The dual hit hypothesis of schizophrenia
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DOI:
10.33612/diss.791866719

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2023

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 1

General Introduction
1. A brief history of schizophrenia

Schizophrenia is a major psychiatric disorder that has already been described in ancient Mesopotamian Old Babylonian period (the first half of the second millennium BC), ancient Egyptian texts and in the Holy Bible, where it is described as “unclean spirit in the man, demon-possessed, demoniac, madness” (Bark, 1988; Jablensky, 2010; Okasha, 2004). Consequently, schizophrenia is often considered to be an ancient disorder that has accompanied mankind through its existence. In the Nineteenth century, the German psychiatrist Emil Kraepelin called the disorder “dementia praecox”, meaning “premature dementia”, as he believed that the condition is worsening with time due to a neurodegenerative process (Heckers, 2011; Jablensky, 2010). Dementia praecox was diagnosed based on deficits in cognitive and executive functioning that led to “impoverishment and devastation of the whole psychic life”. While the term dementia suggests a degeneration, it is now recognized that schizophrenia is a neurodevelopmental disorder with a genetic and environmental etiology (Stachowiak et al., 2013), rather than a progressive neurodegeneration. At the beginning of the Twentieth century, the Swiss psychiatrist Eugen Bleuler termed the disorder schizophrenia, or rather, the schizophrenias, as he believed that the symptomatology of schizophrenia is a spectrum and that no two patients experience it in the same way (Heckers, 2011; Jablensky, 2010; Moskowitz and Heim, 2011). The word schizophrenia has Greek roots combining σχίζω (skhizo), meaning “I split”, and φρένα (phrena), meaning “mind”, to describe a splitting of psychological functions. Nowadays, this name is considered stigmatizing and sometimes replaced with psychosis, or even psychosis-susceptibility, which is a bit of an euphemism, as it does not quite cover all aspects of the disorder (Sommer and Kahn, 2014).

2. Clinical aspects of schizophrenia

Schizophrenia affects about 0.7% of the world’s population and is an important contributor to the global health economic and societal burden, due to high healthcare costs and the impact on the quality of life of affected individuals and their surroundings. The onset of schizophrenia is different in men and women, with a majority of women having an age of onset in their 30s while the onset for men is usually in their 20s. These differences have been suggested to be partly attributable to the protective effects of estrogens (Brand et al., 2021).

Schizophrenia symptomatology is classified into positive, negative, and cognitive symptoms (Tandon et al., 2009). The positive symptoms of schizophrenia include all psychosis-related symptoms, such as delusions, hallucinations, and disorganized thinking. The most common delusions are paranoid, somatic, religious or bizarre. An intriguing symptom is that patients think that their thoughts are audible (hearing thoughts aloud) and controlled by a foreign entity (alienation). Hallucinations in all perceptive domains have been reported, with the most common one being auditory, most often consisting of hearing voices commenting on the patient’s behavior. Disorganized thinking highlights the fractionation of the normal thought process and refers to an inability to keep a train of thought or dissolution of logical goal-directed thought processes reflected in incoherent speech. Negative symptoms include loss of motivation, low energy social withdrawal, and lack of emotional expression. Cognitive symptoms include dysfunction of attention, memory, executive functioning, and processing speed. Affective symptoms such as anxiety, depression and mania are often present but are not considered core symptoms of schizophrenia. Furthermore, catatonia (abnormality of movement and behavior arising from a disturbed mental state) are a frequent co-occurring symptom cluster (Hirjak et al., 2015). According to the DSM-V, a patient must fulfill at least two of the following five symptoms for
a significant portion of time within one month to be diagnosed with schizophrenia: delusions, hallucinations, disorganized or incoherent speech, disorganized or unusual movements and negative symptoms. In addition, daily functioning must be impaired for at least six months, unless successful therapy has been started early.

The most common pharmacological treatment is antipsychotic medication targeting primarily the dopaminergic system. However, it mainly ameliorates the positive symptoms, while having little or even a negative impact on the negative and cognitive ones (Patel et al., 2014). A better understanding of the etiology and pathophysiology of schizophrenia, in particular of its molecular and cellular pathophysiology, is pivotal for the discovery of new therapeutic targets and the development of drugs that can improve all symptoms. However, despite the extensive research on the pathophysiology of schizophrenia, the exact mechanisms underlying the syndrome remain unclear.

3. The pathophysiology of schizophrenia

3.1. Brain changes in schizophrenia

In an attempt to explain the pathophysiology of schizophrenia, many hypotheses suggesting dysregulations in specific neurotransmitter systems have been developed over the years. The first hypothesis, inspired by the success of the dopamine blocking antipsychotic drugs, was the dopaminergic hypothesis established in the 1970s that stated that dopamine levels were increased in the brain. This hypothesis was latter updated as it was found that dopamine levels are higher in striatal areas but lower in the frontal cortex of schizophrenia patients (Davis et al., 1991). Other hypotheses suggest dysregulation in for example the glutamatergic and GABAergic neurotransmission. More recently, genetic discoveries in the HLA genome zone inspired the hypothesis of immune dysregulation. All these hypotheses were supported by pharmacological, brain imaging and post-mortem studies that have observed increased presynaptic dopamine production (Howes and Murray, 2014), prefrontal and hippocampal GABAergic deficits (Glausier and Lewis, 2017; Schmidt and Mirnics, 2014), glutamatergic hyperexcitability (Barch and Ceaser, 2012; Kantrowitz and Javitt, 2010; Moghaddam and Javitt, 2011), low-grade immune activation (Doorduin et al., 2009; Fillman et al., 2016; Horváth and Mirnics, 2014), and the presence of increased oxidative stress (Do et al., 2009; Fillman et al., 2016; Horváth and Mirnics, 2014) as important contributing mechanism in schizophrenia. Furthermore, a meta-analysis of genome-wide association studies (GWAS) showed that schizophrenia was associated with over 100 loci, related to dopamine synthesis, glutamate receptors, calcium channel regulation, and the immune system, amongst others (Ripke et al., 2014). While none of these genes alone have a major impact on the risk to develop schizophrenia, the combination of several risk genes in so called polygenic risk scores can increase the risk for specific aspects of schizophrenia, for example, treatment resistance (Gasse et al., 2019). These biological systems likely interact with each other. For example, increased immune activation can induce oxidative stress and dysregulate the dopaminergic system (Howes et al., 2013; Oskvig et al., 2012; Purves-Tyson et al., 2019). As a reflection of the pathophysiology of schizophrenia, imaging studies showed changes in cerebral glucose metabolism/uptake in patients with schizophrenia (Seethalakshmi et al., 2006; Soyka et al., 2005), which is believed to be an indicator of underlying pathology and may therefore indicate early neurodevelopmental changes that could have contributed to the development of behavioral alterations later in life. The pathophysiology of schizophrenia is likely different per person, involving a mix in alterations in some or many of these interconnected biological systems that could originate from genetic vulnerability, early changes in neurodevelopmental processes and/or triggering mechanisms in the second or third decade of life.
3.2. Neurodevelopment in schizophrenia

Neurodevelopmental alterations such as neurogenesis, neuronal migration and differentiation, axonal outgrowth, myelination, synaptogenesis, and synaptic pruning have been proposed to underly the pathology of schizophrenia (Germann et al., 2021; Howes and McCutcheon, 2017). For example, in 1982 Feinberg stated that faulty programmed synaptic elimination and excessive pruning was an important step in the development of schizophrenia (Cardozo et al., 2019; Germann et al., 2021; Paolicelli et al., 2011). Synaptic pruning is a neurodevelopmental process enabling the proper establishment and maturation of functional neuronal networks by eliminating infrequently used synapses, while reinforcing frequently used connections. Synaptic pruning occurs throughout early development and continues throughout life. Synaptic pruning peaks at toddler age, and again during adolescence, in the second period particularly in brain regions involved in higher cognitive functions, such as the frontal cortex (Cardozo et al., 2019). This excessive pruning during adolescence may be related to the late adolescence-early adulthood age of onset of schizophrenia. This hypothesis is supported by a recent positron emission tomography study employing the novel tracer \([^{11}\text{C}]\text{UCB-J}\) to image synaptic vesicle glycoprotein 2A (SV2A) that reported a significant and large decrease in synaptic density in patients with schizophrenia in vivo (Onwordi et al., 2020). Furthermore, post-mortem studies observed a reduction in SV2A, synaptophysin, synaptosomal-associated protein (SNAP25) and post synaptic density 95 (PSD-95) protein, all markers of synaptic density, in the frontal cortex and hippocampus of schizophrenia patients (Corradini et al., 2009; Onwordi et al., 2020; Osimo et al., 2019). In line with these findings, schizophrenia patients show many structural abnormalities, including a reduction in grey matter volumes (an indirect measure of synaptic density), ventricular enlargement, hypoconnectivities in the insula and somatosensory areas, in some individuals misplaced and clustered neurons, neurons with smaller cell bodies and fewer dendritic spines, and reduced neuronal connections and neurotransmission (Li et al., 2019; Powell, 2010). Alterations in synaptic pruning have been suggested to be orchestrated by a deviant activity of the immune system characterized by an abnormal number and activity status of microglia, and increased opsonization of synapses by the complement system (Germann et al., 2021; Kim et al., 2021; Koyama and Ikegaya, 2015).

3.3. Immune system in schizophrenia

Microglia are the resident macrophages of the brain, aiming to maintain order not only by protecting the brain against infection, but also by determining the strength of brain connectivity, neurogenesis, and synapse maturation or elimination (Dietz et al., 2020; Thion et al., 2018). Microglia can adopt different morphologies ranging from hyper-ramified with long processes and small soma size (surveillant) to a reactive amoeboid shape (activated), with many intermediate stages. Microglia are involved in the surveillance of their microenvironment and the phagocytosis of pathogens and disrupted or apoptotic neuronal parts, such as infrequently used synapses, in order to transform the raising chaos into order (Marques et al., 2019). However, alterations in microglial functions, notably during crucial developmental periods such as early life and adolescence, may lead to excessive pruning of functional and healthy synapses, eventually resulting in a reduction in synaptic density later in life. Postmortem studies reported significantly higher microglial density and activity, increased blood and brain acute phase C-reactive protein, and increased blood and brain pro-inflammatory cytokine (IL-6, TNFα, IFN, IL-1β, IL-33) levels in schizophrenia patients compared to healthy controls (Fond et al., 2020; Van Kesteren et al., 2017). PET imaging studies investigating the involvement of microglia in schizophrenia gave conflicting results as some studies showed low to moderate levels of microglial
activation, whereas other studies did not detect any change at all (Conen et al., 2020; Reis Marques et al., 2018). This discrepancy could be due to differences in methodology, technique, patient population, or disease stage.

The complement system may be involved in the increase in microglial activation and synaptic pruning (Mongan et al., 2020). Particularly, C3 and C4 complement proteins trigger the opsonization of pathogens, abnormal cell parts and synapses, thereby inducing phagocytosis by activated microglial cells (Lee et al., 2019). Schizophrenia has been associated with structurally altered alleles on the short arm of chromosome 6, acting on complement factor 4. Such alterations induce not only a more susceptible complement system, but also an enhanced expression of the complement factor 4a (Germann et al., 2021; Kim et al., 2021; Sekar et al., 2016), which could possibly induce excessive opsonization of synapses. This would result in increased synapses phagocytosis by activated microglial cells, and ultimately in lower synaptic connectivity (Druart et al., 2021; Germann et al., 2021; Koyama and Ikegaya, 2015). However, despite suggesting mechanisms of actions that could underly the development of schizophrenia, the findings previously mentioned do not explain the etiology per se and a gap in our understanding of the relationship between abnormalities in (and the interaction between) these affected systems, and the behavioral symptom of schizophrenia remains. Understanding the risk factors at the origin of these brain abnormalities may provide better insight in the disorder.

4. Risk factors for schizophrenia

The etiology of schizophrenia is multifactorial, comprising a combination of genetic vulnerability and environmental risk factors early and later in life (Modai and Shomron, 2016). Individual environmental factors alone seem to have a relatively weak impact on the individual, despite a modest effect size in large populations, and may not be sufficient to induce the development of schizophrenia (Selten et al., 2010; Stepiak et al., 2014; Stilo and Murray, 2010; Varese et al., 2012). A concept called the dual hit hypothesis of schizophrenia proposes that genetic predisposition or perinatal environmental risk factors may prime the individual to become more susceptible to the pathological effects of a second environmental risk factor later in life (Bayer et al., 1999; Guerrin et al., 2021; Maynard et al., 2001).

Environmental factors early and later in life have been associated with an increased risk of developing schizophrenia. Early environmental risk factors include compromised prenatal environment (infection, malnutrition, antibiotic treatment, and psychosocial stress of the mother during pregnancy) (al-Haddad et al., 2019; Cattane et al., 2020; King et al., 2010), abnormal fetal growth and development (Cannon et al., 2002), complications during pregnancy (bleeding, preeclampsia, and diabetes) or delivery (preterm birth, birth complications and caesarean section) (Cannon et al., 2002), winter birth (Tochigi et al., 2004), childhood adversity and trauma (sexual, physical, and psychological abuse, neglect, parental death) (Varese et al., 2012), and growing up in an urban environment (Vassos et al., 2012). Later in life, environmental risk factors include bullying at school and discrimination (Varese et al., 2012), early-onset drug abuse (Forti et al., 2014; Marconi et al., 2016), (a family history of) immigration (Bourque et al., 2020; Cantor-Graae and Pedersen, 2013), socioeconomic factors (Allardyce and Boydell, 2006), social isolation and social defeat (social adversities, social embarrassment) (Stowkowy and Addington, 2012).

Epidemiological studies suggest a combined effect of polygenic risk score, early and later in life environmental risk factors on symptoms’ intensity of schizophrenia (Stilo and Murray, 2010; Zwicker
et al., 2018). More specifically, recent epidemiological studies observed that the polygenic risk score and prenatal infection can act in synergy with psychological trauma in peripubertal life to increase the risk of schizophrenia (Aas et al., 2021; Debost et al., 2017; Woolway et al., 2022). Furthermore, recent clinical studies observed an additive effect of genetic risk for schizophrenia and regular cannabis use, sexual abuse, emotional abuse, emotional neglect, bullying, and psychological trauma in peripubertal life to increase the risk of experiencing schizophrenia symptoms (Aas et al., 2021; Guloksuz et al., 2019; Woolway et al., 2022). A Danish study, including almost 1,000,000 individuals and about 10,000 schizophrenia patients, observed a significant synergistic interaction between prenatal infection and peripubertal psychological trauma, which was stronger in men than in women (Debost et al., 2017). A major limitation of epidemiological and clinical studies is that they cannot provide clues regarding the underlying mechanisms involved in the etiology of schizophrenia. This is especially the case for studies on the dual hit hypothesis, as there are multiple variables for researchers to account for (e.g., severity of stress, the timing of exposure, number of stressors).

Animal studies enable the investigation of specific genetic and environmental risk factors and their underlying mechanisms in the etiology and pathophysiology of schizophrenia, while controlling for both genetic and environmental variation. Moreover, experimental variables (e.g., stimuli, severity, timing) can be controlled in rodent models. Therefore, several dual-hit rodent models, using different combinations of genetic and environmental risk factors with different severities and at different ages (prenatal, early postnatal, adolescence, or adulthood), have been utilized for research on the etiology of schizophrenia (Guerin et al., 2021). An extensive review on the dual-hit hypothesis of schizophrenia from studies in which animals were exposed to a combination of multiple genetic or environmental risk factors can be found in Chapter 2.

5. Aims and outline of the thesis

Driven by the dual-hit hypothesis, the aim of this thesis was to investigate the effect of single and combined risk factors for schizophrenia on brain and behavior in rodent models. Furthermore, I wanted to investigate the hypothesis that changes in microglial activity may result in alterations in synaptic density and eventually behavior. To do so, I investigated prenatal infection, social adversity, and a genetic component of schizophrenia in rats. I also measured various behaviors related to psychiatric disorders including anhedonia, anxiety, sociability, recognition and working memory, locomotion, and anticipatory reward. In addition, I used PET imaging with the TSPO tracers $[^{11}C]$PK11195 or $[^{11}C]$PBR28 to longitudinally measure possible changes in microglial reactivity and postmortem analysis to measure brain pro and anti-inflammatory cytokines and synaptic proteins.

This thesis consists of 9 chapters, with the following content.

Chapter 2 summarizes animal studies regarding molecular and cellular mechanisms through which genetic and environmental factors may affect brain development, ultimately causing schizophrenia. One of the environmental risk factors frequently investigated in animal models and suggested to increase the vulnerability of the immune system to an adolescence stressor is prenatal infection during pregnancy.

In Chapters 3, 4, and 5, we aimed to determine the effect of prenatal infection alone and social adversity alone as they are two important risk factors for psychiatric disorders. Determining the effect of these important risk factors alone was the first step before being able to combine them.
The work described in Chapter 3 investigates the effect of prenatal infection on brain glucose consumption, microglial activation, anxiety, and recognition memory in male rats. We aimed to determine whether prenatal infection alone can induce changes in neurodevelopment (brain glucose), neuroinflammation (microglia), and behavior (anxiety and recognition memory).

The studies described in Chapters 4 and 5 investigate the effects of social adversity on neuroinflammation, glucocorticoids, social behavior, anhedonia, and anxiety in male rats. Vulnerability to social adversity seems to depend on age. Social adversity experienced during adolescence, a critical phase during which the brain undergoes many neurodevelopmental changes, is believed to have more detrimental effects than trauma experienced in adulthood when the brain is fully developed. Therefore, in chapter 4, we tested whether exposure to social adversity during adolescence or adulthood affects glial reactivity, glucocorticoid levels, and behavior differently and whether such adversity alters the response to infection later in life. In chapter 5, we tested the effect of a single injection of the fast antidepressant ketamine on the social adversity-induced neuroinflammation, increase blood glucocorticoids, and anhedonia.

Once the effect of a single hit was investigated, we wanted to determine their possible combined effects. Chapters 6 and 7 aimed to investigate the combined effects of prenatal infection and a second hit during adolescence.

The study described in Chapter 6 investigates the single and combined effect of prenatal immune activation and adolescent social adversity on microglial activation, synaptic density, anhedonia, social behavior, anxiety, locomotion, and working memory in male rats. More specifically, we aimed to investigate whether the combination of environmental risk factors would increase the symptoms’ intensity and whether this would be related to changes in microglial cells and synaptic density in adulthood.

The study described in Chapter 7 investigates the single and combined effect of prenatal immune activation and adolescent immune challenge on microglial activation, anhedonia, social behavior, anxiety, locomotion, and working memory in female rats. More specifically, we aimed to investigate whether the combination of a prenatal and adolescent postnatal infection would increase the symptoms’ intensity and whether this combination would result in changes in microglial cells in adulthood. We decided to perform an immune challenge using LPS during adolescence instead of social adversity because female rats do not display as much territoriality behavior as males, making it more difficult to develop a social adversity model in female rats.

A limitation of the previous chapters is that they do not consider the genetic component of schizophrenia. On the other hand, most studies investigating the genetic component of schizophrenia only focused on a single gene mutation and did not show major impact on the susceptibility to an environmental risk factor. This parallels the human situation where a single risk gene has hardly any impact on the individual risk to develop schizophrenia. Only many genes acting in concert in polygenic risk profiles will impact an individual’s risk status. We aimed to overcome this limitation in chapter 8. To do so, we used the apomorphine susceptible rats (APO-SUS), a rat strain selectively bred for many generations based on their exaggerated behavioral response to apomorphine (dopamine agonist). These rats display brain and behavioral features relevant to schizophrenia.

The study described in Chapter 8 aimed to investigate the effect of a prenatal infection of APO-SUS rats on heart rate variability, ultrasonic vocalizations, anticipatory locomotor pleasure,
neuroinflammation and synaptic density. We investigated heart rate variability and ultrasonic vocalizations early in life as they are believed to be potential biomarkers of altered autonomic functioning related to the development of psychiatric disorders.

In Chapter 9 the findings described in this thesis are discussed and the results are put in a broader perspective.

By studying the relationship between genetic and environmental on neuroinflammation, synaptic density and behavior, I aimed to contribute to the understanding of the etiology of schizophrenia.

6. References


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