to calculate volume was as follows: volume = \( \frac{4}{3} \pi r^3 \), where \( r \) equals the mean segment length from the nucleolus to the cell boundary, and \( n \) equals the number of segments, in this case, 5.

Only cells within the 3-dimensional sampling box meeting the following criteria for pyramidal neurons were measured: (1) a clearly identifiable nucleolus, (2) an abundance of Nissl-stained cytoplasm, (3) a clearly visible vertical apical dendrite, and (4) a triangular shape. The mean±SD number of neurons measured for each subject did not differ between the control subjects (265±44) and the subjects with schizophrenia (261±45). The mean±SD coefficients of error for average somal volume were 4.5%±0.6% for comparison subjects and 4.6%±0.6% for subjects with schizophrenia.

**STATISTICAL METHODS**

Individual and group somal volume distributions for all measured neurons in each diagnostic group showed a skew toward the larger somal sizes (Figure 3A). To normalize the data, all single neuron volume estimates were transformed using a natural log function31 (Figure 3B). The operator clicked on the nucleolus, which brought up a set of 5 two-dimensional isotropic random rays (Figure 2D). The operator clicked on each ray where it intersected the boundaries of the soma. The formula used to calculate volume was as follows: volume = \( \frac{4}{3} \pi r^3 \), where \( r \) equals the mean segment length from the nucleolus to the cell boundary, and \( n \) equals the number of segments, in this case, 5.

For each subject, estimates of somal volume were averaged over the neurons within each of the 4 sections. These averages were treated as 4 correlated observations. Exploratory regression analyses done to assess the effects of sex, age, PMI, and tissue storage time on somal volume indicated a potential effect of PMI on somal volume, which was confirmed in formal modeling. To examine a main effect of diagnosis, a multivariate analysis of covariance (MANCOVA) model assuming a compound symmetric covariance structure32 was used. To test for a main effect of diagnosis, pair was used as a blocking factor and tissue storage time as a covariate. In this model, the pair factor accounts for the effect of PMI, as subjects were matched, on a pairwise basis, for sex, age, and PMI. This model was validated by using the MANCOVA procedure to assess both diagnosis and pair factors, with tissue storage time and PMI as covariates, as well as by including all pairing factors (age, sex, and PMI) and tissue storage time as covariates in the MANCOVA model. As all 3 models yielded similar results, only the results of the primary model are reported.

Analyses were implemented in SAS PROC Mixed. All statistical tests were performed on the log-transformed estimates of somal volume, and summary descriptions of somal volume were obtained by back transformation of the summary statistics of log-transformed somal volume. Back transformation yields geometric means that are estimates of median somal volume. The distributions of somal size for the 2 diagnostic groups are described using 25% and 75% quartiles, obtained through back transformation.

To examine changes in the distribution of somal volumes between diagnostic groups, arbitrary cutoff values were used to create 4 somal size categories. The mean percentages of neurons for each subject in the 3 smallest size categories were compared across diagnostic groups using a MANCOVA model that assumed an intraclass covariance matrix with pair as a blocking variable.

Within the schizophrenia group, we also used an intraclass covariance matrix MANCOVA model to evaluate the effects of age of onset and duration of illness on somal volume. Because age at the time of death was correlated with both age of onset and duration of illness, it was included as a covariate for these analyses. In exploratory analyses, we used an analysis of covariance model with PMI as the covariate to evaluate the effects of suicide, substance abuse history, and medication status at the time of death on differences in log-transformed somal volumes between subjects with schizophrenia and comparison subjects.

All statistical tests were conducted with \( \alpha = .05 \).

(993.1±81.7 µm) and the subjects with schizophrenia (1003.7±86.3 µm).

We found no significant associations between age of onset \((F_{1,24} = 0.11; P = .75)\) or duration of illness \((F_{1,24} = 0.09; P = .77)\) and somal size. In addition, differences in log-transformed somal volumes between the subjects with schizophrenia and their matched comparison controls did not vary \((F_{1,25} < 0.10; P > .75)\) as a function of sex, suicide, or history of alcohol and/or substance abuse (Figure 6A-C). Finally, differences in somal size between subjects with schizophrenia and the their matched controls were not affected by antipsychotic medication treatment status at the time of death \((F_{1,25} = 0.25; P = .62)\) (Figure 6D).

**COMMENT**

In this study, subjects with schizophrenia showed a significant 9.2% decrease in the somal volume of pyramidal neurons in deep layer 3 of PFC area 9, with a shift in the somal volume distribution toward smaller cell sizes. Decreased somal size was not related to duration or age of onset of illness, sex, death by suicide, history of alcohol or substance use, or antipsychotic medication treatment at the time of death.

The strengths of this study include (1) the sample size; (2) the use of an optical fractionator design, which enabled systematic random sampling within area 9; and (3) the use of the nucleator, which permitted estimates of somal volume.29 However, several potential confounds must be considered in interpreting the pathophysiological significance of our observations.

First, the stereological design of this study has limitations. Although a relatively large volume of area 9 was sampled, we did not sample throughout the region of interest. Consequently, caution must be used when generalizing findings from our sampling scheme to all deep layer 3 pyramidal neurons in area 9. Also, our method...
### Subjects Examined in This Study

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Mean: 52.9, SD: 13.3

*PMI indicates postmortem interval; ASCVD, atherosclerotic coronary vascular disease; CD, carbon monoxide; SA, schizoaffective disorder; CUS, chronic undifferentiated schizophrenia; RS, residual schizophrenia; CPS, chronic paranoid schizophrenia; CDS, chronic disorganized schizophrenia; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; and MCA, middle cerebral artery.

†Superscripts are as follows: a, alcohol dependence, current at time of death; b, alcohol dependence, in remission at time of death; c, alcohol abuse, current at time of death; d, alcohol abuse, in remission at time of death; e, other substance dependence, current at time of death; f, other substance dependence, in remission at time of death; g, other substance abuse, in remission at time of death; †, schizophrenic subjects not taking medications at time of death; and ‡, schizophrenic subject who never received antipsychotic medication.

for estimating somal volume did not take into account the possibility that alterations in the shape or orientation of pyramidal neurons in schizophrenia could lead to underestimates or overestimates of somal volume. However, despite these caveats, the fact that a decrease in somal size of deep layer 3 pyramidal neurons has been reported by another independent group, suggest that decreased somal size of deep layer 3 pyramidal neurons in schizophrenia is unlikely to be the result of biased measurement methods.

Second, terminal conditions associated with changes in brain volume, such as prolonged hypoxemia, could result in altered somal size. However, in this study, 92% of the subjects died suddenly, out of hospital, consistent with a limited agonal state.

Third, neuronal size may change as a function of PMI, as observed in this study. However, subject pairs were closely matched for PMI, and inclusion of PMI as a covariate revealed that subjects with schizophrenia still showed a significant decrease in somal volume.

Fourth, the duration of brain tissue fixation represents a potential confound since tissue volume shrinks as much as 14% after prolonged fixation. In the present study, each specimen was processed following a standard protocol, with a brief fixation time (48 hours), that was identical for both diagnostic groups.

Finally, long-term exposure to antipsychotic medications might confound estimates of somal volume, as dose of antipsychotic medication has been found to be negatively associated with frontal lobe volume in schizophrenia. In the present study, the somal volumes of subjects with schizophrenia who were not taking antipsychotic medications at the time of death did not differ from subjects who were (Figure 6D). Moreover, the somal volume of the subject with schizophrenia who had never been treated with antipsychotic medications was less than that of the matched comparison subject.
Decreased somal size is consistent with and possibly related to other reported abnormalities of dPFC layer 3 pyramidal neurons in schizophrenia, such as decreased dendritic spine density.\textsuperscript{9,10} Specifically, a decrease in the density of basilar dendritic spines on deep layer 3 pyramidal neurons was associated with a decrease in the total length of these dendrites, as well as a nonsignificant decrease in somal size.\textsuperscript{10}

Similar to previous studies of PFC pyramidal neurons,\textsuperscript{19,20} we also found no relationship between somal size and age at time of death in our subjects, confirming that somal size is stable across adulthood. Furthermore, somal volume in schizophrenia was not related to age of onset or duration of illness, suggesting that reductions in somal volume may not progress with time, and that the events leading to decreased somal size may have occurred during development before illness onset or during a limited progressive phase of the illness.\textsuperscript{38}

Alterations in deep layer 3 pyramidal neurons may be related to changes in chandelier cells, a specific subset of cortical inhibitory neurons. Chandelier neuron axon terminals (termed "cartridges") synapse on pyramidal neuron axon initial segments, providing powerful regulation of pyramidal neuron output.\textsuperscript{39} In the dPFC, subjects with schizophrenia exhibit a decrease in the density of cartridges immunoreactive for the \( \gamma \)-aminobutyric acid transporter (GAT-1)\textsuperscript{40,41}; interestingly, this decrease appeared to be greatest in deep layers 3 and 4 of the same subjects studied herein.\textsuperscript{41}

Our findings also suggest that a reduction in somal volume may contribute to the subtle reductions in dPFC volume found in neuroimaging studies of schizophrenia.\textsuperscript{42-47} In addition, our findings may account for the reports of decreased concentrations of dPFC \( N \)-acetylaspartate\textsuperscript{48-51} in schizophrenia, since pyramidal cells make up the majority of neurons in the cortex,\textsuperscript{8} and since the concentration of \( N \)-acetylaspartate may be greatest in these neurons.\textsuperscript{52} Interestingly, in subjects with schizophrenia, \( N \)-acetylaspartate levels in the dPFC were positively correlated with changes in cortical activation, as measured by regional blood flow, in the prefrontal, temporal, and parietal association cortices during performance of a working memory task.\textsuperscript{53} This relationship, found only for the dPFC, suggests that activation of the working memory network may be determined by the integrity of cortico-cortically projecting dPFC pyramidal neurons.\textsuperscript{13,53}
Understanding the pathophysiological significance of a decrease in the somal size of pyramidal neurons in deep layer 3 in persons with schizophrenia depends on whether this abnormality reflects a defect intrinsic to deep layer 3 pyramidal neurons or an extrinsic defect in the inputs they receive. An intrinsic defect may be manifest in a decrease in the capacity of these neurons to form and maintain appropriate afferent and efferent connections. For example, in certain neuronal populations, somal size correlates with the extent of a neuron’s dendritic and axonal arbor. If these correlations apply to the findings of the present study, we would expect evidence for decreases in these components of pyramidal neuron architecture. Indeed, this possibility is suggested by reported abnormalities in spine density and total dendritic length. Interestingly, for 12 subjects with schizophrenia in the present study, who were also examined in a previous study of spine density, average total dendritic length, measured in Golgi-stained deep layer 3 pyramidal neurons, and average somal volumes, esti-
mated herein, are significantly correlated ($r=0.64$, $P=.02$). In contrast to measures of a neuron’s dendritic arbor, the axonal arbor of a neuron is much more difficult to measure in the postmortem state. However, given that layer 3 pyramidal neurons participate in reciprocal short- and long-range circuits intrinsic to the dPFC, a proportion of the decrease in pyramidal neuron spine density in schizophrenia may be due to a decrease in axonal arbor.

On the other hand, a decrease in somal size could result from a loss of input from other brain areas, such as the thalamus. Recent studies of the mediodorsal thalamic nucleus, which projects to the dPFC, have reported a decrease in the number of neurons in subjects with schizophrenia. These findings suggest that, in schizophrenia, deep layer 3 pyramidal neurons may receive less excitatory drive from the thalamus, and consequently, they are less active and hypotrophic. This possibility of “denervation atrophy” is supported by experiments in which lesioning a subset of afferent inputs to the PFC,"denervation atrophy" is supported by experiments in which lesioning a subset of afferent inputs to the PFC,18 a proportion of the decrease in pyramidal neuron spine density in schizophrenia may be due to a decrease in axonal arbor.

Further studies are needed to determine whether abnormalities in dPFC deep layer 3 pyramidal neurons in schizophrenia reflect an intrinsic defect or are the result of altered inputs from other brain regions. Either possibility would support the hypothesis that abnormal thalamocortical and corticocortical circuitry underlie dPFC dysfunction in schizophrenia.

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