Neurotrophic factors in the peripheral blood of male schizophrenia patients

Neurotrofe factoren in perifeer bloed van mannelijke patiënten met schizofrenie

Nico van Beveren

The studies reported in chapters 7, 8 and 9 were made possible by an unrestricted personal grant to N.J.M. van Beveren by AstraZeneca, Netherlands.

Cover design & lay-out pages: Stichting Mandarijn/Clementien Heim

Printed by Ridderprint, Ridderkerk

ISBN/EAN 978-90-5335-282-3

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Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

Prof. dr. H.G. Schmidt

en volgens het besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 18 mei 2010 om 15.30 uur

door

Jacobus Martinus van Beveren
geboren te Amsterdam

Promotiecommissie

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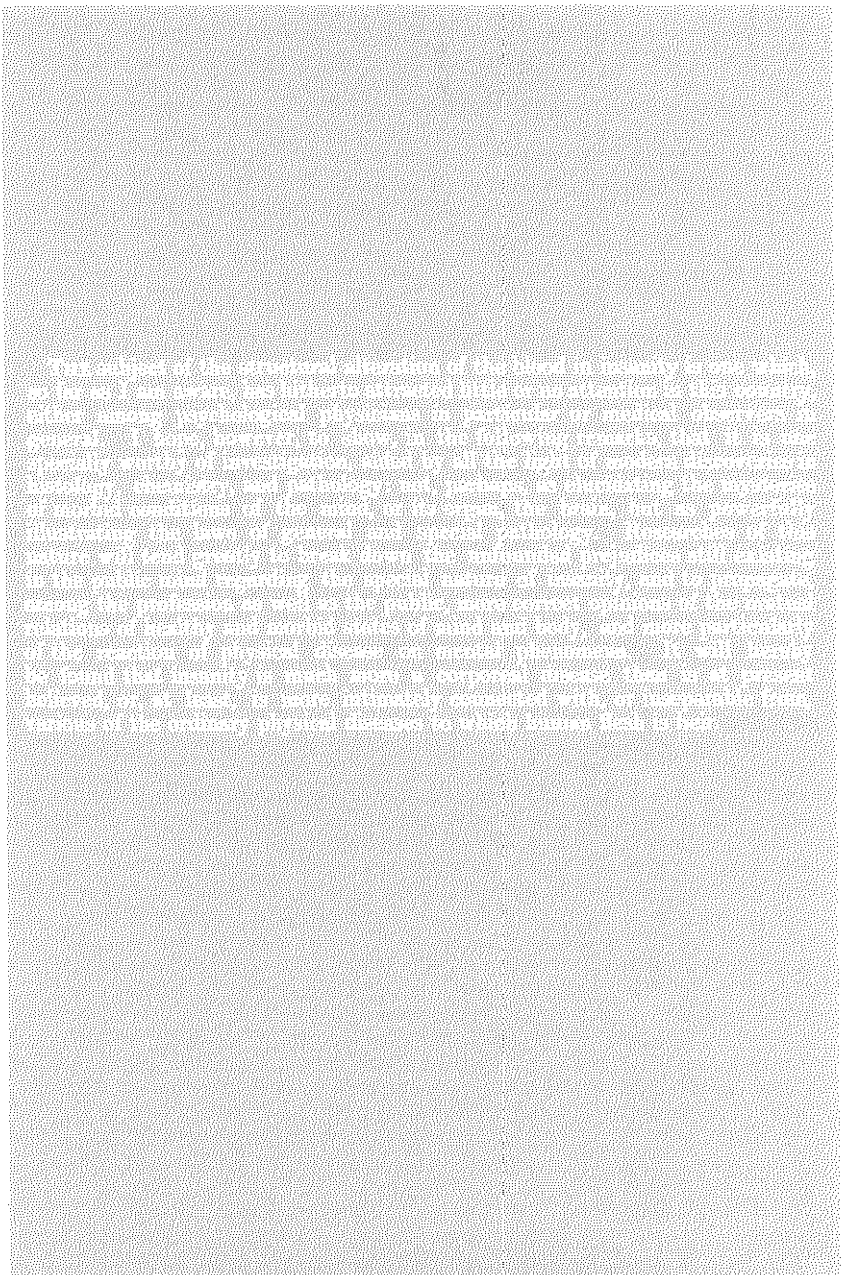

Lauder Lindsay, W., 1855. ***The histology of the blood in the insane.*** *The Journal of Psychological Medicine and Mental Psychology, VIII, p. 78.*

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Chapter 1

Introduction

General aspects

The term schizophrenia denotes a psychiatric syndrome of which the delineation is historically attributed to the work of Emil Kraepelin (Kraepelin, 1971), who lived from 1856 to 1926. Kraepelin described a disorder that starts at a young age and is characterized by clinical and cognitive deterioration that progresses during the course of the disease. For this syndrome he used the terms 'dementia praecox'. The concept was later elaborated upon by Eugen Bleuler (1857-1939) who emphasized core symptoms of the disorder come to be known as Bleuler's '4 A-s': Ambivalence (loss of goal directed behaviour), loosening of Associations, (difficulties in goal-directed thinking), flat Affect, and Autism (predominance of inner thought and concepts over external realities - as a symptom, and not to be confused with the similarly named syndrome). It was Bleuler who gave the disorder its present name, 'schizophrenia', as a general description of the loss of integrated psychological functioning which arises as part of the phenomenology of the disorder.
Over the years, many attempts have been made to more stringently and objectively define the diagnostic criteria of schizophrenia. At present, most clinicians and researchers use the criteria as described in the DSM IV. See **table 1** for the complete list of criteria.

Close inspection of the DSM IV criteria as shown in table 1 shows some key elements of the present conception of the schizophrenia syndrome. Criterium A lists symptoms required to be/have been present for at least one month. Among these one will recognize the influence of Bleuler's '4 A-s': delusions and hallucinations as reflections of the predominance of inner thoughts and concepts over external realities (Autism); disorganized speech and -behaviour as reflections of difficulties in goal-directed thinking and behaviour (Ambivalence and loosening of Associations); and a complex of deficit symptoms, called negative symptoms, broadly reflecting Bleuler's 'flat Affect'.
The influence of Kraepelin is seen in the criteria listed under B: the disorder is required to persistently and negatively affect major areas of psychological and social functioning. Clearly, the way the presence of the A criteria have been defined allows for a heterogeneous expression of these symptoms in schizophrenia. In fact, 23 valid combinations of criterium A symptoms exist.
Taken together, the DSM IV criteria conceptualize schizophrenia as a syndrome with a chronic course, characterized by the presence of heterogeneously defined

Table 1: Diagnostic criteria for schizophrenia

A. **Characteristic symptoms:** Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
   1. Delusions
   2. Hallucinations
   3. Disorganized speech (e.g., frequent derailment or incoherence)
   4. Grossly disorganized or catatonic behaviour
   5. Negative symptoms, i.e., affective flattening, alogia, or avolition

   Note: Only one criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behaviour or thoughts, or two or more voices conversing with each other.

B. Social/occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Duration: Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meets criterion A (i.e. active phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (i.e. odd beliefs, unusual perceptual experiences).

D. Schizoaffective and Mood Disorder exclusion: Schizoaffective Disorder and Mood Disorder With Psychotic Features have been ruled out because either (1) no Major Depressive, Manic, or Mixed Episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

F. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

G. Relationship to a Pervasive Developmental Disorder: If there is a history of Autistic Disorder, or another Pervasive Developmental Disorder, the additional diagnosis of Schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

psychotic episodes, accompanied by a marked decline in social and occupational functioning.

From a clinical perspective schizophrenia is a devastating disorder, destroying uniquely human functions of abstract thinking and conceptualization in predominantly young people. The life time risk which varies with the absence or

presence of risk factors, such as a family history of schizophrenia, living in an urban environment, low social-economical status, and drug-abuse, but is estimated to be on average about 1% (Mueser and Gurk, 2004).

Etiology

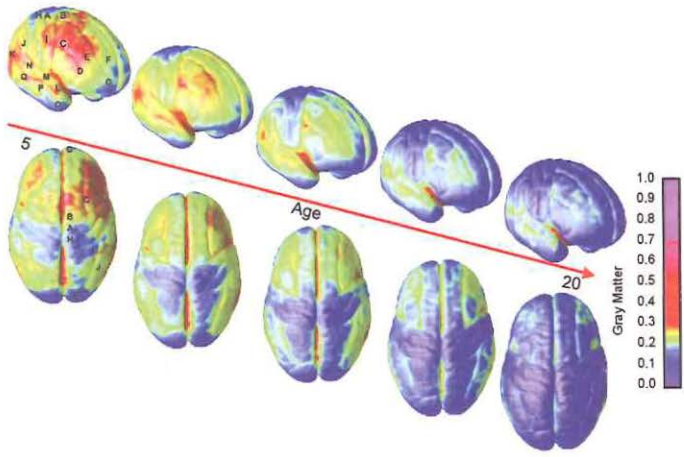
Although the specific etiology of schizophrenia is unclear, during the last decade significant advances have been made in understanding the chain of causes and effects which may lead to the emergence of symptoms within the context of the schizophrenia syndrome. The so-called neurodevelopmental model is at present the most dominant paradigm. It assumes that the syndrome is related to subtle aberrations in perinatal (cortical) brain architecture, resulting in the emergence of symtoms at the end of puberty when normal or abnormal brain maturational processes interact with the already pathologically configured brain. The process may be modulated by the presence of environmental risk factors which interact with brain development, such as drug abuse, or persistent (social) stress. The neurodevelopmental model is based on several isolated findings from epidemiology, neuro-imaging, genetics, developmental neuroscience, pharmacology, and information-processing psychology, as well as the obvious clinical fact that the majority of patients present with symptoms at the end of puberty.

Cortical maturation and brain connectivity and schizophrenia

In the late 1970's Huttenlocher (Huttenlocher, 1979) reported that, in normal individuals, cortical synaptic density diminishes developmentally, especially during adolescence. This relationship between normal adolescent reduction of cortical synaptic density and the typical age of onset of schizophrenia led Feinberg (Feinberg, 1982) to suggest that aberrations in this normal developmental process may underlie schizophrenia . This suggestion has over the years been supported by subsequent postmortem histopathologic studies demonstrating reduced spine densities and smaller dendritic arbors on the pyramidal cells of the prefrontal cortex in schizophrenia (Garey et al., 1998; Lewis et al., 1999; Glantz and Lewis, 2000). Other findings relate to changes in synaptic "products," including reduced glutamate and γ-aminobutyric acid synaptosome release in schizophrenia (Deakin et al., 1989; Sherman et al., 1991), decreased synaptic protein mRNA expression in schizophrenic temporal cortex, (Sokolov et al., 2000) and reduced synaptophysin in

the dorsolateral prefrontal cortex (Perrone-Bizzozero et al., 1996; Glantz and Lewis, 1997; Honer et al., 1999; Karson et al., 1999; Glantz and Lewis, 2000) and thalamus (Landen et al., 1999). All these findings point towards reduced synaptic density or synaptic stability in schizophrenia. The most replicated postmortem finding has been increased cortical neuronal density seen on histological examination as reduced neuropil (Pakkenberg, 1987; Daviss and Lewis, 1995; Selemon et al., 1995; Selemon et al., 1998; Selemon and Goldman-Rakic, 1999) without neuronal loss (Harrison and Law, 2006). This has been interpreted as neurodevelopmental failure and/or neurodegeneration insofar as the decreased neuropil represents a loss of connections between neurons (McGlashan and Hoffman, 2000).
Recent advanced neuroimaging techniques have provided additional insight into the processes present in the normally developing brain, as well as those present in schizophrenia. Gogtay et al. (2004; 2006) investigated cortical maturation in healthy, young individuals, aged 4-21 years. They found that normal cortical maturation proceed in a dorsal-to-ventral sequence, with the occipital visual areas maturing first, and prefrontal and temporal cortical area last. **Figure 1** shows this normal maturational process.

Figure 1



Right lateral and top views of the dynamic sequence of grey matter maturation over the cortical surface. (Gogtay et al., 2004)
The side bar shows a color representation in units of grey matter volume. The initial frames depict regions of interest in the cortex: A, precentral gyrus and

primary motor cortex; B, superior frontal gyrus, posterior end near central sulcus; C, inferior frontal gyrus, posterior end; D, inferior frontal sulcus, anterior end in the ventrolateral prefrontal cortex; E, inferior frontal sulcus in the dorsolateral prefrontal cortex; F, anterior limit of superior frontal sulcus; G, frontal pole; H, primary sensory cortex in postcentral gyrus; I, supramarginal gyrus (area 40); J, angular gyrus (area 39); K, occipital pole; L–N, anterior, middle, and posterior portions of STG; O–Q, anterior, middle, and posterior points along the inferior temporal gyrus anterior end.

Figure 1 clearly shows a reduction of grey matter over time, spreading from the occipital cortex over the midline to the frontal areas, with the dorsolateral prefrontal- and temporo-parietal cortices maturing last.
Later research by the same group (Gogtay et al., 2008; Gogtay, 2008; Vidal et al., 2006) showed that this normal process of coordinated dorsal-to-ventral maturation is disturbed in childhood onset schizophrenia (COS).
Taken together, the findings described in the previous paragraphs have led to the conclusion that postnatal mammalian brain development is characterized by synaptogenic overelaboration of neuritic processes, ie, axons and dendrites, in the cortex followed by a gradual reduction of synaptic density to about 60% of maximum levels (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997). In humans this process is largely complete by age 2 in sensory areas such as the occipital cortex but is not complete until midadolescence in prefrontal and association areas, as illustrated in figure 1 (Huttenlocher and Dabholkar, 1997; Gogtay et al., 2004). Early in development, synaptogenesis likely creates connections more or less randomly, with subsequent selective elimination of weaker connections based on experience, (Murphy and Regan, 1998) as well as endogenous (genetic) factors (Etienne and Baudry, 1990; Sestan et al., 1999). In adulthood, production of new synapses is matched by a similar rate of synaptic elimination.
In schizophrenia, it is assumed that the overt symptoms characterizing the syndrome arise once the normal reduction of (prefrontal and/or parietotemporal) synaptic connectivity falls below a certain critical level. In other words, the syndrome, in a general sense, is caused by *developmentally reduced synaptic connectivity* (McGlashan and Hoffman, 2000).

Dopamine and schizophrenia

Since the introduction of chlorpromazine in the 1950s as an effective antipsychotic

agent dozens of antipsychotics have been developed and introduced in clinical practice (Kapur and Mamo, 2003). In the 1960s, the idea that these antipsychotics were acting on the dopamine system took hold, and this was finally confirmed in the 1970s by the finding that the antipsychotics act on the dopamine D2 receptors. In the 1980's and 1990's the role of dopamine in psychosis, and especially that antipsychotics block the dopamine D2 receptors, was firmly established using neuroimaging studies. Although several efforts have been made to develop antipsychotics which bypass the dopamine system, a blockade of the dopamine D2 receptor remains a necessary and sufficient condition for antipsychotic activity to this day (Kapur and Mamo, 2003; Sanger, 2004).

Animal research at the interface between behaviour and molecular-biology has shown that increases in dopamine have been linked to rewarding stimuli. Dopamine is thought to mediate the "*motivational salience*" of environmental stimuli and their associations (Berridge and Robinson, 1998). The term motivational salience refers to the process whereby reward-associated stimuli come to be "attention grabbing" (salient) to the animal, and become the focus of goal-directed behavior. This release of dopamine is thought to not only direct and accentuate the animals responses in the present situation, it is also thought to lead to memorisation of new associations and reward learning that guides future behavior (Wise, 2004). Thus, the dopamine system is involved in detecting new rewards in the environment, enhancing the animals learning about the rewards and their associations, and by marking these stimuli as being salient to the animal and as a result driving goal-directed behaviour (Berridge and Robinson, 1998).

Under normal circumstances, it is the context-driven activity of the dopamine system that mediates the experience of novelty and the acquisition of appropriate motivational salience (Shizgal, 1997; Berridge and Robinson, 1998). It has been hypothesized, most prominently by Kapur and colleagues (Howes and Kapur, 2009; Kapur, 2003), that in schizophrenia, a series of genetic and environmental predispositions (Lewis and Levitt, 2002), possibly an altered neurodevelopmental status result in a dysregulated (limbic) dopamine system which fires and releases dopamine independent of cue and context. The normal process of context-driven novelty and salience attribution is then replaced by an endogenously driven assignment of novelty and salience to stimuli. Thus, the dopamine system which, under normal conditions, is a mediator of context-driven novelty/salience in the psychotic state becomes a creator of aberrant novelty and salience (Kapur, 2003).

Another aspect of dopamine in schizophrenia is its role in the prefrontal cortex associated with working memory disorders.

Evidence for the presence of reduced levels of dopamine in schizophrenia initially came from the finding of reduced cerebrospinal fluid levels of the principal dopamine metabolite homovanillic acid (Weinberger et al., 1988), and higher dopamine receptor availability in the dorsolateral prefrontal cortex (DLPFC) (Abi-Dargam et al., 2002).

Whereas hyperdopaminergic states in the striatum have been associated with delusions, as outlined above, reduced dopaminergic activity in the prefrontal cortex has been associated with disorders of working memory (Cohen and Servan-Schreiber, 1992; Winterer and Weinberger, 2004). It is assumed that the prefrontal cortex is responsible for representing and maintaining task-relevant information. In this context, dopamine is involved in modulating the presence of task-relevant information in the prefrontal cortex (Cohen and Servan-Schreiber, 1993). Cohen & Servan-Schreiber (1993) concluded that performance deficits in schizophrenia are due to a degradation in the internal representation required as context for processing stimuli. In a state of reduced prefrontal dopaminergic activity noise (i.e. competing, but not task-related environmental stimuli), interferes with the ability of the system to maintain a representation of task-relevant information. The resulting inability of the brain-system to maintain a stable state over time shows itself at a psychological level as the observed disorders of working memory. More generally, this will lead to an uncontrolled spread of activation and an increase of spontaneous neuronal activity, hence, to a decreased signal-to-noise ratio. (Seamans et al., 2001; Winterer and Weinberger, 2004).

The interaction between aberrant cortical maturation and altered dopamine neuromodulation

The processes outlined previously enable us to sketch a general pathological model of the schizophrenia syndrome, which is in part hypothetical, but provides a clinically useful working model of the disorder:

1. Altered cortical maturational processes will critically reduce the connectivity of prefrontal and parietotemporal cortical areas at the end of puberty: *developmentally reduced synaptic connectivity* (Hoffman and McGlashan, 2000).
2. Altered prefrontal cortical connectivity is associated with *decreased prefrontal dopaminergic activity*, and this accounts for the presence of working mem-

ory disorders (it is however unclear whether decreased prefrontal dopamine is secondary to altered cortical connectivity, or whether there is a more complex relationship between the two. In animal studies, diminished cortical dopamine innervation is associated with reduced spine density and dendritic arborization of target neurons, both of which are also found in the post-mortem schizophrenia samples).

3. Low prefrontal dopamine activity leads to *increased striatal dopaminergic activity*. The striatum as a result becomes 'hypersalient' to stimuli, accounting for the development of delusions. As there is evidence in animals that, mesostriatal dopamine activity is regulated by feedback from the prefrontal cortex (Sesack and Carr, 2002), this raises the possibility that striatal dopamine activity might be increased as result of a downstream effect of a primary prefrontal abnormality.

So, there is considerable evidence that in schizophrenia, the initial disturbance underlying the disorder is the presence of altered cortical maturation.
Indeed, there is evidence from genetic studies showing that a considerable number of genes associated with schizophrenia are related to brain maturation, neural connectivity, and myelination (Carter, 2006; Harris et al., 2009).

Neurotrophic proteins in schizophrenia

So, from where we stand now, the term schizophrenia denotes a clinically heterogeneous syndrome of which in, a majority of cases, deregulated brain development may be the central biological theme. The term "neural growth factors" denotes a heterogeneous group of neurotrophic proteins which influence brain development through their actions on plastic processes such as proliferation, survival and differentiation of precursor cells, apoptosis, axon growth, axon collateralization, the synthesis of peptides, transmitter enzymes and calcium-binding proteins, synaptic organization, dendritic arborization and sprouting and myelination (Durany and Thome, 2004). With respect to schizophrenia this has raised the question as to whether these proteins are aberrantly present in schizophrenia. Abnormalities in neural growth factors may involve alterations in their production and secretion, receptor sensitivity and signal transduction processes in the target cells. Neurotrophin-related brain maldevelopment and dysfunction may then pave the

way for neuropsychiatric pathologies to erupt in later life (Shoval & Weizman, 2005); see **figure 2**.Indeed, several post-mortem studies have reported alterations in the levels of neurotrophic factors in selected brain regions.

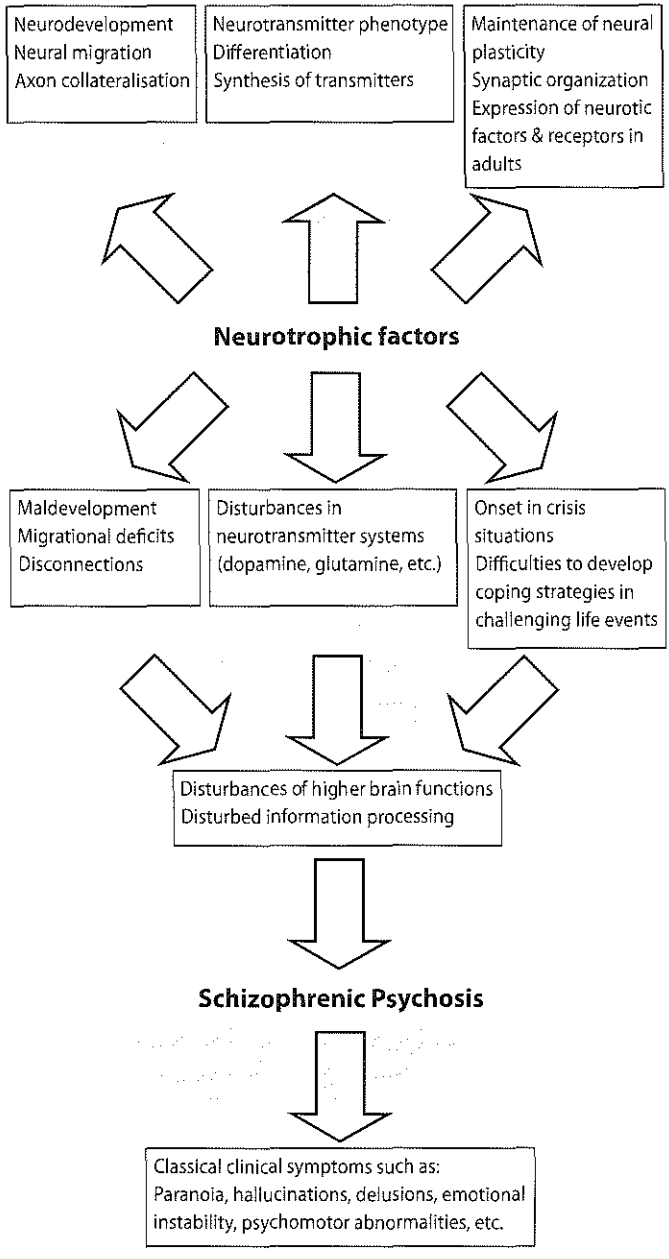
Takahashi et al. (2000) measured Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Nerve Growth Factor (NGF) and their receptors in various brain regions of schizophrenic patients and control subjects. They reported high levels of BDNF in the anterior cingulate cortex and hippocampus of patients with schizophrenia. Durany et al. (2001) compared BDNF and NT-3 levels in different regions of post-mortem brain tissue from patients with schizophrenic psychoses with those of individuals without neuro-psychiatric disorders. In cortical areas of the patients' brains, BDNF content was significantly higher than in age-matched controls, whereas in the hippocampus the opposite was observed. The cingulate gyrus exhibited a tendency towards higher BDNF immunoreactivity in the patient group. In the thalamus, the BDNF content in both groups was similar. Whereas the hippocampus was the area with the highest BDNF content in the control samples, in the schizophrenic patients, BDNF content in the hippocampus was similar as in other brain areas except cingulate gyrus.

Iritani et al. (2003) reported intensive staining of BDNF and its receptor trkB in the hippocampus of schizophrenic patients. Finally, Weickert et al. (2003) reported significant reduction of BDNF mRNA and protein in the dorsolateral prefrontal cortex of schizophrenic patients, and decreased neuronal BDNF expression in pyramidal neurons throughout layers II, III, V and VI of the dorsolateral prefrontal cortex, suggesting that, intrinsic cortical neurons, afferent neurons and target neurons may receive less trophic support in schizophrenic patients.

Aims and scope of this thesis

In schizophrenia, an impediment in further understanding the underlying biology is the inaccessibility of the living brain. However, several neurotrophic factors are present in peripheral blood (i.e serum or plasma) and measurements of neurotrophic factors in peripheral blood may provide insight into the underlying neurobiology, additional to neuroimaging and genetic research. Moreover, as compared to the traditional genetic approach, peripheral blood neurotrophic factors may theoretically change over time and thus reflect dynamic changes associated with illness progression, treatment effects, and, possibly, cortical maturation.

Figure 2 **The relationship between neurotrphic factors and psychosis** (after Durany & Thome, 2004)



Another way to overcome the inaccessibility of the living brain is to investigate the gene expression of some kind of peripheral tissue. White blood cells express many brain-relevant genes (Sullivan et al., 2006), some of which are involved in neurotrophic processes. So, gene expression in white blood cells may also reflect alterations in neurotrophic processes.

At the time we started this study, limited data was available on the presence of peripheral levels of neurotrophic factors in schizophrenia and their possible change over time during initial treatment. No data was available on alterations in whole-genome gene expression status in white blood cells. This formed the impetus for the present thesis. Our research question were: (1) are there aberrant levels of neurotrophic factors in peripheral blood of schizophrenia patients, and do they change during the course of initial treatment? (2) Is there altered expression of sets of genes related to neurotrophic processes in white blood cells?

With our project underway, some intererest emerged in the field in investigating peripheral proteins, not only from the basic science perspective of gaining insight in their relationship with developmental brain processes, but also from the clinical perspective of obtaining a biomarker for the schizophrenia syndrome (Bahn and Schwartz, 2008; Reckow et al., 2008; Singh and Rose, 2009). Recent developments in the field enabled the measurement of many proteins at the same time, analogue to whole-genome high-throughput methods. This development, concomitant to the execution of our research project, led us to add a research question, namely, (3) to explore whether neurotrophic factors (either individually or as multi-protein arrays) have the future potential to serve as biomarkers for the early schizophrenia syndrome?

Finally, as outlined in the previous paragraphs, schizophrenia is a heterogeneous disorder. To reduce heterogeneity in our sample, we limited our research to young patients, with recent onset of the disorder, which we defined as shorter than 5 years. Moreover, in the majority of the studies presented, inclusion was limited to male patients.

Description of chapters

The main theme of this thesis is a biological one. However, a limitation of many biological studies is that the relationship between biological aberrations and the resulting overt pathology is not always clear. In other words, it is not readily understandable how, for example, psychotic symptoms are related to disturbances on the molecular-biological level. To provide a certain level of insight into the relationship

between biology and overt pathology we have provided in **chapter 2** what we have called 'a visual metaphor', that is intended to serve as a heuristic framework to relate biological alterations with overt pathology.

Chapter 3 is a review of the at the time available literature on the subject of neurotrophic proteins in the peripheral blood of schizophrenia patients. **Chapter 4** presents the results of research into the presence of altered levels of the neurotrophic protein S100B in the serum of two independent, recent-onset, male schizophrenia patients, at baseline and after 8 weeks of naturalistic treatment. **Chapter 5** presents the results of research into the presence of altered levels of the neurotrophic protein Brain-Derived Neurotrophic Factor (BDNF) in the serum of the same two independent patient samples as presented in chapter 4, again at baseline and after 8 weeks of naturalistic treatment.

Chapter 6 is an introductory chapter on the subject of (whole-genome) gene expression. It describes the technique of micro-array based whole-genome gene expression, some specific analytic problems and the methods to solve those, as well as a review of micro-array gene-expression findings in post-mortem brain material in schizophrenia, at the time virtually the only available source of gene expression studies. **Chapter 7** presents the results of a pilot study of whole-genome Peripheral Blood Mononuclear Cells (PBMCs) gene expression in recent-onset, partly stabilized schizophrenia patients, which serves as a hypothesis generating study. Based on the results of the study described in chapter 7 we conducted a much larger study of whole-genome PBMC gene expression, which is described in **chapter 8**. In **chapter 9** we move from the subject of whole-genome PBMC gene expression in the classical schizophrenia syndrome towards PBMC gene expression in patients with the 22q11 deletion syndrome, a disorder which is thought to be a genetic model of schizophrenia.

In **chapter 10** we move to the subject of identifying protein biomarkers based on a non-hypothesis driven approach, investigating a large array of proteins in five independent patients cohorts, and seeking to identify a subset of proteins that distinguishes asymptomatic subjects that later developed schizophrenia from controls and from relevant other disease entities.

Chapter 11 finally, presents a general discussion of the findings, and their implications for future studies.

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Chapter 2

A visual metaphor describing neural dynamics in schizophrenia

PLoS One (2008) 3(7): e2577

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Abstract

Background

In many scientific disciplines the use of a metaphor as an heuristic aid is not uncommon. A well known example in somatic medicine is the 'defense army metaphor' used to characterize the immune system. In fact, probably a large part of the everyday work of doctors consists of 'translating' scientific and clinical information (i.e. causes of disease, percentage of succes versus risk of side-effects) into information tailored to the needs and capacities of the individual patient. The ability to do so in an effective way is at least partly what makes a clinician a good communicator. Schizophrenia is a severe psychiatric disorder which affects approximately 1% of the population. Over the last two decades a large amount of molecular-biological, imaging and genetic data have been accumulated regarding the biological underpinnings of schizophrenia. However, it remains difficult to understand how the characteristic symptoms of schizophrenia such as hallucinations and delusions are related to disturbances on the molecular-biological level. In general, psychiatry seems to lack a conceptual framework with sufficient explanatory power to link the mental- and molecular-biological domains.

Methodology/principal findings

Here, we present an essay-like study in which we propose to use visualized concepts stemming from the theory on dynamical complex systems as a 'visual metaphor' to bridge the mental- and molecular-biological domains in schizophrenia.

We first describe a computer model of neural information processing; we show how the information processing in this model can be visualized, using concepts from the theory on complex systems. We then describe two computer models which have been used to investigate the primary theory on schizophrenia, the neurodevelopmental model, and show how disturbed information processing in these two computer models can be presented in terms of the visual metaphor previously described.

Finally, we describe the effects of dopamine neuromodulation, of which disturbances have been frequently described in schizophrenia, in terms of the same visualized metaphor.

Conclusions/significance

The conceptual framework and metaphor described offers a heuristic tool to understand the relationship between the mental- and molecular-biological domains in an intuitive way. The concepts we present may serve to facilitate communication between researchers, clinicians and patients.

Introduction

Schizophrenia is a severe psychiatric disorder, characterized by the emergence at adolescence of psychotic phenomena: hallucinations, delusions and bizarre behavior. The neurodevelopmental hypothesis, which proposes a leading role for early aberrant brain development on which normal and/or abnormal brain maturation is superimposed has become the dominant paradigm for understanding the development of schizophrenia. The neurodevelopmental theory is usually underscored by a large amount of molecular-biological, imaging and genetic data which have been accumulated over the last two decades. Taken together, these findings point to reduced neuronal connectivity and synaptic stability. Psychotic symptoms are considered to be emergent properties on the psychological and behavioral level of the aberrantly developed neural system which start when during brain development a critical threshold is passed.
However, it remains difficult to understand how psychotic symptoms are related to disturbances on the molecular-biological level. Kapur (2003) described this as: "doctor-patient interaction proceeds mainly at a 'mind' or 'behavioral' level of description. On the other hand, the preeminent theories regarding psychosis (...) are mainly neurobiological". We think this is a major problem in contemporary psychiatry because it impedes researchers to convey findings to patients, clinicians, and the general community. The problem is that psychiatry as a science seems to lack a coherent system of terms linking the mental and molecular-biological domains (Goodman, 1991).

In somatic medicine the use of some kind of metaphor to bridge the biological and phenomenological domains is not uncommon. For instance, the immune system is characterized by the defense-army metaphor. Though the immune system is notoriously difficult on a molecular level, and the specific molecular phenomena which happen during, say, an HIV infection, may be only within the grasp of experts, the defense-army metaphor functions as a bridge between lay and professionals and facilitates understanding in an intuitive way.

In this article we attempt to outline a heuristic framework that could provide a basis for uniting clinical phenomena and neurobiological theories. We will introduce what we have coined a 'visual metaphor' which is supposed to bridge the mental and neural domains. To this end we will combine two fields of research, namely the study of the behavior of complex systems and computer models of schizophrenia.
During the last two decades a large body of literature has appeared concerning the study of complex systems and its associated theoretical framework 'complexity theory' (Prigogine et al., 1984; Madore and Freeman, 1987). Complex systems consist

of a set of simple elements which interact with each other and the environment and change in time as result of these mutual interaction. In psychiatry and the life sciences the study of complex systems has been identified as a potential source of new ideas and viewpoints (Mandell and Selz, 1992; Globus and Arpaia, 1994; Ehlers, 1995; Robertson and Combs, 1995; Port and van Gelder, 1995; Kelso, 1995; Beer, 2000; Freeman, 2000; Tretter and Scherer, 2006) as the brain can also be seen as a complex system (Pritchard and Duke, 1992).

The neurodevelopmental theory of schizophrenia has been supported by computer simulations of neural information processing. Hoffman and McGlashan reported a body of research on schizophrenia (Hoffman, 1987; Hoffman and Dobscha, 1989; Hoffman and McGlashan, 1997), resulting in a pathophysiological model coined Developmentally Reduced Synaptic Connectivity (McGlashan and Hoffman, 2000). This model specifically posits that schizophrenia arises from critically reduced synaptic connectivity as a result of developmental disturbances of synaptogenesis and supports the neurodevelopmental theory.

Both research efforts depend greatly on computer models and complicated mathematical ideas which are unfamiliar to most psychiatrists. However, the ideas developed in both fields have great heuristic value. In this paper we will try to visualize findings using concepts from the behavior of complex systems.

This article will first briefly introduce some basic principles of computer models of mental processes, so-called Artificial Neural Networks (ANN). A conceptual framework which is related to the functioning of ANN is described and we show how this conceptual framework can be visualized. This is the visual metaphor we seek to describe. Subsequently, we describe how implementing developmental disturbances in ANN give rise to pathological phenomena, what their relationship is with 'real-life' pathology and how these phenomena can be understood in terms of the visual metaphor we introduced. Finally, we will show how psychotic symptoms originating from dopamine disturbances can be understood in terms of this visual metaphor.

Methods

This is an essay-like study aimed to contruct visualized concepts stemming from the theory on dynamical complex systems to be used as a 'visual metaphor' to bridge the mental- and molecular-biological domains in schizophrenia.

The background for this study has been formed by a PubMed search with the terms ('neural network' OR 'connectionism' OR 'parallel distributed processing' OR 'nonlinear systems' OR 'complex systems') AND ('schizophren*' OR 'psychosis' OR 'psychotic' OR 'psychiatry*').
The general concept of a (visual) metaphor is inspired by the work of Globus & Arpaia (1994), Draaisma (1995; 2001), and Ouzounis & Maziere (2006).
To introduce the sought-after metaphor we first describe a computer model of neural information processing. We then show how the information processing in this model can be visualized, using concepts from the theory on complex systems. Finally, we use the visual metaphor to describe disturbed information processing and dopamine neuromodulation in schizophrenia.

Results

Introducing the metaphor (1): artificial neural networks and attractors

There is an extensive body of literature on the principles of ANN. We describe a certain class of ANN, *the attractor neural network*.
Figure 1 shows a network consisting of 100 artificial neurons (AN's) (figure 1, top, left). Each AN is connected with every other AN by means of connections of random strength (For clarity, only connections between neighboring AN's are shown). Each AN receives input from each of the 99 other AN's. Each AN can be active ('1') or inactive ('0'). For example, the influence of each set of 99 AN's on the remaining one is given by the sum of the products of the number representing active or inactive and the strength of the corresponding connection. The remaining AN is itself active or inactive depending on whether the sum exceeds a certain threshold value or not. In this way, all the AN's continuously and mutually influence each other making this ANN a dynamic system. The functioning of this network can be simulated with a computer.
It is possible to present this ANN a certain pattern, for instance, a pattern that symbolizes the letter 'A' (figure 1, top, middle). The network is constructed such that connections between AN's which are simultaneously active become stronger; connections which are not simultaneously active become weaker.
After pattern 'A' has been presented, this mechanism will have strengthened the connections in the ANN in such a way that pattern 'A' has become imprinted in the connections between the AN's (figure 1, top, right). Pattern 'A' has now become an *attractor* of the network dynamics: the network has learned 'A' ('learning can be regarded as collecting new attractors' (Hoffman and McGlashan, 1993). An attractor implies a preferent state of a dynamic system.

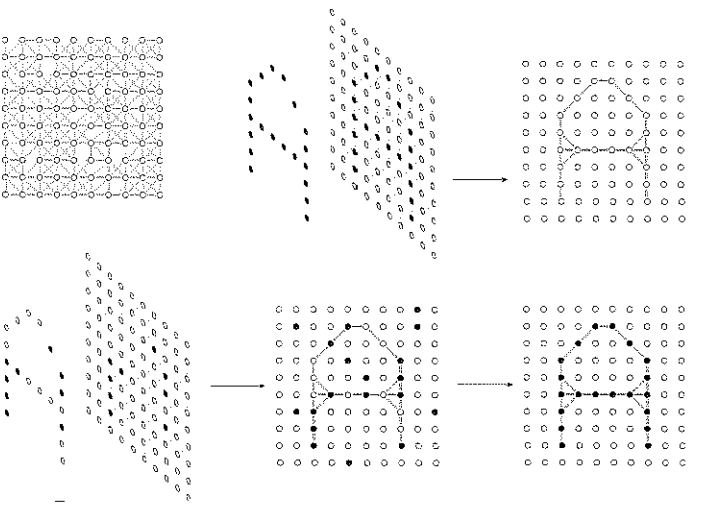
What this means is shown in figure 1 (bottom, left). In the left-hand side of the figure, some part of 'A' is presented. The activity of the ANN is continuously updated (figure 1, bottom, middle). The dynamic behavior of the network will finally be attracted towards pattern 'A', the attractor of the network (figure 1, bottom, right). The ANN is now said to have recognized the 'A' pattern. An ANN can have several attractors.

Introducing the metaphor (2): visualizing the behavior of neural systems as a trajectory through state space

The behavior of systems like an ANN can be described with so-called 'state space' representations and from this concept we derive the 'visual metaphor' we like to introduce.

Mathematically, all the states in which the ANN can exist can be represented by a multidimensional 'space'. The basic idea can be introduced by considering one neuron with two possible states, 'firing' ('1') and 'non-firing' ('0'). These two states can be graphically depicted as two points. Over time, the dynamical behavior of this

Figure 1



Learning an attractor network to recognize patterns.

Figure 1, top, (left): a network with 100 units with connections of random strength and with no specific pattern learned (for clarity only neighbouring connections are shown).

Figure 1, top (middle and right): learning pattern 'A'. Top (middle): presenting 'A'; top (right): connections between units related to 'A' are strengthened.

Figure 1, bottom, (left): presenting part of 'A'. **Bottom (right):** the network has succeeded in retrieving 'A'

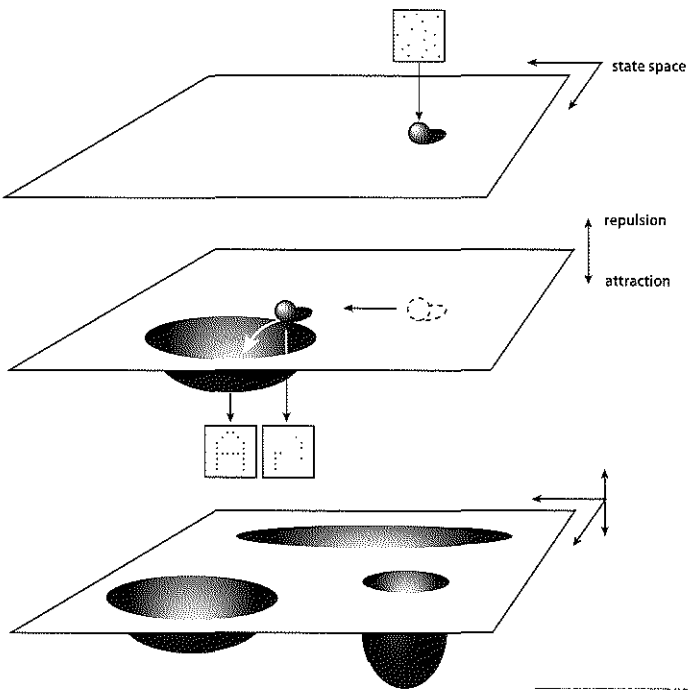
one-neuron 'system' can be represented by a *trajectory* that jumps between the two possible states. Similarly, a two-neuron system can exist in four different states and performs a trajectory that moves between these four points in a two-dimensional space; hence the term 'state space'.
From a mathematical point of view, there is no difference with respect to the situation describing systems consisting of N neurons. They describe a trajectory through a N-dimensional space. Unfortunately, this situation cannot be visualized.
We propose to simplify this high-dimensional situation to the depiction of a plane with the different states as points in the plane (see figure 2, top; see also Globus and Arpaia (1994) and Beer (2000). In fact we performed some kind of intuitive principal component analysis by assuming that relevant variance occurs in limited directions (Beer, 2000).
This plane represents the collection of states in which the system can exist. (Throughout we should keep in mind that in fact we are dealing with a multi-dimensional space which cannot be graphically shown).
An attractor (a learned pattern) can be represented as a dent in this plane (figure 2, center). The magnitude of the attractor, commonly described as *the basin of attraction*, represents the influence of the attractor over its surroundings. In psychological terms, it represents how much of a stored memory has to be 'fed into' the neural system in order for the ANN to perform an associative recollection of the memory. Changing from one state to another can be conceptualized as the movement of a ball over the surface (Jeffery and Reid, 1997). In time, the system 'moves over the plane': it takes different states and by doing so performs a trajectory through the state space. With this conceptual framework we have a visual tool to aid our understanding of the functioning of the ANN. Because of its analogy with the movement of a ball over a landscape we have coined it a 'visual metaphor'.

Presenting a pattern to an ANN can be likened to moving a ball over the surface and letting it loose. The system will then seek some state guided by the attractors which dominate the systems' behavior and will settle in one of its attractors (figure 2, center). A situation in which several attractors are present is shown in figure 2 (bottom). Throughout, we must keep in mind that this visual representation is a metaphor for what are essentially abstract mathematical concepts.

For a proper understanding, one has to imagine the dynamic aspects of the whole process. So, the state of the ANN is continuously moving through the space of its possible states guided by external influences and attractors with the ANN simultaneously creating new attractors which can be regarded as memory traces (Hoffman and McGlashan, 1993).
Moreover, in this kind of system, small changes in the state of the system can result

in major changes in the final state of the system. These changes are known as *phase transitions* and appear when the system is near bifurcation points: unstable states in which the system chooses between one or another final state to evolve to. Such sudden changes in the state of the system can be imagined as popcorn, 'popping' from one attractor to another (Hoffman, 1987).

Figure 2



Dynamical behavior of a network as a trajectory in state space guided by attractors (for clarity repulsors are not shown).

Figure 2, top: different states of a network represented by points in a plane: the state space. A random situation is shown.

Figure 2, center: an attractor representing 'A' symbolised as a dent in state space. Changes in the state of the network can be regarded as a trajectory through state space.

Figure 2, bottom: attractors with variably sized basins (representing the influence of the attractor) and variable depths (representing the preference of the system to stay in the attractor).

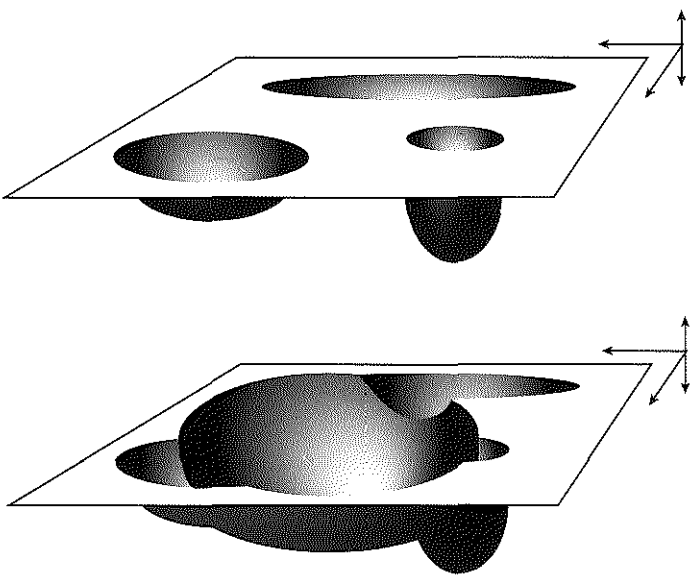
Using the metaphor (1): the neurodevelopmental theory, ANN and psychopathology

Efforts to simulate schizophrenic pathology using ANN assumed that schizophrenia arises from reductions in connectivity between brain regions as a result of developmental disturbances during synaptogenesis (Feinberg, 1982; Friston and Frith, 1995). A first effort used the 10x10 ANN described earlier. After learning this ANN a number of symbols, a pruning rule was imposed on the ANN, removing weak connections (Hoffman 1987). However, at higher levels of pruning, the ANN demonstrated pathology. One of the findings was that parts of the network would tend to re-create a pattern of activation that did not correspond to any particular stored memory. It was hypothesized that autonomous activation of cortical areas arising independent of activity in other areas would be subjectively experienced as hallucinations or the experience that one's thoughts are being controlled by outside forces.
We can visualize this situation using the introduced metaphor (see figure 3, bottom).
Excessive pruning has created a so-called 'parasitic' attractor. Input will not bring the ANN into the regions in state space which correspond to a previously learned symbol but to a newly emerged attractor. The re-creation of the pattern can be interpreted as autonomous activation of the ANN. In terms of dynamical behavior the pathologically configured ANN is performing an inappropriate trajectory through its state space. (Through a region of its state space not corresponding to any previously learned pattern, hence 'inappropriate').

Later studies involved a more complex ANN (a back-propagation network with a recurrent layer) focusing on developmentally disturbed speech processing as a source of auditory hallucinations (Hoffman and McGlashan, 1997; Hoffman et al., 1995). Normal human speech is a complex task given the high level of acoustic ambiguity. Normal perception of a word depends not only on acoustic input corresponding to the word itself but also on previously perceived words and intrinsic knowledge of how words are sequenced into larger messages. The process involves verbal working memory that uses expectations based on prior words and phrases.
This ANN used featured a verbal working memory with linguistic expectations built up from prior exposure to a training set of grammatical correct sentences. The training set consisted of sentences like "Jane kiss girl" or "Cop chase man". The ANN was programmed to process degraded input signals into identifiable words. The ANN was posited to 'hallucinate' when it recognized words during periods of input silence.
Pruning of connections initially improved detection rates of phonetically degraded words. Higher levels of pruning however were associated with progressive impairment in word recognition and the emergence of hallucinations.

Turning to the visual metaphor to describe what is happening (see figure 4) we should conceptualize the state space and its 'attractor landscape' as to a certain extent dynamical itself.
Figure 4 (top) depicts the starting situation in which linguistic concepts are depicted as attractors. The basins of attraction influence the trajectory through the state space as we have described for the simple ANN.
Figure 4 (center) shows the system 'hearing' a degraded acoustic stimulus ('J?ne'). Due to the attractor dynamics (these depent on the basin of attraction of 'Jane' as well as the state the system is in) the network might succeed in recognizing 'J?ne' as 'Jane'.
Perceiving a word (like "Jane") will change basins of attraction (depending on prior expectations, i.e. the training set of sentences), and as a consequence the most likely trajectories that subsequently will be followed. Thus perceiving "Jane" will enlarge the basin of attraction of "kiss" and other words the ANN has learned to associate with "Jane" and at the same time shrink the basin of attraction of concepts associated with, for instance, "cop". This situation is shown in figure 4 (bottom).

Figure 3



Pathology shown as disturbances in state space (see text).
Figure 3, top: 'normally' sized attractors (cf figure 2, bottom).
Figure 3, bottom: aberrant neurodevelopmental processes (i.e. excessive pruning) give rise to a spurious attractor: situations and concepts become malclassified (interpreted after Hoffman (1987)). This may lead to the subjective experience of delusions or hallucinations

As a result, after perceiving "Jane", the most likely trajectories will lead to regions of state space associated with "Jane" (like "kiss" or whatever other words the ANN due to its prior expectations expects to follow "Jane").
The pruning process facilitates this process by improving the capacity to recognize degraded input, probably by enlarging basins of attraction and facilitating common trajectories (like "Jane" ▸ "kiss" ▸ "girl"). However, the drawback is that after a certain point recognizing becomes 'too good', and the system starts off to follow trajectories without any input: the system starts 'hallucinating'.

Using the metaphor (2): dopamine and schizophrenia

There is considerable evidence suggesting a role for dopamine involvement in schizophrenia, particularly motivated by the efficacy of antipsychotic medication, which derive their therapeutic effects from dopamine D2 antagonism (Kapur and Seeman, 2001). Usually, research focusing on the role of dopamine in schizophrenia adresses two aspects of dopamine neuromodulation, namely (1) the role of exess dopaminergic stimulation in delusions, and (2) the role of low levels of prefrontal dopamine in working memory. The metaphor we present should be able to capture aspects of these roles of dopamine in schizophrenia.

General aspects of the role of dopamine in neural system dynamics.

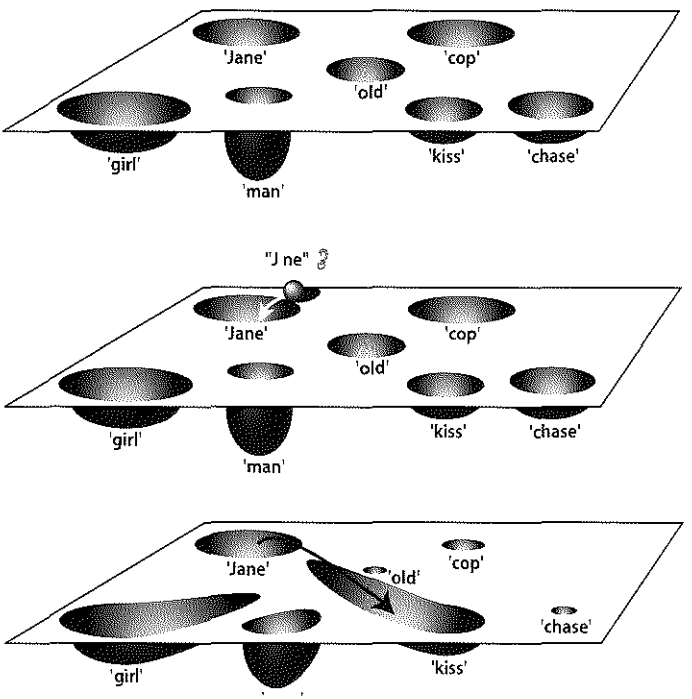
An increasing body of research suggests that dopamine is a modulator of the signal-to-noise ratio of the neural system. One of the first to suggest this role were Servan-Schreiber et al. (1990). In the metaphor presented, a crucial role is played by the attractor concept. The way in which information is processed is influenced by the shape and depth of the attractors and by the ease in which the system can settle into an attractor and perform transitions from one attractor into another. Changing the signal-to-noise ratio, in terms of 'information processing with attractors', comes down to changing the depth of the attractors and changing the magnitude of the basins of attraction.Low levels of dopamine are associated with a low signal-to-noise ratio and in attractors terms this is equivalent to shallow attractors with large basins of attractions. In this situation frequent transitions from one attractor to another can take place (see figure 5, bottom). Servan-Schreiber at al. (1990) suggested that this is the psychological equivalent of relatively unfocused, associative thinking.
High levels of dopamine are associated with a large signal-to-noise ratio and this comes down to the existence of a limited number of deep atractors with relatively large basins of attraction which dominate the information processing of the neural system (figure 5, top). Such a situation will be present in a situation of focused attention.

Excess dopaminergic stimulation in delusions.

Recently, Kapur (2003) presented an heuristic framework on the role of dopamine in delusions. In Kapur's model a central role of dopamine is to mediate the salience of environmental events. A (striatal) hyperdopaminergic state would lead, at a psychological level, to the aberrant assignment of salience to one's experience. Aberrant, because salience is assigned to experiences which (in fact) have none or only minor salience. Subsequently, delusions are the cognitive effort to make sense of aberrantly salient experiences.

Kapur (2003) provides a psychological description of the role of dopamine. Within

Figure 4



Dynamical aspects of the 'attractor landscape' (see also text).

Figure 4, top: meaningful acoustical signals have created an 'attractor landscape'.

Figure 4, center: recognizing a (degraded) signal, ic 'Jane'.

Figure 4, bottom: after recognizing 'Jane' the basins of attraction of concepts associated with 'Jane' (acoustical signals, likely to follow 'Jane') temporarily increase, facilitating the capacity to recognize subsequent input (i.e. the most likely words to follow Jane).

the framework we presented (based on 'information-processing with attractors') an abormal hyperdopaminergic state is equivalent to the emergence of an attractor with a large basin of attraction which dominates the striatal information processing of the neural system. This dominance translates itself in 'aberrant salience'. This is the situation depicted in figure 5 (top).

Low prefrontal dopamine in working memory.

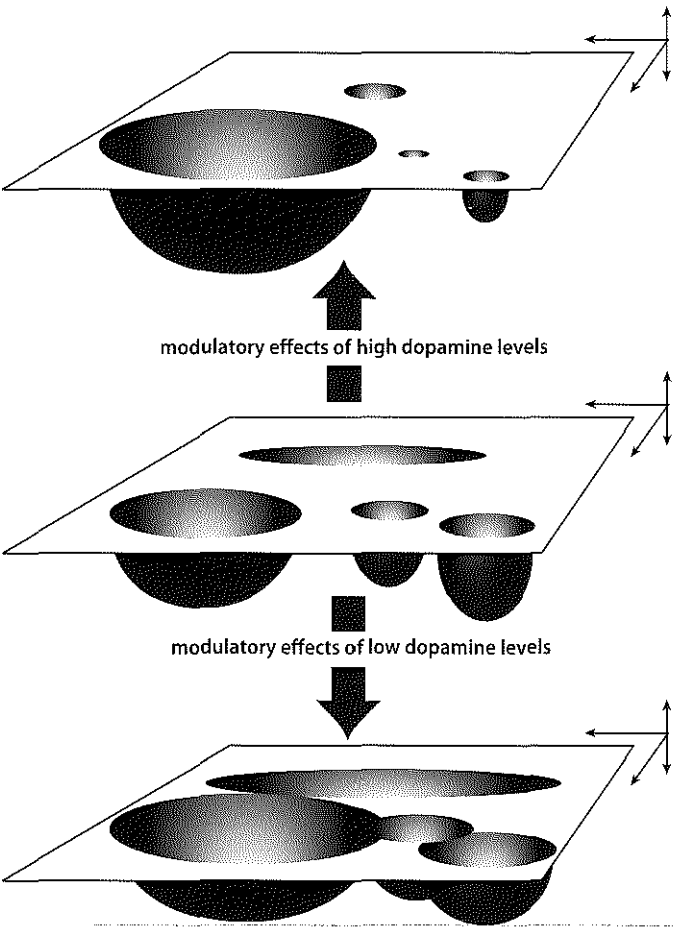
Whereas hyperdopaminergic states in the striatum have been associated with delusions, reduced dopaminergic activity in the prefronal cortex has been associated with disorders of working memory (Cohen and Servan-Schreiber, 1992; Winterer and Weinberger, 2004). Usually it is assumed that the prefrontal cortex is responsible for representing and maintaining task-relevant information, an idea related to the ideas of Goldman-Rakic concerning the role of the prefrontal cortex in working memory. Dopamine is involved in maintaining task-relevant information in the prefrontal cortex (Cohen and Servan-Schreiber, 1992; Cohen and Servan-Schreiber, 1993).

Schizophrenia patients show several deficits in psychological tasks, specifically in tasks that place a demand on the active maintenance of internal representations of the context of the task. Cohen and Servan-Schreiber (1992) concluded that performance deficits in schizophrenia are due to a degradation in the internal representation required as context for processing stimuli. In a state of reduced prefrontal dopaminergic activity noise interferes with the ability of the system to maintain a representation of task-relevant information. In the framework we presented this comes down to the situation depicted in figure 5 (bottom) where the system is liable to transitions to other attractors due to small internal or external perturbations of the system. The resulting inability of the system to maintain a stable attractor state over time shows itself at a psychological level as the observed disorders of working memory. More generally, this will lead to an uncontrolled spread of activation and an increase of spontaneous neuronal activity, hence, to a decreased signal-to-noise ratio (Winterer and Weinberger, 2004).

More recent insights have focused on the differential effects of D1- and D2-dopamine receptor stimulation in the prefrontal cortex (Barch, 2006; Durstewitz, 2006). Durstewitz (2006) argues, partly based on neural network simulations (Durstewitz et al., 2000; Durstewitz et al., 2002), that D1-stimulation could increase the energy barrier between different network states, making it harder to switch from one state to the other. Due to this, active memory states become more robust to distractors and interference. In dynamical terms, D1-induced changes come down to a deepening and widening of the basins of attraction of prefrontal cortex attractor states (figure 5, top).

The combined effects of D1- and D2-receptor stimulation cause dopamine to drive

Figure 5



Modulatory effects of dopamine shown as changes in state space (interpreted after Servan-Schreiber et al. (1990))(see also text)

Figure 5, top: increasing levels of dopamine augment the signal-to-noise ratio: the basin of the attractor associated with the most salient signal increases while the basins of attraction of the other attractors decrease. Note also the increase respectively decrease of the depth of the attractors reflecting a increased respectively decreased tendency of the system to stay in the attractor.

Figure 5, bottom: decreasing levels of dopamine reduce the signal-to-noise ratio: the basins of attraction and the depth of the attractors become more similar resulting in an enhanced tendency of the system to make transitions from one attractor to another.

neural networks through a sequence of phases with opposing characteristics. In the case of prefrontal cortex this may consist of an initial phase where basins of attraction are flattened out (a D2-dominated situation making networks highly susceptible to newly incoming information -see figure 5, bottom), and a late phase (D1-dominated) where network activity is focused on a few relevant states (figure 5, top). Thus, this mechanism is used to 'protect' task relevant information against the interfering, and cumulative effects of noise over time (Winterer and Weinberger, 2004).

The metaphor as part of a broader information processing concept

The terms we used are derived from the theory on complex dynamical systems. In this concept the brain is seen as a system that is in constant interaction with its environment, possessing a great number of preferential states in which the system sequentially relaxes (Hoffman, 1987; Freeman, 1991). These preferential states are attractors of the systems' dynamics. They are the result of the physical structure of the system and past experiences which have influenced the system. The outside world influences the present situation of the system persistently, causing the system to temporarily relax in a state most fit to this ongoing process of mutual influence.
Homeostatic mechanisms produce stability as well as phase transitions, making the healthy system adaptive to its environment. Psychopathology arises when the system becomes either too stable or too unstable, whether by (acute or chronic) external influences or by limitations in the system itself. In most pathological circumstances, there will be a constant interaction between the system and the environment, resulting in a more or less pathological equilibrium.
It has been suggested to name these aspects of neural functioning which focuses on the system properties of neural ensembles *neurodynamics* (Freeman, 2000; Hoffman, 1987; Hoffman and McGlashan, 1993; Erdi, 1993).
Our approach has been to simplify and visualize these processes, in order to produce an intuitive understanding.

Discussion

Ideally, medical thinking depends on the understanding of both physical reality and the availability of a metaconcept describing reality in a more abstract way. For instance, in cancer research, the metaconcept is 'regulation of cell proliferation', with 'misregulation' as its associated pathological state (Andreasen, 2000). The

physical reality is the way in which the bio-physical apparatus of genes and proteins perform regulation of cell proliferation.
The line of reasoning introduced in this paper tries to approach this situation. The metaconcept defined is based on information processing theory (namely, information processing with attractors (Wang and Blum, 1995; Hertz, 1995)), the neurobiological process described (pruning of the dendritic tree) is functionally related with the metaconcept, (pruning optimizes information processing), pathology is understood as arising from disturbances of this neurobiological process (excessive pruning) and the associated pathological phenomena are expressed in terms of the metaconceptual framework (emergence of pathological attractors). Moreover, neuromodulatory influences (dopamine) can be described in terms of the metaconcepts as well.
In psychiatry, there is a large conceptual gap between empirical research and theory which -especially in brain research- must be bridged by an interdisciplinary formal language (Bender et al., 2006). In this respect, 'systems science' or 'computational neuroscience' offers a conceptual and methodological basis for integrating the various data within a sophisticated system framework.
In this context, we think that some 'metaphorization' of psychological categories into cybernetic language might be useful to bridge this gap.
Throughout history, the working of the brain has been compared to that of a clock-work, a steam engine and, most recently, to a digital computer or a hologram (Draaisma, 1995; Draaisma, 2001). The metaphor presented in this article is derived from dynamical systems theory and is a visual representation of the mathematical concepts underlining this theory.
We expect that the conceptual framework and metaphor described in this article offers a tool to understand psychiatric phenomena from a systems perspective. At the same time, we are dealing with a line of thought which is related with the actual behavior of neural systems, so the terminology used, though of an abstract nature, has a close relationship with the processes in the brain. Moreover, it facilitates to appreciate relationships between related phenomena in different fields of science, describing properties and phenomena of neural systems as more specific instances of general principles underlying behavior of all kinds of complex dynamical systems. It therefore satisfies the scientific endeavour to recognize specific behavior (like the behavior of neural sytems) as instances of more general behavior (like the behavior of complex systems) and to construct a conceptual framework describing this general behavior.
Finally, it should be borne in mind that theories and models need not be 'explanations' of observed phenomena but can also be useful as exploratory 'heuristics' (Bender et al., 2006). The presented model might serve this purpose.

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Chapter 3

Schizophrenia-associated neural growth factors in peripheral blood. A review

European Neuropsychopharmacology (2006) 16: 469-480

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Abstract

In this paper we review the findings on neural growth factors in the peripheral blood of schizophrenia patients. The studies we review provide evidence for the fact that in schizophrenia the levels of growth factors in peripheral blood are disturbed. The most robust results (7 studies) are reported for S100B protein, which seems to be elevated in acute psychosis and in patients with predominant negative symptoms.

We conclude that there are aberrant levels of growth factors in peripheral blood in schizophrenia patients, probably most notably in patients with negative symptoms. Large-scale longitudinal multivariate studies, investigating the levels of several growth factors at the same time might give insight in etiological processes and identify clinically useful subsets of patients within the heterogeneous schizophrenia sample.

Introduction

The term "neural growth factors" denotes a heterogeneous group of neurotrophic agents which participate in a wide range of actions such as proliferation, survival and differentiation of precursor cells, apoptosis, axon growth, axon collateralization, the synthesis of peptides, transmitter enzymes and calcium-binding proteins, synaptic organization, dendritic arborization and sprouting and myelination (Durany and Thome, 2004). In the light of the neurodevelopmental hypothesis of schizophrenia (Raedler et al., 1998) it can be expected that the expression of growth factors is altered in schizophrenia, either primary or secondary to the disease. Indeed, many studies report alterations in the expression of neurotrophic factors in post-mortem brains of schizophrenic patients (Durany and Thome, 2004).
Some neural growth factors can be detected in peripheral blood either because they cross the blood-brain barrier or because they are produced in peripheral tissue. This opens up the possibility to use peripheral measures as an adjunct to post-mortem studies and investigate the disease in earlier stages as well as its temporal dynamics. In this paper we review the findings on neural growth factors in schizophrenia in peripheral blood.

Methods

We performed a pubmed search with the search term *schizophrenia* (title), combined with the terms *serum*, *plasma*, *growth factor* *neurotroph*, *peripheral marker*, *peripheral blood* (title). In addition the reference sections of identified articles were screened (last search: june 2005).

Results

Table 1 shows 24 studies examining the level of growth factors in the peripheral blood of schizophrenia patients. Combined, 23 studies reported on 1124 individuals with schizophrenia. One older study (Perez-Polo et al., 1978) examined 58 subjects but did not specify the fraction of patients nor the male/female ratio. Most studies were of a case-control design. Some studies investigated correlations between growth factor levels and variables defining subsets within the patient samples, such as duration of

illness, medication status, drug abuse, or symptom dimensions. A limited number of studies performed a follow-up measurement, either after treatment as usual, or as part of a clinical trial. The growth factors investigated were S100B protein, brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), glutamate, basic Fibroblast Growth Factor (bFGF), and Epidermal Growth Factor (EGF).

S100B (7 studies)

S100B is a calcium-binding protein, which is produced primarily by astrocytes. In vitro experiments showed that S100B is involved in the regulation of energy metabolism in brain cells. It modulates the proliferation and differentiation of neurons and glia (Rothermundt et al., 2004a), maybe through its action on the aggregation of microtubuli associated protein. S100B exerts a proliferative effect as long as it is kept within the cells at physiological levels. Once it is released, nanomolecular concentrations appear to have neuroprotective effects, whereas micromolecular concentrations produce neurodegenerative- or apoptosis-inducing effects. In vivo studies with mice under- or overexpressing S100B suggest that this protein is involved in cognitive functions such as spatial and nonspatial memory and learning (Nishiyama et al., 2002). There is evidence that serotonin is involved in the regulation of S100B release via the 5-HT1a receptor (Whitaker-Azmitia and Azmitia, 1994).

BDNF (6 studies)

BDNF regulates neuronal survival, migration, morphological and biochemical differentiation and synaptic function (Huang and Reichardt, 2001). BDNF is expressed at low levels in the rodent cortex during prenatal development, expression rises during the postnatal period and BDNF is the most abundant neurotrophin in both the adult rodent and human cortex (Gorski et al., 2003). In the CNS BDNF, among other neurotrophins, has been shown to increase the length and the complexity of dendrites of pyramidal neurons. Some effects depend on co-regulation with other neurotrophic factors. In mice overexpressing BDNF in sympathetic neurons, increased numbers of synapses were observed, whereas BDNF knock-out mice showed a decreased number of synapses (Bibel and Barde, 2000). Gorski et al. (2003) reported that BDNF is required for the maintenance of cortical dendrites. More specifically, BDNF appears to support the survival of the dendritic structure that is generated earlier in the development through BDNF-independent mechanisms.

Glutamate (6 studies)

There is extensive evidence for the involvement of the excitatory amino acid gluta-

Table 1: Growth Factor examined

Growth Factor examined	Reference	Design and subject characteristics CC: case control; CT: clinical trial MA: mean age; M/F: # male vs # female DUP: duration untreated psychosis DUI: duration illness	Measurement technique and type of sample	Results nsd: non significant difference
S100B	Wiesmann et al. 1999	CC: 20 patients (M/F: 8/12; MA: 35.710.7 y; DUI: 3 months-19 years; various antipsychotics) vs 20 controls age- and gender matched	Microtiterbased immunofluormetric sandwich assay Lower detection limit: 0.015g/L Plasma	S100B increased in patients (p<=0.001) No correlation with age at onset or DUI; Trend toward higher levels in patients with residual symptoms and with long-term continuous psychotic symptoms
S100B	Gattaz et al. 2000	CC: 23 patients (M/F: 16/7; MA: 369 y; DUI: 177 y; all patients used antipsychotics (mean chlorpromazine eq: 710340 mg/day) -16 patients used clozapine) vs 23 controls age- and gender matched (MA: 4417 y)	Immunolumino-metric assay, Lower detection limit: 0.02 g/L Plasma	S100B decreased in patients (p=0.003) Remark: this study used citrated blood which may disturb the assay
S100B	Lara et al. 2001	CC: 20 patients (M/F: 13/7; MA: 318 y; DUI: 97 y; all medication free > 1 week; no recent (4 months) depot antipsychotics) vs 20 controls age- and gender matched	Immunolumino-metric assay, Lower detection limit: 0.02 g/L Serum	**S100B increased in patients** (p=0.014) Trends: association between S100B and medication -free time and negative correlation between S100B and DUI No correlation with PANSS total or subscales.

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Table 1: *(continued)*

Growth Factor examined	Reference	Design and subject characteristics CC: case control; CT: clinical trial MA: mean age; M/F: # male vs # female DUP: duration untreated psychosis DUI: duration illness	Measurement technique and type of sample	Results nsd: non significant difference
S100B	Rothermundt et al. 2001	CC; CT: measurement at baseline and after 6 weeks of open-label treatment. 26 patients (M/F: 10/16; MA: 3712.9 y; DUI: 9.9610.35) all acute episode of paranoid type schizophrenia; all patients medication free > 6 months at study entry: 7 drug-naive) vs 26 controls (age- gender matched)	Immunofluormetric sandwich assay Lower detection limit: 0.015 g/L Plasma	**S100B increased in patients at T0** (p=0.001) **Patients T6 vs controls: nsd** A subgroup of 15 patients (S100B > 2 standard deviations of the controls mean at T0) had significantly higher PANSS negative scores than the other patients. A subgroup of 7 patients (S100B > 2 standard deviations of the controls mean at T6) showed significantly less improvement in PANSS negative scores (p=0.01)
S100B	Schroeter et al. 2003	CC: 30 patients (M/F: 14/16; MA: 34.8 12.4 y; DUI: 8.9 8.8 y; 16 medicated (7 patients received typical ap, 4 atypical ap, and 5 patients both typical and atypical ap) 14 unmedicated vs 15 controls (M/F: 8/7; MA: 34.25.6)	Immunolumino-metric assay Lower detection limit: 0.1 g/L, serum	**S100B increased** in medicated pt's vs unmedicated pt's (p<0.005) **S100B increased** in medicated pt's vs controls (p<0.001) **S100B nsd** in unmedicated patients vs controls **S100B increased** in deficit syndrome patients vs non-deficit syndrome patients

				(p<0.05)
S100B	Rothermundt et al. 2004b	CC; CT: all previous ap's were discontinued 1 week prior to T0; then randomised controlled trial comparing risperidone (1-6 mg) vs flupenthixol (4-12 mg; measurements after 12 and 24 weeks) 98 patients M/F: 56/42; MA: 42.111.1 y; DUI: all > 2 y; 70.4% typical drugs,19.4% atypical drugs and 10.2% drug-free. All drugs were used up to 1 week before the study; This group does not contain the 26 patients investigated in Rothermundt 2001) vs 98 controls: (age- and gender matched; MA: 42.111.1)	Immunolumino-metric assay Lower detection limit: 0.02 g/L, Serum	**S100B increased in patients** at all three time points compared to the controls (p<0.0001, for the three time points) **Nsd** between the two treatment groups Significant correlation between the S100B levels and the PANSS negative subscale at T0
S100B	Schmitt et al. 2005	CC: 41 patients (chronic, long-term hospitalized) (M/F: 24/17; MA: 63.37.0 y; DUI: 35.311.4 y; vs 23 controls (M/F: 15/8; MA: 64.59.8 y)	Immunolumino-metric assay Lower detection limit: 0.02 g/L, Serum	**S100B increased in patients** (p<0.001) Significant correlation between S100B and age and significant negative correlation between S100B and deficit symptoms
Brain-derived Neurotrophic Factor	Toyooka et al. 2002	CC: two separate comparison groups, one for comparison of serum BDNF levels, one for comparison of whole blood BDNF levels : Group I: 34 chronic schizophrenia patients (MA: 48.614 y) vs 35 controls (MA: 45.611.3 y)	Two site EIA (measurements in serum and in whole blood) Group I: serum Group II: whole blood	**BDNF decreased in patients (serum)** (p<0.005) **Nsd in whole blood** (BDNF concentrations in whole blood not specified)

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Table 1: *(continued)*

Growth Factor examined	Reference	Design and subject characteristics CC: case control; CT: clinical trial MA: mean age; M/F: # male vs # female DUP: duration untreated psychosis DUI: duration illness	Measurement technique and type of sample	Results nsd: non significant difference
		Group II: 34 chronic schizophrenia patients (MA: 52.911.3 y) vs 27 controls (MA: 3811.3 y)		The authors conclude that the lower serum BDNF in patients is not due to reduced production but to decreased release from platelets.
Brain-derived Neurotrophic Factor	Pirildar et al. 2004	CC with a follow-up measurement after 6 weeks of treatment: 22 patients (MA: 27.89.5 y; DUI: 15.213 months) vs 22 controls (age and gender matched)	Commercial ELISA (Promega Corporation) Serum	**BDNF decreased in patients vs controls** ($p<0.001$) **Nsd between post-treatment and baseline** (NB nonreponders to the treatment (3) were excluded from the posttreatment analysis)
Brain-derived Neurotrophic Factor	Shimizu et al. 2003	CC: 40 patients: 15 drug naive (M/F: 7/8; MA: 34.716 y; DUI: 1.091.36 y) 25 medicated (M/F: 13/12; MA: 3613.2 y; DUI: 14.19.87 y) vs 40 controls (M/F:20/20; MA: 36.514.6)	EMAX Immunoassay (Promega Corporation) Serum	**All comparisons nsd**
Brain-derived Neurotrophic Factor	Tan et al. 2004	CC: 40 patients: 15 drug naive (M/F: 7/8; MA: 34.716 y; DUI: 1.091.36 y) 25 medicated (M/F: 13/12; MA: 3613.2 y; DUI: 14.19.87 y) vs 40 controls (M/F:20/20; MA: 36.514.6)	Commercially available ELISA (Banding Biomedical Inc, Beijing, China) Plasma	**BDNF decreased in TD pos patients vs TD neg patients** ($p=0.042$) **BDNF decreased in TD pos patients vs controls** ($p<0.001$) **BDNF decreased in TD neg patients vs controls** ($p=0.002$)

Brain-derived Neurotrophic Factor	Tan et al. 2004	CC: 81 patients: (M/F: 63/18; MA: 48.15.8 y; DUI: 22.67.7 y; all patients used antipsychotic medication) vs 45 controls (M/F:34/11; MA: 45.64.5)	Commercially available ELISA (Banding Biomedical Inc, Beijing, China) Serum	**BDNF decreased in patients vs controls** ($p<0.001$) Significant negative correlation between BDNF levels and PANSS negative subscale ($p=0.005$)
Brain-derived Neurotrophic Factor	Jockers-Scherubl, et al., 2004	CC with both patients and controls stratified for cannabis abuse and multiple drug abuse: 157 patients : 102 cannabis neg patients(M/F: 50/52; MA: 33.3 y) 35 cannabis pos patients (M/F: 28/7; MA: 28.1 y)20 multiple abuse patients (M/F: 12/8; MA: 30.5 y) vs 72 controls 61 cannabis neg ctrls (M/F: 28/33; MA: 32.3 y) 11 cannabis pos ctrls (M/F: 9/2; MA: 29.6 y)	Commercially available ELISA (Promega Corporation) Serum	BDNF increased in patients,cann+ vs patients,cann- ($p<0.0003$) BDNF increased in patients,cann+ vs controls,cann- ($p<0.0062$) BDNF nsd in patients,cann+ vs controls, cann+ BDNF nsd in patients,cann- vs controls, cann- BDNF nsd in patients,cann- vs controls, cann+
Glutamate	Macciardi et al. 1990	CC: 43 patients (M/F: 15/28) 32 controls (M/F: 16/16; age matched	Ion-exchange chromatography	**Glutamate increased in patients** ($p<0.0000$)
Glutamate	Altamura et al. 1993	CC: 13 patients (M/F: 10/3; MA: 32.213.4) vs 9 controls (M/F: 7/2; MA: 36.58.3)	Liquid chromatography with flurescence spectrometry Plasma	**Glutamate probably increased in patients** (significance not specified): Patients: 47.1618.18 nmol/ml Controls: 21.587.61 nmol/ml
Glutamate	Evins et al. 1997	CT: patients treated with conventional antipsychotics were switched to clozapine. Measurements at baseline and after stable clozapine dose was reached (85 months). No comparison with a healthy control group	Quantitative ion exchange column Serum	**Glutamate increased after clozapine treatment** ($p=0.03$, one tailed)

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Table 1: *(continued)*

Growth Factor examined	Reference	Design and subject characteristics CC: case control; CT: clinical trial MA: mean age; M/F: # male vs # female DUP: duration untreated psychosis DUI: duration illness	Measurement technique and type of sample	Results nsd: non significant difference
		7 Patients (M/F: 6/1; mean dose conventional antipsychotics: 629474 chlorpromazine eq/d; mean dose clozapine 39393 mg/d)		
Glutamate	Tortorella et al. 2001	CC study at baseline in mediaction free patients followed by 12 weeks of treatment with clozapinel antipsychotics with amino acid (incl glutamate) measurements at 8 and 12 weeks. 11 patients (MA: 24.514.7 y; M/F: 6/4; DUI 7.64.7 y) vs 11 controls (age- and gender matched)	Liquid chromatography with flurescence spectrometry Serum	**Glutamate increased at baseline compared to controls** ($p<0.001$) **Glutamate decreased progressively during clozapine treatment** ($p<0.05$)
Glutamate	Goff et al. 2003	CT: patients treated with conventional antipsychotics were switched to olanzapine. Measurements at baseline and after 8 weeks. No comparison with a healthy control group. 17 Patients (M/F:13/4; other characteristics not specified)	O-phtaldehyde precolumn derivatization reverse-phase carbon-18 high-performance liquid chromatography separation Plasma	**Glutamate increased after 8 weeks of olanzapine treatment** ($p=0.003$)
Glutamate	Van Der Heijden et al. 2004	CC study at baseline followed by 12 weeks of treatment with atypical	Reversed phase HPLC after automated precolumn	**Glutamate increased in patients at baseline** ($p<0.01$)

		antipsychotics with glutamate measurements at 3, 6 and 14 weeks. 66 patients (MA: 32.513.4 y; M/F: 42/24) vs 73 controls (age- and gender matched)	deriva-tization with o-phthaldialdehyde Plasma	**Significant increase in glutamate during treatment.** ($p<0.05$)
basic Fibroblast Growth Factor	Hashimoto et al. 2003	CC: 40 patients: 15 drug-naive(M/F: 7/8; MA: 34.716.0; DUI: 1.091.36 y) 25 medicated (M/F: 13/12; MA: 36.013.2; DUI: 14.19.87 y; various antipsychotics, chlorpromazine eq: 727412) vs 40 controls (M/F: 20/20; MA: 36.514.6 y)	Commercial quantitative sandwich enzyme immunoassay (Quantikine) Lower detection limit 0.5 pg/ml Serum	**bFGF increased in medicated patients** ($p=0.003$) **drug-naive patients: nsd significant correlation between bFGF and the BPRS negative subscale**
Epidermal Growth Factor	Futamura et al. 2002	CC: 51 patients: 45 chronic (M/F:22/23; MA: 47.09.7) 6 drug free (4 drug-naive, 2 drug free>14 months; M/F: 2/4; MA: 32.212.4) vs 45 and 14 controls (2 groups, age- and gender matched to the 2 patient subgroups)	sandwich enzyme immunoassay serum	**EGF decreased in both chronic patients** ($p<0.001$) **and drug-free patients** ($p<0.05$)

mate in schizophrenia. Antagonism of the glutamatergic NMDA receptor complex produces behavioral and cognitive deficits resembling the symptoms seen in schizophrenia and long-term abuse of phencyclidine, a NMDA receptor antagonist, produces a schizophrenia-like syndrome (for a review of these and other findings, see Goff and Coyle (2001)).

The glutamatergic system could also play a role in neuronal migration, neurite outgrowth, synaptogenesis and apoptosis (Kerwin, 1993). Recently, it has been argued that glutamate which is released by astrocytes contributes to synaptic plasticity (Mazzanti et al., 2001). In vitro studies show that high glutamate levels decrease S100B secretion in astrocytes (Goncalves et al., 2002).

NGF (4 studies)

NGF plays an important role in the development and maintenance of the sensory and sympathetic nervous system and the cholinergic function of the central nervous system, where it is mainly produced in the hippocampus, cortex and olfactory bulb. Changes of NGF concentrations are implicated in alterations of cognitive functioning following brain damage and aging, and NGF seems to play a crucial role in the plasticity of central nervous system neurons (Lang et al., 2004). Cells of the immune and endocrine system are responsive to NGF (Levi-Montalcini et al., 1995) and NGF has been found to increase in mice with a subordinate social status. Recently, NGF has been found to modulate the receptivity of axons to myelination (Chan et al., 2004).

bFGF (1 study)

bFGF is the most abundant member of the FGF group of cytokines. It has been shown that bFGF is generally expressed throughout the developing central nervous system and in platelets (Molteni et al., 2001). bFGF promotes the survival and neurite growth of brain neurons. bFGF has been implicated in neuro- and gliogenesis during development and adulthood (Wagner et al., 1999).

EGF (1 study)

EGF receptors are widely present in both the developing and the mature central nervous system. EGF is a protein that regulates the development of dopaminergic neurons as well as monoamine metabolism. It enhances stem cell proliferation and neural differentiation and is involved in neural plasticity (Futamura et al., 2002)

The relationship between peripheral and central nervous system (CNS) levels of neural growth factors.

The relationship between peripheral levels and CNS involvement can be explored by investigating the relationship between cerebrospinal fluid (CSF) and peripheral blood levels. Another possibility is to investigate post-mortem brain levels (see Krystal et al. (2003) for a review). Attempts to measure levels of glutamate in the living brain with MRS are probably unreliable due to technical limitations (Deicken et al., 2000).
The findings on brain and CSF levels of the growth factors reported in this review and their possible relationship with peripheral levels are described in table 2 and briefly outlined hereafter.

For S100B, a positive correlation between CSF and serum S100B levels in healthy controls have been reported (Missler and Wiesmann, 1995). In neurotrauma patients a clear time-dependent relation exists between changes in CSF S100B and subsequent changes in peripheral blood S100B has been reported (Reiber, 2003). In schizophrenia patients S100B levels in CSF have found to be increased (Rothermundt et al., 2004c), and correlated with serum levels.
Disturbed levels of BDNF have been reported in post-mortem brains of schizophrenia patients (Takahashi et al., 2000; Durany et al., 2001; Iritani et al., 2003; Weickert et al., 2003). NGF levels have reported to be unchanged (Takahashi et al., 2000). There are to our best knowledge no studies on CSF levels of BDNF and NGF in schizophrenia.
For glutamate, a correlation between CSF and peripheral blood has been established (Alfredson et al., 1988; Castillo et al., 1997). Findings on CSF glutamate levels in schizophrenia have been inconsistent: decreased (Kim et al., 1980), increased (Gattaz et al., 1982) as well as non-significant differences (Perry, 1982; Korpi et al., 1987) have been reported, reflecting the inconsistent findings in peripheral blood also found in this review.

Discussion

Although the number of studies identified is relatively small they provide convincing evidence for the fact that in schizophrenia the levels of growth factors in peripheral blood are disturbed. The most robust results are reported for S100B pro-

Table 2: The relationship between peripheral and CNS levels of neural growth factors

Neural growth factor	Findings in the brain in schizophrenia patients	Findings in CSF in schizophrenia patients	Relationship between CSF and peripheral findings (in both schizophrenia patients and other subjects)	General conclusion inferred from the combined findings
S100B	No findings reported	Increased CSF S100B levels (Rothermundt et al. 2004)	CSF S100B levels and peripheral blood levels correlate in healthy controls (Missler and Wiesmann 1995) The CSF/serum S100B ratio in patients is comparable to that of healthy controls. (Rothermundt et al. 2004)	Suggestive evidence that peripheral S100B levels reflect CNS (both brain and CSF) levels (Rothermundt et al. 2004)
BDNF	Elevated BDNF in anterior cingulate cortex and hippocampus of patients (Takahashi et al. 2000) Decreased BDNF in cortical areas and hippocampus of patients (Durany et al. 2001; Iritani et al. 2003). Reduced BDNF in dorsolateral prefrontal cortex of patients (Weickert, et al. 2003)	No findings reported	No relationship between BDNF CSF and plasma levels in children with encephalitis (Chiaretti et al. 2004)	Unclear relationship between peripheral and CNS BDNF levels
NGF	No change in NGF levels in any brain region in patients vs controls (Takahashi ea 2000, ref in Shoval)	No findings reported	No relationship between NGF CSF and plasma levels in children with encephalitis (Chiaretti et al. 2004)	Unclear relationship between peripheral and CNS NGF levels
glutamate	Extensive evidence for disturbed glutamate meta-	Decreased levels of CSF glutamate (Kim et al. 1980)	Positive correlation between CSF and plasma glutamate in	Some evidence for a relationship between peripheral

bolism in schizophrenia patients (reviewed in Krystal et al. 2003) Technical difficulties limit reliable measurement of MRS glutamate (Deicken et al. (2000)	Increased levels of CSF glutamate in medicated, but not in non-medicated patients (Gattaz et al. 1982). No significant difference in CSF glutamate (Perry et al. 1982; Korpi et al. 1987)	healthy volunteers (Alfredson et al. 1988) Strong linear correlation between CSF and plasma in stroke (Castillo et al. 1997)	and CSF glutamate levels Contradicting findings on CSF glutamate levels in schizophrenia

tein, which seems to be elevated in acute psychosis and in patients with predominant negative symptoms (Rothermundt et al., 2001; Schroeter et al., 2003). There is some evidence that BDNF and NGF are decreased and glutamate is increased in schizophrenia and, as for S100B, also for NGF and glutamate a relationship with negative symptoms has been reported. However, in spite of the large interest for the role of glutamate in schizophrenia there are surprisingly few studies investigating glutamate levels in peripheral blood. Reports on EGF and bFGF are interesting but need to be replicated.

At present, there are no sufficient data on the development in time of growth factors. S100B might decrease with illness progression, and seems to be more of a state marker for the early psychotic phase than a trait marker. It is unknown whether S100B is raised in the prodromal phase preceding the onset of frank psychosis; if so, S100B might serve as a marker aiding early recognition efforts.

Although treatment changes the reported levels of some growth factors, it is unclear whether these changes are related to medication effects or to the development of the disease. Also, no clear relations yet emerge between S100B levels and duration of illness, duration of untreated psychosis, type of antipsychotics or cumulative antipsychotic usage.

Two studies by the same group (Jockers-Scherubl et al., 2003; Jockers-Scherubl et al., 2005) focus on drug abuse in schizophrenia and report very disturbed levels of BDNF and NGF in patients with cannabis abuse and even more so in multiple drug abuse patients. However, Jockers-Scherubl and co-workers do not report aberrant levels of BDNF and NGF in patients without drug abuse, as reported by several others groups (see table 1).

With the present knowledge it is difficult to infer etiological hypotheses from the data. It is unclear whether aberrant growth factor levels are the result of primary or secundary disease processes, or of compensatory mechanisms. Imbalance between different growth factors, abnormalities in the control and timing of their production, secretion and signaling pathways may play a role (Shoval and Weizman, 2005). Longitudinal multivariate studies, investigating the levels of several growth factors at the same time are needed, but have yet not been performed as far as we know. Also, no studies have been performed combining peripheral measurements of growth factors with neuro-imaging or genetic data.
It should furthermore be emphasized that peripheral levels of neural growth factors do not necesssarily reflect CNS levels. Peripheral levels may be influenced by aberrant peripheral production, aberrant blood-brain-barrier function or altered CSF flow (Reiber, 2003) with subsequently altered patterns of absorption and distribution of proteins. Finally, in schizophrenia subtle metabolic disturbances may exist in otherwise normally functioning peripheral tissue and this may influence peripheral levels. The only study that directly adresses this question is the one by Toyooka et al. (2002), who conclude that low serum BDNF in patients is due to decreased platelet release. The results presented in table 2 suggest the most convincing relationship between central and peripheral levels for S100B protein. Based on their S100B findings in CSF and serum Rothermundt et al (2004) explicitly state that "serum S100B levels in schizophrenic patients can most likely be used to infer S100B concentrations in the brain"

We conclude that the studies presented show that there are aberrant levels of neural growth factors in peripheral blood in schizophrenia patients, most notably in patients with negative symptoms. These findings should be regarded as indicative of an association between the schizophrenia phenotype and aberrant peripheral levels, not as indicative of aberrant CNS levels per se. Nevertheless, due to their possible association with negative symptoms (and possibly other symptom subsets), growth factors might be candidates for prognostically and diagnostically useful markers during the early phase of the disease. Aberrant levels of peripheral growth factors might be candidate biochemical endophenotypes. Therefore, studies are needed to assess disturbances of peripheral growth factors in first degree family members of patients with schizophrenia. Large-scale studies might identify subsets of patients within the heterogeneous schizophrenia sample. Research on the relationship between peripheral growth factors and genetic variables can be fruitful.

A strong argument for investigating peripheral blood growth factors is that blood is easily accessible, the measurements are easy to perform in large patient samples and comparatively cheap.

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Chapter 4

Increased levels of serum S100B in young, recent-onset, male schizophrenia patients do not normalize after treatment

Submitted

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Abstract

In schizophrenia, elevated serum levels of the neurotrophic protein S100B are reported in patients with a chronic or intermediate duration of illness. S100B levels in young, recent-onset patients are not clearly established. We performed a case-control study of two independent cohorts (N=101 and N=49, 16-35 years) of young, recent onset (< 5 years of illness) patients vs age-mached controls. Cohort I consisted of acutely psychotic patients, cohort II of party stabilized patients. In cohort I S100B was also measured after 8 weeks of treatment. S100B levels were significantly elevated in the patients ($p < 0.001$ and $p = 0.01$, cohorts I and II respectively). S100B remained elevated after eight weeks. In cohort I, serum S100B was negatively correlated with age ($r = -0.251$, $p = 0.011$). S100B levels could not be clearly related to clinically meaningful subgroups. We conclude that, in men, elevated levels of serum S100B do not normalize after treatment, and are thus a trait marker in early schizophrenic psychosis.

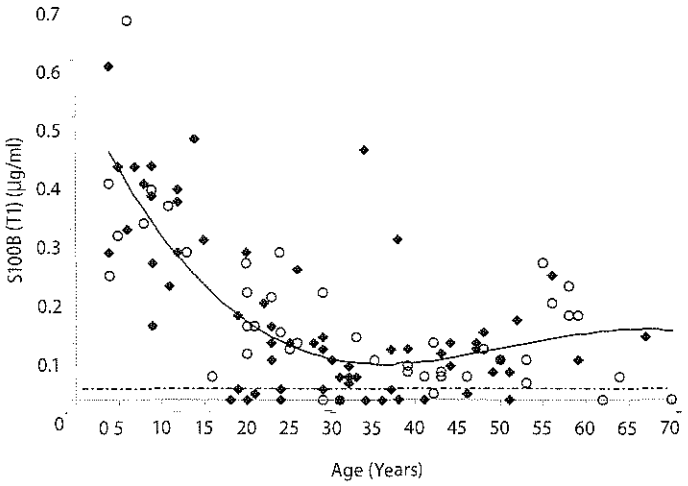
Introduction

Normal cortical maturation is a highly complex process which involves major changes in grey and white matter (especially during puberty and adolescence), and with specific temporal dynamics (Gogtay et al., 2004). These normal early and late maturational processes result in dynamic changes in the structure of the brain, well into the second and third decade of life and even later. In schizophrenia, it is thought that normal processes of brain maturation are disturbed (De Haan and Bakker, 2004); indeed, imaging studies have shown brain changes in schizophrenia (Arango et al., 2008; Van Haren et al., 2008a; 2008b). These findings have led to the leading paradigm regarding the etiology of schizophrenia, the neurodevelopmental hypothesis, which proposes a role for early aberrant brain development on which normal and/or abnormal brain development is superimposed (see McGlashan and Hoffman(2000)).

On the neurochemical level, cortical maturation is influenced by neurotrophic factors, which are a heterogeneous group of proteins participating in a wide range of actions involved in (among others) cell survival, apoptosis and axon sprouting. One such neurotrophic factor is the S100B protein. S100B is a calcium-binding protein found in the central nervous system (CNS), especially in the cytoplasm of astrocytes. S100B regulates cell shape, energy metabolism, cell-to-cell communication, intracellular signal transduction, cell growth, and can be released by astrocytes (Donato et al., 2001). The effects of extracellular S100B depend on its concentration (Hu et al., 1996). Nanomolar concentrations act as a growth and/or differentiation factor for neurons and astrocytes, whereas micromolar concentrations may induce apoptosis. Some in vitro and in vivo findings also suggest that brain metabolic stress is associated with elevated levels of serum S100B (Sarandol et al., 2007; Steiner et al., 2008a; 2008b); this is interesting in the light of recent reports associating schizophrenia with increased brain oxidative stress (Prabakaran et al., 2004; Khaitovich et al., 2008). S100B inhibits protein phosphorylation in a number of enzyme substrates, suggesting a role in fine-tuning specific steps in these pathways (Donato, 2001). Also, S100B interacts with cytoskeletal elements, and is suggested to play a role in the assembly of important components of cell cytoarchitecture.

Portela et al. (2002) report that serum S100B levels show a steady decline from relatively high levels during infancy and puberty to adult levels of S100B (reached at the end of adolescence), and then again show a modest increase beginning at mid-age (Figure 1).

Figure 1



Correlations of serum S100B with age in healthy subjects (after Portela et al. (2002).

Black diamonds: males; open diamonds: females. A significant negative correlation was observed ($r = -0.70$; $p<0.001$). The fitted curve (solid line) was obtained using a third-degree polynomial regression. The dashed line represents the detection limit of the assay (0.02 ng/ml).

Portela et al. (2002) suggest that these age-dependent serum S100B levels reflect ongoing brain processes, possibly related to cortical maturation. Thus, S100B might be a peripheral marker for studying brain maturational processes possibly involved in schizophrenia.

Indeed, elevated blood or cerebrospinal fluid levels of S100B have been reported in schizophrenia (Wiesmann et al., 1999; Rothermundt et al., 2001; 2004a; 2004b; Lara et al., 2001; Schroeter et al., 2003; Schmitt et al., 2005; Van Beveren et al., 2006; Steiner et al., 2006; Sarandol et al., 2007; Ling et al., 2007;). It was also suggested that these altered levels either reflect ongoing brain damage, or compensatory processes (Wiesmann et al., 1999; Rothermundt et al., 2001; 2004a; 2004b; Lara et al., 2001; Schroeter et al., 2003; Schmitt et al., 2005; Steiner et al., 2006; Sarandol et al., 2007; Ling et al., 2007). However, these earlier studies have predominantly investigated older patients (mean age 31.8-63.3 years)(Van Beveren et al., 2006), in the later phase of the disease, with the exception of Steiner et al. (2006) who investigated a sample

(N=12) of young schizophrenia patients (mean age 24.7 years. Thus, data on serum S100B levels in younger, adolescent, recent-onset schizophrenia patients, and on the effects of treatment on S100B levels in this patient population are scarce. Insight into the levels and dynamics of serum S100B at the end of puberty and in adolescence may increase insight into the neurodevelopmental processes present in the early phase of schizophrenia. This is all the more important because cortical developmental processes are thought to be highly dynamical, specifically in this age-category (Gogtay and Thompson, 2009).
Moreover, if elevated levels of S100B are also present in the early phase of the disorder, S100B may be a candidate protein biomarker for the early schizophrenia syndrome; this may be useful in identifying subjects at-risk for schizophrenia in the prodromal state, before the onset of frank psychotic symptoms.

To investigate serum S100B levels in young schizophrenia patients, we performed a case-control study investigating serum S100B levels in two independent cohorts (N=101 and N=49) of young (16-35 years), recent-onset, male, schizophrenia patients. In cohort I (N=101), we investigated serum S100B levels at study entry (T1) and after 8 weeks of treatment (T2); in cohort II (N=49) the measurement was cross-sectional. Recent-onset was defined as duration of illness (DUI) < 5 years.
Our primary focus was to investigate whether elevated levels of serum S100B are present in both cohorts, and to investigate a possible treatment effect (in cohort I) on serum S100B levels. Secondary aims were to explore the putative age-dependency of serum S100B levels, and to identify clinically meaningful subgroups among the patients based on initial S100B levels in both patients and controls, and to investigate possible differences between schizophrenia subtypes.

Methods

Patients

Patients participating in cohort I were included at the clinic for psychotic disorders of the Dept of Psychiatry of the Erasmus University Medical Center (EMC), Rotterdam. Patients are referred to the EMC by primary caretakers and general mental healthcare facilities throughout the Rotterdam region (1.2 million inhabitants), an industrialized area with a mixed population of Dutch ancestry and immigrants.

Patients are referred to the EMC either with acute psychotic symptoms (including emergency referrals from the general community) or with treatment-resistant positive or negative symptoms.
Patients participating in cohort II were recruited at the Dept of Psychiatry of the Academic Medical Centre (AMC) in Amsterdam, an area similar to the Rotterdam region. The AMC also operates a clinic for young patients with psychotic symptoms; however, most of the AMC patients are partly stabilized.
Eligible for inclusion were all male patients diagnosed with schizophrenia according to the DSM-IV criteria after a Comprehensive Assessment of Symptoms and History (CASH) interview (Andreasen et al., 1992) and by consensus between two senior psychiatrists who were blinded to the S100B results. For patients with symptoms for less than 6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterium. Additional criteria were recent onset (defined as DUI <5 years) and age >15 and <36 years. As starting point of the illness, either the time of occurrence of positive symptoms was taken, or the occurrence of clear limitations in social or occupational functioning (if these latter symptoms were the first to emerge). Exclusion criteria were the presence of any somatic or neurological disorders, as investigated by routine clinical and laboratory examination performed at admission.
The clinical and demographic state of the patients was evaluated at baseline and after 8 weeks of treatment. Severity of symptoms was assessed by the Positive And Negative Syndrome Scale (PANNS) by trained investigators.
Patients in the EMC are usually treated as inpatients, with daycare treatment options in the later phase of the treatment. AMC patients are daycare patients.

Deferred consent procedure (cohort I)

In cohort I, for acutely admitted patients who were severely psychotic and too disturbed to provide consent, a separate procedure was applied in which informed consent was initially given by a first-degree relative, and final written consent was sought within 6 weeks from the patients themselves ('deferred consent'). Blood samples for these patients were collected with a vacutainer system, together with the samples which are part of the regular clinical screening, so that no additional venapunction was needed. This procedure also enabled us to include the most severely psychotic patients. Blood samples and data from patients who withheld consent after the stipulated 6 weeks were destroyed.

Control groups

Age-matched controls for cohort I were recruited among students and staff of the EMC faculty and hospital, and for cohort II from the community (mainly from high schools and colleges). Exclusion criteria for controls were a personal history of psychiatric illness, or a first-degree family member with a history of psychiatric illness, as reported by the control person. All controls were recruited at the same time as the patients from the corresponding two geographic locations.

Consent

After receiving a complete description of the study all participants provided written informed consent. Study I (with cohort I), was approved by the Erasmus University MC Institutional Review Board (including the deferred consent procedure). Study II (with cohort II) was approved by the UMC Utrecht and the Academic Medical Center Amsterdam Institutional Review Boards. Both studies were conducted according to the standards of the Declaration of Helsinki.

Samples and S100B measurement

In cohort I, blood was drawn on admission and after 8 weeks, on both occasions between 08.00 and 10.00 am. In cohort II, blood was drawn during admission. Blood was allowed to clot and, after centrifugation for 20 minutes at 2650 g, serum was stored at -80°C until analysis. S100B was analyzed using a commercially available monoclonal two-site immunoluminometric assay (Sangtec Medical, Dietzenbach, Germany) according to the manufacturer's instructions by using a Liaison automated analyzer (Byk Sangtec, Dietzenbach). See Vos et al. (2004) for further details.

Statistical analyses

Analyses were performed with SPSS 16.0 statistical software. Graphs were created using GraphPrism 5.0 software.

Non-Gaussian distributed variables were logarithmically-transformed (base 10) to achieve normality, but for clarity of interpretation means and standard deviations (SD) of untransformed variables are shown; 95% Confidence Intervals are shown log-transformed (table 2 shows which variables were transformed). Non-parametric analyses showed highly similar results (data not shown).

Student's t-test was used to analyze the difference in serum S100B levels between patients and controls, and between subgroups within the patients. The Kruskal-

Wallis test was used to investigate differences in serum S100B levels between schizophrenia subtypes.
The change in serum S100B levels between baseline and after 8 weeks was analysed with a paired samples t-test.
Pearson's product moment was used to investigate correlations between serum S100B and quantitative clinical variables (i.e. age and PANSS scales).

All analyses were two-tailed. Significance levels for between-group comparisons

Table 1: Characteristics of the patients (at T1)

	Cohort I (N=101)	Cohort II (N=49)
Smoking	yes: 73 (72.3%) no: 28 (27.7%)	yes: 34 (69.4%) no: 15 (30.6%)
Cannabis abuse	yes: 47 (46.5%) no: 54 (53.5%)	yes: 16 (32.7%) no: 33 (67.3%)
Duration of Untreated Psychosis (DUP) (N = 80)†	46.3 (SD 59.5) weeks min-max: 0-260 median: 19.0	not specified
Duration of illness (DUI) (N=86)‡	85.3 (SD 78.9) weeks min-max: 1-260 median: 60.0	103.6 (SD 74.7) weeks min-max: 9-240 median: 76.7
Ethnicity		
Caucasian (mostly Dutch descent)	49 (48.5%)	28 (57.1%)
Surinamese / African descent	13 (12.9%)	2 (4.1%)
Cape Verdian	10 (9.9%)	0 (0.0%)
Surinamese / Hindustani	8 (7.9%)	0 (0.0%)
Moroccan / North African	6 (5.9%)	3 (6.1%)
Turkish	4 (4.0%)	2 (4.1%)
Asian	2 (2.0%)	1 (2.0%)
Mixed	5 (5.0%)	4 (8.1%)
Could not be reliably assessed	4 (4.0%)	9 (18.4%)
Antipsychotics used at study entry		
naive (mean age naive: 22.6 (SD 2.8) years)	26 (25.7%)	
free > 2 weeks, not naive (mean age free > 2 weeks: 24.6 (SD 5.3) years)	17 (16.8%)	

total free > 2 weeks	43 (42.6%)		
free unknown duration	3 (3.0%)		8 (16.3%)
haloperidol	17 (16.8%)		3 (6.1%)
risperidone	17 (16.8%)		14 (24.5%)
olanzapine	9 (8.9%)		19 (34.7%)
quetiapine	1 (1.0%)		1 (2.0%)
aripiprazole	4 (4.0%)		1 (2.0%)
clozapine	7 (6.9%)		4 (8.2%)
other			3 (6.1%)
PANSS scores	**T1, cohort I**	**T2, cohort I**	**T1, cohort II**
total score	82.1 (SD16.3)	66.6 (SD 13.0) *	56.8 (SD 17.8)
positive subcale	20.0 (SD 7.31)	14.5 (SD 4.6) *	12.1 (SD 5.1)
negative subscale	21.1 (SD 6.7)	19.2 (SD 5.9) *	16.2 (SD 8.0)
general psychopathology	40.1 (SD 9.2)	33.3 (SD 8.5) *	28.5 (SD 8.2)

* $p < 0.001$ (paired t-test);
†: of 21 patients DUP could not be reliably estimated;
‡: of 15 patients DUI could not be reliably estimated, but was certainly < 260 weeks.

were set at 0.05, but the significance levels for correlations were set at 0.01 to correct for multiple comparisons, except for the correlations between S100B and age, and between S100B and the PANSS negative subscale, as these are based on *a priori* evidence from previous findings (Portela et al., 2002; Rothermundt et al., 2001; 2004b; Van Beveren et al., 2006).
All results are shown as mean (±SD).

Results

Subjects

In cohort I, 101 patients and 86 controls (all males) were enrolled in the study. Patients had a mean age of 22.79 (±4.49; range 16-35) years, and the controls 22.85 (±4.41; range 16-35) years ($p = 0.93$). Of patients in cohort I, 14 were ‹18 years, and 28 ‹20 years; 26 patients (25.7%) were antipsychotic-naive, and 17 (16.8%) were free of antipsychotic medication for ›2 weeks; 27 patients (26.7%) were lost to follow-up for the measurement after 8 weeks.
Cohort II consists of 49 male patients, and 42 age-matched controls. These patients had a mean age of 22.37 (±3.37; range 17-29) years, and the controls 22.33(±4.61; range 17-29) years ($p=0.97$). Table 1 presents characteristics of both cohorts.

Serum S100B levels at study entry:

Serum S100B was significantly elevated in the patients of cohort I group: mean (±SD) serum S100B patients vs controls: 0.085 (±0.036) vs 0.066 (±0.029) ng/ml; t = 4.86; df = 185; p < 0.001; 95%CI = 0.069; 0.164. The magnitude of the differences in the means is moderate to large (eta squared = 0.114). We could not identify altered levels of serum S100B between schizophrenia subtypes (paranoid (N=59), disorganized (N=30), catatonic (N=7), undifferentiated (N=6), or resttype (N=6)); df=4, p=0.229.

Also in cohort II, we found significantly elevated serum S100B levels as compared to the controls (mean serum S100B patients vs controls: 0.0831 (±0.042) vs 0.064 (±0.024) ng/ml; t=2.64; df = 89; p = 0.01; 95%CI = 0.023; 0.166). The effect size is moderate (eta squared = 0.071).

The mean levels of S100B for cohorts I and II are strikingly similar (0.0855 ng/ml and 0.0831 ng/ml, respectively).

To investigate antipsychotic medication use, smoking, and cannabis abuse as possible confounders for patients serum S100B levels, we compared serum S100B levels in smoking vs non-smoking patients, cannabis abuse vs non-cannabis abuse patients, antipsychotic-naive vs medicated patients, and antipsychotic-free >2 weeks vs medicated patients.

In cohort I the smoking patients (N=73) showed significantly decreased levels of serum S100B compared with the non-smokers (N=28): mean serum S100B smokers vs non-smokers: 0.081 (±0.031) vs 0.100 (±0.045) ng/ml; t = 2.46; df = 96; p = 0.018. There were no differences in the other groups of cohort I.

In cohort II there were no differences in serum S100B between patients and controls in all investigated subgroups.

An overview of serum S100B levels between various subgroups is given in **table 2**.

We found a significant negative correlation between S100B and age for cohort I as a whole (r = -0.255, p <0.001). We also found a significant negative correlation between S100B and age for the controls (r = -0.258, p = 0.008) and for the patients (r = -0.251, p = 0.011) separately (Figure 2).

Because Portela et al (2002) described a non-linear relationship between serum S100B and age specifically at the end of puberty (Figure 1), we made a similar investigation. A fourth-order polynomial regression provided the best fit, i.e. patients and controls together: R^2 = 0.10; patients: R^2 = 0.11; controls: R^2 = 0.17 (Figure 2).

Table 2: Mean levels of serum S100B (ng/ml) between patient subgroups

	Controls	Patients	Patients (antipsychotic naive at T1) vs medicated	Patients (antipsychotic free > 2 weeks at T1) vs medicated	Patients using nicotine vs non-nicotine	Patients using cannabis vs non-cannabis
S100B COHORT I (T1) (ng/ml) (mean SD)	0.0660.030 (N=86)	0.0850.036 * (N=101)	0.0850.035* ‡ (N= 26)	0.0840.038* ‡ N= 43	0.0810.031 vs 0.1000.046 †† N= 73, N= 28)	0.0830.033 vs 0.0890.040 ‡‡ (N= 47, N= 54)
S100B COHORT I (T2) (ng/ml) mean SD)		0.0880.040 † (N=73)	0.0820.036 † (N = 19)	0.0870.042 † (N = 30)		
S100B COHORT II (ng/ml) mean SD)	0.0640.024 (N= 42)	0.0830.043 * (N= 49)		0.0850.034 ‡ (N= 8)	0.0840.047 vs 0.081 0.031 ‡ (N= 34, N= 15)	0.0960.047 vs 0.0770.039 ‡‡ (N= 16, N= 33)

* $p < 0.001$ as compared to the controls (t-test)
† non-significant difference as compared to T1 (paired t-test)
†† significantly decreased as compared to non-smokers (t-test), p=0.016)
‡ non-significant difference as compared to medicated patients (t-test)
‡‡ non-significant difference between cannabis abuse vs non-cannabis abuse

In cohort II we did not obtain a significant correlation between age and S100B ($r = -0.109$, $p=0.304$), neither for the patients and controls as a whole, nor for patients and controls separately. A fourth-order polynomial regression did not converge for the sample as a whole, or for the patients and controls separately.

In cohort I we did not find a significant correlation between serum S100B and the PANSS negative subscale ($r = 0.121$, $p = 0.233$); a trend towards a negative correlation

was found between S100B and DUI (r = -0.230, p = 0.03). All other correlations were non-significant.
In contrast, in cohort II we did find a positive significant correlation between S100B and the PANSS negative subscale (r = 0.292, p = 0.042). See Table 3 for an overview of the correlations.

Effects of treatment on serum S100B levels (only cohort I)
After 8 weeks of treatment all PANSS scores showed a significant improvement (paired t-test). PANSS total score: t = 8.477, p <0.001; PANSS positive subscale: t =

Figure 2a

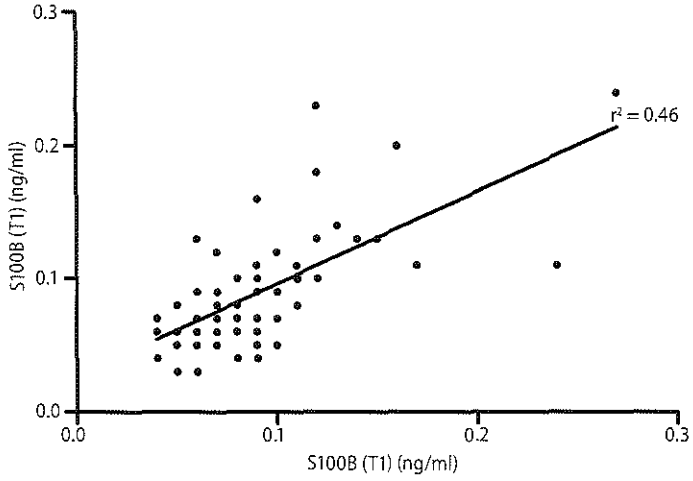


Figure 2b

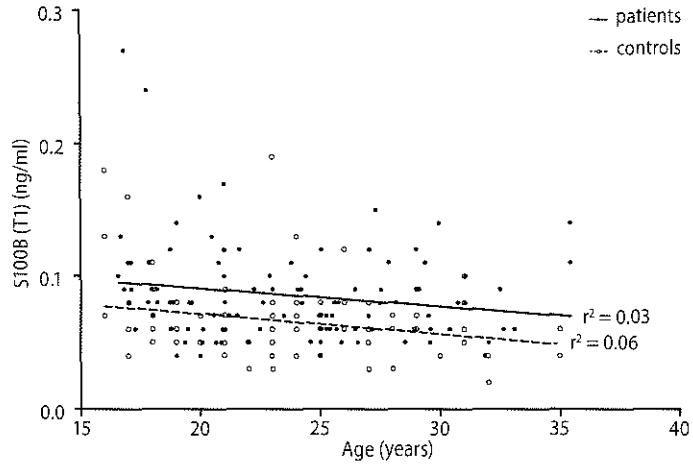
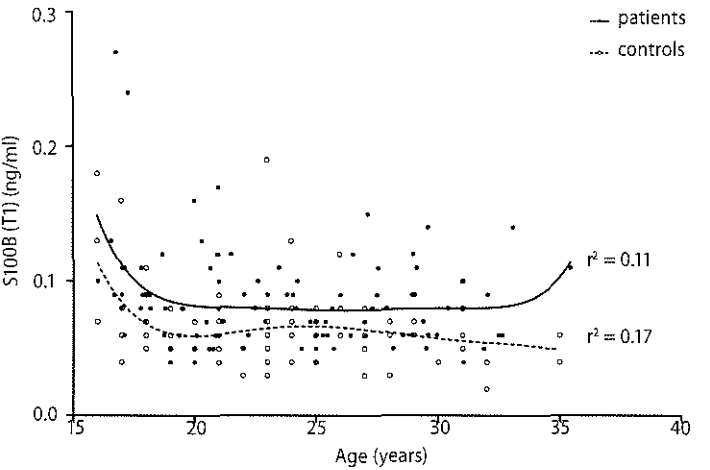


Figure 2c



Correlations of serum S100B and subject parameters (cohort I)

a. Relationship between serum S100B at study entry (T1) and after 8 weeks of treatment (T2); there is a significant positive correlation ($r = 0.686$; $p<0.001$).

b. Effect of age on serum S100B (T1) in patients and controls (linear analysis); there is a significant negative correlation for the sample as a whole ($r = -0.255$; $p < 0.001$), for controls ($r = -0.258$; $p=0.008$), and for patients ($r = -0.251$; $p=0.011$). The variables used for obtaining the graph are not log transformed.

c. Effect of age on serum S100B (T1) in patients and controls, with fitted curves obtained by a fourth-degree polynomial regression (solid line: patients; dotted line: controls). Note the similarity of the curve with that depicted in Figure 1 reported by Portela et al. (2002). The variables used for obtaining the graph are not log transformed.

7.746, p <0.001; PANSS negative subscale: t = 4.019, p <0.001; PANSS general subscale: t = 6.285, p <0.001 (see table 1). There was however no change in serum S100B after 8 weeks of treatment: paired t-test (N=74): t = 0.143, df = 72, p = 0.97; mean S100B (T1): 0.085 (±0.036) ng/ml; mean S100B T2: 0.088 (±0.040) ng/ml.

S100B levels at T1 and T2 showed a strong and significant correlation (r = 0.680, p <0.001) (Figure 2).

Figure 3 summarizes our results: serum S100B is consistently elevated in both cohorts as compared to their respective controls; moreover, there is no effect of treatment on serum S100B levels.

Table 3: Correlations between S100B (at T1) and patient parameters (at T1) and overview of logarithmically transformed variables.

		S100B T1, cohort I [1]	S100B T1, cohort II [2]
Age [1]	r	-0.251	-0.133
	p	0.011*	0.363
	N	101	49
PANSS positive scale T[1]	r	-0.079	0.140
	p	0.435	0.336
	N	101	49
PANSS negative scale T1[2]	r	0.140	0.292
	p	0.168	0.042*
	N	101	49
Duration of untreated psychosis T1 (weeks) [1]	r	-0.131	Not specified
	p	0.245	
	N	80	
Duration of illness T1 (weeks)	r	-0.230	-0.035
	p	0.033	0.812
	N	86	49

* $p < 0.05$

1 Analysis of this variable in cohort I is performed after log transformation (base 10).

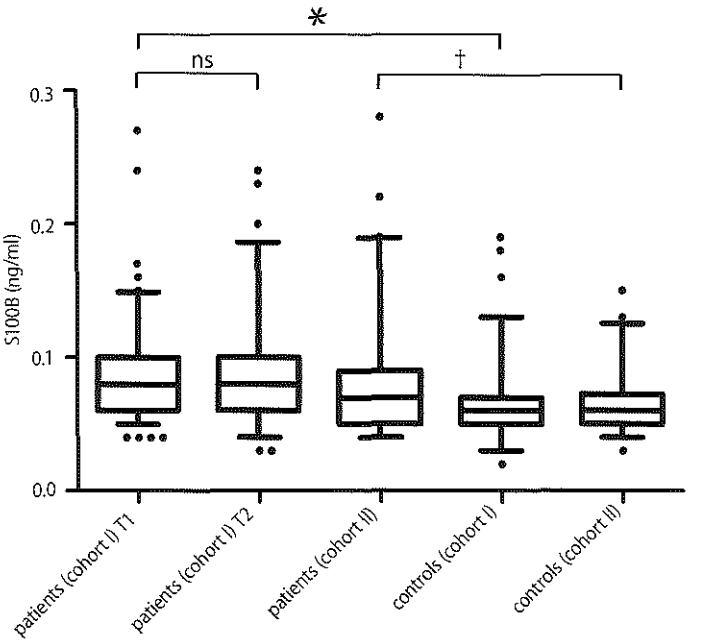
2 Analysis of this variable in cohort II is performed after log transformation (base 10).

At T1, serum S100B levels of the patients lost to follow-up (LFU) at T2 (N=27) did not differ significantly from those who remained in the study [LFU: 0.077 (± 0.023) ng/ml; remaining in the study: 0.088 (± 0.040) ng/ml; t = -1.327; df = 100; p = 0.187].

Post-hoc analyses for S100B (cohort I)

Because our findings suggest that serum S100B may also be elevated in patients with a short DUI, we investigated patients with DUI ‹10 weeks. For comparison, we selected controls aged ‹30 years, to compensate for the younger mean age of the patient group. This patient group (N=19; mean age 21.21 (± 4.49) years vs 22.27 (± 3.84) years for the controls (N=80) (p = 0.34)) also showed significantly elevated levels of serum S100B: 0.092 (± 0.025) vs 0.067 (± 0.030) ng/ml; t = 3.83; df = 97; p ‹ 0.001; 95%CI = 0.073; 0.229.

Figure 3



Boxplot figures of serum S100B levels between the cohorts I and II and their respective control groups.

Cohort I (T1; N=101) consists of florid psychotic patients. Cohort I (T2; N=74) have been naturalistically treated for 8 weeks. Cohort II (N=49) consists of (partly) stabilized patients.

Serum S100B levels between patients and controls are consistently elevated, and do not normalize after treatment.

* $p<0.001$

To investigate whether serum S100B levels predict treatment response, we divided the patient sample into two subgroups, High-S100B (patients with serum S100B higher than the mean plus 1 SD of the controls, N=27) versus Low-S100B (N=74) and investigated the T1 PANSS scores in High-S100B vs Low-S100B, as well as ΔS100B, ΔPANSS-positive and ΔPANSS-negative in High-S100B vs Low-S100B. No significant differences were found on these scores between the High-S100B vs the Low-S100B groups.

Discussion

This study shows that serum S100B levels are increased in young, recent-onset, male schizophrenia patients (cohort I), suffering from a psychotic episode; we confirmed this finding in an independent sample of schizophrenia patients, with similar age and demographic background. S100B levels did not decrease after an 8-week treatment period. Cohort II consisted of not-acutely psychotic, partly stabilized patients; therefore, the finding of elevated serum S100B in this group is in line with the persistent presence of elevated levels of serum S100B found in cohort I. In both cohorts, the patients showed highly similar concentrations of serum S100B.
It was also shown that the elevated levels of S100B could not be attributed to the effects of antipsychotic medication, nicotine or cannabis abuse. Also, elevated levels of serum S100B seem to be independent of DUI, and are also present in patients with a short (<10 weeks) DUI. Taken together, these results strongly suggest that elevated levels of serum S100B are a trait factor in early schizophrenic psychosis in males.
Our findings of increased levels of serum S100B (and the specific concentrations found) are consistent with other studies (Wiesmann et al., 1999; Rothermundt et al., 2001; 2004b; Lara et al., 2001; Schroeter et al., 2003 ;Schmitt et al., 2005; Steiner et al., 2006; Van Beveren et al., 2006; Sarandol et al., 2007; Ling et al., 2007;). The present study extends these findings to young (as young as 16 years), recent-onset schizophrenia, male patients. Moreover, use of the 'deferred consent' procedure enabled us to also include severely disturbed patients (in cohort I). Thus, we could study both young and florid psychotic patients, as well as (partly) stabilized patients.

A positive correlation between serum S100B and negative symptoms (or deficit state) is emerging as a consistent finding and has been reported by others (Rothermundt et al., 2001; 2004b; Ling et al., 2007). In cohort I, we found no correlation between serum S100B and negative symptoms. In contrast, in cohort II there was a significant positive correlation between S100B and negative symptoms.
An explanation for the less clear association found in cohort I might be that these patients showed severe negative symptoms with only modest variance, which reduces the power to find a correlation. In cohort II the patients showed less severe negative symptoms with more variance, which could have contributed to the significant correlation between serum S100B and negative symptoms in this sample.
Positive symptoms seem to be independent of serum S100B levels, as previously reported (Rothermundt et al., 2001; 2004b; Ling et al., 2007).

In cohort I, we found a negative correlation between age and serum S100B levels, both for controls and patients. We also found a trend towards a negative correlation between DUI and S100B. Because age and DUI are positively correlated, we cannot unravel which of these two factors is the main factor behind the negative correlation between DUI and S100B.
In cohort I, a fourth-order polynomial regression shows that serum S100B seems to decline toward adult levels at the end of puberty. Moreover, this mechanism seems to be present in both patients and controls, albeit the patients show consistently elevated levels throughout all age ranges. Generally, the trend of the curve seems to be consistent with the relationship reported by Portela et al (2002) (Figure 1). Specifically, they reported that normal S100B levels are age-dependent, with high levels during infancy and a gradual decline during puberty and basic adult levels (Portela et al., 2002). Lara et al. (2001) reported a negative correlation between serum S100B and DUI; this correlation might reflect an association between S100B and age. Schmitt et al. (2005) reported a positive correlation between age and S100B in their sample of old, chronic patients; this association may reflect the normal age-dependent increase in serum S100B in later life, as described by Portela et al. (2002). Most studies investigating serum S100B in schizophrenia report no correlation between serum S100B and age; this might be because these studies investigated patients (and controls) in the intermediate age range, when the slope of the curve described by Portela et al. (2002) is almost flat. We studied younger patients (some as young as 16 years), giving us a window on the steeper decline in S100B present at the end of puberty; this probably enabled us to identify the reported negative correlation.
That we could not replicate a clear age-dependency for serum S100B in cohort II may be a power problem, as the youngest group (< 20 years) in cohort II is considerably smaller than in cohort I.

We could not identify a treatment effect on serum S100B levels; serum S100B was elevated at study entry and remained so. However, several reports mention a decrease in serum S100B after treatment. (Rothermundt et al., 2001; 2004b; Ling et al., 2007). For example, two studies by Rothermundt et al. (2001; 2004b) suggest that elevated levels of serum S100B (as well as failure of elevated levels to normalize during treatment) seem to identify a specific subgroup of patients with persistent negative symptoms and an unfavourable prognosis; we could not identify such a subgroup in cohort I.

The lack of treatment effect in cohort I could be because this group was more severe than the cohorts in other studies, due to referral bias. The majority of subjects in cohort I would then fail to normalize after treatment, similar to the subgroup described by Rothermundt et al (2001; 2004b). Indeed the PANNS negative score (T1) of cohort I reflects the presence of severe negative symptoms.
At study entry, there was a strong positive correlation between a change in serum S100B and the PANSS negative score, but the most likely explanation for this seems to be regression to the mean.

At study entry, nicotine and antipsychotic medication use were initially considered as possible confounders; however, these have been ruled out. Nicotine abuse is associated with *lower* levels of serum S100B in cohort I, and thus can not contribute to the *elevated* levels in the patient group. At study entry, antipsychotic-naive and antipsychotic-free patients show similar levels of serum S100B to patients who do use antipsychotics.
It is not easy to make a straightforward interpretation of the elevated levels of serum S100B in schizophrenia. One of the main reasons is the differential effect S100B has on neural cells, depending on its concentration. In cell culture and animal experiments, micromolar concentrations of S100B stopped proliferation and induced differentiation of neurons finally leading to apoptosis. However, nanomolar concentrations of S100B appear to boost proliferation of neurons, dendrites and neurites, inducing an increase in synaptic contacts between cells. Thus, elevated levels of serum S100B could either reflect neuronal damage or a compensatory process. In other words, they may indicate primary astrocytic injury or astrocytic response to neuronal injury or aberrant developmental processes.
Primary astrocytic injury may support the hypothesis of a possible progressive degenerative component in the early phase of the disease. However, another explanation might be that elevated concentrations of serum S100B shown in schizophrenic patients reflect secondary S100B upregulation by astrocyte stimulation in an attempt to counter a (so far, unknown) neurodegenerative process. In accordance with the latter hypothesis is the finding that the elevated levels of serum S100B found in schizophrenia are lower than the S100B concentrations found in disorders with obvious CNS damage.
Even though S100B is predominantly expressed in astrocytes, S100B is also present in other brain cells, such as oligodendrocytes, microglia, or neuronal cells. Therefore, these cells may also contribute to elevated levels of serum S100B. Indeed, there

is increasing evidence for an involvement of oligodendrocytes and white matter abnormalities in schizophrenia (Tkachev et al., 2003; 2007; Uranova et al., 2004). Another possibility, is that serum S100B is elevated due to astrocyte activation induced by increased brain oxidative stress in patients (Sarandol et al., 2007). Elevated serum S100B levels may also result from an increased blood-brain barrier permeability (Kapural et al., 2002; Schroeter et al., 2003) in the presence of normal secretion from astrocytes. Incidental reports describe the disrupted integrity of the blood-brain barrier in schizophrenia (Axelsson et al., 1982).

An important issue related to the origin of serum S100B is that the source of serum S100B is not exclusively cerebral. Production of S100B has been detected in adipocytes, smooth muscle cells and lymphocytes (Steiner et al., 2007). Nevertheless, some evidence points to a profound cerebral influence on serum S100B levels. Serum S100B reliably reflects cerebrospinal fluid concentration of S100B in healthy subjects (Nygaard et al., 1997), in patients with neurological diseases, and in patients with schizophrenia (Rothermundt et al., 2004a). In addition, elevated levels of serum S100B were associated with increased brain myo-inositol concentrations, as measured by Magnetic Resonance Spectography (Rothermundt et al., 2007). Memory impairment in chronic schizophrenia is reported to be associated with serum S100B (Pedersen et al., 2008). Moreover, S100B-immunopositive glia is elevated in post-mortem brain tissue of paranoid schizophrenia patients (Steiner et al., 2008a). Taken together, there is sufficient evidence to speculate that the increased levels of serum S100B to some extent reflect CNS levels and, thus, might reflect disturbances in normal cortical maturation in line with the neurodevelopmental hypothesis of schizophrenia.
Combining our findings with those of other studies investigating serum S100B in schizophrenia, the overall picture seems to be that serum S100B is consistently elevated in all patient groups investigated, regardless of age. In the present study, patients with a very short DUI (<10 weeks) also show elevated levels of serum S100B. In this respect it is tempting to speculate that serum S100B levels may be elevated before the onset of frank psychotic symptoms; our results do not contradict this possibility. If this is so, serum S100B levels may be measured in prodromal patients as an objective biological aid to identify patients at risk for psychosis.

To summarize, we found elevated levels of serum S100B in two independent cohorts of young, male, recent-onset schizophrenia patients compared with healthy

controls. Additionally, we found serum S100B levels to be persistently elevated after 8 weeks of treatment. None of the subgroups showed a specific pattern of S100B response to treatment. Our findings strongly suggest that serum S100B levels are elevated, even in the youngest group of schizophrenia patients. We recommend that serum S100B levels be investigated in prodomal patients, because serum S100B might be a biomarker predicting future conversion to a fully psychotic state. Finally, it is worth noting that serum is easy to collect, and measurement of serum S100B can be performed inexpensively.

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Chapter 5

Brain-derived neurotrophic factor is decreased in serum of young, recent-onset, male schizophrenia patients with active psychosis, and normalizes after treatment

Submitted

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Abstract

Objective

Brain-derived neurotrophic factor (BDNF) has emerged as a key mediator for synaptic efficacy, neuronal connectivity and neuroplasticity. In schizophrenia, several reports have shown decreased levels of serum BDNF in patients. However, results are inconsistent; moreover, only limited data are available on BDNF levels in young, recent-onset patients. We asked: are serum BDNF levels in young, recent-onset, male schizophrenia altered as compared to controls, and what is the influence of treatment on BDNF levels.

Methods

Two independent cohorts (cohort I: N=101, age 16-35; cohort II: N=55, age 17-25) were compared with two independent groups of age- matched controls (N= 86 and 42 respectively). Cohort I consists of acutely psychotic patients, cohort II of stabilized patients. BDNF was measured at study entry and after eight weeks in cohort I. In cohort II the measurement was cross-sectional.

Results

Cohort I showed significantly decreased levels of serum BDNF ($p < 0.001$) as compared to the controls. BDNF levels after eight weeks of treatment increased significantly as compared to baseline ($p = 0.049$; paired t-test), but compared to the controls the level of significance serum BDNF was still decreased ($p = 0.017$). In cohort II altered levels in the patients as compared to the controls were not present ($p = 0.20$). BDNF was significantly and negatively correlated with postive symptoms at study entry in cohort I.

Conclusions

Serum BDNF levels are decreased in young, recent-onset, male schizophrenia patients suffering a psychotic episode. BDNF levels show a trend towards normalizing after an eight-week treatment period, a result which is underscored by the absence of altered BDNF levels in in the (partly) stabilized patients of cohort II.
Taken together, these results suggest that decreased levels of serum BDNF are a state marker for a psychotic episode in schizophrenia patients.

Introduction

Normal cortical maturation is a highly complex process which involves major changes in grey and white matter, especially during puberty and adolescence, and with specific temporal dynamics (Shaw et al., 2008; Gogtay et al., 2004). These normal early and late maturational processes result in dynamic changes in the structure of the brain well into the second and third decade of life and even later. In schizophrenia, it is thought that normal processes of brain maturation are disturbed (van Haren et al., 2008a; van Haren et al., 2008b), and, possibly, in brain maturation (de Haan and Bakker 2004). Indeed, several imaging studies have shown changes maturational dynamics in schizophrenia (Arango et al., 2008; Gogtay 2008; Vidal et al., 2006). These findings have led to the leading paradigm concerning the etiology of schizophrenia, the neurodevelopmental hypothesis, which proposes a role for early aberrant brain development on which normal and /or abnormal brain development is superimposed (i.e. see McGlashan and Hoffman (2000)).
On the neurochemical level, cortical maturation is influenced by neurotrophic factors, which are a heterogeneous group of proteins participating in a wide range of actions involved in (among others) cell survival, apoptosis and axon sprouting. One such neurotrophic factor is Brain-Derived Neurotrophic Factor (BDNF).
BDNF has emerged as a key mediator for synaptic efficacy, neuronal connectivity and neuroplasticity. BDNF regulates neuronal development and survival and controls the activity of many neurotransmitters systems, including the serotoninergic and glutamatergic systems (Durany et al., 2001; Machado-Vieira et al., 2007a). BDNF is produced by central nervous system (CNS) cells and by peripheral platelets. However, because it has been described that intact BDNF in the peripheral circulation crosses the blood-brain barrier two-directionally (Pan et al., 1998), BDNF blood concentrations might reflect central BDNF concentrations. Indeed, a positive correlation between central and peripheral levels of BDNF was recently demonstrated (Karege et al., 2002).

Several studies have demonstrated that changes in central and peripheral BDNF levels may be involved in the pathophysiology of mood disorders (Fukumoto et al., 2001; Chen, 2001; Karege et al., 2002; Mai et al., 2002; Hashimoto et al., 2004; Machado-Vieira et al., 2007b). In schizophrenia, several reports have shown decreased levels of BDNF (Toyooka et al., 2002; Pirildar et al., 2004; Grillo et al., 2006; Palomino et al., 2006; Buckley et al., 2007). Specifically, Buckley et al. (2007) reported decreased

levels of plasma BDNF in young (mean age 21.8 (SD 8.83) years), never-medicated schizophrenia patients, and a positive correlation between plasma BDNF levels and positive. Buckley et al. (2007) suggested that decreased levels of BDNF may be a candidate biological marker for predicting a psychotic epispode or relapse.

However, these findings are far from consistent. Unaltered levels have also been reported (Shimizu et al., 2003; Jockers-Scherubl et al., 2004; Huang and Lee 2006), and even increased levels (Gama et al., 2007). See Van Beveren et al. (2006) for a review. Moreover, these previous studies have either mostly investigated older patients in the later phase of the disease, with mean ages of the patients ranging from 33.3 (Shimizu et al., 2003) to 48.6 years (Toyooka et al., 2002), or, when investigating younger patients, had comparatively small sample size (Pirildar et al. (2004): N=22; Palomino et al (2006): N=21; Buckley et al. (2007): N=15). Two studies performed a follow-up measurement after treatment (Pirildar et al., 2004; Palomino et al., 2006). Pirildar et al. (2004) reported persistently decreased BDNF levels after treatment, Palomino et al (2006) reported a steady restoration of normal BDNF levels.

So, at present only limited data are available on serum BDNF levels in younger, recent-onset schizophrenia patients Also, the effects of treatment on BDNF levels are unclear. Insight into the levels and dynamics of blood BDNF at the end of puberty and in adolescence may increase our insight in the neurodevelopmental processes present in the early phase of schizophrenia. Moreover, it has been suggested that serum BDNF might be marker for the early psychotic episode in schizophrenia (Buckley et al 2007). Clearly, this last suggestion needs to be investigated in much larger samples.

These considerations led us to perform a case-control study, investigating serum BDNF levels in two independent cohorts (N=101 and N=55) of young (16-35 years), recent onset, male schizophrenia patients. In cohort I (N=101), we investigated serum BDNF levels at study entry (T1) and after eight weeks of treatment (T2); in cohort II (N=55) the measurement was cross-sectional. Recent-onset was defined as duration of illness (DUI) < 5 years.
Our primary research question was to investigate whether elevated levels of serum BDNF are present in both cohorts, as well as to investigate the effects of an eight-week treatment period (in cohort I) on serum BDNF levels. Our secondary research question was to investigate if we could identify clinically meaningful subgroups

within the patient samples, based on initial BDNF values and/or its development over the eight week period.

Methods

Patients

Patients participating in cohort I were included at the clinic for psychotic disorders of the Department of Psychiatry of the Erasmus University Medical Center (EMC), Rotterdam. There is unlimited referral of patients to the EMC by primary caretakers and general mental healthcare facilities throughout the Rotterdam region (1.2 million inhabitants), an industrialized area with a mixed population of Dutch ancestry and immigrants. Patients are referred to the EMC either with acute psychotic symptoms (including emergency referrals from the general community) or with treatment-resistant positive or negative symptoms.

Patients participating in cohort II were recruited at the Department of Psychiatry of the Academic Medical Centre (AMC) in Amsterdam, an area similar to the Rotterdam region. The AMC also operates a clinic for young patients with psychotic symptoms; however, the AMC patients are mostly stabilized and participating in a day-care program aimed at rehabilation.

Eligible for inclusion in cohort I were all male patients, consecutively brought into the care of the EMC in the period between june 2004 and january 2007 (2.5 year inclusion period), which were diagnosed with schizophrenia according to the DSM-IV criteria after a Comprehensive Assessment of Symptoms and History (CASH) interview (referentie) and by consensus between two senior psychiatrists who were blinded to the BDNF results. For patients with symptoms for less than 6 months, a final diagnosis was made after 6 months to comply with the DSM-IV criterium. Additional criteria were recent onset (defined as DUI ‹5 years) and age ›15 and ‹36 years. As starting point of the illness, either the time of occurrence of positive symptoms was taken, or the occurrence of clear limitations in social or occupational functioning (if these latter symptoms were the first to emerge). Exclusion criteria were the presence of any somatic or neurological disorders, as investigated by routine clinical and laboratory examination performed at admission.

The clinical and demographic state of the patients was evaluated at baseline and after 8 weeks of treatment. Severity of symptoms was assessed by the Positive And Negative Syndrome Scale (PANNS) by trained investigators.

Patients in the EMC are usually treated as inpatients, with daycare treatment options in the later phase of the treatment. AMC patients are daycare patients.

Deferred consent procedure (cohort I)

In cohort I, for acutely admitted patients who were severely psychotic and too disturbed to provide consent, a separate procedure was applied in which informed consent was initially given by a first-degree relative, and final written consent was sought within 6 weeks from the patients themselves ('deferred consent'). Blood samples for these patients were collected with a vacutainer system, together with the samples which are part of the regular clinical screening, so that no additional venapunction was needed. This procedure also enabled us to include the most severely psychotic patients. Blood samples and data from patients who withheld consent after the stipulated 6 weeks were destroyed.

Control groups

Age-matched controls for cohort I were recruited among students and staff of the EMC faculty and hospital, and for cohort II from the community (mainly from high schools and colleges). Controls were in self-proclaimed good health. The presence of indicators of general somatic disorders was checked by measuring C-reactive protein and white blood cell count. Exclusion criteria for controls were any somatic disorder, personal history of psychiatric illness, or a first-degree family member with a history of psychiatric illness, as reported by the control person. All controls were recruited during the same time-period as the patients and from the corresponding geographic locations.

Consent

After receiving a complete description of the study all participants provided written informed consent. Study I (with cohort I), was approved by the Erasmus University MC Institutional Review Board (including the deferred consent procedure). Study II (with cohort II) was approved by the UMC Utrecht and the Academic Medical Center Amsterdam Institutional Review Boards. Both studies were conducted according to the standards of the Declaration of Helsinki.

Samples and BDNF measurement

Blood was drawn at admission and after 8 weeks at both occasions between 08.00 and 10.00 am. Blood was allowed to clot and, after centrifugation for 20 minutes at 2650

g, serum was stored at -80 °C until analysis. Serum BDNF was analyzed with a commercial ELISA Kit (Promega, Madison, WI, USA) as described by the manufacturer. To control for microplate variance merged batches of patients and controls were used, as well as repeated measurements of individuals over the subsequently used microplates. The absorbances were measured with an automated microplate reader at 450 nm.

Statisical analyses

Analyses were performed with SPSS 16.0 statistical software. Graphs were created using GraphPrism 5.0 software.

Student's t-test was used to analyze the difference in serum BDNF levels between patients and controls, and between subgroups within the patients.

The change in serum BDNF levels between baseline and after 8 weeks was analysed with a paired samples t-test. Pearson's product moment was used to investigate correlations between serum BDNF, as well as between the change in serum BDNF from T1 to T2 (ΔBDNF), and quantitative clinical variables.

All analyses were two-tailed. Significance levels for between-group comparisons were set at 0.05, but the significance levels for correlations were set at 0.01 to correct for multiple comparisons, except for the correlations between BDNF and the PANSS positive symptoms subscale, and between BDNF and the PANSS negative subscale, as these correlations are based on *a priori* evidence from previous findings (Buckley, et al., 2007; Huang and Lee 2006) respectively.

All results are shown as mean (±SD).

Results

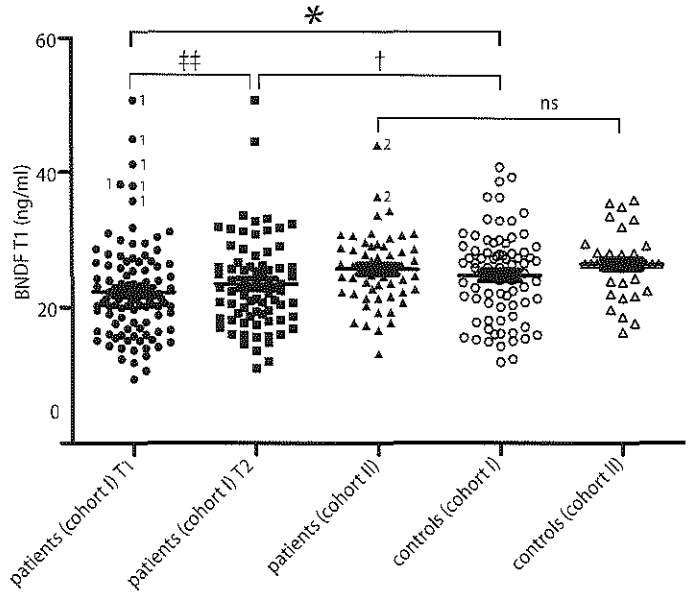
Cohort I

Subjects

In cohort I, 101 patients and 86 controls (all males) were enrolled in the study. Initial inspection of the dsitribution of the data showed 6 clear outliers in the patient group (serum BDNF > mean + 2SD of the patients; 5 of these are also above the mean + 2SD of the controls)(See **figure 1**).

We removed these 6 outliers from the main analyses, which were thus performed on 95 patients. Characteristics of the outliers will be described at the end of the results section, and are also shown in **table 1**.

Figure 1



Scattterplots of serum BDNF levels between patients and controls.

Cross-lines show mean and standard error of the mean.

Dots indicated with [1] are outliers of cohort I; dots indicated with [2] are outliers of cohort II.

* $p < 0.001$ (independent samples t-test, without the outliers marked [1])

†† p=0.049 (paired samples t-test)

† p=0.017 (independent samples t-test)

The remaining 95 patients had a mean age of 22.78 (±4.45; range 16-35) years, and the controls 22.85 (±4.41; range 16-35) years (p = 0.93). Table 1 presents the patient characteristics. Of the patients, 12 were ‹18 years, and 26 ‹20 years; 24 patients (25.3%) were antipsychotic-naive, and an additional 15 (15.8%) were free of antipsychotic medication for ›2 weeks; 25 patients (27.7%) were lost to follow-up for the measurement after 8 weeks.

At study entry: Serum BDNF levels compared to controls (cohort I)

We found the patients to have significantly decreased levels of BDNF (mean serum BDNF patients vs controls: 21.13 (±5.11) vs 24.68 (±6.16) ng/ml; t = -4.24; df = 179; p‹

0.001; 95%CI = -5.21; -1.9. The magnitude of the differences in the means large (eta squared = 0.30). With the outliers retained in the sample there is still a significant decrease in serum BDNF (t = -2.25; p = 0.026)).

Change in serum BDNF after eight weeks of treatment (cohort I).

There was a significant increase in serum BDNF after 8 weeks of treatment: paired

Figure 2a

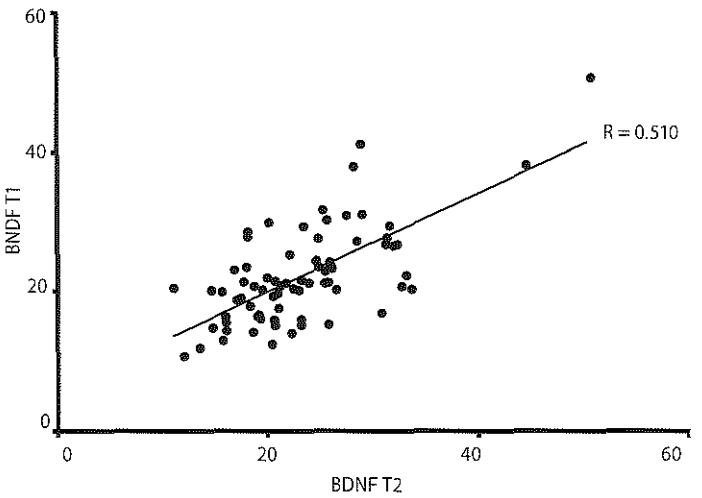


Figure 2b

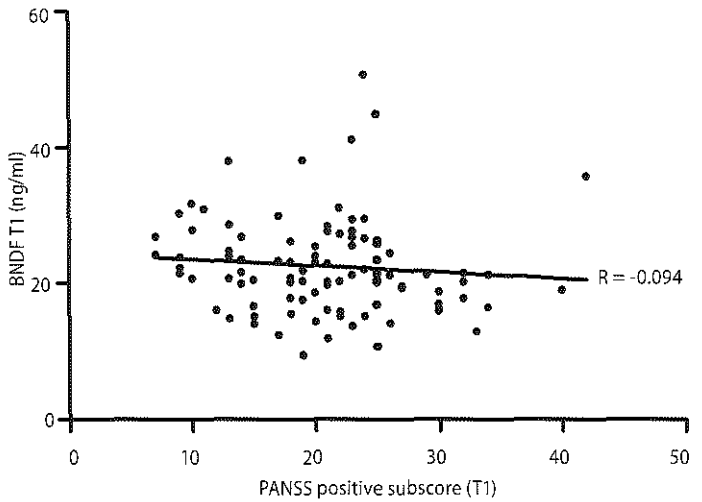
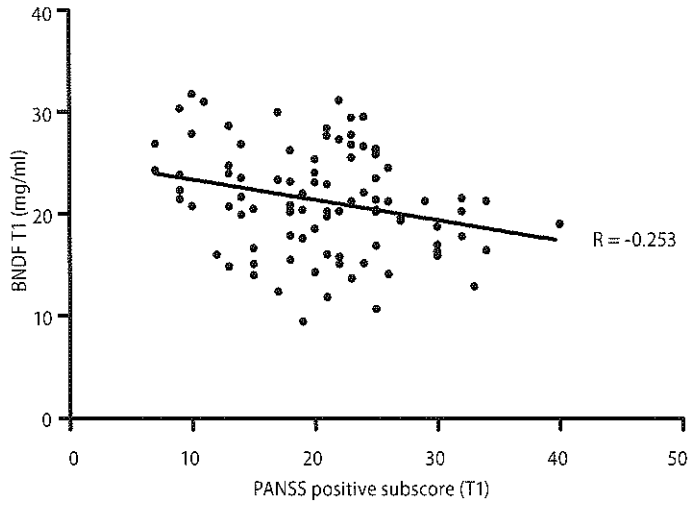


Figure 2c



Correlations of serum BDNF and subject parameters (cohort I)

a) Relationship between serum BDNF at study entry (T1) and after 8 weeks of treatment (T2); there is a significant positive correlation ($r = 0.510$; $p<0.001$).

b) Relationship between serum BDNF (T1) and the PANSS positive symptoms subscale with the outliers retained in the sample; the correlation is not significant ($r = -0.094$; $p=0.36$).

c) Relationship between serum BDNF (T1) and the PANSS positive symptoms subscale after removing the outliers; the correlation is now significant ($r = -0.253$; $p=0.014$)

t-test (N=69): $t = -2.005$, $df = 68$, $p = 0.049$; mean BDNF (T1): 21.12 (±5.24) ng/ml; mean BDNF T2: 22.40(±5.45) ng/ml. BDNF levels at T1 and T2 showed a strong and significant correlation ($r = 0.510$, $p < 0.001$) (see **Figure 2a**).

However, the difference between patient serum BDNF levels at T2 (22.40(±5.45) ng/ml)) and the controls (24.68 (±6.16) ng/ml) was still significant; $t = -2.409$; $df = 153$; $p = 0.017$; 95%CI = -4.15; -0.41.

At T1, serum BDNF levels of the patients lost to follow-up (LFU) at T2 (N=25) did not differ significantly from those who remained in the study (LFU: 20.98(±4.87) ng/ml; remaining in the study: 21.17 (±5.22) ng/ml; $p = 0.876$).

Table 1: Characteristics of the patients (at T1)

	Cohort I (N=95) *(6 outliers removed: their data is given in italics)* mean age 22.78 (SD 4.45) (16-35) years		Cohort II (N=53) *(2 outliers removed; data not given)* mean age 22.60 (SD 3.29) (17-29) years
Smoking	yes: 68 (71.6%) (5 - 83.3%) no: 27 (28.4%) (1 - 16.7%)		yes: 40 (75.5%) no: 13 (24.5%)
Cannabis abuse	yes: 50 (52.6%) (5 - 83.3%) no: 45 (47.4%) (1 - 16.7%)		yes: 15 (28.3%) no: 38 (71.7%)
Duration of Untreated Psychosis (DUP)	47.8 (SD 60.9) weeks (N = 75)† min-max: 0-260 median: 19.0 *23.60 (SD 22.46) (N=5)* *min-max: 4-60*		not specified
Duration of Illness (DUI)	84.6 (SD 79.0) weeks (N=80)‡ min-max: 1-260 median: 60.0 *95.3 (SD 84.9) (N=5)* *min-max: 12-260*		103.6 (SD 74.7) weeks min-max: 9-240 median: 76.7
Ethnicity			
Caucasian (mostly Dutch descent)	47 (49.5%)	*2*	29 (54.7%)
Surinamese / African descent	12 (12.6%)	*2*	2 (4.1%)
Cape Verdian	7 (7.4%)	*2*	0 (0.0%)
Surinamese / Hindustani	8 (8.4%)		0 (0.0%)
Moroccan / North African	6 (6.3%)		4 (7.5%)
Turkish	4 (4.2%)		2 (4.1%)
Asian	2 (2.1%)		1 (2.0%)
Mixed	5 (5.3%)		5 (9.3%)
Could not be reliably assessed	4 (4.2%)		10 (18.9%)
Antipsychotics used at study entry			
naive (mean age naive: 22.6 (SD 2.8) years)	24 (25.3%)	*2 (33.3%)*	
free > 2 weeks, not naive (mean age free > 2 weeks: 24.6 (SD 5.3) years)	15 (15.8%)	*2(33.3%)*	
total free > 2 weeks	39 (41.1%)	*4(66.6%)*	
	3 (3.2%)		8 (16.3%)
haloperidol	16 (16.8%)	*1(16.6%)*	3 (6.1%)

(continued on next page)

Table 1: *(continued)*

	Cohort I (N=95) *(6 outliers removed: their data is given in italics)* **mean age 22.78 (SD 4.45)** **(16-35) years**		**Cohort II (N=53)** *(2 outliers removed; data not given)* **mean age 22.60 (SD 3.29** **(17-29) years**
risperidone	16 (16.8%)	*1(16.6%)*	14 (26.4%)
olanzapine	9 (9.5%)		19 (35.8%)
quetiapine	1 (1.1%)		1 (2.0%)
aripiprazole	4 (4.2%)		1 (2.0%)
clozapine	7 (7.4%)		4 (8.2%)
other			3 (6.1%)
PANSS scores	**T1, cohort I**	**T2, cohort I**	**T1, cohort II**
total score	81.3 (SD 16.0) *94.7 (SD 14.9)*	66.4 (SD 14.0) *	56.8 (SD 17.8)
positive subscale	20.8 (SD 7.14) *24.3 (SD 9.7)*	14.5 (SD 4.6) *	12.1 (SD 5.1)
negative subscale	21.8 (SD 6.7) *25.7 (SD 7.7)*	19.1 (SD 6.1) *	16.2 (SD 8.0)
general psychopathology	39.8 (SD 9.0) *44.7 (SD 11.5)*	33.2 (SD 8.6) *	28.5 (SD 8.2)

* $p < 0.001$ (paired t-test);
†: of 21 patients DUP could not be reliably estimated;
‡: of 15 patients DUI could not be reliably estimated, but was certainly < 260 weeks.

After 8 weeks of treatment all PANSS scores showed a significant improvement (paired t-test). PANSS total score: $t = 7.63$, $p < 0.001$; PANSS positive subscale: $t = 7.30$, $p < 0.001$; PANSS negative subscale: $t = 3.21$, $p < 0.001$; PANSS general subscale: $t = 5.74$, $p < 0.001$ (see table 1).

At study entry: serum BDNF levels in patient subgroups (cohort I)

Serum BDNF levels were compared in four patient subgroups, i.e. smoking (N=68) vs non-smoking (N=27)($p = 0.336$), cannabis abuse (N=50) vs non-cannabis abuse (N=45)($p = 0.216$), antipsychotic-naive (N=24) vs medicated (N=53)($p = 0.994$), and antipsychotic-free >2 weeks (N=39) vs medicated (N=53)($p = 0.596$) (see Table 2 for the subgroup means). As shown, all comparisons were non-significant.

Correlation of BDNF with subject parameters

We investigated correlations between BDNF and age, PANSS positive subscore,

Table 2: Overview of mean levels of serum BDNF (ng/ml) between patients (outliers removed) and controls, and between patient subgroups

	Controls	Patients	Patients (antipsychotic naive at T1) vs medicated	Patients (antipsychotic free > 2 weeks at T1) vs medicated	Patients using nicotine vs non-nicotine	Patients using cannabis vs non-cannabis
BDNF COHORT I (T1) (ng/ml) (mean sd)	24.686.16 (N=86)	21.135.11* (N=95)	20.974.96 vs 20.985.47 (N= 24 vs 53)	21.574.75 vs 20.985.47‡ (N=39 vs 53)	21.264.93 vs 20.805.70†† (N= 68 vs 27)	21.194.74 vs 21.065.59‡‡ (N= 50, N= 45)
BDNF COHORT I (T2) (ng/ml) mean sd)		22.405.45† (N=67)	21.434.53 † (N = 17)	22.394.54 † (N = 26)		
BDNF COHORT II (ng/ml) mean sd)	26.414.47 (N= 42)	25.184.55 (N= 53)	Not specified	24.286.55 ‡ (N= 8)	24.694.66 vs 25.73 4.50 †† (N= 40, N= 13)	24.415.14 vs 25.394.31 ‡‡ (N= 15, N= 38)

* $p < 0.001$ as compared to the controls (t-test)

** $p < 0.05$ as compared to the controls (t-test)

† $p < 0.05$ as compared to T1 (paired t-test)

†† non-significant difference between nicotine and non-nicotine (t-test)

‡ non-significant difference as compared to medicated patients (t-test)

‡‡ non-significant difference between cannabis abuse vs non- cannabis abuse

PANSS negative subscore, and duration of illness (DUI). We identified a significant negative correlation between serum BDNF and the PANSS positive symptom score (r: -0.253, $p = 0.014$), and a trend towards a significant positive correlation between serum BDNF and DUI (r: 0.219, $p = 0.05$) (see figure 2b and 2c). All other correlations were not significant.

Correlation of ΔBDNF with subject parameters

We investigated correlations between the change in BDNF from T1 to T2 (ΔBDNF)

and age, duration of illness, PANSS positive - and PANSS negative subscore (at T1), and change in PANSS positive (ΔPANSS-positive) and change in PANSS negative subscore (ΔPANSS-negative).
None of these correlations reached the level of significance.

Post- hoc analyses regarding BDNF and clinical improvement.
We conducted a post-hoc analysis to further investigate possible relationships between high levels of serum BDNF and clinical parameters
We divided the patient sample into two subgroups, Low-BDNF (patients with serum BDNF lower than the mean minus one standard deviation of the controls, (BDNF < 18.37, N=27) versus High-BDNF (N=74) and investigated the T1 PANSS scores in High-BDNF vs Low-BDNF as well as ΔBDNF, ΔPANSS-positive and ΔPANSS-negative in High-BDNF vs Low-BDNF.
ΔBDNF was significantly larger ($p<0.001$) in the Low-BDNF group as compared to the High-BDNF group. All other comparisons were not significant.
We also investigated the relationship between serum BDNF levels and treatment response as assessed by the change in PANSS positive and negative subscales. We defined treatment response as 25% or greater improvement in PANSS-pos, or PANSS-neg. We found no differences in serum BDNF levels at T1 between responders and non-responders. Neither did we find differences in ΔBDNF from T1 to T2 between responders and non-responders.

Cohort II

Subjects
Sample II consists of 55 exclusively male patients with a DSM IV diagnosis of schizophrenia and 42 age-matched controls. In this sample we identified 2 outlyers among the patients (BDNF > mean + 2SD), which were removed from further analyses.
The patients were of mean age 22.60 years (±3.29)(17 - 29), the controls were of mean age 22.30 years (±4.67)(17-29) ($p=0.72$).
In cohort II serum BDNF levels were not altered as compared to the controls (mean serum BDNF patients vs controls: 25.18 (±4.55) vs 26.41 (±4.47) ng/ml; $df = 93$, $t = -1.291$; $p = 0.20$; 95%CI = -3.11; 0.66. No significant correlations between BDNF and age, PANSS positive subscale, PANSS negative subscale, and DUI could be identified in cohort II.

The outliers of cohort I

Serum BDNF levels were significantly elevated in the outlying patients as compared to the controls (mean serum BDNF of the outlying patients: 41.50 (±5.54); Mann-Whitney U, Z: -3.907, p < 0.001). The mean age of the outliers is 23.00 (±5.51) years (p=0.93, as compared to the main group of patients). Two of the patients were antipsychotic-naïve, and 2 were antipsychotic-free for more than 2 weeks. The remaining 2 patients used haloperidol and risperidone (both 3 mg daily). Five of the patients used nicotine, as well as cannabis. Two of the patients had a caucasian background, 4 were of African descent. PANSS total, and PANSS positive, negative, and general psychopathology subscales were not significantly different between the outliers and the main group of patients (Mann-Whitney test).

Discussion

In this study we show that serum BDNF is decreased in recent-onset, male schizophrenia patients with active psychosis (cohort I). We also show that BDNF levels have a tendency to normalize after treatment in cohort I. This finding of restoration to normal levels is underscored by the absence of altered levels of serum BDNF in cohort II, which consists of remitted male patients of a similar age and demographic background. Our results also show that individual levels of serum BDNF are reasonably stable over the eight week interval we investigated, as indicated by the strong correlation between serum BDNF levels at study entry and after eight weeks. We also report a negative correlation between BDNF and positive symptoms in cohort I, in line with the finding reported by Buckley et al. (2007).
Jockers-Scherubl et al. (2004) reported that BDNF levels are increased in schizophrenia patients with cannabis abuse. We cannot confirm the presence of altered levels of BDNF in cannabis abusers for the patients in cohort I. However, we identified a small subgroup of 6 patients presenting with elevated levels of serum BDNF. Interestingly, 5 of these patients used cannabis (83%). This raises the possibility that in a small subset of patients cannabis abuse significantly raises serum BDNF levels.

The majority of other studies investigating BDNF levels in schizophrenia find decreased levels of BDNF (Toyooka et al., 2002) (Pirildar et al., 2004; Palomino et al., 2006; Grillo et al., 2007), in line with our findings; however, also unaltered (Shimizu

et al., 2003; Jockers-Scherubl et al., 2004) (only for patients without drug abuse), and even increased levels (Gama et al., 2007) have been reported. Huang and Lee (2006) found no differences between patients and controls, but report only decreased levels of BDNF exclusively in patients with chronic catatonia. Due to publication bias the number of studies with negative results may be even larger.

A factor complicating a straightforward comparison between these studies and ours is that studies differ from one another with respect to age, clinical phase of the disorder (i.e. recent onset vs chronic, actively psychotic vs stabilized), and predominance of subtypes (i.e paranoid vs deficit). The studies that compare best to the one presented here are the ones by Pirildar et al. (2004), Buckley et al. (2007), and Palomino et al. (2006), all investigating relatively small numbers of both male and female, young, first-episode patients (mean age 27,8, 25.3 and 23.7 years, respectively); both Pirildar et al. (2004) and Palomino el al. (2006) provide follow-up measurements, after six weeks, and after one, six and twelve months respectively.

All these three studies report decreased levels of BDNF at baseline. Pirildar et al. (2004) fail to find an increase towards normal levels after six weeks of treatment. But, Pirildar et al. (2004) limited their follow-up period to 6 weeks. In contrast, Palomino et al. (2006) provide a one-year follow-up period and they report a clear gradual restoration of BDNF levels over this period towards those of controls.

Our study underscores the results of these three studies, albeit in a much larger sample. We also find decreased levels of BDNF, as well as suggestive evidence that BDNF levels tend to elevate towards normal levels after treatment. BDNF levels increase slightly in cohort I, and more clearly in cohort II, in line with Palomino et al. (2006). Taken together, our findings, combined with those by reported by Buckley et al. (2007), Pirildar et al. (2004), and Palomino et al. (2006) strongly suggest the presence of reduced levels of BDNF in acutely psychotic patients which gradually restore to normal after treatment. It also explains why some studies investigating (partly) remitted patients do not report decreased levels of BDNF.

It should be emphasized that the three studies by Pirildar et al. (2004), Buckley et al. (2007), and Palomino et al. (2006) in combination investigated a total of 56 patients with schizophrenia, whilst in our study 101 patients were included in cohort I alone. The effect size we find (eta squared 0.30) is large. For Pirildar et al. (2004) we calculated an eta squared of 0.33, based on the data given in their report. Buckley et al. (2007) and Palomina et al. (2006) report reduction in mean BDNF levels of the patients as compared to the controls of 66% and 46% respectively. We find a reduction of 10%.

We identified 6 outliers which we removed from the main analyses in cohort I. We argue that these 6 outliers are not likely to be 'inclusion-error false-positives' (i.e. patients with presenting with a phenotype on the borderline between patients and controls), as these patients show serum BDNF levels which are not only elevated as compared to the BDNF levels of the patients, but are also significantly higher then the BDNF levels of the controls (see figure 1a); moreover, also these patients show severe psychopathology, as identified by the PANSS scores (see table 1).

Buckley et al. (2007) report a strong negative correlation between BDNF and positive symptoms. Palomino et al. (2006) and Pirildar et al. (2004) do not report on correlations with BDNF and patient parameters. We also report a significant negative correlation between serum BDNF levels at study entry and the PANSS positive subscale in cohort I. As there is evidence, from our present study and others, that decreased levels of serum BDNF are associated with a psychotic episode and normalize after clinical improvement, an association between BDNF levels and (positive) symptom severity is what might be expected.

Generally, there is an emerging body of converging evidence that points to a relation between schizophrenia and disrupted levels of BDNF, both in the central nervous system and in peripheral blood (Shoval and Weizman 2005). Among postmortem studies, BDNF levels are decreased in the hippocampus and increased in the cerebral cortex of patients with schizophrenia (Durany et al., 2001). Iritani et al. (2003) report an increase in BDNF and in its receptor, TrkB, in neurons in the hippocampus in accordance with another study by Takahashi et al. (2000), who find hippocampal BDNF is elevated, but its receptor down-regulated in schizophrenia. Further, both BDNF and TrkB mRNA levels were decreased in prefrontal cortex of subjects with schizophrenia (Weickert et al.,2003; Weickert et al., 2005).
Genetic research has shown a reducing effect of Val/Met heterozygosity on hippocampal volume as measured with MRI in both normal subjects and patients with schizophrenia, but with the reduction more pronounced in the patients (Szeszko et al., 2005). Wassink et al. (1999) and Ho et al. (2007) found a similar effect of BDNF genotype in schizophrenia (Buckley et al., 2007).
Pharmacologically, there is evidence that typical and atypical neuroleptics influence brain neurotrophins (Shoval and Weizman 2005). Three days of haloperidol administration caused a BDNF decrease in the prefrontal cortex, hippocampus, amygdala and ventral tegmental area (Dawson et al., 2001). The effect was even

stronger in the neostriatum and nucleus accumbens. However, subchronic treatment of 21 days with haloperidol consequently led to a rebound in BDNF in most cell bodies (Dawson et al., 2001). Chronic administration (29 days) of haloperidol and risperidone (Angelucci et al., 2000) as well as olanzapine (Angelucci et al., 2005) triggered each a decrease in BDNF levels in the prefrontal cortex, occipital cortex and hippocampus of rats. Haloperidol and risperidone also altered the expression of the BDNF receptor, TrkB (Angelucci et al., 2000).

Altered levels of serum BDNF are not specific for schizophrenia. Other studies evaluated serum BDNF levels in unmedicated patients presenting with major depression and bipolar disorder. Shimizu et al. (2003) and Karege et al. (2002) observed decreased levels of BDNF during depression and showed a negative correlation between BDNF levels and severity of depressive symptoms. Lang et al. (2004) observed that decreased serum BDNF levels were associated with depressive traits of personality in volunteers. A recent study by Machado-Vieira et al. (2007b) showed decreased levels of BDNF in medication free patients with bipolar disorder.

It has been suggested by Kapczinski et al. (2009) that changes in peripheral BDNF levels are the result of environmental stress as well as the strain imposed by affective episodes accompanying psychiatric disorders of various kind. Kapczinski et al. (2009) claim data that suggest that peripheral BDNF levels are lower in bipolar patients who suffered multiple mood epsiodes as compared to those who had a first episode. They conclude that, for bipolar disorder, aberrant levels of serum BDNF should not be interpreted in the traditional trait-state dichotomy, but rather as a composite of changes related to the trait of having a bipolar disorder and state changes imposed by environmental factors and repeated mood episodes. A similar environment X episode interaction might influence peripheral BDNF levels in schizophrenia. All in all, this considerations raise the possibility that altered levels of peripheral BDNF are not related to a specific diagnostic category (Monteleone et al., 2008), but instead reflect a final common pathway in the development of several major psychiatric disorders.

An important question relates to the relationship between peripheral and CNS BDNF levels. A complete passage of intact BDNF from brain to blood occurs by a high capacity and saturable transport system (Pan et al., 1998). A positive correlation between central and peripheral BDNF levels in rats was observed by Karege et al. (2002). Lang et al. (2007) found a positive correlation between in-vivo cortical N-acetylaspartate concentration in the anterior cingulate cortex (ACC) as measured with proton magnetic resonance spectroscopy and serum BDNF levels in healthy

subjects, which led them to conclude that serum BDNF levels might reflect the neuronal activity of the ACC. Although platelets are known to be a source of BDNF, it has been reported that changes in its serum or plasma levels are not accompanied by changes in the whole blood BDNF levels (Karege et al., 2005) and it is postulated that the observed changes in serum or plasma levels are probably related to aberrant BDNF release mechanisms (Machado-Vieira et al., 2007b). Taken together, these findings suggest at least some relationship between peripheral BDNF levels and CNS BDNF levels and, maybe, even with cortical activity (Lang et al., 2007). Accordingly, our findings may be related to changes in BDNF brain metabolism in schizophrenia patients with active psychosis.

From a clinical perspective, Buckley et al. (2007) suggested that decreased levels of peripheral BDNF might serve as a biomarker for identifying those high-risk individuals who are specifically at risk for conversion to a fully psychotic state. This is certainly an interesting suggestion, and our findings do not exclude this possibility. However, we think it is appropiate to posit some caveats. First, as shown in this study, in young, male patients, peripheral BDNF levels are state-dependent, related to the presence of positive symptoms. It is unclear at which point during the development of positive symptoms decreased levels of BDNF emerge. If decreased levels of BDNF are limited to the florid emergence of positive symptoms, this would clearly limit the value of BDNF as a predictive marker in high-risk patients. Second, as discussed, aberrant serum BDNF levels are not specific to schizophrenia, but may also be present in major depression and bipolar disorder. In that case, aberrant levels of peripheral BDNF in young, 'at risk' patients (when present) may serve as no more than to flag 'a possible development towards a major psychiatric disorder', not unlike the way an elevated erythrocyte sedimentation rate indicates the possible presence of a range of somatic disorders. Moreover, at present there are no data are available on peripheral BDNF levels in diagnostic categories which sometimes pose a differential diagnostic challenge when differentiating from schizophrenia, such as ADHD, autism spectrum disorders, brief psychotic disorder, and (stress related) psychotic phenomena which sometimes accompany personality disorders.

So, taken together, we show the presence of decreased levels of serum BDNF in recent-onset male schizophrenia patients with active psychosis. Serum BDNF levels restore to normal levels after treatment. Our findings point towards a correlation with positive symptoms, suggesting that decreased levels of BDNF are a state marker for psychosis in this patient group. Also, our findings suggest the presence of a small subset of patients with high levels of serum BDNF, as compared

to the majority of the patients which show decreased levels. Future research might focus on investigating peripheral BDNF levels in prodromal patients, to elucidate whether altered levels of BDNF might serve as a marker predictive of conversion to a fully psychotic state. It also might be fruitful to further investigate the suggested presence of a subset of patients with high BDNF levels.

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Chapter 6

Gen-expressie profilering bij schizofrenie: een overzicht

Tijdschrift voor Psychiatrie (2007) 49 (1): 7-16

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Samenvatting

Achtergrond

Een recente ontwikkeling in het genetisch onderzoek is om de activiteit van genen te bestuderen. Kenmerkend voor gen-expressie is dat deze variabel is en onder meer afhankelijk van de ontwikkelingsfase van een organisme, van het weefsel- en celtype en van omgevingsfactoren. Tegenwoordig is het mogelijk om de activiteit van de meer dan 30.000 genen die het volletige menselijke genoom vormen in één enkele keer te bepalen. Deze techniek staat in de engelstalige literatuur bekend als 'micro-array screening', 'high-throughput analyse' of in het neterlands: 'gen-expressie profilering'.

Doel

Het geven van een beschrijving van enkele basiselementen van de gen-expressie techniek en het geven van een overzicht van de resultaten gen-expressie van post-mortem verkregen hersenweefsel bij schizofrenie.

Methode

In 'PubMet' is gezocht naar relevante artikelen met behulp van de zoektermen: "schizophrenia", "microarray", "gene expression".

Resultaten en conclusie

In totaal vonden wij 10 onderzoeken. Gen-expressie profilering geeft aanwijzingen dat verschillende functionele gengroepen (zoals synaps-, metabolisme- myelinisatie- en oligodendrocyt gerelateerde genen) betrokken zijn bij de pathogenese van schizofrenie. Verschillende van deze gengroepen zijn gelocaliseerd op bekende chromosomale risicolocaties voor schizofrenie. Tezamen vormen zij ondersteuning voor die theorieën die postuleren dat schizofrenie verzoorzaakt wordt door verstoringen in de synaptische stabiliteit en plasticiteit. Er zijn aanwijzingen dat verstoringen in de myelinisatie en in vetzuurmetabolisme eveneens een rol kunnen spelen.

Inleiding

Het meeste genetisch onderzoek dat op dit moment bij psychiatrische aandoeningen verricht wordt bestudeert de aan- of afwezigheid van varianten van genen. Zulke varianten staan bekend als polymorfismen. Een recente ontwikkeling is echter om niet zozeer naar het vóórkomen van bepaalde genen te kijken, maar om de activiteit van genen, ook wel de expressie genoemd, te bestuderen. Kenmerkend voor gen-expressie is dat deze variabel is en onder meer afhankelijk van de ontwikkelingsfase van een organisme, van het weefsel- en celtype en van omgevingsfactoren. Tegenwoordig is het mogelijk om de activiteit van de meer dan 30.000 genen die het volletige menselijke genoom vormen in één enkele keer te bepalen. Deze techniek staat in de engelstalige literatuur bekend als 'micro-array screening', 'high-throughput analyse' of in het nederlands: 'gen-expressie profilering'.
Een voordeel van deze techniek is dat de interacties van genen met elkaar kunnen worden bestudeerd. Dit is vooral van belang bij die aandoeningen waarbij verondersteld wordt dat niet één gen, maar een (zeer) groot aantal genen, al dan niet in interactie met de omgeving, betrokken zijn bij het ontstaan van de onderzochte aandoening. Dit geldt waarschijnlijk met name voor veel psychiatrische aandoeningen, waaronder schizofrenie.
Tot nu toe zijn de meest indrukwekkende resultaten met gen-expressie profilering bereikt binnen de oncologie, waar het met behulp van gen-expressie profilering mogelijk is gebleken prognostisch betekenisvolle subgroepen te onderscheiden bij leukaemie. (zie bijvoorbeeld Valk et al. (2004)).
De laatste jaren zijn ook in toenemende mate onderzoeken verschenen waarin gen-expressie profilering gebruikt wordt bij onderzoek naar schizofrenie. Dit artikel beschrijft de techniek en geeft een overzicht van de publicaties op dit gebiet.

Methode en opbouw het artikel

Dit artikel geeft eerst een beschrijving van enkele basiselementen van de gen-expressie techniek.
Daarna worden de resultaten van een literatuuronderzoek gepresenteerd.
In juli 2005 is in 'PubMed' (www.pubmed.com) gezocht naar relevante artikelen met behulp van de zoektermen: "schizophrenia", "microarray", "gene expression". De gevonden artikelen werden tevens onderzocht op relevante referenties.

De gen-expressie bepaling

Technische achtergrond

De techniek van de bepaling is gebaseerd op het volgende principe. Een actief gen produceert mRNA. De technische term hiervoor is dat het gen 'afgelezen' wordt, of ook wel dat er 'expressie' of 'transcriptie' plaats vindt. mRNA wordt op zijn beurt weer omgezet in een eiwit. Naarmate een gen actiever is produceert het meer mRNA. Wij wijzen er nogmaals op dat dit een dynamisch proces is: sommige genen zijn altijd actief, sommige alleen gedurende een bepaalde fase tijdens de ontwikkeling van een organisme, en weer andere onder invloed van omgevingsfactoren, zoals stress, het innemen van voedsel of licht.

Voor het bepalen van de activiteit van genen wordt van weefsel waarin men geïnteresseerd is (bij schizofrenie meestal hersenweefsel, maar dat is niet altijd het geval) het mRNA geïsoleerd. Dit is dus een verzameling verschillend mRNA afkomstig van de corresponderende genen.

Het mRNA wordt voorzien van een fluorescerende kleurstof. Daarna wordt het mRNA aangebracht ('gehybridiseerd') op een plaatje van circa 1 cm2.

Op dit plaatje is op bekende lokaties complementair DNA aangebracht, in feite zijn dit genfragmenten. Een dergelijk plaatje heet een micro-array en dit is waar de techniek zijn naam aan ontleent.

Elk soort mRNA hecht zich nu aan de zijn bijbehorende gen.

Met een scanner (die qua principe te vergelijken valt met een cd-speler) wordt de lokatie en de intensiteit van de fluorescerende markers die aan het mRNA gehecht zijn gemeten . De lokatie geeft aan om wélk gen het gaat. De intensiteit van de fluorescentie geeft aan hóéveel mRNA er aanwezig is en dit is weer een maat voor de expressie van de betreffende genen.

Uiteindelijk levert dit concreet een computerbestand op met voor elk gen een getal dat de mate van expressie weerspiegelt. De gehele verzameling van getallen is het gen-expressie profiel.

Micro-arrays zijn commercieel verkrijgbaar. Sommige onderzoeksgroepen geven er de voorkeur aan zelf micro-arrays te fabriceren. Zie voor een uitgebreid overzicht van de techniek Konradi (2005) of www.affymetrix.com.

Interpretatie van gegevens verkregen uit genexpressie onderzoek

Statistische aspecten

Het meest op de voorgrond staande kenmerk van onderzoek naar gen-expressie

profilering is de zeer grote hoeveelheid gegevens die verzameld wordt, ook reeds bij kleine patiënten aantallen. Dit maakt dit type onderzoek vanuit statistisch oogpunt complex. Immers, meestal is bij patiëntgebonden onderzoek de hoeveelheid deelnemers groter dan de hoeveelheid uitkomstvariabelen (bv 'verbeterd' of 'ernst van de bijwerkingen'). Bij gen-expressie profilering is dit anders. Een rekenvoorbeeld: het door Affymetrix geproduceerde 'Human 233U Plus 2.0 GeneChip®' micro-array levert per sample ruim 50.000 gegevens op. Wanneer we ieder gegeven als een uitkomstvariabele zien, levert een studie met 20 deelnemers 1.000.000 uitkomstvariabelen. Enerzijds wordt het hierdoor mogelijk niet zozeer naar individuele genen te kijken, maar naar de interactie van genen in complexe biologische ketens waarbij vaak tientalllen tot honderden genen betrokken zijn. Anderzijds vormt de grote hoeveelheid gegevens een probleem omdat de kans op het vinden van significant afwijkende gen-expressie op basis van toeval groot is.

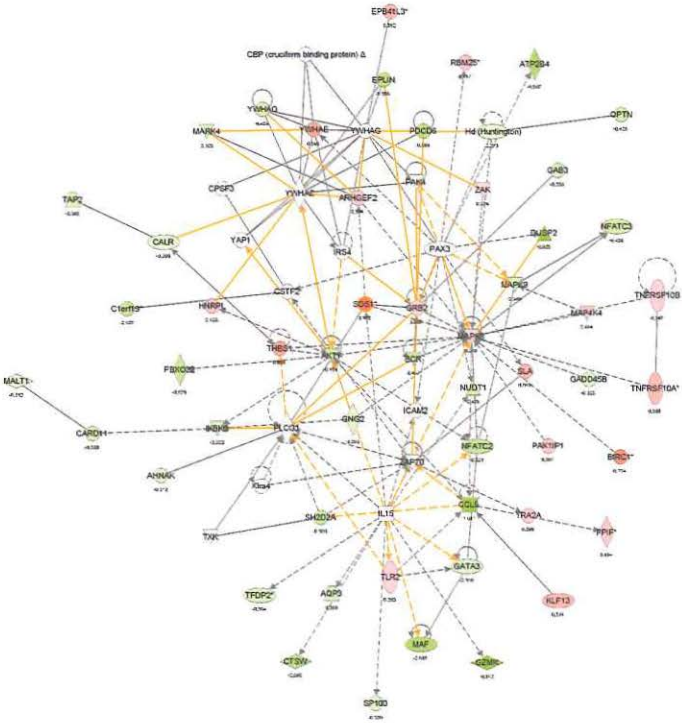
Dit onderzoek is dan ook niet mogelijk zonder hulp van bijzondere statistische methodes die deel uitmaken van een vakgebied dat bekend is komen te staan als 'bioinformatica'. De essentie van de bioinformatica is dat statistische technieken gecombineerd worden met de biologische kennis die omtrent de genen bestaat, met name de interacties die genen met elkaar aangaan. Hierbij wordt gebruik gemaakt van databases gevuld met gegevens over de biologische functies van genen.
Een voorbeeld van een dergelijke database is de 'Ingenuity Pathway Analysis' software (Ingenuity Systems, Mountain View CA; www.ingenuity.com) die informatie bevat over de interactie van genen met elkaar.
Deze database kan gevuld worden met een aantal (enkele honderden) genen, die bij een onderzoek als afwijkend actief naar voren zijn gekomen. De database genereert dan het biologische netwerk dat het beste past bij die verzameling genen. Figuur 1 laat een voorbeeld van een aldus gegenereerd netwerk zien.

Resultaten van genexpressie onderzoek wordt dan ook meestal in twee vormen gepresenteerd: een lijst met (losse) genen die significant veranderd zijn (verhoogde- of verlaagde expressie) met voor ieder gen de significantie en een overzicht van biologische processen waarbij die genen betrokken kunnen zijn.

Technische aspecten

Veel onderzoek gebruikt post-mortem materiaal. Postmortem materiaal is moeilijk

Figuur 1



Een met de Ingenuity Pathway Analysis (IPA) Software Tool geconstrueerd biologisch netwerk.

Op basis van de aanwezigheid van gedisreguleerde genen (groen: downregulatie; rood: upregulatie) die met de genexpressie analyse zijn angetoond wordt door de IPA Software Tool een netwerk gezocht (uit een databank) waarbij de groep gedisreguleerde genen zo goet mogelijk past. In het wit aangegeven genen waren niet gedisreguleerd of waren niet aanwezig op de genchip.

Het programma kan weergeven welke metabole processen door dit netwerk verricht worden

Door te klikken op de genen kan aanvullende informatie worden opgevraagd.

Het gebruik van geavanceerde gecomputeriseerde analysemethodes is onlosmakelijk verbonden met de grote hoeveelheid data die verzameld worden met gen-expressie onderzoek.

te verkrijgen en vaak zijn de patiënten niet goed gekarakteriseerd, met mogelijk verschillende symptomatologie of co-morbiditeit. Het materiaal komt meestal van ouderen en de vroege ontwikkelingsstadia zijn minder makkelijk toegankelijk voor onderzoek. De periode voorafgaand aan het overlijden (plotseling of na lang ziektebed, na coma met wellicht cerebrale hypoxie, na suicide) kan invloed hebben op de gen-expressie (Marcotte et al. 2003).
Voor micro-array onderzoek zijn grote hoeveelheden mRNA nodig, waar soms moeilijk is aan te komen. Sommige onderzoekers vergroten de hoeveelheid mRNA in vitro ('amplificatie') maar dit proces kan de onderlinge verhoudingen tussen de soorten mRNA verstoren. mRNA is in zekere zin een 'levend' product dat in tegenstelling tot DNA snel in kwaliteit achteruit kan gaan.

De onderlinge vergelijkbaarheid tussen monsters kan moeilijk zijn. Een stukje weefsel uit de prefrontale cortex bevat in principe zeer veel celsoorten en -fragmenten: stukjes axon en soma, witte- en grijze stof, glia en verschillende soorten corticale neuronen wat tot verschillende resultaten kan leiden zonder dat er daadwerkelijk expressieverschillen (Mirnics 2001) aanwezig zijn.

Experimentele artefacten kunnen leiden tot problemen. Er kunnen bijvoorbeeld variaties tussen monsters ontstaan tijden het bewerken of het merken. De grote hoeveelheid tussenstappen tijdens de bewerking en de hiermee verbonden noodzaak om met verschillende analisten te werken kan leiden tot methodologische variatie. Verschillen in micro-array's en apparatuur kunnen bestaan (Marcotte et al. 2003) en de resultaten beïnvloeden.

Aanwijzingen voor het beoordelen van onderzoeksresulaten
Bij het beoordelen van onderzoek naar genexpressie kan de geïnteresseerde psychiater zich oriënteren op de volgende elementen.
Welke patiënten zijn gebruikt? Als het gaat om post-mortem onderzoek: zijn de patiënten homogeen, bijvoorbeeld met betrekking tot leeftijd, medicatiegebruik en ziekteduur? Zijn de controles vergelijkbaar?
Welk weefsel is gebruikt voor de analyse? Welke methode is gebruikt voor het verwerven van weefsel? Is het weefsel homogeen of bevat het meerdere celtypen? Welke voorbereidende handelingen zijn getroffen om cellen te isoleren? Kunnen deze handelingen de hoeveelheid mRNA beïnvloeden?
Welke statistische methode is gebruikt? en dan met name: geeft deze methode

veel significante resultaten (met de kans op fout positieve uitslagen) of juist weinig significante resultaten (met de kans dat weinig afwijkende biologische processen worden gevonden?
Welke resultaten worden gepresenteerd? Alleen losse genen of biologische processen? Worden de resultaten aannemelijk gemaakt door validatie in andere groepen, door andere processen te onderzoeken, of door diermodellen?
Zijn de resultaten biologisch en heuristisch zinvol, met andere woorden, sluiten de resultaten aan bij reeds bekende bevindingen of theoretische concepten?

Resultaten literatuuronderzoek

In de tabel 1 zijn de resultaten van ons literatuuronderzoek naar gen-expressie profilering bij schizofrenie vermeld.

In totaal vonden wij 10 onderzoeken. Alle onderzoeken zijn van een case-control design, wat betekent dat de resultaten van patiënten worden vergeleken met die van een groep niet-zieke personen. Zo mogelijk zijn de bevindingen samengevoegd naar onderzoeksgroep.

Mirnics et al. (2000) beschrijven dat genen betrokken bij het coderen van eiwitten die de presynaptische aktiviteit reguleren (PSYN-genen) verlaagd waren in alle patiënten met schizofrenie in vergelijking met controles. Bij de verschillende patiënten was er een wisselende combinatie van verlaagde expressie van PSYN-genen, maar twee genen, namelijk N-ethylmaleimide sensitive factor (NFS) en synapsin II waren verlaagd bij bijna alle patiënten (respectievelijk 10 uit 10 en 9 uit 10). NSF is een eiwit betrokken bij neuronale presynaptische secretoire processen. De gegevens lijken er op te wijzen dat er een gemeenschappelijke abnormaliteit in het presynaptisch functioneren bestaat bij schizofrenie.
In een vervolg beschrijft dezelfde groep (Mirnics et al. 2001) dat het gen dat codeert voor de regulator van G-protein signalling 4 (RGS4) het meest consistent en significant verlaagd was in de dorsolaterale prefrontale cortex (DLPFC) van alle onderzochte patiënten. De gegevens lijken er op te wijzen dat een verlaging van RGS4 expressie een gemeenschappelijke en specifieke eigenschap is van schizofrenie. Dit zou kunnen komen door genetische factoren maar het zou ook een adaptatie aan de ziekte kunnen zijn. Verlaging van RGS4 beïnvloedt de neuronale signaaloverdracht.
In een derde publicatie (Middleton et al. 2002) vond de groep van Mirnics verlaging

in expressie van de DLPFC bij genen betrokken bij de regulatie van het ornithine en polyamine metabolisme, het mitochondriale malaat shuttle systeem, de transcarboxylische zuurcyclus, het aspartaat en alanine metabolisme en het ubiquitine metabolisme. Deze dysfuncties kunnen samenhangen met veranderingen in de regulatie van het metabolisme in de hersenen.

Tabel 1: Overzicht van de gegevens uit onderzoeken naar gen-expressie profilering

Auteurs	Gebruikt weefsel	Patiënten/ Controles/	Beschrijving van de patienten	Significante veranderingen in expressie NB indien mogelijk zijn metabole processen in plaats van losse genen vermeld; waar de auteurs geen metabole paden beschreven zijn de losse genen vermeld. Betekenis gen-afkortingen: zie einde tabel;	Belangrijkste conclusie van dit onderzoek
Mirnics e.a. (2000)	DLPFC	11:11 gematchde paren	lft: 45 + 10.7 jaar ft: 8x schizofrenie, 3x schizoaffectief M+/M-: 9:2 intox: 5x alcoholabusus bijz: -	**Verlaagd:** PSYN-genen (genen betrokken bij presynaptische activiteit) Groei Receptoren GABA overdracht Glutamaat overdracht Energie metabolisme	De presynaptische aktiviteit is verlaagd bij schizofrenie
Mirnics e.a. (2001)	DLPFC	6:6 gematchde paren	lft: 46.5 + 10.7 ft: niet bekend M+/M-: 9:2 intox: 5x alcoholabusus	**Verlaagd:** RGS4	De neuronale signalering is verlaagd bij schizofrenie

(continued on next page)

Table 1: *(continued)*

Auteurs	Gebruikt weefsel	Patiënten/ Controles/	Beschrijving van de patienten	Significante veranderingen in expressie	Belangrijkste conclusie van dit onderzoek
			bijz: 1x suicide		
Middleton e.a. (2002)	DLPFC	10:10 gematchde paren	lft: 46.0 + 12.6 ft: niet bekend M+/M-: 8:2 intox: 3x alcohol-abusus bijz: 2x suicide	**Verlaagd:** Maleaat shuttle Tricarboxylic acid cyclus Asp/Ala metabolisme Ornithine metabolisme Ubiquitine metabolisme	Er is een verlaging van gen-expressie voor de regulering van 5 metabole pathways
Hemby e.a. (2002)	Entorhinale cortex	8:8 gematchde paren	lft: 83.9 + 3.5 ft: voornamelijk negatieve symptomen en langdurige hospitalisatie M+/M-: 0:8 intox: niet bekend bijz: -	**Verlaagd:** NMDA receptor 1 Synaptophysin SNAP23 SNAP25 SVAT Synaptotagmin 1&4 **Verhoogd:** GABA A_1subunit _7 subunit syntaxin	In deze studie werd niet gezocht naar een biologisch pad, maar werd gepoogd een aanzet te geven voor een profiel
(Mimmack e.a. (2002)	DLPFC	20:20 gematchde paren	lft: 65.3 ft: langdurige opname M+/M-: niet bekend intox: niet bekend bijz: -	**Verhoogd:** ApoL1 ApoL2 ApoL4	Een verhoging van apolipoproteines, dicht bij elkaar gelocaliseerd op chromosoom 22q12
Vawter e.a. (2002)	DLPFC	5:5	lft: 46.5 + 5.9 ft: niet bekend M+/M-: 5:0 intox: niet bekend bijz: -	**Verlaagd:** CALM3 UCHL1 NF2 GNL1 PI12 PSMA1 SLC10A1 POR MINK USP9X **Verhoogd:** RFXANK	Een veranderde functie van de synaptische signalering en proteolitische functies

Vawter e.a. (2001)	Pooled samples of cerebellum and DLPFC; Separate cohort of the middele temporal gyrus	15:15	lft: 49 ft: niet bekend M+/M-: niet bekend Intox: niet bekend Bijz: 3x suicide	**Verlaagd:** HINT UBE2N GRIA2	
Hakak e.a. (2001)	DLPFC	12:12 gematchde paren	lft: 44.2 + 11.7 ft: opgenomen, chronische symptomen M+/M-: 12:0 Intox: niet bekend Bijz: 0x suicide	**Verlaagd:** Myelinisatie **Verhoogd:** Ontwikkeling/ Plasticiteit Signaal transductie GABA transmissie Neurotransmissie Receptoren/Ionkanalen/Transporters	Veranderingen van expressie werden gevonden bij genen die te maken hadden met synaptische plasticiteit, neuronale ontwikkeling, neurotransmissie en signaal-geleiding.
Tkachev e.a. (2003)	DLPFC	15:15 gematchde paren	lft: 44.2 ft: niet bekend M+/M-: niet bekend intox: niet bekend bijz: -	**Verlaagd:** Oligodendrocyten cellijn Groei en differentiatie Volwassen myelinerende oligodendrocyten	Er werd een verminderde expressie gevonden van belangrijke oligodendrocytaire, myeline gerelateerde genen en transcriptiefactoren betrokken bij de regulatie van deze genen
Prabakaran e.a. (2004)	DLPFC (dit onderzoek vermeld behalve het genexpressie onderzoek ook de resultaten van eiwit en metabool onderzoek met behulp van resp. 2D-gele electroforese en	54:50 gematchde paren	Lft: 42.4 Ft: niet gespecificeerd M+/M-: allen medicatie Intox: niet gespecificeerd Bijz:-	**Verlaagd:** Glycolyse Electron transport Lipeiden synthese Cholesterol synthese Oxidatieve fosforilatie ATP synthese **Verhoogd:** Glycogeen gebruik Insuline afhankelijke signaalwegen	Er zijn aanwijzingen voor een chronisch hypoxische toestand in de perfrontale cortex wellicht ten gevolge van fundamentele mitochondriale stoornissen. Als gevolg hiervan is de

(continued on next page)

Table 1: *(continued)*

Auteurs	Gebruikt weefsel	Patiënten/ Controles/	Beschrijving van de patienten	Significante veranderingen in expressie	Belangrijkste conclusie van dit onderzoek
	NMR spectroscopie)			Carnitine transport systeem	glycogenolyse verrhoogd en is er een verstoord vetzuurmetabolisme.

Legenda gen symbolen:

ApoL1	Apolipoprotein L, 1	POR	P450 (cytochrome) oxidoreductase
ApoL2	Apolipoprotein L, 2	PSMA1	Proteasome subunit alphatype 1
ApoL4	Apolipoprotein L, 4	RFXANK	Regulatory factor X-associated ankyrin containing protein
CALM3	Calmodulin 3		
GNL1	Guanine nucleotide binding protein-like 1	SLC10A1	Solute carrier family 10, member 1
GRIA2	Glutamate receptor, ionotropic ampa 2	SNAP23	Synaptosomal associated protein, 23 kDa
HINT	Histidinen triad nucleotide binding protein	SNAP25	Synaptosomal associated protein, 25 kDa
MINK	Misshapen-like kinase 1	SVAT	Solute carrier family 18
NF2	Neurofibromin 2	UBE2N	Ubiquitin conjugating enzyme E2N
NSF	N-ethylmaleimide -sensitive factor	UCHL1	Ubiquitin carboxyl-terminal esterase L1

lftd leeftijd in jaren
ft fenotypische gegevens
m+/m- gebruikt medicatie/medicatievrij
intox intoxicatie
bijz bijzonderheden

Hemby et al. (2002) onderzochten meer dan 18.000 mRNA transcripten afkomstig uit de entorhinale cortex uit postmortemweefsel van patiënten met schizofrenie en controle personen.
Er werd een verschil gevonden in expressie van G-protein subunit i(alpha)1, glutamaat receptor 3, N-methyl-D-aspartaat receptor 1 en synaptophysine.

Vawter et al. (2001) vonden afwijkende expressie voor 21 genen bij patiënten met medicatie en 5 genen bij medicatie vrije patienten. Veel van de genen waren betrokken bij synaptische signalering en proteolytische functies.
In een vervolg onderzochten Vawter et al. (2002) de expressie in de DLPFC. Drie

genen lieten een consistente verlaging in expressie zien bij patiënten met schizofrenie in vergelijking met controles. Dit waren histidine triad nucleotide-binding protein (HINT), ubiquitin conjugating enzyme E2N (UBE2N) en de ionotrope AMPA glutamaat receptor.

Hakak et al. (2001) onderzochten eveneens de gen-expressie in de DLPFC van patiënten met schizofrenie en controles. Veranderingen van expressie werden gevonden bij genen die te maken hadden de synaptische plasticiteit, neuronale ontwikkeling, neurotransmissie en signaalgeleiding. Opmerkelijk is de veranderde expressie van genen betrokken bij myelinisatie wat er op lijkt te wijzen dat er een verstoring is in de functie van oligodendrocyten bij schizofrenie.

Drie onderzoeken zijn afkomstig van de groep van Bahn uit Cambridge:
Mimmack et al. (2002) onderzochten genen die eerder verbonden waren met schizofrenie, die conceptueel logisch zijn in het licht van onze huidige kennis van schizofrenie of die gelegen zijn op bekende chromosomale risico locaties. Er werd gebruik gemaakt van post-mortem weefsel van de DLPFC. Er werd een robuuste verhoging gevonden van apolipoprotein L1 (apo-L1) apo-L2 en apo-L4. Zes apo-L genen zijn dicht bij elkaar gelocaliseerd op chromosoom 22q12, gelegen bij de regio 22q11 die geassocieerd is met het velo-cardio-faciale syndroom en dat in 30% van de gevallen gepaard gaat met schizofreniforme symptomen.
Tkachev et al. (2003) onderzochten 15 patienten met schizofrenie, 15 met een bipolaire affectieve stoornis en 15 controles. Er werd een verminderde expressie gevonden van belangrijke oligodendrocytaire- en myeline gerelateerde genen en van transcriptiefactoren betrokken bij de regulatie van deze genen, zowel bij de bipolaire patienten, als bij die met schizofrenie. Tkachev e.a (2003) concluderen dat de veranderingen in expressie bij beide aandoeningen een hoge mate van overeenkomst vertonen wat er op kan duiden dat ze een gemeenschappelijke oorzakelijke en pathofysiologische achtergrond hebben.
Prabakaran et al. (2004) voerden een groot onderzoek uit met post-mortem weefsel van 52 patiënten. Zij vonden een sterke aanwijzingen voor een verlaagde glycolyse, verhoogde glycogeen depletie en een verstoord vetzuurmetabolisme. Gecombineerd met verstoringen in individuele genen concluderen zij dat er sprake is van gestoord glucose metabolisme, wellicht ten gevolge van verstoord mitochondriaal functioneren.

Conclusie

Onderzoek naar gen-expressie profilering bij schziofrenie is nog van recente datum. Tot nu toe heeft er nog geen standaardisatie van het onderzoek plaatsgevonden. Er worden verschillende micro-array's gebruikt, zijn er verschillende opwerkingstechnieken voor de monsters, verschillende scanners en verschillende statistische methodes. Deze situatie, die niet ongebruikelijk is voor een zich ontwikkelend vakgebied maakt het soms moeilijk de resultaten van de diverse studies onderling te vergelijken.
Ondanks deze beperkingen is het ons inziens mogelijk voorlopige aanwijzingen te vinden in de beschreven onderzoeken.
Mirnics et al. (2000) vinden dat genen die betrokken zijn bij presynaptische activiteit sterker verlaagd zijn dan anderen. Dit ondersteunt de hypothese dat schizofrenie gedeeltijk of primair een synaptische aandoening is.
Ook Hemby et al. (2002) vinden diverse veranderingen in de expressie van aan de synaptische functie gerelateerde genen, naast veranderingen in het G-eiwit gekoppelde signaleringsmechnisme.
Hemby ea (2002) en Vawter e.a (2002) vinden veranderingen in bij het glutamaatsysteem betrokken genen. Een relatie tussen glutamaterge dysfuncties en schizofrenie is frekwent beschreven.
Mimmack et al. (2002) heeft een verhoging in expressie gevonden voor Apo-L1, -L2 en -L4 die zijn gelegen op chromosoom 22q12.3, vlakbij de lokatie 22q11 die is geassocieerd met het velo-cardio-faciale syndroom. Een derde van de patienten met dit syndroom heeft schizofreniforme symptomen. Genen in de buurt van dit chromosoom zijn daarom uitermate geschikt als kandidaatgen.
De functie van deze apolipoproteinen in het centraal zenuwstelsel is nog onduidelijk, maar ze zijn waarschijnlijk betrokken bij het transport van cholesterol. Wellicht bestaat er een relatie tussen deze bevinding en die van Hakak et al. (2001) en Tkachev et al. (2003). Zij vonden afwijkingen in myelinisatie en oligodendrocytaire functie. Oligodendrocyten zorgen voor de myelinisatie in het centraal zenuwstel en zijn waarschijnlijk betrokken bij de overleving van axonen.
Een opvallende bevinding is dat geen van de onderzoeken veranderingen vindt in de expressie van genen gerelateerd aan dopaminerge functie en -metabolisme.

De meest coherente hypothese op basis van genexpressie onderzoek is naar onze mening geformuleerd door de groep van Bahn (zie Prabakaran et al. (2004)). Zij

concluderen dat er aanwijzingen zijn voor een chronisch hypoxische toestand in de prefrontale cortex, wellicht ten gevolge van fundamentele mitochondriale stoornissen. Als gevolg hiervan is de glycogenolyse verhoogd en bestaat er een verstoord vetzuurmetabolisme. Door vrije radicalen zouden beschadigingen in myelinisatie ontstaan.

We concluderen dat gen-expressie profilering interessante, maar zeer voorlopige resultaten heeft opgeleverd. Het heeft geleid tot het signaleren van verschillende functionele gengroepen (synaps-, glutamaat-, myelinisatie- en oligodendrocyt, en metabolisme gerelateerde genen) die een rol kunnen spelen in de pathogenese van schizofrenie. Verschillende van deze gengroepen zijn gelocaliseerd op bekende chromosomale risicolocaties voor schizofrenie. Tezamen vormen zij ondersteuning voor die theorieën die postuleren dat schizofrenie verzoorzaakt wordt door verstoringen in de synaptische stabiliteit en plasticiteit.
Er zijn aanwijzingen dat verstoringen in de myelinisatie en in vetmetabolisme eveneens een rol kunnen spelen.

Een aantal beperkingen zijn op dit moment aanwezig. De techniek is kwetsbaar voor variatie in afname en bewerking van lichaamsmateriaal en voor technische verschillen tussen laboratoria. De statistische methodes voor dit soort onderzoek zijn nog niet goed ontwikkeld. Voor toekomstig onderzoek is aan te bevelen dat standaardisatie van de methodes voor gegevensanalyse plaatsvindt. Van belang is vooral replicatie van bevindingen, met name vanwege het grote aantal genen dat onderzocht wordt en de daarmee geassocieerde kans op fout positieve uitslagen. Er zou ook meer uitwisseling van materiaal moeten komen zodat de onderzoekspopulaties aanzienlijk groter en statistisch meer relevant worden. Uiteindelijk zou de combinatie van dergelijke onderzoeken met de reeds bestaande kennis over chromosomale risico locaties en bestaande hypotheses kunnen leiden tot de formulering van nieuwe inzichten over schizofrenie.

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Chapter 7

Gene expression profiling of peripheral blood mononuclear cell's supports involvement of Akt1 and the apoptotic pathway PI3K/Akt in male schizophrenia patients with deficit syndrome. A pilot study

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Abstract

Objective

The authors' goal was to explore the potential utility of microarray gene expression of peripheral blood monunuclear cells (PBMC) by seeking to replicate findings of individual gene expression alterations in blood cells in schizophrenia and/or to identify aberrant expression values of known candidate genes for schizophrenia. Subsequently we would seek to identify deregulated metabolic- or signaling pathways associated with these genes.

Method

PBMC gene expression of 8 male subjects with recent-onset deficit syndrome schizophrenia were compared with 8 controls. Microarray screening was used to determine the gene expression profile. Differentially expressed genes were identified by three criteria: present in all subjects, $p<0.05$ for the mean difference between groups, fold change in expression $>= 1.2$. Of this initial group of deregulated genes, using an internet-based database (Ingenuity Pathway Knowledge Base (IPKB)), the functional relationships were investigated.

Results

Of the 54,675 probe sets 564 probe sets were significantly deregulated. Using this set of 564 deregulated probe sets the IPKB identified 16 significant functionally related networks. Nine canonical pathways are significantly deregulated below the $p=0.001$ level. These pathways include the phosphatidylinositide 3-kinase/AKT (PI3K/AKT) pathway ($p=0.0001$; including a significant decrease of AKT1 itself) and pathways involved in immune processes.

Conclusions

Our microarray findings confirm previous studies showing changes in individual AKT1 expression and changes in immune responses in schizophrenia. We extend these findings by showing involvement of associated pathways. Thus, microarray gene expression of PBMC's may indeed be useful in providing additional information about the molecular biological background of schizophrenia.

Introduction

The combination of gene expression profiling and biomics has been shown to be a powerful technology to identify changes in biological networks (Hood et al., 2004). Therefore, this technique holds great promise to unravel the biological basis of complex diseases such as psychiatric disorders like schizophrenia. However, its application for these studies is limited by the impossibility to obtain fresh brain tissue. Since there are major limitations of using post-mortem samples (Bahn et al., 2001) several researchers have turned to peripheral tissue to investigate the molecular basis of schizophrenia. A number of recent studies have explored the use of various types of white blood cell's (Bowden et al., 2006; Glatt et al., 2005; Middleton et al., 2005; Tsuang et al., 2005; Vawter et al., 2004). Taken together, these studies have shown interesting, but inconclusive results. Specifically, these studies have not yet shown converging (reproducible) results, probably due to methodological variation and heterogeneous patient samples. In addition, they have not clearly confirmed candidate genes and pathways that have previously been implicated in schizophrenia (Iwamoto and Kato 2006).
We reasoned that peripheral blood gene expression studies, in order to perform its putative 'window-on-the-brain' function, should be able to either replicate previous findings of *individual* gene expression alterations in blood cells in schizophrenia and/or identify aberrant expression values of known candidate genes for schizophrenia. Furthermore, as the strength of micro-array profiling lies in its potential to investigate pathways instead of single genes, findings of single genes which were previously associated with schizophrenia should be accompanied by changes in deregulated functional networks in which these genes participate. Moreover, these deregulated networks should be plausible in the light of current theories on schizophrenia pathology.
We therefore focused on changes in signaling- and metabolic pathways rather than changes in expression of single genes.

Based on these considerations we performed a preliminary study investigating the potential utility of whole-genome gene expression of peripheral blood mononuclear cell's (PBMC's) in schizophrenia. To limit sample heterogeneity, we only included male, recent onset schizophrenia patients, with deficit syndrome.

Methods

Subject selection

We limited inclusion to male patients (n=8) with recent onset (< 5 yr) schizophrenia, and all patients satisfied deficit syndrome criteria (Kirkpatrick et al., 1989) and exhibited considerable negative symptoms. Patient diagnosis was confirmed by the CASH interview (Andreasen et al., 1992). Six patients used clozapine, one used risperidone, and one was medication-free for more than two weeks.

We included 8 healty male, age-matched controls who were recruited among students of the Erasmus MC. See table 1 for a description of the subject characteristics. This study was approved by the Erasmus MC Ethics Committee. All participating subjects provided written informed consent after complete description of the study.

Samples

We paid meticulous attention to the processing of the blood samples, isolation of RNA and preparation of the microarray in order to minimize methodological variability.

30 ml of blood were drawn by single needlestick into heparinized tubes. All blood samples were obtained between 10.00 and 11.00 am to minimize diurnal variation.

Table 1: Subject characteristics

	Patients (N=8)	Controls (N=8)	
Mean age (sd)	24.0 (5.1)	23.1 (1.2)	p=.643
Etnicity	Caucasian (N=7)	Caucasian (N=7)	
	Asian (N=1)	Asian (N=1)	
Duration of untreated psychosis (weeks (sd))	69.8 (67.5) weeks		
Duration of illness (weeks (sd))	152.6 (84.9) weeks		
Medication	Clozapine (N=6)		
	Risperidone (N=1)		
	Medication free > 2weeks (N=1)		
Cumulative lifetime antipsychotic usage (haloperidol eq (sd))	3463 (1835) mg		
PANSS total (sd)	68.8 (9.6)		
PANSS positive (sd)	10.3 (1.5)		
PANSS negative (sd)	25.7 (6.2)		
PANSS general (sd)	32.8 (4.1)		

PBMC's were isolated by Ficoll gradient separation, started within 20 minutes after the drawing of blood and performed with minimum time variation.
RNA was immediately isolated, stabilized and frozen at -80 C until further analysis. Before freezing, total RNA quality and integrity was assessed.
After thawing the isolated mRNA was biotinylated. Biotinylated cRNA was hybridized to the Affymetrix Human Genome U133 plus 2.0 GeneChip© microarray containing 54,675 probe sets (Affymetrix Co.)
The arrays were scanned and analyzed using Affymetrix Microarray Suite 4.2 software.

Quality control: Methodological variation and environmental effect on PBMC gene expression.

To explore the reproducibility of the complete procedure we investigated a sample taken in duplo at the same time-point from the same subject. To explore the effects of intra-individual variation in PBMC gene expression within a limited time frame, we investigated the same individual at two time points (with one week interval). To explore the variation on PBMC gene expression in this age group due to environmental (i.e. non-genetic) factors, we measured the PBMC gene expression correlation of one pair of monozygotic twins. Correlation of the expression profiles was performed with the OmniViz software package.

Statistical analysis

Data were normalized by quantile normalization. Minimum thresholds were set at 90. Expression levels from each probe set in every sample were calculated relative to the geometric mean and logarithmically transformed (base 2) to ascribe equal weight to gene expression levels with equal relative distance to the geometric mean. We applied three criteria to identify differentially expressed genes between patients and controls: (1) all calls present in both patients and controls (thus in 16 out of the 16 samples), or present in all patients, or present in all controls, (2) t-test $p<0.05$ for the mean difference between groups, (3) fold change for the ratio median schizophrenia vs controls equals or exceeds 1.2.
We did not apply a correction for repeated measurements, as we find this approach to be overly conservative for microarray experiments (see discussion). Instead, we opted for the initial identification of a large group of deregulated genes, probably including false positives. We would then narrow down this initial group of deregulated genes by a three tier approach: (1) investigating their functional relationship

by pathway analysis; (2) identify focus genes within these functionally related genes which are known high risk genes for schizophrenia; (3) linking the initially identified set of genes by investigating their involvement in metabolic and signaling pathways.

Pathway analysis.

The genes considered to be differentially expressed according to the initial three criteria were used for network analysis.

Probe set id's were imported into the Ingenuity Pathway Analysis (IPA) software tool (Ingenuity Systems, Mountain View, CA; www.ingenuity.com; version october 2006). The IPA defines genetic networks which describe functional relationships between gene products based on known interactions in the literature (Kasamatsu et al., 2005). The genes we identified as differentially expressed were mapped to genetic networks available in the IPA database.

The IPA calculates a significance score (using the right-tailed Fisher's exact test and expressed as a p-value) for networks and pathways by computing the number of deregulated genes that participate in a network or pathway relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the IPA. The significance value associated with networks and pathways is a measure for how likely it is that genes from the dataset participate in that function or pathway.

Results

Quality control: test for variation in PBMC gene expression and methodological variation

In order to use PBMC gene expression profiling as a tool to identify signaling pathways involved in schizophrenia, we reasoned that it should meet three requirements: 1) Samples take at the same time-point but processed independently of each other should be highly correlated (methodological variation). 2) Samples taken from the same individual within a short time span (1 week) should be highly correlated (within subject variation). 3) Samples taken from a pair of monozygotic twins should be highly correlated (environmental variation on PBMC gene expression). We examined the correlation of the expression profiles of the three paired 'quality control' samples, the patients, the controls with the OmniViz software package.

Indeed all three paired samples correlated more with each other than with the other samples (monozygote twins: r=0.522; same time-point: r=0.796; 1-week interval: r=0.356)

Global significance analysis of gene expression

Of the 54,675 probe sets on the array 565 probe sets were significantly deregulated between patients and controls (**supplementary data file** - accessible through www.psych.nl ▸ 'people' ▸ 'beveren' ▸ 'list of deregulated genes chapter 7') according to the three defined criteria. Of these, 273 were upregulated, 291 were downregulated. These 564 probe set id's were uploaded into the IPA. 212 genes were eligible for generating networks.

Pathway analysis

Following the three tier approach described we first investigated the functional relationship of the initial group of 564 differentially expressed genes. The IPA identified 16 networks which are significant according to the IPA criteria.
The most significant network consists of 35 genes (see **figure 1**).
The focus genes in this network are involved in immune response (N=18 genes), cell-to-cell signaling and interaction (N=15 genes), and cellular development (N=22).
We then investigated this most significant network for the presence of known candidate genes for schizophrenia. The only gene fulfilling this criterium is AKT1(figure 1). We found AKT1 expression to be decreased in the patient group (t-test p=0.008; log fold change= -0.045; relative expression levels patients vs controls (log2 median(sd): 313±(51) vs 401±(63)).

Figure 1

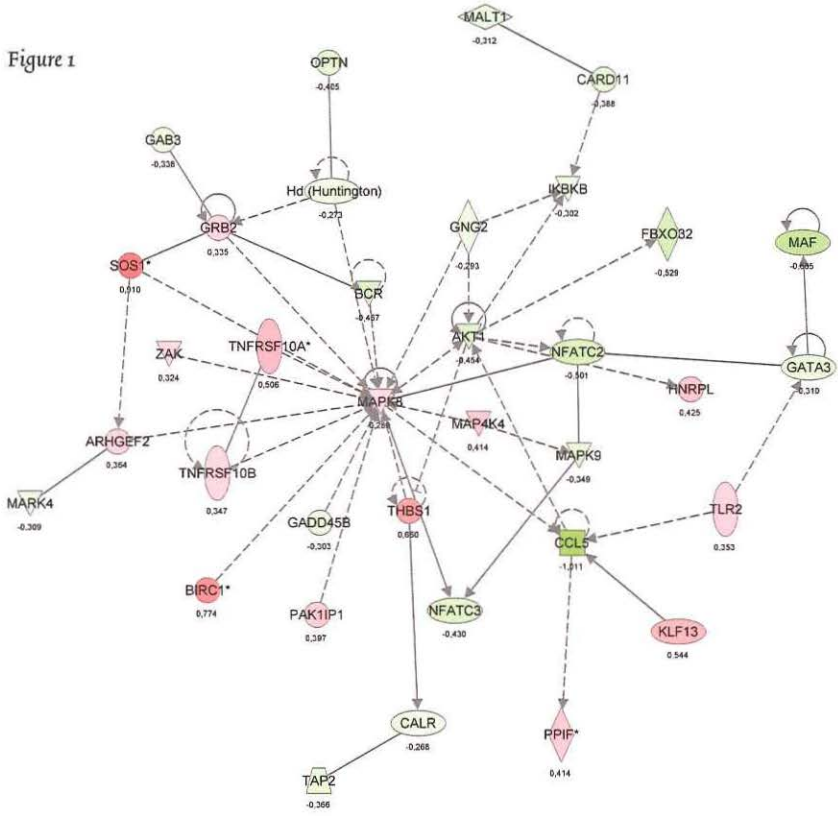

Figure 1. The most significant deregulated network (IPA score 52) as identified by the IPA (version october 2006).

The network shows functional relationships between genes. Network annotation in terms of node shape:

circle: other; hashed square: growth factor; hashed rectangle: ion channel; diamond: enzyme; oval: transcription factor; triangle facing down: kinase; and triangle facing up: phosphatase.

Edge types: line with arrow, acts on and line without arrow, binds to.

*: multiple probe sets in dataset for this gene.

The figures within within the nodes represent log2 fold change in expression in the patients vs the controls; negative values (green nodes) imply decreased expression in the patients and positive values (red nodes) imply increased expression in the patients.

Gene abbreviations: AKT1: v-akt murine thymoma viral oncogene homolog 1- ARHGEF2: rho/rac guanine nucleotide exchange factor (GEF) 2 - BCR: breakpoint cluster region - BIRC1: baculoviral IAP repeat-containing 1 - CALR: calreticulin - CARD11: caspase recruitment domain family, member 11 - CCL5: chemokine (C-C motif) ligand 5 - FBXO32: F-box protein 32 - GAB3: GRB2-associated binding protein 3 - GADD45B: growth arrest and DNA-damage-inducible, beta - GATA3: GATA binding protein 3 - GNG2: Guanine nucleotide binding protein (G protein), gamma 2 - GRB2: growth factor receptor-bound protein 2- HD: huntingtin (Huntington disease) - HNRPL: heterogeneous nuclear ribonucleoprotein L - IKBKB: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta - KLF13: Kruppel-like factor 13 - MAF: v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) - MALT1: mucosa associated lymphoid tissue lymphoma translocation gene 1 - MAP4K4: mitogen-activated protein kinase kinase kinase kinase 4 - MAPK8: mitogen-activated protein kinase 8 - MAPK9: Mitogen-activated protein kinase 9 - MARK4: MAP/microtubule affinity-regulating kinase 4 - NFATC2: Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2- NFATC3: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3 - OPTN: optineurin - PAK1IP1: PAK1 interacting protein 1 - PPIF: peptidylprolyl isomerase F (cyclophilin F) - SOS1: son of sevenless homolog 1 (Drosophila) - TAP2: transporter 2, ATP-binding cassette, sub-family B (MDR/TAP) - THBS1: thrombospondin 1 - TLR2: toll-like receptor 2 - TNFRSF10A: tumor necrosis factor receptor superfamily, member 10a: TNFRSF10B: tumor necrosis factor receptor superfamily, member 10b - ZAK: sterile alpha motif and leucine zipper containing kinase AZK region -

As a third step we investigated the involvement of all 565 initially identified probe sets in metabolic and signaling pathways using the 'canonical pathway' function of the IPKB. This identified 9 pathways in which more probe sets from the 565-probe dataset participated than expected by chance ($p<0.001$) (see **table 2**).

Among these is the PI3K/AKT pathway ($p=0.0001$) which is involved in growth factor mediated apoptosis and cell-survival. The other 8 pathways are mainly involved in immune processes. **Figure 2** shows the PI3K/AKT canonical pathway in detail and the deregulated genes involved in its action.

Table 2: Disregulated canonical pathways as identified by the IPKB. Significances as provided by the IPKB.

canonical pathway	Significance
Interferon signaling	0.000005
EGF signaling	0.00006
IL-6 signaling	0.00008
PI3K/Akt signaling	0.00001
B cell receptor signaling	0.0002
IL-4 signaling	0.0004
SAP/JNK signaling	0.0006
IL-2 signaling	0.0006
T cell receptor signaling	0.0007

Figure 2

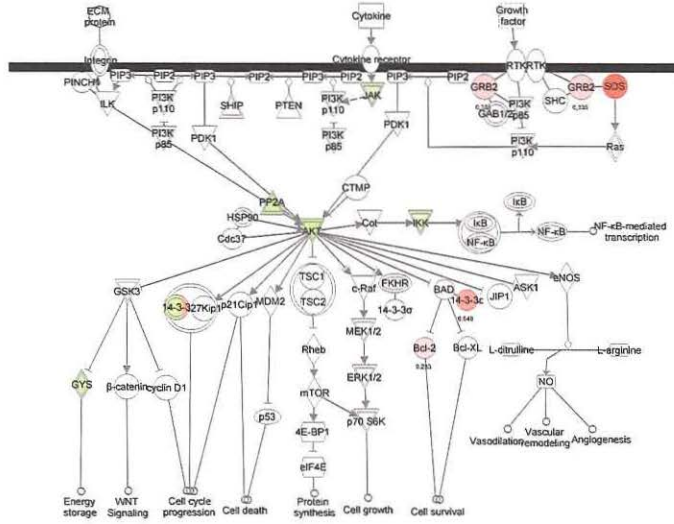

Figure 2. The PI3K/Akt canonical pathway.

Genes with aberrant expresson values are shown coloured. Green: decreased expression in the patients and red: increased expression in the patients. White, no significant change in expression between patients and controls. Criteria for significance: see text.

abbreviations: 14-3-3ε (YWHAE): tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide - 14-3-3θ (YWHAQ): tyrosine-3 monooxygenase/tryptophan-5 monooxygenase activation protein, theta polypeptide - 14-3-3σ (SFN): stratifin - 4E-BP1 (EIF4BP1): eukaryotic translation initiation factor 4E binding protein - AKT1: v-akt murine thymoma

viral oncogene homolog 1 ARHGEF2: rho/rac guanine nucleotide exchange factor (GEF) 2 - ASK1 (MAP3K5): mitogen-activated protein kinase kinase kinase 5 - BAD: Bcl-2 antagonist of cell death - Bcl-2: B-cell CLL/lymphoma 2 - Bcl-XL (BCL2L1): Bcl 2-like 1 - BCR: breakpoint cluster region - BIRC1: baculoviral IAP repeat-containing 1 - CALR: calreticulin - CARD11: caspase recruitment domain family, member 11 - CCL5: chemokine (C-C motif) ligand 5 - c-RAF (RAF1): v-raf-1 murine leukemia viral oncogene homolog - eIF4E: eukaryotic translation initiation factor 4E - eNOS (NOS3): nitric oxide synthase 3 - RK1/2 (MAPK1): mitogen-activated protein kinase 1 - FBXO32: F-box protein 32 - FKHR (FOX01A): forkhead box 01A - GAB3: GRB2-associated binding protein 3 - GAB1/2: GBR associated binding protein - GADD45B: growth arrest and DNA-damage-inducible, beta - GATA3: GATA binding protein 3 - GNG2: Guanine nucleotide binding protein (G protein), gamma 2 - GRB2: growth factor receptor-bound protein 2 - HD: huntingtin (Huntington disease) - HNRPL: heterogeneous nuclear ribonucleoprotein L - IKBKB: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta - JAK1: janus kinase 1 - JIP1 (MAPK8IP1): mitogen-activated protein kinase 8 interacting protein 1 - KLF13: Kruppel-like factor 13 - MAF: v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) - MALT1: mucosa associated lymphoid tissue lymphoma translocation gene 1 - MAP4K4: mitogen-activated protein kinase kinase kinase kinase 4 - MAPK8: mitogen-activated protein kinase 8 - MAPK9: Mitogen-activated protein kinase 9 - ARK4: MAP/microtubule affinity-regulating kinase 4 - MDM2: transformed mouse 3T3 cell double minute 2 -MEK1/2 (MAP2K1/2): mitogen-activated protein kinase kinase 1/2 - mTOR (FRAD1): FK506 binding protein 12-rapamycin associated protein 1 - NFATC2: Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 - NFATC3: nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3 - NO: nitric oxide - PTN: optineurin - P21Cip1 (CDKN1A): cyclin-dependent kinase inhibitor 1A - p27Kip1 (CDKN1B): cyclin-dependent kinase inhibitor 1B - p53: tumor protein p53 - p70S6K (RPS6KB1): ribosomal protein S6 kinase, 70 kDa, polypeptide 1 - PAK1IP1: PAK1 interacting protein 1 - PI3K: phosphoinositide-3 kinase - PINCH1 (LIMS1): LIM and senescent cell antigen-like domains - PPIF: peptidylprolyl isomerase F (cyclophilin F) - PTEN: phoshatase and tensin homolog - Rheb: ras homolog enriched in brain - SHIP (INPP5D): inositol polyphosphatase-5-phosphatase - SOS1: son of sevenless homolog 1 (Drosophila) - TAP2: transporter 2, ATP-binding cassette, sub-family B (MDR/TAP) - THBS1: thrombospondin 1 - TLR2: toll-like receptor 2 - TNFRSF10A: tumor necrosis factor receptor superfamily, member 10a - TSC1: tuberous sclerosis

1 - TNFRSF10B: tumor necrosis factor receptor superfamily, member 10b - ZAK: sterile alpha motif and leucine zipper containing kinase AZK.

Discussion

In this study we provide evidence that PBMC gene expression profiling is a useful tool to get more insight in the signaling pathways involved in schizophrenia. We have chosen to investigate the specific deficit syndrome subtype of schizophrenia in male, recent onset patients in order to homogenize our sample. Gene expression alterations in peripheral blood samples have recently been used to differentiate individuals with schizophrenia from healthy controls and discordant twins (Bowden et al., 2006; Glatt et al., 2005; Middleton et al., 2005; Tsuang et al., 2005; Vawter et al., 2004). However, these studies have not yet given uniform results. This may be caused by methodological differences, but more importantly, these studies have not considered schizophrenia as a heterogeneous disorder. Combining gene expression profiles and phenotypic data may be a step towards specifically identifying subtypes of schizophrenia based on gene expression profiles (Bowden et al., 2006).

We found deregulation of the PI3K/AKT pathway (p=0.0001; including a significant decrease of AKT1 itself) and of pathways involved in immune processes in PBMC's of recent-onset male schizophrenia patients with deficit syndrome.
Our findings confirm the previously reported decreased expression of AKT1 in lymphocyte derived cell-lines (Emamian et al., 2004). They further supported the role of impaired AKT signaling by showing decreased expression of cortical AKT1 in post-mortem samples of a separate cohort of schizophrenia patients, association of AKT1 haplotypes in a separate large patient sample and prepulse inhibition disturbances in AKT1 -/- mice. They also showed decreased phosphorylation of the AKT1 substrate GSK3β, with unchanged expression values of GSK3β. After these initial findings by Emamian et al. (2004), two studies confirmed the association of AKT1 haplotypes and schizophrenia (Ikeda et al., 2004; Schwab et al., 2005) while two other studies failed to find an association (Ohtsuki et al., 2004; Liu et al., 2006).
To our best knowledge no confirmation of aberrant AKT1 expression in lymphocytes as reported in the key study by Emamian et al. (2004) has been reported yet.

It should be noted that we can not exclude the possibility that the decreased expres-

sion of AKT1 in our sample may be caused by the use of antipsychotic medication (mostly clozapine in our sample). Emamian et al. (2004) could not identify an effect of the antipsychotic haloperidol on AKT1 protein levels in vivo, though they did find a haloperidol effect on the phosphorylation status of the three AKT isoforms. Zhao et al. (2006) reported that mice treated with clozapine showed a decrease in AKT levels without a change in AKT phosphorylation status. However, Basta-Kaim et al. (2006) reported the opposite finding in mouse splenocytes, namely that clozapine changed the phosphorylation status of AKT, but the total AKT level remained unchanged.

Three isoforms of AKT encoded by three separate genes have been found in mammalian cells, AKT1, AKT2 and AKT3. Congruent with the findings of Emamian et al (2004) we find only decreased expression of AKT1, not of the isoforms AKT2 and AKT3. We also do not find altered expression of GSK3β.
AKT is a key element in the PI3K/AKT signaling pathway. Genes that are present in the initially identified deregulated dataset, and which were found to be functionally related as well, are associated with PI3K/AKT signaling as well as with immune processes (interferon-, IL-6-, and B- and T-lymphocyte signaling; see table 2).

Apart from the findings by Emamiam et al (2004), the circumstantial evidence for a role of the PI3K/AKT pathway in schizophrenia is considerable (Kalkman 2006). In the past years evidence has been collected that one of the major functions of AKT is to promote growth-factor mediated cell survival and to block programmed cell death or apoptosis (Kandel and Hay 1999). Specifically, AKT functions as the downstream effector of growth factor mediated cell-survival. Inadequate apoptotic neuronal processes form the key element of the leading etiological theory on schizophrenia, the neurodevelopmental hypothesis (Marenco and Weinberger 2000). Growth factors like insulin, Insulin-like Growth Factor-1 (IGF-1), Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neuregulin-1, and Epidermal Growth Factor (EGF) lead to phosphorylation of AKT (Huang et al 2005; De Sarno et al., 2005; Nitta et al., 2004; Subramamiam et al., 2005; Li et al., 2004; Gambarotta et al., 2004). In schizophrenia, aberrant levels of NGF and BDNF have been found present in both brain and peripheral blood (Shoval and Weizman 2005; van Beveren et al., 2006). Aberrant levels in schizophrenia of EGF have been found present in blood (Futamura et al., 2002). Neuregulin-1 is considered a strong candidate gene for schizophrenia (Stefansson et al., 2002).

Moreover, the PI3K/AKT pathway is involved in glucose metabolism (Kalkman 2006; Kandel and Hay 1999). Disturbances in glucose metabolism have been repeatedly associated with schizophrenia (Bushe and Holt 2004).

Apart from these two processes (growth-factor mediated cell survival and glucose metabolism), the PI3K/AKT pathway is involved in a number of other processes which may be linked to schizophrenia.
For an extensive argumentation on the putative relationship between PI3/AKT signaling and schizophrenia, see Kalkman (2006).

Our data also suggest altered gene expression in immune pathways, most notably the interferon and IL-6 canonical pathways. Several reports have shown alterations in immune-related functions in schizophrenia (reviewed in Gladkevich et al. (2004)). Most studies describe a group of schizophrenic patients characterized by elevated IL-6 production.This shift seems to be markedly distinct in patients with predominant negative symptoms (Schwarz et al., 2001). We do not find altered IL-6 expression in our samples; however, we do find involvement of deregulated genes in the canonical IL-6 signaling pathway.
Decreased production of interferon-gamma (INF-γ) has also been reported in schizophrenia (Rothermundt et al., 1996). Specifically negative symptoms have been associated with decreased INF-γ levels in blood (Inglot et al., 1994). Like it is the case for IL-6, we do not find altered INF-γ expression but we do find a highly significant change in the canonical INF-signaling pathway. Thus, our findings of immune alterations in a deficit syndrome patient sample (in which negative symptoms are a predominant feature) are congruent with the existing literature.

With the present knowledge it is virtually impossible to make sensible power calculations for the optimal sample size for this type of research as the number of genes involved in schizophrenia and their variance is unclear. This is particularly relevant in our approach investigating a cell-type that not exhibits the primary pathology (such as PBMC's), as some or most of the disregulations we seek to identify will be of small effect size. This is why we chose the relatively small 1.2 fold change as the cutoff criterium and to investigate a phenotypically specific subtype.

When studying an acccessible tissue such as PBMC's as a potential useful surrogate for gene expression in the central nervous system the correlation between PBMC

and central nervous system gene expression should be taken into consideration. A recent analysis has compared brain and blood gene expression (Sullivan et al., 2006) and reported a high correlation between brain and blood expression for the Gene Ontology (GO) biological process 'intracellular signaling cascade', of which PI3K/AKT signaling is a subprocess. This makes the PI3K/AKT signaling pathway probably more suitable as a general marker of the disease and as a 'window on the brain' process as compared to immunological processes. Immunological disturbances may be more linked to the specific tissue we examined, namely PBMC's.

Taken together our study confirms decreased expression of AKT1, suggests deregulation of the PI3K/AKT pathway and supports previous findings regarding involvement of IFN and IL-6 in schizophrenia. Furthermore, our approach suggests that homogenizing the hetereogeneous schizophrenia syndrome based on phenotypic phenomena (such as the deficit syndrome) may be a feasible strategy to reduce sample variance.
As our primary goal was to explore the potential utility of gene expression of PBMC's to investigate schizophrenia we conclude that our findings suggest that PBMC gene expression indeed may provide additional information about the molecular biological background of schizophrenia.

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Chapter 8

Marked reduction of AKT1 expression and deregulation of AKT1-associated pathways in Peripheral Blood Mononuclear Cells of schizophrenia patients

Submitted

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Abstract

Background

Schizophrenia is caused by environmental factors as well as complex genetic factors. This has hampered our understanding of the molecular basis of schizophrenia. However recent studies have suggested that deregulated AKT1 signaling is associated with schizophrenia. We hypothesized that if this is indeed the case, we should observe decreased AKT1 expression and deregulation of AKT1 regulated pathways in Peripheral Blood Mononuclear Cells (PBMCs) of schizophrenia patients.

Objectives

To examine PBMC expression levels of AKT1 in schizophrenia patients (treated, medication-free or -naive) versus controls, and to examine whether functional biological processes in which AKT1 plays an important role are deregulated in schizophrenia patients.

Methods

We first performed a small pilot study of patients (N=8) and controls to investigate the feasibility of PBMC gene expression analysis to identify previously identified schizophrenia risk-genes. Even though this study was small, it indicated that AKT1 was differentially expressed in PBMCs. A main follow-up study was then initiated using PBMCs of male, recent onset (<5 years) schizophrenia patients, to test our a-priori hypothesis that AKT1 expression and AKT regulated pathways are deregulated in these patients.

Results

The pilot study (N= 8 patients) showed significantly decreased PBMC expression of AKT1 ($p=0.008$). The main study (N=41 patients) replicated this finding ($t= -4.25$, $p<0.001$). Importantly, AKT1 expression was decreased in antipsychotic-free/naive patients (N=11), in florid psychotic (N=20) and in remitted (N=21) patients. A total of 1224 genes were differentially expressed between patients and controls (False Discovery Rate (FDR) =0.05). Combining the deregulated geneset with AKT1 signaling showed deregulated pathways involved in a large number of cellular processes: immune system, cell adhesion and neuronal guidance, neurotrophins and (neural) growth factors, oxidative stress and glucose metabolism, and apoptosis and cell-cycle regulation.

Conclusions

We show significantly decreased PBMC gene expression of AKT1 in male, recent-onset schizophrenia patients. Decreased AKT1 was observed in antipsychotic-free or -naive, florid psychotic, remitted, and smoking or non-smoking schizophrenia patients. In addition, we could not identify a correlation between AKT1 expression, and age, duration of illness, and clinical symptom severity (PANSS positive and negative subscales). Taken together, our observations suggest that decreased PBMC AKT1 expression is a stable trait in recent onset, male schizophrenia patients. We identified several AKT related cellular processes which were deregulated in these patients, a majority of which play a prominent role in current schizophrenia hypotheses. Hence, our findings supports the notion that PBMCs are useful to investigate the molecular biology of schizophrenia.

Introduction

Whole-genome gene expression studies using peripheral blood cells, have yielded an extensive list of deregulated genes in schizophrenia patients (Vawter et al., 2004; Middleton et al., 2005; Tsuang et al., 2005; Glatt et al., 2005;). Although there are obvious limitations to using these cells to investigate a CNS disease, they do circumvent some of the confounding factors present in post-mortem brain samples of schizophrenia patients, (e.g. life-long exposure to psychotropic medication and drugs of abuse, and the impact of long-term hospitalization). Moreover, lymphocytes can be collected in the early phases of the disorder. Peripheral gene expression can be informative about gene expression in the central nervous system as whole blood shows significant gene expression similarities with multiple central nervous system tissues, and the expression levels of many classes of biologically relevant processes are not significantly different between whole blood and prefrontal cortex (Sullivan et al., 2006; Naydenov et al., 2007). In patients with schizophrenia, a comparison of gene expression profiles from brain tissue with profiles from peripheral blood cells, identified genes that were common to both tissues, confirming the validity of gene expression profiling of blood for detecting schizophrenia biomarkers (Tsuang et al., 2005; Glatt et al., 2005). Specifically, the combination of gene expression data and bioinformatics provides an opportunity to investigate perturbations in functional processes over alterations in individual gene expression (Konradi, 2005).

Converging evidence from several studies has resulted in several candidate risk-genes for schizophrenia (REF toevoegen). Among these is the serine-threonine protein kinase AKT gene family (also known as protein kinase B (PKB) which includes three members (AKT1, AKT2, and AKT3), that possess partially redundant functions and contribute to several cellular functions including cell growth, survival, and metabolism. AKT has several substrates, most notable among them glycogen synthase kinase 3α (GSK-3α) and GSK-3β, both of which are inhibited by AKT in response to various external cellular stimuli (Woodgett, 2005).
Gain or loss of AKT activity has been associated with several human diseases, including cancer and type 2 diabetes (Woodgett, 2005; Arguello and Gogos, 2008). An influential article by Emamian and colleagues (Emamian et al., 2004) showed a 68% reduction of AKT1 protein levels in lymphocyte-derived cell lines of schizophrenia patients, and presented several additional converging lines of evidence suggesting involvement of AKT1 in schizophrenia, including disturbed prepulse inhibition in

Akt-/- mice, decreased expression of AKT1 in post-mortem brains of schizophrenia patients, and association of AKT1 variants with schizophrenia.

To test whether previously identified candidate risk-genes are indeed deregulated in Peripheral Blood Mononuclear Cells (PBMCs) from schizophrenia patients, we undertook a two-step approach. First, we performed a small pilot study in which we investigated whether known schizophrenia risk-genes genes are indeed differentially expressed between PBMCs of patients and controls. This pilot study identified AKT1 as such a gene.
For the second phase of the study, we used a larger, carefully selected homogenous sample of only young, adult men The patients were selected in such a way that half of them were suffering from a florid psychois, the other half were remitted patients. In this second study we tested whether AKT1 expression is decreased in PBMCs of schizophrenia patients as compared to controls (*hypothesis 1*), and whether lower AKT1 expression in patients will lead to deregulation of functional biological processes in which AKT1 plays an important role (*hypothesis 2*). In addition, we reasoned that a meaningful risk-gene should also be deregulated in antipsychotic-free patients (*hypothesis 3*).
Finally, the underlying hypothesis of all studies investigating a peripheral tissue in schizophrenia is the existence of subtle, disease-related effects in all tissues of the body, but which only exert a detrimental/disease-causing effect in the brain, ultimately resulting in the (heterogeneous) schizophrenia phenotype. This is an important hypothesis, warranting validation: non-neural tissues are clearly convenient alternatives to post-mortem brain samples, but questions remain as to how informative gene expression studies based on peripheral samples will be (Matigian et al., 2008). So, our general objective was to provide a proof-of-principle study on the subject of PBMC gene expression in schizophrenia, by looking at known risk-genes.

Methods

Subject selection (pilot study and main study)

Male patients presenting with recent-onset schizophrenia at the department of psychiatry of the Erasmus University Medical Center, Rotterdam (EMC) were candidate to participate in the study. The EMC operates an early recognition and treatment clinic for patients with psychotic symptoms. Patients are referred to the

EMC by primary caretakers and general mental health care facilities throughout the Rotterdam region (1.2 million inhabitants), an industrialized area with a mixed population of Dutch ancestry and immigrants, mainly from Northern-Africa, Turkey and the Dutch West-Indies. Patients are referred to the EMC, with acute psychotic symptoms. The EMC also offers prolonged ambulatory treatment for patients with schizophrenia after remission of acutely psychotic symptoms.
Eligible for inclusion were male patients diagnosed with schizophrenia or schizophreniphorm disorder according to DSM IV criteria after a Comprehensive Assessment of Symptoms and History interview (CASH)(Andreasen et al., 1992) and by consensus between two senior psychiatrists who were blind to the expression results at the time of diagnosis. Additional criteria were recent onset (defined as duration of illness < 5 years) and age > 15 and < 36 years.
Exclusion criteria were the presence of any somatic or neurological disorders as investigated by routine clinical and laboratory examination performed at admission, and abuse of heroin, cocaine, or alcohol. Cannabis abuse was not an exclusion criterium, to warrant the generalizability of our results, as patients often use cannabis. Concomitant use of mood-stabilizers and/or antidepressants was an exclusion criterium, as these agents are known to influence AKT expression and/or phosphorylation status.

For the pilot study we included stabilized schizophrenia patients (N=8).
For the main study (N=46) we included two groups of patients; group 1: stabilized patients from our outpatient clinic; group 2: acutely psychotic recent-onset patients from our inpatient clinic. In this way we would be able to assess the impact of variable Duration of Illness (DUI) on AKT1 expression, and to assess the effect of prolonged exposure to antipsychotic medication on AKT1 expression. Moreover, inclusion of acutely psychotic, never- or minimally treated patients enabled us to include antipsychotic-naïve and -free patients (see also description of the deferred consent procedure).
Age- matched controls (N=8 and N= 30 for the pilot and main study respectively) were recruited from the students and staff of the EMC medical school and hospital. Patients and controls participating in the main study did not participate in the pilot.
All participating subjects were diagnosed and screened by a single senior psychiatrist (NJMvB) who is trained in the CASH interview, and final inclusion was reached based on consensus between NJMvB and another senior psychiatrist (CR).

Consent and deferred consent

All participating subjects provided written informed consent after complete description of the study.

For those patients in the main study who were severely psychotic and too disturbed to provide consent (group 2), a separate procedure was applied in which informed consent was initially given by a first-degree relative and final written consent was sought within six week from the patients themselves ('deferred consent'). Blood samples for these patients were collected with a vacutainer system together with the samples which are part of the regular clinical screening, so no additional venapunction was needed. Blood samples and data from patients who withheld consent after six weeks were destroyed. This procedure enabled us to include also the most severely psychotic patients.

This study was approved (including the deferred consent procedure) by the Erasmus University Medical Center Institutional Review Board and was conducted according to the declaration of Helsinki.

Sample preparation

We paid meticulous attention to the processing of the blood samples, isolation of RNA and preparation of the microarray in order to minimize methodological variability. Samples from both patients and controls were taken in the same room, with venapunction performed by the same (experienced) research nurse. From each participant 30 ml of blood was drawn into heparinized tubes. All blood samples were non-fasting and obtained between 10.00 and 11.00 am to minimize diurnal variation. Samples were transported immediately to the laboratory, each time by the same individual. All subsequent laboratory procedures including biotinylation and hybridization to the microarrays were performed by the same analyst.

PBMC's were isolated by Ficoll gradient separation, started within 20 minutes after the drawing of blood and performed with minimum time variation. Cells were subsequently disrupted (Qiashredder kit; Qiagen), and RNA was isolated (RNeasy minikit; Qiagen) with an additional DNAse digestion step (RNase-free DNase set; Qiagen), all according to the manufacturer's protocol, diluted in nuclease free water, and frozen at -80 C before use. Before freezing, RNA purity and quantity was assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). After thawing the isolated RNA was biotinylated into cRNA using the One-Cycle Target Labeling and Control Reagents Kit (Affymetric Co) according to the manufacturer's protocol. Before hybridisation RNA quality and integrity was assessed

using the Agilent 2100 BioAnalyzer (Agilent). Biotinylated cRNA was hybridized to the Affymetrix Human Genome U133 plus 2.0 GeneChip© microarray containing 54,675 probe sets (Affymetrix Co). Each sample was individually biotinylated and hybridized to an individual microarray. Biotinylation was performed in batches with randomisation of patients and controls according to their ratios across the batches. The arrays were scanned and analyzed using Affymetrix Microarray Suite 4.2 software.

Samples of the pilot study were scanned in one single batch. Scanning for the main study was performed in three batches with randomization of patients and controls according to their ratios across the batches.

Statistical- and functional analysis

Pilot study

Raw intensities were normalized by quantile normalization. Minimum thresholds were set at 90.

We applied three criteria to identify differentially expressed genes between patients and controls: (1) all calls present in both patients and controls, or present in all patients, or present in all controls, (2) t-test $p<0.05$ for the mean difference between groups, and (3) fold change for the ratio mdian schizophrenia vs controls equals or exceeds 1.2. We did not apply a correction for repeated measurements for the pilot, as this is a hypothesis generating study. Instead, we opted for the initial identification of a large group of deregulated genes, probably including false positives. We would then narrow down this initial group of deregulated genes by investigating their functional relationship by pathway analysis, eventually leading to identify focus genes within functionally related genes known to be high-risk genes for schizophrenia.

Main study

Raw intensities were normalized by quantile normalization. Initial quality control investigated the effect of scanning date and array intensities. Further data analysis was done using OmniViz version 5.0 (Biowisdom), Partek, and R program. Minimum thresholds were set at 30. Scanning data effects on expression values were removed using Partek's software package.

As the hypothesis following the pilot data is that AKT1 expression is altered in PBMC's of patients, we investigated AKT1 expression levels between patients and controls, using students t-test (SPSS 16.0 statistical software). To identify transcripts

that were differentially expressed patients and controls without an a priori hypothesis we used analysis of variance (ANOVA) with Benjamini-Hochberg correction for multiple comparisons, as provided by Partek's software package, with False Discovery Rate (FDR) set to 0.05. We did not specify a minimal Fold-Change (FC) cut-off value, as it has been shown that alterations in gene expression in Akt-/- mice can be significant and informative, though with minimal FC (Lai et al., 2006)

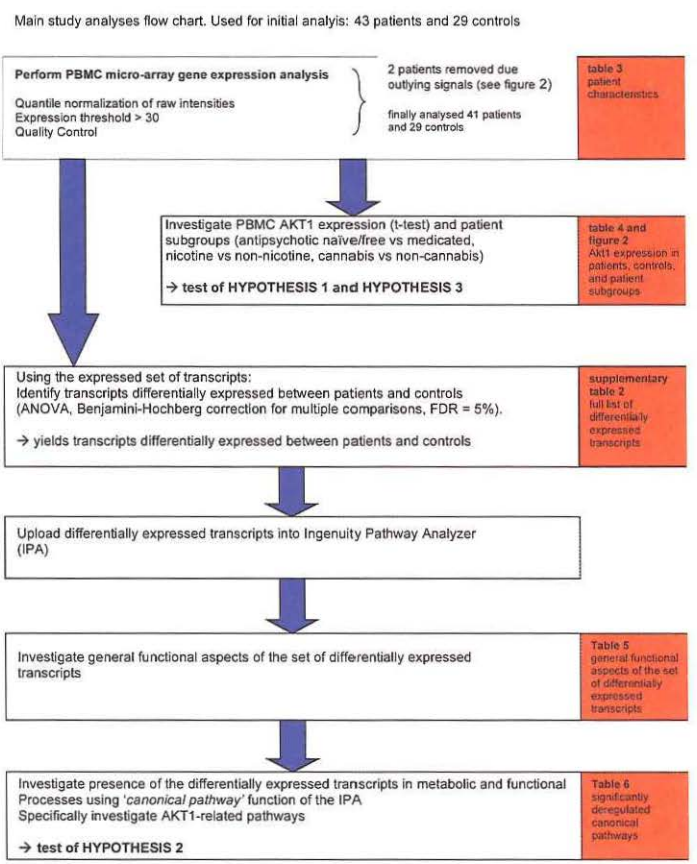
For both the pilot and the main study functional relationships between genes which were differentially expressed between patients and controls were investigated using the Ingenuity Pathway Analysis software tool (IPA -Ingenuity Systems®; www. ingenuity.com). The IPA version of October 2006 and January 2009 were used for the pilot study and the main study respectively.

The IPA is an internet-accessible database in which knowledge about the interaction between genes and gene products is stored based on known interactions in the literature (Sakamoto et al., 2005). The IPA defines genetic networks, functions, and metabolic- and signaling pathways which describe functional relationships between gene products and presents as output networks, functions and metabolic- and signaling pathways in which the genes in the dataset participate more than can be expected by change. It does so by calculating a significance score (using the right-tailed Fisher's exact test and expressed as a p-value) for each process by computing the number of deregulated genes that participate in a network or pathway relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the IPA. The significance value associated with networks and pathways is a measure for how likely it is that genes from a dataset participate in that function or pathway. For genetic networks an we considered and IPA score ≥ 3 as significant, for canonical pathways we considered a p-value < 0.05 as significant, both in accordance with IPA guidelines.

Based on our hypothesis we specifically investigated the presence of genetic networks and canonical pathways in which AKT1 plays a role.

Figure 1 gives a flow-chart overview of the analyses performed for the main study, and in which tables the results of the analyses can be found.
All raw data obtained are available as a MIAMExpress submission. All raw .CELL file can be obtained from the corresponding author on request.

Figuur 1



Results

Pilot study

Eight male patients with recent onset (‹ 5 yr) schizophrenia were included, as well as 8 age-matched controls. All patients exhibited considerable negative symptoms. Six patients used clozapine, one used risperidone, and one was medication-free for more than two weeks. See table 1 for a description of the pilot study subject characteristics.

Of the 54,675 probe sets on the array 565 probe sets were significantly deregulated

Table 1: Subject characteristics, pilot study

	Patients (N=8)	Controls (N=8)	
Mean age (sd)	24.0 (5.1)	23.1 (1.2)	p=.643
Etnicity	Caucasian (N=7) Asian (N=1)	Caucasian (N=7) Asian (N=1)	
Duration of untreated psychosis (weeks (sd))	69.8 (67.5) weeks		
Duration of illness (weeks (sd))	152.6 (84.9) weeks		
Medication	Clozapine (N=6) Risperidone (N=1) Medication free > 2weeks (N=1)		
Cumulative lifetime antipsychotic usage (haloperidol eq (sd))	3463 (1835) mg		
PANSS total (sd)	68.8 (9.6)		
PANSS positive (sd)	10.3 (1.5)		
PANSS negative (sd)	25.7 (6.2)		
PANSS general (sd)	32.8 (4.1)		

(t-test $p<0.05$ and $FC > 1.2$) between patients and controls. Of these, 273 were upregulated, 291 were downregulated.

These 564 probe set id's were uploaded into the IPA (October 2006). 212 genes were eligible for generating networks.

The IPA identified 16 networks which are significant according to the IPA criteria. We show only the most significant network consisting of 35 genes (see **table 2**).

The focus genes in this network are involved in immune response (N=18 genes), cell-to-cell signaling and interaction (N=15 genes), and cellular development (N=22).

Table 2: Network pilot study

id	genes	score	focus genes	top functions
1	AKT1, ARHGEF2, BCR, BIRC1, CALR, CARD11, CCL5, FBXO32, GAB3, GADD45B, GATA3, GNG2, GRB2, Hd (Huntington), HNRPL, IKBKB, KLF13, MAF, MALT1, MAP4K4, MAPK8, MAPK9, MARK4, NFATC2, NFATC3, OPTN, PAK1IP1, PPIF, SOS1, TAP2, THBS1, TLR2, TNFRSF10A, TNFRSF10B, ZAK	56	35	Immune Response, Cell-To-Cell Signaling and Interaction, Cellular Development

We then investigated this most significant network for the presence of known candidate genes for schizophrenia. The only gene fulfilling this criterium is AKT1 (figure 1). We found AKT1 expression to be decreased in the patient group (log-transformed relative intensity values: 313 (SD 51) vs 401(SD 63); t-test p=0.008;).

Main study: subjects

We initially included 46 patients, and 30 controls in the study. Of these, a total of 5 patients and 1 control were excluded: two patients were excluded because of somatic disorders (one patient suffered from microcytic anaemia, one other patient of nephrotic syndrome); after thawing RNA quantity of 1 patient and 1 control were not measurable; RNA quality of all other sample was satisfactory. Post-scanning we had to exclude 2 more patients, because quality control showed aberrant signal intensities as compared to the other samples.

So, in the main study we finally analyzed 41 patients and 29 controls. Eleven patients were free of antipsychotic medication for more than 2 weeks. See **table 3** for the complete patient characteristics.

The mean ages of patients (23.02 (SD 4.03)) and controls (23.90 (SD 4.08) years) were not significantly different: $p = 0.378$.

Main study: AKT1 expression between patients and controls

We found decreased levels of expression of AKT1 (log-transformed relative intensity values) in patients (518.58 (SD 61.46) vs controls (580.48 (SD 57.91)); $t = -4.250$, $df = 68$, $p < 0.001$; 95% CI -90.97; -32.84. The effect size of the difference in the means is large (eta squared: 0.21).

Expression levels of AKT 1 between acutely admitted (florid psychotic) and remitted patients were not significantly different (528.92 (SD 67.34 vs 508.73 (SD 55.13)), $p = 0.299$. See **figure 2**.

Expression levels of AKT1 in antipsychotic-free patients (495.91 (SD 52.96) were not significantly different from the medicated patients (526.89 (SD 63.06); $t = 1.449$, $df = 39$, $p = 0.155$; 95% CI -12.25; 74.21, and still significantly different from the controls (N=29; 580.48 (SD 57.91); $t = -4.220$, $df = 38$, $p < 0.001$; 95% CI -125.18; -43.96. Also, AKT1 expression in antipsychotic-naïve patients (N=6) was not significantly different as compared to medicated patients (N=35) (p=0.114).

Indeed, if any, AKT1 expression levels tended to be higher in the medicated patients. See figure 2.

Table 3: Patient characteristics main study

	Patients (N=41)
Smoking	yes: 26 (63.4%) no: 15 (36.6%)
Cannabis abuse	yes: 18 (43.9%) no: 22 (56.1%)
Duration of illness (DUI) (weeks) (N=36)†	116.22 (SD 99.89) weeks min-max: 1-250 weeks median: 92.50 weeks
Ethnicity	
Caucasian (mostly Dutch descent)	20 (48.8%)
Surinamese / African descent	6 (14.6%)
Cape Verdian	1 (2.4%)
Surinamese / Hindustani	6 (14.6%)
Moroccan / North African	2 (4.9%)
Asian	1 (2.4%)
Mixed	3 (7.3%)
Could not be reliably assessed	2 (4.9%)
Antipsychotics used at study entry	
naive (mean age naive: 25.0 (SD 4.7) years))	6 (14.6%)
free > 2 weeks, not naive (mean age free > 2 weeks: 25.2 (SD 3.9) years)	5 (12.2%)
total free > 2 weeks	11 (26.8%)
haloperidol	9 (22.0%)
risperidone	1 (2.4%)
olanzapine	4 (9.8%)
quetiapine	1 (2.4%)
clozapine	15 (36.6%)
PANSS scores	
total score	77.7 (SD 14.4)
positive subcale	18.5 (SD 6.6)
negative subscale	22.0 (SD 5.8)
general psychopathology	37.3 (SD 8.0)

† of 5 patients DUI could not be reliably assessed

Also, AKT1 expression in patients using nicotine (N=26; 530.45 (SD 58.31)) was not significantly different from those who did not smoke (N=15; 498.00 (SD 63.29): p=0.104. Neither was AKT1 expression in patients using cannabis significantly different as compared to patients not using cannabis (p=0.691). Also in these cases, if any, patients with nicotine or cannabis abuse tended towards higher level of AKT1 expression. Table 4 gives a complete overview of AKT1 expression values in patient subgroups. AKT1 expression did not correlate with age (r: -0.046, p=0.704), duration of illness (r: 0.242, p=0.155), PANSS positive- (r: 0.120, p=0.501), or PANSS negative subscales (r: 0.129, p=0.466).

Figure 2

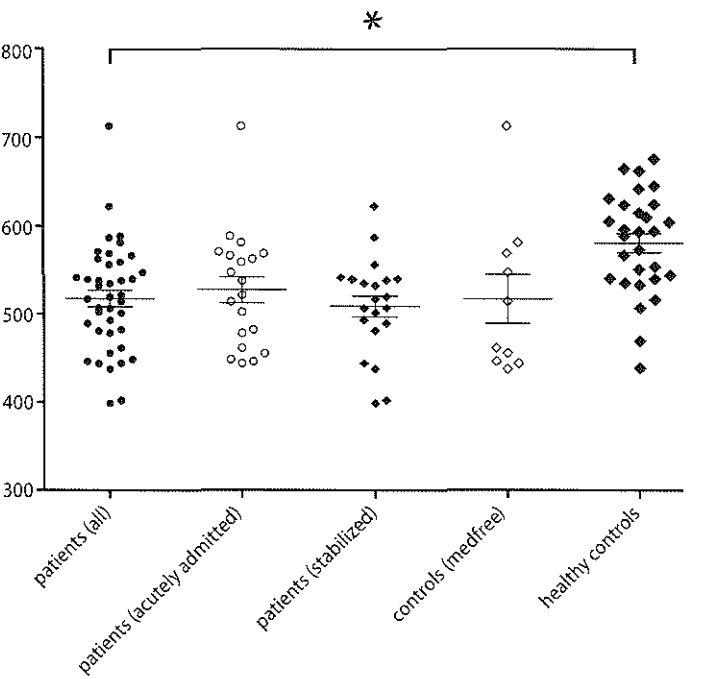


Figure 2. Scattterplots of PBMC AKT1 expression levels between patients (subgroups) and controls.

Cross-lines show mean and standard error of the mean.

* $p < 0.001$ (independent samples t-test)

Table 4: Overview of mean levels of PBMC AKT1 expression levels (log-transformed relative intensity values) between patients and controls and between patient subgroups

	Controls	Patients (all)	Patients (acute) vs patients (stabilized)	Patients (antipsychotic naïve)	Patients (antipsychotic free > 2 weeks)	Patients using nicotine vs non-nicotine	Patients using cannabis vs non-cannabis
AKT1 expression (mean sd)	580.4857.91 N=29)	518.5861.46* (N=41)	528.9267.34 vs 508.7355.13 (N=20, N= 21)	481.8141.23 ‡ (N= 6)	493.3355.09‡ (N= 11)	530.4558.31 vs 498.0063.29 †† (N= 26, N= 15)	522.9765.88 vs 515.1459.05 ‡‡ (N= 18, N= 23)

* $p < 0.001$ as compared to the controls (t-test)

†† non-significant difference between nicotine and non-nicotine

‡ non-significant difference as compared to medicated patients

‡‡ non-significant difference between cannabis abuse vs non- cannabis abuse

Main study: significance analysis of the complete set of genes

ANOVA showed 1224 transcripts that were significantly deregulated between patients and controls, after Benjamini-Hochberg correction for multiple comparisons (FDR = 5%). See **supplementary data file** (accessible through www. psych.nl

Table 5: General aspects of the deregulated set of transcripts

Diseases and disorders Name	p-value	# Molecules
Cancer	1,01E-06 - 4,54E-03	197
Neurological disease	3,82E-05 - 2,04E-03	33
Reproductive system disaese	6,78E-05 - 3,42E-03	46
Dermatological diseases and conditions	7,22E-05 - 3,75E-03	13
Connective tissue disorders	8,23E-05 - 4,07E-03	50
Molecular and cellular functions **Name**	**p-value**	**# Molecules**
Cellular growth and proliferation	2,48E-14 - 4,54E-03	182

▸ 'people' ▸ 'beveren' ▸ 'list of deregulated genes chapter 8') for the complete list of differentially expressed genes. Of the 1224 deregulated transcripts, 272 were upregulated, 952 were downregulated.

AKT1 is among these 1224 transcripts, so AKT1 remains differentially expressed between patients and controls also after Benjamini-Hochberg correction.

The 1224 differentially expressed transcripts were uploaded in the IPA for pathways analyses.

Main study: pathway analyses (see also figure 1: flowchart of the analyses)

We first investigated the general aspects of the set of differentially expressed transcripts.

According to the IPA, the disorders most significantly related to the deregulated transcripts are *cancer, neurological disease, reproductive system disease, dermatological diseases and conditions,* and *connective tissue disorders.* The molecular and cellular functions most significantly related to the deregulated transcripts are *cellular growth and proliferation, cell signaling, gene expression, cell cycle,* and *cell death.* The physiological systems most significantly related to the deregulated transcripts are *immune response, hematological system development and function, immune and lymphatic system development and function, tissue morphology,* and *tissue development.* See **table 5**

Then, we investigated the involvement of the 1224 initially identified probe sets in metabolic and signaling pathways using the 'canonical pathway' function of the IPA. This identified 35 canonical pathways in which more transcripts from the 1224 probe dataset participated than expected by chance ($p < 0.01$), and 13 pathways us-

ing a more stringent criterium ($p<0.001$). Of the 35 deregulated pathways, AKT1 is involved in 25 pathways (71%). Using the more stringent criterium of $p < 0.001$ as significance threshold, AKT1 is involved in 6 of the 11 deregulated pathways (55%). Table 6 shows the deregulated canonical pathways, its significance, and in which pathways AKT1 participates.

Table 6: Canonical pathways, main study; pathways in which AKT1 participates are shown in bold

Canonical pathway	Significance (IPA)
Natural killer cell signaling	**0.00000036**
SAP/JNK Signaling	0.00000283
Leucocyte Extravasation Signaling	0.00000304
T Cell Receptor Signaling	0.0000178
Huntington's disease Signaling	**0.0000334**
Inositol Phosphate Metabolism	0.000105
Cell Cycle: G1/S Checkpoint Regulation	0.000114
Integrin Signaling	**0.00019**
Fc Epsilon RI Signaling	**0.00021**
B Cell Receptor Signaling	**0.00025**
Insulin Receptor Signaling	**0.00054**
JAK/Stat Signaling	**0.001**
p53 Signaling	**0.001**
PDGF Signaling	0.001
PTEN Signaling	**0.001**
Apoptosis Signaling	0.002
Nicotinate and Nicotinamide Metabolism	0.002
Axonal guidance Signaling	**0.003**
Ephrin receptor Signaling	**0.004**
Neurotrophin/Trk Signaling	**0.004**
PPARα/RXRα activation	0.005
NRF2-mediated oxidative stress response	**0.008**
TGF-β Signaling	0.01
Hypoxia Signaling in the Cardiovascular System	**0.01**
FGF Signaling	**0.01**
IL-2 Signaling	**0.01**
Wnt/β-catenin Signaling	**0.01**
ERK/MAPK Signaling	**0.01**
IGF-1 Signaling	**0.01**
Xenobiotic metabolism Signaling	**0.02**
IL-4 Signaling	**0.03**
Aryl Hydrocarbon Receptor Signaling	**0.03**
VEGF Signaling	**0.03**
Neuregulin Signaling	**0.04**
Protein Ubiquitination Pathway	**0.04**

Further inspection shows that deregulated canonical pathways are related to immune processes, cell adhesion and neuronal guidance, neurotrophin signalling and (neural) growth factors, oxidative stress and glucose metabolism, and apoptosis and cell-cycle regulation.

Discussion

In this study we show decreased expression of AKT1 in PBMC's of young, recent-onset, male schizophrenia patients. We initially showed decreased PBMC AKT1 expression in a small pilot sample, and confirmed decreased AKT1 expression in a larger sample consisting of both acutely psychotic patients and stabilized patients. AKT1 expression was decreased in both the acutely admitted florid psychotic patients, as well as in the (partly) remitted patients. AKT1 expression was decreased in both medicated and unmedicated patients. We could not identify a correlation between AKT1 expression, and age, duration of illness, and clinical symptom severity (PANSS positive and negative subscales). Taken together, our observations suggest that decreased PBMC AKT1 expression is a stable trait in recent onset, male schizophrenia patients.
To our best knowledge this is the first confirmation of aberrant AKT1 expression in PBMC's of schizophrenia patients after the initial report (Emamian et al., 2004). We extend their findings by showing that AKT1 is also decreased in non-cultured PBMC's of schizophrenic patients, and that this is also observed in medication-free/-naive patients. In addition we demonstrate significantly deregulated canonical pathways in PBMC's of patients, in a majority of which AKT1 is a key enzyme. We also show that deregulated pathways tend to cluster in families that are related to immune processes, cell adhesion and neuronal guidance as well as apoptosis and cell-cycle regulation, neurotrophin signalling and (neural) growth factors, and oxidative stress and glucose metabolism.

Antipsychotic medication and nicotine or cannabis abuse were initially considered as possible confounders affecting the expression of AKT1. However, we could not identify significantly different levels of AKT1 expression between patients using antipsychotic medication and those who were antipsychotic free or -naïve, nor between drug abuse and non-abuse patients. If any, patients using medication or drugs tended towards higher AKT1 expression levels. So, antipsychotic medication

or substance use cannot account for the decreased levels of PBMC AKT1 expression we found.

Our findings compare well to other studies investigating the possible involvement of AKT1 in schizophrenia. Since the initial report of deregulated AKT1 in schizophrenia (Emamian et al., 2004), several case/control samples and family cohorts showed an association between schizophrenia and AKT1 genetic variants (Ikeda et al., 2004; Schwab et al., 2005; Bajestan et al., 2006; Xu et al., 2007; Thiselton et al., 2008). However, as usual in association studies in schizophrenia, a number of negative findings have also been reported (Ohtsuki et al., 2004; Turunen et al., 2007; Norton et al., 2007; Liu et al., 2008; Sanders et al., 2008) (for an updated compilation al all studies; see http://www.schizophreniaforum.org/res/sczgene/geneoverview.asp?geneid=6). Also a number of post-mortem studies show evidence of a decrease in AKT1 expression in brains of individuals with schizophrenia (Emamian et al., 2004; Thiselton et al., 2008; Zhao et al., 2006).
Our observations showing decreased PBMC AKT1 expression do not only compare well with these other studies, but our findings are also specific, inasmuch as we do nót find altered expression of the AKT isoforms AKT2 and AKT3, in complete concordance with Emamian et al (Emamian et al., 2004). Neither do we find altered expression of the main downstream target of AKT, GSK-3, and also this finding is in concordance with previous reports (Nadri et al., 2002; Kozlovsky et al., 2005).

From a theoretical perspective, the involvement of AKT in schizophrenia is plausible. Generally, one of the major functions of AKT is to promote growth-factor mediated cell survival and to block programmed cell death or apoptosis (Kandel and Hay, 1999; Brazil et al., 2004; Brazil et al., 2002). Altered apoptotic processes have repeatedly been proposed to underlie schizophrenia, and are part of the influential so-called 'neurodevelopmental model of schizophrenia' (Jakob and Beckmann, 1986; Murray and Lewis, 1987; Weinberger, 1987; McGlashan and Hoffman, 2000).
Recently, it has also been shown that AKT is a key signaling enzyme downstream of dopamine D2 receptors (Beaulieu et al., 2005; Beaulieu et al., 2007a; Beaulieu et al., 2007b). Aberrant dopaminergic neuromodulation is one of the most well-established findings in schizophrenia and is thought to underlie psychotic phenomena as well as cognitive disorders present in schizophrena (i.e. see Kapur 2003; De Haan and Bakker, 2004). Interestingly in this respect, the expression of AKT1 in lymphoblasts was recently reported to be positively correlated with performance

on a dopamine-dependent cognitive tasks in healthy individuals (Tan et al., 2008).

Besides AKT1, we found a total of 1224 transcripts deregulated between patients and controls in the main study. Of these, the down-regulated transcripts greatly outnumber the up-regulated ones, in an approximately 80:20% ratio. This finding is in line with previous reports that (post-mortem) gene expression changes in subjects with schizophrenia are predominantly characterized by transcript reductions, rather than increases(Mirnics et al., 2000; Iwamoto and Kato, 2006; Arion et al., 2007). The magnitude of change however, although significantly altered, is limited for the vast majority of transcripts, pointing towards only subtle alterations in expression status. As PBMCs in schizophrenia do not show overt pathology, subtle alterations in gene-expression are what might be expected.
The results of the pathway analyses we performed show aberrantly regulated processes in patients to be related to a number of biological themes: immune processes, cell adhesion and neuronal guidance, neurotrophins and (neural) growth factors, oxidative stress and glucose metabolism, and apoptosis and cell-cycle regulation (see table 5 and 6).

A considerable number of significantly changed canonical pathways point towards the presence of an altered immune status in patients (Natural killer cell signaling, Leucocyte Extravasation Signaling, T Cell Receptor Signaling, Fc Epsilon RI Signaling, B Cell Receptor Signaling, IL-2 Signaling, Xenobiotic metabolism Signaling, and IL-4 Signaling). The presence of an immunological component in schizophrenia has been hypothesized, based on several convergent findings (Hanson and Gottesman, 2005; Jones et al., 2005; Muller and Schwarz, 2006; Rothermundt et al., 2001; Arion et al., 2007) Recently, it has been suggested that alterations in schizophrenia susceptibility genes can be related to infections with cytomegalovirus, influenza, herpes simplex, rubella, and toxoplasma gondii (Carter, 2008). The transcriptional changes related to immune function we find may be a consequence of (early life) activation of various immune-related pathways in schizophrenia.
Although most theories relating immunological processes with schizophrenia share the assumption that immunological aberrations are somehow primarily related to schizophrenia pathology, we point out that there is another possibility, namely that the finding of immune perturbations are an epiphenomenon of the specific tissue under investigation. It is possible that in schizophrenia molecular-biological aberrations are subtly present in áll tissues (as has been previously shown in liver

cells (Prabakaran et al., 2007)), but only give rise to overt functional disturbances in the brain. In that case, when investigating a non-neural tissue (like PBMCs) disturbances in the primary function (i.e. immune processes) may become manifest, without necessarily implying a causative relationship with schizophrenia.

Aberrant development of the nervous system in concert with altered apoptotic processes in later life have repeatedly proposed to underlie schizophrenia, and form the hallmark of the influential neurodevelopmental model of schizophrenia (Jakob and Beckmann, 1986; Murray and Lewis, 1987; Weinberger, 1987; McGlashan and Hoffman, 2000). In normal human brain development, programmed cell death plays an important role. (Jernigan et al., 1991; Jernigan and Tallal, 1990; Gogtay et al., 2004; Shaw et al., 2006). It has been argued that in schizophrenia overly active apoptotic processes drive the maturing adolescent brain beyond a critical limit, finally resulting in overt pathology.
The presence of deregulation of Integrin Signaling, Axonal guidance Signaling, Ephrin receptor Signaling, and Neuregulin Signaling (table 6), all of which are associated with neuronal outgrowth, cellular patterning during development, and cell adhesion, combined with the presence of deregulated apoptotic and cell-cycle regulation processes (G1/S Checkpoint Regulation, p53 Signaling, PTEN Signaling, Apoptosis Signaling)(see table 5) may indeed point to the presence of aberrant neurodevelopental processes.
Integrins are a class of cell surface receptors that play important roles in a vast array of cellular and physiological processes, ranging from embryonic development to immunity to tumor metastasis (Liddington and Ginsberg, 2002; Ginsberg et al., 2005). Integrins also have been implicated as playing key functions in mediating neuronal migration (Galileo et al., 1992) and synapse formation and maintenance. Integrins and Neuregulin (NRG1) converge on various developmental processes linked to neuronal migration and to synapse biology (Kanakry et al., 2007). NRG1 is at present considered to be one of the candidate genes most clearly associated with schizophrenia (Arnold et al., 2005). Ephrin receptor signaling has been implicated in pathway selection by axons, the guidance of cell migration, and the establishment of regional patterns in the developing nervous system. (Flanagan and Vanderhaeghen, 1998). In addition, ephrin- and integrin dependent processes are mediated by axonal guidance signaling.

We also find the neurotrophin/Trk signaling pathway deregulated, as well as some

other growth factor and cytokine related pathways (PDGF Signaling, TGF-β Signaling, FGF Signaling, IGF-1 Signaling, VEGF Signaling) (table 6) . Neurotrophins are a specific subset of growth factors, most notably among them Brain-Derived Neurotrophic Factor (BDNF). There is an emerging body of converging evidence that points to a relation between schizophrenia and disrupted levels of BDNF, both in the central nervous system and in peripheral blood (see (Shoval, 2005; Van Beveren et al., 2006; Buckley et al., 2007) for a review). The biological responses of neurotrophins after activating the neurotrophin/trk pathway include among others proliferation and survival,and axonal and dendritic growth and remodeling (Shoval, 2005).
Interestingly, we find involvement of IGF-1 signaling. On theoretical grounds, IGF-1 involvement in schizophrenia has been suggested, and recenty, reduced levels of IFG-1 in antipsychotic-naïve schizophrenia patients have been reported (Venkatasubramanian et al., 2007). IGF-1 is an important growth factor, which also regulates energy metabolism. Low birth weight, leanness, and short stature (which are linked to decreased IGF-1 levels) are shown to be associated with an increased risk for developing schizophrenia.
Moreover, schizophrenia is associated with an increased risk to develop impaired glucose tolerance, insulin resistance, and type II diabetes mellitus, not only secondary to the use of antipsychotics, but also in antipsychotic-naïve patients subjects (Zhao et al., 2006; van Nimwegen et al., 2008). Reduced levels of IGF-1 in schizophrenia may play role in the association of schizophrenia with type II diabetes and insulin insensitivity. Additionally, a number of publications suggest that increased oxidative stress is implicated in schizophrenia pathology (i.e. (Mahadik and Scheffer, 1996; Reddy and Yao, 1996; Prabakaran et al., 2004; Do et al., 2000). Apart from IGF-1 signaling we indeed find several other deregulated canonical pathways which may point towards the presence of altered insulin signaling and oxidative stress (Inositol Phosphate Metabolism, Insulin Receptor Signaling, NRF2-mediated oxidative stress response, IGF-1 Signaling, and Hypoxia Signaling in the Cardiovascular System) (see table 6).

A striking and for us unexpected finding is the magnitude of decreased expression of AKT1 in PBMCs . When we set out to perform this project, we expected to find only limited effect sizes for alterations in individual gene expression, based on the limited effect sizes generally reported for gene association studies in schizophrenia. However, we report decreased AKT1 expression with a large effect size (eta squared = 0.21). Such a magnitude cannot be exclusively attributed to the putative presence

of schizophrenia-associated AKT1 haplotypes, which individually decrease AKT1 expression. It can only be understood as the result of the presence of multiple pathological processes involved in schizophrenia of which the majority converge on AKT1-associated signaling pathways, which then in concert considerably alter the PBMC expression of AKT1. Indeed, of the broad themes which can be identified in schizophrenia research (disturbed dopaminergic neuromodulation, disturbed glutamate signaling, disturbances in myelination and oligodendrocyte function, disturbed neuronal migration, disturbed neurotrophic signaling, altered immune status, and the presence of oxidative stress as well as insulin insensitivity), a considerable number are reflected in the pathways and processes we find deregulated between patients and controls, as discussed in the previous paragraphs.

There are several strengths and limitations to our study. We consider a specific strength the deferred consent procedure we used, which enabled us to include also the most severely disturbed, acutely psychotic patients. This also enabled us to include several antipsychotic free, or even -naïve patients. Furthermore, we included patients after thorough standardized diagnostics with consensus by two senior psychiatrists which were all personally screened by one. So, our sample does not consist of a plethora of subjects recruited from various sites, or with a various background, long duration of illness with concomitant exposure to antipsychotics and/or drugs of abuse, such as might occur in brain-bank obtained post-mortem samples.
We also rigorously eliminated confounders during the laboratory procedures, such as blood sampling by various staff members, inclusion at several sites with associated multiple transport, storage and/or laboratory procedures, and bench work by multiple scientists. There are however, limitations of our study. First, the majority of patients were on antipsychotic medication. Several reports show that antipsychotics (haloperidol as well as olanzapine and clozapine) influence AKT. However, the majority of reports show that antipsychotics do not influence expression levels of AKT, but only increase the phosphorylation status of AKT, thus increasing AKT1 activity without influencing its expression levels. Indeed we showed that AKT1 expression is also decreased in the antipsychotic-free patients, in line with these findings. However, we did not perform separate pathway analyses comparing the medicated with the medication-free patients, as this would imply performing separate Benjamini-Hochberg corrected ANOVA's on these two separate groups, which are uneven in size, with the medication-free group being relatively small.

So, the pathway analyses probably reflect the combined effects of possessing the schizophrenia phenotype, with its associated decreased AKT1 levels, and the effects of antipsychotic medication use.
Second, we investigated PBMCs, which consist of a mixture of white blood cells. Investigating a single cell type might give a more 'pure' signal, specific to the particular cell type investigated. However, obtaining PBMCs implies the least manipulating of the cells, as compared by obtaining single cell types, i.e. those obtained by flow cytometry, or by Epstein-Barr virus activated lymphoblasts. Moreover, investigating multiple cell types may have its specific advantages because the underlying genetic dysfunctions common to all cell types may be brought forward, while at the same time the variance between the cell types may be canceled out as noise (i.e. see Pintilie et al. (Pintilie 2009)).

Our findings are important for a number of reasons. First, we find clearly decreased expression of AKT1. This suggests that multiple processes present in schizophrenia alter PBMC AKT1 expression. Moreover, these processes may individually be rather subtle, but their concerted action shows itself quite clearly, as indicated by the large effect-size of decreased AKT1 expression.
Second, the presence of decreased AKT1 irrespective of clinical phase of the disorder suggests that it is a trait marker for the (early) schizophrenia syndrome in males. As it is plausible that at least some of the processes that are associated with AKT1 may be disturbed in schizophrenia before the frank onset of the disorder, decreased AKT1 expression may be present in prodromal patients. If so, decreased AKT1 expression in PBMCs might be a biomarker for identifying patients at risk for conversion to a fully psychotic state.
Third, we show that several schizophrenia-related pathways are deregulated between patients and controls. Thus, PBMCs may be a suitable, relatively easy obtainable type of cells to study aspects of the molecular biology of schizophrenia. We do not claim that the processes in PBMCs are the same as found in the nervous system. But, findings in PBMCs can be compared with post-mortem brain findings. In older patients, PBMC gene expression obtained during life, might be compared with later obtained post-mortem brain samples. Cultured PBMCs may be exposed to specific stressors, like in vitro low-glucose to investigate differentiated gene expression reactivity between patients and controls (i.e. see Konradi (2005)).
Fourth, and this is a general methodological aspect, our findings suggest that whole genome gene expression studies may benefit from an approach in which a

specific hypothesis (i.e. 'decreased PBMC AKT1 expression') is combined with an non-hypothesis driven approach (investigating functional processes related to the differentially expressed gene set).

In conclusion, we show decreased PBMC gene expression of AKT1 in schizophrenia patients, with a considerable effect size. A large number of AKT-related functions, processes and canonical pathways are deregulated between patients and controls. A majority of these have previously been associated with schizophrenia. Specific attention merits the presence of an altered immune status in the patients as well as a deregulated status of genes associated with cell growth, cell guidance, cell adhesion, (developmental) spatial patterning, and energy metabolism. Decreased PBMC AKT1 expression may serve as a biomarker signaling the early schizophrenia syndrome, or even flag a future conversion to a fully psychotic state in prodromal patients. This merits further investigation. (Cultured) PBMCs may be useful tissue in which to investigate aspects of the molecular biology of schizophrenia.

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Chapter 9

Functional gene-expression analysis of peripheral blood mononuclear cells shows involvement of several schizophrenia-relevant signalling pathways in adult patients with 22q11 deletion syndrome

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Abstract

22q11 Deletion Syndrome (22q11DS) is associated with dysmorphology and a high prevalence of schizophrenia-like symptoms. Several genes located on 22q11 have been linked to schizophrenia. The deletion is thought to disrupt the expression of multiple genes involved in maturation and development of neurons and neuronal circuits, and neurotransmission. We investigated whole-genome gene expression of Peripheral Blood Mononuclear Cells (PBMC's) of 8 22q11DS patients and 8 age- and gender matched controls, to (1) investigate the expression levels of 22q11 genes and (2) to investigate whether 22q11 genes participate in functional genetic networks relevant to schizophrenia. Functional relationships between genes differentially expressed in patients (as identified by Locally Adaptive Statistical procedure (LAP) or satisfying $p<0.05$ and fold-change > 1.5) were investigated with the Ingenuity Pathways Analysis (IPA).

14 samples (7 patients, 7 controls) passed quality controls. 14 genes were deregulated in patients (10 located on 22q11). LAP identified 29 deregulated genes. 262 transcripts satisfied $p < 0.05$ and fold-change > 1.5. Functional pathways most disturbed were cell death, cell morphology, cellular assembly and organisation, and cell-to-cell signalling. In addition, 10 canonical pathways were identified, among which the signal pathways for Natural Killer-cells, neurotrophin/Trk, neuregulin, axonal guidance, and Huntington's disease.

Our findings support the use of the 22q11DS as a research model for schizophrenia. We identified decreased expression of several genes (among which COMT, Ufd1L, PCQAP, and GNB1L) previously linked to schizophrenia as well as involvement of signalling pathways relevant to schizophrenia, of which Neurotrophin/Trk and neuregulin signalling seems to be especially notable.

Introduction

Deletions of chromosome 22q11 are associated with a high prevalence of dysmorphology (velo-cardio-facial syndrome; 22q11DS), cognitive and behavioral disorders and schizophrenia-like psychosis (Van Amelsvoort et al., 2004). It is thought that a hemizygous 22q11 deletion disrupts the expression of multiple genes involved in maturation, development of neurons and neuronal circuits, and neurotransmission (Meechan et al., 2007). Several genes located in the 22q11 region have been linked to schizophrenia, including COMT, ProDH, Ufd1L, PCQAP (Maynard et al., 2003), and, recently, GNB1L (Williams et al., 2007). It is likely that genes present in the 22q11 deleted region form functional networks (i.e. metabolic- and signalling pathways) with other genes outside this region involved in neuronal functioning, and that the decreased expression of 22q11 genes alters the functional activity of these pathways. It is however unclear which pathways these are, and what their relationship is with pathways involved in schizophrenia not related to 22q11DS. More insight into the nature of these functional networks may increase our understanding in the genetic networks involved in the psychiatric and cognitive and behavioral disturbances seen in 22q11DS including schizophrenia. Moreover, it will provide more understanding of the relationship between 22q11DS and schizophrenia and the value of 22q11DS for investigating schizophrenia pathology.

The combination of gene expression profiling and biomics is proven to be a powerful technology to identify functional changes in genetic networks (Konradi 2005). However, for neuropsychiatric disorders a major limitation is the inaccessibility of the living brain and the heterogeneity of many psychiatric disorders. This is especially relevant for disorders such as schizophrenia, which show a prolonged lifelong course during which the effects of normal and abnormal brain development, drug abuse and medication influence the results of post-mortem gene-expression studies (Konradi 2005; Mirnics et al., 2006).
Peripheral Blood Mononuclear Cells (PBMC's) express many brain relevant genes (Sullivan et al., 2006) and have been suggested as 'window-on-the brain' surrogate tissue to investigate neuropsychiatric disorders. In schizophrenia, a number of recent studies have explored the use of various types of PBMC's (Vawter et al., 2004; Middleton et al., 2005; Glatt et al., 2005; Tsuang et al., 2005; Bowden et al., 2006). The key assumption of these studies is that although peripheral blood cells in schizophrenia show no clear aberrations in function or morphology, subtle

molecular-biological aberrations are present and can be informative about the molecular-biological underpinnings of schizophrenia. However, in non-22q11DS schizophrenia a multivariate genetic and environmental cause is suspected. Therefore PBMC gene expression of patients with schizophrenia not related to 22q11DS may be largely variable. We reasoned that in 22q11DS the deletion will most likely result in decreased PBMC expression of 22q11 genes which may in turn influence the expression of other genes outside the deleted region more robustly and persistently than in non-22q11DS schizophrenia. Furthermore, using functional pathway analysis, 22q11DS might offer a view of the concerted altered expression of genes both within and without the 22q11 deleted region that may alter the activity of functional genetic networks.

PBMC gene expression in 22q11DS patients has not yet been investigated, neither the individual expression levels of genes in the 22q11 deleted region, nor the influence of the deletion on functional genetic pathways. Thus, we investigated gene expression in PBMC's of VCFS patients, with the objective to (1) investigate the expression levels of 22q11 genes and to (2) investigate whether 22q11 genes, together with genes outside this region, participate in functional networks relevant for VCFS and schizophrenia.

Materials and methods

Subjects

8 22q11DS subjects and 8 age- and gender matched controls were included in this study. Deletions at chromosome 22q11 were previously identified in all subjects by Fluoresence In-Situ Hybridization (FISH).

This study was approved by the institutional review boards of the two participating centers (Erasmus University Medical Center, Rotterdam, and Academic Medical Center, Amsterdam) and was performed in accordance with the declaration of Helsinki. All subjects provided written informed consent.

Samples

From each participant 30 ml of blood was drawn into heparinized tubes. PBMC's were isolated by Ficoll-gradient separation starting 90 minutes after the drawing of blood. Cells were subsequently disrupted (Qiashredder kit; Qiagen), and RNA was isolated (RNeasy minikit; Qiagen) with an additional DNAse digestion step

(RNase-free DNase set; Qiagen), all according to the manufacturer's protocol, diluted in nuclease free water and frozen at -80 C before use. After thawing the isolated RNA was biotinylated into cRNA using the One-Cycle Target Labeling and Control Reagents Kit (Affymetric Co) according to the manufacturer's protocol. Before hybridisation RNA quality and integrity was assessed using the Agilent 2100 BioAnalyzer (Agilent) and RNA purity and quantity with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Biotinylated cRNA was hybridized to the Affymetrix Human Genome U133 plus 2.0 GeneChip© microarray containing 54,675 probe sets (Affymetrix Co). Each sample was individually biotinylated and hybridized to an individual microarray. Biotinylation was performed in two batches with randomisation of samples across the batches. Hybridisation was performed in one batch. The arrays were scanned and analyzed using Affymetrix Microarray Suite 4.2 software

Statistical analysis

Probe sets that were absent in all samples (according to Affymetrix software) were omitted from further analysis. Raw intensities (33,196 probe sets) were normalized by quantile normalization. Data analysis was done using OmniViz version 5.0 (Biowisdom) and R program. Minimum thresholds were set at 30. To investigate the expression levels of genes in the 22q11 region between patients and controls we applied two separate methods, Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) (OmniViz) and Locally Adaptive Statistical procedure (LAP)(Callegaro et al., 2006) (R program). SAM uses permutations of the repeated measurements of the expression levels to estimate the false discovery rate (FDR). LAP combines a FDR approach with information about chromosomal location of the genes and is specifically suited to identify differentially expressed regions that are involved in known chromosomal aberrations (Callegaro et al., 2006). It should be noted that LAP may identify significantly decreased expression of genes which are actually not expressed at all, due to way the LAP algorithm uses information about chromosomal location. These not expressed genes were removed from further analyses.

To investigate the functional relationships between genes which are differentially expressed between patients and controls we used the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems®; www.ingenuity.com; october 2007). The IPA is an internet-accessible database in which knowledge about the interaction between genes and gene products is stored based on known interactions in the literature (Kasamatsu et al., 2005). Thus, the IPA defines genetic networks, functions, and

metabolic- and signalling pathways which describe functional relationships between gene products and presents as output networks, functions and metabolic- and signalling pathways in which the genes in the dataset participate more than can be expected by change. It does so by calculating a significance score (using the right-tailed Fisher's exact test and expressed as a p-value) for each process by computing the number of deregulated genes that participate in a network or pathway relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the IPA. The significance value associated with networks and pathways is a measure for how likely it is that genes from a dataset participate in that function or pathway. For genetic networks an IPA score >= 3 is considered significant, for canonical pathways a p-value <.05 is considered significant.

We uploaded into the IPA a dataset which consisted of the genes identified by LAP and of genes with different expression levels between patients and controls according to the criterium: t-test between patients and controls significant ($p<0.05$) and a 1.5 or more fold-change (FC) in expression level in patients compared to controls.

Results

Subjects

8 VCFS patients and 8 controls were included. Three patients had a previous history of psychotic symptoms, whereas four did not (further characteristics see **table 1**).

Table 1: Subject characteristics

Pair	Patients				Controls	
	Psychosis	Antipsychotic use	Age	Gender	Age	Gender
1	no	none	18	F	19	F
2	no	none	39	F	39	F
3	no	none	25	M	25	M
4	no	none	17	F	18	F
5	yes	zuclopentixole	27	M	27	M
6	yes *	risperidone	32	F	30*	F
7	yes	risperidone	25	M	26	M
8	yes	risperidone	21	M	21	M

* excluded cases because of bad signal-to-noise ratio of the expression data; coincidentally, the excluded cases form a matched pair.

Routine quality controls of the gene expression results showed sub-optimal signal-to-noise ratios in two subjects (one patient and one control). These two samples were excluded from further analyses.

Table 2: SAM results

Probe Set ID	Gene title	Gene symbol	Entrez gene	Chromosomal location
229906_at	Armadillo repeat containing 7	ARMC7	79637	chr17q25.1
217427_s_at	HIR histone cell regulation defective homolog A (S.cerevisiae)	HIRA	7290	chr22q11.21
1553974_at	hypothetical protein LOC 128977	LOC128977	128977	chr22q11.21
202483_s_at	Ran binding protein 1	RANBP1	5902	chr22q11.21
203152_at	mitochondrial ribosomal protein L40	MRPL40	64976	chr22q11.21
209103_s_at	ubiquitin fusion degradation 1 like (yeast)	UFD1L	7353	chr22q11.21
210010_s_at	solute carrieer 25, member 1	SLC25A1	6576	chr22q11.21
32032_at	DiGeorge syndrome critical region gene 14	DGCR14	8220	chr22q11.21
208818_s_at	catechol-O-methyltransferase	COMT	1312	chr22q11.21-q11.23
206184_at	v-crk sarcoma virus CT 10 oncogene homolog (avian)-like	CRKL	1399	chr22q11.21
212180_at	v-crk sarcoma virus CT 10 oncogene homolog (avian)-like	CRKL	1399	chr22q11.21
202206_at	ADP-ribosylation-like 4C	ARL4C	10123	chr2q37.1
235289_at	eukaryotic translation initiation factor 5A2	EIF5A2	56648	chr3q26.2
230685_at	hypothetical protein LOC644873	FLJ33630	644873	chr5q23.3

False Discovery Rate=6.6%; falsely called < 1

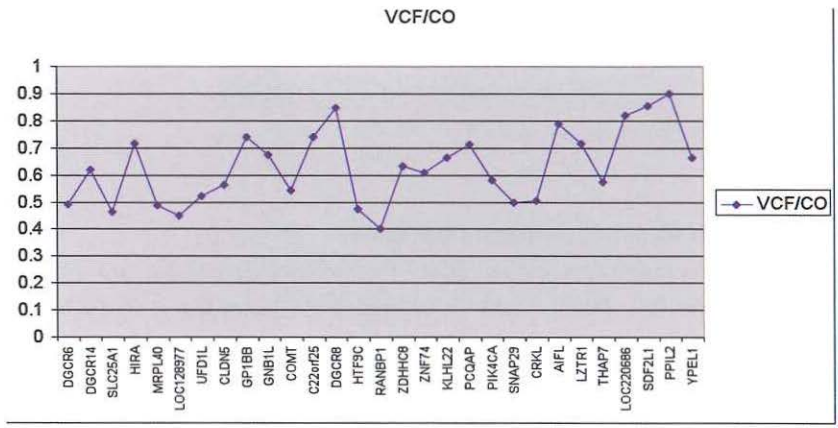
Global significance analysis of gene expression

SAM showed 14 transcripts differentially expressed between patients and controls (False Discovery Rate (FDR) = 6.6%; falsely called < 1) (see **table 2**). Ten of these transcripts are located in the 22q11 region.

LAP identified 44 genes (q value<0.05), all in the 22q11 region (see **table 3**).
Of these 44 genes, 9 were not expressed in blood and 6 were not differentially expressed between patients and controls, resulting in 29 differentially expressed genes.

Table 3: LAP analysis and expression ratio's of patients versus controls

gene.id	locuslink	symbol	score	smoothed.score	chromosome	gene.map	gene.position	q.value	median CO	median VCF	VCF/CO	log2 VCF/co
219180_s_at	55670	PEX26	0.31485	1.793781252	22	22q11.21	16940704	0	87.6	86.7	0.989726	-0.0149
220069_at	51807	TUBA8	0.981132	1.917861395	22	22q11.1	16973558	0	30	30	1	0
219211_at	11274	USP18	0.446857	2.066129479	22	22q11.21	17012757	0	76	65.1	0.856579	-0.22334
215603_x_at	2679	GGT2	-0.02505	2.523903834	22	c("22q11.2	17141174	0	62.9	66.1	1.050874	0.07159
208024_s_at	8214	DGCR6	4.862316	2.949790973	22	c("22q11.2	17273778	0	300.4	147.4	0.490679	-1.02715
227028_s_at	9993	DGCR2	3.990962	3.346799347	22	22q11.21	17403800	0	38.9	30	0.771208	-0.37481
239730_at	26221	DGCR13	0.928571	3.624496017	22	22q11	17497792	0	30	30	1	0
204383_at	8220	DGCR14	14.39046	3.634961642	22	c("22q11.2	17501451	0	134	83.1	0.620149	-0.68931
210010_s_at	6576	SLC25A1	6.782165	3.750364612	22	22q11.21	17543094	0	145.7	67.4	0.462594	-1.11218
240451_at	7290	HIRA	7.138001	4.105658037	22	c("22q11.2	17698223	0	41.8	30	0.717703	-0.47854
203152_at	64976	MRPL40	8.159832	4.260705025	22	22q11.21	17800035	0	400.8	194.9	0.486277	-1.04015
1553974_at	128977	LOC12897	12.51765	4.273502747	22	22q11.21	17810894	0	309.2	139.5	0.451164	-1.14828
209103_s_at	7353	UFD1L	7.190132	4.281149677	22	22q11.21	17817701	0	822.9	429.7	0.522178	-0.93739
204482_at	7122	CLDN5	0.416221	4.347187431	22	22q11.21	17890549	0	54	30.5	0.564815	-0.82415
206655_s_at	2812	GP1BB	1.82882	4.422411971	22	c("22q11.2	18091065	0	184.1	136.2	0.739815	-0.43476
207662_at	6899	TBX1	0	4.419058183	22	22q11.21	18124225	0	30	30	1	0
226204_at	54584	GNB1L	5.191292	4.40950642	22	22q11.2	18155939	0	103.3	69.7	0.674734	-0.56761
219560_at	79680	C22orf29	1.410881	4.373725262	22	22q11.21	18213670	0	37.1	30	0.808625	-0.30646
210803_at	10587	TXNRD2	4.941368	4.347271321	22	22q11.21	18243039	0	30	30.1	1.003333	0.004801
208818_s_at	1312	COMT	6.993038	4.275342606	22	c("22q11.2	18309308	0	849.2	461.3	0.543217	-0.8804
235396_at	128989	C22orf25	4.892061	4.161717579	22	22q11.21	18388630	0	244.9	181.4	0.74071	-0.43302
218650_at	54487	DGCR8	3.752243	4.0744775	22	22q11.2	18447833	0	120.9	102.7	0.849462	-0.23538
218475_at	27037	HTF9C	4.688983	4.030278781	22	c("22q11.1	18479397	0	63.4	30	0.473186	-1.07952
202483_s_at	5902	RANBP1	5.80296	4.022109915	22	22q11.21	18485023	0	715.2	287	0.401286	-1.3173
225744_at	29801	ZDHHC8	4.862845	4.000747456	22	22q11.21	18499364	0	112.9	71.3	0.631532	-0.66307
228550_at	65078	RTN4R	1.149472	3.832453675	22	22q11.21	18608939	0	30	30	1	0
205881_at	7625	ZNF74	2.551791	3.174154163	22	c("22q11.2	19078477	0	49.4	30	0.607287	-0.71955
221838_at	84861	KLHL22	4.842764	3.127260579	22	22q11.21	19125805	0	88.2	58.7	0.665533	-0.58742
222175_s_at	51586	PCQAP	3.315321	3.044264972	22	22q11.2	19191885	0	319.7	228.4	0.71442	-0.48516
223628_at	84222	DKFZp434	0.899982	2.722664167	22	22q11.21	19385401	0	56.6	53.7	0.948763	-0.07588
207081_s_at	5297	PIK4CA	4.708394	2.709945032	22	22q11.21	19391990	0	410.5	239	0.582217	-0.78037
218327_s_at	9342	SNAP29	3.712754	2.398500785	22	22q11.21	19543291	0	309.4	154.1	0.498061	-1.00561
212180_at	1399	CRKL	6.762857	2.285366627	22	c("22q11",	19601713	0	658.4	332.6	0.505164	-0.98518
244084_at	150209	AIFL	0.948051	2.19852904	22	22q11.21	19649433	0	60.9	48	0.788177	-0.34341
203412_at	8216	LZTR1	3.832558	2.169034501	22	c("22q11.2	19666655	0	137.6	98.6	0.71657	-0.48082
218492_s_at	80764	THAP7	5.002873	2.140414446	22	22q11.2	19684060	0	215.3	123.8	0.575012	-0.79834
206880_at	9127	P2RXL1	-0.19305	2.11589133	22	22q11.21	19699448	0	31.4	34	1.082803	0.11477
233374_at	23119	HIC2	-1.45296	1.454573723	22	22q11.21	20101692	0	30	30	1	0
213408_s_at	220686	LOC22068	2.219998	1.357961705	22	22q11.21	20157288	0	1127.7	926.3	0.821406	-0.28383
200682_s_at	7332	UBE2L3	1.152725	1.200855685	22	22q11.21	20251956	0.026938	2243.6	2215.5	0.987475	-0.01818
227042_at	150223	LOC15022	-0.14804	1.111671641	22	22q11.21	20312380	0.048977	144.3	143	0.990991	-0.01306
218681_s_at	23753	SDF2L1	1.915884	1.092181796	22	22q11.21	20326541	0.048977	214.4	183.6	0.856343	-0.22374
214986_x_at	23759	PPIL2	-1.20472	1.060760572	22	22q11.21	20350275	0.048977	123.6	111.4	0.901294	-0.14993
213996_at	29799	YPEL1	3.27043	1.021279608	22	22q11.2	20381825	0.048977	218.4	145.2	0.664835	-0.58893



The top table displays the results of the LAP analysis.

Green rows show genes which are not expressed in PBMC's; red rows show genes

not differentially expressed in PBMC's between patients and controls. Columms respectively show (from left to right): Probe Set ID; Entrez Gene ID; Gene Symbol; SAM score (di); smoothed score (Si); chromosomal localization and position; qvalues; median intensity of the controls (CO); median intensity of the VCF patients; ratio VCF/CO and log2 ratio VCF/CO.

The bottom figure shows the ratio median patients vs median controls for each of the genes shown along the x-axis.

Pathway analysis

The criterium $p<0.05$ and $FC>1.5$ showed 262 transcripts differentially expressed between patients and controls. These 262 transcripts are described in **supplementary dat file** (Accessible through www. psych.nl > 'people' > 'beveren' > 'list of deregulated genes chapter 9). Combining these with the genes identified by LAP ($n = 29$) and uploading these in the IPA generated 128 genes available for building networks and investigating relationships.

Seven functional networks are considered to be significantly associated with the uploaded geneset acoording to IPA criteria (IPA score > 3) These networks are involved in (among others) cardiovascular disease, cardiovascular system development and function, nervous system development and function, connective tissue development and function, cell signalling, and cell-to-cell signalling and interaction. **Table 4** shows the seven highest ranking networks, and the biological processes associated with these networks.

Canonical pathways identified by the IPA in which the uploaded geneset participates more than can be expected by chance ($p < .05$) are Natural Killer (NK)-cell signalling (p=.0004), neurotrophin/Trk signalling (p=.003), Fibroblast Growth Factor (FGF) signalling (p=.006), leukocyte extravasation signalling (p=.007), neuregulin signalling (p=.002), complement and coagulation cascades (p=.002), Platelet Derived Growth Factor (PDGF) signalling (p=.003), ERK/MAPK signalling (p=.003), axonal guidance signalling (p=.047), and huntington's disease signalling (p=.049).

Comparison of PBMC expression levels with brain expression levels in mouse models

The Df1/+ heterozygous mice (Df1/+), a model for 22q11DS, displays specific deficits in hippocampus-dependent functions. Sivagnanasundaram et al. (2007) analysed

Table 4: Significantly deregulated genetic networks as identified by the Ingenuity Pathways Analysis (IPA) (version october 2007).

ID	Molecules in Network	Score	# Focus molecules	Top functions
1	ARNT2, B2M, ↑**BASP1**, ↓CDC42, CYBA, H2-Q1, HLA-E, JAK2, ↑**KCNJ15**, KLRC1, ↓**KLRC2**,↓**KLRC3**,↓**KLRD1**, ↑**MEF2C**, ↑**MXD1**, MYCN, AK1, ↓**PARD6A**, PARD6G, ,**PIK3R3**, ↑**PPM1F**, ↓**PTPN4**, QDM, RAC2,,**RANBP1**, Rb, RB1, RBL2,↓**SEPT5**,↓**SNAP29**, ↑**SNX5**, STX1A, ↑**STXBP2**, ↑**TLR4**, VAMP2	26	18	Cell Cycle, Connective Tissue Development and Function, Nervous System Development and Function
2	↓**ACVR2B**,↓**CD2**, ↓**COMT**, CRK,↓**CRKL**,↓**CXCL10**, ↑**DDEF1**, ↑**DOCK4**, E2F4, EPHB6, ESR1, ↑**ETV6**, ↓**EXOSC2**, EXOSC7, EXOSC8, EXOSC9, EXOSC10, ↑**HSF1**, Hsp70, HSPA7, ↑**MAFG**, MAP4K5, MYC, NFE2L3, ↓**NQO1**, ↑**P2RX1**, ↑**PCID1**, PIK3R1, PLAG1, ↓**PPP2R2B**, RICS, ↑**SENP6**, ↑**SLC11A1**, SUMO1, XRN2	24	17	Cancer, Hematological Disease, Cell-To-Cell Signaling and Interaction
3	APEX1, ATF3, CARM1, ↓**CCL5**, CCR4, ↑**CD24**, CD63, CKM, ↓**CPT1A**,,**DGCR6**, EP300, ↑**GK**, ↑**HK2**, MDM2 (includes EG:4193), ↓**MYO6**, NEUROG3, ↑**OLIG1**, ↓**PIK4CA**, PPARGC1A, ↓**PRF1**, PTGDS, ↓**PYHIN1**, RELA, SELP, SLC2A4, ↑**SLC2A5**, ↓**TBX21**, TFDP1, ↑**TNFRSF10C**, TP53, TRIM28, TSC22D3, ZBTB17, ↓**ZDHHC8**, ↓**ZNF74**	22	16	Gene Expression, Cancer, Respiratory Disease
4	↑**ABCA1**,,**API5**, CDKN2A, CKB, ↑**CR1**, CR2, ↓**4S234E**, ↓**DGCR14**, DLG4, ↓**F2R**, FGF2, FLT1, ↓**GZMA**, HMGB2, ↑**HPSE**, Hsp27, KCNJ4, KCNJ12, KPNB1, LAMA1, ↑**LIN7A**, ↑**NAB2**, ↓**PCQAP**, RNA polymerase II, ↓**RNGTT**, RNMT, SMAD3, Smad2/3, SP1, STX12, SUPT5H, ↑**TFE3**,↓**UFD1L**, ↓**ZNF83**, ↑**ZNF451**	22	16	RNA Post-Transcriptional Modification, Cell Signaling, Cardiovascular System Development and Function
5	↑**ADAM9 (includes EG:8754)**, APOB,↓**APOBEC3G**, ↓**ARL4C**, CEBPA, CIAA1, CIAA2, COL10A1, COL11A2, COL18A1, COL1A1, COL2A1, COL3A1,,**COL5A3**, ↑**CSF2RA**, EGR2, GRM5, ↑**HES7**, HMGB2, Homer, HOMER1, **HOMER3**, ↑**HP**, ITGA2, ITGA9 (includes EG:3680), ITGB1, ITPR, NFYB, NFYC, ↑**PTPRE**, SHANK3, ↑**THBD**, ↑**TNFRSF8**,,**TXNRD2**, ↑**ZBTB7B**	17	13	Dermatological Diseases and Conditions, Cardiovascular Disease, Cell Cycle
6	↑**AQP9**, ARF1, CARM1, CBX1, CBX5, ↑**CD53**, ↓**CENTG2**,,**CHAF1A**, Creb, ↑**CREB5**, CREM, ↑**ERAF**, GRIA2, GRM7, H3F3B,↓**HIRA**, HIRIP3, Histone h3, KIR3DL1, MBD1, NCAPD2, NCOA1, NCOA2, PICK1, Pkc(s), PLD1, ↑**PRKCA**, ↓**RORA**, ↓**RORC (includes EG:6097)**, SETDB1, SFTPA1, ↓**SMC2**, SMC4,↓**THAP7**, TRIM24	15	12	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cell Morphology

7	↑**ACSL4**, Actin, Arp2/3, ↑**BACH2** (includes EG:60468), ↓**C19ORF12**, CAM, CD44, ,**CLDN5**, ↓**CLDN19**, ↓**CLIC5**, DNMBP, HDAC4, MARK4 (includes EG:57787), ↑NCOR2, PARD3, ↓**PARD6A**, PARD6B, PARD6G, ↑**PDK1**, PFN1, ↓**PHLDB2**, PKC (λ,ζ), PRKCI, ↑**RAPGEF2**, SMARCA4, STK11, TJP1, TJP2, TJP3, VIM, WAS, ↑**WASF1**, WASF2, YWHAQ, YWHAZ	13	11	Cell Morphology, Cellular Development, Cell-To-Cell Signaling and Interaction

The table displays the seven functional networks identified by the IPA as significantly (score > 3) associated with the geneset defined by the two critera (1) deregulated between patients and controls according to LAP or (2) t-test < 0.05 and Fold Change > 1.5. The table displays the genes associated with the functional networks (genes which are present in the uploaded gene set are in bold). The arrows behind each gene indicate the direction of change (arrow pointing upwards: increased expression in patients, arrow pointing downwards: decreased expression in patients).The column 'score' gives the significance score for the network . The column '# of genes' gives the number of genes in the network. Each network consists of 35 genes.
The last column shows the functions in which each network is predominantly involved..

the hippocampal gene expression of genes mapping to the deleted region as compared to wild type (WT) mice. Twelve genes were differentially expressed in the hippocampus. Of these twelve genes seven were also expressed in our PBMC samples (DGCR6, RANBP1, ZDHHC8, HTF9C, COMT, CLDN5, and UFD1L). The relative expression levels of these genes (hippocampal levels of Df1/+ vs WT mice (after Sivagnanasundaram et al (Sivagnanasundaram et al., 2007)) and PBMC levels of 22q11DS patients vs controls) are shown in **table 5**.

The relative expression levels of mice and humans correlated strongly and significantly (r: 0.677, p = 0.05, one sided).
In another study, Meechan et al (Meechan et al., 2006) investigated the expression levels of nine 22q11 orthologues in a 22q11DS mouse model (IDD, PRODH2, ZDHHC8, RANBP1, T10, COMT-MB, TBX1, UFD1L, and HIRA) in the developing and adult mouse brain. They also found diminished expression for the entire set of orthologues. Their findings indicated a fairly consistent decrease in expression lev-

Table 5: Relative hippocampal gene expression levels of Df1/+ vs WT mice (after Sivagnanasundaram et al, 2007) and PBMC gene expression levels of 22q11DS patients vs controls (this study) for seven genes expessed in both samples (mice and humans)

Gene	Relative hippocampal expression Df1/+ vs WT	Relative PBMC expression 22q11DS patients vs controls
DGCR6	0,45	0,49
RANBP1	0,53	0,40
ZDHHC8	0,86	0,63
HTF9C	0,63	0,47
COMT	0,54	0,54
CLDN5	0,68	0,56
UFD1L	0,78	0,52

els with a magnitude between 40% and 60%, quite similar to our PBMC findings. Taken together, these results in mice cautiously suggest that dimished expression levels of 22q11 genes in PBMC's in humans might reflect decreased expression levels in the mouse brain for those genes which are also expressed in neuronal tissue.

Discussion

To our best knowledge our study is the first to examine gene expression of VCFS patients. We show decreased expression of several genes present in the 22q11 deleted region. Among these are the genes which have been previously associated with schizophrenia, COMT, Ufd1L, PCQAP (Maynard et al., 2003), and GNB1L (Williams et al., 2007).

Canonical pathway analyses show the significant involvement of the canonical pathways NK-cell signalling, neurotrophin/Trk signalling, FGF signalling, leukocyte extravasation signalling, neuregulin signalling, complement and coagulation cascades, PDGF signalling, ERK/MAPK signalling, axonal guidance signalling, and Huntington's disease signalling. Many of these seem relevant for schizophrenia pathology or, more generally, for neuronal development and neuropsychiatric disorders.

The most significantly involved canonical pathway in our study is NK-cell signalling. Altered NK-cell activity in schizophrenia has been reported by some studies (Yovel et al., 2000) and is hypothesized to be related to the altered immunity and the reduced occurrence of autoimmune diseases and malignancies that has been observed in schizophrenia (Kobrynski and Sullivan, 2007). An increased proportion of NK-cells have been described in patients with 22q11DS (Kornfeld et al., 2000; Jawad et al., 2001; Kanaya et al., 2006). However, generally there is no clear agreement among studies on the involvement of NK cell activity in schizophrenia, with some studies showing lower activity, some higher activity, and the majority no change in activity between schizophrenia patients and controls (Yovel et al., 2000).

There is far more support for the involvement of neurotrophin/Trk signalling in schizophrenia. This pathway is activated via the Trk family of receptors by various neurotrophic factors. The neurotrophic factors best known for their association with schizophrenia are Brain-derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), and neurotrophin-3 (NT-3). BDNF, NGF, and NT-3 are involved in a range of neuroplastic processes. Disturbed neurotrophin signalling is thought to underlie the neurodevelopmental disturbances seen in schizophrenia. Altered brain- (reviewed in Shoval and Weizmann (2005)) and serum levels (reviewed in Van Beveren et al (2006)) of neurotrophins have repeatedly been reported in schizophrenia.

There is also considerable support for an association of neuregulin with schizophrenia. Neuregulin is considered one of the genes most strongly associated

with schizophrenia (Carter, 2006; Straub and Weinberger, 2006; Ross et al., 2006). Neuregulin has been implicated in neuronal differentiation and migration. However, no consistent changes in neuregulin expression have been detected in schizophrenia.
Disturbances in axonal guidance signalling and Huntington's disease signalling are at present not specifically associated with schizophrenia but may underlie the general cognitive and behavioral disturbances found in schizophrenia. Moreover, in 22q11DS frequently motor disturbances, obsessive-compulsive symptoms, tics, and neurological aberrations are being observed (Kobrynski and Sullivan, 2007), which are also found in Huntington's disease.
There is incidental evidence for an association of FGF signalling with schizophrenia. One publication reports decreased serum levels of FGF in schizophrenia (Hashimoto et al., 2003; Klejbor et al., 2006). We could not identify reports describing involvement of PDGF signalling in schizophrenia. However, both FGF and PDGF are important growth factors of which involvement in schizophrenia has been suggested on theoretical grounds (Ross et al., 2006).
Disturbances in leucocyte extravasation signalling, and complement and coagulation cascades cannot be clearly related with 22q11DS and schizophrenia pathology. It has been postulated that subtle abberations in cellular machinery may be present in all organs and cell types in schizophrenia (Prabakaran et al., 2007), but only give rise to overt pathology in the nervous system. Such subtle abberations may however become present in in-vitro situations, explaining differences in coagulation and leucocyte functions between patients and controls.

Taken together, our pathway analyses show a number of pathways previously associated with schizophrenia among the pathways significantly associated with the deregulated gene set in our DS sample. Most notably for their involvement in schizophrenia are neurotrophin/Trk signalling and neuregulin signalling. Our findings cautiously suggest that the molecular-biological underpinnings of the psychotic phenomena observed in DS are at least partly related with those seen in 'regular' schizophrenia. Moreover, our findings are present even though only three patients show psychotic phenomena, and four do not. This suggests that molecular-biological pathways involved schizophrenia are deregulated in DS patients both with and without psychosis, and that, like in schizophrenia, environmental factors modulate the expression of the psychosis phenotype in the 22q11DS.

Our study has a number of limitations; first, the number of subjects is small and heterogeneous (ie with- and without psychosis). Second: to identify functional networks we used a liberal statistical approach by not correcting for repeated measurements to obtain the geneset with criterium $p<0.05$ and $FC>1.5$, with a risk of identifying false-positives. However, we assume that combining this set with the genes present in the 22q11 deleted region (the set identified by LAP), which are certainly no false-positives, and investigating the functional relationships of combined geneset will 'pull out' the true positive genes as those will be the ones that predominantly have functional relationships with the 22q11 genes.
Not so much a limitation as well as a caveat is that the approach presented here (investigating PBMC gene expression to gain insight in neuropsychiatric phenomena) relies on the assumption that molecular-biological aberrations observed in peripheral tissue can be informative about brain molecular-biological processes. Though may signalling pathways are similar in peripheral tissue and brain, and there are some reports that this approach is feasible (Sullivan et al., 2006; Gladkevich et al., 2004), the precise validity of this assumption remains to be further elucidated.

In summary, this study (1) shows decreased expression of genes in the 22q11 deleted region in PBMC's of 22q11DS patients and (2) suggests the presence of deregulated signalling pathways relevant for schizophrenia pathology in PBMC's of 22q11DS patients.
Generally, our findings support the use of the 22q11DS syndrome as a suitable, more homogeneous research model for schizophrenia. Future studies should replicate our findings using larger samples.

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Chapter 10

A Biomarker Fingerprint for Schizophrenia in Blood

Submitted

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Abstract

A key challenge for modern medicine is to develop accurate molecular tests for early diagnosis of debilitating psychiatric disorders such as schizophrenia. In this study, we have identified a biological signature in serum of first and recent onset schizophrenia subjects using a multiplex molecular profiling approach. This signature, comprised of 13 molecules implicated in inflammation, was replicated across five independent cohorts of schizophrenia patients and was capable of identifying unaffected individuals who later went on to develop schizophrenia. Importantly, this signature was not apparent in related psychiatric disorders such as major depressive disorder, bipolar disorder and Asperger syndrome, suggesting that these findings hold promise for future development of a rapid, non-invasive blood test to facilitate early or even pre-symptomatic diagnosis of schizophrenia. It is hoped that this will help clinicians to identify vulnerable patients early in the disease process, allowing for earlier therapeutic intervention and better outcomes.

Schizophrenia is a debilitating psychiatric disorder affecting around 1% of the world population (Saha et al., 2005). It negatively impacts on quality of life for patients and their families and costs hundreds of billions of U.S. dollars in healthcare provision and lost earnings (National Institute of Mental Health, 2008). Although advances have been made over recent decades in quality and reliability of psychiatric diagnoses using standardized systems and structured interviews such as DSM-IV (American Psychiatric Institute, 2000) or SCID (First et al., 1996), clinical diagnostics still rely on subjective assessment and categorization of overt symptoms. The accuracy of diagnosis is complicated further by the presence of overlapping symptoms with other psychiatric illnesses such as bipolar disorder (BD), major depressive disorder (MDD) and Autism Spectrum Disorders [e.g. Asperger syndrome (AS)], and by the presence of potential confounding factors including substance abuse (Csernansky et al., 2002). Patients often present initially with atypical mood and cognitive symptoms leading to uncertainty and delay in final diagnosis which can hinder early therapeutic intervention, resulting in a poorer outcome (Moller, 2004). Here, we have attempted to identify serum biomarkers which have proven crucial for aiding clinical diagnosis and effective treatment of many disorders but are still

Supplementary table 1: List of analytes measure by the Rules Based Medicine HumanMAP® assays.

Alpha-1 Antitrypsin	FGF-4	MIP-1beta
Angiotensin Converting Enzyme	Fibrinogen	MIP-3 alpha
Adrenocorticotropic Hormone	Follicle Stimulation Hormone	MMP-1
Adiponectin	G-CSF	MMP10
Agouti-Related Protein	Growth Hormone	MMP-2
Alpha-2 Macroglobulin	GLP-1 total	MMP-3
Alpha-Fetoprotein	Glucagon	MMP7
Alpha-1-Microglobulin	Glutathione S-Transferase-α	MMP9 (Total)
Amphiregulin	GM-CSF	MMP-9
Angiopoietin 2 (ANG-2)	GRO-alpha	Myeloid Progenitor Inhibitory Factor 1
Angiotensinogen	Haptoglobin	Myeloperoxidase
Apolipoprotein A1	HB-EGF	Myoglobin
Apolipoprotein A2	HCC-4	Neutrophil Gelatinase-Associated Lipocalin
Apolipoprotein CI	Hepatocyte Growth Factor	NGFb
Apolipoprotein CIII	I-309	NrCAM
Apolipoprotein H	ICAM-1	Osteopontin
Apolipoprotein B	IFN-gamma	PAI-1

(continued on next page)

Supplementary table 1: *(continued)*

Apolipoprotein D	IgA	Pancreatic polypeptide
Apolipoprotein E	IgE	Prostatic Acid Phosphatase
Clusterin (Apo J)	IGF-1	PAPP-A
AXL	IGF BP-2	Pulmonary and Activation-Regulated Chemokine
Beta-2 Microglobulin	IgM	PDGF
B-Lymphocyte Chemoattractant	IL-10	Progesterone
BMP-6	IL-11	Prolactin
Brain-Derived Neurotrophic Factor	IL-12p40	Protein S
Betacellulin	IL-12p70	Prostate Specific Antigen, Free
Complement 3	IL-13	PYY
Cancer Antigen 125	IL-15	RANTES
Cancer Antigen 19-9	IL-16	Resistin
Calbindin	IL-17	S100b
Calcitonin	IL-17E	Serum Amyloid P
CD40	IL-18	Stem Cell Factor
CD40 Ligand	IL-1alpha	Secretin
CD5L	IL-1beta	SGOT
Carcinoembryonic Antigen	IL-1ra	SHBG
CgA	IL-2	SOD
Creatine Kinase-MB	IL-23	Sortilin
Ciliary Neurotrophic Factor	IL-3	sRAGE
Complement Factor H	IL-4	Tamm-Horsfall Protein (THP)
Connective Tissue Growth Factor	IL-5	Thyroxine Binding Globulin
Cortisol	IL-6	Thymus-Expressed Chemokine (TECK)
C Reactive Protein	IL-6 Receptor	Tenascin C
Cystatin C	IL-7	Testosterone
EGF	IL-8	Tissue Factor
EGF-R	Insulin	TGF-alpha
ENA-78	IP-10 (Inducible Protein-10)	TGF-beta 3
Endothelin-1	Kidney Injury Molecule-1	TIMP-1
EN-RAGE	Leptin	TNF RII
Eotaxin	LH (Luteinizing Hormone)	TNF-alpha
Eotaxin-3	Lipoprotein (a)	TNF-beta
Epiregulin	Lymphotactin	Thrombopoietin
Erythropoietin	MCP-1	TRAIL-R3
E-Selectin	MCP-2	Transferrin
Fatty Acid Binding Protein	MCP-3	Trefoil Factor 3 (TFF3)
Factor VII	MCP-4	Thyroid Stimulating Hormone
FAS	M-CSF	Thrombospondin-1
Fas-Ligand	MDC	TTR (prealbumin)
Ferritin	MIF	VCAM-1
Fetuin A	Gamma-Interferon-induced-Monokine	VEGF
FGF basic	MIP-1alpha	Vitronectin
		von Willebrand Factor

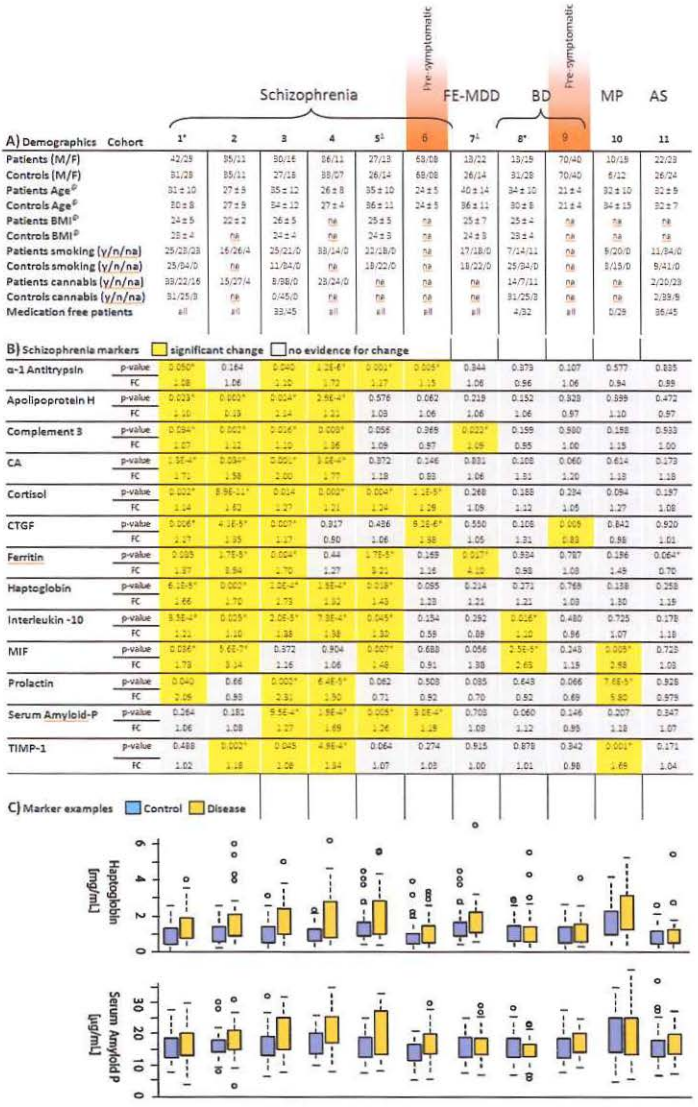
lacking for psychiatric illnesses such as schizophrenia(7). One reason for this is that it is not known whether psychiatric disorders can be identified by a biological signal in the peripheral circulation. The recent introduction of fluorescent bead-based technologies allows the simultaneous measurement of multiple analytes in small-volume samples, revolutionizing these analyses. Such analytical platforms are also suitable for further development of rapid, sensitive and specific diagnostic assays.

With this in mind, we have used the HumanMAP ® Multi-Analyte Profiling platform (**Supp. Table 1**) in collaboration with Rules Based Medicine (Austin, TX, USA) to analyze serum samples from 1061 individuals comprised of 326 first, recent onset and pre-symptomatic schizophrenia, 206 affective disorder (35 first episode major depressive disorder (MDD), 142 bipolar disorder (BD), 29 manic psychosis), 45 Asperger syndrome and 484 control subjects, recruited from psychiatric centres in Germany, Holland, the United Kingdom and the United States. The HumanMAP ® technology has been shown to be reproducible and robust and has already been applied successfully in numerous clinical studies or biomarker discovery projects of diseases such as epithelial ovarian cancer (Bertenshaw et al., 2008), coronary artery disease (Gurbel et al., 2008), myocardial infarction (Escobar et al., 2007) and autoimmune disorders (Delaleu et al., 2008).

The first stage of this study was aimed at identifying a reproducible pattern of molecular changes in schizophrenia (n=250) compared to control subjects (n=230) across 5 independent cohorts (**Fig. 1A**). Analytes were selected for the final panel based on biological reproducibility. This required that they had two-tailed p-values less than 0.05 in 3 or more cohorts and showed consistent directional changes. Cohorts 1, 2 and 4 were comprised of first onset antipsychotic-naïve subjects and most patients from cohort 3 and all patients from cohort 5 were antipsychotic-naïve or had been off medication for at least six weeks prior to sample collection. The remaining 12 patients from cohort 3 were medicated. All cohorts were matched for age and gender and only subjects with no medical co-morbidities or substance abuse were included. Cohort 1 was also matched for body mass index, smoking, cannabis consumption and date of sample collection and some of these parameters were also matched across the other cohorts (Fig. 1A).

Analysis using the Human MAP ® platform resulted in identification of a signature consisting of 13 analytes using the above criteria (**Fig. 1B**). At least 7 of these markers were altered consistently across all schizophrenia cohorts. An algorithm comprising the 13 analytes was trained on data from cohort 1 and tested blindly

Figure 1



HumanMap® profiling of clinical serum samples resulting identification of a molecular signature for schizophrenia.

A) Demographic details. Cohorts 1-5: schizophrenia - paranoid subtype (295.30). Cohort 6: pre-symptomatic subjects later diagnosed with schizophrenia (295.1-295.3, 295.6, 295.7, 295.9). Cohort 7: acutely ill major depressive disorder (FE-MDD).

Cohort 8: bipolar disorder (BD) euthymic [types I (296.4) and II (296.89)]. Cohort 9: BD pre-symptomatic (296.00–296.06; 296.40–296.7, 296.89). Cohort 10: BD subjects in mania phase (manic psychosis; MP). Cohort 11: Asperger syndrome (AS). Subjects were matched for the indicated parameters and the medication status of each patient group is indicated. M/F = male/female; BMI = body mass index; Y/N = yes/no; na = not available. @Values are shown as mean ± sd. #, & Control groups of cohorts 1 and 8, and those of cohorts 5 and 7 were identical.

B) Expression profiles of 13 serum analytes in schizophrenia compared to MDD, BD, MP and AS subjects. Significant differences (two-tailed parametric t-test; yellow) and no change (grey) are shown for all patient and control comparisons. Measurements that were also significant using non-parametric Wilcoxon rank sum tests are indicated by an asterisk. CA = carcinoembryonic antigen, CTGF = connective tissue growth factor; MIF = macrophage migration inhibitory factor. Timp 1 = tissue inhibitor of metalloproteases 1. Prolactin was also changed in cohort 7, but the observed fold change was opposite to the one observed in the schizophrenia cohorts.

C) Expression profile changes of serum amyloid P and haptoglobin in patient and control populations across the 11 cohorts. The expression levels are given as box plots for patients (yellow) and controls (blue).

across cohorts 2-5, yielding an unweighted average sensitivity of 0.82 and specificity of 0.79 (Fig. 2). We also assessed whether there was any effect on biomarker profiles from any of the recorded demographic parameters. This analysis showed that only 14% of the observed changes in cohorts 1-5 were affected, suggesting that potential confounding factors did not influence the results significantly.

For the second stage of the study, we tested serum samples (cohort 6) collected by the United States military within one month of schizophrenic subjects first coming to the attention of clinical psychiatrists. As such, these individuals were also antipsychotic naïve. It should be noted that these samples were selected from a bank comprising approximately 43 million sera, which facilitated excellent matching for age, gender, ethnicity and lifestyle between diseased and control subjects. The use of illicit drugs was not a factor due to regular military screening procedures and, as with the other cohorts, only those individuals with no co-morbid medical histories were included in the study. HumanMAP® profiling showed that α-1 antitrypsin, cortisol, connective tissue growth factor and serum amyloid P were altered significantly in these subjects (**Fig. 1B**). The finding that these markers were present

before overt clinical presentation of the disorder suggests that they could represent trait or early state markers for schizophrenia, whereas other analytes from the panel may represent state markers since these were present during the acute stage of the illness.

One factor which renders diagnosis of schizophrenia difficult is the overlap of symptoms with other neuropsychiatric disorders. For this reason, we also tested the 13 analyte panel using sera from 35 MDD (cohort 7), 142 BD (cohorts 8 and 9), 29 manic psychosis (cohort 10), 45 AS (cohort 11) and 277 control subjects. MDD subjects were acutely ill, drug naïve (n=22) or drug free (n=13) for at least 6 weeks prior to sample collection, and chosen due to the overlap of negative symptoms between depression and schizophrenia (Fleischhacker, 2000). Of the 32 euthymic BD patients (cohort 8), 28 were medicated and chosen for this study as such patients can experience disruptions in cognitive behaviours as seen in schizophrenia (Ferrier et al., 1999). The 110 BD patients (cohort 9) were medication-naïve and, similar to cohort 6, collected through the United States military before coming to the attention of clinical psychiatrists. Mania subjects were all medicated and selected for the study as they can display behaviours similar to the psychotic symptoms of schizophrenia (Dunayevich and Peck, 2000). AS subjects were mostly un-medicated (36 out of 45 individuals) and were analyzed since they also overlap with schizophrenia for such symptoms as emotional lability, anxiety and poor social functioning (Raja and Azzoni, 2001). The results of the HumanMAP® analysis showed that the biological signature was lower, with only 0 to 3 analytes altered in the non-schizophrenia cohorts (Fig. 1B). Interestingly, the presymptomatic BD subjects (cohort 9) showed a significant alteration in only one of the analytes, suggesting that the panel was specific for schizophrenia. We also performed power analysis to investigate potential effects of group size on ability to detect significant analyte differences between patients and controls in each cohort. This analysis revealed that for all 13 analytes, the statistical power was similar across all cohorts adding further support to the specificity of the results (**Supp. Table 2**). An example of the biological profile of two of the analytes (haptoglobin and serum amyloid P) is shown across all 11 cohorts (**Fig. 1C**). This illustrates the biological specificity of the signal for schizophrenia which is not apparent for the non-schizophrenia conditions.

To gain further insight into the differential diagnostic capability of the 13 analyte signature, we trained an algorithm on data from cohort 6 (schizophrenia) and tested this on cohort 9 (BD). The two military cohorts were selected for this comparison as the subjects were matched closely across all parameters. Testing the algorithm on

Supplementary table 2.

Cohort	1	2	3	4	5	6	7	8	9	10	11
α-1 Antitrypsin	0.41	0.31	0.31	0.30	0.28	0.48	**0.26**	**0.28**	**0.63**	**0.17**	**0.32**
Apolipoprotein H	0.67	0.53	0.52	0.50	**0.47**	**0.74**	**0.44**	**0.48**	**0.88**	**0.29**	**0.54**
Complement 3	0.63	0.49	0.48	0.46	**0.43**	**0.70**	0.41	**0.44**	**0.85**	**0.26**	**0.50**
CA	>0.99	>0.99	>0.99	>0.99	**>0.99**	**>0.99**	**>0.99**	**>0.99**	**>0.99**	**0.97**	**>0.99**
Cortisol	0.60	0.47	0.46	0.44	0.42	0.68	**0.39**	**0.42**	**0.83**	**0.25**	**0.48**
CTGF	0.83	0.70	0.69	**0.67**	**0.63**	0.89	**0.60**	**0.64**	0.97	**0.4**	**0.71**
Ferritin	0.73	0.59	0.58	**0.56**	0.53	**0.80**	0.50	**0.53**	**0.92**	**0.32**	**0.60**
Haptoglobin	>0.99	0.99	0.99	0.99	0.99	**>0.99**	**0.98**	**0.99**	**>0.99**	**0.87**	**>0.99**
Interleukin-10	>0.99	>0.99	>0.99	>0.99	>0.99	**>0.99**	**>0.99**	>0.99	**>0.99**	**0.99**	**>0.99**
MIF	>0.99	0.99	**0.99**	**0.99**	0.99	**>0.99**	**0.98**	0.99	**>0.99**	0.87	**>0.99**
Prolactin	>0.99	**0.99**	0.99	0.98	**0.98**	**>0.99**	**0.97**	**0.98**	**>0.99**	0.83	**0.99**
Serum Amyloid-P	**>0.99**	>0.99	>0.99	>0.99	>0.99	>0.99	**>0.99**	**>0.99**	**>0.99**	**0.97**	**>0.99**
TIMP-1	**>0.99**	0.99	0.99	0.98	**0.97**	**>0.99**	**0.96**	**0.98**	**>0.99**	0.82	**0.99**

Power calculations for two-tailed T-tests (alpha=0.05, unequal group sizes) were performed based on the effect size in cohort 4 (serum amyloid P and TIMP-1) and cohort 1 (remaining analytes). Significant differences in analyte levels between patients and controls are indicated in light. Non-significant differences are shown in bold.

cohort 6 identified 56 out of the 76 schizophrenia subjects (sensitivity 0.74) and 60 of 76 controls (specificity 0.79) correctly (**Fig. 2**). The accuracy of the signature for schizophrenia was shown by the finding that only 13 of the 110 BD patients (sensitivity = 0.12) had a signature similar to schizophrenia, and 85 of the 110 controls (specificity = 0.79) were identified correctly (Fig. 2). To rule out any gender effects, we retrained and retested the algorithm using data from male subjects only. This gave essentially the same results with a good sensitivity for schizophrenia (sensitivity 0.75, specificity 0.79) and low sensitivity for this signal in BD subjects (sensitivity 0.11, specificity 0.77).

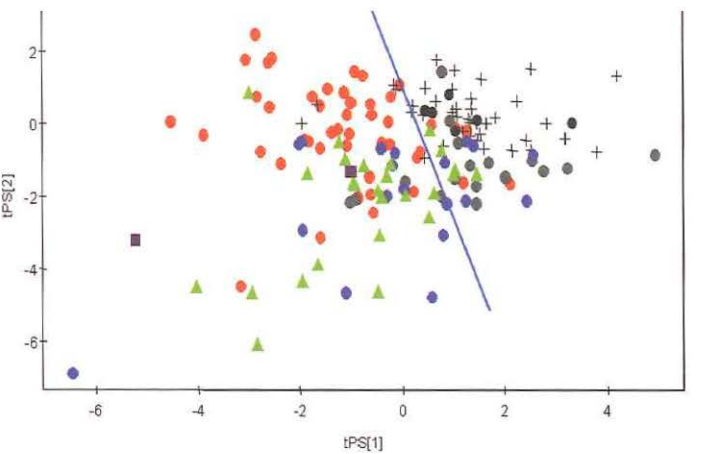
To investigate whether the 13 analyte signature was comprised of trait and/or state biomarkers, we tested the performance of the panel using samples from concordant (n=26) and discordant (n=36) twins for schizophrenia (**Supp. Fig. 1**). Partial Least Squares analysis showed that 75% of all affected twins clustered with schizophrenia subjects, consistent with the results of cohorts 1-5. Two subjects from discordant pairs who were unaffected at the time of sample collection and later developed schizophrenia, also showed a schizophrenia-like profile, as seen for cohort 6. However, only 22% of the remaining unaffected twins of discordant pairs showed a pattern similar to the schizophrenia signature, suggesting that genetic

Figure 2

Stage
Study design
Diagnostic accuracy
I
First and recent onset schizophrenia
First presentation with psychosis
Final Diagnosis within 6 months (according to DSM IV)
Sample collection
*
II
Pre-symptomatic schizophrenia/ bipolar disorder (US Military)
30 days
no overt impairment
First clinical encounter
Final diagnosis and retirement from service
Cohort
Sens
Spec
1 73 80
2 89 76
3* 93 76
4 77 85
5* 68 78
SZ 6 74 78
BD 9 12 77
Traning set
Test set
LDA coefficients - Cohort 1
coefficient
Analyte number
Cohort 2
control
SZ
Density
Probability for schizophrenia
Cohort 6
control
SZ
Density
Probability for schizophrenia

Diagnostic accuracy of the 13 analyte panel for schizophrenia.
The study design indicates times of sera collection (red arrows). Final DSM-IV diagnosis was made at first presentation or within 6 months after manifestation. Red stars indicates samples collected before or after final diagnosis. Sensitivity and specificity values (%) were determined for distinguishing schizophrenia (SZ) or BD subjects from controls using Linear Discriminant Analysis. The algorithm was trained on cohort 1 and tested blindly on cohorts 2-5. The algorithm was also trained on the presymptomatic schizophrenia subjects in cohort 6 and compared to presymptomatic BD by blind testing on cohort 9. Sensitivity and specificity were estimated in cohort 6 using leave one out cross validation. Coefficients of the linear discriminants are shown for each analyte on the top right (algorithm built on cohort 1). The density distributions describe the output of the algorithm for cohort 2 (blinded prediction using the algorithm trained on cohort 1) and for cohort 6 (leave one out cross-validation estimate). The algorithm output ranged from 0-1 and was smoothed for illustration purposes. For the calculation of classification accuracy, a cut point of 0.5 was used.

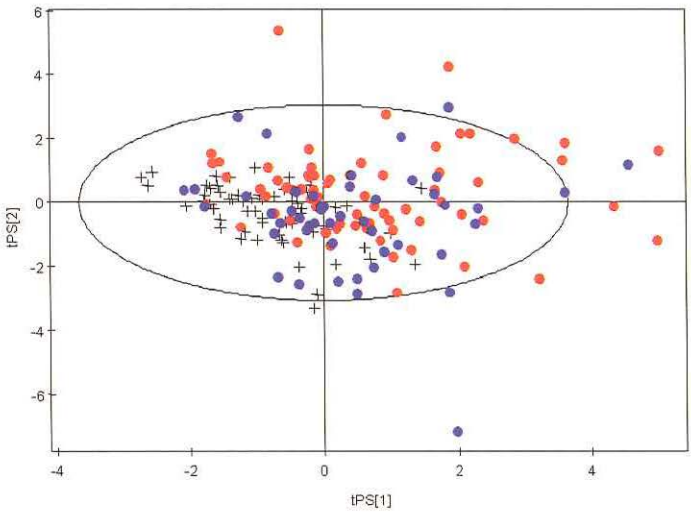
Supplementary figure 1



Analysis of serum/plasma from twins concordant and discordant for schizophrenia using the 13 analyte schizophrenia panel.
Partial Least Squares (PLS) scores plot with model built using antipsychotic naïve first onset schizophrenia patients (red circles) and controls (black crosses) from cohort 4. Scores were predicted for twins concordant for schizophrenia

(green triangles), twins discordant for schizophrenia (affected = blue circles; non-affected = grey circles), non-affected twins of schizophrenia patients who developed schizophrenia after sample collection (purple squares) and healthy control twins (black circles). One potential caveat is that cohort 4 samples were sera and the twin study was composed of plasma. Therefore, to increase comparability between sample sets, the ratio between the average measurement in controls from cohort 4 and healthy control twins was calculated and used to correct all measurements of twin samples accordingly. The linear separator (blue line) was chosen subjectively to approximate the maximal separation between schizophrenia patients and controls.

Supplementary figure 2



Investigation of drug effects on the analyte profile.
PLS scores plot with model built using drug naïve, first onset schizophrenia patients (red dots; n=71) and controls (black crosses; n=59) from cohort 1. Based on this model, scores were predicted for schizophrenia patients treated short term with antipsychotic medications (blue circles; n=48). Only 12 analytes of the panel were used for this analysis since sample volume restrictions prevented the measurement of cortisol in treated patients.

predisposition alone is not sufficient to cause significant serum abnormalities. To test this further, we also analyzed samples collected from patients after short term

treatment with antipsychotics that resulted in improved symptoms (**Supp. Fig. 2**). Interesting, 60% of these subjects showed a shift from the schizophrenia signature to a more control-like pattern, suggesting that a significant proportion of the panel may contain biomarkers which reflect the state of the disorder.

All of the analytes identified as schizophrenia biomarkers have also been implicated in acute or chronic inflammatory conditions such as systemic lupus erythematosus (SLE). This is intriguing as around 22% of SLE patients also show a variety of neuropsychiatric symptoms similar to those in schizophrenia (Fessel and Solomon, 1960). This link is thought to arise from common inflammatory abnormalities in brain microvasculature, resulting in blood-brain barrier disturbances (Johnson and Richardson, 1968; Bresnihan et al., 1977). However, it should be stressed that there are several markers on the HumanMAP® panel which have been associated previously with SLE (IL-2, IL-4, IL-6, TNF-alpha, MMP-9, and others) which showed no significant alterations in the present schizophrenia cohorts. This suggests that schizophrenia and SLE may share some aspects of an inflammatory component. Consistent with this, analysis of the 13 analytes in silico using the Ingenuity Pathways Knowledge Base (www.ingenuity.com) confirmed that the most significant functional pathway was "inflammatory response" ($p=5.04E-9 - 4.10E-3$). An altered inflammatory response has been associated with a number of other psychiatric disorders, as has dysregulation of the adrenal cortex hormone cortisol (Rybakowsky et al., 2006). It was interesting in this regard that cortisol was elevated significantly across all schizophrenia cohorts and showed a trend for increase across all other non-schizophrenia cohorts ($p=0.094-0.298$).

Hypercortisolemia and hypothalamic-pituitary-adrenal hyperactivity may also be linked to the observed increase in macrophage migration inhibitory factor (MIF) levels. MIF has been shown to play a central role in the progression of immunological disturbances of SLE associated with atherosclerotic plaque development (Burger-Kentischer et al., 2002; Santos and Morand, 2009). SLE and schizophrenia also share an increased prevalence of insulin resistance, metabolic syndrome and type II diabetes (Wayed et al., 2004; De Hert et al., 2006; Shoelson et al., 2006; Chung et al., 2007). This is consistent with our previous findings of such abnormalities in the brain vasculature of patients and changes in glucoregulation in the schizophrenia brain and periphery (Prabakaran et al., 2004; Harris et al., 2008; Guest et al., 2010).

Further support of an inflammatory component in schizophrenia has been demonstrated by epidemiological studies which found that history of autoimmune disease was associated with a 45% increased risk for schizophrenia (Eaton et al.,

2006). A recent study published in Nature has shown that there is a significant association of single nucleotide polymorphisms and copy number variation within the major histocompatibility region, with an increased schizophrenia risk (Stefansson et al., 2009). In addition, inflammation-related gene products have been shown to be altered in schizophrenia post-mortem brain studies and in plasma of living schizophrenia patients (Saetre et al., 2007; Potvin et al., 2008). Taken together, these findings suggest that inflammation may be a converging pathophysiological process in schizophrenia and other psychiatric and non-psychiatric disorders such as metabolic syndrome (Steiner et al., 2009). The biomarker signature comprising inflammatory biomarkers that we have identified, already shows promise for distinguishing schizophrenia from controls and other psychiatric disorders. Therefore, further expansion of the signature by targeting hormonal, metabolic and neurotrophic pathways in larger population studies, may lead to a more robust diagnostic panel for identification of schizophrenia and provide improved classification of this disorder which is known to be comprised of overlapping subtypes (Seaton et al., 2001).

One strong point of the present study is that samples were obtained from first onset antipsychotic naïve subjects who were well matched to their respective control populations with regards to such factors as age, gender, substance abuse and lifestyle. Almost all previous schizophrenia studies have investigated chronic patients who have been treated with antipsychotic medications, and often have multiple co-morbidities which can confound biomarker investigations. The reason for the scarcity of studies including first-onset antipsychotic naïve patients is that such patients are difficult to recruit. Even large specialized centres can only recruit around 20-30 such patients each year and few centres follow strict standard operating procedures for collection of samples. In this study, patients and controls were acquired from multiple independent cohorts and underwent extensive clinical characterization. In addition, sera were collected and stored according to strict standard operating procedures to maximize reliability and accuracy of the results.

In summary, this is the first study showing a reproducible biological signature in sera of schizophrenia patients. A remarkable finding of this study was that the schizophrenia disease signature was also apparent in individuals who underwent routine blood screening in the United States military, prior to a subsequent diagnosis of schizophrenia, and before overt symptoms of mental disorder had emerged. As this signature was not apparent in other related psychiatric disorders, these findings hold promise for the future development of a rapid, specific and non-invasive

blood test for schizophrenia. It should be noted that tests for disorders with a low incidence such as schizophrenia require exceptionally high specificities if used in the general population. For this reason, the most effective use of such tests would be as a confirmatory diagnostic aid by a psychiatric specialist in conjunction with a clinical assessment. In this way, the test would be used in populations already enriched for schizophrenia with the purpose of establishing and confirming a diagnosis more rapidly, as compared to the requirement for 6 months duration of continuous symptoms in a DSM IV-based diagnosis. Such an application of a biomarker test would help to initiate treatment of patients more rapidly and, therefore, reduce the duration of untreated psychosis and, in turn, improve outcomes. This would be an important breakthrough by helping clinical psychiatrists to identify vulnerable patients early in the disease process, allowing for earlier or even preventative therapeutic intervention.

Methods

Clinical samples.

The institutional ethical committees approved the protocols of the study, informed written consent was given by all participants and studies were conducted according to the Declaration of Helsinki. All diagnoses (DSM-IV) and clinical tests were performed by psychiatrists under Good Clinical Practice-compliance to minimize variability. Cohorts 1 and 8 were from the University of Cologne (Germany), cohort 2 from the University of Muenster (Germany), cohorts 3, 5 and 7 from the University of Magdeburg (Germany), cohort 4 from Erasmus University (Netherlands), cohorts 6 and 9 from the United States military (facilitated by the Stanley Medical Research Institute, MD, USA), cohort 10 from the Sheppard Pratt hospital (Baltimore, USA) and cohort 11 from the Department of Psychiatry, University of Cambridge (UK). Controls were recruited from the same geographical areas or institutes and matched to the respective patient populations as indicated. Blood samples were collected from all subjects into S-Monovette 7.5mL serum tubes (Sarstedt; Numbrecht, Germany) and serum prepared and stored at -80°C in Low Binding Eppendorf tubes (Hamburg, Germany).

Multiplexed immunoassay. Approximately 180 analytes were measured in sera using the HumanMAP® multiplexed antigen immunoassays in a CLIA-certified laboratory at Rules Based Medicine. Assays were calibrated using standards, raw in-

tensity measurements converted to absolute protein concentrations, and performance verified using quality control samples. Data analyses were performed using the statistical software package R (www.r-project.org). The protocol for the study participants, clinical samples and test methods was carried out in compliance with the Standards for Reporting of Diagnostic Accuracy (STARD) initiative (Bossuyt et al., 2003).

Acknowledgements

This study was supported by the Stanley Medical Research Institute (SMRI) and Psynova Neurotech Inc. We want to thank Anke Dudeck, Jeanette Schadow, Dr. Wolfgang Jordan, Dr. Bernd Hahndorf, Dr. Florian Kästner, Dr. Anya Pedersen, Dr. Ansgar Siegmund, Dr. Katja Kölkebeck, Torsten Schoenborn, Dr. Christoph W. Gerth, Dr. Christian Mauss, Dr. Brit M. Nolden, Dr. M. A. Neatby, Dr. Liliana Ruta and Dr. Erin Ingudomnukul for their participation in sample characterization and collection. We want to thank Michael G. Walker, Ph.D. for suggestions concerning data analysis. Thanks to all members of the Bahn Laboratory for discussions, help, and encouragement. Most of all, thanks to all patients and healthy volunteers for their selfless donation of samples used in this study.

Author Contributions

E.S., P.C.G., H.R., N.v.B. and S.B wrote the manuscript. E.S. analyzed the data. F.M.L., M.R., J.S., D.G., L.K., P.O., T.S., B.B., N.v.B, S.B-C., N.W., D.N., D.C. and R.H.Y were responsible for patient recruitment, patient characterization and sample collection. G.M., E.S. and S.B. facilitated the communication to clinical centers and Rules Based Medicine, Inc. M.S. supervised the data acquisition at Rules Based Medicine, Inc. L.W. was responsible for sample organization and shipment. S.B. supervised the project.

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Chapter 11

General discussion

Conclusions and discussion

In this thesis we describe studies that explore the altered presence of the neurotrophic factors S100B and Brain-Derived Neurotrophic Factor (BDNF) in the peripheral blood of male recent-onset schizophrenia patients. We also investigated the gene expression profiles of Peripheral Blood Mononuclear Cells (PBMCs) of male recent onset schizophrenia patients with a focus on the altered expression of AKT1, a key enzyme involved in the regulation of trophic and metabolic cell processes. Additionally, we investigated whether alterations in a large array of neurotrophic proteins obtained via an innovative high-throughput methodology would be able to separate patients from controls.
The research presented in this thesis adds to the evidence that aberrancies in neurotrophic factors and in signalling pathways involved in cellular growth and energy metabolism are present in schizophrenia.
The main results are visually depicted in **figure 1.** They show:

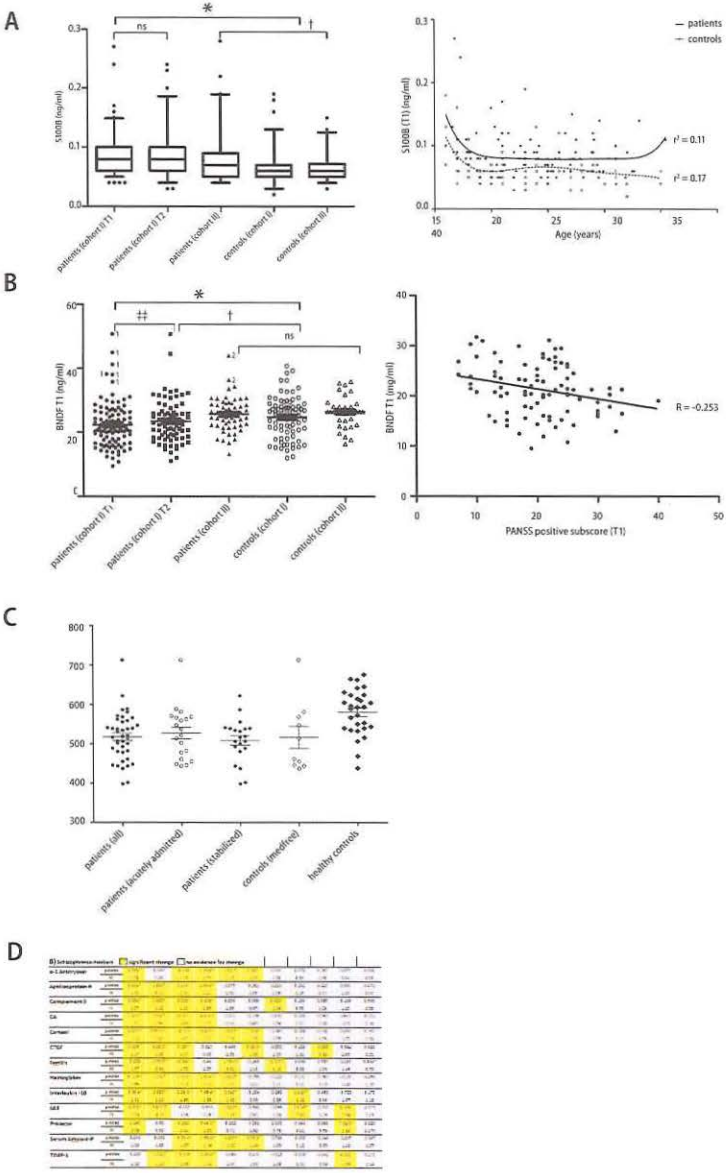
1. Serum S100B is elevated in serum of recent-onset male schizophrenia patients, and remain so after treatment. Elevated serum S100B levels are a trait marker for the early schizophrenia syndrome in males (chapters 3 and 4).

2. Serum BDNF is decreased in male recent-onset schizophrenia patients with active psychosis. Serum BDNF levels restore to normal after treatment. Decreased serum BDNF levels are a state marker for the psychotic phase in this patient group (chapters 3 and 5).

3. The expression of AKT1 is decreased in PBMCs of male schizophrenia pa
tients, both acutely psychotic as well as (partly) stabilized. Thus, decreased expression of AKT1 is a stable trait in this group of patients. Moreover, we report altered activity in patients in many canonical pathways in which AKT1 plays an important role (chapter 7 and 8), most notably immune-,neurotrophin-, and metabolic pathways.

4. Using the HumanMAP platform, a signature consisting of thirteen analytes was identified that is capable of distinguishing 5 indepenedent patient cohorts from their respective control groups; for of these thirteen analytes are altered significantly in asymptomatic subjects, later diagnosed with schizophrenia. The profiles

Figure 1



Visual summary of the main findings of this thesis.

A (chapter 4): serum S100B levels are elevated in young, male schizophrenia patients, and remain elevated after treatment (*: p<0.001); the age-related decline

of serum S100B, observed in normal adolescents, and thought to be related to brain maturational processes, seems to be also present in schizophrenia patients.

B (chapter 5): serum BDNF levels are decreased in young, male schizophrenia patients suffering a psychotic episode, and normalize after remission of psychotic symptoms (*: p<0.001; +: p=0.049; ++: p=0.017) . A negative correlation between serum BDNF levels at admission and the PANSS positive subscale was observed (r: -0.25; p=0.014).

C (chapter 8): PBMC AKT1 expression is decreased in young, male schizophrenia patients (*: p<0.001), irresepctive of antipsychotic status or the presence of active psychosis.

D (chapter 10): a panel of thirteen analytes separates patients from 5 independent cohorts from their respective control groups (columns 1-5, significant analytes indicated in yellow); four of these thirteen analytes are altered significantly in asymptomatic subjects, later diagnosed with schizophrenia (column 6). The profiles formed by the thirteen, resp four analytes separate the schizophrenia subjects from other clinical entities (columns 7-11).

formed by the thirteen, resp. four analytes separate the schizophrenia subjects from relevant other clinical entities.

Neurotrophic factors S100B, BDNF, and AKT1

S100B

It is not easy to make a straightforward interpretation of the elevated levels of serum S100B in schizophrenia. One of the main reasons is the differential effect S100B has on neural cells, depending on its concentration. In cell culture and animal experiments, micromolar concentrations of S100B micromolar doses of S100B cause neuronal apoptosis via excess production of reactive oxygen species. However, nanomolar concentrations of S100B appear to boost proliferation of neurons, dendrites and neurites, inducing an increase in synaptic contacts between cells (Businaro et al., 2006). Thus, elevated levels of serum S100B could either reflect neuronal damage or

a compensatory process. In other words, they may indicate primary astrocytic injury or astrocytic response to neuronal injury or aberrant developmental processes. Primary astrocytic injury may support the hypothesis of a possible progressive degenerative component in the early phase of the disease. However, another explanation might be that elevated concentrations of serum S100B shown in schizophrenic patients reflect secondary S100B upregulation by astrocyte stimulation in an attempt to counter a (so far, unknown) neurodegenerative process. In accordance with the latter hypothesis is the finding that the elevated levels of serum S100B found in schizophrenia are lower than the S100B concentrations found in disorders with obvious CNS damage.

Even though S100B is predominantly expressed in astrocytes, S100B is also present in other brain cells, such as oligodendrocytes, microglia, or neuronal cells. Therefore, these cellsmay also contribute to elevated levels of serum S100B. Indeed, there is increasing evidence for an involvement of oligodendrocytes and white matter abnormalities in schizophrenia (Tkachev et al., 2003; Uranova et al., 2004; Tkachev et al., 2007).

Another possibility, is that serum S100B is elevated due to astrocyte activation induced by increased brain oxidative stress in patients (Sarandol et al., 2007).

Elevated serum S100B levels may also result from an increased blood-brain barrier permeability (Kapural et al., 2002; Schroeter et al., 2003) in the presence of normal secretion from astrocytes. Incidental reports describe the disrupted integrity of the blood-brain barrier in schizophrenia (Axelsson et al., 1982).

BDNF

Generally, there is an emerging body of converging evidence that points to a relation between schizophrenia and disrupted levels of BDNF, both in the central nervous system and in peripheral blood (Shoval and Weizman, 2005). Among post-mortem studies, BDNF levels are decreased in the hippocampus and increased in the cerebral cortex of patients with schizophrenia (Durany et al., 2001). Iritani et al (Iritani et al., 2003) report an increase in BDNF and in its receptor, TrkB, in neurons in the hippocampus in accordance with another study by Takahashi et al (Takahashi et al., 2000), who find hippocampal BDNF is elevated, but its receptor down-regulated in schizophrenia. Further, both BDNF and TrkB mRNA levels were decreased in prefrontal cortex of subjects with schizophrenia (Weickert et al., 2003; Weickert et al., 2005).

Genetic research has shown a reducing effect of Val/Met heterozygosity on hippoc-

ampal volume as measured with MRI in both normal subjects and patients with schizophrenia, but with the reduction more pronounced in the patients (Szeszko et al., 2005). Wassink et al (1999) and Ho et al (2007) found a similar effect of BDNF genotype in schizophrenia (Buckley et al., 2007).

AKT1

The AKT gene family includes three members (AKT1, AKT2, and AKT3), that possess partially redundant functions and contribute to several cellular functions including cell growth, survival, and metabolism. AKT has several substrates, most notable among them glycogen synthase kinase 3α (GSK-3α) and GSK-3β, both of which are inhibited by AKT in response to various external cellular stimuli (Woodgett, 2005). AKT is activated by insulin as well as by a variety of growth factors such as platelet derived growth factor, epidermal growth factor, basic fibroblast growth factor and insulin-like growth factor I and plays a major role in signalling effects on metabolism, cell growth, cell cycle and apoptosis. In the past years evidence has been collected that one of the major functions of AKT is to promote growth-factor mediated cell survival and to block programmed cell death or apoptosis (Kandel and Hay, 1999). Specifically, AKT functions as the downstream effector of growth factor mediated cell-survival.

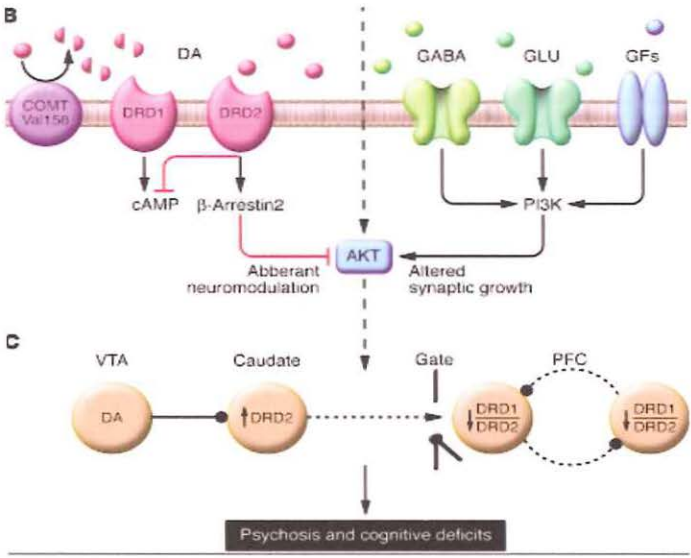
Gain or loss of AKT activity has been associated with several human diseases, including cancer and type 2 diabetes (Woodgett, 2005; Arguello and Gogos, 2008). Since the initial report in 2004 from Emamian et al. (2004), accumulating evidence suggests that impaired AKT signaling also plays a role in the pathogenesis of schizophrenia. First, an association between schizophrenia and AKT1 genetic variants has been reported in several case/control samples and family cohorts (an updated compilation of such studies is available; (see Schizophrenia Research Forum 2009). Second, a number of studies have provided convergent evidence of a decrease in AKT1 mRNA, protein, and activity levels (reflected by changes in substrate phosphorylation) in brains of some individuals with schizophrenia (Emamian et al., 2004; Zhao et al., 2006; Thiselton et al., 2008). Overall, an increasingly strong link between a dysregulation of AKT signaling and schizophrenia is emerging.

Very importantly, pharmacological evidence indicates that drugs used in the management of psychosis, such as the typical antipsychotic haloperidol as well as several atypical antipsychotics, can act as enhancers of AKT signaling in vivo or in vitro by directly activating AKT or by increasing the phosphorylation of its substrates GSK-3α and GSK-3β (reviewed in (Beaulieu et al., 2007b).

Metaphor revisited

In chapter 2 we presented what we called a 'visual metaphor' and suggested that this metaphor might outline an heuristic framework that *'could provide a basis for uniting clinical phenomena and neurobiological theories'*, and might be able to *'bridge the mental and neural domain'*. Here we show how the findings we presented in this thesis can be incorporated into the metaphor we described in chapter 2. We focus on disturbed AKT1 signalling, its influence on dopamine neuromodulation, and how this relates to the visualization of dopamine signalling as presented in figure 5 of chapter 2.

Figure 2



AKT is a key node for various signalling systems, among which is dopamine. (after Argello & Gogos, 2008)

(**B**) Dopamine in the cortex is inactivated by Catechol-O-Methyl Transferase (COMT) and activates Dopamine Receptor D1-(DRD1) coupled cAMP signalling whereas Dopamine Receptor D2 (DRD2) decreases cAMP levels and inhibits AKT activity via β-arrestin2. GABA, glutamate (GLU), and various growth factors (GFs) also modulate AKT activity via PI3K, resulting in alterations in synaptic growth and transmission. (**C**) These combined effects on dopamine modulation and synaptic connectivity may alter the function

of cells (circles) within neuronal circuits important for cognitive function. The caudate of the basal ganglia plays a critical role in gating information and restricting access to working memory, which relies on proper connections among cortical neurons in the prefrontal cortex. The gating of information itself is heavily dependent on dopamine transmission, which originates from the ventral tegmental area (VTA). One possible, although probably oversimplified, scenario is that sensitization to dopamine via DRD2 may lead to psychotic symptoms and, coupled with altered neuronal connectivity and decreased DRD1 signaling, also contribute to cognitive dysfunction. Dashed lines represent the functional impairments resulting from attenuation of *AKT1* expression and signaling.growth factors (GFs) also modulate AKT activity via PI3K, resulting in alterations in synaptic growth and transmission.

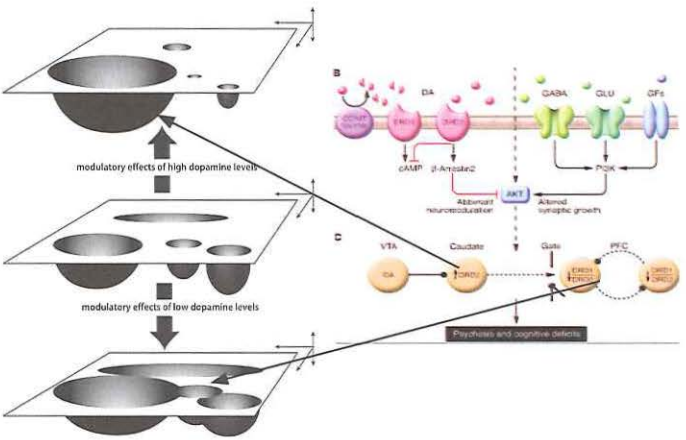
Mice lacking the AKT1 gene show enhanced sensitivity to disruption of sensory motor gating by amphetamine (Emamian et al., 2004). One action of this drug is to cause the release of dopamine (DA) at nerve terminals and thus increase DA transmission via G protein–coupled DA receptors. The two major classes of DA receptors, the DA receptor 1 (DRD1) class and the DA receptor 2 (DRD2) class, signal through increases and decreases, respectively, in cAMP levels within nerve cells. Sensitivity to amphetamine is widely used to model psychosis in rodents and is efficiently prevented by blocking DRD2, the best-established target of antipsychotic drugs (Beaulieu et al., 2004; Beaulieu et al., 2005; Beaulieu et al., 2007b). Moreover, AKT1-knockout mice show a more pronounced deficit in response to DRD2 class but not DRD1 class agonists in working memory tests that depend on proper function of prefrontal cortex (PFC) (Lai et al., 2006). In a series of experiments, Caron and colleagues have established that AKT is a novel and key signaling intermediary downstream of DRD2 class receptors (Beaulieu et al., 2005; Beaulieu et al., 2007a; Beaulieu et al., 2007b). These experiments demonstrate that DRD2 class receptors are essential for the inhibition of AKT by DA and that AKT function is important for normal dopaminergic transmission and expression of DA-associated behaviors in a manner distinct from cAMP-dependent signaling. Thus, a hypothesis has been formulated according to which a partial loss of function of AKT1 in schizophrenia, mimicking DA overactivity, results in exacerbated responses to DRD2 class receptor stimulation (**Figure 2** bottom part left, 'caudate'). Antipsychotics acting by blocking DRD2 class receptors could correct this imbalance by preventing further

reductions of AKT activity by these receptors (Lai et al., 2006; Beaulieu et al., 2007a). AKT signaling is also known to modulate the development of neuronal connectivity in vitro (Jaworski et al., 2009) as well as in the human brain (Boland et al., 2009). AKT1-deficient mice show changes in neuronal connectivity of PFC layer V neurons (Lai et al., 2006), the output neurons of the cortex. Transcriptional profiling in the PFCs of these mice identified concerted changes in genes controlling synaptic function, neuronal development, myelination, and actin polymerization. Thus, impaired AKT and, in particular, AKT1 signaling may have an additive effect on the properties of neural networks, resulting in neuronal disconnectivity as well as aberrant neuromodulation (Figure 2, bottom part, right ('PFC').

So, decreased expression of AKT1 may lead to psychosis through caudal upregulation of DRD2 mediated DA neuromodulation and to cognitive deficits through PFC DRD1/DRD2 disbalance.
The complete process is shown in figure 2 (adapted after Arguello and Gogos (2008)).

Combining these neurobiological findings with figure 5 in chapter 2, which shows the effects of altered dopamine neuromodulation from a systems' perspective leads to the following integrative visualization with our metaphor framework. (**Figure 3**): low levels of AKT1 are hypothesized to lead to increased DRD2 neuromodulation in the caudate nucleus, leading to an abormal hyperdopaminergic state which is equivalent to the emergence of an attractor with a large basin of attraction which dominates the striatal information processing of the neural system. This dominance translates itself in 'aberrant salience'. This is the situation depicted in figure 3 (top). In contrast, low levels of AKT1 will lead to deregulated DRD1/DRD2 neuromodulation, leading to with a low signal-to-noise ratio. In attractors terms this is equivalent to shallow attractors with large basins of attractions. In this situation frequent transitions from one attractor to another can take place (see figure 3, bottom). Performance deficits in schizophrenia are due to a degradation in the internal representation required as context for processing stimuli. In a state of reduced prefrontal deregulated DRD1/DRD2 neuromodulation noise interferes with the ability of the system to maintain a representation of task-relevant information. This comes down to the situation depicted in figure 5 (bottom) where the system is liable to transitions to other attractors due to small internal or external perturbations of the system. The resulting inability of the system to maintain a stable attractor state over time shows itself at a psychological level as the observed disorders of working memory.

Figure 3 **The effects of decreased AKT1 activity on dopamine neurodulation**



Top: low levels of AKT1 are hypothesized to lead to increased DRD2 neuromodulation in the caudate nucleus, leading to an abnormal hypodopaminergic state, which is equivalent to the emergence of an attractor with a large basin of attraction dominating the striatal information processing. This dominance translates itself on the psychological level in 'aberrant salience', ultimately leading to psychosis.

Bottom: in contrast, in prefrontal areas, low levels of AKT1 will lead to deregulated DRD1/DRD2 neuromodulation, leading to a low signal-to-noise ratio. In attractor terms this is equivalent to shallow attractors with large basins of attraction. In this situation frequent transitions from one attractor to another take place. This situation results in cognitive deficits, i.e. impairments in working memory.

Biomarkers for the schizophrenia syndrome

In recent years, some interest has emerged in the field in investigating peripheral proteins, from the clinical perspective of obtaining a biomarker for the schizophre-

nia syndrome (Bahn and Schwarz, 2008;Reckow et al., 2008;Singh and Rose, 2009). Recent developments in the field enabled the measurement of many proteins at the same time, analogue to whole-genome high-throughput methods. Biomarkers are molecular readouts that correlate with a certain disease state and may have utility in the development of diagnostics and therapeutics. If biomarkers are predictive of the onset of a disease, early intervention becomes feasible which can improve clinical outcome. Another important application is the monitoring of treatment progress.

It makes intuitive sense to analyse samples that are most closely connected with the disease process, e.g. biopsies of tumour tissue for the identification of cancer biomarkers, as these are most likely to show the highest degree of disease related alterations. However, for biomarker discovery in neuropsychiatric disorders the sample choice is limited. For obvious reasons, the brain is not readily accessible for diagnostic approaches. For the development of clinically useful diagnostics, the aberrant state of the proteome ultimately has to be reflected in readily accessible body fluids or peripheral cells. Suitable samples include serum, plasma, cerebrospinal fluid (CSF), saliva, urine and peripheral blood cells.

The identification of disease-related biomarkers is needed to advance the diagnosis, treatment and management of complex psychiatric disorders such as schizophrenia. Current diagnosis of schizophrenia relies on subjective patient interviews, whilst 'at risk individuals' or the so called prodromal patients can at present not be objectively identified. Increasing evidence suggests that early intervention is crucial in the case of schizophrenia, as early intervention is likely to improve disease outcome.

Our findings show that serum S100B levels in young, male schizophrenia patients are elevated, irrespective of age, duration of illness, and medication status. Thus, it might be that serum S100B levels are also elevated in (some) in some at-risk individuals exhibiting prodromal signs. If so, S100B might serve as a biomarker for identifying those high-risk individuals who are specifically at risk for conversion to a fully psychotic state. It should however be kept in mind that aberrant serum S100b levels seem not to be specific to schizophrenia. There is some evidence that elevated serum S100B levels may also be present in major depression and bipolar disorder. In that case, aberrant levels of peripheral S100B in young, 'at risk' patients (when present) may serve as no more than to flag 'a possible development towards a major psychiatric disorder', not unlike the way an elevated erythrocyte sedimentation rate

indicates the possible presence of a range of somatic disorders. Moreover, at present there are no data are available on peripheral S100B levels in diagnostic categories which sometimes pose a differential diagnostic challenge when differentiating from schizophrenia, such as ADHD, autism spectrum disorders, brief psychotic disorder, and (stress related) psychotic phenomena which sometimes accompany personality disorders.

Strengths and limitations

Strengths of this study are the relative homogeneity of the patient samples with respect to gender, age, and onset of the disorder. A substantial number of patients were young; of the two cohorts described in chapter four and five, 56 (approx. 30%) patients are younger than 21 years, 21 (approx. 15%) are younger than 18 years. We closely age-matched the controls. Patients were primarily recruited from the Erasmus MC department of psychiatry ward for recent-onset psychotic disorders. The use of the deferred consent procedure enabled us to include severely psychotic patients, too disturbed to give regular consent. The patients therefore confidently reflect the population referred for diagnosis and treatment of acute psychotic disorders in a Dutch urban environment.
All of our findings are replicated in independent cohorts either recruited from a similar clinical facility, the 'adolescentenkliniek' of the Academic Medical Center Amsterdam (for S100B and BDNF), or in an independent sample recruited in the Erasmus MC department of psychiatry (decreases AKT1 expression, as described in chapters 7 and 8).
We designed our studies in such a way that both acutely psychotic patients are present, as well as (partly) stabilized patients, allowing for some differentiation between state and trait parameters.

However, some limitations must be discussed. Controls samples have been mainly recruited from Erasmus MC students. Therefore, our controls are not matched for IQ and, more generally, for level of education. Also, the controls are not matched for nicotine and cannabis abuse. We were not able to provide follow-up measurements covering a considerable time frame, enabling a more profound assessment of the development over time of levels of neurotrophic factors. We did not perform

imaging measurements or neuropsychological testing, which would allow for a direct assessment of the relationship between serum proteins and structural or psychological parameters. We also did not specifically address the relationship between peripheral measurements and brain levels, for instance by measuring cerebro-spinal fluid levels of S100B and BDNF. We did not investigate the relationship between S100B, BDNF and AKT1 polymorphisms and their corresponding protein/mRNA levels.
A specific limitation is that we did not address the issue of specificity of our findings with respect to other (major) psychiatric disorders. This is of course especially relevant in the context of the biomarker concept.

Suggestions for future research

The findings presented in this thesis suggest several future lines of research.

S100B and BDNF as possible biomarkers for identifying 'at risk patients'

Patients who later develop schizophrenia often initially present with atypical mood and cognitive symptoms (Moller, 2004). Recently, in clinical psychiatry, a lot of interest has been raised by attempts to confidently identify such patients (usually referred to as 'prodromal' patients, or 'at risk patients'), with the aim to ameliorate delay and uncertainty in the final diagnosis, and thus improving early therapeutic intervention and outcome (Yung and McGorry, 1996). At present, young patients deemed to be at risk for psychosis, are identified based on the traditional psychiatric approach of applying the (subjective) assessment and categorization of overt symptoms by using clinical interviews and questionnaires (i.e. see Cannon et al., 2008). The elevated levels of serum S100B we found in young schizophrenia patients, which seem independent on treatment status, age, and duration of illness, suggest that elevated levels of serum S100B may also be present before the overt onset of schizophrenia symptoms. If so, serum S100B levels may be measured in prodromal patients as an (objective) biomarker, additional to questionnaires, to identify patients at risk for psychosis. So, an important next step of research would be to investigate serum S100B levels in prodromal patients, with the aim to identify whether serum S100B levels are also elevated in these patients, and, if so, to calculate the

predictive value of above-threshold values for future conversion to a fully psychotic state. However, an important issue in this respect should immediately be kept in kind. Elevated levels of serum S100B may not be specific to schizophrenia, but may also be present in major depression and bipolar disorder (Machado-Vieira et al., 2002; Schroeter et al., 2002; Andreazza et al., 2007; Schroeter et al., 2008). In that case, aberrant levels of peripheral S100B in young, 'at risk' patients (when present) may serve as no more than to flag 'a possible development towards a major psychiatric disorder', not unlike the way an elevated erythrocyte sedimentation rate indicates the possible presence of a range of somatic disorders.

With respect to serum BDNF levels, it has been suggested that also decreased levels of serum BDNF may serve as a biomarker for either the schizophrenia syndrome, or for prodromal states. So, future research may also imply investigating serum BDNF levels in prodromal patients. However, the same caveat as outlined in the previous paragraph holds even more in the case of BDNF: decreased levels of serum S100B may not be specific to schizophrenia, but may also be present in major depression and bipolar disorder (Fukumoto et al., 2001; Karege et al., 2002; Mai et al., 2002; Hashimoto et al., 2004; Machado-Vieira et al., 2007). Moreover, decreased serum BDNF levels seem to be dependent on the psychotic state (this thesis, but also Palomino et al., 2006), and may only alter when overt (popsitive) symptoms arise, which obviously would limit the value of BDNF as a biomarker for the prodromal state of schizophrenia.

S100B and BDNF in diagnostic categories related to schizophrenia

At present there are no data available on peripheral serum S100B and BDNF levels in diagnostic categories which are conceptually related to schizophrenia, such as brief psychotic disorder, or in clinical entities which sometimes pose a differential diagnostic challenge in the early phases of schizophrenia, such as ADHD, autism spectrum disorders, and (stress related) psychotic phenomena which sometimes accompany personality disorders. Some of these categories have recently been shown to be also genetically related to schizophrenia, such as autism spectrum disorders and bipolar disorder (Gill et al., 2009; Guilmatre et al., 2009; McCarthy et al., 2009; O'Donovan et al., 2009). Investigating serum S100B and BDNF levels in these categores may provide more insight in common as well as differential biological mechanisms in these disorders as compared to schizophrenia.

Multi-protein arrays

Apart from S100B and BDNF, several other neurotrophic proteins may be altered in the peripheral blood of schizophrenia patients. I.e. altered levels of Nerve-Growth Factor, Epidermal Growth Factor, and basic Fibroblast Growth Factor, have been incidentally identified in schizophrenia (see chapter 3). Decreased levels of the important growth factor Insulin-like Growth Factor-1 have been described (Venkatasubramanian et al., 2008).

Recent technical developments enable the measurement of a large number of proteins (approximately 200) in parallel, and allowing for the profiling of serum proteins, and the subsequent identification of altered protein profiles in patients. This is the approach that has been described in chapter 10.

Future research using this method can take tow forms. First, an obvious possible approach would be to investigate the identified profile in young, 'at-risk' individuals, showing cognitive and behavioral changes suspect for emerging schizophrenia. Furthermore, an attempt may be made to move towards developing subgroup diagnostics within the schizophrenia syndrome, using serum of a (very) large sample of schizophrenia and psychosis patients. In such a large sample, it is hypothesized that, using unsupervised clustering techniques, subgroups with similar protein profiles may emerge within the (heterogeneous) schizophrenia/psychosis sample. If so, the next step would be to investigate whther the identifoed subgroups represent clinically (i.e. prognostically) useful entities.

This approach may also allow for a more differentiated biomarker profiling for schizophrenia and other neuropsychiatric disorders, identifying subgroups within the schizophrenia syndrome, or, possibly, showing subgroups with overlap between individual disorders. We argue that such an approach will probably be most fruitful for those psychiatric disorders which have a strong 'complex genetic' background, and/or known non-cerebral manifestations (i.e. such as an association between diabetes and schizophrenia), as these disorders are the most likely ones to have non-cerebral protein alterations based on a subtly altered gene-expression status throughout the whole body.

S100B and BDNF as biological endophenotypes for genetic association studies

The classical approach in genetic association studies is to identify patients, based on their (endo)phenotypical characteristics (i.e. based on the symptom profile that is defined as 'schizophrenia', or based on neuropsychologically characterized subgroups

within the schizophrenia syndrome), and then to investigate the presence of gene variants of interest as compared to a control group. This approach identified (among numerous other associations) the val66met variant of the BDNF gene (Ho et al., 2007; Gratacòs et al., 2007; Takahashi et al., 2008; Koolschijn et al., 2009) as a gene-variant associated with schizophrenia. An interesting approach would be to investigate a possible influence of BDNF variants on serum BDNF levels. Also, the influence of genes in epistasis with BDNF on serum BDNF levels may be investigated.
Although the S100B gene is less clearly associated with schizophrenia, the same approach may be taken for S100B gene variants. More insight into the genetic backgrounds of altered levels of neurotrophic factors may significantly contribute to our understanding of the underlying processes involved.
Generally, the use of altered levels of biochemical parameters as an endophenotypical trait may increase the power to find a genetic association when examining genes related to the aberrant biochemical compound.

PBMC gene-expression

The results of this thesis show that the gene-expression status of PBMCs is (subtly) altered in male schizophrenia patients as compared to controls. The conceptual value of this finding is that altered patterns of gene expression may be present in peripheral tissue in schziophrenia, even when this particular peripheral tissue is not showing overt disfunctions. This finding suggests several lines of research.
Cultured PBMCs may be used as a surrogate tissue to investigate more specifically the altered gene-expression status in schizophrenia, and the way gene-expression signature alter under environmental influnces in schizophrenia as compared to controls. It has been suggested that in schizophrenia the utilisation of glucose in the brain is suboptimal (Prabakaran et al., 2004; Khaitovich et al., 2008; Somel et al., 2009). Such a metabolically compromised state may show itself in cultured PBMCs of schizophrenia patients as compared to controls, especially under increased metabolic stress, or under another suitable experimental condition. Interestingly, such an effect has already been demonstrated in cultured PBMCs of bipolar patients, which show altered regulation of metabolic pathways under low glucose as compared to controls (Naydenov et al., 2007).
Drug abuse, especially of cannabis and nicotine, is a common problem in schizophrenia. An ongoing discussion is related to the question as to whether cannabis or nicotine have a differential effect in schizophrenia patients as compared to healthy

controls. So, the influence of nicotine or cannabis on the gene-expression profiles between patients and controls of cultured PBMCs may be investigated.
AKT1 has been shown to be an important intermediate signaling protein in the action of several neurotrophic pharmacological agents, most notable antipsychotics and mood stabilizers (Beaulieu et al., 2009). The influence of antipsychotics on (AKT-related) signaling pathways may be investigated in cultured PBMCs of schizophrenia patients.

Finally, an exciting opportunity is suggested by the finding by Tan et al. (2008), who showed that the expression of AKT1 in lymphoblasts of healthy individuals was positively correlated with their performance on a dopamine-dependent cognitive task. This suggests the possibility to study the relationship between neuropsychological performance, imaging data, and (PBMC) gene expression levels of either specific genes or of functional pathways related to these genes (Tan et al. 2009).

Then, in the last paragraph, as a tribute to patients who lived more than a century ago in England, I would like to return to the work of W. Lauder Lindsay, of which a facsimile is printed on one of the first pages of this thesis. Lauder Linday examined the blood of his patients under a microscope. A little further on in the work, he makes the following remark:

"some presented their fingers, under the impression that, from the single drop of blood, the state of their constitution, the chances of cure, and the period of their removal, could infallibly be predicted;

So: nothing new under the sun! Lauder Lindsay's patients defined the biomarker concept already in 1855, and there is still a long way to go.

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Samenvatting (summary in dutch)

Schizofrenie is een ernstige psychiatrische aandoening die gekenmerkt wordt door verstoringen van de inhoud van het normale denken en van het waarnemen, gecombineerd met verstoringen in het initiëren van handelingen, in de aandacht, en in het ervaren en uiten van emoties.
De aandoening openbaart zich veelal op jong-volwassen leeftijd. Centraal in het huidige wetenschappelijk denken over schizofrenie staan een drietal elementen. Ten eerste dat de term schizofrenie waarschijnlijk een (groot) aantal subsyndromen omvat, en dat de term als zodanig geen eenduidige oorzaak of eenduidig beloop beschrijft. Ten tweede, dat de aandoening een genetische component heeft, en veroorzaakt wordt door een groot aantal genen, elk afzonderlijk met een klein effect, en geen van alen als noodzakelijk voorwaarde voor het ontstaan van schizofrenie. Ten derde, dat een verstoorde ontwikkeling van het brein (en dan met name van de prefrontale cortex) een belangrijke rol speelt bij het ontstaan van schizofrenie.
De ontwikkeling van het brein wordt door een groot aantal stoffen benvloedt, waaronder een heterogene groep stoffen die bekend staan als neurotrofe factoren.

Centraal in dit proefschrift staat de vraag of zulke neurotrofe factoren afwijkend aanwezig zijn in het perifere bloed van patiënten met schizofrenie, en of de hoeveelheid van neurotrofe factoren verandert gedurende de behandeling. Naast onderzoek naar de aanwezigheid van neurotrofe eiwitten in perifeer bloed, hebben we onderzocht of in witte bloedcellen de expressie van genen, betrokken bij neurotrofe processen, anders is bij patiënten vergeleken met controles. Bovendien hebben we verkend of (combinaties) van neurotrofe factoren kunnen dienen als diagnostische markers (zogenaamde 'biomarkers') voor schizofrenie.
We hebben ons daarbij gefocused op mannelijke patiënten. Alleen aan de onderzoeken beschreven in de hoofdstukken 9 en 10 hebben ook vrouwelijke patiënten meegedaan. Verder bestond de meerderheid van de patiënten uit jonge mensen, bij wie de aandoening zich relatief kortgeleden (<5 jaar) had geopenbaard. Een groot deel van de patiënten was opgenomen op de afdeling voor vroege psychoses van het Grasmus MC, en leed aan een floride psychose.

Alvorens over te gaan tot de daadwerkelijke behandeling van de vraagstellingen, presenteren we in **hoofdstuk 2** een heuristisch model. Dit model poogt een veelvoorkomend probleem in de moderne, biologisch georiënteerde psychiatrie te verhelderen, namelijk dat het soms onduidelijk wat de relatie is tussen verschijnselen

op gedragsniveau (zoals wanen of hallucinaties) enerzijds, en verstoringen op het moleculair-biologische niveau anderzijds. In hoofdstuk 2 wordt gepoogd deze relatie te verduidelijken, weliswaar op een abstract niveau, door inzichten uit de theorie van complexe systemen en uit onderzoek op het gebied van kunstmatige neurale netwerken te combineren met resultaten van moleculair-biologisch onderzoek naar schizofrenie.
We laten zien dat de toestand op enig moment van een neuraal systeem kan worden beschreven als een punt in een N-dimensionele ruimte. Vereenvoudigd ka deze ruimte gevisualiseerd worden als een plat vlak. 'Kuilen' in het vlak, of *attractors*, zijn een visualisatie van de tendentie van het neurale systeem om zich naar een bepaalde regio binnen de multi-dimensionele ruimte te bewegen. Attractors kunnen beschouwd worden als herinneringen.
De bijbehorende visualisatie hebben we een 'visuele metafoor' genoemd. We bespreken hoe symptomen van schizofrenie, en de effecten van dopaminerge neuromodulatie, kunnen worden begrepen in termen van de geïntroduceerde visuele metafoor.

Om inzicht te verkrijgen in eerder verricht onderzoek naar de aanwezigheid van neurotrofe factoren bij schizofrenie, werd een literatuuronderzoek uitgevoerd, waarvan de resultaten in **hoofdstuk 3** worden gerapporteerd.
We vonden 24 onderzoeken die aan de zoekcriteria voldeden. Meerdere (gerepliceerde) onderzoeken vonden we voor *S100B*, Brain-Derived Neurotrophic Factor (BDNF), *glutamaat*, en Nerve-Growth Factor (NGF). Een tweetal niet-gerepliceerde onderzoeken beschrijft de aanwezigheid in serum bij schizofrenie van basic Fibroblast Growth Factor (bFGF) en Epidermal Growth Factor (EGF).
We concluderen dat de meest robuuste aanwijzingen voor veranderingen in het serum of plasma van patiënten met schizofrenie bestaan voor S100B (verhoogd bij schizofrenie), en BDNF (verlaagd). Bovendien suggereert dit literatuur-onderzoek dat meerdere groeifactoren veranderd aanwezig kunnen zijn.
S100B is een eiwit dat voornamelijk wordt geproduceerd door astrocyten. In nanomolaire hoeveelheden stimuleert het in vitro de uitgroei van neuriten. Micromolaire hoeveelheden daarentegen, induceren in-vitro celdood.
Veranderde hoeveelheden in serum S100B bij patiënten met schizofrenie kunnen dus passen bij een primair degeneratief proces, of bij een compensatoire poging neuronale schade te beperken.
BDNF reguleert neuronale overleving, migratie van neuronen, morfologische en

biologische differentiatie van zenuwcellen. Het is betrokken bij de regulatie van dopaminerge neuronen. De gevonden verlaging in de hoeveelheid serum BDNF kunnen aanwijzingen zijn voor veranderde neurotrofe processen bij patiënten, of wijzen op een mogelijke betrokkenheid van BDNF bij veranderingen in de dopaminerge neuromodulatie.
Op basis van de bevindingen van onze literatuurstudie onderzochten we het vóórkomen van veranderingen in de hoeveelheid S100B en BDNF in het serum van patiënten met schizofrenie.

In **hoofdstuk 4** rapporteren we onderzoek naar de hoeveelheid S100B in het serum van jonge (< 36 jaar), mannelijke patiënten met schizofrenie, die korter dan 5 jaar lijden aan de aandoening. In het onderzoek werden twee onafhankelijke groepen patiënten geïncludeerd, en vergeleken met twee eveneens onafhankelijke controlegroepen. Groep I bestaat uit floride psychotische patiënten, opgenomen in het Erasmus MC; bij deze patiënten werd ook het serum S100B gemeten na 8 weken naturalistische behandeling. Groep II bestaat uit grotendeels gestabiliseerde patiënten die deelnamen aan vervolg-(dag)behandeling in het Academisch Medisch Centrum Amsterdam.
We vonden dat serum S100B verhoogd is bij patiënten van groep I ten opzichte van de controlegroep. Ook patiënten die nog nooit eerder met antispychotica behandeld waren vertoonden een verhoogd serum S100B. Serum S100B bleef verhoogd, ook na 8 weken naturalistische behandeling. In de gestabiliseerde patiënten van groep II was eveneens een verhoogde hoeveelheid S100B in het serum aanwezig, vergeleken met de bij groep II horende controlegroep.
We concludeerden dat serum S100B persisterend verhoogd is bij deze patiënten, onafhankelijk van de aanwezigheid van psychotische verschijnselen, of van het gebruik van antipsychotica. In overeenstemming hiermee vonden we in beide groepen geen correlatie tussen serum S100B en positieve symptomen, gemeten met de Positive And Negative Syndrome Scale (PANSS).
Uit eerder onderzoek was reeds bekend dat serum S100B bij gezonde proefpersonen een leeftijdsafhankelijke daling vertoont tot aan het einde van de adolescentie. Van deze leeftijdsafhankelijke relatie wordt gedacht dat deze gerelateerd is aan rijpingprocessen van het brein. Wij vonden ook bij de patiënten aanwijzingen voor een niet-lineaire negatieve relatie tussen serum S100B en leeftijd. Echter, de gemiddelde waarden van S100B liggen bij voor alle patiënten, in ieder geval na het begin van de ziekte, persisterend hoger dan bij de controles.

Wij vonden geen aanwijzingen dat op basis van de hoeveelheid S100B bij het begin van het onderzoek, of van de verandering van S100B gedurende het onderzoek, subgroepen binnen de patiënten-populatie gedefinieerd kunnen worden met een specifieke respons of prognose.
Bij elkaar genomen suggereren deze bevindingen dat de hoeveelheid S100B in het serum van jonge, mannelijke patiënten, met recent ontstane schizofrenie, hoger is dan bij gezonde controles, voor alle onderzochte leeftijdscategorieën. Of de hoeveelheid serum S100B ook voorafgaand aan het ontstaan van schizofrenie verhoogd is (bijvoorbeeld bij zogenaamde prodromale of 'risico-patiënten') moet nog onderzocht worden. Onze bevindingen sluiten het in ieder geval niet uit. Dit betekend dat serum S100B een potentiële biomarker kan zijn voor de herkenning van prodromale patiënten.

In **hoofdstuk 5** presenteren we onderzoek naar de hoeveelheid BDNF in serum, bij dezelfde twee groepen patiënten en controles als beschreven in hoofdstuk 4.
We vonden dat serum BDNF verlaagd is bij patiënten van groep I ten opzichte van de controlegroep. Na 8 weken naturalistische behandeling was de hoeveelheid serum BDNF nog steeds enigszins verlaagd, maar ten opzichte van de eerste meting had zich een stijging voorgedaan. We vinden een positieve correlatie vinden tussen serum BDNF en positieve symptomen op het moment van de eerste meting. In de gestabiliseerde patiënten van groep II was geen afwijkende hoeveelheid BDNF in het serum aanwezig, vergeleken met de bij groep II horende controlegroep.
Wij vonden geen aanwijzingen dat de hoeveelheid BDNF bij het begin van het onderzoek, of de verandering van BDNF gedurende het onderzoek, subgroepen binnen de patiënten-populatie definieert met een specifieke respons of prognose. Wel vonden wij 6 patiënten met sterk afwijkende (verhoogde) waarden van BDNF. Wij vonden echter geen klinische of demografische variabelen die deze patiënten gemeenschappelijk hadden. In hoeverre deze patiënten dus een betekenisvolle subgroep vormen (klinisch of anderszins), dient nader onderzocht te worden.
We concluderen dat de hoeveelheid BDNF in het serum van patiënten met schizofrenie alleen verlaagd is tijdens de acute (psychotische) fase, maar tijdens behandeling geleidelijk verhoogd, en uiteindelijk niet meer verlaagd is na remissie van (positieve) psychotische verschijnselen. Deze bevinding wordt ondersteund door het feit dat we ten tijde van de eerste meting bij de patiënten van groep I een positieve correlatie vinden tussen serum BDNF en positieve symptomen.

In de hoofdstukken 6, 7, 8, en 9 richtten we onze aandacht op onderzoek naar gen-expressie.

Chapter 6 presents the results of a review of the at the time available literature on the subject of micro-array gene-expression studies conducted in post-mortem material of schizophrenia patiënts.
The initial part of the study presents an overview of the techniques and methods for data-analysis aimed at the unacquainted but interested psychiatrist.
The second part of the study presents the results of the review. We found 10 studies which satisfied our inclusion criteria. The studies identified several functionally related groups of genes of which the expression was altered in post-mortem brain tissue, mostly of the prefrontal cortex. Most prominent were alterations in genes involved in synaptic stability, glutamate metabolism, myelination, and energy metabolism.
Taken together, the reviewed papers underscore the hypothesis that schizophrenia is associated with difficulties in maintaining an appropriate level of synaptic connectivity and -stability. Possibly, inadequate levels of energy metabolism, resulting in increased oxidative stress and subsequent damage to fatty acids and myelin may also play a role. However, sound replications were found to be scarce, and the both analytical and data-analysis techniques were still under development. Also, an issue in post-mortem studies is the fact that brain material is obtained from older patiënts, resulting in the possible confounding effects of long-term hospitalisation and subsequent social deprivation, antipsychotic drug use, and drug abuse.

Zoals beschreven in **hoofdstuk 6** kan analyse van gen-expressie een interessante toevoeging kan zijn aan klassiek genetisch onderzoek. Dit onderzoek biedt de mogelijkheid verstoringen van functionele processen te onderzoeken. Bovendien is het binnen het psychiatrisch veld relatief nieuw
De onderzoeken gepresenteerd in hoofdstuk 6 suggereren dat de expressie van genen die functioneel verbonden zijn aan neurotrofe processen verstoord zijn bij schizofrenie. Wij hypothetiseerden dat afwijkende gen-expressie ook aantoonbaar is in perifeer weefsel, in casu in witte bloedcellen. De basis voor deze aanname is de hypothese dat bij schizofrenie een afwijkende verzameling van genen aanwezig is, die weliswaar alleen in het brein pathologie veroorzaakt, maar ook tot een subtiel veranderde expressie van het genoom leidt (zonder evidente pathologie) in (wellicht álle) perifere weefsels.

In **hoofdstuk 7** presenteren we de resultaten van een pilot onderzoek op dit gebied. We onderzochten de expressie van het gehele genoom (in de vakliteratuur bekend als ('whole-genome gene expression') van witte bloedcellen, bij 8 mannelijke patiënten met schizofrenie, en vergeleken met 8 controles.
Alle patiënten waren jonger dan 36 jaar, en leden korter dan 5 jaar aan schizofrenie. In tegenstelling tot de onderzoeken gepresenteerd in de hoofdstukken 4 en 5, is er bij hen geen sprake van een floride psychotische episode. Wel vertoonden zij allen aanzienlijke negative symptomen. Alle patiënten voldeden aan de criteria voor het 'deficit syndrome'. Alle patiënten gebruikten antipsychotica.
Aangezien dit een explorerend onderzoek was, werden de resultaten voor wat betreft de betrokkenheid van genen op een niet-hypothese gestuurde basis geanalyseerd. Om dezelfde reden pastten we geen statistische correctie voor meervoudig testen ('multiple comparisons') toe.
We vonden in totaal 565 genen die verschillend tot expressie kwamen in de witte bloedcellen van patiënten vergeleken met de gezonde controles. Nadere analyses brachten naar voren dat het recent eerder ontdekte kandidaat gen *AKT1* verminderd tot expressie komt in witte bloedcellen van patiënten. Verder, dat significant meer bij genen die bij de patiënten veranderd tot expressie komen, betrokken zijn bij de intracellulaire signaalketen die bekend staat onder de naam '*PI3K/AKT signaling*'.
Het enzym *AKT1*, en de '*PI3K/AKT signaling*' signaalketen zijn betrokken bij celgroei en (geprogrammeerde) celdood, en bij de cellulaire energiehuishouding.
Eveneens vonden wij aanwijzingen voor betrokkenheid van signaalketens betrokken bij immuun processen.

Aangezien *AKT1* betrokken is bij neurotrofe processen werd vanwege het focus van dit proefschrift de mogelijke verminderde expressie van *AKT1* tot centrale hypothese van een uitgebreider vervolgonderzoek gekozen, dat beschreven is in **hoofdstuk 8**.
Bovendien hypothetiseerden we dat verlaagde *AKT1* expressie bij patiënten zal leiden tot deregulatie van signaalketens waarbij *AKT1* betrokken is (waaronder '*PI3K/AKT signaling*').
We includeerden 41 patiënten en 29 gezonde controles in dit onderzoek. De patiënten waren zo gekozen dat 21 van hen voldeden aan de criteria zoals vermeld bij hoofdstuk 7 (gestabiliseerde patiënten, jonger dan 36 jaar, korter dan 5 jaar lijdend aan schizofrenie, die voldoen aan de criteria voor het 'deficit syndrome'), en dat 20 van hen leden aan een acute psychotische episode. Elf van deze 20 acuut psychotishe patiënten gebruikten geen antipsychotische medicijnen ten tijde van het onderzoek.

Ook nu vonden we verlaagde expressie van *AKT1* bij de patiënten, onafhankelijk van fase van de ziekte (floride psychotisch of in remissie) en van medicatiegebruik. De effect-grootte van het verschil in *AKT1* expressie tussen patiënten en controles is groot (eta2 = 0.21).
Voor de niet-hypothese gestuurde analyse van de gehele verzameling genen pasten we, in tegenstelling tot de aanpak beschreven in hoofdstuk 7, nu wél correcties voor meervoudig testen toe (volgens Benjamini-Hochberg). We vonden in totaal 1224 genen die bij de patiënten veranderd tot expressie komen; 272 komen verhoogd tot expressie bij de patiënten, 952 verlaagd. Ook bij deze aanpak vinden we verlaagde expressie van *AKT1*.
We vonden 35 signaalketens waaraan de bij de patiënten veranderd tot expressie komende genen -meer dan op basis van toeval verwacht kan worden- deelnemen. Deze signaalketens zijn betrokken bij immuun-processen, cellulaire hechting, neurotrofe processen, oxidatieve stress en glucose metabolisme, en geprogrammeerde celdood. De op basis van hoofdstuk 7 gehypothetiseerde betrokkenheid van de '*PI3K/AKT*' signaalketen kon echter niet bevestigd worden; deregulatie van deze signaalketen viel juist onder de grens van significantie.
We concluderen op basis van deze bevindingen dat de expressie van *AKT1* in witte bloedcellen verlaagd is. Processen betrokken bij celgroei en energiehuishouding zijn verstoord, evenals processen die betrokken zijn bij de immuunstatus. De verstoringen in signaalketens die betrokken zijn bij de immuunstatus kunnen duiden op een primaire of secundaire immunologische component in de pathogenese van schizofrenie, het kan echter ook een epifenomeen zijn ten gevolge van het gebruikte weefsel.
Vanuit een conceptueel oogpunt is de bevinding belangrijk dat het mogelijk is bij schizofrenie (robuuste) afwijkingen in de gen-expressie van perifere weefsels te vinden.

In **hoofdstuk 9** presenteren we de resultaten van een pilot onderzoek naar de gen-expressie in witte bloedcellen bij patiënten met het velo-cardio-faciaal syndroom (VCFS). Patiënten die lijden aan dit syndroom bezitten een deletie op positie 11 van de lange arm van chromosoom 22 (vandaar ook: 22q11-deletie syndroom). Patiënten met een 22q11 deletie lijden een groot aantal (ontwikkelings-) stoornissen, die niet noodzakelijkerwijs allen tegelijk tot uiting hoeven te komen. Veelvuldig komen dysmorfologieën voor (met name van het gelaat en van het hart), en cognitieve beperkingen, waaronder gedragsstoornissen en verminderde intelligentie. Vanuit het perspectief van dit proefschrift is van belang dat bij patiënten met een 22q11

deletie op schizofrenie gelijkende psychotische verschijnselen vorkomen, én dat in de 22q11 regio het gen gelegen is dat codeert voor Catecol-O-Methyl Transferase (COMT). COMT is betrokken bij het dopamine metabolisme, en dopamine is op haar beurt betrokken bij schizofrenie. Het 22q11 syndroom geldt derhalve als een genetisch model voor schizofrenie.
We hypothetiseerden dat de expressie van genen gelegen in de 22q11 regio significant verminderd zou zijn. Bovendien hypothetiseerden we dat door interactie (epistasis) van genen gelegen in de 22q11 regio met genen buiten die regio, ook buiten de 22q11 regio gelegen genen afwijkend tot expressie zouden komen. Tenslotte hypothetiseerden we dat de gezamenlijke verzameling afwijkend tot expressie komende genen -zowel in als buiten de 22q11 regio- betrokken zou zijn bij processen die relevant zijn voor schizofrenie.
We onderzochten de gen-expressie in witte bloedcellen van 7 patiënten met een bekende 22q11 deletie vergeleken met 7 gezonde controles. In dit onderzoek includeerden we, in tegenstelling tot zoals beschreven in de voorafgaande onderzoeken, wel vrouwelijke patiënten.
Negentwintig genen, gelegen in de 22q11 regio, kwamen verminderd tot expressie; zes waren niet afwijkend. Negen genen, gelegen in de 22q11 regio komen niet in witte bloedcellen tot expressie. We vonden 262 genen (niet gecorrigeerd voor meervoudig testen) die buiten de 22q11 regio lagen en eveneens verminderd tot expressie komen. Signaalketens waaraan de bij de patiënten veranderd tot expressie komende genen -meer dan op basis van toeval verwacht kan worden- deelnemen, zijn betrokken bij immuun-, en neurotrofe processen.
We concluderen dat dit pilot onderzoek toont dat deels dezelfde signaalketens als gevonden in de hoofdstukken 7 en 8 afwijkend gereguleerd zijn.

Hoofdstuk 10 presenteert de resultaten van een multi-center onderzoek. Het onderzoek bestaat uit drie fases.
In fase 1 ligt het focus op een poging om, uitgaande van een uit 189 serumcomponenten bestaand spectrum, te komen tot een deelverzameling die in staat is vijf onafhankelijke cohorten van patiënten (totaal N=250) met schizofrenie te scheiden van vijf bijbehorende controle groepen. Deze fase van het onderzoek heeft als hypothese dat een dergelijke deelverzameling (van onbekende grootte) bestaat.
Op basis van lineaire discriminant analyse werd een subgroep bestaande uit 13 componenten geïdentificeerd die het meest optimaal de patiënten van elk cohort scheidt van de corresponderende controles (gemiddelde sensitiviteit cohorten 2-5

ten opzichte van de respectievelijke controle groepen: 0.82; gemiddelde specificiteit: 0.79).
In de tweede fase wordt onderzocht of een deelverzameling van de in fase 1 geïdentificeerde serum-componenten in staat is om presymptomatische personen die later schizofrenie ontwikkelden te scheiden van een bijbehorende groep controles. Op basis hiervan werden 4 serum-componenten geidentificeerd (*Alfa-1 Antitrypsin, Cortisol, Connective Tissue Growth Factor (CTGF), and Serum Amyloid-P*).
In de derde fase werd onderzocht of de 13, respectievelijk 4 gevonden componenten voldoende specifiek patiënten met schizofrenie, respectievelijk presymptomatische personen die later schizofrenie ontwikkelden, scheiden van differentiaal diagnostisch relevante andere groepen. Voor dit doel werden de patiënten met schizofrenie vergeleken met patiënten met een depressieve stoornis, een bipolaire stoornis (zowel euthym, als lijdend aan een manische episode), en het Syndroom van Asperger. In deze groepen waren slechts 0-3 van het uit 13 elementen bestaande 'schizofrenie-signatuur' veranderd.
De presymptomatische personen die later schizofrenie ontwikkelden werden vergeleken met presymptomatische personen die later een bipolaire stoornis ontwikkelden.
We concluderen dat een uit 13 serum-componenten bestaande, reproduceerbare biologische signatuur, in staat is patiënten met schizofrenie te scheiden van controles, en van relevante differentiaal diagnostische categorieën. Bovendien, dat een uit 4 serum-componenten bestaande subgroep presymptomatische personen die later schizofrenie ontwikkelden scheidt van controles, die later geen schizofrenie ontwikkelden.
Onze bevindingen suggereren dat er specifieke en reproduceerbare afwijkingen in de samenstelling van het serum zijn bij schizofrenie, en dat een deel van deze afwijkingen reeds aanwezig zijn voor het openlijk begin van de aandoening.

In **hoofdstuk 11** ten slotte, worden de bevindingen van dit proefschrift samengevat, de implicaties besproken, de beperkingen belicht, en aanbevelingen gedaan voor toekomstig onderzoek.
De belangrijkste bevindingen zijn:

1. In het serum van jonge, mannelijke patiënten met schizofrenie is een verhoogde hoeveelheid van het eiwit S100B aanwezig; de hoeveelheid S100B normaliseert bij deze patiënten niet na behandeling en remissie van symptomen. De normale leeftijdsafhankelijke daling van serum S100B is ook bij patiënten aanwezig.

2. In het serum van jonge, mannelijke patiënten met schizofrenie is een verlaagde hoeveelheid BDNF aanwezig, die normaliseert na remissie van (positieve) psychotische symptomen; we vinden een positieve correlatie tussen serum BDNF en positieve symptomen tijdens een psychotische episode.
3. De expressie van het bij trofische cellulaire processen en energiehuishouding betrokken enzym AKT1 is verminderd bij jonge, mannelijke patiënten met schizofrenie; deze verminderde expressie is zowel aanwezig bij floride psychotische patiënten, als bij patiënten in remissie, en zowel bij patiënten die geen antipsychotica gebruiken, als bij patiënten die dat wel doen. Bovendien vinden we deregulatie van signaalketens die betrokken zijn bij immuunprocessen, celhechting en -groei, en bij energiehuishouding.
4. Een panel bestaande uit 13 serum componenten is in staat om 5 cohorten van schizofrenie patiënten te scheiden van controles, en van relevante differentiaal diagnostische groepen.; een sub-groep van 4 componenten is in staat presymptomatische personen die later schizofrenie ontwikkelden te scheiden van bijbehorende controles, die later geen schizofrenie ontwikkelden.

Bij elkaar genomen vinden wij derhalve aanwijzingen dat er veranderde niveaus van de neurotrofe eiwitten S100B en BDNF zijn in het serum van patiënten met schizofrenie. Voor S100B is deze verandering, in iedere geval bij jonge, mannelijke patiënten, in de vroege fase van de ziekte, een persisterende trek; voor BDNF is deze verandering verbonden aan het bestaan van psychotische verschijnselen, dus toestandsafhankelijk.
Eveneens is het aannemelijk dat veranderingen in de expressie van het totale genoom aanwezig zijn in witte bloedcellen, dus in een perifeer weefsel dat geen openlijke pathologie vertoont. Dit is in ieder geval waarschijnlijk voor de expressie van het enzym AKT1.
Bovendien vinden we op basis van een analyse van circa 200 serum bestanddelen aanwijzingen dat 13 componenten in staat zijn patienten met schizofrenie te scheiden van controles, en dat een subgroep van 4 componenten in staat is asymptomatische personen die later schizofrenie ontwikkelden te scheiden van controles. Deze 4 componenten lijken bovendien specifiek te zijn voor deze groep, ten opzichte van asymptomatische personen die later een bipolaire stoornis ontwikkelden.

Dankwoord

De onderzoeken die in dit proefschrift beschreven zijn, zijn het resultaat van de inspanningen van veel verschillende mensen.

Ik denk dat ik allereerst de patiënten moet bedanken voor het meedoen aan het onderzoek. Jonge mannen houden in het algemeen niet van bloed prikken. Jullie hebben het toch gedaan.

Verder de verpleegkundigen van de zorglijn psychotische stoornissen. Samen staan jullie voor een humane, integere benadering van onze patienten zoals ik dat niet vaak heb meegemaakt. Regelmatig moesten jullie plotseling bloed prikken, terwijl er eigenlijk andere werkzaamheden waren. Bedankt voor jullie geduld en toewijding. Ik ben er trots op dat ik met jullie samen mag werken.

Mijn promotor Michiel Hengeveld. Michiel, we begonnen ongeveer tegelijkertijd in het Erasmus MC, dat toen nog Dijkzigt heette. Ik dank je voor de mogelijkheid die je me hebt geboden om in een uitdagende stad als Rotterdam een zorglijn voor jonge mensen met een psychose op te zetten. Ook dank ik je voor je ondersteuning bij mijn onderzoek naar 'stofjes in bloed', ofschoon ik me realiseer dat de filosoof in jou daar wel gemengde gevoelens bij had en waarschijnlijk nog heeft.

De leden van de kleine commissie, prof dr RS Kahn, prof dr PJ van der Spek, en prof dr CI de Zeeuw.

Professor Kahn wil ik van harte bedanken voor het zitting nemen in de beoordelingscommissie. Ik heb groot respect voor de manier waarop u zich beijvert voor de emancipatie van de psychiatrie als medische discipline, en voor het inzichtelijk maken van ons vak bij het publiek.

Peter van der Spek. Zonder jouw inspanningen waren er geen hoofdstukken verschenen over genexpressie. Onmiddellijk heb je jouw afdeling, met al haar deskundigheid op het gebied van complexe data-analyse, aan mij ter beschikking gesteld. Je enorme enthousiasme wekt aanstekelijk. Met jou moet ik ook Sigrid Swagemakers bedanken. Sigrid, altijd wist je mijn vragen op het gebied van de biostatistiek te beantwoorden. Een mailtje en powerpoints met de meest fantastische plaatjes en analyses stroomden binnen. Ik hoop dat ik nog regelmatig een beroep op je kan doen, want ik heb nog niet eens alles in dit boekje kunnen stoppen.

Chris de Zeeuw. Voor een drukbezet hoofd van een florerende onderzoeksafdeling ben je enorm toegankelijk. Altijd kun je binnenlopen om even samen na te denken. Al vanaf onze eerste ontmoeting was het duidelijk dat je een grote belangstelling hebt voor de psychiatrie. Jij bent degene die mijn rudimentaire ideeen over genexpressie en proteomics momentum gaf door me in contact te brengen met Peter van der Spek, Ype Elgersma en Sabine Bahn. Je heb van een afstand dus een belangrijke

bijdrage geleverd aan mijn onderzoek. Bovendien bedank ik je voor het vervullen van de rol van secretaris van de kleine commissie.
Mijn copromotores, Durk Fekkes en Lieuwe de Haan.
Durk, ik heb veel om je voor de bedanken. Je analytische geest bij het doorspreken van de resultaten van onderzoek; je zorgvuldige metingen; de beschikbaarheid van lab en vriezers voor de soms wel erg grote hoeveelheden monsters; de flexibiliteit van jou en je medewerkers als ik weer eens een monsters uitgevuld wilde hebben. Ik hoop dat we nog meer metingen gaan doen. Mijn dank is er dus ook voor je medewerkers, Elly Taal, Ans Kooyman en Marieke Mulder. Geduldig ontvingen jullie mijn verzoeken om, liefst gauw, monsters te meten. Gelukkig maar dat jullie de administratie (ook) bijhielden!
Lieuwe. Los van alle wetenschap lijkt het me dat ik je allereerst moet bedanken voor het feit dat je me hebt leren schaatsen. Ik ben bang dat het omgekeerde niet voor skiën geldt. Nou ja en verder: je stuurt prompt alle manuscripten terug met zinnige aanwijzingen, en geeft me daarbij het gevoel dat ik slimme dingen heb bedacht. Als bonus verzamel je ook nog allerlei sera voor mij. Je inspanningen voor mensen met schizofrenie, en hun familie, zijn een voorbeeld. Ik zou zeggen, laten we samen eens een bijscholing voor huisartsen organiseren, op een plek met veel bergen of zo.
Ype Elgersma. Aanvankelijk wilde ik van je faciliteiten gebruik maken om de genexpressie in neusbiopten te onderzoeken. Ik meende toen een zeker afgrijzen te bespeuren ten opzichte van psychiaters die moleculair-biologisch onderzoek willen doen. Maar daarna heb je mij je geweldige lab ter beschikking gesteld voor het onderzoek naar de genexpressie in witte bloedcellen. Ik heb je leren kennen als iemand die precies is, en kritisch, maar eenmaal overtuigd van iets ga je door roeien en ruiten en is zelfs de editor van de American Journal of Psychiatry niet veilig voor je. Dank voor het zitting nemen in de grote commissie. Met jou bedank ik ook Lianne Krab en Gabriëlle Buitendijk, die de genexpressie bepalingen hebben gedaan. Lianne, voor jou was dit project een grote zijstap van je eigen promotieonderzoek. Toch heb je de techniek aan het draaien gekregen, en de eerste analyses gedaan. Jij was ook de eerste die bij het kijken naar een hele lijst genen riep: "AKT en schizofrenie, daar heb ik eens iets over gelezen". Onderwijsvernieuwers kunnen hieruit opmaken dat parate kennis ook zijn nut heeft. Gabriëlle, jij hebt de grote bulk van de genexpressiebepalingen gedaan. Hoe goed je ze hebt gedaan blijkt wel uit het feit dat er bijna geen 'bad samples' tussen zaten. Ook heb je een groot aantal jonge controles voor het onderzoek verzameld. Verder heb ik kunnen vaststellen dat je er goed in bent ze op te vangen als ze flauw vallen bij het bloedprikken.

Sabine Bahn. Sabine, I think you're an extraordinary person. I greatly admire you're innovative ideas concerning mental illnesses and their diagnostic challenges. It is an honor for me to collaborate with you and your lab members. Thank you for so spontaneously allowing me to incorparate the 'blood fingerprint' paper in my thesis. I sincerely hope we will perform many more projects, possibly with an even more close cooperation between you and the Erasmus MC.

Therese van Amelsfoort. Toen ik geld overhad om nog een beperkt aantal genexpressie bepalingen te doen, reageerde je onmiddellijk enthousiast toen ik voorstelde om de techniek uit voeren bij patienten met het 22q11 deletie syndroom. Nu de druk van het beëindigen van het 'boekje' weg is hoop ik dat we eens kunnen kijken of we hier een vervolg aan kunnen geven.

Even terug in de tijd: Ernst Colon, jij hebt me als oudere, en behoorlijk eigenwijze assistent in opleiding genomen. Mijn ideeën over neurale netwerken heb je meteen gestimuleerd. Bedankt.

En dan mijn collega's. Vaak hebben jullie me moeten missen omdat ik bezig was met het onderzoek, of moest er juist wat op stel en sprong geregeld worden. Reinier van der Lugt, Hermien van der Wilt, Odilia Streep, Monica Baldini en Wim Verveer hebben in belangrijke mate bijgedragen aan het identificeren van patienten voor het onderzoek. Speciaal wil ik Barbara van Iersel bedanken. Barbara, wij hebben in de eerste jaren samen 'onze' afdeling opgezet, en ik bewaar daar hele goed herinneringen aan. Jammer dat je weg bent. Misschien kunnen we samen ooit weer eens iets opzetten.

Mijn collega psychiaters, Robert Jan Osse, Walter van den Broek ('bedankt voor de wijn'), Tom Birkenhager, Esther Pluims, Monique Raems, Veerle Bergink, Meike van den Berg, Christian Röder, en, in het verleden, Jan Bruijn en Anne-Marie van Hulst bedank ik voor hun collegialiteit. Met name Walter, Robert Jan, Tom, en Esther hebben regelmatig klinisch werk overgenomen. In het bijzonder wil ik Christian Röder bedanken. Sinds 2005 doen we samen de 'zorglijn psychotische stoornissen'. Als nieuwkomer in Nederland moet het niet gemakkelijk voor je zijn geweest. Nog regelmatig verbaas je jezelf over Nederlandse gewoontes. Maar je bent een van de meest collegiale en integere personen die ik ken. Bedankt dat je mijn paranimf wilt zijn. Wel de hele tijd blijven staan!

Kees, jij bent mijn andere paranimf. Het is goed dat er bij al het Rotterdamse geweld een echte Amsterdammer naast me staat, en dan nog wel een die ik al 34 jaar ken. Ik hoop dat jij en Brigitte nog heel vaak komen eten. Volgens mij hebben wij alle uitschakelingen van het Nederlands elftal gezamenlijk gezien. Dat moet de komende zomer maar eens stoppen.

Clementien, bedankt voor het maken van de prachtige lay-out en de illustraties, en vooral voor het geduld mijn voortdurende verzoeken om wijzigingen te doorstaan. Het is knap dat je je niet klein laat krijgen door problemen met je gezondheid, maar volop van het leven blijft genieten.

Lieve Martijn, wij zijn al 19 jaar samen. Misschien heb jij me wel schaatsen geleerd! En nog een heleboel andere dingen, zoals hoe belangrijk het is om gewoon rustig je gang te gaan. Ik hou van je. En wat hebben we een leuke kinderen. Fien en Daan, het is leuk om aan zo'n promotie te werken, maar nog veel leuker om naar jullie wedstrijden te kijken. Wel mijn aanwijzingen opvolgen!, ik roep ze hard genoeg. Sluit ik af met een uitspraak van Christian: "KlaarKees!"

Curriculum vitae

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Nico van Beveren is geboren op 25 oktober 1958 te Amsterdam. Hij slaagde in 1978 voor het examen gymnasium B. Na twee jaar scheikunde te hebben gestudeerd ving hij in 1980 aan met de studie geneeskunde. Tijdens de studie geneeskunde werkte hij als fysiologie-assistent, gaf hij computerlessen aan een school voor doktersassistenten, en deed hij vruchteloze pogingen om een goede schaakspeler te worden. In 1989 behaalde hij het artsexamen. In 1989 werkte hij als arts-assistent op de intensive care van het Catharina ziekenhuis te Eindhoven. In 1990 was hij kortstondig in opleiding tot anesthesioloog. In 1991 maakte hij een definitieve, en nooit betreurde switch naar de psychiatrie. Van 1991 tot 1995 was hij werkzaam als arts ten behoeve van de verblijfspsychiatrie bij het APZ Bavo te Noordwijkerhout/ Rotterdam. In 1995 was hij werkzaam bij de stichting Adhesie te Deventer, alwaar hij in 1996 aanving met de opleiding tot psychiater. De stage ziekenhuispsychiatrie deed hij in 2000 in het latere Erasmus MC te Rotterdam. Aldaar is hij vanaf 2001 werkzaam als psychiater en medisch coordinator van de door hem opgezette zorglijn psychotische stoornissen. Hij is lid van het platform biologische psychiatrie, en was in de periode 2004-2009 namens het platform lid van de Commissie Wetenschappelijke Activiteiten (CWA) van de Nederlandse Vereniging voor Psychiatrie.
Hij is getrouwd met Martijne Kruse; samen hebben ze twee kinderen, Fien (14) en Daan (12).