Neurodevelopment, brain vasculature and schizophrenia

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DOI:
10.33612/diss.582204366

Chapter 1

General introduction and outline of the thesis

Preface

Schizophrenia is a mental disorder that arises from abnormal neurodevelopment, led by genetic and environmental factors. Several lines of evidence suggest the involvement of both the nervous and vascular system in the etiology and pathophysiology of schizophrenia. The nervous and vascular system are intimately related in terms of development, establishment, and function. Therefore, alterations in either of them would potentially affect the correct development and functioning of both systems. In this thesis, human-induced pluripotent stem cell-derived neurons were used to study early neurodevelopment in schizophrenia. To investigate physiological adult neurogenesis and the possible contribution of the cerebral vasculature to the pathophysiology of schizophrenia, single-cell gene expression profiling of *post-mortem* brain tissues was performed. Together, our data and observations contribute to the understanding of schizophrenia brain pathology, as well as to our knowledge of human neurodevelopment and brain vasculature heterogeneity.

The Brain

The brain and the spinal cord form the central nervous system (CNS). The brain participates in all cognitive functions, such as perception, thinking, and memory [1], as well as in movement control and regulation of vital parameters, such as breathing and body temperature [2]. Although the cellular composition of the brain varies through different regions [3], some major cell types can be found throughout the entire brain:

Neurons

Neurons are electrically excitable cells with a highly specialized morphology that mediates their communication. Neurons exhibit extended cellular processes, the dendrites and axons, connecting them across distant brain regions. The dendrites can receive information in the form of electrical and/or chemical signals, from a large number of neurons. These signals are integrated and translated into voltage changes that, upon reaching a certain threshold, can trigger action potentials. Action potentials travel through the neuronal body and the axon, inducing neurotransmitter release at the axon terminal [4]. At the synapse, the axonal plasma membrane of the sending neuron approaches the dendritic membrane of the receiving neuron, facilitating neuronal communication. Thus, the synapse may be considered the elementary structural and functional unit that supports the flow of information between individual neurons throughout the brain.

Astrocytes

Astrocytes are star-shaped cells with great morphological and functional heterogeneity [5], which has led to their classification into different subpopulations. Protoplasmic astrocytes are enriched in the grey matter of the CNS, in close structural and functional association with neurons. Protoplasmic astrocytes are highly ramified, facilitating their contact with the synapses [6]. They modulate synaptic transmission by synthesis and clearance of neurotransmitters and neuromodulators [7]. In addition, their end-feet wrap the brain vasculature [8] and contribute to the regulation of regional blood flow in response to changes in neuronal activity [9]. Conversely, fibrous astrocytes are in the brain white matter, are less branched, and express higher levels of the glial fibrillary acid protein (GFAP), as compared to protoplasmic astrocytes [10]. Astrocytes also regulate immune responses in the brain. For instance, in response to disease or injury,

astrocytes undergo morphological, molecular, and functional remodeling, including higher proliferation rates, production of cytokines and recruitment of immune cells [11, 12].

Oligodendrocytes

Oligodendrocytes are the myelinating cells of the CNS. Extension of the oligodendrocyte plasma membrane form the myelin sheets that wrap around the axons, acting as electric insulators that allow saltatory propagation of action potentials [13].

Immune cells of the brain

Microglia are tissue-resident macrophages in the brain parenchyma. Microglia exhibit functions associated with innate immune response, such as antigen presentation, cytokines release and phagocytosis [14]. Microglia also display CNS-tailored functions, such as maturation of synapses and synaptic pruning associated with synaptic activity [15]. CNS-associated macrophages (CAMs) are localized at the borders of the CNS, such as in the perivascular space, choroid plexus, and meninges [16]. Altogether, these cells surveil and protect the CNS against invading pathogens and injury.

Neurovasculature

The brain is a highly vascularized organ. The vasculature of the brain is arranged in the neurovascular unit (NVU). The NVU is comprised of different cell types, including endothelial, smooth muscle cells (SMSc), pericytes [17], fibroblasts, astrocytes, and neurons [18]. Endothelial cells line the inner wall of the blood vessels. SMSc and pericytes are contractile cells that regulate local blood flow, whereas fibroblasts give structural and trophic support to vessels.

Development of the nervous and vascular system

The vascular network develops in temporal and spatial synchrony with the nervous system, probably to fulfill the high energy and oxygen demands of the brain [19]. The vascular and nervous system share molecular guiding cues and exhibit a fine-tuned crosstalk that allows the establishment of an intimately coordinated neurovascular network [19, 20].

Neuro and gliogenesis

In humans, cortical neurogenesis starts at the end of the first month of embryonic development, along with the closure of the neural tube [21]. Neuroepithelial cells lining the luminal wall of the neural tube differentiate into the radial glia cells, constituting the first neural stem cells (NSCs) population [22]. These cells divide asymmetrically, to self-renew and to generate excitatory projecting neurons. Neurogenesis from radial glial occurs both directly and through intermediate progenitors, also known as transient amplifying cells [23]. Radial glial exhibit long radial processes with apical-basal polarity. Apically, radial glia contact the lumen of the neural tube (Figure 1), which later becomes the ventricular system of the developing brain [24]. The soma of radial glia are located apically, in the surrounding area of the ventricle. The basal process of the radial glial projects to the meninges, the basal lamina and blood vessels. The newly generated neurons migrate towards the basal side, guided by the long process of the radial glia [23], populating upper cortical layers.

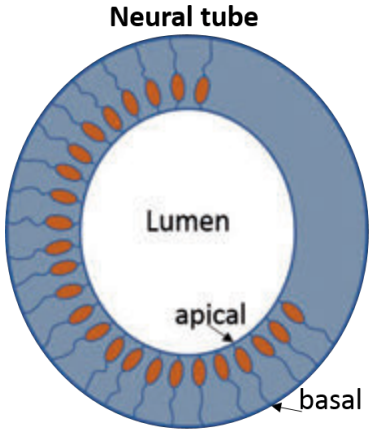

Figure 1. Scheme of radial glia in the neural tube.
The orange ovals represent the soma of radial glia cells, from which two processes extend. The apical process contacts the lumen of the tube, while a basal process extends in the opposite direction.

Some intermediate progenitors delaminate from the ventricular zone and colonize the adjacent region, establishing another neurogenic niche in the superficial layer of the ventricular zone, which is known as the subventricular or subependymal zone [25].

In humans, around the gestational week 10, GABAergic interneuron genesis starts in the ganglionic eminence, a transitory structure in the embryonic ventral telencephalon.

These neurons migrate tangentially from the ganglionic eminence to the dorsal telencephalon. Later on, around the 15th week of gestation, GABAergic interneurons also originate in the subventricular zone. These neurons will migrate to the cerebral cortex [26, 27].

Following on and perhaps overlapping with neurogenesis, radial glial initiate gliogenesis, giving rise to non-neuronal glial populations, such as astrocytes and oligo progenitor cells (OPCs) [28]. Afterward, around the time of birth, the radial glial differentiate into both type B cells, which are astrocytes that retain NSCs properties, and into ependymal cells that line the luminal wall of the subventricular zone [29]. Type B cells in the subependymal zone divide slowly, generating rapidly dividing intermediate progenitors that differentiate into migrating neuroblasts, which slowly proliferate and differentiate into neurons [30]. In humans, there is extensive migration of neuroblasts from the subventricular zone to cortical areas during the first months after birth. Both neurogenesis and migration of neuroblasts sharply decline with age and seems lost by adulthood [31, 32]. Nonetheless, some studies have suggested the presence of proliferating cells in the adult human subependymal zone [33-35]; therefore, the existence and the rate of human adult neurogenesis is still on debate. Single-cell transcriptomic profiling, such as single-nucleus or single-cell RNA sequencing, enables simultaneous profiling of the different cell types composing a tissue [36]. Using single-cell transcriptomics to characterize the different cell types in the adult human subventricular zone may help to answer whether there is ongoing CNS development and neurogenesis in the adult human.

CNS vascularization and blood-brain barrier establishment

The vascularization of the CNS is concomitant to neurogenesis, starting at the fourth gestational week in humans [37]. Endothelial precursors, derived from the mesodermal layer adjacent to the neural tube, are recruited through signaling factors secreted by the neuroepithelial cells, such as vascular endothelial growth factor A (VEGF-A) [38] and WNT ligands [39]. The recruited endothelial precursors form a perineural vascular plexus (PNVP) that surrounds the neural tube. Subsequently, sprouting vessels from the PNVP invade the neural tube radially from the basal surface and extend towards the ventricle. The vessels reaching the ventricle then give off branches that merge with each other, forming a capillary plexus known as the intraneural vascular plexus (INVP) [40]. Signals derived from the INVP, such as oxygen and Laminin, modulate

neurogenesis [41]. In addition, the INVP supports and guides migration of newly formed neurons in the ventricular and subventricular zone to the cerebral cortex [42].

During CNS vascularization, the endothelial cells recruit the pericytes, forming a pericyte-ensheathed vascular network. Then, the pericyte-ensheathed vascular network interacts with the neurons and the end-feet of astrocytes, forming the NVU [43]. Around gestational week 15 [44, 45], the cells of the NVU induce and modulate the expression of adherent and tight junction proteins in the CNS endothelium, forming a blood-brain barrier (BBB) that provides a dynamic interface between the CNS and the periphery [46]. This barrier restricts the passage of cells and molecules [47], conferring an immune privileged environment to the CNS.

Of note, colonization of the brain by microglia precedes cerebral vascular development and BBB formation, indicating an important role of microglia in the development of the nervous and vascular system [48-50].

Schizophrenia

The activity and performance of the brain are based on proper interaction and communication between the different types of cells that form it [51]. Thus, disturbances in brain cell interactions can lead to psychiatric diseases. Schizophrenia is a heterogeneous psychiatric disorder, affecting around 20 million people worldwide. It is characterized by positive symptoms, such as hallucinations and delusions, and negative symptoms, such as social and emotional withdrawal, and cognitive impairment [52].

The etiology of schizophrenia seems to rely on abnormal neurodevelopment, influenced by the interaction of diverse genetic and environmental factors [53]. An altered developmental trajectory may converge into vulnerable brain circuits that lead the organism prone to a higher probability of developing psychosis, resulting in the onset of schizophrenia around early adulthood [54].

In schizophrenia, alterations in nervous system functioning have been described in terms of neurogenesis [34], brain activity [55, 56], brain connectivity [57-60], and neurotransmission signalling [61]. For instance, the glutamate hypothesis of schizophrenia posits that hypo-functioning of NMDA glutamate receptors on GABAergic interneurons reduces inhibition of glutamatergic cortical pyramidal

neurons, leading to excessive glutamate release and disrupting the excitatory/inhibitory brain balance [62]. A downstream increase of glutamatergic signaling to the ventral tegmental area can induce hyper-activity of midbrain dopaminergic neurons and excessive striatal dopamine release [63], which associates with the presence of positive symptoms in patients with schizophrenia [64].

In the context of brain connectivity, functional networks involve different brain regions that exhibit correlated activity, both at rest and during cognitive tasks. This reflects that the brain regions integrating the network communicate and are, thereby, functionally connected. During rest, or in the absence of external stimuli, the human brain exhibits a particular spatial activation pattern that involves the medial prefrontal cortex, the posterior cingulate cortex and the angular gyrus. This activation pattern is conserved across different individuals and it is known as the default mode or resting-state network [65]. Compared to controls, schizophrenia patients exhibit increased activation of the resting-state functional network [65, 66], which might be a consequence of the increased excitatory/inhibitory balance associated with schizophrenia and may lead to cognitive impairment [65]. In addition, patients with schizophrenia exhibit a more restricted repertoire of different brain activity patterns during rest, indicating alterations in the dynamics of resting-state functional connectivity in schizophrenia [67].

Generally, measurements of brain activity and connectivity analyses in patients with schizophrenia are performed using functional magnetic resonance imaging (fMRI). With this technique, the hemodynamic variation in response to changes in brain activity is registered [68]. Thereby, the spatial and temporal resolution of fMRI is low, and the cellular mechanisms involved in brain functional connectivity anomalies associated with schizophrenia remain to be understood. Furthermore, most connectivity studies have been performed with adults already diagnosed with the disease; therefore, the timing of brain connectivity disturbances in schizophrenia is not entirely clear. Nonetheless, some longitudinal imaging studies indicated changes in brain connectivity before the onset of schizophrenia [69, 70], suggesting a developmental origin of brain connectivity alterations in schizophrenia.

The underlying polygenic architecture of schizophrenia, in addition to the human-specific nature of the traits associated with the disease, have precluded the design of reliable animal models for schizophrenia, limiting the study of the cellular mechanisms related to it. However, advances in stem-cell culture techniques enabled the generation

of human-induced pluripotent stem cells (hiPSCs) [71] from patients' somatic cells obtained from the skin, blood, or urine. Thereby, hiPSCs harbor the complete genetic information of the patients. [72]. Differentiation of hiPSCs into neurons allows recapitulating neurogenesis and the evolution of spontaneous network activity during brain development [73-78]. Therefore, hiPSCs represent a novel strategy to obtain patient-derived neurons and may be useful to study the cellular mechanisms and the timing of brain connectivity alterations in schizophrenia.

Besides the impairments in nervous system functioning related to schizophrenia, the largest reduction in life expectancy associated with schizophrenia comes from cardiovascular morbidity [79], illustrating the importance of the vasculature for these patients. Evidence derived from various imaging techniques indicated alterations in blood perfusion, across different brain regions, associated with schizophrenia [80-86]. In addition, *post-mortem* and *in-vivo* studies suggested a potential increase in the permeability of the BBB [34, 87-92] in patients with schizophrenia (detailed in chapters 4 and 5 of this thesis). Nonetheless, proper brain perfusion and BBB functioning involves a variety of cell types, and it is not known which of them might be particularly affected in schizophrenia. Transcriptional profiling of the different cell types comprising the NVU may help identify the cellular mechanisms contributing to brain vascular alterations in schizophrenia.

As mentioned above, the development of the nervous system and the development of the vascular system are coordinated phenomena that influence each other [38, 39, 41, 42, 46]. In addition, changes in neural activity of the already developed brain are sufficient to trigger modifications in the brain vascular network establishment and function [93, 94], suggesting that the influence of the nervous system on the brain vasculature continues beyond neurodevelopment. As a consequence, in patients with schizophrenia, alterations in the brain vasculature and nervous system functioning could share the same origin and/or could mutually influence the performance of both systems.

Alongside the mentioned potential alterations in the functioning of nervous and vascular system, a relatively consistent finding is the higher inflammatory status of the brain, seen at *post-mortem* inspections of brain tissue in schizophrenia [95-99]. This suggested the implication of the immune system in schizophrenia brain pathophysiology [96, 100, 101]. Increased acute or chronic inflammation may have

detrimental consequences on the brain vasculature of patients with schizophrenia [102]. On the other hand, intrinsic alterations in the brain vasculature and the functioning of the BBB may impair toxin efflux and facilitate the ingression of toxic material and circulating immune cells into the brain parenchyma, leading to neuroinflammation (discussed in [103-105]). Interestingly, neuroinflammation has been associated with increased glutamatergic signaling, establishing a bridge between the inflammation hypothesis and the glutamate hypothesis of schizophrenia [106]. Thus, the nervous, vascular, and immune system are strongly interconnected and all of them may contribute to the complexity of schizophrenia pathophysiology.

In summary, schizophrenia pathology seems to involve the functioning of the nervous, vascular, and immune system of the brain, which in turn are closely related from the beginning of development. Nonetheless, the specific mechanisms involved in the etiology, triggering and progression of the disease remain poorly understood. Further studying the trajectory of human neurodevelopment and the heterogeneity of the brain vasculature in schizophrenia may broaden our vision and knowledge of the different cellular and molecular mechanisms that contribute to the etiology and progression of schizophrenia.

Outline of the thesis

Schizophrenia associates with a heterogeneous pathology, which can affect the nervous, immune, and vascular system. This is possibly related to the neurodevelopmental origin of schizophrenia, which could affect diverse aspects of the developing organism. The goal of this thesis was to gain more insight into the etiology and pathophysiology of schizophrenia, as well as deepen the knowledge of human neurodevelopment and brain vasculature heterogeneity under physiological condition.

In **chapter 1**, we gave a brief description of nervous and brain vascular system development, including a presentation of the different cell types forming the brain. Then, schizophrenia was introduced, a neurodevelopmental disorder possibly involving alterations in both the nervous and vascular system. In **chapter 2**, we profiled the cellular composition of *post-mortem* human subependymal zone (SEZ), in early and middle adulthood, using snRNAseq. We found that the NSCs of the adult SEZ are in a quiescent state. Nonetheless, the expression of genes related to nervous system development was higher in early than middle adulthood, suggesting ongoing central

nervous system development in early adulthood that may decline in middle age, probably decreasing the potential to restart the cell cycle in aged NSCs. In addition, we used this dataset to identify the cell types potentially affected by the genetic variation associated with different neurodevelopmental disorders, and identified several neuronal sub-types that would likely be affected by a genetic predisposition to schizophrenia. In **chapter 3**, we modeled neurogenesis with hiPSCs and studied neuronal functional connectivity dynamics during early development of schizophrenia. We identified reduced dynamism and flexibility of functional connectivity in schizophrenia patients-derived emerging neuronal networks, which may contribute to brain functional connectivity anomalies related to schizophrenia. In **chapter 4**, recent studies addressing the status of the brain vasculature and BBB in schizophrenia were reviewed, whereas in **chapter 5**, potential alteration in the BBB of patients with schizophrenia was investigated with snRNAseq of *post-mortem* midbrain tissue. We found that transcriptional changes in the BBB of schizophrenia patients are limited and specific to the ependymal cells and pericytes, suggesting that the cell types of the BBB are not broadly affected in schizophrenia. In **chapter 6**, the findings reported in this thesis are summarized and discussed, including future prospects and possible follow-up studies.

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