Neuropathology of schizophrenia : a new vistas

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Neuropathology of schizophrenia: a new vistas

Pawel Kreczmanski

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Pawel Kreczmanski

Promotor

Prof. dr. W.H.M. Steinbusch

Copromotor

Dr. C. Schmitz

Beoordelingscommissie/Evaluation committee

Prof. dr. JHS. Vles (voorzitter)

Dr. T. Wierzba-Bobrowicz Institute of Psychiatry and Neurology, Warsaw Poland

Prof. dr. M Lammens (Universiteit Nijmegen)

Prof. dr. G. Hopkins (Universiteit Maastricht)

Prof. dr. rer. H. Korr. (universiteit Aachen)

*I came to this world with nothing,
And I leave with nothing but love.
Anything else is just borrowed.
The streets*

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Introduction

Schizophrenia is a devastating neuropsychiatric disorder characterized by psychosis, cognitive impairment and social withdrawal (Mueser and McGurk, 2004). It is the most disabling disorder among mental illnesses (Mueser and McGurk, 2004), being listed as the one of the top ten leading causes of disease related disability in the world (WHO, 2001). The molecular and cellular mechanisms involved in schizophrenia are still remaining largely illusive (Tsuang, 2000; Harrison and Weinberger, 2005). A combination of genetic susceptibility and environmental perturbations appears to be necessary for the expression of the disease (Tsuang, 2000; Siever and Davis, 2004; Harrison and Weinberger, 2005). Possibilities range from alterations of highly specific developmental processes at the synaptic level (Harrison, 1999; Lewis and Lewitt, 2002) and abnormalities in myelination (Hakak et al., 2001; Tkachev et al., 2003) to global metabolic changes of brain function (Berman et al., 1986; Andreasen et al., 1992; Middleton et al., 2002). As the function of neurons depends on adequate oxygen and substrate supply, subtle changes in vascular integrity may also be important for the brain of patients with schizophrenia.

Clinical picture and historical perspective of schizophrenia

The course and outcome of schizophrenia were for the first time systematically investigated by Emil Kraepelin (Kraepelin, 1919). The concept of dementia praecox developed by Emil Kraepelin was based on linking together specific “Bilder” or concepts of madness and making them uniformed. At that time a rapid development of bacteriology gave a useful paradigm for a model of the disease. It was based on the finding that almost every investigated disease was caused by external factors such as bacteria or later discovered viruses. There were then two elements of the equation: the disease and its cause. The same paradigm was considered in psychiatry motivated by spectacular success in the treatment of cerebral syphilis. Kraepelin’s original idea was to complete it by adding two important elements that for a long time dominated the traditional view of mental disorders. The first one implies that the mental disease is also a disease of the brain. In other words, there is no mental disease without neuropathological alterations in the brain. The second one was more illusory and called “eigentümliche Schwächezustände” (“peculiar states of mental weakness”) by Kraepelin (1919). “Eigentümlich” (which can be translated into English as “peculiar”) means in an original sense a state or feature exclusively belonging to something, in this context to an abstractive object or definition. Kraepelin (1919) used the word “eigentümlich” always with respect to “Schwächezustände”. Thus, “eigentümliche Schwächezustände” was considered by Kraepelin (1919) as unique only for schizophrenia. Kraepelin (1919) also grouped and simply listed the features of the psychopathology of schizophrenia. Although the importance of Kraepelin’s work cannot be underestimated, it was Bleuler (1911), who pushed the concept furthermore and gave it the more adequate name “schizophrenia” (a “shattered mind”; *σχίζω*-schizo, to split or to divide and *φρήν*-phren, mind). In his ideas, deeply rooted in a German Enlightenment philosophy, Bleuler acquired and fused essence of the Germanic tradition, the idea of the disturbance of the reason as a basis of the disease and association theory of Locke (Locke, 1841). Using associations to determine the classification of symptomatology of

schizophrenia, Bleuler (1911) rejected the holistic idea of grouping the symptoms without any distinction among them and proposed a system based on two groups of symptoms: basic and accessory symptoms. According to Bleuler (1911) the basic symptoms (known as 4A's: flattened Affect, Autism, impaired Association of ideas and Ambivalence) are "as far as we know typical for schizophrenia while the accessory symptoms can also appear in other diseases".

The current valid diagnostic criteria of schizophrenia are listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR, Fourth Edition) and include two or more of the following basic symptoms present for a period of at least one month duration or less if successfully treated.

The "positive" symptoms include:

- Delusions (false beliefs) despite of invalidating evidence, including:
 - delusions of persecution (also known as paranoid; for example, the patient is being watched or followed and someone is trying "to get her/him"),
 - delusions of reference (people or other creatures communicating with an affected individual through TV or radio),
 - somatic delusions (for example, that something foreign is passing through the body), and
 - delusions of grandeur, exaltation or majesty (i.e., that an individual possesses special powers or abilities). An example of a grandiose delusion is believing that the patient is God or Napoleon;
- Hallucinations: visual (seeing things that are not there), auditory (hearing voices that other people cannot hear), tactile (something is touching your skin that isn't there), olfactory (not smelling the same that other people smell) or gustatory (tasting things that do not exist) experiences;
- Disorganized speech in form of incoherence and frequent derailment (also described as "word salads" or rambling monologues); and
- Grossly disorganized or catatonic behaviour (patients demonstrate variable conditions of stupor or inactivity and either rigidity or flexibility of the extremities).

The "negative" symptoms include:

- Alogia, or the poverty of speech;
- Affective flattening (which is the reduction or difficulty to initiate and persist in goal directed behaviour; for example, no longer being interested in activities that the person used to show enthusiasm for).

In addition, lack of emotion, low energy, lack of interest in life, low motivation, inappropriate social skills or lack of interest or ability to socialize with other people and social isolation has been observed in schizophrenia.

The third group of symptoms refers to intellectual and cognitive impairments and include disorganized thinking, slow thinking, difficulty in understanding, poor concentration, poor memory, difficulty in expressing thoughts and difficulty integrating thoughts, feelings and behaviour.

Although the symptomatology of schizophrenia includes a wide spectrum of cognitive alterations there is no pathognomonic sign of the disease (Kraepelin, 1919). Rather the

diagnosis is based on a profile of symptoms and the clinical course of the patient. Moreover, the severity of different symptoms can vary across patients and through the course of the illness (Harrison et al., 2001). The significant heterogeneity in clinical manifestations and the course of schizophrenia is also reflected in the complexity of its epidemiology.

Epidemiology

In general, epidemiological data raise a very important issue over the variations in the diagnostic criteria and ascertainment methods which are often the source of observed differences and discrepancies. Accordingly, epidemiological data about neuropsychiatric disorders should be analyzed with some caution.

The incidence of schizophrenia is approximately 8–40/100,000/year and is relatively similar across continents and cultures (Sartorius et al., 1986). There is a higher incidence associated with migration and urbanity (Jablensky et al., 1992). The point of prevalence reaches the value of 2–10/1,000 including pockets of high and low prevalence (Dohrenwend et al., 1992), with a higher prevalence among lower socio-economical classes (Crow, 2007).

The risk of inception lies between 25 and 35 years for men and women (Haefner et al., 1989) and usually does not extend beyond the age cut-off at 54–59 years for the late-onset schizophrenia (Jablensky, 2000). In approximately 75% of the cases, the first non-specific psychotic episode, diagnosed at mean age of 24.0 years, is preceded by a prodromal phase of several years' duration (Hambrecht et al., 1994). During this phase non-specific symptoms are predominant. At a mean age of 25.5 years delusions of reference and negative symptoms appear. The first positive symptoms are usually present at mean age of 29.0 years, reaching clinical climax at mean age of 30.1 years, just before the first hospitalization at mean age of 30.3 years (Haefner et al., 1992).

Sex differences, reported for the first time by Kraepelin (1919), are manifested in several different ways. In general, the earlier age at onset and incidence of brain abnormalities are more frequent in men while women have better premorbid performance, less disability and higher possibility for a remitting course (Jablensky, 2000). According to the Jablensky et al., (1992), men are 3–4 years younger than women at the time of the first hospitalization. Nevertheless, sex differences at onset as well as during the course of the disease seem to reflect normal sexual brain dimorphism rather than sex-linked etiological mechanism (Jablensky, 2000).

Genetics

Schizophrenia is a highly heritable mental illness. Genetic factors contribute up to 80% to the susceptibility of the disease (Cannon et al., 1998). Gottesman (1991) showed that three degree relatives share 12.5% of their genetic material and show a risk of 2% to develop schizophrenia (see also Fig. 1). Half-siblings share respectively 25% of their genome and show a risk of 6% to develop schizophrenia. Dizygotic twins with 50% of shared genes show 9% of risk to develop the disease, whereas monozygotic twins show a risk near 50% (Fig. 1). Comparison of the rates between monozygotic vs. Dizygotic twins (48% vs. 16%)

in a meta-analysis based on the data from ongoing 30 years of European studies (Gottesman, 1993) clearly demonstrates genetic heterogeneity of schizophrenia.

The molecular genetic approaches which emerged in the late 1980's failed to find allelic variants conferring susceptibility of schizophrenia (Owen et al., 2004). No single gene, even with an incomplete penetration, was found. Because the recurrence risk decreases too rapidly with an increase of the genetic distance, the transmission of schizophrenia, like other common disorders, is complex and non-Mendelian (McGue and Gottesman, 1989). However, analyses of positional genes produced promising results in linkage genes studies. Two of those, dysbindin (DTNBP1) (Straub et al., 2002) and neuregulin (NRG1) (Stefansson et al., 2002) have been replicated in at least two independent studies (Schwab et al., 2003, Williams et al., 2003).

Fig. 1 Population genetics of schizophrenia (taken from Gottesman, 1991).

Environmental risk factors

The examination of genetic risk factors in association with environmental risk factors, especially developmental abnormalities such as pregnancy and delivery complications, provides valuable information on antecedent states for schizophrenia (Buka and Fan, 1999). Patients with schizophrenia encounter a significantly greater number of labour and delivery complications at birth than in the control group (Geddes and Lawrie, 1995). Among those complications one of the most significant is pre-eclampsia which is responsible for inducing foetal hypoxia. A nine-fold increase in the risk of subsequent schizophrenia has been associated with pre-eclampsia (Kendell et al., 1996; Susser et al., 1996). Moreover, the severity of prenatal hypoxia and the risk of schizophrenia seem to be correlated in a dose-dependent manner in the offspring of mothers with schizophrenia (Cannon, 1996). On

the other hand, delivery complications were unrelated to the development of schizophrenia in the offspring of parents who did not have schizophrenia (Cannon et al., 1993). Several other environmental factors have been associated with schizophrenia, including first and second trimester maternal starvation (Susser et al., 1996), anemia (Kendell 1996), chronic pulmonary disease (Kendell et al., 1996), diabetes (Kendell et al., 1996), and rhesus incompatibility (Dalman et al., 1999) as well as low birth weight and premature delivery (Dalman et al., 1999). Maternal viral infections are also known to be associated with increased risk of schizophrenia (Mednick et al., 1988).

Neurodevelopmental and neurodegenerative hypotheses of schizophrenia

Alterations of the highly specific developmental processes have generated a lot of interest among neuroscientists investigating schizophrenia (Harrison, 1999; Lieberman, 1999; Tsuang, 2000; Lewis and Lewitt, 2002; Murray et al., 2004). Many genetic and environmental risk factors of schizophrenia appear either prior to or around the time of birth (Jones and Cannon, 1998) leading to disruption of early postnatal brain development and causing expression of the symptoms in adolescence and adulthood. This assumption was developed into the neurodevelopmental hypothesis of schizophrenia.

According to the neurodevelopmental hypothesis of schizophrenia abnormal genetics and epigenetics together with gestational and/or perinatal trauma amplify and influence abnormalities in development of the brain of patients with schizophrenia (Weinberger, 1987). Thus, postpubertal onset of the disease is attributed to the continuing development of brain throughout adolescence. Neural networks do not develop properly at a certain time point during pregnancy and patients develop psychosis as a result of long standing abnormalities interacting with environmental factors. Evidence suggesting the existence of neuropathology before the onset of the disease include:

- congenital and minor physical alterations such as abnormalities in the capillary system of the fingernail folds which have been found in patients affected by the disorder (Curtis et al., 1999) and reduction of the vasodilatory response to methyl nicotinate application in the skin of patients with schizophrenia (Ross et al., 2004);
- childhood neuromotor, social and cognitive abnormalities;
- enlargement of the lateral ventricles (Harrison, 1999; Wright et al., 2000; Shenton et al., 2001) as well as bilateral reduction of the total volume of the cerebral cortex (Harrison, 1999; Shenton et al., 2001) which is not related to the patient's age;
- absence of gliosis and cytoarchitectural abnormalities in cortical and subcortical regions of the brain of patients with schizophrenia in postmortem studies (Falkai et al., 1999).

One of the underlying mechanisms causing these cytoarchitectural abnormalities, which have long been suspected to cause widespread neuronal dysfunction in schizophrenia, is hypoxia (Kety, 1959a; 1959b).

Despite plethora of data there is however no direct evidence for neurodevelopmental lesions directly responsible for schizophrenia. The disease is also not inevitably irreversible.

Antipsychotic medication can successfully improve symptoms and change the course and duration of the illness. Moreover, there is no explanation how an developmental event, latent for two decades, can not only trigger schizophrenia but also be responsible for heterogeneous and often progressive clinical courses (McClure and Lieberman, 2003). Thus a restricted neurodegenerative process cannot be fully rejected.

In a neurodegenerative approach abnormalities in brain maturation and genetic and/or epigenetic events lead to neurodegenerative process that can be associated with progressive course of the illness. Evidence includes:

- progressive clinical course;
- age related progressive loss of synapses (Lieberman, 1999); and
- progression of cognitive dysfunction.

In this context loss of cognitive functions, chronicity and onset of schizophrenia resemble other neurodegenerative diseases such as Alzheimer's disease or Huntington's disease. These two alternative hypotheses (i.e., neurodevelopmental vs. Neurodegenerative), although regarded incompatible, are not mutually exclusive. Rather the possibility that neurodegeneration occurs after the onset of the disease cannot be completely ruled out. Since psychotic episodes that are manifested after the onset can produce a neurotoxic effect, they could also have a detrimental effect on the brain triggering another, yet unknown, process (Wyatt, 1995; McGlashan and Johannessen, 1996) (see Fig. 2).

Fig. 2. Processes that have been indicated to play a pivotal role in development, onset and course of schizophrenia (according to Tsuang, 2000).

Hypoxia and schizophrenia

As the function of neurons depends on adequate oxygen and substrate supply, subtle changes in vasculature integrity may also be important for the brain of patients with schizophrenia. It has been argued that a subtle metabolic deficit such as hypoxia would be sufficient to cause widespread neuronal dysfunction (Kety, 1959a; 1959b). Furthermore, a number of abnormalities in the coupling between neuronal activity and cerebral blood flow were described in patients with schizophrenia (Meyer-Lindenberg et al., 2002; Hill et al., 2004). These changes were attributed to primary neuronal dysfunction, although subtle changes in the capillary system were not excluded. Abnormalities in the capillary system of the fingernail folds was found in patients affected by the disorder (Curtis et al., 1999), and the vasodilatory response to methyl nicotinate application in the skin is reduced in patients with schizophrenia (Ross et al., 2004). A study employing a combined transcriptomics, proteomics and metabolomics approach found evidence for mitochondrial dysfunction, compromised brain metabolism, and oxidative stress in the frontal cortex of patients with schizophrenia (Prabakaran et al., 2004) thereby confirming and expanding earlier reports (Berman et al., 1986; Andreasen et al., 1999; Middleton et al., 2002). Prabakaran et al. (2004) hypothesized that the observed alterations can be explained by abnormalities of the microvasculature in schizophrenia.

Postmortem studies and modern imaging techniques

Modern imaging techniques such as computerized tomography (Johnstone et al., 1976) and particularly magnetic resonance imaging (MRI) have led to a revival of postmortem investigations on alterations in the brain of patients with schizophrenia over the last decades. Based on meta-analyses of several MRI and CT investigations there is now a strong body of evidence in favor of an enlargement of the lateral ventricles (Harrison, 1999; Wright et al., 2000; Shenton et al., 2001) as well as of a bilateral reduction of the total volume of the cerebral cortex (Harrison, 1999; Shenton et al., 2001).

Postmortem investigations revealed subtle alterations in several cortical regions in schizophrenia such as the prefrontal, entorhinal and anterior cingulate cortices (Harrison, 1999; Tamminga et al., 2000; Selemon, 2001) as well as in subcortical regions such as the caudate nucleus, putamen and nucleus accumbens (Bogerts, 1984; Beckmann and Lauer, 1997; Lauer and Beckmann, 1997), the mediodorsal nucleus of the thalamus (Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000; Byne et al., 2002; Cullen et al., 2003; Dorph-Petersen et al., 2004; Danos et al., 2005) and the amygdala (Bogerts, 1984) with respect to volume, total neuron number and neuron density. However, methodological differences such as the lack of standardization and normalization of applied procedures, investigated parameters and above all availability of the investigated samples from post-mortem brains of patients with schizophrenia and matched controls were to blame for the discrepancy and divergence of published results (Harrison, 1999). A possible solution to this unsatisfactory situation is the analysis of several regions in brains from patients with schizophrenia and matched controls with state-of-the-art quantitative histological tech-

niques in one study. Moreover, general observations on the macrovasculature in the brain of patients with schizophrenia were performed (Arnold and Trojanowski, 1996), but there are no systematic, design-based stereological studies on the vascular system in this disease. For this purpose I performed capillary length density measurements within several cortical and subcortical regions in postmortem brains from patients with schizophrenia and matched controls with the recently developed "Space balls" method (Calhoun and Mouton, 2000; Mouton et al., 2002; Schmitz and Hof, 2005).

Accordingly, a new methodological approach was performed in the present study to shed new light on the neuropathology of schizophrenia, including (among many others) the following innovative key elements:

- A large and carefully selected sample of postmortem brains from 13 male patients with schizophrenia and age-matched male controls was analyzed, including full medical records and autopsy records. All patients with schizophrenia met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria.
- Tissue processing was performed as follows: both hemispheres were cut into serial 600-700 μm -thick coronal sections (Heinsen and Heinsen, 1991). From each hemisphere, every second or third section was stained with galloxyanin. Adjacent sections not stained with galloxyanin were selected, and small pieces containing the desired brain region were cut out for investigation with immunohistochemistry (Kreczman-ski et al., 2005; 2007).
- "Space balls" technology (Schmitz and Hof, 2005): after delineation of the investigated brain regions, the sections processed with immunohistochemistry were brought into focus. Then hemispheres with radius of 30 μm (generated with a special stereology software [Stereoinvestigator; MBF Bioscience, Williston, VT, USA] while focusing through the section thickness) were systematically and randomly placed within the sections through the regions of interest. The capillary length density was obtained from the total number of intersections between the hemispheres and the capillaries as described considering the area of the delineated region of interest and the actual section thickness after tissue processing using the following equation:

$$CLD = \frac{2 \times \sum is(D_x \times D_y \times t)}{2 \times \pi \times r^3} \times \frac{1}{V}$$

where CLD represents the capillary length density, $\sum$ is the total number of intersections between the hemispheres and the capillaries, D_x and D_y are the distances between the midpoints of the centers of the hemispheres in directions X and Y , t is the actual average section thickness after histologic processing, r the radius of the hemispheres and V the investigated tissue volume.

- Minicolumn analysis: minicolumns are thought to be the basic cytoarchitectural entity of the mammalian neocortex, also known as cortical microdomain. Although the functional significance of minicolumns remains controversial (Jones, 2000; Hutslar and Galuske, 2003) they have been proposed as a primary cortical information processing unit (Mountcastle, 1997). Minicolumns are (two-dimensionally defined) detected in Nissl-stained sections in linear arrangements, perpendicular to the pial surface, of the neurons and a presence of a cell-free space on both sides of the column core (Buxhoeveden and Casanova, 2002b) creating a basic input/output unit that overlaps internal processing chains (Mountcastle, 1997). It has been hypothesised that middle layered neurons excited by incoming stimuli from thalamic afferents with only small differences in latency are tapered in vertical connections to both superficial and deep layers (Jones, 2000).

The analyses were performed under the hypothesis that a distinct pattern of histological alterations would emerge from these cortical and subcortical areas known to be involved in the neuropathology of schizophrenia. Specifically,

- Chapter 2 of this thesis describes morphological alterations of the cytoarchitecture in five subcortical regions of the brain of patients with schizophrenia (caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala), investigated with state-of-the-art design-based stereological techniques (focussing on volumes, neuron densities and total neuron numbers) (published in Kreczmanski et al., 2007);
- Chapters 3 and 4 describe results of investigations on microvasculature abnormalities suggested in the brain of patients with schizophrenia (published in Kreczmanski et al., 2005; 2008); and
- Chapters 5 and 6 describe minicolumnar abnormalities in the neocortex of the patients with schizophrenia, focussing on alterations in mean cell spacing (published in Casanova et al., 2005) and in the distribution of neurons in the neocortex (published in Casanova et al., 2008).

CHAPTER 2

Volume, neuron density and total neuron number in five subcortical regions in schizophrenia

Pawel Kreczmanski^{1,2}, Helmut Heinsen³, Valentina Mantua^{1,6,7}, Fritz Woltersdorf⁴
Thorsten Masson⁵, Norbert Ulfing⁴, Rainald Schmidt-Kastner¹, Hubert Korr^{1,2,5}
Harry W. M. Steinbusch^{1,2}, Patrick R. Hof⁸ and Christoph Schmitz^{1,2}

¹ Department of Psychiatry and Neuropsychology, Division of Cellular Neuroscience,
Maastricht University

² European Graduate School of Neuroscience (EURON), Maastricht, The Netherlands

³ Morphological Brain Research Unit, University of Wuerzburg, Wuerzburg

⁴ Department of Anatomy, University of Rostock, Rostock, Germany,

⁵ Department of Anatomy and Cell Biology, RWTH Aachen University, Aachen

⁶ Department of Psychiatry, Section of Clinical Neuropharmacology, Institute of Psychiatry,
Kings College London, London, UK

⁷ Department of Psychiatric Sciences and Psychological Medicine, Psychiatric Clinic III,
University of Rome La Sapienza, Rome, Italy

⁸ Department of Neuroscience, Mount Sinai School of Medicine, New York, NY, USA

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Abstract

Several studies have pointed to alterations in mean volumes, neuron densities and total neuron numbers in the caudate nucleus (CN), putamen, nucleus accumbens (NA), mediodorsal nucleus of the thalamus (MDNT) and lateral nucleus of the amygdala (LNA) in schizophrenia. However, the results of these studies are conflicting and no clear pattern of alterations has yet been established in these subcortical regions, possibly due to differences in quantitative histological methods used as well as differences in the investigated case series. The present study investigates these subcortical regions in both hemispheres of the same post-mortem brains for volume, neuron density and total neuron number with high-precision design-based stereology. The analysed case series consisted of 13 post-mortem brains from male schizophrenic patients [age range: 22–64 years; mean age 51.5 ± 3.3 years (mean $\pm$ SEM)] and 13 age-matched male controls (age range: 25–65 years; mean age 51.9 ± 3.1 years). A general linear model multivariate analysis of variance with diagnosis and hemisphere as fixed factors and illness duration (schizophrenic patients) or age (controls), post-mortem interval and fixation time as covariates showed a number of statistically significant alterations in the brains from schizophrenic patients compared with the controls. There was a reduced mean volume of the putamen [-5.0% on the left side (l) and -4.1% on the right side (r)] and the LNA (l: -12.1%, r: -17.6%), and a reduced mean total neuron number in the CN (l: -10.4%, r: -10.2%), putamen (l: -8.1%, r: -11.6%) and the LNA (l: -15.9%, r: -16.2%). These data show a previously unreported, distinct pattern of alterations in mean total neuron numbers in identified subcortical brain regions in a carefully selected sample of brains from schizophrenic patients. The rigorous quantitative analysis of several regions in brains from schizophrenic patients and matched controls is crucial to provide reliable information on the neuropathology of schizophrenia as well as insights about its pathogenesis.

Abbreviations

- ABNA – accessory basal nuclei of the amygdala
- BNA – basal nuclei of the amygdala
- CGM – cortical grey matter
- CN – caudate nucleus
- LNA – lateral nucleus of the amygdala
- MDNT – mediodorsal nucleus of the thalamus;
- NA – nucleus accumbens

Introduction

Schizophrenia is a devastating neuropsychiatric disorder that presents with a variety of cognitive disturbances in attention, working memory, verbal production and response monitoring and inhibition. Equally diverse are the putative mechanisms which translate deficits into schizophrenic pathology, and the anatomical substrates of neuron number in schizophrenia both the deficits and the pathology (Andreasen et al., 1999). Modern imaging techniques have indicated various discrete morphological alterations in the brain from schizophrenic patients. Based on meta-analyses, there is now a strong body of evidence in favour of an enlargement of the lateral ventricles (Harrison, 1999; Wright et al., 2000; Shenton et al., 2001; Kasai et al., 2002), as well as of a reduction of the total volume of the cerebral cortex bilaterally in schizophrenia (Harrison, 1999; Shenton et al., 2001).

Post-mortem studies have revealed subtle neuropathological abnormalities in several cortical regions in schizophrenia such as the prefrontal, entorhinal and anterior cingulate cortices (for review see Harrison, 1999; Tamminga et al., 2000; Selemon, 2001), as well as in subcortical regions such as the caudate nucleus (CN), putamen and nucleus accumbens (NA) (Bogerts, 1984; Beckmann and Lauer, 1997; Lauer and Beckmann, 1997), the mediodorsal nucleus of the thalamus (MDNT) (Pakkenberg, 1990; Popken et al., 2000; Young et al., 2000; Byne et al., 2002; Cullen et al., 2003; Dorph-Petersen et al., 2004; Danos et al., 2005) and the amygdala (Bogerts, 1984). However, many of the reported data are divergent if not mutually contradictory and no clear pattern of histological alterations has yet been established in schizophrenia. Such discrepancies can be explained by methodological differences, investigated parameters and particularly the available samples of brains from schizophrenic patients and controls (Harrison, 1999).

A possible solution to this situation is the analysis of several regions in brains from schizophrenic patients and matched controls with state-of-the-art quantitative histological techniques. We have used this approach recently in investigations of mean cell spacing abnormalities in the neocortex (Casanova et al., 2005) and capillary length densities in the frontal cortex (Kreczmanski et al., 2005) in a carefully selected sample of post-mortem brains from male schizophrenic patients and age/gender-matched controls. In the pres-

ent study, we have continued the analysis of these brains by investigating five subcortical regions [CN, putamen, NA, MDNT and lateral nucleus of the amygdala (LNA)] with a high-precision design-based stereology approach for possible alterations in volumes, neuron densities and total neuron numbers. The analyses were performed under the hypothesis that a distinct pattern of histological alterations would emerge from these subcortical areas known to be involved in the neuropathology of schizophrenia.

Material and methods

Brain specimens

This study was performed on the same post-mortem brains (both hemispheres) from 13 male schizophrenic patients [mean age 51.5 ± 3.3 years; mean post-mortem interval (time between death and autopsy) 27.5 ± 6.0 h; mean fixation time 912 ± 372 days; data given as mean $\pm$ SEM] and from 13 age-matched male controls (mean age 51.9 ± 3.1 years; mean post-mortem interval 23.7 ± 3.8 h; mean fixation time 247 ± 53 days) that were investigated in our previous studies for mean cell spacing abnormalities in the neocortex (Casanova et al., 2005) and for capillary length densities in the frontal cortex (Kreczmanski et al., 2005). The age of the patients, illness duration, clinical diagnoses, causes of death, the post-mortem interval and the fixation time are summarized in Table 1. The schizophrenic patients did not differ from the controls with respect to mean age (Student's two-tailed t-test; $P = 0.946$), mean post-mortem interval ($P = 0.581$) and mean fixation time ($P = 0.089$). All schizophrenic cases had been patients either in German university hospitals or in German State psychiatric hospitals (six hospitals including one university hospital in which some control patients were also treated for non-psychiatric or neurological illnesses) and full clinical records were available. All controls had been patients either in German university hospitals or in German local district hospitals (five hospitals including one university hospital in which schizophrenic patients were also treated). Records from autopsy (including a summary of the medical history) were available for all schizophrenic cases and all controls. All pathologists involved in the autopsies were instructed by H.H. and adhered to identical handling and processing conditions of the brains. All schizophrenic patients met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria. The clinical notes were assessed by two experienced clinical psychiatrists to ensure that the brains from the controls were free from psychopathology, and for clear evidence that the diagnosis of schizophrenic patients was concordant with DSM-IV criteria for schizophrenia. The mean age at onset was 22.9 ± 1.5 years. Exclusion criteria for both schizophrenic patients and controls comprised neurological problems that required intervention or interfered with cognitive assessment (e.g. stroke with aphasia), history of recurrent seizure disorder, history of severe head injury with loss of consciousness, diabetes

mellitus with free plasma glucose 4200 mg/dl and history of self-administered intoxication. Schizophrenic patients and controls were similar in terms of the ethnic backgrounds. However, they were not fully matched for socioeconomic status and education, which would have placed severe constraints on our sample. Moreover, all schizophrenic patients were subjected to long-term treatment with typical neuroleptics (because of the fact that most of the patients were not hospitalized throughout the duration of their illness, and, therefore, the clinical records did not cover fully the entire medication histories, it was not possible to calculate lifetime medication exposures). In all of the cases, autopsy was performed after consent was obtained from a relative according to the laws of the Federal Republic of Germany. The use of these autopsy cases for scientific investigations as outlined here has been approved by the relevant institutional review boards. Brains were fixed by immersion in 10% formalin (one part commercial 40% aqueous formaldehyde in nine parts H₂O) prior to histological processing.

Tissue processing

The brainstem with the cerebellum was separated from the forebrain at the level of the rostral pons, and the hemispheres were divided mediosagittally. Then, both hemispheres were cut into serial 600–700 µm-thick coronal sections as previously described (Heinsen and Heinsen, 1991). Briefly, the hemispheres were cryoprotected in a mixture of glycerol-dimethylsulphoxide-formalin after carefully removing the meninges and pial vessels, embedded in gelatin, deeply frozen at -60° C and serially sectioned using a cryomicrotome (Jung, Nussloch, Germany). One brain (C7) was embedded in celloidin as recently described (Heinsen et al., 2000) and was cut into serial 440–500 µm-thick coronal sections using a sliding microtome (Polycut, Cambridge Instruments, UK). From each hemisphere, every second or third section was stained with galloxyanin (a Nissl stain) as previously described (Heinsen and Heinsen, 1991). The remaining sections were stored in formalin for up to 10 years. From all the brains of the schizophrenic patients and controls older than 40 years, sections through the central portion of the entorhinal and transentorhinal cortex that were not stained with galloxyanin were labelled with the Gallyas method to detect neurofibrillary changes (Heinsen et al., 1989). Neurofibrillary tangles were very rarely detected in the transentorhinal and entorhinal cortex on Gallyas-stained sections, compatible with Braak's stage I (Braak and Braak, 1995). Furthermore, each section was coded and controlled for the absence of tumours, infarcts, heterotopias, signs of autolysis, staining artefacts and gliosis. Fixation and tissue processing were performed at the Morphological Brain Research Unit, University of Würzburg (Germany) under identical conditions for all brains (except for brain C7 that was embedded in celloidin instead of gelatin).

Investigated brain regions

The CN, putamen, NA, MDNT and LNA were investigated for their volume, total number of neurons and neuron density. Delineations of these regions were performed according to established criteria in the literature (CN, putamen and NA: Brockhaus, 1942; Lauer

and Heinsen, 1996; Holt et al., 1999; Lauer et al., 2001; MDNT: Dewulf, 1971; Hirai and Jones, 1989; Jones, 1997; Heinsen et al., 1999; Popken et al., 2000; Dorph-Petersen et al., 2004; LNA: Braak and Braak, 1983; Sims and Williams, 1990; Sorvari et al., 1996; Schumann and Amaral, 2005). In the case of the MDNT, the magnocellular, parvocellular and densocellular regions as well as the caudodorsal subdivision were included in the delineations, as also done by Popken et al. (2000), Byne et al. (2002) and Dorph-Petersen et al. (2004) (Fig. 1). In addition, the volumes of the cortical grey matter (CGM), the basal and accessory basal nuclei of the amygdala (BNA and ABNA) were determined. Delineations of the BNA and ABNA also followed the criteria established by Braak and Braak (1983), Sims and Williams (1990), Sorvari et al. (1996) and Schumann and Amaral (2005).

The boundaries of the investigated brain regions were identified on all sections showing the corresponding region using an Olympus SZX9 stereo microscope (Olympus; Tokyo, Japan) and were marked on the back side of the glass slides with a felt-tip pen. Identification and delineation of boundaries was performed by H.H. (CGM, CN, putamen), V.M. (NA), T.M. (MDNT) and F.W. (LNA, BNA and ABNA). Cross-validation (and, if necessary, slight modifications of the delineations) was performed by C.S. (CGM, CN, putamen) and C.S., H.H. and P.R.H. (all other brain regions).

Stereological analyses

Stereological analyses were performed with a stereology workstation, consisting of a modified light microscope [Olympus BX50 with PlanApo objective 1.25× [numerical aperture (N.A.) = 0.04] and UPlanApo objectives 10× (N.A. = 0.4), 20× (oil; N.A. = 0.8) and 40× (oil; N.A. = 1.0); Olympus, Tokyo, Japan, motorized specimen stage for automatic sampling (Ludl Electronics; Hawthorne, NY, USA) CCD colour video camera (HV-C20AMP; Hitachi, Tokyo, Japan) and stereology software StereoInvestigator, MicroBrightField, Williston, VT, USA). Volumes of brain regions were analysed using Cavalieri's principle [Cavalieri, 1635; see also Fig. 2 in Schmitz and Hof (2005)], by determining the projection area of a given brain region on each section showing this region, summing the data from all sections, and multiplying this value with the interval of selecting sections for staining with galloxyanin (two or three; see above) and the average actual section thickness after tissue processing [determined with the calibrated fine adjustment knob of an Olympus BH microscope and a Planapo objective (40×; N.A. $\frac{1}{4}$ 1.0) as described in Heinsen et al. (1994)]. The projection areas of the CGM were determined with point counting [Gundersen and Jensen, 1987; see also Fig. 1 in Schmitz and Hof (2005)] as already described in Kreczmanski et al. (2005). In contrast, the projection areas of the subcortical regions were determined by tracing their boundaries on each section on video images displayed on the monitor of the stereology workstation.

Total neuron numbers were evaluated with the optical fractionator [West et al., 1991; see also Figs 1 and 4 in Schmitz and Hof (2005)]. All neurons whose nucleus top came into focus within unbiased virtual counting spaces distributed in a systematic-random fashion throughout the delineated regions were counted. Then, total neuron numbers

were calculated from the numbers of counted neurons and the corresponding sampling probability. Details of the counting procedure for all investigated brain regions are summarized in Table 2.

In addition, the nearest neighbour distance distribution functions were determined for the neurons in the lateral nucleus of the right amygdala with the nearest neighbour method of the StereoInvestigator software. For each neuron in the lateral nucleus of the right amygdala that was counted with the unbiased virtual counting spaces, the neuron positioned closest to the counted neuron was detected [as explained in Schmitz et al. (2002)1]. Then, the distance between these neurons was calculated in three dimensions. From these individual nearest neighbour distances, the nearest neighbour distance distribution function in the lateral nucleus of the right amygdala was calculated for each investigated brain. Finally, neuron densities were calculated individually for each subcortical region as the ratio of the total neuron number in and the volume of this region.

Statistical analysis

For both schizophrenic patients and controls, mean and standard error of the mean were calculated for all investigated variables (except for the nearest neighbour distance distribution functions), separately for the left and the right hemispheres. Comparisons between schizophrenic patients and controls were performed using generalized linear model multivariate analysis (MANOVA), with diagnosis and hemisphere as fixed factors and the following variables as covariates: (i) the adjusted illness duration of the schizophrenic patients (calculated as individual age at death minus age at onset plus the mean age at onset of all schizophrenic patients) or the age of the controls, respectively, (ii) the post-mortem interval and (iii) the fixation time [note that use of the actual individual illness duration of the schizophrenic patients instead of the adjusted ones as covariate would have caused invalid results of the MANOVA model because there was no illness duration of the controls, and the mean illness duration of the schizophrenic patients was significantly different from the mean age of the controls (Student's two-tailed t-test; $P < 0.001$) whereas the mean adjusted illness duration was not ($P = 0.974$)]. For each investigated variable, all investigated brain regions were tested simultaneously. Post hoc tests in the analyses of covariance were performed with linear regression analysis. In all analyses an effect was considered statistically significant if its associated P-value was < 0.05 . Calculations were performed using SPSS (Version 12.0.1 for Windows, SPSS, Chicago, IL, USA). The nearest neighbour distance distribution functions of the neurons in the lateral nucleus of the right amygdala from the schizophrenic patients and the controls were graphically analysed with empirical distribution function (EDF) plots as explained in detail in Schmitz et al. (2002). The computer simulations necessary to perform these EDF plot analyses were carried out with Microsoft Visual Basic (version 6.0; Microsoft, Redmond, WA, USA); graphical analysis was done with GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA, USA).

Photography

Photomicrographs shown in Fig. 1A–F and N–S were produced by digital photography using the stereology workstation described above. On average, 120 images were captured for the composite in each Fig. 1A–C and Q–S, and 16 images for the composite in each Fig. 1D–F and N–P. These images were made into one montage using the Virtual Slice module of the StereoInvestigator software. Photomicrographs shown in Fig. 1G–M were produced by digital photography using an Olympus DP 70 digital camera attached to an Olympus AX 70 microscope and cellP software (version 2.3; Soft Imaging System, Muenster, Germany). The final figure was constructed using Corel Photo-Paint v.11 and Corel Draw v.11 (Corel, Ottawa, Canada). Only minor adjustments of contrast and brightness were made, without altering the appearance of the original materials.

Three-dimensional reconstructions

Reconstructions of LNA, BNA and ABNA in the right hemispheres were performed by digitally photographing close-up views of serial sections of the temporal lobe enclosing the amygdaloid complex with a digital single lens reflex camera (Fuji FinePix S2 Pro; Fuji Photo Film Co., Tokyo, Japan) and a 50 mm macro objective (1:2.8; Sigma, Kanagawa, Japan). Then the stacked photos were imported into Amira software (version 3.1; Mercury Computer Systems; San Diego, CA, USA). The serial sections were aligned manually in the editor window of Amira with the contours of the parahippocampal gyrus, fusiform gyrus, and the surface of the anterior perforated substance serving as reference structures in the alignment of the individual sections. This coarse alignment was supplemented by a computer-assisted fine-tuned automatic alignment. Afterwards, the outlines of the LNA, BNA and ABNA were identified and manually traced in the image segmentation editor of Amira. With these outlines and the average section thickness, the software generated surface views of the investigated nuclei of the amygdala. Irregular contours were smoothed by additional editors.

Results

Compared with controls the schizophrenic patients showed a significantly reduced mean volume of the fixed factor Diagnosis in the MANOVA1, the putamen [l: -5.0%, r: -4.1%; $F(1) = 5.864$, $P = 0.0201$, the BNA [l: -10.6%, r: -11.9%; $F(1) = 4.447$, $P = 0.0421$ and the LNA [l: -12.1%, r: -17.6%; $F(1) = 8.661$, $P = 0.006$] (Fig. 2; Table 3 shows all P-values of the MANOVAs performed). The smaller volumes of the BNA and LNA in the brains from the schizophrenic patients were also visible in three-dimensional reconstructions of the investigated nuclei of the amygdala (Fig. 3). Notably, not only the volume of the BNA and LNA was smaller in the schizophrenic patients than in the controls but also the combined volume of the BNA, LNA and accessory BNA {-11.3% on the left side [schizophrenic patients: $621 \pm 24 \text{ mm}^3$;

controls: $700 \pm 28 \text{ mm}^3$; mean $\pm$ SEM; P (Student's t -test) = 0.0421 and -14.7 on the right side [schizophrenic patients: $603 \pm 23 \text{ mm}^3$; controls: $706 \pm 29 \text{ mm}^3$; $P = 0.011$].

With respect to the mean neuron density in the investigated subcortical regions, there were no significant differences between the schizophrenic patients and the controls (Fig. 4). In contrast, the schizophrenic patients had a significantly reduced mean total neuron number in the CN [l: -10.4%, r: -10.2%; $F(1) = 7.088$, $P = 0.011$], the putamen [l: -8.1%, r: -11.6%; $F(1) = 8.733$, $P = 0.005$] and the LNA [l: -15.9%, r: -16.2%; $F(1) = 6.498$, $P = 0.015$] compared with controls (Fig. 5).

Furthermore, no significant differences between the left and the right hemispheres were found for the investigated variables in both schizophrenic and control cases ($P > 0.05$ for the fixed factor Hemisphere in all MANOVAs performed). The post-mortem interval had a significant effect on the volume of the putamen [$F(1) = 9.553$, $P = 0.004$] and the total neuron number in this brain region [$F(1) = 6.899$; $P = 0.012$] (see Fig. S1 in the Supplementary online material). A significant effect was also found for the control patients' age (or the illness duration of the schizophrenic patients, respectively) on the total neuron number in the CN [$F(1) = 4.197$, $P = 0.0471$ and the NA [$F(1) = 4.224$, $P = 0.0461$]. However, post hoc linear regression analysis revealed only a significant, positive correlation between the illness duration and the total neuron number in the left CN in the brains from the schizophrenic cases [$r^2 = 0.349$, $F(1,11) = 5.893$, $P = 0.034$]. Furthermore, disregarding the total neuron numbers in the CN from the 22-year-old schizophrenic patient, S1 had no significant effect on the results from the MANOVA but abolished the significant effects in the post hoc linear regression analysis concerning the correlation between the illness duration and the total neuron number in the left CN in the brains from the schizophrenic cases [$r^2 = 0.213$, $F(1, 10) = 2.704$, $P = 0.131$]. It can be therefore concluded that the alterations in mean volumes and mean total neuron numbers found in the investigated subcortical regions in the brains from the schizophrenic patients were not caused by the control patients' age (or the illness duration of the schizophrenic patients, respectively), the post-mortem interval or the fixation time of the brains.

The nearest neighbour distance distribution function analysis of neurons in the lateral nucleus of the right amygdala showed no distinct differences between the schizophrenic patients and the controls (see Fig. S2 in the Supplementary online material). Finally it should be mentioned that the results obtained for the single brain embedded in celloidin (C7) showed no systematic deviation from the results obtained for the other brains (Figs 2, 4 and 5).

Discussion

The present study revealed a distinct pattern of subtle neuropathological alterations in a carefully selected sample of brains from schizophrenic patients. This previously unrecognized set of alterations comprises reduced volumes of the total CGM, putamen and LNA as

well as reduced total neuron numbers in the CN, putamen and LNA (note that the analysis of neuron densities showed no differences between schizophrenic patients and controls). Reports in the literature of reduced volumes of the NA and MDNT as well as reduced total neuron numbers in these subcortical regions in schizophrenia could not be confirmed. Furthermore, no correlation between the illness duration of the schizophrenic patients and the observed neuropathological alterations was found. The results from our control cases are in line with previous studies (summarized in Tables 4 and 5). The results obtained for the schizophrenic patients can be compared with data in the literature as discussed below (see also Tables 4 and 5). In addition, a significant decrease in the total CGM volume (-9.3%; averaged for the left and right hemispheres) was found when comparing the schizophrenic patients with the controls, in agreement with several reports in the literature (e.g. Zipurski et al., 1992, 1997; McCarley et al., 1999; Shenton et al., 2001).

Clinical neuroimaging studies have shown an enlargement of the striatum in relation to treatment with typical neuroleptics (Chakos et al., 1994; Shenton et al., 2001; Lang et al., 2004). This is in contrast to the results of a post-mortem study by Beckmann and Lauer (1997) who reported no differences in mean volumes of the CN and the putamen when comparing schizophrenic patients treated with typical neuroleptics to controls (-0.3% and +1.9%, respectively; $P > 0.05$;) as well as to a report by Bogerts (1984) who found a trend towards reduced mean volumes of the CN (-4.5%; $P > 0.05$) and the putamen (-4.1%; $P > 0.05$) when comparing schizophrenic neuroleptic-naïve patients with controls. Notably, several clinical neuroimaging studies reported a decrease in volume of the striatum in drug-naïve schizophrenic patients [reviewed in Shenton et al. (2001)], as well as reduced basal ganglia volumes after switching to olanzapine in schizophrenic patients chronically treated with typical neuroleptics (Chakos et al., 1994; Frazier et al., 1996; Lang et al., 2004). Accordingly, increased volume of the striatum in schizophrenia as found in certain clinical neuroimaging studies might reflect a specific, currently unknown action of typical neuroleptics that is reversible by switching to atypical neuroleptics or during histological processing. In a post-mortem study, Beckmann and Lauer (1997) compared nine schizophrenic patients with nine matched controls, and found an increased total neuron number in the CN [$P < 0.05$ only for the right hemisphere (difference: +17.5%) but not for the left hemisphere (difference: +15.8%)]¹ and a trend towards an increased total neuron number in the putamen (+9.8%; $P > 0.05$) in the brains of the schizophrenic patients. This is in contrast to the finding of reduced total neuron numbers in the CN (-10.2%) and putamen -9.9%) in the brain of the schizophrenic patients in the present study. The reason for this discrepancy is unknown and might be related to differences in the stereological analysis [the number of unbiased virtual counting spaces used to estimate total neuron numbers was considerably lower in the study of Beckmann and Lauer (1997) than in the present study, implying a considerably higher variation in estimated total neuron numbers (Schmitz, 1998; Schmitz and Hof, 2000, 2005)].

Pakkenberg's (1990) early report of a reduction in the mean volume of the NA in schizophrenia by 42% (and in brain regions in a three-dimensional impression during

microscopic inspection. The MDNT has been the focus of several studies applying design-based stereological techniques over the last 20 years (Table 5). Pakkenberg (1990), Popken et al. (2000) and Byne et al. (2002) reported a reduced mean volume and a reduced mean total neuron number in the MDNT in the brains of schizophrenic patients compared with controls (the mean total neuron number in this nucleus by 50%) should be taken cautiously, as was already discussed by Lauer et al. (2001). Pakkenberg's (1990) data are also in contrast to Bogerts's (1984) finding of almost no difference in mean volumes of the NA between brains from 14 schizophrenic patients and 13 controls (+1.2%; $P > 0.05$). The discrepancy between the results of Pakkenberg (1990), those of Bogerts (1984), Lauer et al. (2001) and the present study might be due to differences in stereological design. For instance, Pakkenberg (1990) performed microscopic investigations on 4 μm -thick paraffin sections stained with galloxyanin. However, exact delineations of the borders of subcortical brain regions on such sections might be much more difficult than on 20 μm -thick paraffin sections processed with a myelin stain [as performed by Bogerts (1984)], on 500 μm -thick (Lauer et al., 2001) or on 440–700 μm -thick (present study) sections stained with galloxyanin, facilitating the identification of borders of brain regions in a three-dimensional impression during microscopic inspection.

The MDNT has been the focus of several studies applying design-based stereological techniques over the last 20 years (Table 5). Pakkenberg (1990), Popken et al. (2000) and Byne et al. (2002) reported a reduced mean volume and a reduced mean total neuron number in the MDNT in the brains of schizophrenic patients compared with controls (studies with positive outcome; PO studies), whereas Cullen et al. (2003), Kemether et al. (2003), Dorph-Petersen et al. (2004), Danos et al. (2005) and the present study could not confirm these findings (studies with negative outcome; NO studies). Differences in study design may again explain this discrepancy (details are provided in Table 5). First, the PO studies had on average smaller sample sizes than the NO studies (PO studies: 9.3 schizophrenic patients versus 7.7 controls; NO studies: 21.2 versus 25.4); second, except in the study by Dorph-Petersen et al. (2004), in all NO studies, both left and right MDNTs were investigated, whereas in the PO studies, only either the left or the right MDNTs were analysed, and third, the average age of the schizophrenic patients and controls was higher in the PO studies than in the NO studies [PO studies: 65 years (schizophrenic patients)] versus 65 years (controls); NO studies: 49 versus 51 years). However, small sample sizes and additional, confounding pathologies (in this case possible age-related neurodegeneration) have been identified as major potential shortcomings in most studies addressing the neuropathology of schizophrenia (Harrison, 1999).

Meta-analyses of clinical neuroimaging studies found reductions in the mean volume of the amygdala in schizophrenic patients compared with controls in the range of 5–10% (Lawrie and Abukmeil, 1998; Nelson et al., 1998; Wright et al., 2000; see also Honea et al., 2005). Although these clinical neuroimaging studies did not differentiate between the nuclei of the amygdala, these data are in line with the results of the present study as well as with results from a post-mortem study by Bogerts (1984) who found a significantly re-

duced volume of the amygdala in schizophrenic patients compared with controls (-22%). In contrast, Heckers et al. (1990) and Chance et al. (2002) did not observe volume reductions of the amygdala in schizophrenia in post-mortem studies; the reason for this discrepancy is not known. Nevertheless, Chance et al. (2002) suggested that there may be alterations in other morphological aspects of the amygdala such as cellular composition, as found in the present study.

It should be mentioned that several reports have suggested schizophrenia being associated with a disturbance of cerebral asymmetry (for review see Crow, 1990, 1997; Petty, 1999). This did not apply to the findings of the present study in which no significant differences between the left and the right hemispheres were found for the schizophrenic patients and the controls. This is in agreement with other studies of subcortical pathology in schizophrenia. For instance, a meta-analysis of clinical neuroimaging studies, in which a volume of 100% in the comparison group was assumed, found for patients with schizophrenia an overall volume of 94% in the left and right amygdala (Wright et al., 2000). Disturbance of cerebral asymmetry in schizophrenia might therefore be restricted to discrete cortical regions such as the dorsolateral prefrontal cortex (Cullen et al., 2006).

What then, in this context, does the pattern of rather subtle neuropathological alterations reported in the present study contribute to our understanding of the pathogenesis of schizophrenia? Generally, two non-exclusive hypotheses for the development of schizophrenia have been proposed, a neurodevelopmental one (Lieberman, 1999; Tsuang, 2000; Lewis and Levitt, 2002; Murray et al., 2004) and a neurodegenerative one (Ashe et al., 2001; Sawa and Snyder, 2002; Church et al., 2002). A neurodevelopmental origin for schizophrenia has become the prevailing pathogenic hypothesis in recent decades (Weinberger, 1987; Lewis and Levitt, 2002; Murray et al., 2004). Specifically, genetic and non-genetic mechanisms are thought to interact, in as yet not understood ways, to affect the developing brain, resulting in a predisposition to schizophrenia (see, e.g. Schmidt-Kastner et al., 2006). Although several genes have been proposed as candidates for schizophrenia (for review see Davis et al., 2003; Harrison and Owen, 2003; Owen et al., 2004; Harrison and Weinberger, 2005; Kirov et al., 2005), genetic defects may not account for all aspects of the pathogenesis of schizophrenia (Kendler, 2005). There is no direct evidence for a neurodevelopmental or a neurodegenerative origin of the results of the present study. Yet, considering that the reductions in both volumes of brain regions (CGM, putamen, LNA) and total neuron numbers (CN, putamen and LNA) did not correlate with illness duration, it is tempting to speculate that such negative correlation is indicative of a neurodevelopmental deficit. Particularly in respect to the alterations found in the LNA, it is noteworthy that early postnatal lesions of the amygdala in rats lead to a combination of behavioural changes which share features with symptoms of schizophrenia, whereas adult amygdala lesions do not produce such changes (Wolterink et al., 2001). Furthermore, tract-tracing studies have shown that connections between the basolateral amygdala and the prefrontal cortex (including the anterior cingulate cortex) mature slowly during postnatal development in rats (Bouwmeester et al., 2002; Cunningham et al., 2002). A slow maturation of connections

may also be important for the primate brain, because lesions of mesial temporal limbic structures, including the amygdala, in postnatal monkeys (but not in mature monkeys) are associated with abnormal function of the prefrontal cortex of adult animals (Bertolino et al., 1997). Accordingly, alterations in the prolonged maturation of connections between the amygdala and the prefrontal cortex have been hypothesized to be involved during the onset of schizophrenia in adolescence (Benes, 2003). Reduced total neuron numbers in the LNA based on a neurodevelopmental deficit could play an important role in these pathological processes and deserves further investigation.

Deficiencies in limbic functions which many researchers believe are the cause of the productive symptoms of schizophrenia such as paranoia and psychosis (Alexander et al., 1990; Bogerts, 1997; Weinberger, 1997) must have another neuropathological basis than reductions in the total neuron number in the NA [as suggested by Thune and Pakkenberg (2000)]. Likewise, involvement of MDNT in positive symptoms of schizophrenia [as discussed by, e.g. Andreasen et al. (1994), Friston (1999) and Stephan et al. (2006)] must have another neuropathological basis than reductions in the total neuron number in this nucleus [as suggested by, e.g. Thune and Pakkenberg (2000)]. Abnormal anatomical connections (i.e. 'miswiring' of association fibres), impairments in synaptic transmission and plasticity or complex combinations of both are the most relevant candidates in this regard (Stephan et al., 2006).

Furthermore, the hyperdopaminergic state of the striatum is one of the most important aspects of the pathophysiology and treatment of schizophrenia, but the mechanisms leading to increased dopamine levels in the basal ganglia are more complex than initially thought (Abi-Dargham et al., 2000; Carlsson et al., 2001; Winterer and Weinberger, 2004). The small decrease in neurons found in the present study could be due to a prolonged neurodevelopmental perturbation affecting several processes in the striatum, including neurogenesis and the innervation by dopaminergic mesencephalic fibres. A simple mismatch between neuronal numbers and dopaminergic innervation, however, is unlikely to explain long-lasting effects on neurotransmission, because glutamatergic cortical inputs and transmitters of striatal interneurons also come into play. Finally, the reduced number of neurons in LNA found in the present study could be involved in emotional disturbance in schizophrenia. According to Yaniv et al. (2004), LNA is a secondary interface limited to relatively simple, unimodal conditioned stimulus features, whereas BNA serves as an amygdalar sensory interface for complex, configural stimulus information. On the other hand, at least in rat, LNA is reciprocally connected with both BNA and ABNA (Savander et al., 1997). Accordingly, a reduced number of neurons in the LNA could result in impaired function of BNA in schizophrenia, with potential influence on at least two functional systems. First, the BNA interacts with striatal and cortical motor circuits, allowing active response to emotional arousing stimuli which is impaired in schizophrenia (Aleman and Kahn, 2005). Second, impaired output of the basolateral amygdala (including LNA and BNA) to the central nucleus of the amygdala (CNA) could disturb information flow from the CNA to brainstem areas involved in controlling specific involuntary components of emotional reac-

tion (such as autonomic and endocrine responses) that are also disturbed in schizophrenia (Aleman and Kahn, 2005). Notably, the reduced number of neurons in the LNA could even be involved in alterations of the dopaminergic system in schizophrenia. This is due to the fact that all subdivisions of the amygdala project to the ventral striatum and therefore can indirectly influence dopaminergic neurons through amygdalo-striato-nigral loops (Haber and Fudge, 1997; Fudge et al., 2002; Fudge and Emiliano, 2003).

In summary, the results of the present study support a neurodevelopmental deficit in certain subcortical regions in schizophrenia, primarily affecting the development of the basolateral amygdala and the dorsal striatum. Further studies are required to address other neuropathological alterations in the prefrontal cortex and the dopaminergic system in schizophrenia and their possible interactions with disturbances of the development of the basolateral amygdala and dorsal striatum.

Supplementary material

Supplementary material is available at Brain Online.

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Tables

Table 1. Clinical characteristics of all cases included in this study

No	A	O	Cause of death	PMI	Fix	Diagnosis	
						DSM-IV	ICD-10
	(y)	(y)		(h)	(d)		
S1	22	19	Suicidea	88	130	295.30	F20.00
S2	36	28	Suicidea	<72	115	295.30	F20.00
S3	46	24	Systemic hypothermia	<24	327	295.30	F20.01
S4	50	17	Peritonitis	<24	203	295.30	F20.00
S5	50	22	Suicide	18	170	295.30	F20.00
S6	51	17	Septicaemia	33	127	295.60	F20.50
S7	54	20	Septicaemia	27	250	295.60	F20.50
S8	55	22	Right-sided heart failure	25	84	295.30	F20.00
S9	57	37	Septicaemia	76	163	295.30	F20.00
S10	60	24	Pulmonary embolism	<48	311	295.30	F20.01
S11 ^b	62	19	Aspiration	7	171	295.30	F20.00
S12	63	22	Acute myocardial infarct	15	338	295.60	F20.50
S13	64	26	Pulmonary embolism	6	817	295.60	F20.50
C1	25	–	Cardiac tamponade	14	119	–	–
C2	36	–	Gunshot	24	143	–	–
C3	47	–	Acute myocardial infarct	<24	133	–	–
C4	50	–	Acute myocardial infarct	35	433	–	–
C5	50	–	Avalanche accident	23	498	–	–
C6	51	–	Septicaemia	7	285	–	–
C7	54	–	Acute myocardial infarct	18	168	–	–
C8	56	–	Acute myocardial infarct	60	3570	–	–
C9	58	–	Acute myocardial infarct	28	126	–	–
C10	60	–	Gastrointestinal haemorrhage	18	101	–	–
C11 ^b	60	–	Gastrointestinal haemorrhage	27	302	–	–
C12	62	–	Acute myocardial infarct	<24	3696	–	–
C13	65	–	Bronchopneumonia	6	2289	–	–

S = schizophrenic patient; C = control; A = age at death; O = age at onset; PMI = post-mortem interval (time between death and autopsy); Fix = fixation time; y = years; h = hours; d = days. ^aThese two patients had relatively long post-mortem intervals. However, both had committed suicide (one by hanging, the other by jumping from a building), were found within 1h of death and were kept at 4°C until autopsy. Accordingly, the post-mortem intervals between death and autopsy of these patients cannot be compared with the corresponding intervals of the other cases and were thus excluded from the calculation of the mean post-mortem intervals. ^bThe volume of the CGM of these cases could not be analysed because sections from the frontal as well as the occipital poles were missing.

Table 2. Details of the stereological counting procedures

	CN	P	NA	MDNT	LNA
Obj.	40	40	40	20	40
B (μm^2)	10000	10000	3600	15298	3600
H(mm)	30	30	20	15	10
D(mm)	3000	3000	600	800	600
ΣOD	171	196	461	626	767
ΣQ	755	761	499	1071	1110
CE pred (n)	0.036	0.036	0.048	0.031	0.031

CN = caudate nucleus; P = putamen; NA = nucleus accumbens; MDNT = mediodorsal nucleus of the thalamus; LNA = lateral nucleus of the amygdala; Obj. = objective used; B and H = base and height of the unbiased virtual counting spaces; D = distance between the unbiased virtual counting spaces in mutually orthogonal directions x and y; ΣOD = average sum of unbiased virtual counting spaces used; ΣQ = average number of counted neurons; CEpred(n) = average predicted coefficient of error of the estimated total numbers of neurons using the prediction method described by Schmitz (1998) and Schmitz and Hof (2000).]

Table 3. Results of statistical analysis (P-values) with generalized linear model MANOVA

Variable	Brain region	A/ID	PMI	Fix	D	H	D x H
Volume	CGM	0.299	0.609	0.223	0.008	0.392	0.601
	CN	0.143	0.973	0.961	0.209	0.436	0.860
	P	0.082	0.004	0.169	0.020	0.782	0.929
	NA	0.600	0.136	0.572	0.299	0.394	0.842
	MDNT	0.581	0.125	0.574	0.161	0.225	0.546
	BNA	0.335	0.851	0.422	0.042	0.404	0.926
	ABNA	0.515	0.463	0.416	0.858	0.053	0.497
	LNA	0.900	0.628	0.462	0.006	0.988	0.520
Neuron density	CN	0.916	0.617	0.727	0.866	0.627	0.676
	P	0.929	0.893	0.834	0.393	0.897	0.559
	NA	0.054	0.204	0.118	0.735	0.209	0.955
	MDNT	0.442	0.188	0.800	0.105	0.781	0.987
	LNA	0.126	0.892	0.507	0.786	0.835	0.389
Total neuron number	CN	0.047	0.861	0.297	0.011	0.764	0.932
	P	0.062	0.012	0.129	0.005	0.792	0.707
	NA	0.046	0.856	0.089	0.200	0.893	0.933
	MDNT	0.818	0.555	0.975	0.419	0.529	0.698
	LNA	0.170	0.608	0.898	0.015	0.591	0.926

CGM = cortical grey matter; CN = caudate nucleus; P = putamen; NA = nucleus accumbens; MDNT = mediodorsal nucleus of the thalamus; BNA = basal nucleus of the amygdala; ABNA = accessory basal nucleus of the amygdala; LNA = lateral nucleus of the amygdala; A/ID = age (controls) or adjusted illness duration (schizophrenic patients), respectively (adjusted illness duration calculated as age at death minus age at onset plus mean age at onset; details are provided in Statistical analysis); PMI = post-mortem interval (time between death and autopsy); Fix = fixation time; D = diagnosis; H = hemisphere. P-values smaller than 0.05 are shown in boldface.

Table 4. Reports in the literature of estimated mean volumes of and estimated total neuron numbers in four subcortical regions in the brains of controls and schizophrenic patients (averaged for left and right hemispheres)

Source	CN	P	NA	LNA
Volume data for all control casus (all values in mm ³)				
Present study	2212	2713	204	414
1	2043a	2681a	522a	
2	2565 b,c	2979b		
3			147d	
4			138	
5				452
Volume data for schizophrenic patients (compared with controls)				
Present study	-9,1%(ns)	-4,6% (ns)*	-8,8% (ns)	-15,0%*
1	-4,5% (ns)	-4,1% (ns)	+1,2% (ns)	
2	-0.3% (ns)	+1.9% (ns)		
3			+10.5% (ns)	
4			-42.0%*	
Total neuron number data for control cases (all values in millions)				
Present study	32,7	35,4	2,43	4,43
4	28,2e	30,0		
5			2,93	
6				4,00
Total neuron number data for schizophrenic patients compared with controls)				
Present study	-10.2%*	-9.9%*	-2.1% (ns)	-16.0%*
6	+16.6%*,f	+9.8% (ns)		
4			-49,5%*	

CN = caudate nucleus; P = putamen; NA = nucleus accumbens; LNA = lateral nucleus of the amygdala. 1 = Borgerts (1984), a considering an average shrinkage factor of 0.53 reported in this study; 2 = Lauer and Beckmann (1997), b considering an average shrinkage factor of 0.53 reported in this study, c including the NA; 3 = Lauer et al. (2001), d considering an average shrinkage factor of 0.47 reported in this study; 4 = Pakkenberg et al. (1990); 5 = Schumann and Amaral (2006); 6 = Beckmann and Lauer (1997), e including the NA, f P<0.05 only for the right hemisphere (difference: +17.5%) but not for the left hemisphere (difference: +15.8%). ns = not significant (i.e. P>0.05), *P<0.05.

Table 5. Reports in the literature of estimated mean volumes of and estimated total neuron numbers in the MDNT in the brains of controls and schizophrenic patients (averaged for left and right hemispheres)

Source	V (mm ³)	NN (x106)	n (S/C)	H	Sex S (M/F)	C (M/F)	Age (S/C)
Data for controls							
1	360	1.81	12 (C)	l		6/6	62 (C)
2	863	3.48	6 (C)	l		5/1	64 (C)
3	701a	3.17a	5a (C)	r		4/1a	75 (C)
4	680	3.88	27 (C)	l+r		14/13	71 (C)
5	675b	-	60 (C)	l+r		45/15	40 (C)
6	916	7.29	9 (C)	l		6/3	54 (C)
7	956	6.22	18 (C)	l+r		10/8	53 (C)
8	569	3.79	13 (C)			13/0	52 (C)
Data for schizophrenic patients (compared to controls)							
1	-28.9%*	-40.3%*	12/12	l	8/4	6/6	63/62
2	-17.8%*	-27.0%*	6/6	l	5/1	5/1	66/64
3	-15.0%a,*	-29.7% a,*	10/5a	r	4/6a	4/1a	67/75
4	+4.7% (ns)	+5.2% (ns)	21/27	l+r	11/10	14/13	68/71
5	-6.7% (ns)	-	41/60	l+r	32/9	45/15	37/40
6	+2.1% (ns)	-6.5%(ns)	11/9	l	7/4	6/3	48/54
7	-6.9%(ns)	-6.8%(ns)	20/18	l+r	10/10	10/8	53/53
8	-8.8%(ns)	+4.6%(ns)	13/13	l+r	13/0	13/0	52/52

V = volume; NN = total neuron number; n = number of analysed brains; H = hemisphere; S = schizophrenic patients; C = controls; M = male; F = female; l = left; r = right. 1 = Pakkenberg et al. (1990); 2 = Popken et al., 2000; 3 = Byne et al. (2002), a without the schizophrenic patients and controls suffering from Alzheimer's disease analysed in this study; 4 = Cullen et al. (2003); 5 = Kemether et al. (2003), b MRI study; 6 = Dorph-Petersen et al. (2004); 7 = Danos et al. (2005); 8 = present study. Ns = not significant (i.e. P>0.05), *P<0.05. Data of the present study are given boldfaced.

Figures

Fig. 1. Photomicrographs of 600 μm -thick frontal sections of the right hemisphere from the control CII (60 years old; A-I) and from the schizophrenic patient S13 (64 years old; K-S), showing the striatum (A, D, G, K, N, Q), the amygdala (B, E, H, L, O, R) and the thalamus (C, F, I, M, P, S). In D-F and N-P, respectively, the borders of the CN, putamen (P), NA (D, N), ABNA, BNA and LNA (E, O), and MDNT (F, P) are indicated. The high-power photomicrographs showing neurons in the NA (G, K), LNA (H, L) and MDNT (I, M) are representative of the magnification at which the stereological estimates were obtained. Scale bar = 23 mm in A-C and Q-S, 10 mm in D-F and N-P, and 50 μm in G-M.

Fig. 2. Volumes of CGM (A, I, R), CN (B, K, S), putamen (P; C, L, T), NA (D, M, U), MDNT (E, N, V), BNA (F, O, W), ABNA (G, P, X) and LNA (H, Q, Z) in both hemispheres of the brains from 13 male schizophrenic patients (S; open bars in A-H, and dots in R-Z) and 13 age-matched controls [C; closed bars in A-H, and dots in I-Q; squares in case of the results obtained for the single brain embedded in celloidin (C7)]. In A-H, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from schizophrenic patients (S-l and S-r) and controls (C-l and C-r). In I-Z, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (I-Q) and schizophrenic patients (R-Z) are shown as function of the control patients' age (or the illness duration of the schizophrenic patients, respectively). *P<0.05 for the fixed factor Diagnosis in general linear model MANOVA.

Fig. 3. Reconstructions of the LNA (green), BNA (red) and ABNA (magenta) in the right hemisphere from the control C11 (60 years old) and from the schizophrenic patient S13 (64 years old) in rostral -caudal view (left column), caudal - rostral view (middle column) and dorsal - ventral view (right column). Compared with the volume of the LNA from C11 (set to 100%), the relative volume of this nucleus was 71.2% in S13. For the BNA, the corresponding values were 76.9% (S13/C4) and for the ABNA, 62.8%. Scale bar = 1 cm.

Fig. 4. Neuron density in CN (A, F, L), putamen (P; B, G, M), NA (C, H, N), MDNT (D, I, O) and LNA (E, K, P) in both hemispheres of the brains from 13 male schizophrenic patients (S; open bars in A-E, and dots in L-P) and 13 age-matched controls [C; closed bars in A-E, and dots in F-K; squares in case of the results obtained for the single brain embedded in celloidin (C7)]. In A-E, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from schizophrenic patients (S-l and S-r) and controls (C-l and C-r). In F-P, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (F-K) and schizophrenic patients (L-P) are shown as function of the control patients' age (or the illness duration of the schizophrenic patients, respectively).

Fig. 5. Total neuron number in CN (A, F, L), putamen (P; B, G, M), NA (C, H, N), MDNT (D, I, O) and LNA (E, K, P) in both hemispheres of the brains from 13 male schizophrenic patients (S; open bars in A-E, and dots in L-P) and 13 age-matched controls [C; closed bars in A-E, and dots in F-K; squares in case of the results obtained for the single brain embedded in celloidin (C7)]. In A-E, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from schizophrenic patients (S-l and S-r) and controls (C-l and C-r). In F-P, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (F-K) and schizophrenic patients (L-P) are shown as function of the control patients' age (or the illness duration of the schizophrenic patients, respectively). * $P < 0.05$ for the fixed factor diagnosis in general linear model MANOVA.

CHAPTER 3

Microvessel length density, total length, and length per neuron in five subcortical regions in schizophrenia

Pawel Kreczmanski^{1,2}, Helmut Heinsen³, Valentina Mantua^{1,4,5}
Fritz Woltersdorf⁶, Thorsten Masson⁷, Norbert Ulfing⁶, Rainald Schmidt-Kastner¹
Hubert Korr^{1,2}, Harry W.M. Steinbusch^{1,2}, Patrick R. Hof⁸, and Christoph Schmitz^{1,2}

¹School for Mental Health and Neurosciences, Division of Cellular Neuroscience, Maastricht University, Maastricht, The Netherlands

²European Graduate School of Neuroscience (EURON), Maastricht, The Netherlands

³Morphological Brain Research Unit,
Psychiatric Clinic, University of Wuerzburg, Wuerzburg, Germany

⁴Department of Psychiatry, Section of Clinical Neuropharmacology,
Institute of Psychiatry, Kings College London, London, United Kingdom

⁵Department of Psychiatric Sciences and Psychological Medicine,
Psychiatric Clinic III, University of Rome La Sapienza, Rome, Italy

⁶Department of Anatomy, University of Rostock, Rostock, Germany

⁷Department of Anatomy and Cell Biology,
RWTH Aachen University, Aachen, Germany

⁸Department of Neuroscience, Mount Sinai School of Medicine,
New York, NY, USA

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Abstract

Previous studies (Prabakaran et al. [2004] *Mol Psychiat* 9:684-697; Hanson and Gottesman [2005] *BMC Med Genet* 6:7) have suggested that microvascular abnormalities occur in the brain of patients with schizophrenia. To assess the integrity of the microvasculature in sub-cortical brain regions in schizophrenia, we investigated the microvessel length density, total microvessel length, and microvessel length per neuron using design-based stereologic methods in the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala in both hemispheres of 13 postmortem brains from male patients with schizophrenia and 13 age-matched male controls. A general linear model multivariate analysis of variance with diagnosis and hemisphere as fixed factors and illness duration (patients with schizophrenia) or age (controls), postmortem interval and fixation time as covariates showed a number of statistically significant differences in the brains from the patients with schizophrenia compared to the controls. There was an increased mean microvessel length density in the caudate nucleus (+6.3% on the left side [l] and +5.8% on the right side [r]) and the mediodorsal nucleus of the thalamus (l: +4.3%, r: +5.1%), and a reduced total microvessel length in the lateral nucleus of the amygdala (l: -17.5%, r: -10.8%). No significant differences between patients with schizophrenia and controls were found for the microvessel length per neuron in any of the investigated regions. These data extend our earlier findings in prefrontal cortex area 9 and anterior cingulate cortex area 24 from the same brains (Kreczmanski et al. [2005] *Acta Neuropathol* 109:510-518), that alterations in microvessel length density, total length, and particularly length per neuron cannot be considered characteristic features of schizophrenia. As such, compromised brain metabolism and occurrence of oxidative stress in the brains of patients with schizophrenia are likely caused by other mechanisms.

Introduction

Schizophrenia is a mental illness characterized by psychosis, cognitive impairment and social withdrawal, and is the most disabling among psychiatric disorders [58]. A combination of genetic susceptibility and environmental perturbations appears to be necessary for the expression of the disease [1, 30, 66, 69, 73]. However, the mechanisms involved in the pathogenesis of schizophrenia remain elusive [29, 30, 69, 73]. Possibilities include alterations of highly specific developmental processes at the synaptic level [28, 52], abnormalities in myelination [26, 71], as well as global metabolic changes of brain function and oxidative stress [2, 8, 56]. Subtle metabolic deficits such as hypoxia have long been suspected to cause widespread neuronal dysfunction in schizophrenia [42, 43] (see also [44]). Because the function of neurons depends on adequate oxygen and energy substrate supply, subtle changes in vascular integrity may play a role in the development of schizophrenia. Routine neuropathological observations indicate that there are no differences in the frequency of large or small blood vessel alterations in the brain of patients with chronic schizophrenia compared to controls [3] but there are no systematic studies of the brain microvasculature system in this disease. Whereas a genetic and inflammatory vascular mechanism has been proposed for schizophrenia [27], neuropathological evidence for inflammatory vascular changes in the brain of patients with schizophrenia is lacking. Furthermore, studies of regional cerebral blood flow (rCBF) in the frontal cortex of patients with schizophrenia under resting conditions have yielded inconsistent results [16, 54, 64]. Following to the pioneering studies of Ingvar and Franzen [39] and Franzen and Ingvar [24] a number of abnormalities in the coupling between task-related increases in brain activity and rCBF have been described in frontal cortical regions of patients with schizophrenia [2, 33, 55, 76] but these changes were generally attributed to primary neuronal dysfunction. Imaging studies with dynamic susceptibility contrast MRI pointed to an increase of cerebral vascular volume in patients with schizophrenia and postulated abnormalities of the configuration of cerebral vessels [17] whereas a more recent study found a decrease of cerebral vascular volume in schizophrenia [11].

A recent study employing a combined transcriptomics, proteomics, and metabolomics approach found evidence for mitochondrial dysfunction, compromised brain metabolism, and oxidative stress in the frontal cortex of patients with schizophrenia [61], thereby confirming and expanding earlier reports [8, 56]. Prabakaran and colleagues [61] hypothesized that the observed alterations can be explained by abnormalities of the microvasculature in schizophrenia.

In a previous study, we analyzed the microvessel length density in prefrontal cortex area 9 and anterior cingulate cortex area 24 (both areas are involved in the neuropathology of schizophrenia, as reviewed in [28]), with a design-based stereology approach, and found no differences between patients with schizophrenia and controls [46]. In the present study we extended these investigations by analyzing the microvessel length density in the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral

nucleus of the amygdala, structures that are also considered to be involved in the neuropathology of schizophrenia [7, 9, 13, 18, 19, 22, 47, 50, 59, 60, 77]. To this end, we used the same sample of brains from patients with schizophrenia and controls that was used in our earlier study [46]. In a parallel study, we have already investigated these five subcortical regions for their volume, total number of neurons, and neuron density [47]. Based on these data we could also assess possible changes in total microvessel length and length per neuron in these five subcortical regions.

Materials and Methods

Brain specimens

This study was performed on postmortem brains (both hemispheres) from 13 male patients with schizophrenia (mean age 51.5 ± 3.3 years; mean postmortem interval [time between death and autopsy] 27.5 ± 6.0 hours; mean fixation time 912 ± 372 days; data given as mean $\pm$ SEM) and from 13 age-matched male controls (mean age 51.9 ± 3.1 years; mean postmortem interval 23.7 ± 3.8 hours; mean fixation time 247 ± 53 days). The same brains were investigated in our previous studies for mean cell spacing abnormalities in the neocortex [15], microvessel length densities in the frontal cortex [46], and volume, neuron density, and total neuron number in the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala [47]. The age of the patients, illness duration, clinical diagnoses, causes of death, the postmortem interval and the fixation time are summarized in Table 1. The patients with schizophrenia did not differ from the controls with respect to mean age (Student's two-tailed *t* test; $p = 0.946$), mean postmortem interval ($p = 0.581$), and mean fixation time ($p = 0.089$). All patients with schizophrenia had been patients either in German university hospitals or in German State psychiatric hospitals (6 hospitals including one university hospital in which some control patients were also treated for nonpsychiatric or neurologic illnesses) and full clinical records were available. All controls had been patients either in German university hospitals or in German local district hospitals (5 hospitals including one university hospital in which patients with schizophrenia were also treated). Records from autopsy (including a summary of the medical history) were available for all patients with schizophrenia and all controls. All pathologists involved in the autopsies were instructed by H.H. and adhered to identical handling and processing conditions of the brains. All patients with schizophrenia met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria. The clinical notes were assessed by two experienced clinical psychiatrists to ensure that the brains from the controls were free from psychopathology, and for clear evidence that the diagnosis of patients with schizophrenia was concordant with DSM-IV criteria for schizophrenia. The mean age at onset was 22.9 ± 1.5 years. Exclusion criteria for both patients with schizophrenia and controls comprised neurological problems that required

intervention or interfered with cognitive assessment (e.g., stroke with aphasia), history of recurrent seizure disorder, history of severe head injury with loss of consciousness, diabetes mellitus with free plasma glucose >200 mg/dl, and history of drug or alcohol abuse. Patients with schizophrenia and controls were similar in terms of the ethnic backgrounds. However, they were not fully matched for socio-economical status and education, which would have placed severe constraints on our sample. Moreover, all patients with schizophrenia were subjected to long-term treatment with typical neuroleptics (because most of the patients were not hospitalized throughout the duration of their illness and their clinical records did not cover fully the entire medication histories, it was not possible to calculate lifetime medication exposures). In all of the cases, autopsy was performed after consent was obtained from a relative according to the laws of the Federal Republic of Germany. The use of these autopsy cases for scientific investigations as outlined here has been approved by the relevant Institutional Review Boards. Brains were fixed by immersion in 10% formalin (1 part commercial 40% aqueous formaldehyde in 9 parts H₂O) prior to histologic processing. Fixation and tissue processing was performed at the Morphological Brain Research Unit, University of Wuerzburg (Germany) under identical conditions for all brains (except for brain C7 that was embedded in celloidin instead of gelatin).

Tissue processing and immunohistochemistry

After separating the brainstem with the cerebellum from the forebrain and dividing the hemispheres mediosagittally, both hemispheres were cut into entire series of 440-to 700- μ m thick coronal sections (for details see [47]). Every second or third section was stained with gallocyanin (a Nissl stain) as previously described [31]. From the remaining sections, two each showing the striatum, the thalamus and/or the amygdala were randomly selected, and approximately 5×5-cm small pieces containing these regions were cut out. After rinsing in tap water for several hours, these pieces were stored in Tris-buffered saline (TBS) overnight (pH = 9.0) at room temperature. Then they were placed in TBS (pH = 9.0) and heated in a microwave oven for 15 min at 90° C for antigen retrieval. After cooling down tissue pieces were rinsed in TBS twice (pH = 7.4) and were placed in TBS based 30% sucrose for cryoprotection. Pieces were then frozen and cut into 50 μ m-thick coronal sections on a cryostat (Type HM 500 OMV, Microm, Walldorf, Germany). One 50 μ m-thick section per piece was mounted on a glass slide, dried, and stained with cresyl violet (0.01%, 25 min). Slides were dehydrated and coverslipped using DPX (Serva, Heidelberg, Germany). These 50 μ m-thick sections stained with cresyl violet as well as the adjacent 440-700 μ m-thick sections stained with gallocyanin were used to identify the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala according to established criteria in the literature (caudate nucleus and nucleus accumbens: [12, 37, 49, 51]; mediodorsal nucleus of the thalamus: [21, 22, 32, 34, 41, 60]; lateral nucleus of the amygdala: [10, 65, 67, 68]. In the case of the mediodorsal nucleus of the thalamus, the magnocellular, parvocellular and densocellular regions as well as the caudodorsal subdivision were included in the delineations, as

also done in other studies [13, 22, 60]. The boundaries of the investigated brain regions were identified on all sections showing the corresponding region using an Olympus SZX9 stereo microscope (Olympus; Tokyo, Japan) and were marked on the back side of the glass slides with a felt-tip pen. Identification and delineation of boundaries was performed by H.H. (caudate nucleus, putamen), V.M. (nucleus accumbens), T.M. (mediodorsal nucleus of the thalamus) and F.W. (lateral, basal and accessory basal nuclei of the amygdala). Cross-validation (and, if necessary, slight modifications of the delineations) was performed by C.S. (caudate nucleus, putamen) and C.S., H.H. and P.R.H. (all other brain regions). The remaining 50 μm -thick sections were stored in TBS (pH = 7.4) at 4° C.

For immunohistochemical processing, another 50 μm -thick section per piece (free-floating sections) was rinsed in TBS after each step of the incubation procedure. Non-specific background staining was blocked by two preincubation steps: first, 10% methanol and 7% hydrogen peroxide in TBS (30 min), and then 1.5% lysine, 0.25% Triton X-100 and 10% bovine serum albumin (BSA) in TBS (60 min) (all from Sigma-Aldrich; St. Louis, MO, USA). Then, sections were incubated with mouse monoclonal anti-collagen IV primary antibody (#C1926, clone COL-94; Sigma) at 4° C for 48 hours. Antibody dilution was 1:500 in TBS containing 2% BSA. Afterwards sections were incubated with secondary antibody (biotinylated anti-mouse IgG 1:200, Jackson ImmunoResearch, West Grove, PA, USA; secondary antibody diluted in TBS containing 0.2% donkey serum, Sigma) for 2 hours at room temperature, and then with avidinbiotin peroxidase complex (ABC Vectastain; 1:25 in TBS; Sigma) for another 2 hours. 3,3'-diaminobenzidine (DAB; 0.8 mg/ml with 0.003% hydrogen peroxide; Sigma) was used as a chromogen. To prevent shrinkage in section thickness, sections were not dehydrated but mounted on gelatin-coated slides and coverslipped with 80% glycerol in TBS. Note that the actual average section thickness after immunohistochemical processing was 45.7 μm (see also Table 2) as determined during the stereologic analyses described in detail below. Accordingly, shrinkage in section thickness was less than 10%. This was important because the space balls method used to estimate microvessel length density is sensitive to shrinkage in section thickness [25].

Stereologic analyses

Stereologic analyses were performed with a stereology workstation, consisting of a modified light microscope (Olympus BX50 with PlanApo objective 1.25 $\times$ [N.A. = 0.04] and UPlanApo objectives 10 $\times$ [N.A. = 0.4], 20 $\times$ [oil; N.A. = 0.8] and 40 $\times$ [oil; N.A. = 1.0]; Olympus, Tokyo, Japan), motorized specimen stage for automatic sampling (Ludl Electronics; Hawthorne, NY, USA), electronic microcator (Heidenhain, Traunreut, Germany), CCD colour video camera (HVC20AMP; Hitachi, Tokyo, Japan), and stereology software (StereoInvestigator; MicroBrightField, Williston, VT, USA).

Microvessel length density was investigated with the Space Balls method [14, 57] (see also Fig. 8 in [63]) (Fig. 1). Briefly, the 50 μm -thick sections stained with cresyl violet were laid onto the corresponding adjacent sections processed with immunohistochemistry and were exactly aligned under the microscope (the 50 μm -thick sections stained with cresyl

violet did not show differences in shrinkage in direction X and Y compared to the sections processed with immunohistochemistry). Then the sections stained with cresyl violet were brought into focus, and the five regions of interest were delineated by tracing their boundaries on each section on video images displayed on the monitor of the stereology workstation. Afterwards the sections stained with cresyl violet were removed, and the sections processed with immunohistochemistry were brought into focus. Then hemispheres with a radius of 30 μm (generated by the stereology software while focussing through the section thickness) were systematically and randomly placed within the sections through the regions of interest (with a guard zone of 5 μm between the upper surface of the section and the top of the hemispheres; details are provided in Table 2). The microvessel length density was obtained from the total number of intersections between the hemispheres and the microvessels (data given in Table 2) using the following formula derived from the description of the Space Balls method in the literature [14, 57] according to the sampling scheme applied in the present study and considering the area of the delineated regions of interest and the actual section thickness after tissue processing (see also [46]):

$$CLD = \frac{2 \times \sum is(D_X \times D_Y \times t)}{2 \times \pi \times r^3} \times \frac{1}{V}$$

where CLD represents the microvessel length density, $\sum is$ is the total number of intersections between the hemispheres and the microvessels, D_X and D_Y the distance between the mid-points of the centers of the hemispheres in the X and Y axes, t the actual average section thickness after histologic processing, r the radius of the hemispheres and V the investigated tissue volume. It is important to note that these microvessel length density estimates were based on the analysis of thick sections in three dimensions (3D) and therefore not dependent on the plane of section, the spatial arrangement of the microvessels or their diameter. To focus the analysis on microvessels and to exclude arterioles and venules, the diameter of all vessels coming into view during the space ball analysis was measured with the "Fast measure line" tool of the stereology software, and intersections between the (space ball) hemispheres and vessels with a diameter greater than 9 μm were not counted. In addition, we always focused on the middle of a given vessel's diameter when recording intersections between the Space Ball hemispheres and the microvessel in order to minimize the Space Ball bias due to the non-zero diameter of the vessels [25]. The actual section thickness after immunohistochemical processing was measured at the position of every 10th Space Ball, yielding on average approximately 40 measures per section. Note that the actual section thickness after immunohistochemical processing (on average 45.7 μm) guaranteed that the Space Balls were placed always fully within the section thickness. Finally, total microvessel lengths and lengths per neuron were calculated individually for each subcortical region

as the product of the microvessel length density in and the volume of the region (total microvessel lengths), and as the ratio of the total microvessel length and the total neuron number in the region (microvessel lengths per neuron), respectively. Volume and total neuron number data were taken from [47]. Furthermore, it was tested whether there was a principal correlation between the neuron density and microvessel length density when all subcortical structures were combined.

Statistical analysis

For both patients with schizophrenia and controls, mean and standard error of the mean were calculated for all investigated variables separately for the left and the right hemispheres. Comparisons between patients with schizophrenia and controls were performed using generalized linear model multivariate analysis (MANOVA), with diagnosis and hemisphere as fixed factors and the following variables as covariates: (i) the adjusted illness duration of the patients with schizophrenia (calculated as individual age at death minus age at onset plus the mean age at onset of all patients with schizophrenia) or the age of the controls, respectively, (ii) the postmortem interval and (iii) the fixation time (note that use of the actual individual illness duration of the patients with schizophrenia instead of the adjusted ones as covariate would have caused invalid results of the MANOVA model because there was no illness duration of the controls, and the mean illness duration of the patients with schizophrenia was significantly different from the mean age of the controls [Student's two-tailed t test; $p < 0.001$] whereas the mean adjusted illness duration was not [$p = 0.974$]). For each investigated variable all investigated brain regions were tested simultaneously. Post-hoc tests in the analyses of covariance were performed with linear regression analysis. In addition, the microvessel length per neuron data from both hemispheres of patients with schizophrenia and controls were pooled for each subcortical region, and a comparison among the five investigated regions was performed with one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni tests for pairwise comparisons. In all analyses an effect was considered statistically significant if its associated p value was smaller than 0.05. Linear regression analysis was carried out between neuronal densities and microvessel length densities across the five subcortical structures. Calculations were performed using SPSS (Version 12.0.1 for Windows, SPSS, Chicago, IL).

Photography

Photomicrographs shown in Fig. 1 and 2 were produced using an Olympus DP 70 digital camera attached to an Olympus AX 70 microscope and cellP software Version 2.3 (Soft Imaging System, Münster, Germany). The final figures were constructed using Corel Photo-Paint v.11 and Corel Draw v.11 (Corel, Ottawa, Canada). Adjustments of contrast and brightness were made to facilitate recognition of the immunohistochemical signal at low and high magnification, without altering the appearance of the original materials.

Results

No major abnormalities such as irregular size of the vascular wall, stenosis, or dilation were noted in the immunohistochemical preparations for collagen IV in the materials from the patients with schizophrenia (Fig. 2). Compared to the controls, the patients with schizophrenia showed a statistically significant increase in mean microvessel length density in the caudate nucleus (+6.3% on the left side [l] and +5.8% on the right side [r]; $F(1)$ [one degree of freedom] = 4.869 and $p = 0.034$ for the fixed factor diagnosis in the MANOVA), and in the mediodorsal nucleus of the thalamus (l: +4.3%, r: +5.1%; $F(1) = 4.857$, $p = 0.034$) (Fig. 3; Table 3 shows all P values of the MANOVAs performed).

The patients with schizophrenia had a significantly reduced mean total microvessel length in the lateral nucleus of the amygdala (l: -17.5%, r: -10.8%; $F(1) = 6.553$, $p = 0.015$; Fig. 4). In contrast, there were no significant differences between the patients with schizophrenia and the controls with respect to the mean microvessel length per neuron in the investigated subcortical regions (Fig. 5). Also, no significant differences between the left and the right hemispheres were found for the investigated variables in both patients with schizophrenia and controls ($p > 0.05$ for the fixed factor hemisphere in all MANOVAs performed). The mean microvessel length per neuron differed among the five investigated subcortical regions, in the order mediodorsal nucleus of the thalamus > lateral nucleus of the amygdala > putamen > caudate nucleus > nucleus accumbens ($p < 0.001$). Except for the comparison between the caudate nucleus and the nucleus accumbens, all pairwise comparisons showed significant differences between the regions ($p < 0.01$ for the comparison between caudate nucleus and putamen, and $p < 0.001$ for all other comparisons).

Postmortem changes are known to affect stereologic estimates of cerebral blood vessels [38]. In the present study, the postmortem interval had a significant effect on the total microvessel length in the putamen ($F(1) = 4.864$, $p = 0.034$; Fig. 6). A significant effect of the brain's fixation time on the total microvessel length in the mediodorsal nucleus of the thalamus was also found ($F(1) = 4.812$, $p = 0.035$). However, post-hoc linear regression analysis revealed no significant correlations between the postmortem interval and the fixation time and any of the investigated variables. These results suggest that the alterations in mean microvessel length densities and mean total microvessel lengths found in the investigated subcortical regions in the patients with schizophrenia were not caused by the control patients' age (or the illness duration of the patients with schizophrenia), the postmortem interval or the fixation time of the brains. In addition, no correlation was found between the microvessel length density and the neuron density in the five investigated subcortical regions, either in the patients with schizophrenia, or in the controls (controls: $r^2 = 0.006$; $p = 0.900$; patients with schizophrenia: $r^2 = 0.054$; $p = 0.708$; Fig. 7). Finally it should be mentioned that the results obtained for the single brain embedded in celloidin (C7) showed no systematic deviation from the results obtained for the other brains (Figs. 3, 4 and 5).

Discussion

The present data reveal a small increase in microvessel length density in the caudate nucleus and in the mediodorsal nucleus of the thalamus, and a decrease in mean total microvessel length in the lateral nucleus of the amygdala in schizophrenia. Also, as the total neuron numbers were assessed in the same subcortical regions in this brain series [47], microvessel length per neuron could be calculated. There were no changes in the mean microvessel length per neuron in any of the five subcortical structures in the brains from the patients with schizophrenia, suggesting that modest changes in neuron number are likely to be paralleled by minor vascular changes. It should be noted that the results reported in the present study do not reflect absolute values found in the brain of living humans because postmortem brains show shrinkage during formalin fixation. Accordingly, the results presented here should only be used to interpret potential differences between patients with schizophrenia and matched controls whose postmortem brains were processed under exactly identical conditions. In this regard one could speculate that postmortem brains from patients with schizophrenia may shrink differently than postmortem brains from controls, but there is no available evidence for this in the literature.

The present study confirms and extends observations of normal microvessel lengths in the prefrontal cortex area 9 and anterior cingulate cortex area 24 in schizophrenia [46]. These data do not support the hypothesis of alterations in the microvasculature in chronic schizophrenia. Because these studies were performed in regions considered to represent "hot spots" for structural pathology in schizophrenia, the present negative outcome indicates that there might be no anatomical substrate at the microvascular level for abnormal rCBF and cerebral blood volume, alterations reported in the brains of patients afflicted by schizophrenia [2, 11, 17, 24, 33, 55, 76].

A survey of gene expression studies with microarrays in the brain of patients with schizophrenia showed changes in a few genes primarily associated with the vasculature, although no consistent directional trends emerged [62]. A hypothesis paper suggested that genetic factors and inflammatory responses of cerebral vessels could underlie some of the changes seen in schizophrenia [27]. However, the data of the present study altogether do not support the hypothesis of involvement of brain microvessels that would be otherwise expected if a major inflammatory process took place in schizophrenia. Because only vessels with a maximal diameter of 9 μm were counted in the present study, subtle abnormalities may still occur upstream in arterioles, involving vascular smooth muscle cells and regulation of tissue perfusion. Indeed, changes in the activity-dependent regulation of rCBF have been observed in the prefrontal cortex in schizophrenia [24, 76]. However, hemodynamic responses appear to be normal in visual, motor and somatosensory cortices in this disorder [5]. It is unlikely that primary differences in the structure and function of arterioles occur selectively in some cortical regions, and therefore changes in rCBF activation most likely are due to region-specific neuronal or glial factors. In addition, it appears that changes in gene and protein expression found in cortical samples from chronic schizo-

phrenia patients probably reflect an abnormal metabolic state [61]. This could be equally due to the primary disease process and to secondary long-term neuronal dysfunction, chronic medication, or imbalances in agonal events influencing microarray measurements [40, 53, 72, 74]. It remains to be shown whether the microvasculature of the white matter in schizophrenia is normal, as several recent findings point to subtle white matter changes in schizophrenia [20, 35, 36], and in view of the vulnerability of the white matter to prolonged hypoperfusion [23]. Furthermore, an abnormal decrease in perivascular labeling of GFAP in astrocytes has been reported in the white matter in schizophrenia [75].

As a side observation it is of interest that the neuron density was *not* correlated with the microvessels length density when five subcortical regions were combined for analysis in controls or in patients with schizophrenia. Baborie and Kuschinsky [4] did not find a correlation between the density of all nuclei and microvessel densities across multiple areas of the rat brain. It is likely that the microvessel length density is linked to metabolic expenditures in neuronal and glial processes whereby neuron density (measured in the present study) or overall cell density (measured in [4]) do not tightly correlate with the total amount of functional processes in each structure. As all patients were treated with neuroleptics, we cannot exclude that the lack of changes in microvessel measurements was due to the treatment; in other words, untreated patients with schizophrenia could have an abnormal microvasculature. However, the influence of neuroleptics on brain microvessels in chronically treated patients with schizophrenia is presently unknown. Dopaminergic processes make close contacts with capillaries in the prefrontal cortex and dopamine reduces local perfusion [48]. Dopamine has been shown to have anti-angiogenic properties in peripheral tissues, tumors, and placenta [6, 45, 70]. Thus, one cannot exclude that abnormal dopamine levels in schizophrenia may have functional and structural effects on cerebral microvasculature before the onset of clinical symptoms that may be masked by chronic dopamine receptor blockade with neuroleptics. Further research will be necessary in this regard.

Finally it should be mentioned that the present observations in chronic schizophrenia cases must be separated from the concept that abnormal interactions between hypoxia, the microvasculature and developing neurons could be important for the neurodevelopmental perturbations that are thought to underlie schizophrenia [62]. Specifically, one cannot exclude the possible occurrence of alterations in microvessel length and microvessel length per neuron in the fetal brain of people who will develop schizophrenia later in life. If this were the case, however, these alterations do not persist during postnatal life in the brains from patients with schizophrenia. Rather, compromised brain metabolism and occurrence of oxidative stress in the brains from patients with schizophrenia are likely caused by other mechanisms.

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Tables

Table 1. Clinical characteristics of all cases included in this study.

No	Age [years]	Cause of death	PMI [hours]	Fix [days]	Diagnosis	
					DSM-IV	ICD-10
S1	22	Suicide†	88	130	295.30	F20.00
S2†	36	Suicide†	<72	115	295.30	F20.00
S3	46	Systemic hypothermia	<24	327	295.30	F20.01
S4	50	Peritonitis	<24	203	295.30	F20.00
S5	50	Suicide	18	170	295.30	F20.00
S6	51	Septicemia	33	127	295.60	F20.50
S7	54	Septicemia	27	250	295.60	F20.50
S8	55	Right-sided heart failure	25	84	295.30	F20.00
S9	57	Septicemia	76	163	295.30	F20.00
S10	60	Pulmonary embolism	<48	311	295.30	F20.01
S11	62	Aspiration	7	171	295.30	F20.00
S12	63	Acute myocardial infarct	15	338	295.60	F20.50
S13	64	Pulmonary embolism	6	817	295.60	F20.50
C1†	25	Cardiac tamponade	14	119	-	-
C2†	36	Gunshot	24	143	-	-
C3	47	Acute myocardial infarct	<24	133	-	-
C4	50	Acute myocardial infarct	35	433	-	-
C5	50	Avalanche accident	23	498	-	-
C6†	51	Septicemia	7	285	-	-
C7	54	Acute myocardial infarct	18	168	-	-
C8	56	Acute myocardial infarct	60	3570	-	-
C9	58	Acute myocardial infarct	28	126	-	-
C10	60	Gastrointestinal hemorrhage	18	101	-	-
C11	60	Gastrointestinal hemorrhage	27	302	-	-
C12†	62	Acute myocardial infarct	<24	3696	-	-
C13	65	Bronchopneumonia	6	2289	-	-

Variable	Brain region	A/ID	PMI	Fix	D	H	DxH
Microvessel length density	CN	0.399	0.977	0.990	0.034	0.434	0.838
	P NA	0.926	0.509	0.605	0.077	0.101	0.727
		0.434	0.320	0.842	0.231	0.863	0.738
	MDNT	0.991	0.697	0.251	0.034	0.541	0.719
	LNA	0.129	0.844	0.709	0.607	0.426	0.057
Total microvessel length	CN	0.276	0.788	0.436	0.312	0.273	0.998
	P NA	0.697	0.034	0.342	0.409	0.417	0.833
		0.236	0.949	0.381	0.839	0.512	0.850
	MDNT	0.099	0.825	0.035	0.951	0.209	0.419
	LNA	0.446	0.661	0.403	0.015	0.942	0.837
Microvessel length per neuron	CN	0.104	0.328	0.592	0.225	0.427	0.751
	P NA	0.434	0.670	0.677	0.086	0.578	0.623
	MDNT	0.075	0.130	0.280	0.391	0.095	0.682
		0.052	0.621	0.052	0.645	0.849	0.926
	LNA	0.677	0.782	0.365	0.870	0.378	0.969

S, patient with schizophrenia; C, control. PMI, postmortem interval (time between death and autopsy); Fix, fixation time. †The microvessel length densities in the left and right hemispheres of the cases C1 and C2 (as well

as in the right hemispheres in C6, C12 and S2, respectively) could not be determined because the corresponding sections not stained with gallicyanin were missing. ‡ These two patients had relatively long postmortem intervals. However, both had committed suicide (one by hanging, the other by jumping from a building), were found within one hour of death and were kept at 4° C until autopsy. Accordingly, the postmortem intervals between death and autopsy of these patients cannot be compared to the corresponding intervals of the other cases and were thus excluded from the calculation of the mean postmortem intervals.

Table 2. Details of the stereologic procedure to measure microvessel length densities with the Space Balls method.

	CN	P	NA	MDNT	LNA
Obj.	40x	40x	40x	40x	40x
r (µm)	30	30	30	30	30
t [µm]	45.6	45.7	45.6	46.2	45.5
D (µm)	725	925	550	600	400
Σ hs	388	398	390	396	497
Σ is	500	550	376	507	374

CN, caudate nucleus; P, putamen; NA, nucleus accumbens; MDNT, mediodorsal nucleus of the thalamus; LNA, lateral nucleus of the amygdala. Obj., objective used; r, radius of the hemispheres; D, distance between the midpoints of the centers of the hemispheres in directions X and Y; Σ hs, total number of hemispheres used; Σ is, total number of intersections between the hemispheres and the capillaries.

Table 3. Results of statistical analysis (p values) with generalized linear model multivariate analysis of variance (MANOVA).

Variable	Brain region	A/ID	PMI	Fix	D	H	DxH
Microvessel length density	CN	0,339	0,977	0,990	0,034	0,434	0,838
	P	0,926	0,509	0,605	0,077	0,101	0,727
	NA	0,434	0,320	0,842	0,231	0,863	0,738
	MDNT	0,991	0,967	0,251	0,034	0,541	0,719
	LNA	0,129	0,844	0,709	0,607	0,426	0,057
Total microvessel length	CN	0,276	0,788	0,436	0,312	0,273	0,998
	P	0,697	0,034	0,342	0,409	0,417	0,833
	NA	0,239	0,949	0,381	0,839	0,512	0,850
	MDNT	0,099	0,825	0,035	0,951	0,209	0,419
	LNA	0,446	0,661	0,403	0,015	0,942	0,837
Microvessel length per neuron	CN	0,104	0,328	0,592	0,645	0,849	0,751
	P	0,434	0,670	0,677	0,086	0,578	0,623
	NA	0,075	0,130	0,280	0,391	0,095	0,682
	MDNT	0,052	0,621	0,052	0,645	0,849	0,926
	LNA	0,677	0,782	0,365	0,870	0,378	0,969

CN, caudate nucleus; P, putamen; NA, nucleus accumbens; MDNT, mediodorsal nucleus of the thalamus; LNA, lateral nucleus of the amygdala. A/ID, age (controls) or adjusted illness duration (patients with schizophrenia), respectively (adjusted illness duration calculated as age at death minus age at onset plus mean age at onset; details are provided in Statistical analysis); PMI, postmortem interval (time between death and autopsy); Fix, fixation time; D, diagnosis; H, hemisphere. P values smaller than 0.05 are shown in boldface.

Figures

Fig. 1. Estimating microvessel length density with the Space Balls method, illustrated for the caudate nucleus of control brain no. C4. (a), Low-power overview of capillaries, superimposed by a rectangular lattice with uniform distance between the lines in the X and Y axes. This lattice defines the position of microscopic fields (indicated as gray rectangles), at which the section is inspected at higher magnification to perform Space Ball analyses. (b), High-power photomicrographs of one of these microscopic fields at the upper surface of the section, at 10 consecutive focal planes below the upper surface with a distance of 4 μm between the focal planes, and at the lower surface of the section that was found 43 μm below the upper surface. Between -8 μm and -36 μm the microscopic field is superimposed by intersections of a hemisphere (a semi-Space Ball) with the focal plane at the investigated focal depth, illustrated as circles. The semi-space ball was centered at a depth of -8 μm and had a radius of 30 μm . Intersections between the semi-Space Ball and the microvessels in focus were found at -12 μm , -16 μm , -24 μm and -28 μm (asterisks). The average microvessel length density within the caudate nucleus was calculated from the total number of intersections and the number and size of the semi-space balls. Scale bar: 700 μm in A, and 40 μm in the high-power photomicrographs.

Fig. 2. Representative high-power photomicrographs of 50 μm -thick coronal sections from the left brain half of a patient with schizophrenia (a,c,e,g,i) and a control (b,d,f,h,k) showing immunohistochemical detection of collagen IV in the caudate nucleus (A,B), putamen (c,d), nucleus accumbens (e,f), mediodorsal nucleus of the thalamus (g,h) and lateral nucleus of the amygdala (i,k). Note that no major abnormalities such as irregular size of the vascular wall, stenosis or dilation were noted in the immunohistochemical preparations for collagen IV in the materials from the patients with schizophrenia. Furthermore, differences in microvessel length density between patients with schizophrenia and controls were not obvious at simple observation of the tissue but necessitated the type of rigorous quantitative analysis undertaken in this study to be revealed. Scale bar: 50 μm .

Fig. 3. Microvessel length density in caudate nucleus (CN; a,f,l), putamen (P; b,g,m), nucleus accumbens (NA; c,h,n), mediodorsal nucleus of the thalamus (MDNT; d,i,o) and lateral nucleus of the amygdala (LNA; e,k,p) in both hemispheres of the brains from 13 male patients with schizophrenia (S; open bars in a to e, and dots in f to p) and 13 age-matched controls (C; closed bars in a to e, and dots in f to p; squares in case of the results obtained for the single brain embedded in celloidin [C7]). In a to e, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from patients with schizophrenia (S-l and S-r) and controls (C-l and C-r). In f to p, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (f to k) and patients with schizophrenia (l to p) are shown as function of the control patients' age (or the illness duration of the patients with schizophrenia, respectively). *, $p < 0.05$ for the fixed factor Diagnosis in general linear model multivariate analysis of variance (MANOVA).

Fig. 4. Microvessel length in caudate nucleus (CN; a,f,l), putamen (P; b,g,m), nucleus accumbens (NA; c,h,n), mediodorsal nucleus of the thalamus (MDNT; d,i,o) and lateral nucleus of the amygdala (LNA; e,k,p) in both hemispheres of the brains from 13 male patients with schizophrenia (S; open bars in a to e, and dots in f to p) and 13 age-matched controls (C; closed bars in a to e, and dots in f to p; squares in case of the results obtained for the single brain embedded in celloidin [C7]). In a to e, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from patients with schizophrenia (S-l and S-r) and controls (C-l and C-r). In f to p, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (f to k) and patients with schizophrenia (l to p) are shown as function of the control patients' age (or the illness duration of the patients with schizophrenia, respectively). *, $p < 0.05$ for the fixed factor Diagnosis in general linear model multivariate analysis of variance (MANOVA).

Fig. 5. Microvessel length per neuron in caudate nucleus (CN; a,f,l), putamen (P; b,g,m), nucleus accumbens (NA; c,h,n), mediodorsal nucleus of the thalamus (MDNT; d,i,o) and lateral nucleus of the amygdala (LNA; e,k,p) in both hemispheres of the brains from 13 male patients with schizophrenia (S; open bars in a to e, and dots in f to p) and 13 age-matched controls (C; closed bars in a to e, and dots in f to p; squares in case of the results obtained for the single brain embedded in celloidin [C7]). In a to e, data are shown as mean and standard error of the mean for the left (l) and right (r) hemispheres from patients with schizophrenia (S-l and S-r) and controls (C-l and C-r). In f to p, individual data for the left hemispheres (closed dots or squares, respectively) and right hemispheres (open dots or squares, respectively) from controls (f to k) and patients with schizophrenia (l to p) are shown as function of the control patients' age (or the illness duration of the patients with schizophrenia, respectively).

Fig. 6. Microvessel length in the putamen as a function of the postmortem interval (PMI; time between death and autopsy) (P-CL; a to d) and microvessel length in the mediodorsal nucleus of the thalamus as a function of the fixation time (MDNT-CL; e to h). The graphs show individual values (dots) for the left (l) and right (r) hemispheres from controls (C-l; a,e, and C-r; b,f, respectively) and patients with schizophrenia (S-l; c,g, and S-r; d,h, respectively) as well as results from linear regression analysis (regression lines with 95% confidence intervals, regression correlation coefficients [r^2 values] and p values). All regression lines are dotted because their slope did not significantly differ from zero ($p > 0.05$).

Fig. 7. Neuron density (ND) in the caudate nucleus (CN), putamen (P), nucleus accumbens (NA), mediodorsal nucleus of the thalamus (MDNT) and lateral nucleus of the amygdala (LNA) as a function of microvessel length density (CLD) in these subcortical regions in both hemispheres of post-mortem brains from 13 male controls (a) and 13 male patients with schizophrenia (b). Region-specific values for microvessel length density and neuron density are shown as mean $\pm$ standard error of the mean (SEM). Linear regression analysis showed no significant correlation between the microvessel length density and the neuron density in these subcortical regions (controls: $r^2 = 0.006$; $p = 0.900$; patients with schizophrenia: $r^2 = 0.054$; $p = 0.708$). The regression lines and their 95% confidence intervals are also shown in the graphs.

CHAPTER 4

Stereological studies of capillary length density in the frontal cortex of schizophrenics

Pawel Kreczmanski¹, Rainald Schmidt-Kastner², Helmut Heinsen³
Harry W. M. Steinbusch¹, Patrick R. Hof⁴, Christoph Schmitz¹

¹European Graduate School of Neuroscience (EURON), Maastricht, The Netherlands

²Department of Neurology, University of Miami School of Medicine, Miami, Florida, USA

³Morphologic Brain Research Unit, University of Wuerzburg, Wuerzburg, Germany

⁴Department of Neuroscience and Department of Geriatrics and Adult Development,
Mount Sinai School of Medicine, New York, New York, USA

Abstract

The presence of microvasculature abnormalities in the prefrontal cortex of schizophrenics was proposed in a recent study of molecular signatures of schizophrenia [Prabakaran et al (2004) *Mol Psychiat* 9:684–697]. To assess this possibility further, we investigated capillary length densities in prefrontal cortex area 9 and anterior cingulate cortex area 24 in post-mortem brains from 13 schizophrenics and 13 age- and sex-matched controls. To check that our sample of brains shared cardinal neuropathological features of schizophrenia with previously reported case studies, we also measured cortical gray matter volumes and cortical thickness in areas 9 and 24. The mean cortical gray matter volume was significantly reduced in brains from schizophrenics compared to controls. Mean cortical thickness was significantly reduced in area 24, but not in area 9, in schizophrenics. There were no differences in mean capillary length densities in either area 9 or 24 between the two groups. Thus, alterations in capillary length density in the prefrontal cortex cannot be considered a general feature of schizophrenia. Compromised brain metabolism and occurrence of oxidative stress in the brain of schizophrenics are likely caused by other mechanisms.

Introduction

Schizophrenia is a mental illness characterized by psychosis, cognitive impairment and social withdrawal [37]. Among psychiatric disorders, schizophrenia is the most disabling [37]. A combination of genetic susceptibility and environmental perturbations appears to be necessary for the expression of the disease [19, 48, 51]. However, the actual mechanisms involved in the pathogenesis of schizophrenia remain elusive [18, 19, 51]. Considerations range from alterations of highly specific developmental processes at the synaptic level [17, 31] and abnormalities in myelination [16, 50] to global metabolic changes of brain function [1, 3, 35].

Anatomic and pathological analyses of brain tissue from schizophrenic patients have focused on neurons, including detailed studies of dendrites and synapses [17]. The present consensus is that a slight reduction of overall cortical volume is caused by a reduction of the neuropil that may be related to some form of abnormal developmental process for synaptic connections [46]. Another possibility is that a progressive loss of synapses occurs due to a degenerative process during the chronic course of the disease [32]. Studies of glial cells provided no evidence for reactive gliosis [13]. However, some deficits of glial cells have recently been detected in the white matter using quantitative methods [12, 25, 26]. Given the possible involvement of glial cells in pathogenetic scenarios of psychiatric disorders [12, 49], it is timely to consider the brain microvasculature as another key factor in the development of schizophrenia. As the function of neurons depends on adequate oxygen and substrate supply, subtle changes in vasculature integrity may also be important for the schizophrenic brain. General observations on the macrovasculature in the brain of schizophrenics have been made [2], but there have been no systematic studies on the vascular system in this disease. It has been argued in earlier studies that a subtle metabolic deficit, such as hypoxia, would be sufficient to cause widespread neuronal dysfunction [28, 29]. However, reports on alterations of regional cerebral blood flow in the frontal cortex of schizophrenics under resting conditions have yielded inconsistent results [8, 33, 45]. A number of abnormalities in the coupling between neuronal activity and cerebral blood flow have been described in schizophrenic patients [24, 34]. These changes were attributed to primary neuronal dysfunction, although subtle changes in the capillary system have not been excluded. Some imaging studies pointed to an abnormal cerebral vascular volume in schizophrenic patients [10]. Furthermore, abnormalities in the capillary system of the fingernail folds have been found in patients affected by the disorder [11], and the vasodilatory response to methylnicotinate application in the skin is reduced in schizophrenics [42]. A very recent study employing a combined transcriptomics, proteomics and metabolomics approach found evidence for mitochondrial dysfunction, compromised brain metabolism, and oxidative stress in the frontal cortex of schizophrenics [39], thereby confirming and expanding earlier reports [1, 3, 35]. Importantly, these authors hypothesized that these alterations can be explained by microvasculature abnormalities in the frontal cortex in schizophrenia [39].

We thus decided to investigate whether the brain microvasculature in the schizophrenic brain is normal. In the present study we examined, for the first time, capillary length densities within the prefrontal cortex (area 9) and anterior cingulate cortex (area 24) in post-mortem brains from schizophrenics and controls with state-of-the-art design-based stereological technology, i.e., the recently developed “Space balls” method [7, 36,44].

Materials and methods

Brain specimens

Postmortem brains (both hemispheres) from 13 male schizophrenics (mean age 51.5 ± 3.3 years; mean interval between death and autopsy 27.5 ± 6.0 h; data given as mean $\pm$ SEM) and from 13 age-matched male controls (mean age 51.9 ± 3.1 years; mean interval between death and autopsy 23.7 ± 3.8 h) were analyzed. Clinical diagnoses, causes of death, the fixation time and the interval between death and autopsy are summarized in Table 1.

Schizophrenics did not differ from controls with respect to mean age (Student's two-tailed t-test; $P=0.946$), mean interval between death and autopsy ($P=0.581$) and mean fixation time ($P=0.089$). All schizophrenics had been patients either in German university hospitals or German State psychiatric hospitals and full clinical records were available. Records from autopsy (including a summary of the medical history) were also available for all controls. All schizophrenics met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria. The clinical notes were assessed by two experienced clinical psychiatrists to ensure that the control brains were free from psychopathology, and for clear evidence that the schizophrenic cases conformed to DSM-IV criteria for schizophrenia. Exclusion criteria for both schizophrenics and controls comprised neurological problems that required intervention or interfered with cognitive assessment (e.g., stroke with aphasia), history of recurrent seizure disorder, history of severe head injury with loss of consciousness, diabetes mellitus with free plasma glucose >200 mg/100 ml, and history of self-administered intoxication. Brains from schizophrenics were also excluded if patients had undergone a leukotomy. The two groups were similar in terms of the ethnic backgrounds of the subjects. However, they were not fully matched for socio-economical status and education, which would have placed severe constraints on our sample. Moreover, all schizophrenics were subjected to long-term treatment with high doses of neuroleptics. In all of the cases, autopsy was performed after consent was obtained from a relative, according to the laws of the Federal Republic of Germany. The use of these autopsy cases for scientific investigations as outlined here has been approved by the relevant Institutional Review Boards. Brains were fixed by immersion in 10% formalin (1 part commercial 40% aqueous formaldehyde in 9 parts H_2O) prior to histological processing.

Tissue processing

The brainstem with the cerebellum was severed at the level of the rostral pons, and the hemispheres were divided medio-sagittally. Both hemispheres were then cut into serial 600- to 700- μm -thick coronal sections as previously described [20]. Briefly, the hemispheres were cryoprotected in a mixture of glycerol-dimethylsulfoxide-formalin after careful removing of the meninges and the pial vessels, embedded in gelatin, deeply frozen at -60°C , and serially sectioned using a cryomicrotome (Jung, Nussloch, Germany). One brain (control case C7) was embedded in celloidin as recently described [23], and was cut into serial 440- μm -thick coronal sections using a sliding microtome (Polycut, Cambridge Instruments, UK). From each hemisphere, every second or third section was stained with gallocyanin as described [20]. The remaining sections were stored in formalin for up to 10 years. From all brains of schizophrenics and controls older than 40 years, sections through the central portion of the entorhinal and transentorhinal cortex that were not stained with gallocyanin were labeled with the Gallyas method to detect neurofibrillary changes [21]. Neurofibrillary tangles were very rarely detected in the transentorhinal and entorhinal cortex on Gallyas-stained sections, compatible with Braak's stage I [5]. Furthermore, each section was coded and controlled for the absence of tumors, infarcts, heterotopias, signs of autolysis, staining artifacts, and gliosis.

Immunohistochemistry

From each brain and hemisphere, the 600- to 700- μm -thick coronal section stained with gallocyanin that showed the rostral tip of the caudate nucleus was selected. The adjacent caudal section not stained with gallocyanin was selected, and an approximately 4.4-cm piece containing the cingulate gyrus and the superior frontal gyrus was cut out to investigate area 24 (the appearance of area 24 at this rostrocaudal level is depicted in Fig. 1E in [14]). Another approximately 4.4 cm piece containing both superior frontal gyrus and medial frontal gyrus was cut out to investigate area 9. After rinsing in tap water for several hours, these pieces were stored in TRIS-buffered saline (TBS) overnight (pH 9.0) at room temperature. They were then placed in TBS (pH 9.0) and heated in a microwave oven for 15 min at 90°C for antigen retrieval. After cooling, tissue pieces were rinsed twice in TBS (pH 7.4) and were placed in TBS-based 30% sucrose for cryoprotection. Pieces were then frozen and cut into 50- μm -thick coronal sections on a cryostat (Type HM 500 OMV, Microm, Walldorf, Germany). One 50- μm -thick section per piece was mounted on a glass slide, dried, and stained with cresyl violet (0.01%, 25 min). Slides were dehydrated and coverslipped using DePeX (Serva, Heidelberg, Germany). These 50- μm -thick sections stained with cresyl violet as well as the adjacent 600- to 700- μm -thick sections stained with gallocyanin were used to identify areas 9 and 24 in both cerebral hemispheres according to cytoarchitectural criteria established in the literature.

With respect to area 9, Rajkowska and Goldman-Rakic [41] showed that lateral parts of area 9 surround area 46 (area FDD of von Economo and Koskinas [54]). It is important to note that Brodmann [6] never described the cytoarchitectural characteristics of this area in the human brain. Rather, he projected his observations in monkey onto the human frontal

lobe. Von Economo and Koskinas [54] provided a detailed cytoarchitectonic description of the human frontal lobe. Their area FDM corresponds to area 9. Its characteristics are a distinct layer II, absence of very large, slender and well-built pyramidal cells in layer IIIc (typical of area 6 and to a lesser extent of area 8), and an uninterrupted layer IV with fairly definite upper and lower boundaries. In thick galloxyanin-stained sections, a cell-poor layer is found between layers IIIc and layer IV. Layer IV is more prominent and cell rich in the frontopolar regions; however, the transition between areas 9 and 10 is gradual. Layer V of area 9 is subdivided into layers Va and Vb, the latter being rather sparse in cells. Most probably the cell-poor layers between layer IIIc and IV and within layer Vb correspond to the upper and lower bands described by Rajkowska and Goldman-Rakic [40] in myelin-stained sections. Finally, layer VI consists of layers VIa and VIb. Layer VIa is rich in cells and its upper border abutting the cell-sparse layer Vb can be easily recognized. The deeper border of layer VIb with the underlying white matter are much more distinct than in the caudally located areas 8 and 6. The overall cortical thickness of the frontopolar area 10 (field FE of von Economo and Koskinas [54]) is considerably less than in area 9, layer VIa is small, rich in darkly stained pyramidal as well as spindle-shaped cells, and its boundary with the medullary layer is even more distinct than in area 9. The presence of the small and cell-rich layer VI facilitates the delineation of area 9 from the rostral area 10 in thick galloxyanin stained sections. Finally, layer IV is more conspicuous in area 10 than in area 9. Area 24 (including subfields 24a,b,c) was identified according to the criteria of Vogt et al. [53] and Nimchinsky et al. [38].

The remaining 50 μ m-thick sections were stored in TBS (pH 7.4) at 4° C. For immunohistochemical processing, free-floating sections were rinsed in TBS after each step of the incubation procedure. Nonspecific background staining was blocked by two preincubation steps: first, 10% methanol and 7% hydrogen peroxide in TBS (30 min), and then 1.5% lysine, 0.25% Triton X-100 and 10% bovine serum albumin (BSA) in TBS (60 min) (all from Sigma-Aldrich; St. Louis, MO). Sections were then incubated with mouse monoclonal anti-collagen IV primary antibody (C1926, clone COL-94; Sigma) at 4° C for 48 h. The antibody was diluted 1:500 in TBS containing 2% BSA. Subsequently, sections were incubated with secondary antibody (biotinylated anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA; secondary antibody diluted 1:200 in TBS containing 0.2% donkey serum; Sigma) for 2 h at room temperature, and then with avidin-biotin peroxidase complex (ABC Vectastain; 1:25 in TBS; Sigma) for another 2 h. 3,3'-Diaminobenzidine (DAB; 0.8 mg/ml with 0.003% hydrogen peroxide; Sigma) was used as a chromogen. To prevent shrinkage in section thickness, sections were not dehydrated but mounted on gelatin-coated slides and coverslipped with 80% glycerol in TBS.

Stereological analysis

Analysis of cortical gray matter volume was performed with the Cavalieri principle [9] and point counting [15, 44]. Briefly, a rectangular grid with uniform side length of 15 mm printed on a transparency was randomly placed on the surface of the 600- to 700- μ m-thick sections,

and all intersections of the grid and the cortical gray matter were counted (on average 477 intersections per hemisphere). Then, the cortical gray matter volume was calculated from the product of the sum of the counted intersections, the uniform area represented by each intersection ($15 \cdot 15 = 225 \text{ mm}^2$), the interval of selecting sections for staining with galloxyanin (2 or 3; see above), and the average actual section thickness after tissue processing. Section thickness was determined with the calibrated fine adjustment knob of an Olympus BH microscope and a Zeiss Planapo objective ($\times 20$) as described [22]. The cortical gray matter volume of cases C11 and S11 could not be analyzed because sections from the frontal as well as the occipital poles were missing. Capillary length density was investigated with the "Space balls" method [7, 36, 44]. Briefly, the 50- μm thick sections stained with cresyl violet were laid onto the corresponding adjacent sections processed with immunohistochemistry and were exactly aligned. The regions of interest (areas 9 and 24) were delineated with a stereology workstation, consisting of a modified light microscope (Olympus BX50 with UPlanApo objectives: 10 $\times$ for delineation of the regions of interest; 40 $\times$, oil, NA=1.0 for Space ball analysis; Olympus, Tokyo, Japan), motorized specimen stage for automatic sampling (Ludl Electronics; Hawthorne, NY), CCD color video camera (HV-C20AMP; Hitachi, Tokyo, Japan) and stereology software (Stereo- Investigator; MicroBrightField, Williston, VT). For this, the sections stained with cresyl violet were brought into focus, these sections were removed, and the sections processed with immunohistochemistry were brought into focus. Hemispheres with radius of 30 μm (generated by the stereology software while focusing through the section thickness) were systematically and randomly placed within the sections through the regions of interest (on average 365 hemispheres per brain half in area 9, and 426 hemispheres per brain half in area 24) (Fig. 1). The capillary length density was obtained from the total number of intersections between the hemispheres and the capillaries (on average 431 intersections per brain half in area 9, and 448 intersections per brain half in area 24) using the following formula derived from the description of the Space balls method [9, 46] according to the sampling scheme applied in the present study, and considering the area of the delineated region of interest and the actual section thickness after tissue processing [55]:

$$CLD = \frac{2 \times \sum is(D_x \times D_y \times t)}{2 \times \pi \times r^3} \times \frac{1}{V}$$

where CLD represents the capillary length density, S_i is the total number of intersections between the hemispheres and the capillaries, D_x and D_y the distance between the midpoints of the centers of the hemispheres in directions X and Y, t the actual average section thickness after histological processing, r the radius of the hemispheres, and V the investigated tissue volume. It is important to note that these capillary length density estimates were based on the analysis of thick sections in three dimensions (3D), and therefore not dependent on the plane of section, the spatial arrangement of the capillaries or their diameter. To focus the analysis on capillaries and to exclude arterioles and venules, the diameter of all vessels coming

into view during the space ball analysis was measured with the “Fast measure line” tool of the stereology software, and vessels with a diameter greater than 9 μm were not counted.

Cortical thickness was measured on the 50- μm -thick sections stained with cresyl violet with the “Fast measure line” tool of the stereology software ($\times 10$ objective). To consider variations in cortical thickness caused by oblique sectioning, equidistant traverses [43] spanning layers I—VI with distance between two neighboring traverses of 1 mm (measured at the top of layer I) were generated, and the mean cortical thickness per brain half was calculated from the mean length of the traverses.

Statistical analysis

For each group (schizophrenics, controls) and hemisphere (left, right), mean and standard error of the mean (SEM) were calculated for all investigated parameters. Comparisons between groups were performed with generalized linear models (GLM) multivariate analysis, with diagnosis and hemisphere as fixed factors, all investigated parameters (cortical gray matter volume, cortical thickness of both areas 9 and 24, and capillary length density within both areas 9 and 24) as dependent variables, and patients' age, postmortem interval and fixation time as covariates. All dependent variables were investigated simultaneously. An effect was considered statistically significant if its associated P value was smaller than 0.05. Calculations were performed using SPSS (Version 11.5 for Windows, SPSS, Chicago, IL).

Photography

Photomicrographs shown in Fig. 1a—d were produced by digital photography using the stereology workstation described above. On average approximately 125 images were captured for the composite in each Fig. 1a—d to represent the entire cortical thickness of area 9 (or area 24, respectively). These images were made into one montage using the Virtual Slice module of the Stereo- Investigator Software (MicroBrightField). Photomicrographs shown in Fig. 1e—h were produced by digital photography using an Olympus U-CMAD-2 digital camera attached to an Olympus AX 70 microscope and image analysis software (analysis; Imaging System, Munster, Germany). The final figures were constructed using Corel Photo-Paint v.11 and Corel Draw v.11 (Corel, Ottawa, Canada). Adjustments of contrast and brightness were made to facilitate recognition of the immunohistochemical signal at low and high magnification, without altering the appearance of the original materials.

Results

In a pilot study, the cortical gray matter volume of the investigated brains was analyzed and found to be on average significantly reduced in brains from schizophrenics compared to controls ($P=0.045$; 9.4% overall difference between schizophrenics and controls; 10.7% difference on the left side and 8.0% difference on the right) (Fig. 2; Table 2 provides an

overview on all *P* values of the multivariate analysis). Cortical thickness (layers I—VI) was found to be on average significantly reduced in area 24 in schizophrenics ($P < 0.001$; 10.6% overall difference between schizophrenics and controls; 11.7% difference on the left side and 10% on the right) but not in area 9 ($P = 0.368$) (Fig. 2). Microscopic inspection of immunohistochemically stained sections showed no overt differences in the pattern and the density of capillaries between schizophrenics and controls (Fig. 1). Stereological analysis revealed that the mean values of all samples from schizophrenics (area 9: 435.9 ± 8.8 mm/mm³; area 24: 391.7 ± 10.5 mm/mm³; mean $\pm$ SEM) did not differ from the corresponding mean values of all samples from controls (area 9: 434.5 ± 12.7 mm/mm³; 0.3% overall difference between schizophrenics and controls; area 24: 380.3 ± 6.5 mm/mm³; 3.0% overall difference between schizophrenics and controls) (Fig. 2). Further analysis using each hemisphere separately indicated that diagnosis had no significant effect on the mean capillary length density within area 9 ($P = 0.498$) and area 24 ($P = 0.056$); only the combination of diagnosis and hemisphere had a significant effect on the mean capillary length density within both areas 9 and 24 ($P = 0.038$ and $P = 0.033$, respectively) (Fig. 2). However, this did not indicate an overall reduction (or increase, respectively) in mean capillary density within the frontal cortex of the schizophrenics compared to the controls. Rather, the schizophrenics differed slightly compared to the controls in the asymmetry of the mean capillary length density between the left and the right hemisphere in both areas 9 and 24 (Fig. 2). Both schizophrenics and controls had a larger mean capillary length density in the right hemisphere compared to the left, which was found to be significant in area 24 ($P = 0.002$; 15.6% difference in schizophrenics and 4.6% difference in controls) but not in area 9 ($P = 0.117$; 2.8% difference in schizophrenics and 13.6% difference in controls). The patients' age, postmortem interval, and fixation time had no significant effect on any of the investigated parameters.

Discussion

This is the first rigorous analysis of capillary length density in the frontal cortex on a well-characterized sample of postmortem brains from schizophrenics in comparison to controls. The finding of a significantly reduced mean cortical gray matter volume in brains from schizophrenics compared to controls indicated that the sample of brains from schizophrenics investigated in this study shares a cardinal neuropathological feature of schizophrenia with previously reported case studies [17]. The slightly reduced mean cortical thickness in area 24 but not in area 9 in brains from schizophrenics was also in line with previous reports [4, 47, 56]. Thus, the schizophrenic brains investigated in this study showed typical features of the neuropathology of schizophrenia, but no significant alterations in the mean capillary length density within the frontal cortex compared to brains from controls.

As the biological reason for the slightly reduced mean cortical thickness in area 24 in

brains from schizophrenics is unknown, one can only speculate why no significant alteration in the mean capillary length density was found within this area. At first glance one could hypothesize that the 10.6% overall reduction in the mean cortical thickness of area 24 and the 3.0% overall increase in the mean capillary length density within this area in brains from schizophrenics compared to controls was due to compression of the brains from schizophrenics during tissue processing. However, the protocols used in the present study did not introduce selective compression of area 24. Furthermore, a reduced mean cortical thickness in area 24 in brains from schizophrenics was also reported in another study [4]. Moreover, on the left side both mean cortical thickness of area 24 and mean capillary length density within this area were reduced in brains from schizophrenics (albeit the latter was not significant), whereas, on the right side, the mean cortical thickness of area 24 was reduced and the mean capillary length density within this area was reduced in brains from schizophrenics. As the same trends were found for area 24 in brains from controls, one could hypothesize a slight compression of area 24 in the right hemisphere in both schizophrenics and controls during neurodevelopment, occurring after formation of the capillary bed, perhaps in connection with the slightly increased mean cortical gray matter volume of the right hemisphere found for both schizophrenics and controls (4.1% difference in schizophrenics and 1.3% difference in controls). However, this will need further investigation of human brain development.

Our data do not support the hypothesis that microvasculature abnormalities are a key alteration in the frontal cortex in schizophrenia [39]. Therefore, changes in capillary length density in the frontal cortex cannot be considered a general feature of schizophrenia, and it is unlikely that some type of chronic hypoxia-ischemia occurs in the frontal cortex due to structural microvascular abnormalities. Vice versa, chronic exposure of the frontal cortex to hypoxia-ischemia during fetal development or later in life, usually resulting in structural microvascular abnormalities [30], is neither a general event in the neuropathology of schizophrenia.

In two recent studies, reductions in resting regional cerebral blood flow (rCBF) within the frontal cortex of schizophrenics were reported [33, 45]. These reports were in contrast to earlier findings of an increased resting rCBF within the frontal cortex of schizophrenics [8] without providing an explanation for this discrepancy. Importantly, the results of these studies are not necessarily contradictory to the findings of the present study. Rather, recent work on transgenic mice expressing human vascular endothelial growth factor (VEGF) has shown that the capillary length density within the cortex does not predict resting rCBF within the cortex [52]. Despite a significantly increased capillary length density within the frontal cortex (approximately 20%) and only 3% of the capillaries not being perfused, the VEGF transgenic mice showed on average less than 10% increase in resting rCBF within the cortex (not significant) [52]. Interestingly, during severe hypercapnia, which was accompanied by maximal vasodilation, the VEGF transgenic mice even showed a slight reduction in rCBF within the cortex compared to non-transgenic controls. These findings point to functional changes in the vasculature itself or in neuronal and glial metabolism, which can be affected in neuropsychiatric disorders [27].

In summary, the results of the present study show that alterations in the capillary length density cannot account for the microvasculature abnormalities in the frontal cortex in schizophrenia hypothesized by Prabakaran et al. [39] as the reason for mitochondrial dysfunction, compromised brain metabolism, and oxidative stress in the frontal cortex of schizophrenics. Rather functional changes in the vasculature itself in schizophrenia or in neuronal and glial metabolism should be addressed in further studies. In this regard it should be noted that collagen IV is present only in the basal membranes of microvessels. Thus, it remains possible that endothelial cells themselves have subtle abnormalities. Pericytes surrounding microvessels and vascular smooth muscle cells of larger arterioles will require further investigation, as these cells are important for the regulation of CBF.

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Tables

Table 1. Clinical characteristics of all cases included in this study

No	A	O	Cause of death	PMI	Fix	Diagnosis	
	(y)	(y)		(h)	(d)	DSM-IV	ICD-10
S1	22	19	Suicidea	88	130	295.30	F20.00
S2	36	28	Suicidea	<72	115	295.30	F20.00
S3	46	24	Systemic hypothermia	<24	327	295.30	F20.01
S4	50	17	Peritonitis	<24	203	295.30	F20.00
S5	50	22	Suicide	18	170	295.30	F20.00
S6	51	17	Septicaemia	33	127	295.60	F20.50
S7	54	20	Septicaemia	27	250	295.60	F20.50
S8	55	22	Right-sided heart failure	25	84	295.30	F20.00
S9	57	37	Septicaemia	76	163	295.30	F20.00
S10	60	24	Pulmonary embolism	<48	311	295.30	F20.01
S11 ^b	62	19	Aspiration	7	171	295.30	F20.00
S12	63	22	Acute myocardial infarct	15	338	295.60	F20.50
S13	64	26	Pulmonary embolism	6	817	295.60	F20.50
C1	25	–	Cardiac tamponade	14	119	–	–
C2	36	–	Gunshot	24	143	–	–
C3	47	–	Acute myocardial infarct	<24	133	–	–
C4	50	–	Acute myocardial infarct	35	433	–	–
C5	50	–	Avalanche accident	23	498	–	–
C6	51	–	Septicaemia	7	285	–	–
C7	54	–	Acute myocardial infarct	18	168	–	–
C8	56	–	Acute myocardial infarct	60	3570	–	–
C9	58	–	Acute myocardial infarct	28	126	–	–
C10	60	–	Gastrointestinal haemorrhage	18	101	–	–
C11 ^b	60	–	Gastrointestinal haemorrhage	27	302	–	–
C12	62	–	Acute myocardial infarct	<24	3696	–	–
C13	65	–	Bronchopneumonia	6	2289	–	–

S = schizophrenic patient; C = control; A = age at death; O = age at onset; PMI = post-mortem interval (time between death and autopsy); Fix = fixation time; y = years; h = hours; d = days. ^aThese two patients had relatively long post-mortem intervals. However, both had committed suicide (one by hanging, the other by jumping from a building), were found within 1 h of death and were kept at 4°C until autopsy. Accordingly, the post-mortem intervals between death and autopsy of these patients cannot be compared with the corresponding intervals of the other cases and were thus excluded from the calculation of the mean post-mortem intervals. ^bThe volume of the CGM of these cases could not be analysed because sections from the frontal as well as the occipital poles were missing.

Table 2. Results of generalized linear models (GLM) multivariate analysis (p values). P values smaller than 0.05 are shown in boldface (CGMV cortical gray matter volume, T cortical thickness, CLD capillary length density, D diagnosis, H hemisphere)

Source	CGMV	9-T	9-CLD	24-T	24-CLD
Age	0.852	0.680	0.821	0.164	0.193
PMI	0.844	0.473	0.804	0.189	0.078
Fix	0.812	0.731	0.217	0.090	0.971
D	0.045	0.368	0.498	<0.001	0.056
H	0.730	0.417	0.117	0.838	0.002
D · H	0.881	0.644	0.038	0.454	0.033

Figures

Fig. 1. Analysis of capillary length density with the “Space balls” method. a–d Representative low-power photomicrographs of 50-µm-thick coronal sections from the left brain half of a schizophrenic patient (a, c) and a control (b, d) showing immunohistochemical detection of collagen IV in area 9 (a, b) and area 24 (c, d). Arrows indicate the border between layer VI and the white matter. The asterisk points to an artifact, which, at high magnification, did not disturb the analysis. e–h Representative high-power photomicrographs showing the same microscopic field at four consecutive focal planes below the upper surface, with a distance of 8 µm between the focal planes. The intersections of a hemisphere (a “space ball”) with a radius of 30 µm and the focal plane at the investigated focal depth are shown as circles. Intersections between the hemisphere and the capillaries in focus at the point of intersection are marked with arrowheads. Bars a–d 1 mm, e–h 60 µm

Fig. 2. Results of the stereological analysis of the total cortical gray matter volume as well of the cortical thickness of areas 9 and 24 and the capillary length density within these areas from 13 male schizophrenics (open bars) and from 13 age-matched male controls (closed bars). On the left side, all data from schizophrenics and controls are shown (left and right hemispheres); on the right side data are shown separately for the left and the right hemispheres. *Significant difference between schizophrenics and controls with uniform alterations on both sides

CHAPTER 5

Neuronal distribution in the neocortex of schizophrenic patients

Manuel F. Casanova^{a,*}, Pawel Kreczmanski^{b,c}, Juan Trippe II^a, Andrew Switala^a
Helmut Heinsen^d, Harry W.M. Steinbusch^{b,c}, and Christoph Schmitz^{b,c}

^aDepartment of Psychiatry and Behavioral Sciences, University of Louisville,
Louisville, KY 40292, USA

^bDepartment of Psychiatry and Neuropsychology, Division of Cellular Neuroscience,
Maastricht University, Maastricht, Netherlands

^cEuropean Graduate School of Neuroscience (EURON), Maastricht, Netherlands

^dMorphological Brain Research Unit, University of Wuerzburg, Wuerzburg, Germany

Abstract

It has been postulated that the prefrontal cortices of schizophrenic patients have significant alterations in their neuropil space. However, previous results have been contradictory and inconclusive, reporting both decreases and increases in the prefrontal neuropil. The present study re-examines these findings based on measurements of cell density, and intercellular distances within and between cell minicolumns. The results indicate alterations in the neuropil of schizophrenic patients according to both the lamina and cortical area examined. Alterations were present in all cortical areas studied. The findings suggest an alteration in the modulatory systems innervating the cell minicolumn. Furthermore, the lack of variation in core columnarity parameters argues in favor of a defect postdating the formation of the cell minicolumn.

Introduction

The minicolumn is a basic architectonic motif of mammalian neocortex, consisting of a core of pyramidal neurons and their associated apical dendritic and myelinated bundles oriented perpendicularly to the pial surface (Mountcastle, 1997; Buxhoeveden and Casanova, 2002). Around the circumference of this core is a cell-sparse peripheral neuropil space containing inhibitory interneurons, glia, basilar dendritic and collateral axonal elements, capillaries and synaptic elements. Changes in minicolumnar morphometry have been associated with neuropsychiatric pathology such as autism (Casanova et al., 2006a). A recent study analyzing mean cell spacing (MCS) in minicolumns of schizophrenic patients indicated significant interactions by both lamina and cortical area examined (Casanova et al., 2005). Parameters in this study also modeled pyramidal cell core integrity with indices of columnar width (CW) and a verticality index (VI) (representing relative distribution of somas along the radial axis). The study found no significant changes in these minicolumnar parameters.

The structure of each neocortical layer reflects a characteristic organization of dendritic arbors, synaptic components, and other elements. Mesocortical and basal forebrain projections of modulatory neurotransmitters ramify tangentially throughout the neocortex. These projections act via lamina-specific distributions of monoamine receptors predominately within neuropil (Benavides-Piccione et al., 2005) the largest compartment of which is the peripheral neuropil space surrounding the pyramidal cell core. Significant MCS changes in the context of stable values for both VI and CW in schizophrenia are consistent with an abnormality of cortical lamination. The implication is that the source(s) of pathology originates outside of the cortex and is independent of the core components of the minicolumn (Casanova et al., 2005).

The minicolumnar pyramidal cell core develops from an ontogenetic cell column in the fetal cortical plate comprising clonally-related neuroblasts migrating radially outward along a common radial glial fiber (Rakic, 1988, 1995). Its morphometry, including column width and radial orientation, is constrained by tangential distribution of progenitors arising from symmetric divisions in the germinal neuroepithelium and asymmetric divisions yielding the full complement of postmitotic daughter neurons within each ontogenetic column and is conserved over the course of prenatal and postnatal development (Casanova et al., 2006b). Lamination arises subsequent to formation of this columnar core cytoarchitecture (Kostović and Rakic, 1990) and concurrent with elaboration of mesocortical monoaminergic inputs ramifying tangentially through the cortical plate (Lambe et al., 2000; Verney, 2003). The largest proportion of local monoaminergic appositions is located in neuropil on dendritic processes (Benavides-Piccione et al., 2005). Monoaminergic inputs vary with regional and laminar cytoarchitecture (Berger et al., 1988), and alterations of monoaminergic input are associated with changes in dendritic structure and neuropil composition in schizophrenics (Benes et al., 2000). Lamina-specific development of neuropil elements, including migration of interneurons and glia, growth of dendritic arborizations and super-

facial axon collaterals affects neuronal spacing both within and between minicolumn cores. Changes in MCS in schizophrenic brains thus may reflect lamina-specific neuropil changes resulting from altered monoaminergic inputs during development.

In the present study we used a Boolean model (Hahn et al., 1999) to calculate the number of cells and the size of the soma from the same tissue slides as previously reported (Casanova et al., 2005). In this method, computer assisted image processing of coronal brain tissue photomicrographic sections generate characteristic radially-oriented periodic texture gradients corresponding to minicolumnar periodicity. Cell density is represented by the parameter λ , which incorporates values for particle surface area and intensity. This allows for comparisons of particle density across core and peripheral neuropil. We also obtained gray-level index values (GLI) corresponding to the relative proportion of somatic staining according to a modified method of Schleicher and colleagues (2005). A Delaunay triangulation method was employed to determine the distribution of intracolumnar relative to intercolumnar edge lengths between particles. Changes in the bimodal distribution for these values reflect lamina- and area-specific changes in density and organization of neuropil versus the minicolumn's pyramidal cell core.

Methods

Brain specimens

We investigated post mortem brains (both hemispheres) from 13 male schizophrenics with a mean age of 51.5 years (standard deviation [s.d.] 11.8 years) and a mean interval between death and autopsy of 27.6 hours (s.d. 19.9 hours). Controls consisted of 13 age-matched males with a mean age of 51.9 years (s.d. 11.1 years) and a mean interval between death and autopsy of 23.7 hours (s.d. 13.6 hours). Clinical diagnoses, causes of death and the interval between death and autopsy are summarized in Table 1. All schizophrenics were long-term patients at German university hospitals or at German state psychiatric hospitals. Complete clinical records were available in all cases. Records from autopsy (including a summary of the medical history) were available for all controls. All of the patients met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria for schizophrenia. The clinical notes were assessed by experienced clinical psychiatrists (mentioned in Acknowledgements) to ensure that the control brains were free from psychopathology, and for clear evidence that the schizophrenic cases conformed to DSM-IV criteria for schizophrenia. In particular, the schizophrenic group included no patient diagnosed with schizoaffective disorder. Exclusion criteria comprised neurological problems that required intervention or interfered with cognitive assessment (e.g., stroke with aphasia), history of recurrent seizure disorder (i.e., epilepsy), history of severe head injury with loss of consciousness, diabetes mellitus with poor glycemic control (free plasma

glucose >200 mg/dl), and history of self-induced water intoxication with hyponatremia. Brains from schizophrenics were also excluded if patients had undergone a leukotomy. The two groups – schizophrenics and controls – were similar in terms of the ethnic backgrounds of the subjects. However, they were not fully matched for socio-economical status and education, which would have placed severe constraints on our sample. Moreover, all schizophrenics were subjected to long-term treatment with high doses of neuroleptics.

In all of the cases, autopsy was performed after consent was obtained from a patient's relative according to the laws of the Federal Republic of Germany. The use of these autopsy cases for scientific investigations as outlined here has been approved by the relevant Institutional Review Boards (IRB). Brains were fixed by immersion in 10% formalin (1 part commercial 40% aqueous formaldehyde in 9 parts H₂O) for at least 3 months and up to 11 years. As the distortion due to fixation stabilizes after no more than approximately 3 weeks (Quester and Schroeder, 1997), all brains would have reached their stable, post-fixation state when studied. Thus, the duration of fixation can be ruled out as a potential confound.

Tissue processing

The brainstem with the cerebellum was severed at the level of the rostral pons, and the hemispheres divided medio-sagittally. Then, both hemispheres were cut into serial 600 µm to 700 µm thick coronal sections as previously described in the literature (Heinsen and Heinsen, 1991). Briefly, the hemispheres were cryoprotected in a mixture of glycerol-dimethylsulfoxide-formalin after carefully removing meninges and pial vessels. They were then embedded in gelatin, frozen at -60° C, and serially sectioned using a cryostat (Jung, Nussloch, Germany). One brain (control case C7) was embedded in celloidin (Heinsen et al., 2000) and cut into serial 440 µm thick coronal sections using a sliding microtome (Polycut, Cambridge Instruments, UK). From each hemisphere, every second or third section was stained with gallocyanin (Heinsen and Heinsen, 1991). The remaining sections were stored in formalin for up to ten years.

From all the brains of schizophrenics and controls older than 40 years, sections through the central parts of the entorhinal and transentorhinal cortices were labeled with the Gal-lyas silver method to detect neurofibrillary changes (Heinsen et al., 1999). Neurofibrillary tangles were rarely detected. In affected patients the findings corresponded to Braak-Braak Alzheimer classification stage I (Braak and Braak, 1995). Furthermore, all of the sections used in this study were coded and controlled for the absence of tumors, infarcts, heterotopias, signs of autolysis, staining artifacts, and gliosis.

Gallocyanin-stained sections were used by two of us (P.K. and C.S.) to identify areas M1, V1, frontal association cortex (areas 4, 17, and 9 of Brodmann [1909], respectively) and S1 (area 3b of Vogt and Vogt [1919]) in both cerebral hemispheres according to anatomical landmarks and cytoarchitectural criteria. Gross anatomical landmarks for M1 include the anterior wall of the central sulcus and adjacent portions of the precentral gyrus. Cytoarchitecturally, the region is clearly demarcated by its giant Betz cells and minimization of layer IV. Area 17 (V1) is located along the walls of the calcarine sulcus in the oc-

cipital lobe and adjacent portions of the cuneus and lingual gyrus (Carpenter, 1985). It is defined histologically by a broad lamina IV divided into 3 sublayers with numerous very small pyramidal cells in layers II and III. It is noted for the dense line of Gennari in myelin stains. Area 9 lies in the superior and middle frontal gyrus. Rajkowska and Goldman-Rakic (1995a, 1995b) found that it was located in the middle third of the superior frontal gyrus in all the cases they examined. It covered both dorsolateral and dorsomedial surfaces of this gyrus and extended in some cases to the depth of the superior frontal gyrus and portions of the middle frontal gyrus.

Concerning area 9, Rajkowska and associates showed that its more lateral portions embrace area 46 (area FΔ of Economo and Koskinas [1925]) (Rajkowska and Goldman-Rakic, 1995a, 1995b). It is important to note that Brodmann (1909) never described the cytoarchitectonic characteristics of area 9 in man. Rather he projected his observations in monkey onto the frontal lobe of man. Economo and Koskinas (1925) provided a detailed cytoarchitectonic description of the human frontal lobe. Their area FΔm corresponds to area 9. It is characterized by a distinct layer II, absence of very large, slender and well-elaborated pyramidal cells in layer IIIc (typical of area 6 and to a lesser extent of area 8), and an uninterrupted layer IV with fairly definite upper and lower boundaries. In thick gallo-cyanin-stained sections a cell-poor layer is found between layers IIIc and layer IV. Layer IV is more prominent and cell-rich in the frontopolar regions; however the transition between areas 9 and 10 is a gradual one. Layer V of area 9 is subdivided into layers Va and Vb, the latter of which is rather cell-sparse. Most probably the cell-poor layers between lamina IIIc and IV and within layer Vb correspond to the upper and lower band described by Rajkowska and associates in myelin-stained sections of area 9 (Rajkowska and Goldman-Rakic, 1995a, 1995b). Finally, layer VI consists of sublayers VIa and VIb. VIa is cell-rich and its upper border to the cell-sparse layer Vb can easily be recognized. The lower boundaries of layer VIb to the underlying white matter are much more distinct than in the caudally ensuing areas 8 and 6. The overall cortical thickness of the frontopolar area 10 (field FE of Economo and Koskinas [1925]) is considerably less than in area 9, lamina VIa is small, rich in darkly-stained pyramidal as well as spindle-shape cells and the boundary of VIa to the medullary layer is even more distinct than in area 9. The presence of the small and cell rich layer VI facilitates the delineation of area 9 from the rostral area 10 in thick gallo-cyanin stained sections. Finally, layer IV is more conspicuous than its corresponding part in area 9.

Regions of interest were removed from adjacent sections stored in formalin and not stained with gallo-cyanin as 2 cm × 2 cm tissue pieces. After rinsing in tap water for several hours, the pieces were stored in tris-buffered saline (TBS) overnight (pH = 9.0). They were then placed in TBS (pH = 9.0) and heated in a microwave oven for 15 min at 90° C for antigen retrieval (results of immunohistochemical analysis will be published elsewhere). After cooling down, the tissue blocks were rinsed in TBS twice (pH = 7.4) and placed in a TBS based 30% sucrose solution for cryoprotection. The pieces were then frozen and cut into 50 μm thick sections using a cryostat (Frigocut 2800E; Jung, Nussloch, Germany). These 50 μm thick sections were stained with cresyl-violet (0.1%, 20 min).

Computerized image analysis

Digital images were taken from laminae III and V in cortical areas M1, S1, V1, and Brodmann area 9 in both cerebral hemispheres. Photomicrographs were taken through the flat face of the gyri with minicolumns perpendicular to the pia. The equipment (Nikon E-800 microscope, using a 10× objective and digital camera) was such that each field of view measured approximately 1.45 mm². Up to three fields were captured per lamina/area/hemisphere as tissue quality allowed. Of the 416 possible regions of interest (16 in each of 26 brains), 90 were completely lacking in usable tissue; the number of images captured per available region numbered 2.7.

Color micrographs were converted to intensity (grayscale) images, which were corrected for uneven illumination and subsequently thresholded to produce binary images using Otsu's (1979) method. Boolean model and GLI parameters (below) were estimated from these bitmaps without further processing. In order to estimate the interneuronal distance distributions, the foreground (dark) regions of the binary images were segmented into connected regions. Clusters of overlapping cell somata, which appear to be single contiguous regions in the binary images, were further separated using the watershed transformation. Connected regions were classified according to size. Objects smaller than 10 μm², assumed to be glia, neuron fragments, or noise, were discarded altogether. Of the remaining objects, those with areas above the 15th percentile were taken to be pyramidal cell somata, while the smaller objects were classified as interneurons. The latter threshold was based on the fraction of all neurons estimated to be inhibitory by Braitenberg and Schüz (1998).

Computerized image analysis produced estimates of the surface fraction (i.e. the proportion A_A) of Nissl-stained tissue within the sections, the boundary length L_A of stained tissue per unit area, and the planar convexity N_A^+ , which quantities suffice to compute parameters of a Boolean model with convex primary grains using the method of moments (Stoyan et al., 1995). In this spatial process, a random closed set (the stained region of an image in our case) is modeled as the union of primary sets (grains) drawn at random from a distribution with mean grain area is $\bar{A}$ and mean grain perimeter is $\bar{U}$. The primary grains, or rather their centroids, are placed at the points of a Poisson process with intensity λ . These three parameters, which completely determine the Boolean model, were obtained algebraically from the following equations (Stoyan et al., 1995), substituting for A_A , L_A , and N_A^+ their respective estimates:

$$A_A = 1 - e^{-\lambda \bar{A}}.$$

$$N_A^+ = \lambda(1 - A_A).$$

$$L_A = N_A^+ \bar{U}.$$

Interactions between points, such as clumping in minicolumns, are not considered in this simple model. Parameter estimates represent an average across an entire microscopic field.

Thresholded images were also analyzed according to the gray level index (GLI) method (Schleicher et al., 2005), slightly modified so that the GLI varied smoothly across a region. Here, the inverse thresholded image, with foreground set equal to one and background set equal to zero, was convolved with a kernel measuring $11\ \mu\text{m}$ by $110\ \mu\text{m}$ at half-maximum. The long axis of the kernel was parallel to the axes of minicolumns, following the image Y-axis. What resulted was an estimate of the GLI, or staining intensity, in the neighborhood of each pixel. Each GLI image so produced was measured in profiles in the X direction to obtain:

- mean (D) and standard deviation (sdD) in distance between local maxima of the GLI,
- amplitude (A) of the local maxima, or difference between peak GLI and the lowest GLI between the peak in question and the next peak in the profile, and
- mean (W) and standard deviation (sdW) in the width (full width at half maximum) of the peaks.

A follow-up study considered the distance between neighboring objects, excepting those below the 15% area threshold for pyramidal cells. Objects were considered neighbors if (a) they were endpoints of an edge of the Delaunay triangulation of object centroids, and (b) they were not further apart than the distance from either of them to the edge of the region of interest. If objects in a single lamina are clustered, as cells in minicolumns, an object's neighbors may include members of the same cluster or members of a nearby cluster (Figure 1). The distribution of distances d between neighbors is then a mixture of intracluster or near distances, and intercluster or far distances. This was modeled as a two component lognormal mixture distribution

$$\log d \sim \alpha N(\mu_{\text{near}}, \sigma_{\text{near}}^2) + (1 - \alpha)N(\mu_{\text{far}}, \sigma_{\text{far}}^2); 0 \leq \alpha \leq 1.$$

Schizophrenic and normal patients were paired for purposes of statistical analysis. Testing employed multivariate ANOVA with fixed effects diagnosis, cortical area, hemisphere, lamina, and corresponding interactions in a full factorial design. Separate multivariate ANOVA tests were performed for each of the three sets of parameters (Boolean model, GLI, and distance distribution). P values were estimated from Wilks's λ , transformed to the equivalent F statistic.

Results

Multivariate testing of the Boolean model parameters was significant in the diagnosis by cortical area interaction ($F = 3.28$; d.f. = 9, 682; $P = 0.0006$), the lamina by cortical area interaction ($F = 3.02$; d.f. = 9, 682; $P = 0.0015$), and the main effects of cortical area ($F = 78.5$; d.f. = 9, 682; $P < 0.0001$) and lamina ($F = 42.0$; d.f. = 3, 682; $P < 0.0001$). Of the individual parameters, only λ depended significantly on the interaction of diagnosis and cortical area ($F = 6.58$; d.f. = 3, 282; $P = 0.0003$). Multivariate testing of the

GLI parameters also revealed a statistically significant diagnosis/cortical area interaction ($F = 1.79$; d.f. = 15, 768; $P = 0.032$), as well as significant dependence on cortical area alone ($F = 23.3$; d.f. = 15, 768; $P < 0.0001$) and on lamina ($F = 11.8$; d.f. = 5, 278; $P < 0.0001$). None of D , sdD , W , sdW , or A , however, individually varied with the diagnosis/cortical area interaction with any significance. Regarding the distribution of interneuronal distances, multivariate tests were again significant for the interaction of diagnosis and cortical area ($F = 3.52$; d.f. = 6, 562; $P = 0.0020$), and additionally diagnosis alone ($F = 4.45$; d.f. = 2, 281; $P = 0.012$), cortical area alone ($F = 76.3$; d.f. = 6, 562; $P < 0.0001$), lamina ($F = 17.0$; d.f. = 2, 281; $P < 0.0001$), and cortical area/lamina interaction ($F = 4.61$; d.f. = 6, 562; $P = 0.0001$). The near and far mean distances each varied significantly with diagnosis/cortical area interaction ($F = 5.38$ and 5.33 , respectively; d.f. = 3, 282; $P = 0.0013$ and 0.0014 , respectively), although no significant diagnosis main effect was found in either parameter.

The potential for distortion due to heating the tissue for antigen retrieval was considered. Samples of the four cortical areas of interest were obtained from both microwave-treated and untreated blocks of tissue from four patients. One sample was accidentally destroyed, leaving 15 pairs of treated/untreated tissue samples. Computerized image analysis of lamina III produced estimates of the Boolean model parameters. Matched pairs testing found no significant effect on $\bar{A}$ ($t = 1.42$; d.f. = 15; $P = 0.0878$) or λ ($t = 0.764$; d.f. = 15; $P = 0.228$) due to heating, although there was a significant effect on $\bar{U}$ ($t = 3.17$; d.f. = 15; $P = 0.0032$). Since a diagnosis-dependence was found in λ only, microwaving the tissue is unlikely to have confounded the results.

Discussion

In this study of post mortem brain tissue obtained from schizophrenic subjects, we demonstrated morphometric changes consistent with altered neocortical neuropil. Indices of intra- and intercluster cellular density varied systematically across all sampled areas according to their relative position along the anteroposterior axis. These data extend long-established findings that the neocortex can be regionally parcellated according to cytoarchitectonic criteria, and confirm that the criteria used in our sampling methods were valid. Diagnosis-by-area interactions were derived for cell intensity modeled as λ , a parameter incorporating local particle density and surface area according to the Boolean method for stereological analysis of periodic gradient textures (Hahn et al., 1999). Regional values for λ were consistent with cell density values established previously in humans and monkeys with stereological cell-counting methods (Selemon, 2001). Values obtained for λ reflected area-specific cytoarchitectonics i.e. increased granular cell density in striate cortex compared with lower density in area 4 (Beaulieu et al., 1992; DeFelipe et al., 2001). The difference in area values of λ between schizophrenic and matched samples varied systematically along the caudorostral axis. Values for λ were decreased in areas 17 and 3 and increased in areas

4 and 9 (Figure 2) relative to matched samples. This finding suggests that earlier reports (Selemon et al., 1995, 1998) of increased cell density in dorsolateral prefrontal cortex in schizophrenics may reflect an accentuation of morphogen patterning gradients over the full rostrocaudal extent of the telencephalic vesicular surface prior to or concurrent with area-specific cytoarchitectural development.

These data complement previous findings by Selemon and colleagues (1995, 1998), obtained by direct cell counting, which demonstrated neuronal density increases of 17% in area 9, 10% in area 17, and 21% in area 46. In view of findings in schizophrenic brains of increased neuronal density and preservation of total neocortical cell count (Pakkenberg, 1993; Selemon et al., 1998), numerous histopathologic and imaging studies documenting cortical volume reduction of 3% to 13% suggest a relative decrease of neuropil in the neocortex of schizophrenics (Selemon and Goldman-Rakic, 1999). To further assess relative proportions of somal area and neuropil, we used an automated imaging method to determine the gray level index (GLI), the area percentage of Nissl-stained perikarya (Wree et al., 1982; Schleicher et al., 2005), for tissue in each sample area. Our results indicated an area-by-diagnosis interaction consistent with those obtained by Vogele and colleagues (2003) who used a similar method to determine GLI in area 10 of schizophrenics' prefrontal cortex. This group observed decreased GLI in all layers, possibly reflecting increased neuropil or a decreased perikaryal fraction. This finding conflicts with Selemon and co-workers' (1995, 1998) stereological data suggesting reduced neuropil in prefrontal areas. Relevant to this finding, the GLI method provides an index for image area percentage of Nissl-stained material above an intensity threshold determined by the model and thus does not provide direct information concerning neuronal density within that area.

In order to investigate this discrepancy, we employed the Delaunay triangulation method to quantify changes in the morphometry of core radial arrays of pyramidal cells and their peripheral neuropil. This method generated a mixed bimodal distribution comprising short inter-particle distances determining mean cell spacing within minicolumnar pyramidal cell cores and long inter-particle distances indicative of the width of neuropil space between cores. Data obtained from brains of our schizophrenic cohort exhibited area-by-diagnosis interactions and a caudorostral gradient of progressively decreased values for distribution means of distances between Nissl-stained particles. These results confirm those of previous studies (Selemon, 2001) showing a comparable caudorostral trend toward increased overall neocortical density in brains of schizophrenics.

Greater reduction in the distribution modes for intra-cluster relative to inter-cluster distances was observed in rostral neocortical Brodmann areas 4 and 9 (Table 2, Table 3). The degree of reduction of mean intercluster distances in the Delaunay model corresponds to the decrease in cortical plate thickness of Brodmann area 9 in schizophrenics (Selemon et al. 2003). Reduction of peripheral neuropil by this measure can thus be accounted for by laminar thinning rather than decreased peripheral neuropil width. A recent study (Casanova et al., 2005) conducted on the same brain tissue series as analyzed in the present investigation demonstrated a significant dependence of mean cell spacing on diagnosis interactions with

area and lamina. However, there was no significant diagnosis effect for parameters of minicolumnar width or vertical orientation of minicolumnar components. The relatively greater degree of decrease in mean intracluster distance, i.e. the Delaunay parameter corresponding to mean distance between cells within the pyramidal cell core, is consistent with previously documented size reduction of basal dendritic arbors contributing to both core and peripheral neuropil volume (Kalus et al., 2000; Broadbelt et al., 2002; Black et al., 2004), core pyramidal somata providing them metabolic support (Rajkowska et al., 1998; Pierri et al., 2001; Cotter et al., 2002) as well as apposed astroglia (Rajkowska et al., 1998). Astroglial cell death (*contributing to reduced GLI*) and *reduced neuronal size without cell death* is evident in dorsolateral prefrontal cortex of schizophrenics (Cotter et al., 2002); these results reconcile findings in prefrontal areas of increased cell density with those of reduced GLI.

Minicolumnar morphometry may be constrained by developmental processes (i.e., coordinated migration of neuronal cohorts), mechanical factors (i.e., gyrification and white matter tension effects) or functional constraints (i.e., macrocolumnar organization). Changes in mean cell spacing within its radial framework likely reflect alterations in peripheral neuropil of intracolumnar circuit features including dendritic arborization, synaptopil and networks of astroglia and inhibitory interneuron connections. These elements are largely distributed in and constitute the bulk of peripheral neuropil. Tangentially oriented basilar dendritic processes, axon collaterals and their synaptic connections also provide the network of connections characteristic of each lamina. The finding of decreased mean cell spacing in this report complements our earlier study demonstrating preserved minicolumnar morphometry by describing changes attributable to the peripheral neuropil compartment.

Numerous reports in recent years have documented schizophrenia-related qualitative/quantitative alterations of neuropil components, varying systematically according to layer and area. In dorsolateral prefrontal cortex (area 46), significantly increased neuronal density was identified specific to layers II/III, IV, and VI (Selemon, 1998). Corresponding layer-specific reductions were demonstrated in pyramidal cell spine density (Glantz and Lewis, 2000; Koluri et al. 2005), GABA-related gene transcripts and molecules (Pierri et al., 1999; Hashimoto et al., 2003) as well as molecules related to synaptic development and transmission (Eastwood and John, 2004). In area 9, a reduced-width layer V yielded decreased immunoreactivity for GFAP, suggesting a diminished density/volume of glial processes in this region (Rajkowska et al., 2002). Decreased glial numbers have been documented in prefrontal and motor (Benes et al., 1986) as well as anterior cingulate (Stark et al., 2004) cortices. Less glia also provide diminished metabolic support for other neuropil components thereby amplifying neuropil loss. These reductions in laminar width may account for the increased pyramidal cell density in minicolumn cores whose widths are unaltered in schizophrenia. The central role of neuropil components in the pathology of schizophrenia is emphasized by the results of microarray studies which implicate regional alterations of mRNA expression for molecules involved in synaptic transmission (Mirnics et al., 2001). Linkage and association studies have identified the largest proportion of schizophrenia-related genes to be involved in mechanisms regulating synaptic development and plasticity (Harrison and Weinberger, 2005).

Mesocortical monoaminergic projections constitute a significant component of neuropil and serve to modulate glutamatergic and GABAergic output (Seamans and Yang, 2004). These projections play a central role in regulating cell arrays subserving working memory (Yang et al., 1999; Gao and Goldman-Rakic, 2003) in dorsolateral prefrontal cortical areas affected in schizophrenia. Catecholaminergic and serotonergic fibers and receptor classes have characteristic area- and layer-specific distributions (Verney, 2003). Innervation of frontal areas in primates by dopaminergic axons exhibits layer-specific post-natal development which anticipates the specific pattern of dopamine receptor expression (Lidow and Rakic, 1992) and synaptic growth and regression in these areas (Rosenberg and Lewis, 1995; Rakic et al. 1996). Middle layers exhibiting greatest relative changes in dopamine innervation and synaptogenesis are those with notable width-reduction in schizophrenia. Axons and varicosities immunolabeled for tyrosine hydroxylase show area- and layer-specific reductions in schizophrenic cases (Akil et al., 1999). Monoaminergic brainstem nuclei develop within the first trimester in humans, and their projections extend through the basal forebrain and ramify tangentially throughout neocortex by midgestation (Nobin and Björklund, 1973; Verney et al., 1993). Monoaminergic innervation is concurrent with emergence of cortical plate lamination from 20 to 30 weeks of gestation in humans (Levitt, 2003). Thus developmental perturbations of monoaminergic nuclei during the first two trimesters may disrupt corticogenesis. Activity of aminergic and cholinergic projections modulates multiple signaling mechanisms thereby influencing early patterning and neuronal specification events. Within the context of a given stochastic and epigenetic history, such activity influences the cascade of cortical developmental events. Data generated from perinatal focal ablation of mesocortical dopamine projections resulted in layer- and area-specific reductions, including cortical width in rodents (Onténiente et al., 1980), decreased dendritic branching (Kalsbeek et al., 1987) and layer-specific reduction of dendritic length and spine densities (Felten et al., 1982).

The neocortex is an assemblage of interdependent modules. Within each module connectivity between its internal elements far exceeds connectivity between homologous modules (Casanova et al., 2003). This bias in the way that neurons connect to each other engenders a canonical circuit with massive ion-gated excitatory connections flowing through vertical strips of the cortex (Braitenberg and Schüz, 1998; Douglas and Martin, 2004). Researchers have traced the developmental transformation of ontogenetic columns into mature elements (Krmpotić-Nemanić et al., 1984; Casanova et al., 2006b). It may be that the early scaffolding of neuronal precursors along radial glia projections during corticogenesis provides the opportunity for close contact and future Hebbian reciprocity. Unsurprisingly neurons with similar stimulus selection are found in close proximity to each other (Reich et al., 2001). Therefore, the minicolumn is a possible way of compartmentalizing neurons into a vertical component that shortens both neuronal processes and conduction times.

Perpendicular to the vertical axis of information processing, slower-onset, second messenger systems provide a modulatory effect on minicolumns, i.e., cholinergic, histaminergic, serotonergic, noradrenergic, and dopaminergic systems. These modulatory systems origi-

nate from groups of neurons that primarily employ small amines to bypass the thalamus and directly innervate large portions of the cortex. The terminal zones of these systems comprise an element of the horizontal or laminar organization of the cortex. Both cytoarchitectonic and myeloarchitectonic studies have used different lamination parameters to define anatomical regions of the cortex. However, the resultant parcellation schemes do not necessarily coincide with the innervation of individual neurochemical systems (Levitt, 2003).

Our results suggest that in schizophrenia the channels that provide for information processing within the neocortex, i.e., minicolumns, are normal. Rather, abnormalities are present in lamina where modulator systems relate information processing to behavioral states, arousal, attention, mood and motivation (Mesulam, 2000). The result may be an alteration in the “state” of information processing manifesting itself as a wide array of manifestations that characterize schizophrenia as a syndrome.

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Tables

Table 1. Clinical characteristics of the cases included in this study. PMI: interval between death and autopsy. Storage: duration of immersion in 10% formalin. All schizophrenic patients and all controls were male.

No.	Age [years]	Cause of death	PMI [hours]	Storage [days]	Diagnosis	
					DSM-IV	ICD-10
S1	22	Suicide	88 ¹	130	295.30	F20.0 ²
S2	36	Suicide	<72 ¹	115	295.30	F20.0 ²
S3	46	Systemic hypothermia	<24	327	295.30	F20.01 ²
S4	50	Peritonitis	<24	203	295.30	F20.0 ²
S5	50	Suicide	18	170	295.30	F20.0 ²
S6	51	Septicemia	33	127	295.60	F20.50 ³
S7	54	Septicemia	27	250	295.60	F20.50 ³
S8	55	Right-sided heart failure	25	84	295.30	F20.00 ²
S9	57	Septicemia	76	163	295.30	F20.00 ²
S10	60	Pulmonary embolism	<48	311	295.30	F20.01 ²
S11	62	Aspiration	7	171	295.30	F20.00 ²
S12	63	Acute myocardial infarct	15	338	295.60	F20.50 ³
S13	64	Pulmonary embolism	6	817	295.60	F20.50 ³
C1	25	Cardiac tamponade	14	119		
C2	36	Gunshot	24	143		
C3	47	Acute myocardial infarct	<24	133		
C4	50	Acute myocardial infarct	35	433		
C5	50	Avalanche accident	23	498		
C6	51	Septicemia	7	285		
C7	54	Acute myocardial infarct	18	168		
C8	56	Acute myocardial infarct	60	3570		
C9	58	Acute myocardial infarct	28	126		
C10	60	Gastrointestinal hemorrhage	18	101		
C11	60	Gastrointestinal hemorrhage	27	302		
C12	62	Acute myocardial infarct	<24	3696		
C13	65	Bronchopneumonia	6	2289		

¹These patients had long post mortem intervals (PMI). However, both patients committed suicide (one by hanging, the other by jumping from an eight story building), were found within 1 h of death, and were kept at 4° C until autopsy. Accordingly, the intervals between death and autopsy of these patients cannot be compared to the corresponding intervals of the other persons and were thus excluded from the calculation of the mean PMI between death and autopsy.

²Paranoid schizophrenia.

³Residual schizophrenia.

PMI: interval between death and autopsy. Storage: duration of immersion in 10% formalin. All schizophrenic patients and all controls were male.

Table 2. Mean values of those measurements with joint dependence on diagnosis and cortical area. S: schizophrenic group; C: normal control group. The mean of both laminae investigated is given. To obtain values specific to lamina III (respectively, lamina V), subtract (resp., add) 179 mm^{-2} to λ , and multiply (resp., divide) m_{near} by 1.016. m_{far} does not differ significantly by lamina. S.D. is the Residual root mean squared error, or pooled standard deviation. Standard deviations for interneuronal distances are given as percentages of the mean value.

Brodmann area		λ (mm ⁻²)		m_{near} (μm)		m_{far} (μm)		$m_{far} - m_{near}$ (μm)	
		S	C	S	C	S	C	S	C
Rostral ↑ Caudal ↓	9	1416	1308	24.3	25.2	48.3	49.8	24.0	24.6
	4	1313	1291	25.1	25.7	51.0	50.9	25.9	25.2
	3	1673	1867	24.2	23.6	48.2	46.5	24.0	22.9
	17	2838	3230	20.8	20.0	40.3	38.3	19.5	18.3
S.D.		387		6.87%		6.98%		10.36%	

Table 3. 95% confidence intervals on the mean values in Table 2. The same remarks apply regarding values in lamina III versus lamina V.

		λ (mm ⁻²)		m_{near} (μm)		m_{far} (μm)	
Brodmann area		S	C	S	C	S	C
Rostral ↑ Caudal ↓	9	[1317,1514]	[1215,1404]	[23.9,24.8]	[24.7,25.6]	[47.5,49.2]	[48.9,50.6]
	4	[1216,1406]	[1185,1398]	[24.7,25.5]	[25.3,26.2]	[50.2,51.9]	[49.9,51.9]
	3	[1578,1767]	[1740,1990]	[23.8,24.7]	[23.1,24.1]	[47.4,49.1]	[45.5,47.5]
	17	[2742,2933]	[3119,3342]	[20.4,21.1]	[19.6,20.4]	[39.6,41.0]	[37.6,39.1]

Figures

Fig. 1. In this schematic, a linear cluster (minicolumnar fragment) is situated between two neighboring clusters. Cells, whether in the same cluster or no, are considered adjacent if they are joined by an edge of the Delaunay triangulation of cell centroids. (Three points comprise the vertices of a Delaunay triangle if and only if the circle through those points has no other point in its interior.) Delaunay edges between cells within a cluster (solid lines) will tend to be shorter than edges between cells in different clusters (broken lines). Not knowing a priori which cells belong to which cluster, one can estimate the intracluster and intercluster distances by considering the lengths of edges to be distributed according to a two-component mixture model. Edges of the triangulation not involving any of the points of the middle cluster in the diagram have been omitted for clarity.

Fig. 2. Increased cell density can be seen in these micrographs of prefrontal area 9 (right hemisphere, lamina III). In the normal patient (left), estimated Boolean model parameters were $\bar{A} = 85.8 \mu\text{m}^2$, $\bar{U} = 31.6 \mu\text{m}$, and $\lambda = 823 \text{ mm}^{-2}$. In the schizophrenic patient (right), $\bar{A} = 73.4 \mu\text{m}^2$, $\bar{U} = 29.4 \mu\text{m}$, and $\lambda = 1640 \text{ mm}^{-2}$. Scalebars measure $250 \mu\text{m}$.

CHAPTER 6

Mean cell spacing abnormalities in the neocortex of patients with schizophrenia

Manuel F. Casanova^{a,*}, Leonie de Zeeuw^b, Andrew Switala^a
Pawel Kreczmanski^{b,c,d,e,f}, Hubert Korr^{e,f}, Norbert Ulfing^c, Helmut Heinsen^g
Harry W.M. Steinbusch^{b,f}, Christoph Schmitz^{b,c,f}

^aDepartment of Psychiatry and Behavioral Sciences, University of Louisville,
Louisville, KY 40292, USA

^bDepartment of Psychiatry and Neuropsychology, Division of Cellular Neuroscience,
University of Maastricht, Maastricht, Netherlands

^cNeuroembryonic Research Laboratory, Department of Anatomy,
University of Rostock, Rostock, Germany

^dDepartment of Anatomy, Center of Biostructure, Medical University of Warsaw,
Warsaw, Poland

^eDepartment of Anatomy and Cell Biology, RWTH Aachen University, Aachen, Germany

^fEuropean Graduate School of Neuroscience (EURON), Maastricht, Netherlands

^gMorphological Brain Research Unit, University of Wuerzburg, Wuerzburg, Germany

Abstract

It has been postulated that the prefrontal cortices of schizophrenic patients have significant alterations in their interneuronal (neuropil) space. The present study re-examines this finding based on measurements of mean cell spacing within the cell minicolumn. The population studied consisted of 13 male schizophrenic patients (DSM-IV criteria) and 13 age-matched controls. Photomicrographs of Brodmann's areas 9, 4 (M1), 3b (S1), and 17 (V1) were analyzed with computerized image analysis to measure parameters of minicolumnar morphometry, i.e., columnarity index (CI), minicolumnar width (CW), dispersion of minicolumnar width (VCW), and mean interneuronal distance (MCS). The results indicate alterations in the mean cell spacing of schizophrenic patients according to both the lamina and cortical area examined. The lack of variation in the columnarity index argues in favor of a defect postdating the formation of the cell minicolumn.

Introduction

It has been noted that the prefrontal cortices of schizophrenic patients have less interneuronal (neuropil) space than controls, specifically in areas 9 and 46 (Selemon et al., 1995). This is thought to reflect an alteration in the neural circuitry of these areas and is a possible component of the pathology of schizophrenia. More recently, other researchers have used an automated image analysis method to scan the whole cortical width (Brodmann area 10) of schizophrenic patients for gray-level index (GLI), i.e., the area percentage covered by perikarya (Vogeley et al., 2003). Results of these experiments – reductions in the gray-level index of all cortical layers – were consistent with a decreased perikaryal fraction or an increased amount of neuropil. All of these studies were based on measures of neuronal density which focus on morphological alterations at the cellular level while disregarding the anatomical interdependence of neurons. The lack of analysis of the anatomical arrangement of neurons into physiological units might compromise any results from neuropathological studies. Neurons are interdependent elements within cortical modules, the smallest of which seems to be the cell minicolumn (Casanova et al., 2003; Mountcastle, 1998). Our approach takes advantage of the capabilities of computerized image analysis to procure adequate information regarding the morphology of cortical minicolumns.

Methods

Brain specimens

We investigated post mortem brains (both hemispheres) from 13 male schizophrenics with a mean age of 51.5 years (standard deviation [s.d.] 11.8 years) and a mean interval between death and autopsy of 27.6 hours (s.d. 19.9 hours). Controls consisted of 13 age-matched males with a mean age of 51.9 years (s.d. 11.1 years) and a mean interval between death and autopsy of 23.7 hours (s.d. 13.6 hours). Clinical diagnoses, causes of death and the interval between death and autopsy are summarized in Table 1. All schizophrenics were long-term patients at German university hospitals or at German state psychiatric hospitals. Complete clinical records were available in all cases. Records from autopsy (including a summary of the medical history) were available for all controls. All of the patients met the Diagnostic Statistical Manual, 4th revision (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) diagnostic criteria for schizophrenia. The clinical notes were assessed by experienced clinical psychiatrists (mentioned in Acknowledgements) to ensure that the control brains were free from psychopathology, and for clear evidence that the schizophrenic cases conformed to DSM-IV criteria for schizophrenia. In particular, the schizophrenic group included no patient diagnosed with schizoaffective disorder. Exclusion criteria comprised neurological problems that required intervention or interfered with cognitive assessment (e.g., stroke

with aphasia), history of recurrent seizure disorder (i.e., epilepsy), history of severe head injury with loss of consciousness, diabetes mellitus with poor glycemic control (free plasma glucose >200 mg/dl), and history of self-induced water intoxication with hyponatremia. Brains from schizophrenics were also excluded if patients had undergone a leukotomy. The two groups – schizophrenics and controls – were similar in terms of the ethnic backgrounds of the subjects. However, they were not fully matched for socio-economical status and education, which would have placed severe constraints on our sample. Moreover, all schizophrenics were subjected to long-term treatment with high doses of neuroleptics.

In all of the cases, autopsy was performed after consent was obtained from a patient's relative according to the laws of the Federal Republic of Germany. The use of these autopsy cases for scientific investigations as outlined here has been approved by the relevant Institutional Review Boards (IRB). Brains were fixed by immersion in 10% formalin (1 part commercial 40% aqueous formaldehyde in 9 parts H₂O) for at least 3 months and up to 11 years.

Tissue processing

The brainstem with the cerebellum was severed at the level of the rostral pons, and the hemispheres divided medio-sagittally. Then, both hemispheres were cut into serial coronal sections 600 µm to 700 µm thick coronal sections as previously described in the literature (Heinsen and Heinsen, 1991). Briefly, the hemispheres were cryoprotected in a mixture of glycerol-dimethylsulfoxide-formalin after carefully removing meninges and pial vessels. They were then embedded in gelatin, frozen at -60° C, and serially sectioned using a cryostat (Jung, Nussloch, Germany). One brain (control case C7) was embedded in celloidin (Heinsen et al., 2000), and cut into serial 440 µm thick coronal sections using a sliding microtome (Polycut, Cambridge Instruments, UK). From each hemisphere, every second or third section was stained with gallocyenin (Heinsen and Heinsen, 1991). The remaining sections were stored in formalin for up to ten years.

From all the brains of schizophrenics and controls older than 40 years, sections through the central parts of the entorhinal and transentorhinal cortices were labeled with the Gal-lyas silver method to detect neurofibrillary changes (Heinsen et al., 1999). Neurofibrillary tangles were rarely detected. In affected patients the findings corresponded to Braak-Braak Alzheimer classification stage I (Braak and Braak, 1995). Furthermore, all of the sections used in this study were coded and controlled for the absence of tumors, infarcts, heterotopias, signs of autolysis, staining artifacts, and gliosis.

Gallocyenin-stained sections were used by L.d.Z., P.K. and C.S. to identify areas M1, V1, frontal association cortex (areas 4, 17, and 9 of Brodmann [1909], respectively) and S1 (area 3b of Vogt and Vogt [1919]) in both cerebral hemispheres according to anatomical landmarks and cytoarchitectural criteria. Gross anatomical landmarks for M1 include the anterior wall of the central sulcus and adjacent portions of the precentral gyrus. Cytoarchitecturally, the region is clearly demarcated by its giant Betz cells and minimization of layer IV. Brodmann area (BA) 17 (V1) is located along the walls of the calcarine sulcus in the occipital lobe and adjacent portions of the cuneus and lingual gyrus (Carpenter,

1985). It is defined histologically by a broad lamina IV divided into 3 sublayers with numerous very small pyramidal cells in layers II and III. It is noted for the dense line of Gennari in myelin stains. Brodmann area 9 lies in the superior and middle frontal gyrus. Rajkowska and Goldman-Rakic (1995a, 1995b) found that it was located in the middle third of the superior frontal gyrus in all the cases they examined. It covered both dorso-lateral and dorsomedial surfaces of gyrus and extended in some cases to the depth of the superior frontal gyrus and portions of the middle frontal gyrus.

Concerning BA 9, Rajkowska and Goldman-Rakic (1995b) showed that its more lateral portions embrace area 46 (area FD delta of Economo and Koskinas [1925]). It is important to note that Brodmann (1909) never described the cytoarchitectonic characteristics of area 9 in man. Rather he projected his observations in monkey onto the frontal lobe of man. Economo and Koskinas (1925) provided a detailed cytoarchitectonic description of the human frontal lobe. Their area FDM corresponds to BA 9. It is characterized by a distinct layer II, absence of very large, slender and well-built pyramidal cells in layer IIIc (typical of BA 6 and to a lesser extent of BA 8), and an uninterrupted layer IV with fairly definite upper and lower boundaries. In thick galloxyanin-stained sections a cell-poor layer is found between layers IIIc and layer IV. Layer IV is more prominent and cell-rich in the frontopolar regions; however the transition between BAs 9 and 10 is a gradual one. Layer V of BA 9 is subdivided into layer Va and Vb, the latter of which is rather cell-sparse. Most probably the cell-poor layers between lamina IIIc and IV and within layer Vb correspond to the upper and lower band described by Rajkowska and Goldman-Rakic (1995a) in myelin-stained sections of BA 9. Finally, layer VI consists of sublayers VIa and VIb. VIa is cell-rich and its upper border to the cell-sparse layer Vb can easily be recognized. The lower boundaries of layer VIb to the underlying white matter are much more distinct than in the caudally ensuing BAs 8 and 6. The overall cortical thickness of the frontopolar area 10 (field FE of Economo and Koskinas [1925]) is considerably less than in BA 9, lamina VIa is small, rich in darkly-stained pyramidal as well as spindle-shape cells and the boundary of VIa to the medullary layer is even more distinct than in BA 9. The presence of the small and cell rich layer VI facilitates the delineation of BA 9 from the rostral area 10 in thick galloxyanin stained sections. Finally, layer IV is more conspicuous than its corresponding part in BA 9.

Regions of interests were removed as 2 cm × 2 cm tissue pieces. After rinsing in tap water for several hours, the pieces were stored in tris-buffered saline (TBS) overnight (pH = 9.0). They were then placed in TBS (pH = 9.0) and heated in a microwave oven for 15 min at 90°C for antigen retrieval (results of immunohistochemical analysis will be published elsewhere). After cooling down, the tissue blocks were rinsed in TBS twice (pH = 7.4) and placed in a TBS based 30% sucrose solution for cryoprotection. The pieces were then frozen and cut into 50 µm thick sections using a cryostat (Frigocut 2800E; Jung, Nussloch, Germany). These 50 µm thick sections were stained with cresyl-violet (0.1%, 20 min).

Computerized image analysis

Digital images were taken from laminae III and V in cortical areas M1, S1, V1, and Brodmann area 9 in both cerebral hemispheres. Photomicrographs were taken through the flat face of the gyri with minicolumns perpendicular to the pia. Each field of view measured approximately 1.45 mm², and up to three fields were captured per lamina/area/hemisphere as tissue quality allowed. Of the 416 possible regions of interest (16 in each of 26 brains), 90 were completely lacking in usable tissue; the number of images captured per available region numbered 2.7.

The columnarity index, CI, a global measure of anisotropy or degree of orientation (Figure 1), was obtained with a Fourier transform algorithm as follows (Casanova and Switala, 2005): The dominant orientation of the image was found using the formula

$$\theta_d = \frac{1}{2} \tan^{-1} \frac{\sum_{i,j} |F_{ij}|^2 \sin 2\theta_{ij}}{\sum_{i,j} |F_{ij}|^2 \cos 2\theta_{ij}},$$

where $|F_{ij}|^2$ is the squared amplitude and $\tan \theta_{ij} = f_y(i,j) / f_x(i,j)$ is the orientation corresponding to the frequency component at line i , sample j of the discrete Fourier transform of the image (Pourdeyhimi et al., 1994). Then the columnarity index was defined by

$$CI = \sqrt{\frac{\sum_{i,j} |F_{ij}|^2 \cos 2(\theta_{ij} - \theta_d)}{\sum_{i,j} |F_{ij}|^2}},$$

which is constrained to lie between zero and one, inclusive.

Computerized image analysis of minicolumnar structure was performed with a modification of algorithms previously described in the literature (Buxhoeveden et al., 2000a): Each image was flattened to correct for uneven illumination by first estimating and then subtracting background. Background estimation involved smoothing the image followed by filling depressions in the image viewed as an intensity surface, i.e. removing any dark regions surrounded by bright pixels. The flattened image was segmented using thresholding with Otsu's method, which maximizes the contrast between the two pixel classes (above and below threshold) with respect to the pooled within-class variance (Otsu, 1979). Nonconvex foreground (dark) patches in the binary image were subdivided by applying the morphological watershed transform to the distance map of the binary image. Excessively oblong watershed regions were identified, and corresponding basins of the distance map were filled in. Then the watershed algorithm was applied to the modified distance map. Each connected region in the resulting image was further classified as a large neuron if its area was greater than 30 μm^2 and as a small neuron if its area was between 10 μm^2 and 30 μm^2 . Smaller objects, assumed to be glia, cell fragments, or noise, were discarded

altogether. In our study small granule cells and glia that may have been omitted would not have significantly affected column location or morphology since cell columns essentially consists of pyramidal cells.

Local cell density was calculated as follows: d was the typical distance between neighboring large neurons. Each image was divided into horizontal bands $2d$ high. Within each band, the observed cell density was smoothed as a function of horizontal position using a bell-shaped kernel of width $(\frac{1}{2})d$ (Figure 2). Local maxima of cell density indicated minicolumn cores, and local minima indicated peripheral neuropil space. Local maxima in each strip were joined with mutual nearest neighbors in the strips above and below to form chains. Minicolumnar width (CW) was defined as the median over all distances between chains adjacent to the left or right, and its dispersion (VCW) was the median absolute deviation of these distances. Mean interneuronal distance (MCS) was calculated as for d , above, including all neurons large and small.

Bootstrap confidence regions

The basic bootstrap method (Davison and Hinkley, 1997) was used to compute confidence intervals on the mean MCS and CW, below. Simulated data sets were generated by resampling cases: each simulated data set was composed of the measurements from thirteen schizophrenic patients and thirteen control cases selected randomly and allowing for duplication (Table 2). These were paired as in the original data set, youngest schizophrenic with youngest control, second-youngest with second-youngest, and so forth. Approximately 10,000 bootstrap samples were thus generated. The linear model described below was fit to each artificial data set in turn and used to estimate the expected values of the dependent variables in each lamina, cortical area, and pathological condition. The orientation and eccentricities of the confidence ellipses are given by the covariance matrix of the bootstrap expected values; the size of the confidence ellipses is the least such that it encloses just over 95% of the bootstrap estimates.

Results

For purposes of statistical analysis each schizophrenic patient was paired with his age-matched normal counterpart. Diagnosis, cortical area, hemisphere, and lamina were included as effects in a full factorial model. All four dependent variables (columnarity index [CI], minicolumnar width [CW], dispersion of minicolumnar width [VCW], and mean interneuronal distance [MCS]) were analyzed simultaneously. The multivariate likelihood ratio statistics supported a relationship between the dependent variables and cortical area ($\chi^2 = 430$, 12 degrees of freedom, $p < 0.001$), lamina ($\chi^2 = 109$, 4 d.f., $p < 0.001$), diagnosis by cortical area interaction ($\chi^2 = 21.2$, 12 d.f., $p = 0.048$), and lamina by cortical area interaction ($\chi^2 = 31.9$, 12 d.f., $p = 0.002$). Each of the four measurements, considered

individually, varied significantly with cortical area ($p < 0.001$). CI, VCW, and MCS varied significantly with lamina ($F_{1,282} > 22$, $p < 0.001$ in each variable), but CW did not ($F_{1,282} = 0.449$, $p = 0.654$).

MCS was the only single measurement with a significant dependence on the diagnosis by cortical area interaction term ($F_{3,282} = 3.26$, $p = 0.022$). There was a suggestion of greater cell packing within the minicolumn in schizophrenia in the prefrontal and motor areas but lesser packing in the primary sensory areas (Figure 3, Table 3). Post-hoc testing, however, did not yield significant results in any of the four areas examined, and there is a good amount of overlap in the confidence intervals in Table 2 and in simultaneous confidence regions for the mean CW and MCS (Figure 4).

Possible influence of the time each brain spent in fixative (Table 1) was tested separately using nonparametric statistics, since the distribution of time in storage is non-normal, as is its logarithm. For the subset of 13 control cases, none of the four dependent variables were significantly correlated with time in storage. The strongest relationship between a dependent variable and time in fixative was observed for CW, with a Spearman rank correlation coefficient $r = -0.032$ ($p = 0.56$).

Discussion

The present study found no differences in the morphometric parameters of minicolumns when considering interactions of hemisphere by diagnosis. This is not surprising as only one area (M1) of the sampled regions exhibits cerebral dominance. A GLI study has shown that the neuropil space is lateralized in human motor cortex (Amunts et al., 1997). Asymmetry in this area may be related to handedness as well as gesture (Hocheberg and LeMay, 1975; Hopkins and Morris, 1989; Hopkins et al., 1990). Our sampling within M1 may have strayed beyond the area controlling handedness thus diluting any findings accrued to cerebral dominance.

The findings with respect to MCS in schizophrenia were not significant on post hoc analysis. Therefore, the findings are considered tentative in nature. One possibility is that interneuronal spacing (mean cell spacing or MCS in our study) is altered in the brains of schizophrenic patients. If a significant reduction of minicolumnar neuropil space had occurred where the majority of cell soma reside, the findings would indicate a defect among the projection systems present in that compartment: apical dendrite clusters and most of the thalamocortical and corticocortical input (Ong and Garey, 1990; Selemon et al., 1995). However, our findings do not indicate changes within the cell core compartment of the minicolumn (e.g., columnar width) but rather within different lamina. The evidence thus suggests a defect within the extrathalamic system that innervates the neocortex in a horizontal or laminar fashion. One implication is that the source of the pathology lies in projections stemming from a source or sources outside of the neocortex.

Overall, transmission of information within the neocortex occurs within vertical strips of grey matter called minicolumns. In reviewing cortical microcircuits, including the minicolumn, Silberberg et al. (2002) observed great variability accrued to species, region, area, and age. Despite this variability, the existence of stereotypical features indicates a deterministic basis for cortical microcircuits. It may be a general principle of cortical organization that neurons with similar stimulus selection are found in close proximity to each other (Reich et al., 2001). If this is the case, the minicolumn is a possible way of organizing them into a vertical component.

Neurons within the cortex participate in canonical circuits (Douglas and Martin, 2004) that exhibit relative independence from neighboring units (Casanova et al., 2003; Silberberg et al., 2002). Unsurprisingly, the vast majority of connections within the minicolumn are intrinsic to the same, giving rise to massive excitatory and inhibitory connections (Braitenberg and Schüz, 1991; Casanova et al., 2003). Slower-onset modulatory effects are provided by horizontal components that innervate different layers of the minicolumn, e.g., monoaminergic agents that bypass the thalamus. The result provides for a Cartesian-like system for the action of neurotransmitters. This fact may help explain why amino acids (e.g., glutamate, GABA) are the most abundant neurotransmitters in the mammalian nervous system. On the other hand, modulatory transmitters stemming primarily from sources outside of the minicolumn (e.g., acetylcholine, norepinephrine, dopamine, histamine, and 5-hydroxytryptamine) account for transmission at only a small percentage of cortical synaptic sites.

If confirmed, alterations in mean cell spacing reported in the present study may be secondary to the loss of modulatory input to the cell minicolumns. Alterations in neuronal spacing might also imply a correlated alteration in the vertical dimension of the involved lamina. The present study did not measure the width of the lamina. However, in a previous result from our group, using a different patient population, the mean vertical height for our column segments (lamina III, BA 9) was reduced in the brains of schizophrenic patients (304 μm versus 348 μm in controls, $p = 0.058$) (Buxhoeveden et al., 2000b). This finding compares favorably with the 7% reduction in cortical thickness reported within the same region reported by Selemon and associates (Selemon et al., 1995).

In our study, variation in MCS as a function of diagnosis was not a uniform feature of the sampled cerebral cortex (see Figures 3 and 4). Studies on the structural pathology of schizophrenia indicate significant but non-uniform reductions in the cortical gray matter. Arguably, changes preferentially affect association cortices such as those located within the superior temporal gyrus and the dorsal prefrontal cortex (McCarley et al., 1999). These abnormalities are present in first-episode patients and prior to clinical onset of the illness thus suggesting that they are a core feature of the pathology of schizophrenia (Lewis and Lieberman, 2000). Consistent with the interpretation that the nonuniform reduction in cortical gray matter reflects alterations in lamination are findings of decreased levels of synaptophysin (a presynaptic terminal protein) (Glantz and Lewis, 1997), lowered density of dendritic spines to which the synapses may be apposed (Garey et al., 1998), and decreased immunoreactivity of tyrosine hydroxylase and dopamine membrane transporter (Akil et al., 1999) in the cerebral cortex of schizophrenic patients.

Assessing the clinical consequences (e.g., onset of manifestations) of a developmental disturbance in lamination may prove a difficult endeavor. Developmental studies have shown that, even within the same cortical area, individual neurochemical systems may exhibit different patterns of lamination. These patterns depend on the specific cortical layer and type of synapse examined. This finding has led researchers to conclude that, “it is not likely that short periods of environmental disruption will uniformly disturb all neurochemical systems or even one system in all brain areas, but rather individual functional domains may be selectively affected” (Levitt, 2003). Furthermore, postnatal remodeling of the ontogenetic laminar organization is a hallmark of the primate brain. Prominent and relatively complex layer-specific remodeling of the dopaminergic system is still patent in the rhesus monkey at 5 years of age (18 to 20 years in human beings) (Levitt, 2003). This time period is marked by life changes, demands for complex problem solving, and stress. In some patients, primarily males, this time frame coincides with the age of onset of schizophrenia.

Schizophrenia has been linked to prenatal risk factors, particularly to influenza epidemics. Offsprings of mothers exposed to the virus during the second trimester have been shown to have an increased rate of becoming schizophrenic (Limosin et al., 2003). Similarly, the rate of schizophrenia is nearly three times greater in Rhesus (Rh) positive infants born to Rh negative mothers (Hollister et al., 1996). Rh incompatibility is associated with the creation and transfer of maternal antibodies to the fetus during the second trimester of gestation. This results in hemolytic anemia and brain damage to the fetus during this period of pregnancy. When starvation is examined as a risk factor, it is found that only the offspring of women exposed to famine during their first trimester of pregnancy have an increased rate of hospitalization for schizophrenia (Susser et al., 1996). Whether first or second trimester, epidemiological studies on schizophrenia have offered conflicting results regarding a window of selective vulnerability for the developing brain.

Studies of the prenatal brain have shown that the more primitive parts (e.g., brainstem) develop before more complex and evolved structures (e.g., cerebral cortex). Whenever any brain region is rapidly growing/developing it is most vulnerable to injury. The disparity in timing among risk factors could be the result of a noxious agent's (e.g., starvation) effect on the brainstem during the first trimester. Contrariwise, another agent (e.g., influenza virus) may selectively target brainstem projection to the cerebral cortex during the second trimester. Despite differences in timing, either lesion would provide a disturbance in cortical lamination and connectivity by targeting the brainstem nuclei or their efferents.

One could argue that any lamination disturbance in schizophrenic patients may represent a side effect of neuroleptics. However, cortical cytoarchitectural studies have found no correlations with treatment exposure (Harrison, 1999), and little or no effects of the drugs upon similar parameters have been found in the rat brain (Harrison, 1993). It is therefore reasonable to assume that the results of the present study do not depend on the antipsychotic medication of the patients, but rather indicate neuropathological abnormalities of the illness itself.

Another possibility to the reported laminar abnormalities relates to alterations in the level of Reelin. Reductions in Reelin mRNA and protein have been shown in frontal, hippocampal and cerebellar cortices of schizophrenic patients by Impagnatiello and associates (Impagnatiello et al., 1995). The results have been reproduced by the same group in additional subjects while another study has shown reduced Reelin immunoreactivity in both schizophrenia and bipolar patients (Fatemi et al., 2000). In the reeler mutant mouse, an absence of Reelin provides for severe alterations that include misrouted fibers forming abnormal patches at their terminal zones (Borrell et al., 1999). Some of these abnormalities are transient and not detectable in the adult reeler mouse. These findings have led researchers to postulate that Reelin has a modulatory role during the formation of layers and specific topographic connections (Borrell et al., 1999).

Currently the basis for laminar abnormalities in schizophrenia, if present, remains unknown. Previous studies that could clarify this putative abnormality have implemented faulty research designs. Thus, reductions in the level of Reelin have been reported following postnatal hypoxia (Currstin et al., 2002). In schizophrenia, the preagonal state of the patients, e.g. suicide by asphyxiation, may therefore offer a confound to significant alteration in Reelin levels. Furthermore, no one during the last century of neuropathological studies in schizophrenia has described the characteristic reversal in the normal pattern of lamination observed in the Reeler mouse cortex. Similarly, older cell counting studies of brainstem nuclei (that provide laminar projections to the cortex) used biased quantitative techniques. More recent studies of cortical lamination in schizophrenia have provided measurements of pseudo-optical density while using techniques that have no relative or absolute quantitative abilities, e.g., immunocytochemistry.

In summary, the data presented in this study indicate a laminar disturbance in the neocortex of schizophrenic patients. Normal measurements in minicolumnar width, variability in minicolumnar width, and verticality index, suggest an abnormality that post-dates the development of the cell minicolumn. Our discussion has therefore expounded on a possible alteration of the modulatory systems of the cell minicolumn. This putative disruption of the systems that modulate information processing through the cell minicolumn may account for the range in both severity and symptoms that defines schizophrenia as a clinical syndrome.

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Tables

Table 1. Clinical characteristics of the cases included in this study. PMI: interval between death and autopsy. Storage: duration of immersion in 10% formalin. All schizophrenic patients and all controls were male.

No.	Age [years]	Cause of death	PMI [hours]	Storage [days]	Diagnosis	
					DSM-IV	ICD-10
S1	22	Suicide	88 ¹	130	295.30	F20.0 ²
S2	36	Suicide	<72 ¹	115	295.30	F20.0 ²
S3	46	Systemic hypothermia	<24	327	295.30	F20.01 ²
S4	50	Peritonitis	<24	203	295.30	F20.0 ²
S5	50	Suicide	18	170	295.30	F20.0 ²
S6	51	Septicemia	33	127	295.60	F20.50 ³
S7	54	Septicemia	27	250	295.60	F20.50 ³
S8	55	Right-sided heart failure	25	84	295.30	F20.00 ²
S9	57	Septicemia	76	163	295.30	F20.00 ²
S10	60	Pulmonary embolism	<48	311	295.30	F20.01 ²
S11	62	Aspiration	7	171	295.30	F20.00 ²
S12	63	Acute myocardial infarct	15	338	295.60	F20.50 ³
S13	64	Pulmonary embolism	6	817	295.60	F20.50 ³
C1	25	Cardiac tamponade	14	119		
C2	36	Gunshot	24	143		
C3	47	Acute myocardial infarct	<24	133		
C4	50	Acute myocardial infarct	35	433		
C5	50	Avalanche accident	23	498		
C6	51	Septicemia	7	285		
C7	54	Acute myocardial infarct	18	168		
C8	56	Acute myocardial infarct	60	3570		
C9	58	Acute myocardial infarct	28	126		
C10	60	Gastrointestinal hemorrhage	18	101		
C11	60	Gastrointestinal hemorrhage	27	302		
C12	62	Acute myocardial infarct	<24	3696		
C13	65	Bronchopneumonia	6	2289		

¹These patients had long post mortem intervals (PMI). However, both patients committed suicide (one by hanging, the other by jumping from an eight story building), were found within 1 h of death, and were kept at 4° C until autopsy. Accordingly, the intervals between death and autopsy of these patients cannot be compared to the corresponding intervals of the other persons and were thus excluded from the calculation of the mean PMI between death and autopsy.

²Paranoid schizophrenia.

³Residual schizophrenia.

PMI: interval between death and autopsy. Storage: duration of immersion in 10% formalin. All schizophrenic patients and all controls were male.

Table 2. An example of resampling with replacement. This simulated data set includes the measurements from patient S1, control C1, three replicates of patient S3, and so forth. It is possible to generate $1326 \approx 1029$ distinct data sets in this fashion.

Group	Case													Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Schizophrenic	1		3	1		2	2	1		1		1	1	13
Control	1		2	1	1	1	1		3	1	2			13

Table 3. Estimated mean, standard deviation, and 95% confidence intervals for mean interneuronal distance (MCS), by diagnosis and cortical area, averaged across laminae III and V. Means and standard deviations are also provided for the other three parameters (CW, VCW, CI), which exhibited no significant diagnosis dependence or diagnosis interaction.

Cortical Area	Group	MCS (μm)			CW (μm)		VCW (μm)		CI	
		Mean	S.D.	Interval	Mean	S.D.	Mean	S.D.	Mean	S.D.
BA 9	S	21.8	4.2	[21.3,22.3]	54.7	7.8	19.4	4.1	0.346	0.168
	C	21.2	4.4	[20.7,21.7]	53.8	8.2	19.5	4.3	0.331	0.176
M1	S	22.4	4.8	[21.9,23.0]	55.6	8.8	19.8	4.6	0.346	0.190
	C	22.0	4.2	[21.5,22.5]	56.0	7.8	20.3	4.1	0.349	0.167
S1	S	19.5	5.5	[18.9,20.2]	52.8	10.3	19.4	5.4	0.358	0.222
	C	20.1	4.2	[19.7,20.6]	53.6	7.8	19.8	4.1	0.355	0.168
V1	S	15.6	5.0	[15.1,16.2]	43.2	9.2	17.0	4.8	0.242	0.198
	C	16.4	4.3	[15.9,16.9]	44.3	8.0	16.7	4.2	0.261	0.171

Mean: estimated population mean; S.D.: estimated population standard deviation; S: schizophrenic patients; C: controls; BA9: Brodmann's area 9; M1, S1, and V1: primary motor, sensory, and visual cortical areas, respectively.

Figures

Fig. 1. An image of a seemingly isotropic patch of cortex (A) has its power, estimated with a Fast Fourier Transform (FFT), distributed evenly about the origin (B). By contrast, an image of a region of cortex showing clear columnar structure (C) has its power distribution (estimated with a FFT) concentrated into two lobes symmetric about the origin (D). This is the rationale for the columnarity index CI: the degree to which the image spectrum is concentrated along one axis. The origin of Fourier space is the center of images B and D.

Fig. 2. Superposed on an image of cortical layer III are the boundaries of cells (dark outlines) as determined by the automatic segmentation algorithm, and the resulting cell density profiles at several depths (smooth curves), which determine the estimate of minicolumnar width (CW).

Fig. 3. Raw measurements of minicolumnar width and interneuronal distance, broken down by cortical area, lamina, and diagnostic group. Observations from the two schizophrenic patients with long post-mortem intervals (S1 and S2 in table 1) are circled. Measurements are given in parentheses for mild outliers in areas 9 and 17, where the axis limits were narrowed to better show the scatter in the bulk of the data.

Fig. 4. Bootstrap 95% confidence ellipses, by cortical area, for the mean minicolumn width (CW) and interneuronal distance (MCS). Also shown are the maximum likelihood estimates for the means (points). Solid lines: control. Broken lines: schizophrenia. Thin lines: lamina III. Bold lines: lamina V.

Chapter 7

General discussion
and future implications

Postmortem studies in the literature of brains of patients with schizophrenia revealed subtle neuropathological abnormalities in several cortical and subcortical regions. However, many of the reported data were divergent if not mutually contradictory. No clear pattern of morphological alterations has yet been established. The problem, according to some researchers (Harrison, 1999) lies mainly within methodological differences, investigated parameters and particularly in the availability of brain samples from patients with schizophrenia and matched controls.

By means of this thesis I am presenting a solution to this situation:

- simultaneous analysis of several cortical and subcortical regions of the same brains;
- application of state-of-the-art quantitative design-based stereologic techniques; and
- analysis of a sample of well documented postmortem brains from 13 male patients with schizophrenia and 13 age-matched male controls.

In Chapter 2 I demonstrated a distinct pattern of subtle neuropathological alterations in several subcortical regions. This previously unrecognized set of alterations comprises of:

- reduced volumes of the total cortical gray matter, putamen and nucleus accumbens;
- reduced total neuron numbers in the caudate nucleus, putamen and the lateral nucleus of the amygdala; and
- no correlation between the illness duration of the patients with schizophrenia and the observed neuropathological alterations.

The results from the control cases were in line with previous studies. The results obtained for the patients with schizophrenia can be compared to data in the literature as follows.

Cortical gray matter

A significant decrease in the total cortical gray matter volume (-9.3%; averaged for the left and right hemispheres) was found when comparing the patients with schizophrenia to the controls. This result was in agreement with several reports in the literature (Zipurski et al., 1992; 1997; McCarley et al., 1999; Shenton et al., 2001)

Caudate nucleus, putamen and nucleus accumbens

Several clinical neuroimaging studies which have shown an enlargement of the striatum are in contrast to a postmortem study of Beckmann and Lauer (1997), who reported no differences in mean volumes of the caudate nucleus and the putamen, as well as Bogerts (1984) who found a trend towards reduced mean volumes of the putamen in schizophrenia. On the other hand, several neuroimaging studies reported a decrease in the mean volume of the striatum in drug-naïve patients with schizophrenia and a reduction of the mean volume in basal ganglia of patients with schizophrenia after switching to olanzapine (Harrison, 1999; Wright et al., 2000; Shenton et al., 2001). An increase of the mean volume of the striatum in schizophrenia in those studies could have been caused by an yet unknown, but reversible action of typical neuroleptics or during histological processing.

The discrepancies of the mean total neuron numbers in the caudate nucleus and the putamen between the present study and the analysis by Beckmann and Lauer (1997) might

be related to differences in the stereological design of the studies and the analyzed samples of postmortem brains. Anyway, the number of unbiased virtual counting spaces used to count neurons by Beckmann and Lauer (1997) was considerably lower than in the present study, which implies a considerably higher variation in estimated total neuron numbers (Schmitz, 1998; Schmitz and Hof, 2000; 2005).

Pakkenberg's (1990) early report of a reduced mean volume of the nucleus accumbens (-42%) and a reduced mean total neuron number (-50%) in postmortem brains of patients with schizophrenia should be taken cautiously as already discussed by Lauer and Beckmann (1997). Bogerts (1984) found almost no differences in the mean volumes of the nucleus accumbens between patients with schizophrenia and controls. The discrepancy between the results of Pakkenberg (1990), Bogerts (1984), Lauer and Beckmann (1997) and the present study might be due to differences in the stereological design. For instance, Pakkenberg (1990) performed microscopic investigations on 4 μm -thick paraffin sections. Delineations of the brain regions on such sections might be much more difficult than on sections of 20 μm (Bogerts, 1984), 500 μm (Lauer and Beckmann, 1997) or 600-700 μm (present study).

Mediodorsal nucleus of the thalamus

The reduction of the mean volume of the mediodorsal nucleus of the thalamus in schizophrenia (as reported in several studies; Pakkenberg, 1990; Popken et al., 2000; Byne et al., 2002; these studies with positive outcome are henceforth referred to as PO studies here) could not be confirmed in the present study as well as in other studies in the literature (Cullen et al., 2003; Kemether et al., 2003; Dorph-Petersen et al., 2004; Danos et al., 2005; these studies with negative outcome are henceforth referred to as NO studies here). Again, differences in the study design may explain this discrepancy. The PO studies had on average smaller sample sizes than the NO studies. Furthermore, except of the study by Dorph-Petersen et al. (2004) in all NO studies both the left and right mediodorsal nucleus of the thalamus were investigated, and the mean age of the patients was higher in the PO studies than in the NO studies. In this regard small sample sizes and confounding pathologies (possible age-related neurodegeneration) cannot be excluded as a major potential obstacle in most neuropathological studies on schizophrenia (Harrison, 1999).

Amygdala

Meta-analyses of clinical neuroimaging studies reported reductions in the mean volume of the amygdala in patients with schizophrenia in the range of 5 to 10% (Lawrie and Abukmeil, 1998; Nelson et al., 1998; Wright et al., 2000; see also Honea et al., 2005). However, no differentiation between the different nuclei of the amygdala were performed in these studies. These data are in line with the results of the present study as well as of a postmortem study by Bogerts (1984). In contrast, no mean volume reduction in schizophrenia was found by Heckers et al. (1990) and Chance et al. (2002). Nevertheless, according to the latter authors alterations of other morphological aspects such as cellular composition of the amygdala cannot be excluded.

Asymmetry of brain regions

Although suggested by several reports in the literature (Crow, 1990; 1997; Patty, 1999) no significant differences between the right and left hemispheres were found in the present study. A meta-analysis of clinical neuroimaging studies, in which a volume of 100% was assumed for the control group, found an overall mean volume of 94% for both the left and right amygdala in patients with schizophrenia. Disturbance of cerebral asymmetry in schizophrenia might therefore be restricted to cortical regions such as the dorsolateral prefrontal cortex (Cullen et al., 2006)

In Chapters 3 and 4 I assessed the integrity of the microvasculature in several subcortical and cortical brain regions in schizophrenia. To this end I investigated variables such as microvessel length density, total microvessel length and microvessel length per neuron in the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala. The patients with schizophrenia showed a statistically significant increase in mean microvessel length density in the caudate nucleus and in the mediodorsal nucleus of the thalamus, and a decrease in mean total microvessel length in the lateral nucleus of the amygdala. The stereological data presented in Chapter 2 allowed then to assess possible changes in total microvessel length and mean microvessel length per neuron in these regions. There were, however, no significant differences between the patients with schizophrenia and the controls with respect to the mean microvessel length per neuron in all investigated subcortical areas. Furthermore, the analysis of the mean capillary length density within cortical areas 9 and 24 also showed no significant differences between patients with schizophrenia and controls. Only the combination of diagnosis and hemisphere had a significant effect on the mean capillary length density within both areas 9 and 24.

The postmortem interval can affect stereologic estimates of cerebral blood vessels (Ingvar and Franzen, 1974). In the present study the postmortem interval had a significant effect on the total microvessel length in the putamen and the mediodorsal nucleus of the thalamus. However, post-hoc linear regression analysis revealed no significant correlations between the postmortem interval (or the fixation time) and any other investigated variable.

In Chapter 5 I demonstrated that mean cell spacing abnormalities in the cerebral cortex of patients with schizophrenia were not significant in post-hoc analyses and therefore tentative in nature. It is however possible that the interneuronal mean cell spacing is altered in the brains of patients with schizophrenia. The findings indicate changes rather within different lamina than within the cell core compartment of minicolumns. Moreover, the alterations in mean cell spacing may be secondary to the loss of modulatory input to the cell minicolumns. Therefore, these alterations do not indicate a single mechanism that can be found in all regions but rather a disruption of the systems that modulate information processing, another aspect of the wiring, cytoarchitecture and cell morphology alterations of the brain of patients with schizophrenia. Direct conclusions from the alterations observed cannot be therefore drawn.

In Chapter 6 I present results of analyses of the neuronal distribution in the neocortex of patients with schizophrenia that are consistent with altered neocortical neuropil reports, a phenomenon well established in the literature (Selemon 1998, Broadbelt 2000 and Kalus 2000). These alterations reflect *de facto* modulatory systems abnormalities innervating the cell minicolumns.

What is the specific contribution of this thesis to a better understanding of schizophrenia? In general, two non-exclusive hypotheses have been proposed, i.e., the neurodevelopmental hypothesis of schizophrenia (Lieberman, 1999; Tsuang, 2000; Lewis and Levitt, 2002; Murray et al., 2004) and the neurodegenerative hypothesis of schizophrenia (Ashe et al., 2001; Church et al., 2002; Sawa and Snyder, 2002). In recent decades the neurodevelopmental hypothesis of schizophrenia has prevailed (Weinberger, 1987; Lewis and Levitt, 2002; Murray et al., 2004). In this hypothesis genetic and non-genetic mechanisms are thought to interact, in yet not fully understood ways, affecting the developing brain and resulting in a predisposition to schizophrenia (Schmidt-Kastner et al., 2006). Yet the several genes proposed as candidates (for review see Davis et al., 2003; Harrison and Owen, 2003; Owen et al., 2004; Harrison and Weinberger, 2005; Kirov et al., 2005) may not account for all aspects of the pathogenesis (Kendler et al., 2005). There is no direct evidence for a neurodevelopmental or a neurodegenerative origin of the results presented in this thesis. Yet, reductions in mean volumes (cortical gray matter, putamen and lateral nucleus of the amygdala) and mean total neuron numbers (caudate nucleus, putamen and lateral nucleus of the amygdala) in the brains of patients with schizophrenia did not correlate with the illness duration. Such lack of correlation might be indicative for a neurodevelopmental deficit, particularly with respect to the alterations found in the lateral nucleus of the amygdala. Moreover, lesions of the amygdala in rats obtained during the early postnatal period (but not during adulthood) lead to a combination of behavioral changes with shared features with schizophrenia (Wolterink et al., 2001). Furthermore, the minicolumn analysis indicated the possibility of laminar disturbances in cortical regions and therefore an alteration of the modulatory systems in the brains of patients with schizophrenia. Together with the suggested alterations in the neuropil (mostly by means of laminar thinning rather than decreased neuropil width) the minicolumns analysis supported the neurodevelopmental hypothesis and was in line with the stereologically obtained data since those alterations also do not correlate with the illness duration. Furthermore, connections between the basolateral amygdala and the prefrontal cortex develop slowly during postnatal development in rats (Bouwmeester et al., 2002; Cunningham et al., 2002). Postnatal lesions of mesial temporal limbic structures (including the amygdala) in monkeys is associated with abnormal function of the prefrontal cortex of adult animals (Bertolino et al., 1997). The prolonged maturation of connections between the amygdala and prefrontal cortex has been hypothesized to be involved during onset of schizophrenia in adolescence (Benes, 2003). Reduction of mean total neuron numbers found in the present study in the amygdala can also play a pivotal role in these processes and requires further investigation.

The small decrease in mean total neuron numbers found in the present study could be due to a prolonged neurodevelopmental perturbation affecting several processes in the striatum, including neurogenesis and the innervation by dopaminergic mesencephalic fibers. Furthermore, the reduced mean total neuron number in the lateral nucleus of the amygdala in schizophrenia could also result in impaired function of the basal nucleus of the amygdala with potential influence on (at least) two functional systems, i.e., the striatal and cortical output of the basal nucleus of the amygdala. This in turn could impair the control of the specific involuntary components of emotional reactions which are impaired in schizophrenia (Aleman and Khan, 2005). Finally, the reduced mean total neuron number in the lateral nucleus of the amygdala in schizophrenia can indirectly (via ventral striatum projections) influence dopaminergic neurons through amygdalo-striato-nigral loops (Haber and Fudge, 1997; Fudge et al. 2002).

Analysis of the integrity of the microvasculature in schizophrenia revealed no changes in the mean microvessel length in cortical brain areas 9 and 24 and in the mean microvessel length per neuron in all investigated subcortical brain areas. These data do not support the hypothesis of (structural) alterations in the microvasculature in schizophrenia. Furthermore, these studies (presented in Chapters 3 and 4) were performed in the areas that represent so called “hot spots” for structural abnormalities in schizophrenia. The present negative outcome indicates that the anatomical substrate for abnormal regional cortical blood flow (rCBF) and cerebral blood volume (Brockhaus 1942; Hirai and Jones, 1989; Arnold and Trojanowski, 1996; Middleton et al., 2002; Cullen et al., 2003,) might be absent. Changes in rCBF activation are most likely due to region-specific neuronal or glial factors. Alterations in gene and protein expression found in cortical samples from patients with schizophrenia probably reflect an abnormal metabolic state (Schmidt-Kastner et al., 2006), caused by the primary disease process, secondary long-term neuronal dysfunction, chronic medication, or imbalances in agonal events influencing microarray measurements (Weinberger 1987; Jones, 1997; Malaspina et al., 2004; Vawter et al., 2006).

A side observation was that the neuron density was not correlated with the microvessels length density within the five investigated subcortical regions both in the brains from patients with schizophrenia and the controls. This can be explained by the fact that the microvessel length density is linked to metabolic expenditures in neuronal and glial processes whereby neuron density (measured in the present study) or overall cell density do not tightly correlate with the total amount of the functional processes in each structure. Nevertheless the possibility of vascular abnormalities in untreated patients with schizophrenia cannot be excluded. Dopaminergic processes make close contacts with capillaries in the prefrontal cortex and dopamine reduces local perfusion (Lauer and Heinsen, 1996). Thus it is tempting to speculate that abnormal dopamine levels in schizophrenia prior to administration of neuroleptics (which are responsible for chronic dopamine receptor blockade) may have functional and structural effects on the microvasculature before the onset of clinical symptoms.

Finally it should be mentioned that the present observations in schizophrenia must not be misinterpreted as evidence against a possible interaction of hypoxia, the microvascula-

ture and neurons within the developing brain. Rather neurodevelopmental perturbations that are thought to underlie schizophrenia could in fact be manifested by the occurrence of alterations in microvessel length and microvessel length per neuron in the fetal brain of people who will later develop schizophrenia. However, those alterations might not necessarily persist in the postnatal life in the brains of patients with schizophrenia.

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Summary

Chapter 1 is a general introduction to this thesis. It elucidates the origins of schizophrenia as a nosological entity, followed by the overview of the current epidemiological, genetical and environmental data and evolution of modern theoretical concepts of the disease. It further explains the consequences of the rapid progress of the technology behind methodology, focusing on post mortem investigations of human brain.

Chapter 2 of this thesis describes morphological alterations of the cytoarchitecture in five subcortical regions of the brain of patients with schizophrenia (caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala), investigated with state-of-the-art design-based stereological techniques (focusing on volumes, neuron densities and total neuron numbers). The previously unrecognized set of alteration comprises of reduced volumes of the total cortical gray matter, putamen and nucleus accumbens, reduced total neuron numbers in the caudate nucleus, putamen and the lateral nucleus of the amygdala and no correlation between the illness duration of the patients with schizophrenia and the observed neuropathological alterations

Chapter 3 and 4 describe results of investigations on microvasculature abnormalities suggested in the brain of patients with schizophrenia. They present data collected during investigation of variables such as microvessel length density, total microvessel length and microvessel length per neuron in the caudate nucleus, putamen, nucleus accumbens, mediodorsal nucleus of the thalamus, and lateral nucleus of the amygdala followed by assessment of possible changes in total microvessel length and mean microvessel length per neuron in these regions. The patients with schizophrenia showed a statistically significant increase in mean microvessel length density in the caudate nucleus and in the mediodorsal nucleus of the thalamus, and a decrease in mean total microvessel length in the lateral nucleus of the amygdala. There were, however, no significant differences between the patients with schizophrenia and the controls with respect to the mean microvessel length per neuron in all investigated subcortical areas. The analysis of the mean capillary length density within cortical areas 9 and 24 also showed no significant differences between patients with schizophrenia and controls. Only the combination of diagnosis and hemisphere had a significant effect on the mean capillary length density within both areas 9 and 24.

Chapters 5 and 6 describe minicolumnar abnormalities in the neocortex of the patients with schizophrenia, focussing on alterations in mean cell spacing and in the distribution

of neurons in the neocortex. Mean cell spacing abnormalities were not significant in post-hoc analyses and therefore tentative in nature. It is however possible that the interneuronal mean cell spacing is altered in the brains of patients with schizophrenia. The findings indicate changes rather within different lamina than within the cell core compartment of minicolumns. Moreover, the alterations in mean cell spacing may be secondary to the loss of modulatory input to the cell minicolumns. Therefore, these alterations do not indicate a single mechanism that can be found in all regions but rather a disruption of the systems that modulate information processing, another aspect of the wiring, cytoarchitecture and cell morphology alterations. The neuronal distribution in the neocortex of patients with schizophrenia are consistent with altered neocortical neuropil reports, a phenomenon well established in the literature. These alterations reflect *de facto* modulatory systems abnormalities innervating the cell minicolumns.

Finally, in the last chapter, results presented in this thesis are discussed with respect to the application of modern theoretical concepts in current post mortem investigations of schizophrenia.

Streszczenie

Rozdział pierwszy zawiera wstęp niniejszej pracy doktorskiej, w którym na pierwszym miejscu przedstawiono zarys historii schizofrenii oraz proces formowania podstawowych pojęć odnoszących się do psychopatologii choroby. Następnie dokonano przeglądu podstawowych danych epidemiologicznych, genetycznych oraz środowiskowych. Rozdział ten kończy się opisem stanu zaawansowania technologicznego w badaniach post mortem z uwzględnieniem metodologii zastosowanej w tej pracy.

W rozdziale drugim umieszczono publikację wyników badań stereologicznych cytoarchitektury pięciu podkorowych struktur mózgowia, elementów prążkowania (jądro ogoniaste, skorupa oraz jądro półleżące), przyśrodkowo-grzbietowego jądra wzgórza oraz jądra bocznego ciała migdałowatego. Mózgowia schizofreników charakteryzowały się zmniejszeniem się całkowitej objętości korowej istoty szarej, skorupy oraz jądra półleżącego, zmniejszoną całkowitą ilością neuronów jądra ogoniastego, skorupy, oraz jądra bocznego ciała migdałowatego. Nie wykazano zależności pomiędzy czasem trwania choroby, liczonym od postawienia rozpoznania do momentu zgonu, a dynamiką zmian parametrów morfologicznych względem czasu.

Rozdziały trzeci i czwarty zawierają opublikowane wyniki badań nad unaczynieniem mózgowia na poziomie kapilar (o średnicy mniejszej niż 9 μm). Zebrane dane obejmują parametry takie, jak gęstość długości naczyń, całkowitą długość naczyń w odniesieniu do objętości badanej struktury oraz długość naczynia przypadająca na jeden neuron w strukturach takich jak jądro ogoniaste, skorupa, jądro półleżące, przyśrodkowo-grzbietowe jądro wzgórza oraz boczne jądro ciała migdałowatego. Badania wykazały statystycznie znamienne większą średnią gęstość naczyń w jądrze ogoniastym oraz we wzgórzu oraz zmniejszoną całkowitą długość naczyń w jądrze bocznym ciała migdałowatego u schizofreników. Co ciekawe, badania nie wykazały znaczących statystycznie różnic grupy kontrolnej względem schizofreników dla parametrów takich, jak średnia długość naczynia przypadająca na jeden neuron we wszystkich badanych strukturach. Analiza średniej gęstości długości naczyń w polach korowych Brodmanna 9 oraz 24 również nie wykazała znaczących statystycznie różnic pomiędzy oba grupami.

Rozdziały piąty oraz szósty opisują wyniki badań nad jednostką funkcjonalną kory nowej: minikolumną. Obejmują one badania średniej odległości pomiędzy neuronami oraz rozkładu przestrzennego neuronów w obrębie jednej minikolumny. Zmiany średniej od-

ległości pomiędzy neuronami nie były znaczące statystycznie i po przeprowadzeniu analizy post-hoc i podlegają dalszej dyskusji i interpretacji. Nie jest jednakże niemożliwe, że średnia odległość pomiędzy neuronami w mózgu schizofrenika różni się od tej w zdrowym mózgu. Wyniki wskazują, że zmiany znajdują się raczej w obrębie poszczególnych warstw kory, niż na całej długości minikolumny. Co więcej, zmiany średniej odległości między neuronami mogą być wtórne do utraty impulsów modulacyjnych w komórek nerwowych w obrębie minikolumny. Tego typu zmiany nie wskazują na jeden, uogólniony mechanizm, którego należałoby poszukiwać we wszystkich strukturach mózgowia, ale raczej na zakłócenie pracy systemów neuronalnych modulujących przetwarzanie informacji, innej konfiguracji sieci połączeń, cytoarchitektury oraz morfologii neuronów. Rozkład neuronów kory nowej schizofreników opublikowanych w niniejszej pracy odpowiada zmianom zaobserwowanym w neuropilu schizofreników, zjawisku dobrze znanym z literatury. Zmiany te odzwierciedlają de facto zmiany systemów modulujących w sieci neuronalnej minikolumny.

Ostatni rozdział zawiera dyskusję na temat wyników zawartych w powyższych publikacjach z uwzględnieniem i zastosowaniem aktualnych poglądów obecnych w środowisku badań post-mortem nad schizofrenią.

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Wujek, będzie dobrze.

Bart, thanks for help, dude.

Eva, thanks for keeping my keys for so long :D

My dear Ladies, Marijke, Annerieke, Imke and Eveline, I am lost in translation.

Curriculum vitae

Paweł Kreczmański was born in Warsaw in 1975. After he finished basic school in 1990, he attended Lyceum "Hugo Kołłątaj" in Warsaw where he graduated in 1994. He was then successfully admitted to study medicine at Warsaw Medical University. Six years later he started to work as a young assistant at the Anatomy Department of the Warsaw Medical University. During this time he also worked at the Central Hospital of the Ministry of the Internal Affairs during his post-graduate training for one year. In 2003 he went to Rostock to study the development of the human fetal brain at the University of Rostock in Germany under supervision of prof. Norbert Ulfig. After three months, in June of 2003 he was offered a PhD position at joined project between Aachen University and Maastricht University under supervision of dr. Christoph Schmitz until the end of 2006. This thesis is the result of that project. After that he worked for one year at the Department of the Neurosurgery at Maastricht University Hospital and one year at the Department of Pathology at Maastricht University Hospital. He is currently working as a doctor in a nursery home in Landgraaf searching for other opportunities. He likes philosophy, history and diplomacy.

Name	Paweł Kreczmański (m)
Date of birth	23-10-1975 (33 years)
Address	Grote Staat 16 C05, 6211 CW Maastricht
Country	Netherlands
Telephone	+31 (0)6 17678855
E-mail	pawel.kre@NP.unimaas.nl
Citizenship	Polish

Education

1994-2000	Medical University of Warsaw in Warsaw, medical degree
1990-1994	Hugo Kollataj Lyceum, Warsaw 10 subjects: Polish, German, English, Russian, Mathematics, Biology, Chemistry, Physics, Geography, History

Occupation

January 2009 – present	Medical doctor at the nursery home De Dormig, Meander Group, Landgraaf, Netherlands
January 2008 – December 2008	Resident Pathology, Department of Pathology at Maastricht University Medical Centre, Maastricht
January 2007 – December 2007	Assistant Neurosurgery, Maastricht University Medical Centre, Maastricht
October 2003 – December 2006	PhD Student. Project Neuropathology of Schizophrenia, Dept. of Psychiatry and Neuropsychology, division Cellular Neuroscience, University Maastricht, Maastricht
Maart 2003 – Oktober 2003	"Wissenschaftlicher Angestellter" (equivalent of the Post-Doc), Department Anatomy, University Rostock, Germany
October 2001 – March 2003	Young-Assistant Department Anatomy, Medical University of Warsaw
October 2000 – Oktober 2001	Post graduate training Central Hospital of the Ministry of the Internal Affairs, Warsaw, Poland

Trainings

April 2005	Stereologie, University Maastricht
October 2005	Writing in English Part I, University Maastricht

List of publications

1. Kreczmanski P, Heinsen H, Mantua V, Woltersdorf F, Masson T, Ulfing N, Schmidt-Kastner R, Korr H, Steinbusch HWM, Hof PR and Schmitz C. Capillary length density, total capillary length and capillary length per neuron in five subcortical regions in schizophrenia. *Acta Neuropathol*, Apr;117(4):409-21. Epub 2009 Feb 6. IF 3.179 (2007)
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6. Yamamoto H, Hofmann S, Hamasaki DI, Yamamoto H, Kreczmanski P, Schmitz C, Parel JM, Schmidt-Kastner R. Wolfram syndrome 1 (WFS1) protein expression in retinal ganglion cells and optic nerve glia of the cynomolgus monkey. *Exp Eye Res*. 2006 Nov;83(5):1303-6. Epub 2006 Aug 22. IF 2.651 (2006)

Presentations

Poster presentations

14 November , 2005: The Society for Neuroscience, 35th Annual Meeting Washington, DC: Stereologic studies of capillary length density in the frontal cortex of schizophrenics Kreczmanski P, Heinsen H, Mantua V, Woltersdorf F, Masson T, Ulfing N, Schmidt-Kastner R, Korr H, Steinbusch HWM, Hof PR and Schmitz C

Oral presentations

11 April 2008, Institute's day, Maastricht, Annual Institute for Neuroscience Meeting, Division 2: Capillary length density, total capillary length and capillary length per neuron in five subcortical regions in schizophrenia: Kreczmanski P, Heinsen H, Mantua V, Woltersdorf F, Masson T, Ulfing N, Schmidt-Kastner R, Korr H, Steinbusch HWM, Hof PR and Schmitz C. Winner of the Second Division Prize for the best publication in 2007.

Languages

Language	in writing	in reading
Polish	Mother tongue	Mother tongue
Dutch	Good	Good
Engels	Fluid	Fluid
Duits	Basic	Basic
Russisch	Basic	Basic

Computer skills

Windows 95/98/ME/XP
Microsoft Office 97/2000/2003 (Word, Excel, PowerPoint, Outlook etc.)
Stereoinvestigator
SPSS, GraphPad
Corel suite 11
Adobe Photoshop 7.0

Hobbies

History of Europe
Film
Diplomacy