On Neuroimmunology and Brain Function: Experimental and Clinical Studies

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Für Elsa, Freya und Selma

there's a whale in the moon when it's clear

Sein Blick ist vom Vorübergehn der Stäbe
so müd geworden, dass er nichts mehr hält.
Ihm ist, als ob es tausend Stäbe gäbe
und hinter tausend Stäben keine Welt.

Der weiche Gang geschmeidig starker Schritte,
der sich im allerkleinsten Kreise dreht,
ist wie ein Tanz von Kraft um eine Mitte,
in der betäubt ein großer Wille steht.

Nur manchmal schiebt der Vorhang der Pupille
sich lautlos auf –. Dann geht ein Bild hinein,
geht durch der Glieder angespannte Stille –
und hört im Herzen auf zu sein.

Rainer Maria Rilke

SAMMANFATTNING PÅ SVENSKA

Immunförsvaret har inte bara till uppgift att skydda mot infektioner utan har också visats vara viktigt för normala hjärnfunktioner och verkar dessutom vara en del i uppkomsten av sjukdomar som rör mentala funktioner. Immunsignalering i centrala nervsystemet spelar en stor roll vid anläggning och utveckling av hjärnan via samspel med bland annat neurotransmittorer, neuroendokrina hormoner, cytokiner och deras respektive receptorer. Att utforska samspelet mellan hjärna, immunförsvar och beteende kan bidra till ökad insikt kring orsaker till uppkomsten av psykiska sjukdomar, samt ge uppslag till behandling av dessa.

Syftet med denna avhandling var att undersöka immunförsvarets roll i dels en djurmodell som används för studier av depression och dels i två kliniska studier rörande autismliknande personlighetsdrag samt bipolär sjukdom.

I djurförsöken användes råttor av typen Flinders sensitive line (FSL), som utgör en genetisk modell där djuren uppvisar ett depressionsliknande beteende. Avsikten var att studera uttrycket av gener i hjärnan med betydelse för immunsystemet efter aktivering av kroppens immunförsvar (Artikel I) och efter behandling med ett antidepressivt läkemedel (Artikel II). Några av dessa gener uttrycktes i en lägre grad hos råttorna jämfört med vanliga kontrolldjur, ett fynd som vi sedan kunde upprepa i nya försök. Aktivering av immunförsvaret gav också upphov till förändringar i uttrycket av vissa immunrelaterade gener i hjärnan. Även antidepressiv behandling med läkemedlet escitalopram förändrade uttrycket av vissa gener, i synnerhet S100B och serotoninreceptor 2A, i hjärndelarna amygdala och hypothalamus som anses vara av betydelse för depression. Denna djurmodell tycks således vara användbar för att studera mekanismer som sammanlänkar immunsystemet med depressionsliknande beteende samt effekten av antidepressiv behandling.

I de kliniska studierna i detta arbete studerade vi huruvida varianter i immunförsvarsrelaterade gener är associerade med psykiatriska tillstånd och volym av olika hjärndelar. Exempelvis såg vi att variationer i genen som kodar för NF-kB inhibitor-like protein 1 (*NFKBIL1*) var associerade med autism-liknande personlighetsdrag och språksvårigheter (Artikel III). Vidare undersökte vi sambandet mellan varianter av genen som kodar för cytokinen interleukin 1beta (IL-1beta) och volym av olika hjärnregioner hos patienter med bipolär sjukdom och friska kontroller (Artikel IV). Vi fann här ingen genetisk skillnad mellan patienter och kontroller. Däremot påvisades ett samband mellan genvariant och volym av putamen i vänstra hjärnhalvan hos både patienter och kontroller, vilket kan tolkas som att IL-1beta är inblandad i utveckling av nervsystemet.

Sammanfattningsvis redovisas i denna avhandling samband mellan komponenter i immunförsvaret och psykiatriska tillstånd samt förändrad utveckling av nervsystemet, vilket ytterligare understryker immunförsvarets roll för mentala funktioner.

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ABSTRACT

The immune system has been implicated in the mechanisms underlying many psychiatric disorders. Immune mediators are expressed in the central nervous system (CNS) not only in response to harmful stimuli, but also in a constitutive manner, and serve as important plasticity factors during development. There is complex bidirectional communication between the immune system and the CNS throughout life which is based on interactions between neurotransmitters, neuroendocrine hormones, cytokines, and their respective receptors. Exploring the interplay between brain, behaviour and immunity is central to our understanding of the pathology of psychiatric morbidity.

The aim of this thesis is to investigate the role of some aspects of the immune system in several psychiatric conditions, both in experimental and clinical contexts.

We used the Flinders sensitive line (FSL), a genetic animal model of depression, to study central gene expression of markers related to immune response and neurotransmission following immune stimulation and antidepressant treatment. Several genes were found to be expressed differently in rats displaying depressive-like behaviour compared to their controls (Paper I), a finding that we replicated in Paper II. Additionally, we showed that antidepressant treatment with escitalopram altered expression of several genes, notably the astrocyte-derived protein S100B, and the serotonin receptor 5-HT_{2A}, in the amygdala and hypothalamus (Paper II), two brain regions that have been shown to be of relevance for the effect of antidepressant treatment. Our results support the use of the FSL model for studying the role of these immune-related markers in depression and antidepressant treatment.

In the clinical studies included in this thesis, we found that genetic variants in immune-related genes were associated with neuropsychiatric traits and the volume of certain brain regions. The gene encoding the NF-kB inhibitor-like protein 1 (*NFKBIL1*) was found to be associated with autistic-like traits, as well as with language impairment in a cohort from the general population (Paper III). We further investigated the effect of genetic variation in the gene coding for interleukin-1beta (*IL1B*) on the volume of several brain regions in a case-control population of patients diagnosed with bipolar disorder (Paper IV). Genotype distribution did not differ between patients and controls, suggesting that variants in *IL1B* may not be associated with bipolar disorder. However, we found associations between *IL1B* polymorphisms and the volume of the putamen in the left hemisphere in patients and controls, suggesting that genetic variation in *IL1B* may influence neurodevelopment.

In conclusion, this thesis demonstrates associations between immune mediators and mental functions, as well as altered brain development in humans. Also, insight is gained into the use of the FSL animal model for investigating the impact of the immune system for depression. Taken together, our findings confirm the importance of the immune system for the development of psychiatric disorders.

Keywords: immune system, mental disorders, polymorphisms, depression, autism, bipolar disorder, SSRI, S100B, 5-HTR2A, Flinders sensitive line

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Nina Strenn**, Petra Suchankova, Staffan Nilsson, Christina Fischer, Gregers Wegener, Aleksander A. Mathé, Agneta Ekman. 2015. Expression of inflammatory markers in a genetic rodent model of depression. *Behavioural Brain Research* 281:348-357

II. **Nina Strenn**, Gregers Wegener, Christina Fischer, Staffan Nilsson, Agneta Ekman. Effects of chronic escitalopram treatment on the expression of inflammatory markers in the Flinders rat model of depression. *Manuscript*.

III. **Nina Strenn**, Daniel Hovey, Lina Jonsson, Henrik Anckarsäter, Sebastian Lundström, Paul Lichtenstein, and Agneta Ekman. Associations between autistic-like traits and polymorphisms in *NFKBIL1*. *Acta Neuropsychiatrica 2019. Manuscript in press. https://doi.org/10.1017/neu.2019.18*

IV. **Nina Strenn**, Erik Pålsson, Benny Liberg, Mikael Landén, Agneta Ekman. Influence of variations in *IL1B* on brain region volumes in bipolar patients and controls. M*anuscript*.

CONTENT

ABBREVIATIONS

A	Adenine
ALT	Autistic-like trait
ASD	Autism spectrum disorder
BBB	Blood brain barrier
C	Cytosine
C3	Complement component 3
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVOs	Circumventricular organs
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
FRL	Flinders resistant line
FSL	Flinders sensitive line
G	Guanine
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary adrenal
5-HT	5-hydroxytryptamin (serotonin)
5-HTR	5-hydroxytryptamine (serotonin) receptor
i.p.	Intraperitoneal
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MET	MET receptor tyrosine kinase
MDD	Major depressive disorder
MHC	Major histocompatibility complex
MMP	Matrix-metalloproteinase

MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor kappa B
NFkBIL	NFkB inhibitor-like protein
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PDD	Pervasive developmental disorder
PDD-NOS	Pervasive developmental disorders not otherwise specified
PFC	Prefrontal cortex
RNA	Ribonucleic acid
SSRI	Selective serotonin reuptake inhibitor
T	Thymine
TIMP	Tissue inhibitor of matrix-metalloproteinases
TNF	Tumor necrosis factor

INTRODUCTION

The distinction between mind and body, and thus between mental and physical health, is a philosophical question that humans have contemplated for thousands of years. The father of modern medicine, Hippocrates, developed the theory of the four humors – blood, yellow bile, black bile, and phlegm – hypothesizing that the relative proportions of these substances regulate human temperament and behaviour, and that their correct balance defines "health". However, more than 2000 years later the neurobiology of emotions and behaviour is far from understood, and there are still many who disregard that disorders of thought, behaviour, or mood have a biological explanation.

One of the first successful pharmacological treatments was of an illness that was then believed to be purely psychiatric, i.e., the treatment of *paralytic dementia*, also known as *general paralysis of the insane*. The progressive course of the disease went from symptoms of mania and euphoria to seizures, cognitive decline and dementia, ultimately leading to paralysis and death, and it affected more than a tenth of all institutionalized psychiatric patients at the turn of the last century [1]. It was later found to be due to chronic meningoencephalitis caused by infection with the bacterium *Treponema pallidum*, also known as syphilis, and successfully treated by the antibiotic penicillin. The increasing knowledge about etiology and pathophysiology of mental disorders will hopefully continue to give rise to preventive strategies and better treatment, and help to relieve us further from the burden they impose.

Our behaviour is determinded by (i) our genes, which form the basis for our mental functions, and (ii) the environment we are exposed to, which determines how that basis is used. Not even identical twins, which have the exact same genotype, think and behave entirely alike, due to differences during embryonic and adult environments. The immune system plays a pivotal role during neurodevelopment and can affect it on several levels; differences in the deoxyribonucleic acid (DNA) coding for immune-related genes, as well as pre- and postnatal immune activation have the ability to alter the structure and

function of the brain and subsequently cause changes in the way we think and feel [2].

THE IMMUNE SYSTEM

Our immune system defends us against a wide variety of harmful organisms, such as pathogenic bacteria, viruses and parasites, and in order to do so, it has to distinguish between foreign material and our own healthy tissue. An imbalance of the immune system can lead to disorders such as immunodeficiencies, where the immune system is less active than normal and thus leaves us susceptible to life-threatening infections; autoimmunity, where an overactive immune system attacks healthy tissues; and inflammatory diseases. The immune system consists of two components in which different cells and molecules work cooperatively to provide host defense and to restore homeostasis. These two components are the innate and the adaptive immune responses.

Innate and adaptive immune responses

The first line of defense against pathogens is provided by the evolutionarily older *innate* immune response. It consists of physical and chemical barriers, including skin, mucosa, antimicrobial molecules (blood proteins such as complement components), and cellular defense mediated through phagocytes (macrophages and neutrophils) and natural killer cells. It specifically targets molecules shared by pathogens such as the bacterial carbohydrate lipopolysaccharide (LPS), and molecules released by damaged host cells. Its diversity is limited, as it is germline encoded and not able to "learn", but it is highly important for the organism, as it is the only immediate protection against pathogens and tissue injury before the adaptive immune response is activated.

The *adaptive* immune system evolved in early vertebrates and is antigen-specific. It requires recognition of specific "non-self" antigens during a process called antigen presentation. There are two types of adaptive immune responses, called

humoral immunity and cell-mediated immunity, which differ in their components and eliminate different types of microbes. Humoral immunity is mediated by antibodies that are produced by B-lymphocytes and is the principle defense mechanism against extracellular microbes and their toxins. The antigen binding fragments of antibodies have a large diversity as they are produced by somatic recombination of their gene segments. Furthermore, the adaptive immune response has a memory function, and is able to remember if it had been presented to a specific antigen before, and if so, can augment the reaction. Cell-mediated immunity is mediated by T-lymphocytes and targets intracellular microbes such as viruses and some bacteria that are inaccessible to circulating antibodies.

Inflammation

Inflammation is an essential response to harmful stimuli such as infection and tissue damage, and is part of the innate immune response. It can be classified as either acute or chronic.

The cardinal symptoms of *acute* inflammation are *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function). Immune molecules and cells (predominantly neutrophils) are recruited to sites of infection or tissue damage in order to eliminate the cause of the inflammatory reaction, and an inflammatory cascade is initiated via the secretion of mediators including cytokines, chemokines, histamines, and complement factors. Neutrophils are activated and start attacking the pathogen by releasing toxic effector molecules. However, these do not distinguish between pathogen and healthy host tissue, which gets damaged in the process. After elimination of the pathogen, further immune cells such as macrophages are recruited to remove cell debris and restore the tissue at the site of inflammation. For restoration to happen, an important switch in secretion from pro-inflammatory mediators to anti-inflammatory ones must take place. Instead of neutrophils, monocytes are then recruited to the tissue, which facilitate the removal of dead cells and initiate tissue remodeling.

While *acute inflammation* is vital to our survival, leading to destruction of invading pathogens and initiating cell recovery, prolonged inflammation known as *chronic inflammation* does not seem to serve a protective function. It is characterized by simultaneous inflammation and repair, and does not appear to be caused by the classic instigators of inflammation—infection and injury—but instead by a homeostatic imbalance that is not directly related to fighting infection or tissue repair. This can happen when the acute inflammatory response was not strong enough and fails to eliminate the irritant, when the persistent irritant is not large enough to elicit an acute inflammatory response, or by autoimmune reactions [3]. The symptoms are much less severe than during acute inflammation but can still cause harm. Chronic inflammation has been recognized as being involved in a wide variety of diseases including cardiovascular diseases [4], diabetes [5] and neurodegenerative diseases [6]. Furthermore, mounting evidence is pointing to a role of inflammation in e.g. neuropsychiatric disorders, as will be discussed in more detail later.

Cytokines

Cytokines are a vast and heterogenous group of small proteins that are secreted by a wide range of immune and non-immune cells. They are of importance in the transmission of information between the immune system, the endocrine system, and the nervous system, and they are involved in both innate and adaptive immune responses via *autocrine* signaling (binding to receptors on the same cell as part of autoregulatory mechanisms), *paracrine* signaling (binding to and inducing changes in nearby cells) and *endocrine* signaling (being secreted to the circulatory system and affecting distant target cells). Cytokines regulate various biological processes and include chemokines, interferons, interleukins (ILs), lymphokines, and tumor necrosis factors (TNFs), that are generally grouped into pro- or anti-inflammatory cytokines. Pro-inflammatory cytokines (e.g. IL-1b, IL-6 and TNF) augment inflammatory responses, while anti-inflammatory cytokines (e.g. IL-4) dampen inflammation, and together they orchestrate initiation, maintenance, and termination of inflammation.

Cytokines are not stored in the cells that secrete them but their synthesis is initiated in response to an external signal, e.g. binding of a ligand to a cell surface receptor that leads to a signaling cascade resulting in the nuclear translocation of transcription factors such as nuclear factor kappa-B (NF-κB), which in turn activates expression of a plethora of inflammatory mediators. Newly transcribed messenger ribonucleic acid (mRNA) of cytokines is often unstable or rapidly degraded, and may require transcriptional or translational processing, like proteolytic cleavage of an inactive precursor molecule into an active product. This shows that cytokine expression is a highly regulated and transient process, but once synthesized, they are rapidly secreted, resulting in a burst of release when needed.

NEUROIMMUNOLOGY

The central nervous system (CNS) has traditionally been viewed as an "immune-privileged" area due to the existence of the blood brain barrier (BBB). The BBB was believed to make the CNS inaccessible to the immune system, with the exception occurring only during disease states, and the two systems were thought to be functionally independent of each other. This notion has been successfully challenged over the last decades. Advances in the field of neuroimmunology have shown that there is a complex bidirectional communication between the CNS and the immune system via multiple neuro-immune pathways, and although the immune-privilege does exist, it is relative.

The blood-brain barrier

The BBB is a complex structure which is built up of several components. The predominant barrier is the vascular barrier, which is composed of endothelial cells, forming elaborate tight junctions and preventing diffusion. Some molecules can be carried from the blood through the endothelium into the cerebrospinal fluid (CSF) via e.g., transport molecules or vesicular transport. The basement membrane forms the second barrier. Its charged pores function as a molecular sieve, either repelling or binding charged molecules. Astrocytes form a layer around the basement membrane and maintain a constant ion-concentration of the extracellular matrix, which is crucial for the electrical activity of neurons and their axons. Furthermore, perivascular macrophages and microglia cells patrol the area surrounding the BBB. If foreign material manages to pass through into the brain, it is taken up and degraded by them.

Neuro-immune pathways

The notion that the immune system and the CNS function in close association with each other was proposed decades ago by Besedovsky and Sorkin [7], but the molecular pathways underlying the intricate communication between the two systems took longer to elucidate.

There are several pathways in which the immune system can relay signals to the brain (*Figure 1*). The first one to be discovered was the *humoral pathway*; although cytokines are too large to pass the BBB freely, there are several proposed ways how they may affect the brain. Slowly diffusing cytokines, including IL-1beta, may pass through leaky regions of the BBB such as the choroid plexus and the circumventricular organs (CVOs) [8]. Brain endothelial cells and perivascular macrophages that line the cerebral vasculature may produce cytokines and other inflammatory mediators such as prostaglandins and nitric oxide (NO) in response to circulating cytokines or pathogen-associated molecular patterns, and then release them into the brain parenchyma [9]. Cytokines and even cells from the periphery can also enter directly into the CNS; cytokines may do so via active transport through saturable transport molecules [10], and activated microglia may recruit peripheral monocytes to the brain via production of monocyte chemoattractants [11], also referred to as the *cellular pathway*. In the *neural pathway*, the peripheral immune signal may be relayed to the brain by afferent nerves, e.g. the vagus nerve. These nerves express cytokine receptors which peripheral cytokines such as IL-1, IL-6 and TNF bind to, and the afferent nerves communicate the cytokine signals to relevant brain regions such as the hypothalamus and the amygdala [8,12]. Once cytokine signals reach the CNS, there is a vast network of cells that express cytokines and their receptors, including microglia, astrocytes and neurons [9]. These cells can cause a cytokine cascade within the CNS and may affect sleep, temperature regulation, food intake, cognition, and behaviour through alterations in neurotransmitter metabolism, neuroendocine function, and neural plasticity.

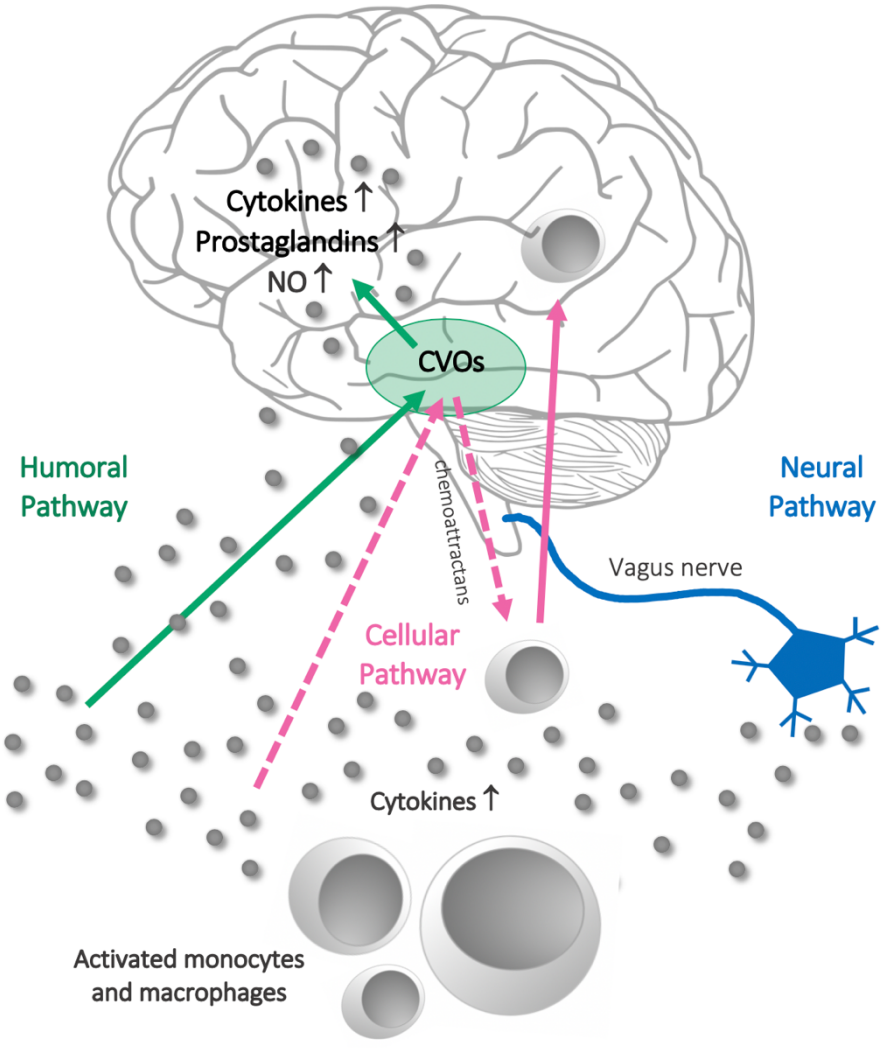

Figure 1. Representation of communication pathways from the peripheral immune system to the brain. Diffusing cytokines released from activated monocytes and macrophages access the brain at leaky regions of the BBB such as the circumventricular organs (CVOs), where they stimulate endothelial cells to release second messengers, e.g., more cytokines, prostaglandins, or nitric oxide (NO) into the brain parenchyma, called the humoral pathway (green). In the cellular pathway (pink) pro-inflammatory cytokines activate microglia to produce chemoattractants, which in turn recruit monocytes from the periphery into the brain. Cytokines can also bind to cytokine receptors on afferent nerve fibers in the vagus nerve, which relay the information to other neural pathways (blue).

There is also brain-to-immune signaling, where the nervous system regulates the immune system. It can do so through neuroendocrine peptide hormones, activation of the sympathetic nerves innervating lymphoid organs, or activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of anti-inflammatory glucocorticoids [9], and all of these can have widespread effects on the immune system. A mechanism known as the inflammatory reflex, proposed and revised by Tracey et al. [13], states that the CNS reflexively regulates the inflammatory response, just as it controls other vital functions such as heart rate. Pro-inflammatory cytokines released by activated innate immune cells (e.g. macrophages) stimulate afferent vagus fibers, which leads to activation of the parasympathetic brainstem regions and the subsequent signaling in efferent vagus fibers in a reflex-like manner. This in turn activates the (sympathetic) splenic nerves, which results in recruitment of acetylcholine-producing T-cells that downregulate inflammation by interacting with cholinergic receptors on macrophages, causing inhibition of cytokine production [14].

Immune mediators in the brain

Cells in the CNS can be divided into neuronal cells and non-neuronal cells, called glia. Glia cells are responsible for homeostasis, myelination, support and protection for neurons, and include microglia, ependymal cells, and the macroglial cells oligodendrocytes and astrocytes. Below, two cell types relevant for this thesis are highlighted.

Astrocytes

Astrocytes are large star-shaped cells that are structurally and functionally associated with neurons and the cerebral microvasculature. They are derived from progenitor cells in the neuroepithelium of the developing CNS, and the genetic mechanisms of astrocytic cell differentiation are similar to those of neurons [15]. Although they are unable to generate action-potentials, they can propagate intercellular Ca^{2+} waves over long distances in response to stimulation, and, similar to neurons, release transmitters in a Ca^{2+} dependent manner. Amongst the glial transmitters that are released by astrocytes are e.g.

classical transmitters (e.g. glutamate), chemokines, cytokines (e.g. TNF), peptides, and the Ca^{2+} binding protein S100B [16]. Apart from their important roles in the maintenance of neural circuits, astrocytes are also modulators of synaptic transmission, and astrocyte dysfunction has been implicated in neurodevelopmental disorders such as autism [17].

Microglia

Microglia are the resident macrophages of the CNS. These immunocompetent cells are derived from yolk sac primitive macrophages during early development, and persist by self-renewal in the CNS. Microglia can be in a ramified "resting state" and an amoeboid "active state". Depending on the type and duration of the activating stimulus, microglia can transform into different phenotypes, including pro-inflammatory (M1) and anti-inflammatory (M2) states [18]. Upon activation, they proliferate and migrate to the site of injury, where they can destroy invading pathogens, remove debris, and promote tissue repair by secreting growth factors, and thus protect the CNS from potentially fatal damage and restore tissue integrity.

In the resting state, however, microglia are far from inactive. They are constantly surveying their surrounding microenvironment for pathogens with their highly branched processes, and their function is modulated by neuronal activity and astrocytes [19]. They are required for the formation of mature synapses during embryogenesis [20] and regulate adult neurogenesis [21]. Microglia have been shown to play an important role in the activity-dependent elimination or pruning of inactive synapses, and thereby the formation of mature neural circuits. They make use of several signaling mechanisms, including chemokines and chemotaxis [20], as well as the classic complement cascade that is part of the innate immune system [22]. Complement proteins such as complement component 3 (C3) bind to immature synapses, being an important part of the developmental pruning of nonfunctional synapses. During the period of synaptic elimination microglia upregulate the expression of receptors that bind to these complement proteins, leading to engulfment and elimination of inactive synapses. Disruption

of the microglia-specific C3 signaling results in reduced microglia phagocytic function and sustained deficits in synaptic connectivity [22].

Microglia and associated cytokines have received a great deal of attention in research regarding depression [23] and have been suggested as a promising target for depression treatment strategies [24]. Increased microglial density has been found in several brain regions of suicide victims diagnosed with depression, bipolar disorder, and schizophrenia [25]. Also, enhanced microglia activation during a major depressive episode has been reported in a positron emission tomography study [26]. Microglia have been implicated as a link between maternal immune activation and disturbed fetal neurodevelopment [27]. Nevertheless, their exact role in the pathophysiology of psychiatric disorders remains unknown.

Brain development

The development of the CNS is a complex series of dynamic and plastic processes. These are under highly constrained genetic regulation in a constantly changing environment. Development of the CNS begins in the third week after conception with the differentiation of neural progenitor cells and extends beyond adolescence [28]. Both genetic factors and environmental input are fundamental for normal brain development, and disruption of either can alter neural outcomes to great extent.

By the end of the embryonic period (i.e. nine weeks after conception) the fundamental structures of the brain have been formed. These will later become the cerebral hemispheres, diencephalon, cerebellum and brain stem. During fetal development (i.e. week 9 until birth) there is rapid growth and specialization of both cortical and subcortical structures, and the rudiments of the major fiber pathways are formed [29]. Neurons are produced from six weeks after conception to midgestation, and immediately migrate to different brain areas where they begin to make connections with other neurons, establishing basic neural networks. Brain development continues after birth, with the brain increasing its size rapidly. Structural as well as functional changes in both grey matter (e.g. cell bodies and glia cells) and white matter (i.e. axons with myelin

sheaths) continue throughout childhood and adolescence and are reflected by changes in e.g. cognition and behaviour.

Healthy brain development does not exclusively mean proliferation and growth; there are two important processes that are essential for CNS development that involve substantial loss of neural elements; naturally occurring cell death in neural populations, and massive overproduction of synaptic connections followed by the systemic elimination of up to 50% of these connections. While neuronal cell death occurs mostly prenatally, natural cell death of glia populations, as well as proliferation followed by pruning of synapses are largely postnatal events [28]. There are far more connections during the early postnatal period compared to the adult brain, and this excessive connectivity is carefully pruned back via competitive processes that are influenced by an individual's genetic make-up, as well as their environment and experiences. This also means that the developing brain is highly sensitive to both endogenous and exogenous signals. Experiences during the pre- and postnatal period, including nutrition, trauma, stress and infection, have been strongly connected to alterations in neural circuits and associated behavioural outcomes [30–33].

Many components of the immune system have been found to play important roles during CNS development [34], including cytokines, complement factors, and members of the major histocompatibility complex class I (MHCI) and their receptors. This is not surprising, as accumulating evidence indicates a pleiotropic nature of proteins, that is to say that the same protein can have multiple, and paradoxically unrelated functions, within and between systems [34]. Many proteins first discovered as a part of the immune system have now been discovered in the healthy CNS where they have non-immune functions. Hence, a vast molecular repertoire seems to be shared by the nervous and the immune systems [35].

Although little is known about the exact role of immune mediators in brain development as of yet, animal studies have uncovered several examples supporting varied roles for these proteins, with microglia playing an important part; differentiation of progenitor cells into neurons and glia is dependent upon

local factors and intrinsic signals, such as IL-1, which is produced by developing microglia [36]. During further development, microglia produce cytokines (including IL-1beta and TNF) which are important for ongoing neurogenesis within the developing brain, as well as chemokines that guide axons of new neurons via chemoattraction toward their new synaptic targets [37]. TNF and MHC class I molecules have been suggested to be important for the strengthening of new synaptic connections within the brain [38,39]. Finally, in the later stages of neurodevelopment, abundant or inappropriate synaptic connections are eliminated (synaptic pruning) and phagocytosed by microglia, as immune proteins such as C3 tag synapses for elimination during this process[40].

Neural plasticity

Neural plasticity is the ability of the nervous system to change throughout an individual's life, i.e. to modify itself, functionally and structurally, in response to changing environment, aging, or pathological insult. It is a key component of development and normal functioning of the brain, and responsive to experience and insult. In addition, it is necessary not only for neural networks to acquire new functional properties, but also for them to remain robust and stable.

Nearly all neurological and psychiatric disorders have been associated with changes in neural plasticity, including reductions of adult hippocampal neurogenesis, diminished cortical dendritic arbors, deficits in long-term potentiation (LTP) and impaired synaptogenesis [41]. Disruptions in neural plasticity have also been shown in depression, and modulation of neuronal adaptation has been implicated in the treatment actions of antidepressants [42]. The immune system plays a central role in various mechanisms underlying neural plasticity, both by communication pathways from the peripheral immune system to the brain and by signals produced by immune-competent cells within the CNS, involving neuro–glial communication [19].

Under normal conditions, immune mechanisms positively regulate neural plasticity and neurogenesis, promoting learning, memory, and hippocampal

LTP. These beneficial effects of the immune system are mediated by interactions between the cellular and the non-cellular components. The cellular components include microglia, astrocytes, neurons, and peripheral immune cells such as T cells and macrophages [19]. Their interplay involves the responsiveness of non-neuronal cells to classical neurotransmitters (e.g., glutamate and monoamines) and hormones (e.g., glucocorticoids), as well as the secretion and responsiveness of neurons and glia to low levels of inflammatory cytokines, such as IL-1beta, IL-6, and TNF, along with other mediators, such as complement factors [43], prostaglandins and neurotrophins [44]. Under inflammatory conditions, the delicate physiological balance between immune and neural processes is disrupted; immune-competent cells within the brain parenchyma become activated and express high levels of pro-inflammatory cytokines and prostaglandins, which may lead to impairments in neural plasticity [19].

In this thesis we studied the Ca^{2+}-binding protein S100B, which has also been implicated in neural plasticity [45]. S100B is mainly produced by astrocytes in the brain, and has both intracellular and extracellular functions, which are dependent on the concentration of this protein [46]; while exerting neurotrophic effects at very low concentrations, it has been shown to have detrimental effects at high concentrations [47]. A study in mice showed that overexpression of S100B resulted in activation of microglia and decreased numbers of astrocytes, as well as changes in hippocampal serotonin innervation [48].

Neurotransmission

Neurotransmission is the process where signaling molecules called neurotransmitters are released from *presynaptic* neurons and bind to receptors on *postsynaptic* cells in order to translate an electrical signal to a chemical. Released neurotransmitters may also activate *autoreceptors* in a feedback-manner among many. In that way, neurons can "talk" to each other and relay information.

Several neurotransmitter systems have been implicated in neuropsychiatric conditions and mood disorders. The one most important for this thesis, and

one of the most excessively studied neurotransmitters in depression, is serotonin[49]. Serotonin, or 5-hydroxytryptamine (5-HT), is highly abundant in the periphery. In the CNS it is only released by serotonergic neurons, which have their cell bodies in the raphe nuclei of the brain stem. These neurons have projections to many different brain regions, including the amygdala, hippocampus, hypothalamus, prefrontal cortex (PFC), and septum. As the widely used antidepressants selective serotonin reuptake inhibitors (SSRIs) increase extracellular serotonin levels by blocking its reuptake from the synaptic cleft back into the presynaptic neuron, mood disorders have long been viewed as consequences of low serotonin levels. The mechanisms by which serotonin regulates mood appear a lot more complex however.

There are several mechanisms by which the immune system has been shown to affect neurotransmission [50]. One example is the activation of the indoleamine-2,3-dioxygenase (IDO) enzyme [51]. IDO is an enzyme expressed in multiple cell types, including macrophages, dendritic cells, microglia, astrocytes, and neurons[52]. The enzyme catabolizes tryptophan, the primary amino-acid precursor of serotonin, into kynurenine, thereby reducing the availability of tryptophan for serotonin synthesis. Pro-inflammatory cytokines have been shown to activate IDO, thereby causing lower levels of serotonin [51].

NEUROIMMUNOLOGY AND PSYCHIATRIC DISORDERS

According to the World Health Organization, "Mental disorders comprise a broad range of problems, with different symptoms. However, they are generally characterized by some combination of abnormal thoughts, emotions, behaviour and relationships with others" [53]. The symptoms in mental disorders can be divided into following categories:

- Emotion (e.g., sadness, irritability, euphoria)
- Somatic (e.g., sleep disturbance, fatigue, headache, pain)
- Behaviour (e.g., tics, agitation, motor slowing)
- Perception (e.g., hallucinations, delusions)
- Cognition (e.g., memory impairment, inattention)

Different types of mental disorders are characterized by a combination of symptoms from most, if not all, of these categories. Most mental disorders are very heterogenous; individuals diagnosed with the same psychiatric condition often display a different combination of symptoms, and the same symptoms can be due to different mental illnesses. Genetic studies suggest high heritability (i.e., the inherited contribution of genetic variance to trait variance) for many mental disorders [54], and several studies have implicated a polygenic overlap in a range of neuropsychiatric and mood disorders, including unipolar and bipolar depression, schizophrenia, and autism [55,56], suggesting shared etiology. These conditions often show nonspecific psychiatric symptoms that cross diagnostic boundaries, including behavioural abnormalities, intellectual disability, mood and anxiety, attention deficit, impulse control deficit, and psychosis [57]. Another common denominator for various mental disorders is aberrations of the immune system; increased levels of pro-inflammatory cytokines, such as IL-1beta, IL-6 and TNF have been reported in patients with schizophrenia, bipolar disorder, and depression [58], and pathway analysis by the Psychiatric Genomics Consortium identified histone modifications, synaptic density, and immune and neuronal signaling pathways in common for schizophrenia, depression and bipolar disorder [59]. Further, genetic correlations between immune-related

disorders and a number of psychiatric conditions have been reported in a recent study combining data from several GWAS [60].

Considering the highly sophisticated processes involved in brain development, any small disturbance may lead to alterations of the developing architecture and function. Infection with the Zika virus has been linked to microcephaly and other serious brain anomalies [61], demonstrating the detrimental effects that infections during pregnancy can have. However, these changes can also be more subtle, leading to neurodevelopmental disorders that are noted early in life, as well as mood disorders such as depression that may not be apparent until adulthood. An increasing body of evidence is connecting maternal immune activation with a broad spectrum of CNS disorders in humans [31,32,62]. Possibly, it is not the specific pathogen causing an infection that determines the neurological and cognitive outcome in the offspring; the diversity of causes associated with increased risks of mental disorders suggests that general immune activation during gestation, rather than the type of pathogen, is associated with disturbances of fetal brain development causing debilitating effects later in life. This notion is supported by a recent study that associated fetal exposure to any maternal infection with increased risk of autism or depression diagnosis in the child [62].

There are various ways in which changes in the immune system may lead to altered neurodevelopment and/or mental disorders (*Figure 2*). Maternal risk factors such as infection [62], psychosocial stress [63], genetic predisposition [64] and autoimmunity [65] can cause immune activation and changes in expression of cytokines, auto-antibodies against fetal proteins and/or other immune-related genes. This may lead to increased amounts of maternal markers crossing the placenta, including cytokines, immune cells, and stress hormones [31], possibly causing effects on the fetus such as aberrant development of the immune, neurotransmitter, and endocrine systems (e.g. altered stress response).

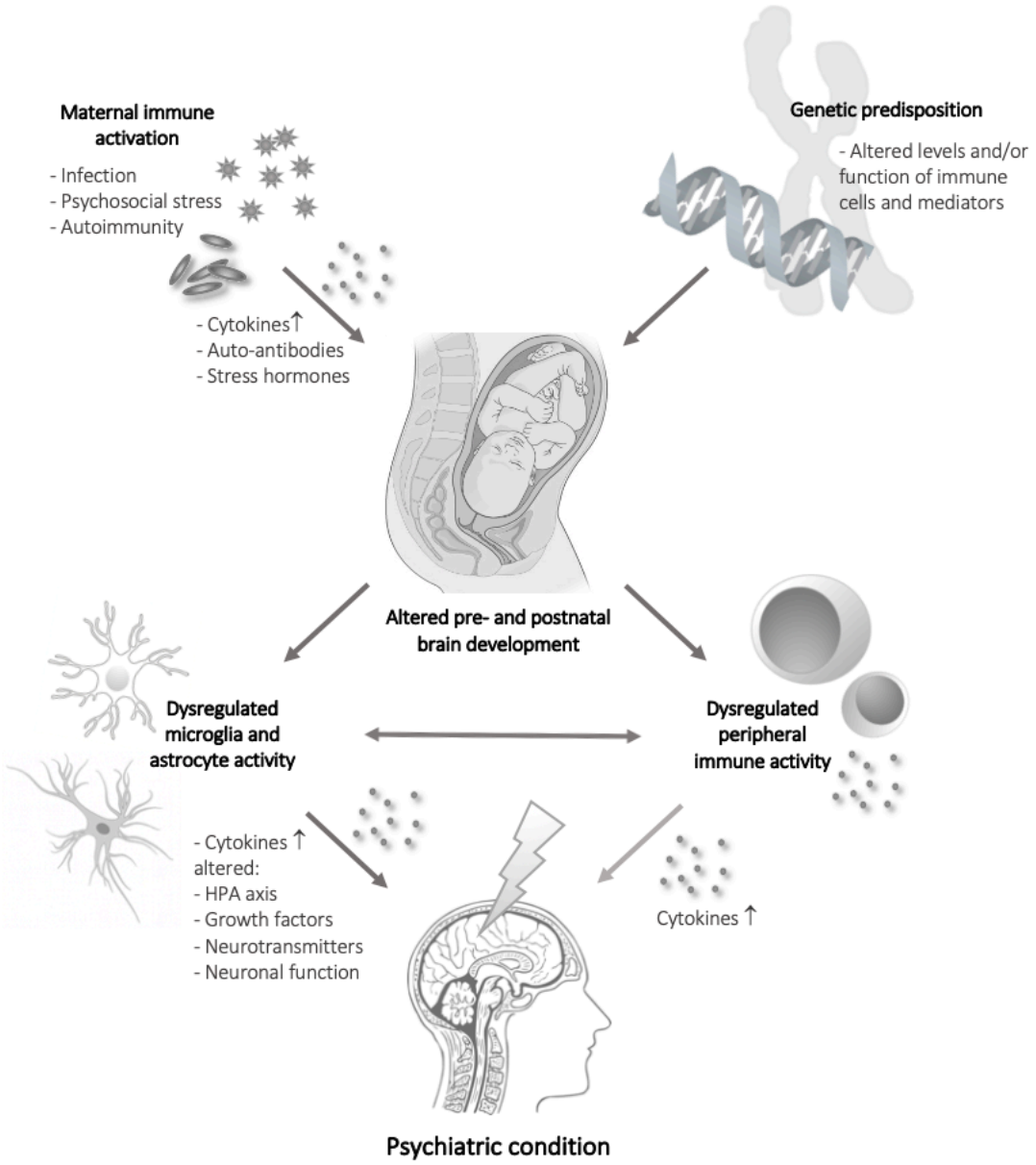

Figure 2. Schematic representation of potential pathways linking a dysregulated immune system to the etiopathogenesis of mental disorders.

Also cellular components of the CNS may be affected during development, including alterations of neurogenesis, (re)myelination, synaptic functions and/or brain homeostasis via astrocyte dysfunction [64].

However, immune challenges during fetal development are not the only way the immune system may cause psychiatric symptoms; infection with streptococcal bacteria during childhood has been shown to elicit a rapid onset of psychiatric symptoms in a subset of children, including symptoms of obsessive compulsive disorder and tic-disorders, which could be relieved by e.g. antibiotic treatment[66]. This implicates also gene-environment interactions, i.e., that given a genetic vulnerability, exposure to certain environmental risk factors will increase the risk for disorder development. Genetic factors and/or prenatal immune challenges may be initial factors, increasing the susceptibility for "second hits", such as immune challenges or psychosocial stress during childhood or adolescence, ultimately leading to changes in cognition and/or behaviour.

Major Depressive Disorder

Affecting more than 300 million people worldwide, major depressive disorder (MDD; in this thesis interchangeably referred to as depression) is among the leading causes of disability worldwide, with a lifetime prevalence of at least 16%[67]; yet the underlying mechanisms are far from understood. The symptoms include depressed mood, anhedonia (the inability to feel joy), feelings of worthlessness and inappropriate guilt, anxiety and recurrent thoughts of death and suicide, as well as psychomotor retardation, sleep disturbance and weight loss (for DSM-5 diagnostic criteria see Supplementary Table A) [68]. The high mortality rate in patients with depression is not only caused by suicide, but also due to an increased risk for a number of other conditions, such as cardiovascular diseases and diabetes [5,69].

Structural changes have been reported in several brain areas of depressed patients, including volumetric reductions of the hippocampus, basal ganglia and cortical regions are consistently found in depressed patients [70], with severity of depression being associated with greater impact on regional brain volumes. Changes in the amygdala have also been observed, but are more ambiguous than the changes in the aforementioned structures; while amygdala volumes earlier in the course of illness tend to be enlarged, a longer illness duration and severity tend to show volumetric reductions [70].

Even though there are different pharmacological treatments available, not all patients can be treated successfully. Many of the current treatments for depression have in common the increase of neurotransmission at central serotonergic or noradrenergic synapses, leading to the *monoamine hypothesis of depression*, which suggests that depression is a direct consequence of an imbalance in monoamine neurotransmitters [71]. Serotonin belongs to the monoamine family of neurotransmitters and is derived from the amino acid tryptophan. In the CNS, serotonin-containing neurons are clustered within the nine raphe nuclei, each of which projects to a different region of the brain. Amongst the most commonly prescribed antidepressants are SSRIs. As the name indicates, they inhibit the reuptake of serotonin from the synaptic cleft, thereby

increasing the availability of the neurotransmitter. Although the increase in serotonin levels is immediate [72], their therapeutic effects develop slowly, and it can take up to several weeks until patients feel relief from their symptoms [73]. Therefore, the mechanism of action that mediates antidepressant effects is not believed to be due to the immediate elevation of extracellular serotonin but rather due to structural or functional adaptations of the CNS to chronically elevated serotonin levels. An increasing amount of evidence has led to several other hypotheses regarding the etiology of depression, involving the hypothalamic-pituitary-adrenal (HPA) axis and stress response, as well as inflammation and the immune system [51].

Genetics and environment

With a heritability estimate of approximately 30%, depression displays less contribution of inherited genetic variance to trait variance than many other mental disorders, e.g., autism or bipolar disorder [54].

Genetic association studies, including linkage analyses, candidate gene studies, and genome-wide association studies (GWAS), have identified several loci for MDD [74]. A plethora of genes have been identified in candidate gene association studies. However, a recent publication by Border et al. investigated 18 candidate genes for depression that have been studied 10 or more times and examined evidence for their relevance to depression phenotypes; they concluded that "the study results do not support previous depression candidate gene findings, in which large genetic effects are frequently reported in samples orders of magnitude smaller than those examined here. Instead, the results suggest that early hypotheses about depression candidate genes were incorrect and that the large number of associations reported in the depression candidate gene literature are likely to be false positives." [75].

With regard to GWAS, three major genome-wide meta-analyses of MDD have been published by the Psychiatric Genomics Consortium [76–78], including a recent study by Wray et al., where they found 44 risk variants for depression, and

furthermore identified cytokine and immune response as one of the major pathways being involved [77].

Environmental factors might play an important role in the etiology of depression, and gene-environment interactions have been implicated. However, also some of these findings were revoked by more recent studies that included significantly larger sample sizes; e.g., in one of the most highly cited papers in psychiatric genetics (>4700 citations), Caspi et al. (2003) reported a gene-environment interaction of a functional polymorphism in the promoter region of the serotonin transporter gene and stressful life events between the age of 21 and 26 in a cohort of Caucasians (n=847). They reported that individuals carrying the allele that is associated with lower transcriptional activity of the promoter exhibited more depressive symptoms, diagnosable depression, and suicidality in relation to stressful life events than individuals that were homozygous for the other allele. However, a study from 2011 using a comparable population (n=893) did not find a gene-environment interaction between this polymorphism, life stress, and mental disorders [79], and a recent publication that investigated the effect of previously implicated candidate gene polymorphisms across multiple, large samples (n>60000), found no effect of any of the "historical" candidate gene variants, including the serotonin transporter polymorphism [75]. Furthermore, in a study from 2014, Peyrot et al. reported the effect of a genetic risk for depression to be significantly increased by exposure to childhood trauma [80], which they later controverted in a study where they found no significant interaction [81]. Nonetheless, the fact that both genetics and environment play an important role in depression remains. Both animal and human studies have shown that early life exposure to prolonged levels of glucocorticoids and/or stress, such as childhood abuse, can induce epigenetic modifications on the glucocorticoid receptor gene that lead to alterations in expression and function of this receptor [82]. Such epigenetic alterations have been associated with childhood abuse and with functional alterations in HPA-axis activity in depressed patients [83]. The glucocorticoid receptor is expressed in almost every cell in the body and regulates the expression of genes controlling development, metabolism, and immune response, including IL-1beta, IL-6 and TNF [84]. This repression of inflammatory cytokine expression causes the

immunosuppressive actions of glucocorticoid hormones. Antidepressants have been shown to activate the glucocorticoid receptor and to increase hippocampal neurogenesis [85], possibly via mechanisms including the immune system [86].

Inflammation in depression

Apart from the genetic findings pointing to a role of the immune system in depression (see above), also epidemiologic studies support that notion; a recent study including more than 1.5 million people found that exposure to any maternal infection during pregnancy increased risk of depression by 24% [62], supporting the role of early immune dysregulation in the etiopathology of depression.

Further, alterations within the peripheral immune system have long been associated with mood disorders [9,87]. Depressed patients have been found to have chronic peripheral immune activation with elevated cytokine levels [58,88,89]. These cytokines can affect the CNS in various ways, as discussed above, causing e.g. elevated levels of pro-inflammatory cytokines in the brain parenchyma [19] and microglia impairment [90]. Also peripheral levels of the Ca^{2+}-binding protein S100B have been shown to be elevated in patients with depression [91,92], but central levels of S100B measured in the CSF of depressed patients provide contradicting results, with both increased [93] and decreased [94] levels being reported.

Almost half of the patients undergoing cytokine treatment for the treatment of e.g. cancer or chronic viral infections have been reported to develop depressive symptoms as well as suicidal ideation [95], and interferon-alpha treatment in rats has been shown to induce depression-like behaviour accompanied by elevated levels of hippocampal quinolinic acid levels [96]. Interestingly, a study in cancer patients receiving interferon (IFN)-alpha treatment showed that antidepressant treatment affected symptoms of depression, anxiety, and cognitive dysfunction more than symptoms of fatigue and anorexia [97], suggesting that sickness behaviour and cytokine-induced depression are mediated by different mechanisms. A proposed mechanism underlying this observation is the

stimulation of the enzyme IDO by pro-inflammatory cytokines [98], subsequently increasing levels of kynurenic acid in astrocytes and quinolinic acid in microglia. Together with glutamate released by activated microglia, quinolinic acid activates the N-methyl-D-aspartate (NMDA) receptors. Also, kynurenic acid has been shown to inhibit the release of glutamate, which, by extension, may downregulate neurotransmission of dopamine, whose release is regulated in part by glutamatergic activity [88]. Activation of NMDA receptors and deficient dopaminergic neurotransmission may both result in depression symptoms and contribute to treatment resistant depression and/or suicidal behaviour.

Several other immune aberrations have been reported in depressed patients, including signs of activation of the monocytic and lymphocytic arms of cell-mediated immunity, characterized by increased numbers of peripheral blood leukocytes, monocytes, and neutrophils, and elevated circulating levels of and acute-phase proteins [99,100].

Another factor that has been implicated in depression is psychosocial stress. As discussed above, hyperactivity of the HPA axis and increased levels of the glucocorticoid hormone cortisol are common observations in depressed patients. Cortisol activates glucocorticoid receptors, which in turn regulate the expression of neurotrophic factors, neuronal survival, neuronal excitability, neurogenesis and memory acquisition. High levels of cortisol may thus contribute to the manifestation of depressive symptoms by impairing these brain functions. Glucocorticoid receptors also regulate immune functions, exerting anti-inflammatory effects, and have been implicated in the therapeutic action of antidepressants [84]. Cytokines can also cause glucocorticoid receptor resistance, i.e. the reduced ability of glucocorticoids to translocate into the nucleus, impeding the normally inhibitory effect of glucocorticoids on cytokine production and action, resulting in an increasing production of pro-inflammatory cytokines and, subsequently, of stress hormones.

Taken together, there are several pathways that can be affected by immune mediators, which in turn can cause depression symptoms. These include activation of IDO and the subsequent generation of neuroactive tryptophan

metabolites, as well as glucocorticoid receptor resistance, which amplifies the inflammatory response and leads to excessive production of stress hormones.

Bipolar Disorder

As in major depressive disorder, individuals suffering from bipolar disorder experience depressive episodes with lowered mood, anhedonia, guilt and thoughts of death and suicide. Unlike unipolar depression, however, bipolar disorder is characterized by episodes of mania or hypomania in addition to depressive episodes. Bipolar disorder has frequent psychiatric comorbidity [101], and is strongly related to both depression and schizophrenia; with the introduction of the DSM-5 in 2013, bipolar disorder has moved further away from MDD and more towards schizophrenia [68]. The data in this thesis was collected before the release of the DSM-5, with diagnosis being based on the fourth text-revised edition from 2000 (DSM-IV) [102]. However, DSM-IV and DSM-5 resemble each other greatly with respect to the diagnosis criteria for bipolar disorder (for DSM-5 diagnostic criteria see Supplementary Table B) [68].

Bipolar disorder affects approximately 60 million people worldwide [53], although this number might be an underestimation, due to misdiagnosis and reluctance of the affected to admit to symptoms. As for most psychiatric disorders, the etiology of bipolar disorder is largely unknown. However, it is highly heritable, with twin studies reporting up to 85% heritability [103]

Depending on the symptoms, bipolar disorder can be divided into, amongst others, type I and II. Bipolar disorder type I is defined as having at least one manic episode, which lasts for at least seven days (or if it leads to hospitalization). Often, patients with bipolar type I have intermittent manic and depressive episodes, although according to the DSM one manic episode without depressive episodes is enough to classify the mood disorder as bipolar type I. Manic episodes are characterized by elated mood which may include euphoria, but the predominant mood is often irritable rather than elevated. In case of psychotic features, the episode is, by definition, manic, and not hypomanic. Bipolar patients experiencing a manic episode may experience inflated self-esteem and engage in e.g. multiple overlapping new projects, which are often initiated with little knowledge of the topic, feel a decreased need for sleep, and display rapid, pressured speech and/or distractibility.

Bipolar type II is defined as intermittent hypomanic and depressive episodes, both being a requirement for diagnosis. Hypomania is characterized by very similar features as mania, but in general has less impact on global functioning and typically, the hypomanic episodes themselves do not cause impairment. However, bipolar type II is no longer thought to be a "milder" condition than bipolar type I, as it is characterized by a significant amount of time spent in depression, as well as a persistent pattern of unpredictable mood changes and fluctuating, unreliable interpersonal or occupational functioning, causing serious impairment in work and social functioning [68].

Depressed mood is the predominant state in bipolar disorder, with depression being three times more prevalent than mania or hypomania over time [104]. Mood episodes usually have a gradual onset over days to weeks, and varying duration; manic episodes may last as long as three months if left untreated, and depressive episodes normally have a duration of at least four months if left untreated. Although alterations in mood and general level of activity are the main features of bipolar disorder, other symptoms include changes in motivation, cognition, perception, and motor activity. Also, patients may display normal mood and be free from symptoms between episodes, a state known as *euthymia*. However, euthymic patients often still display symptoms such as subthreshold mood symptoms, affective dysregulation, psychomotor disturbances, impaired social capacity, and neurocognitive impairments. With regard to cognitive impairments, studies have shown e.g. impaired attention, dysfunction in episodic memory, delayed verbal memory, and impairments in executive functioning, response inhibition, and psychomotor speed [105–107]. A study comparing depressed patients with bipolar disorder to patients with MDD found the same type of cognitive dysfunction in all patients, but to a much higher extent in bipolar patients [108], suggesting that frontal lobe impairment is more marked in bipolar than unipolar patients during depressive episodes.

Structural and neurophysiological changes are also part of bipolar disorder; several studies have reported cortical abnormalities [109–111], and a shape alterations of the striatum have been seen in bipolar patients as compared to healthy controls [112]. A review comparing findings from magnetic resonance

imaging (MRI) studies in patients with bipolar disorder or MDD found several structural and functional alterations in emotion- or reward-processing neural circuits [44]. They concluded that activation patterns differed in several brain regions, including the amygdala, anterior cingulate cortex, PFC, and striatum, during emotion-, reward-, or cognition-related tasks, and that grey matter volume differed in the amygdala, anterior cingulate cortex, hippocampus, and dorsolateral PFC, which was also found to be thinner in bipolar patients compared to patients with unipolar depression [44]. Interestingly, markers of immune activation have been associated with cortical thickness in patients with bipolar depression [113].

In order to explain the progressive pathophysiological processes accompanying bipolar disorder, a neuroprogressive model has been proposed, adding several components to the earlier implicated role of monoamines and messenger systems. These components include i.a. changes in neurotrophins, corticosteroids and inflammatory cytokines [114,115].

Genetics and environment

Heritability measures for bipolar disorder are consistently high; some studies have reported heritability of 85% for the disorder [103], and up to 93% have been reported for bipolar type I [116]. A population-based study of schizophrenia and bipolar disorder found increased risks of both schizophrenia and bipolar disorder to first degree relatives of probands with either disorder, with heritability estimates of 59% for bipolar disorder, and a unique genetic effect (i.e. not shared with schizophrenia) of 31% [117]. Several genome-wide studies have investigated genetic associations in bipolar disorder, describing loci implicated in the disorder. A very recent GWAS by Stahl et al. reports 30 genome-wide significant loci, including 20 newly identified loci, in genes coding for e.g., ion channels, neurotransmitter transporters and synaptic components [118]; they furthermore found bipolar type I to be strongly genetically correlated with schizophrenia, driven by psychosis, whereas bipolar type II was more strongly correlated with MDD. Shared genetic risk factors between bipolar disorder and schizophrenia have also been suggested by other studies; e.g., variants in the

CACNA1C gene encoding a voltage-gated calcium channel subunit have been associated with both disorders [118–120].

Also immune markers have been implicated by genetic studies; haplotype studies have found associations between bipolar disorder and human leucocyte antigen (HLA) genes [121], suggesting that the HLA system may mediate some of the immune dysregulations observed in bipolar patients. The HLA gene locus encodes the human versions of three different classes of MHC proteins, which are involved in various immune functions and have been associated with autoimmune, infectious, and inflammatory diseases [122]. Furthermore, bipolar disorder has been associated with variants in the IL-1beta gene cluster[123].

Inflammation in bipolar disorder

Immune dysfunction has been implicated in the etiology and pathophysiology of bipolar disorder on various levels, including findings in epidemiological studies, as well as peripheral and post-mortem brain studies. Replicated epidemiological studies have demonstrated that bipolar disorder has high rates of inflammatory medical comorbidities, including autoimmune disorders, chronic infections and cardiovascular disease [124].

Increased levels of a plethora of cytokines have been found in both blood and plasma of patients with bipolar disorder [58,114,125], including the proinflammatory cytokines IL-1beta, IL-6 and TNF, but also anti-inflammatory markers. Also increased peripheral levels of the acute phase protein corticotropin releasing protein and complement factor C3 have been associated with bipolar disorder[126,127]. With regard to central cytokine levels, a post-mortem study found significantly higher mRNA and protein levels of IL-1beta and its receptor in the frontal cortex of bipolar individuals [128]. This cytokine has also been shown to be increased in the CSF of the bipolar patients [129] included in this thesis, with levels being even higher in patients that had experienced at least one manic/hypomanic episode during the previous year.

Central levels of neuroinflammatory markers have been associated with cognitive performance in patients with bipolar disorder but not in healthy

controls [130], suggesting that inflammation may be involved in the underlying causes for cognitive decline in bipolar individuals. Bipolar patients are furthermore reported to have higher rates of medical conditions with a serious inflammatory component, including cardiovascular diseases, diabetes, and rheumatoid arthritis [131].

Epidemiological data regarding maternal immune activation and bipolar disorder is controversial however; although an earlier study reported a nearly 4-fold increase in risk of bipolar disorder after exposure to maternal influenza infection at any time during pregnancy [132], a very recent study found no associations between maternal immune activation and bipolar disorder or diagnosis of psychosis, including schizophrenia [62].

Autism Spectrum Disorder

Autism spectrum disorder (ASD), hereafter interchangeably referred to as autism, is a neurodevelopmental disorder characterized by early-onset difficulties in social communication (both verbal and non-verbal) and unusually restricted, repetitive behaviour and interests (for DSM-5 diagnostic criteria see Supplementary Table D). With a worldwide prevalence of about 1% it affects approximately four times more males than females [133], and has a high comorbidity with other neuropsychiatric disorders, such as attention-deficit hyperactivity disorder, tic disorder and anxiety disorders [134,135]. Neuroimaging studies have revealed structural differences in the brains of children with autism; cerebral cortical overgrowth has been reported [136], and a recent mega-analysis, i.e., combining original data of several studies, in a large sample (n=1571 patients, 1651 controls) found autism to be associated with smaller subcortical volumes of the pallidum, putamen, amygdala, and nucleus accumbens, as well as increased cortical thickness in the frontal cortex and decreased cortical thickness in the temporal cortex [137]. A post-mortem study furthermore revealed higher number of neurons in the PFC of male children with autism, although in a very small sample (n=7 patients, 6 controls) [138].

With respect to the etiology of autism, no single cause has been identified. For decades, autism was believed to be caused by lack of affection, also referred to as "emotional refrigeration", of the parents to children with autism. This notion wasn't abandoned until e.g., Michael Rutter and Lorna Wing reviewed the biological basis for autism, renouncing the concept of autism as an infantile psychosis or psychogenic disease caused by emotional coldness of the parents, and emphasizing instead the role of language or coding problems caused by cognitive and perceptual defects, as well as genetic factors [139,140]. Nowadays, both genetic and environmental factors have been attributed influence and will be discussed in more detail in the chapter *Genetics and environment*.

History of autism diagnostics

The term "autism" was introduced in 1911 by the psychiatrist Eugene Bleuler to describe schizophrenic patients who withdrew from reality and became self-absorbed. Childhood conditions that today would be referred to as ASD were labeled "childhood schizophrenia" [141], and the first (1952) and second (1968) editions of the Diagnostic and Statistical Manual of Mental Disorder (DSM)[142,143] viewed autism as a form of childhood psychosis.

In 1943 and 1944, respectively, the Austrian-American psychiatrist Leo Kanner and the Viennese pediatrician Hans Asperger, independently of each other published papers on children displaying symptoms of childhood autism [144,145]. Their descriptions contained several similarities such as higher incidence in males, lack of interest for other people's emotions or thoughts, communication deficits and repetitive behaviours. Asperger´s children were, however, less severely disabled compared to those described by Kanner, and Asperger noted a vast vocabulary and high intelligence in some of the children, coupled with narrow interests.

It was not until 1980 and the publication of the third edition of the diagnostic manual (DSM-III) [146], that *infantile autism* became an independent diagnosis under the general category of pervasive developmental disorder (PDD). When the DSM-IV was released in 1994 [102], infantile autism was renamed autistic disorder, and the umbrella term PDD included the diagnoses autistic disorder, Asperger's syndrome, pervasive developmental disorders not otherwise specified (PDD-NOS), childhood disintegrative disorder and Rett's disorder. In the DSM-IV, the inclusion criteria were broadened compared to DSM-III, and it included twelve diagnostic symptoms for autistic disorder (for DSM-IV diagnostic criteria see Supplementary Table C). These symptoms were organized in accordance with a triad of impairments that had been suggested by Wing et al. in 1981 [147]: (1) impairments in social interaction, (2) impairments in communication, and (3) restricted, repetitive and stereotyped patterns of behaviour, interests and activities, each containing four individual symptoms.

In 2013, the fifth edition of the DSM was released (DSM-5) [68]. In this latest revision of the diagnostic manual, the diagnoses previously assembled under the umbrella term pervasive developmental disorders (PDD) have been merged into the single diagnosis of ASD (for DSM-5 diagnostic criteria see Supplementary Table D), without a definition of subtypes and with severity measured within the broader diagnosis. Furthermore, the triad of symptoms for autism diagnosis was merged into two domains: the social interaction and communication domains were combined into a single domain. Atypical language development was removed from the autism diagnosis criteria and classified as a co-occurring condition, since large variation in language is a characteristic of autism [148]. This new diagnosis of "social (pragmatic) communication disorder" is defined by difficulties with social uses of both verbal and non-verbal communication, but does otherwise not meet criteria for autism.

With the DSM-5, a conceptual change of the diagnostics was introduced: while the previous diagnostics regarded all disorders included under the umbrella term PDD as discrete disorders, the new diagnostics of the DSM-5 include but one ASD diagnosis with varying degrees of severity. This concept was proposed decades ago [149], suggesting a continuum of symptoms that are also present in varying degrees in the general population.

Autistic-like traits

While autism can be construed as the lower-most extreme end of a continuum of social abilities, individuals might display milder phenotypes related to the autism characteristics that do not meet the diagnostic criteria for ASD: these traits are referred to as *autistic-like traits* (ALTs) and represent the boundary between autism and normality. A nation-wide study reported that ALTs occur to some extent in 35% of the whole population [150], and an aggregation of ALTs in close relatives of individuals with autism has been reported [151]. Thus, autism has been suggested to represent the extreme end of a normal distribution of ALTs, with genetic factors playing an important role in both. Although a genome-wide association study failed to identify single nucleotide polymorphisms (SNPs) common to autism and ALTs [152], several studies have

suggested a shared genetic etiology between autism and ALTs across normal variation and the extreme ends [153,154]. Furthermore, a study using a large autism consortium and population-based genomic data sets demonstrated that there is a genetic correlation between certain ALTs concerning social and communication skills and autism [155], and a recent GWAS comparing ALTs in a large cohort from the general population with results from the Psychiatric Genomics Consortium GWAS of autism further supported this notion [156]. Bralten *et al.* identified several polymorphisms in genes encoding proteins involved in neurite outgrowth to be shared between ALTs and autism, including the MET receptor tyrosine kinase gene (*MET)*. These findings suggest that multiple types of genetic risk factors for autism influence a continuum of behavioural and developmental traits, the extreme end of which can result in autism or other neuropsychiatric disorders.

Genetics and environment

Autism has one of the highest heritability estimates amongst mental disorders, with twin studies suggesting a heritability of 80-98% [135,157]. For approximately 5% of autistic cases where autism co-occurs with a known Mendelian genetic syndrome, such as Fragile X syndrome, tuberous sclerosis or Rett syndrome, the genetic cause has been identified. This is known as *syndromic autism*. However, this constitutes only a small proportion of individuals with autism, and for the majority the genetic cause has not been identified – this is referred to as *idiopathic autism*. In autism research, syndromic autism disorders are used as "model disorders" for idiopathic autism. Affected biological pathways are beginning to be elucidated through extensive analyses of these genes. To give an example, the fragile X syndrome is caused by mutations in the FMR1 gene and Rett disorder by mutations in the MECP2. Although the disrupted gene has been identified, different mutations can occur in these genes. Depending on where in the gene the mutation occurs, protein function might be affected differently. Thus, the clinical presentation of the genetic syndromes can be highly heterogeneous, highlighting the complex relationship between genotype and phenotype. If we can understand how the loss of function of the affected genes can lead to

behavioural alterations such as deficits in social communication, it will help us to better understand the underlying biology of autism.

Despite the high heritability estimates, the underlying genetics of the disorder are characterized by large heterogeneity and complexity; autism, as well as virtually all other mental health disorders, are now generally accepted to not be due to one single or even a few genetic causes but precipitated by a plethora of genetic factors and/or largely unknown gene-environment interactions. No single gene leads to autism every time it is mutated, and genetic studies have so far implicated up to 1000 genes in autism [158]. In addition to this locus heterogeneity a high degree of pleiotropy (i.e., one gene affects more than one phenotype) has been shown in variants linked with autism, further adding to the complexity of the genetic etiology. Both rare mutations with large individual effects and common variants with small effect sizes have been implicated [158]. Rare mutations can occur in the form of the already mentioned Mendelian genetic syndromes, chromosomal abnormalities, rare copy number variations and de novo and transmitted single nucleotide variants [159]. Although common variants (e.g. SNPs with an allele frequency of >5% in the general population) have been proposed to account for the majority of autism liability [160], they have small individual effects, and GWAS have, until very recently, been largely unsuccessful at identifying mutations with a large enough effect size to reach genome-wide significance level. This lack of results might possibly have been due to limited sample size, as recent studies with markedly larger samples have found significant associations of several common variants with autism [161,162]; the identified loci were mainly in genes involved in the regulation of brain development and neuronal function, and several of these genes have previously been linked to autism risk in studies of de novo and rare variants, as well as with risk for depression and schizophrenia [77,161]. Their findings furthermore support the notion that the heterogeneity seen in the clinical presentation of autism is reflected in the genetic architecture of subtypes found in the spectrum [163]; common variants may have a greater impact in high-functioning autism, while de novo mutations like copy number variants and gene-disrupting point mutations are more present in subtypes including severe impairments and intellectual disability. The complexity of the genetic etiology for autism has only

been increased by the results from genome wide technologies; however, several autism-related genes encode components of the immune system, such as the MET gene, an important regulator of immunity and brain development [164], and the HLA locus, which encode the MHC genes in humans. Variants in all three classes of MHC genes have been reported to enhance autism risk [165]. However, as with many other autism-related genes, these associations are not unique to autism; they are also associated with other neurodevelopmental disorders and autoimmune disorders [166], which occur at higher rates in the relatives of individuals with autism [167,168].

Although not reaching significance level after correction for whole genome multiple testing, the findings reported by Grove *et al.* point to a role of the immune system in autism; tissue-specific analysis of genes revealed that immune related genes were among the top three affected systems (together with brain tissues and stem cells) before correction for multiple testing [169].

Despite the high heritability suggested by twin studies, the fact that monozygotic concordance rates (i.e. the risk for a monozygotic co-twin receiving the same diagnosis as the proband) never reach 100% suggests an impact of environmental factors. A large role for shared environmental effects has been proposed in a twin study by Hallmayer *et al.* [170], but this is however not supported by all studies [157]. It seems that both the genetic background as well as epigenetic mechanisms and gene-environment interactions, which are dependent on the context of environmental risk factors, play an important role in the etiology and pathophysiology of autism. Environmental risk factors that have been identified include, amongst others, prenatal exposure to toxicants [171] and maternal infections [172], both of which lead to altered levels of inflammatory mediators. Considering the role of the immune system in brain development [34], altered immune function might impair a wide array of neurodevelopmental processes such as neurogenesis, proliferation, apoptosis, synaptogenesis and synaptic pruning, making it a crucial but complex biological pathway in the etiology of autism in at least a subset of cases, which will be discussed in the following chapter.

Inflammation in autism

Almost half a century ago a connection between congenital rubella infection and autism was noted [173] that seemed preventable by rubella vaccination [174]. Since then numerous other epidemiological studies have found associations between maternal immune activation and autism, including a Danish population cohort study linking viral infection in the first trimester and bacterial infection in the second trimester, that required hospitalization, to an increased risk of an autism diagnosis later in life [175]. A meta-analysis of more than 40000 autism cases from 15 different studies [176] found an association between general infection and autism, as well as a more pronounced risk of autism in the offspring of patients who required hospitalization for the treatment of infection during pregnancy, with type of infectious agent and time of infection during pregnancy affecting the risk. Most recently, a study including more than 1.5 million people found that exposure to any maternal infection during pregnancy increased risk of autism by 79% [62]. A plethora of animal maternal infection models have shown consistent results, establishing autism-relevant atypical behaviours [172], further confirming a strong impact of maternal immune activation in the etiology of autism.

However, studies investigating the effect of maternal immune activation are not the only evidence linking the immune system to autism. A number of immunological factors have been linked to autism, including cell-surface proteins such as the HLA complex [177,178], and a variety of clinical studies have linked cytokines to autism, for review see [164,179–181]; elevated levels of several pro-inflammatory cytokines have been reported in the serum and blood of autistic individuals, including IL-1beta, IL-6, TNF, and IFNγ [182–185], and changes in cytokine and other immune mediator marker expression have also been reported in post-mortem brain studies [186–188].

However, lower levels of immune markers have also been reported; a study investigating peripheral cytokine levels in children with autism found that lower cytokine levels were significantly associated with increased severity of autism-related symptoms [189]. They furthermore found that boys and girls had

significantly different immune profiles. These results add a new dimension to the sex-differences observed in autism. In addition to findings showing that females carry significantly more mutations in neurodevelopmental-related genes that males with the same symptoms, supporting a concept referred to as "the female protective model" [190], the study by Masi et al. suggests that also differences in immune activity might be a contributing factor to the higher incidence of autism in males.

To date, there is no genetic test that can diagnose autism. However, specific immunologic assays have been tested in the diagnostic procedure of autism, using a signature of differentially co-expressed genes that were enriched in translation and immune function/inflammation, identifying boys with autism with 83% accuracy [191]. This study suggests that genomic immune markers might contribute to a clinical test that could be implemented in diagnostic settings.

AIMS

The overall aim of this thesis is to investigate the role of the immune system in brain function, both in animal model and in human populations. We hypothesize that inflammatory mediators are significant for psychiatric conditions by modulating e.g. the development of the brain and neurotransmission.

Paper I

The aim of "*Expression of inflammatory markers in a genetic rodent model of depression*" was to study behaviour in the open field test and forced swim test, as well as to investigate central gene expression in a rat model of depression; the Flinders sensitive line (FSL), and its control the Flinders resistant line (FRL); in response to peripheral immune stimulation by the bacterial endotoxin lipopolysaccharide.

Paper II

The aim of "*Effects of chronic escitalopram treatment on the expression of inflammatory markers in the Flinders rat model of depression*" was to study the effects of chronic administration of the antidepressant escitalopram on central expression of immune-related genes in the FSL depression model and its control.

Paper III

The aim of "*Associations between autistic-like traits and polymorphisms in NFKBIL1*" was to investigate associations between autistic-like traits and polymorphisms in two candidate genes that are central to immune functions; *NFKB1* and NF- κ B inhibitor-like protein 1 (*NFKBIL1*).

Paper IV

The aim of "*Influence of variations in IL1B on brain region volumes in bipolar patients and controls*" was to investigate associations between polymorphisms in the gene coding for the pro-inflammatory cytokine interleukin-1 beta (*IL1B*) and bipolar disorder, as well as whole-brain grey matter volume and volumes of several brain regions shown to be of importance in mood disorders.

RESULTS

Paper I

The main findings of this paper are:

- FSL rats displayed higher locomotor activity in the open field test than their controls, and LPS decreased locomotor activity in both rat lines
- FSL rats showed higher immobility in the forced swim test when compared with FRL rats, and LPS increased immobility in both rat lines
- mRNA levels of two genes were lower in FSL compared to FRL rats: *S100b* in all investigated brain regions, and *C3* in all investigated brain areas apart from the hypothalamus
- mRNA levels of *Htr2a* were higher in the hippocampus of FSL rats compared to FRL rats
- LPS had no effect on *S100b* expression levels, but increased levels of *C3*, *Il1b*, *Nfkbia*, *Timp1* and *Tnf* in all investigated brain regions, as well as of *Il6* in the hippocampus and hypothalamus and of *Mmp9* in the hypothalamus and striatum in both rat lines
- there was no interaction effect between rat line and LPS

In Paper I, FSL rats had lower mRNA levels of *S100b* and *C3* in almost all investigated brain regions, as well as higher *Htr2a* mRNA levels in the hippocampus. There was no difference, neither behavioural nor in transcription levels, between FSL and FRL animals in response to peripheral immune stimulation.

Paper II

The main findings of this paper are:

- mRNA levels of *S100b* were lower in FSL compared to FRL animals in all investigated brain regions, replicating our findings from Paper I
- mRNA levels of *Htr2a* were higher in the hippocampus of FSL compared to FRL rats, replicating our findings from Paper I
- The following findings from Paper I were replicated regarding mRNA levels in FSL compared to FRL animals: lower levels of *C3* in the PFC and striatum, *Il1b* in the hippocampus, *Mmp9* in the PFC and *Timp1* in the hippocampus, albeit only nominally significant
- Escitalopram treatment lowered mRNA levels of *S100b* and *Htr2a* in the amygdala and hypothalamus of FSL and FRL animals
- An interaction between rat line and treatment was found for *Il18* in the hippocampus; post-hoc analyses revealed that FSL rats had lower *Il18* levels than FRL rats, and that escitalopram lowered *Il18* expression in FRL but not FSL animals

In Paper II, we replicated findings from Paper I regarding differences in gene expression of e.g., *S100b* and *Htr2a*, between FSL and FRL rats. Escitalopram treatment lowered S100b and *Htr2a* mRNA in the amygdala and hypothalamus.

Paper III

The main findings of this paper are:

- The polymorphisms rs2239707 and rs2230365 in *NFKBIL1* showed associations in the case-control analysis, i.e. with a validated proxy for clinical diagnosis of ASD; for rs2230365, the finding replicates an earlier association found between this variant and autism in an independent genome-wide association study
- rs2230365 was associated with the autistic-like trait of language impairment

In Paper III, two polymorphisms in *NFKBIL1* were associated with a probable diagnosis of ASD, with the finding concerning rs2230365 replicating earlier associations between this variant and autism. This polymorphism was additionally associated with language impairment.

Paper IV

The main findings of this paper are:

- Genotype distribution of the investigated polymorphisms in *IL1B* was not different between bipolar patients and controls
- The T allele at rs16944 and the C allele at rs1143627 were associated with increased volume of the left putamen in patients and controls
- Several of the investigated polymorphisms were nominally associated with volume of the globus pallidus, putamen, and thalamus in both patients and controls
- No associations were seen between *IL1B* variants and volume of the amygdala, caudate nucleus, hippocampus, or nucleus accumbens

In Paper IV, two polymorphisms in *Il1B* were associated with increased volumes of the left putamen in both bipolar patients and controls. No association was found between bipolar disorder and variants in *IL1B*.

DISCUSSION

ANIMAL STUDIES (PAPER I & II)

Behaviour (Paper I)

Although the increased locomotor behaviour of FSL compared to FRL rats in the open field test (Paper I) is not in line with the general understanding that FSL rats move less than FRL rats, the observed increase in immobility of FSL rats in the forced swim test confirms the more depressive-like phenotype of the FSL animals. The behavioural responses to LPS, i.e., reduced locomotor activity in the open field test and higher immobility in the forced swim test, suggest acute sickness behaviour of the animals, as was expected six hours after endotoxin administration [12].

Effect of rat strain on central gene expression (Paper I & II)

The Ca^{2+}-binding, astrocyte specific protein S100B has been associated with a wide spectrum of diseases, such as neurodegenerative diseases, congenital/perinatal disorders, and psychiatric disorders [46]. It is used as a biomarker of active neural distress, although the wide spectrum of pathological conditions in which S100B is involved markedly reduces its specificity.

With regard to psychiatric disorders, altered levels of S100B have been shown in a plethora of studies; increased peripheral levels have been reported in patients with autism [182,192], schizophrenia [193] and mood disorders [93,194,195]. Interestingly, high peripheral S100B baseline levels have been positively associated with response to antidepressant treatment [196]. Post-mortem studies have also reported alterations of central S100B levels in depression, bipolar disorder, and schizophrenia [197–199]. However, the findings are often contradicting, with both higher and lower levels being reported, and it is unclear whether central levels of S100B are correlated with peripheral levels [200].

S100B has also been implicated in neurotransmission and central immune functions. Pro-inflammatory cytokines such as IL-1beta, IL-6 and TNF stimulate secretion of S100B [201], and S100B in turn induces expression of pro-inflammatory cytokines in both neuronal and microglial cells [202,203]. Chronic S100B overexpression resulted in decreased numbers of mature, stable astrocytes, as well as in activation of microglia in mice [48]. These data suggest that S100B may cause cell-type specific gene-regulatory events leading to long-lasting activation of immune cells and cytokine production, further supporting a pro-inflammatory regulatory role of S100B on cells of the CNS [204].

The difference in basal expression levels of S100B between the FSL and FRL rat lines observed in Paper I and II—that is, lower levels in FSL—might be due to a differently regulated immune system in the two rat lines that may lead to glial pathology and neurodevelopmental effects causing functional and/or structural differences between them. However, it is not obvious that S100B is crucial for the behavioural differences seen in the FSL animals and their controls; given the human S100B literature [91], one could hypothesize that the levels of this protein should be higher in the FSL animals, even though the literature regarding S100B and psychiatric disorders is not conclusive yet.

Our findings in Paper I and II suggest that the Flinders rat may be a suitable model in the study of the functional implications of altered S100B levels in depression-like behaviour. However, certain points should be investigated in order to further validate this animal model as a proper tool with which to study the role of S100B in depression. First, peripheral S100B levels have been shown to be elevated in two other rat depression models [200], but need to be investigated in the FSL rat. Second, since we measured mRNA levels, we cannot be certain that these reflect protein levels, and this should be addressed in future studies. Third, the downregulation of S100B mRNA levels in FSL compared to their controls was much stronger in Paper I than Paper II. The reason for this is not known, and this should be investigated in forthcoming studies. Finally, it needs to be established whether the lower levels of S100B are part of the etiology of depressive-like behaviour, a consequence of it, or a finding not related to

behaviour in this animal model. Furthermore, additional studies are warranted with respect to the influence of S100B on neurotransmission.

We also found lower mRNA levels of the complement factor C3 in FSL compared to FRL animals in both Paper I and II. Complement activity and especially C3 are highly involved in pruning of nonfunctional synapses during brain development [40], and disruption of C3 signaling results in sustained deficits in synaptic connectivity [22]. C3 has also been implicated in the pathophysiology of depression in humans, as depressed patients had lower CSF levels of the complement component compared to healthy subjects [205], which is in line with our results. However, it should be noted that we were unable to replicate all findings regarding C3 from Paper I in Paper II.

The upregulation of the serotonin receptor 5-HT_{2A} mRNA in the hippocampus of FSL compared to FRL rats observed in both Paper I and II is in line with earlier experimental studies [206,207] and further supports a role of serotonin and the 5-HT_{2A} receptor in the depression-like behaviour of this animal model. Furthermore, our results are compatible with findings in humans; increased numbers of 5-HT_{2A} receptors have been reported in the brains of depressed patients [208,209] and suicide victims [210–212]. Our results underline the suitability of this model to further study the impact of serotonin and its receptors in the study of depression.

Additionally, we found differences in the expression levels of the matrix metalloproteinase MMP-9 and its inhibitor tissue inhibitor of matrix-metalloproteinases (TIMP-1) between FSL and FRL rats. MMP-9 plays an important role in the remodeling of the extracellular matrix via degradation of proteins, and both MMPs and their physiological antagonists TIMPs are crucial for CNS development and have beneficial roles in the healthy adult brain, such as regulating synaptic and structural plasticity. However, aberrant expression of MMP-9 has been implicated in CNS diseases, contributing to neuroinflammation via several mechanisms such as disruption of the blood-brain barrier, cytotoxicity and demyelination [213]. Candidate gene studies have proposed a role of MMP-9 in psychiatric disorders such as schizophrenia and

bipolar disorder [214], and genetic variability in the MMP-9 gene has been associated with personality traits [215], suggesting its involvement in psychiatric morbidity and mental functions. However, although our finding that MMP-9 is lower in the PFC of FSL than FRL rats is consistent in Paper I and Paper II, it is only borderline significant and should be interpreted with caution.

Moreover, we found lower mRNA levels of IL-1beta in the hippocampus and raphe nuclei, and of IL-18 in the amygdala and hippocampus, of FSL rats compared to their control, but no significant strain effect for any of the other investigated pro-inflammatory cytokines.

IL-1beta has been implicated in the pathophysiology of depression with respect to several mechanisms, including neurogenesis and the serotonergic system [216]. IL-18, also called interferon-gamma-inducing factor, is a potent inducer of TNF and other cytokines and chemokines [217]. Il-18 is produced in the brain by several cell types, such as astrocytes, microglia, and neurons [218] and is tightly regulated[219]. Promoter variants in IL-18 that have been associated with increased levels of IL-18 were more common in individuals that developed a depressive episode in response to stressful life events [220], and central levels of IL-18 have been found to be elevated in a post-mortem study of prefrontal cortex tissue of depressed patients [221], suggesting that this cytokine is regulated differently in the animal model than humans.

It should be noted that the levels of all investigated cytokines, as well as some other immune-related markers, had a tendency to be lower in FSL compared to FRL rats, showing nominal significances in many of the investigated brain regions. This is in contrast with a number of human studies showing elevated levels of e.g. IL-1beta, IL-6, and TNF in the periphery of depressed patients [222,223]. The reason for the discrepancy in these findings is not clear, but, as mentioned above, the expression of cytokines might be regulated differently in the CNS compared to the periphery [224]. However, human data regarding central cytokine levels is more controversial; increased levels of IL-6 have been reported in the CSF of suicide attempters [225], and depressed patients showed both elevated levels of IL-1beta, and lower levels of IL-6 [226]. A possible explanation for the lower

central mRNA levels of various immune mediators in FSL rats might be compensatory mechanisms, or an overall downregulation in central gene expression.

However, our findings that FSL and FRL rats displayed no differences in cytokine mRNA levels, apart from IL-1beta and IL-18, suggest that the depressive-like behaviour of the FSL rats might be caused by other underlying mechanisms that do not involve changes in central mRNA levels of these cytokines. It is questionable whether the Flinders depression model is a suitable tool to study cytokine-related mechanisms in depression, but our findings in Paper I and II indicate that it might be a useful model in the study of the serotonin system and the glial marker S100B in depression. Regarding the reduced expression of *Il1b*, *Il18*, *Mmp9*, and *Timp1* observed in some brain regions, future studies are needed to explore the relevance of these findings for the Flinders depression model.

Effect of LPS on central gene expression (Paper I)

The behavioural data from Paper I, confirming sickness behaviour after LPS administration, are further confirmed by elevated mRNA levels of several immune mediators in response to LPS. These include IL-1beta and TNF, which are the main pro-inflammatory cytokines involved in sickness behaviour. Considering that we found no differences in mRNA levels in response to LPS between FSL and FRL rats, it is unlikely that differences in sensitivity to acute inflammatory mechanisms are involved in the behavioural differences between the rat lines. Also, we found no effect of LPS on the expression of S100B, leading to the conclusion that this substance is not part of the sickness behaviour in this model. This is in contrast to a finding regarding S100B transgenic female mice displaying depressive-like behaviour in the forced swim test [227].

LPS caused significant upregulation of 5-HT_{2A} receptor mRNA levels in the striatum of the FSL/FRL rats, indicating a possible sensitivity of this receptor to immune changes in this brain region. Apart from their function in e.g., monoaminergic signaling [228], 5-HT_{2A} receptors are proposed to play a role in

inflammation [229] although whether this role is pro- or anti-inflammatory is unclear [230–232]. It is suggested that 5-HT_{2A} receptor agonists mediate anti-inflammatory effects through disruption of TNF receptor downstream signaling, thereby inhibiting NF-κB nuclear translocation and transcription of pro-inflammatory markers such as IL-6 and TNF itself [229,231]. These 5-HT_{2A} receptor agonists, also known as psychedelics, have—due to their anti-inflammatory effects—been proposed as treatment for several inflammatory disorders, and are viewed as promising new treatment options for a variety of disorders [233], including anxiety and depression, constituting a renaissance more than half a century after their initial use as treatment for these disorders [234,235]. It is hypothesized that 5-HT_{2A} agonists exert their immediate antidepressant effects through a resetting of resting state functional connectivity to healthy networks, and then produce long-lasting effects by reducing neuroinflammation [233]. Why serotonin acting at 5-HT_{2A} receptors is primarily pro-inflammatory, but other 5-HT_{2A} receptor agonists anti-inflammatory, is yet to be clarified. It is hypothesized that this discrepancy among the effects of substances which all activate the receptor might be due to functional selectivity, that is, serotonin inducing a conformation of the receptor that activates pro-inflammatory signaling pathways, and other 5-HT_{2A} receptor agonists stabilizing the same receptor in another conformation that recruits anti-inflammatory pathways [233].

Effect of chronic SSRI treatment on central gene expression (Paper II)

Our findings that antidepressant treatment with the SSRI escitalopram lowered mRNA levels of S100B and the 5-HT_{2A} receptor in the amygdala and hypothalamus of FSL and FRL rats further confirm the validity of this animal model in studying the action of antidepressants.

Genetic studies of the 5-HT_{2A} receptor have shown significant associations between variants in the gene encoding this receptor and treatment response to antidepressants [236,237]. Interestingly, a down-regulation of 5-HT_{2A} receptors has been reported following long-time treatment with antidepressants in humans [238–240], suggesting that this mechanism might be central for their therapeutic effect.

Antidepressant treatment has been shown to reduce serum levels of S100B in patients with depression [241], and baseline levels of S100B have been associated with treatment response to antidepressants [196,242]. However, studies have also shown no effect of antidepressant treatment on elevated S100B levels despite clinical improvement [91,196,243].

S100B has also been implicated in serotonergic neurotransmission; S100B overexpressing mice showed a significant loss of serotonergic terminals in the hippocampus [48], and S100B secretion in turn has been shown to be induced by binding of serotonin (5-HT_{1A}) receptor agonists and cytokines [244]. Considering that FSL rats showed aberrant mRNA levels of both S100B and the 5-HT_{2A} receptor at baseline, and that escitalopram treatment altered their levels in the same brain regions, one could speculate that S100B might, in part, be involved in 5-HT_{2A} receptor function and that these two proteins constitute a common target for antidepressants. Further studies are warranted to explore the role of these two proteins in depression, and considering our findings in Paper I and II, the Flinders rat may be a suitable model in this regard.

Our findings that escitalopram treatment had no effect on central cytokine levels in FSL rats is in contrast with the notion that antidepressants decrease inflammation; studies in patients with MDD have reported that antidepressants decreased peripheral levels of pro-inflammatory cytokines [245], and an experimental study showed that antidepressant treatment reduced up-regulation of cytokines in response to LPS in the brain and periphery of rats [246]. However, the latter study investigated the effect of antidepressants on an acute inflammatory response, while our study addressed the effect on basal levels in the rat depression model. Alboni et al. also reported no changes in the expression levels of *IL1b* or *Tnf* in the rat hypothalamus after chronic treatment with either an SSRI or a tricyclic antidepressant [247], but a significant decrease in *Il6* mRNA after both types of antidepressants. These differences in gene expression might be attributable to using a different strain of rats, as well as a different SSRI (fluoxetine). Another possible factor influencing results may be the dosages of antidepressants used. Our results suggest that the documented antidepressant effect of escitalopram on the depressive-like behaviour in this

Flinders rat model might be caused by other underlying mechanisms that do not involve changes in mRNA levels of these cytokines.

HUMAN GENETIC ASSOCIATION STUDIES (PAPER III & IV)

Variants in *NFKBIL1* and autistic-like traits (Paper III)

Since it has been difficult to replicate common genetic variant findings in case-control studies of mental disorders, investigations of endophenotypes may be another strategy to gain insight into the influences of common genetic variations in e.g. autism. In Paper III, we investigated common SNPs in immune-related genes in relation to ALTs and also used a dichotomous cutoff that has been used as a validated proxy for a clinical diagnosis of autism [153,248].

We found associations between ALTs and two polymorphisms in *NFKBIL1*, a gene that is located in the HLA region. The HLA region encodes several molecules that play key roles in the immune system, and has been implicated in autism before [165,249,250]. A nominal association between rs2230365 and autism has been reported in an earlier genome-wide autism case-control study by the Autism Genetics Group [250], and our finding that rs2230365 was associated with a clinical proxy for autism replicates this.

Moreover, the same rs2230365 SNP was associated with the ALT module language impairment. Although atypical language development is no longer part of the DSM-5 diagnostic criteria for ASD (see supplementary table D), language anomalies or impairments are a characteristic of autism [148]. Language impairment has been associated with HLA alleles previously [251], and the HLA region has been implicated in dyslexia [252], suggesting a role of HLA loci in language disorders.

Our data suggest that variants in *NFKBIL1* may possibly contribute to ASD risk and some autistic traits. Only a modest genetic overlap has been reported with respect to social and nonsocial ALTs [152], and different genetic influences may be of importance in various autistic endophenotypes. We therefore conclude that

it might be advantageous to consider autistic traits separately in order to elucidate how neuropsychiatric symptoms develop. One could speculate that SNPs in *NFKBIL1* might lead to alterations in immune function, possibly causing developmental dysfunctions in conjunction with other risk factors. Further studies of this gene are therefore warranted in order to elucidate its role in brain development. Moreover, the possible functional implications of the studied polymorphisms should be investigated.

It should be noted that candidate gene studies have been subject to criticism; recent studies investigating the effect of genes that have been previously implicated in disorder phenotype, found that historical candidate gene literature was essentially uninformative for the genetic basis of several mental disorders, including schizophrenia [253], and depression [75]. However, autism has been shown to have a much higher genetic component than depression [67], while epigenetic alterations possibly play a more important role in the mechanisms leading to depression [254]. Also, a point of criticism regarding candidate gene studies is the small sample sizes frequently employed; many candidate gene association studies have likely been severely underpowered, as e.g. discussed by Border et al. [75]. This is unlikely regarding our study, considering the sample size in Paper III (n= 12,319). It should be noted that two more recent GWAS have not shown any association with polymorphisms in *NFKBIL1* and autism [161,162], and, although corrected for multiple testing, our results should be interpreted with caution.

Variants in *IL1B* and volume of brain regions (Paper IV)

The pro-inflammatory cytokine IL-1beta is produced by activated immune cells and plays a key role in the onset of inflammatory processes and the acute phase response. However, it has also been implicated in mood disorders in a number of studies, for a review see Maes et al. [255]. IL-1beta has been shown to be of relevance for embryonic development of the CNS, where it promotes proliferation and production of cytokines and trophic factors [256], together with neurodegeneration [257]. Experimental studies also point to a role of IL-1beta in axonal plasticity after spinal cord injury (Boato et al., 2013), as well as in hippocampal synaptic plasticity (Erion et al., 2014). Patients with bipolar

disorder have higher peripheral IL-1beta levels compared with controls [125], a trend that has also been observed in depression [255]. Also, levels of IL-1beta were elevated in the CSF of bipolar patients compared with controls – an observation that was even more pronounced depending on the manifestation of manic/hypomanic episodes during the year prior to sampling [129].

Our results do not support a role of the studied *IL1B* gene variants in bipolar disorder, as we found no differences in genotype distribution between patients and controls. This is in contrast with a study by Papiol et al. associating *IL1B* polymorphisms with bipolar disorder [123].

Our analyses revealed two *IL1B* polymorphisms (i.e., rs16944 and rs1143627) to be associated with volumes of the putamen of the left hemisphere in patients and controls. Alterations in grey matter brain volume have previously been associated with polymorphisms in cytokine-encoding genes [258,259], and with peripheral cytokine levels [260]. It has been suggested that the size/volume of brain regions is associated with intrinsic brain activity and cognitive functions [261], and with regard to the findings concerning rs16944 and rs1143627 in this thesis, one might speculate that IL-1beta is of relevance for mental functions.

Our findings contradict yet another study by Papiol et al.; while they report associations between the T allele at rs16944 with lower grey matter volumes [262], we find this allele to be associated with increased volume of all investigated areas, although only the association with the left putamen survived correction for multiple testing. However, there are several possible reasons for why our results differ from those of Papiol et al.; first of all, their sample size (n=20) was very small; second, their results were not corrected for multiple testing; and third, they investigated grey matter in the PFC, an area that was not investigated in our study. Nevertheless, associations between this allele and frontal grey matter deficits have also been reported in schizophrenic patients but not in healthy controls [263], with no difference in genotype distribution between patients and controls. Also, a study in patients with depression found no association with this genetic polymorphism [264], suggesting that this variant in the gene coding for IL-1beta might not lead to a pathogenic phenotype per se. However, the

associations seen with alterations in volume of different brain regions indicate that this polymorphism may have an impact on neurodevelopment or other processes affecting brain region volume.

Both rs16944 and rs1143627, two polymorphisms that are in strong linkage with each other, lie in the promoter region of the *IL1B* gene and may affect binding of transcription factors. *In vitro* studies have investigated the functional implications of these variants; a haplotypic combination of rs16944 and rs1143627 has been associated with increased binding of transcription factors to the *IL1B* promoter *in vitro* [265], and the C allele at rs1143627 has been found to inhibit protein complex formation at this site, indicating that some transcription factors may be unable to bind and form the transcription initiation complex [266]. However, the latter study found no differences in binding activity for the T allele at rs16944.

The T allele at rs16944 has also been associated with later onset of depression[267] and with lower severity of depressive symptoms, as well as with faster and more pronounced treatment response [264,268] to antidepressant treatment. Together, these data point to mechanisms involving this *IL1B* polymorphism which may influence disease onset and treatment response in individuals who are perhaps already vulnerable to depression, but do not cause the disorder or affect its severity per se.

Our study adds to the existing literature regarding associations between genetic variants in immune markers and altered brain volume [258,259], further supporting a role of immune mediators, especially IL-1beta, in neurodevelopment.

CONCLUDING REMARKS

The psychiatric disorders discussed in this thesis—depression, bipolar disorder, and autism—are highly heterogenous disorders, both with regard to their phenotypical manifestations, and to the genetic factors that may contribute to their etiology and pathophysiology. Nevertheless, a multitude of studies point to a dysfunctional immune system as a possible common denominator for mental disorders; aberrant cytokine levels have been reported both in the periphery and CNS of patients suffering from these disorders, and immune-related genes and neuro-immune pathways have been implicated in several psychiatric conditions. However, the underlying mechanisms of immune-related factors in mental disorders are not yet fully understood.

This thesis investigates the role of the immune system in the abovementioned psychiatric conditions, utilizing experimental, population-based, and clinical studies.

In the experimental studies, we used the Flinders genetic rodent model of depression, showing differences between the affected animals and their controls in basal expression levels of various immune-related genes—including also a serotonergic receptor that has been linked to the immune system—in several brain regions relevant for depression, thereby increasing our knowledge of this animal model. We believe that the results regarding the glial-specific protein S100B and the serotonergic receptor 5-HT_{2A} may not only be useful for future experiments using Flinders rats, but also in further investigations of the role of these proteins in affective disorders.

As our results regarding immune markers are inconsistent with findings in depressed humans, this model requires further examination to clarify its value in the study of the association between cytokines and depression. Furthermore, we found no dissimilarities between the two rat lines after treatment with an immune stimulant or with the antidepressant escitalopram, suggesting no basic differences in immune response or sensitivity to antidepressant treatment. However, the observation that antidepressant treatment reduced the central

expression of S100B is interesting and valuable since this protein has been linked to the serotonergic system in previous animal and human studies.

To replicate findings in genetic association studies is often difficult and renders a large number of investigations inconclusive. In this thesis, we replicated a previous finding showing an association between a polymorphism in *NFKBIL1* and autism. Even though there is no consensus in the literature about this genetic variant in autism yet, our finding further reinforces the impact of the immune system on neuropsychiatric disorders.

Our finding that the same variant in the *NFKBIL1* gene is specifically associated with language impairment, as opposed to social interactions and restrictive/repetitive behaviour, strengthens the notion that different endophenotypes of complex psychiatric conditions have distinct underlying causes. Our data, together with studies showing that maternal infections during pregnancy increase the risk of an autism diagnosis in the offspring, point to the impact of the immune system during brain development and may lead to a better understanding of the consequences of infections during pregnancy.

In our study of the impact of variants in the cytokine IL-1beta gene (*IL1B*) on brain regional volumes, we found no differences in the studied gene between patients diagnosed with bipolar disorder and controls. Although the literature on this remains unclear, our results suggest that the studied polymorphisms in *IL1B* may not be directly associated with bipolar disorder.

Nonetheless, analyses of the *IL1B* variants and brain volume revealed that this gene is associated with the volume of all studied brain regions, as well as total grey matter volume. Particularly, the association between two polymorphisms and volume of the left putamen survived correction for multiple testing. Again, while previous results are inconsistent, our data imply the influence of the immune system on neurodevelopment. The underlying mechanisms for this association are not obvious yet; IL-beta may directly affect brain development or other systems of relevance. Nevertheless, IL-1beta has previously been associated with both neural plasticity and psychiatric disorders, and our results are in line with the hypothesis that the influence of the immune system on

neurodevelopment may be associated with the origin and pathogenesis of psychiatric symptoms.

The work presented in this thesis confirms the importance of the immune system for mental functions and possibly for brain development. There is an intricate balance of immune-regulated processes in the CNS, and one cannot simply view immune activation in the brain as beneficial or detrimental. Additionally, psychiatric conditions are highly heterogeneous and different causes and mechanisms may underlie the various symptoms; perhaps some of these symptoms may be treated by modulating the immune system. Exploration of the interactions between brain, behaviour, and immunity is central to our understanding of the pathology of psychiatric disorders, and might lead to the development of new treatment strategies for at least a subgroup of the afflicted.

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APPENDIX

DEFINITIONS

Molecular genetics

The instruction manual for the creation of all living organisms is written in their genes in the form of DNA, using only four letters: adenine (A), guanine (G), cytosine (C) and thymine (T). By binding to a pentose sugar (2'-deoxyribose), which forms the backbone of the structure, these bases (then called nucleotides) form hydrogen bonds with each other (A to T and C to G) and form a double helix, which is tightly wrapped and stored as chromosomes. The human genome is made up of 23 chromosome pairs (a total of 46 chromosomes), with one copy from the mother, and one from the father. Thus, each gene is present in two copies, and they can differ to some extent due to e.g. mutations causing genetic variability and subsequent phenotypic variation (see below). Only approximately 1,5% of the human genome consists of protein-coding sequences (called *exons*) (*Figure 3*). The other non-coding sequences are made up of e.g. *introns*, regulatory sequences such as *promoters* and enhancers, repetitive sequences, or encode functional non-coding RNA molecules. It is still not known why there is so much non-coding DNA, but many of these sequences have been found to be involved in the regulation of gene expression.

The synthesis of proteins involves two steps: *transcription* and *translation*. First, the specific part of the chromosome containing the desired genes is unwrapped and transcribed into ribonucleic acid (RNA). These contain both exons and introns, and the latter have to be removed in a process called splicing, leading to *messenger RNA* (mRNA). mRNA is then transported from the nucleus into the cytoplasm of the cell, where it serves as a template for the translation into proteins. The amino acid sequence of a protein is determined by the genetic code, which consists of three letter "words" called codons. Each codon is formed from a sequence of three nucleotides, e.g CGG GAC AGC, which codes for arginine (Arg), aspartic acid (Asp) and serine (Ser), respectively.

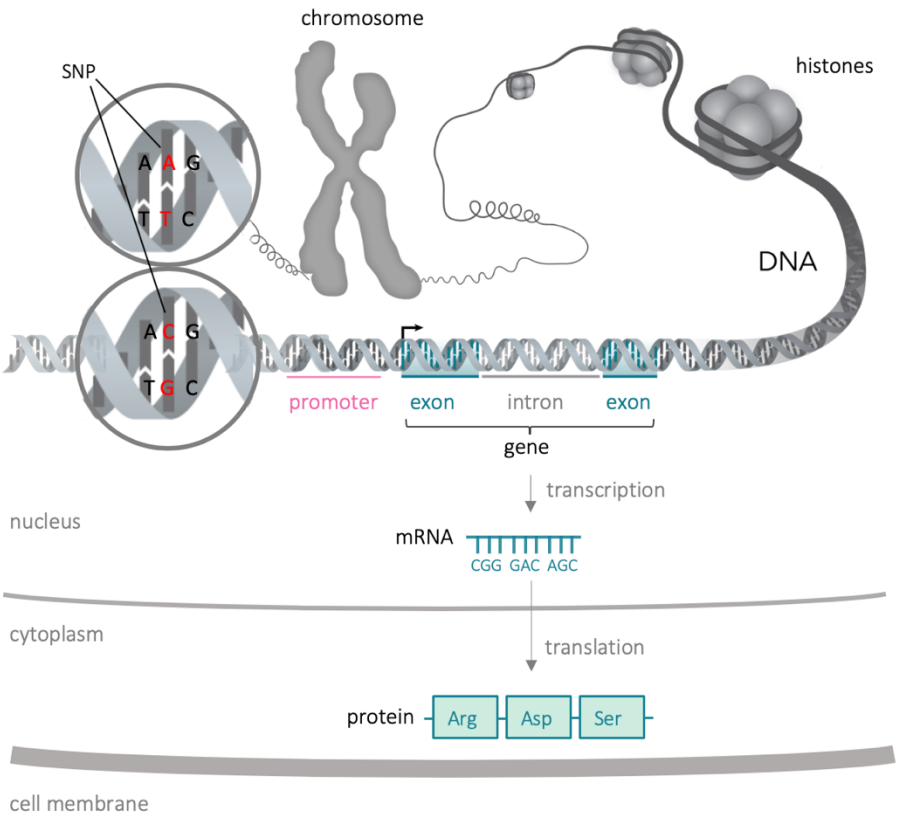

Figure 3. A chromosome pair with tightly packed DNA. Upon transcription, the DNA is uncoiled, revealing the double helix structure and different regions of a gene: the promoter, which initiates transcription, the protein-coding exons, and introns. After transcription, the introns are spliced out and the mRNA is transported from the nucleus into the cytoplasm, where it is translated into protein. Each gene is present in two copies (one from the mother and one from the father), and differences at one base pair are called a single nucleotide polymorphism (SNP), shown in red. Adapted from https://commons.wikimedia.org/wiki/File:Chromosom-DNA-Gen.png

Since three letter words made up of four different letters give rise to 4^3=64 different combinations, with only 20 different amino acids being coded for, plus three stop codons, more than one codon codes for an amino acid. If a mutation occurs at a site in the genetic code (this site is called an *allele*, while the combination of alleles is the genotype), it can either change the encoded amino acid (non-synonymous), introduce a stop codon (nonsense), or have no effect on

the amino acid and be silent (synonymous), or simply occur in the noncoding regions. There it may influence promoter activity and gene expression, mRNA conformation and stability, or subcellular localization of mRNAs and/or proteins and hence may produce a different phenotype or even a disease.

Genotype, phenotype, and epigenetics

The *genotype* is the full genetic hereditary information (i.e. DNA sequence) of a cell, organism or individual. The *phenotype* is all of its observable characteristics, such as morphology, development and behaviour, and is influenced both by genotype and environment. One way how the environment can affect the phenotype is through epigenetic mechanisms. *Epigenetics* is the study of heritable changes in gene expression without a change in DNA sequence, i.e. changing the phenotype without changing the genotype. Epigenetic modifications such as DNA methylation or histone modification change the accessibility of DNA and chromatin structure, thereby regulating patterns of gene expression. To give an analogy; if the DNA is all the books in a library, epigenetic mechanisms are the librarians, deciding which books to lock away and which ones to display. Every single cell in our body contains, or used to contain, that entire library, and epigenetic mechanisms, together with a plethora of signals, causes different genes to be read and expressed, leading to e.g., different cell types and tissues.

Another term often used in psychiatric genetics is *endophenotype*, which can be defined as a heritable, simple component of a disease phenotype. For a behavioural symptom to be called an endophenotype it must have a clear genetic connection and segregate with illness within the population and, to a higher extent, within families of affected individuals, and it must be measurable and specific to the illness of interest. Investigations of endophenotypes are a strategy to gain insight into the genetic components underlying the phenotype heterogeneity of complex psychiatric disorders.

Genetic variation

All humans are approximately 99,9% similar in their genetic code. It is the remaining 0,1% variation, together with environmental factors, that makes each

of us unique. These variations all stem from mutations such as base pair substitutions, deletions, insertions, or copy number variations. If a genetic locus displays different forms of alleles in more than 1% of the population, it is called a polymorphism, and a *single nucleotide polymorphism* (SNP) is a change in one single base-pair.

The first method to sequence DNA was invented in the 1970s and called Sanger sequencing, after its founder Frederick Sanger. It was used to sequence the first gene [269] and more than twenty years later, with an immense workload and costs, the first genome [270]. With the development of next generation sequencing, or massive parallel sequencing, it became possible to sequence the whole genome quickly and at a substantially lower cost. Nowadays, this method is used to sequence large populations in order to search for e.g. the genetic basis of different phenotypes or diseases.

Heritability

Heritability measures to what extent variation in a phenotypic trait in a population is due to genetic variation between individuals in that population. That does not mean that this fraction of a phenotype is genetic. For example, if everyone in a population has the same allele for a trait and shows little variation on that trait, then the heritability for that trait is zero. One example is the number of fingers: 100% due to genetic factors, but 0% heritability (because that trait has no genetic variation). In other words, heritability is the proportion of the variation in a given trait within a population that is not explained by environment or random chance. It is estimated by comparing individual phenotypic variation among related individuals in a population, and one of the most commonly used heritability estimation methods is twin studies, in which the impact of genetic and environmental factors is identified by comparing the concordance between monozygotic (MZ) and dizygotic (DZ) twin pairs. A high concordance means that both subjects in a twin pair will likely have the same phenotype/disease. If the concordance rate for monozygotic twins is twice as high as for dizygotic twins, it is interpreted as a high heritability for the specific disease or trait.

Linkage disequilibrium

Humans receive one set of chromosomes from their mother, and one from their father, and during meiosis (i.e. the creation of egg or sperm cells; DNA replication followed by two rounds of cell division, resulting in four haploid cells) homologous chromosomes exchange parts of their DNA in a process called genetic recombination. This makes it possible that offspring can have traits depending on genes that lie on the same chromosome from all four of their grandparents and leads to greater genetic diversity. Regions that are close together on the same chromosome are often inherited together, as the probability that a recombination occurs between to alleles increases with the distance between them. *Linkage disequilibrium* (LD) is the non-random association of alleles at different loci, i.e. loci are said to be in LD when they are inherited together more often (or seldom) than would be expected from random and independent recombination. If two loci are in complete LD, one can predict the outcome of the other. However, it is possible that one locus may predict the outcome of the other, but not the other way around. A *haplotype* is the specific combination of alleles on a chromosome that are inherited together from a single parent, but also a set of SNP alleles that tend to always occur together (i.e., that are statistically associated). LD-information is used by essentially all genetic studies to establish to what extent genetic variation may influence a complex trait or disease.

Structures and circuits of the brain

The limbic system

Various scientists have attempted to elucidate the neural systems that control human emotions and behaviour throughout the centuries, but there is still much to be found out regarding the neuroanatomy of emotion. In 1878, Paul Broca, a French physician best known for his research on the anatomical location of language and Broca´s area, coined the term *limbic lobe*, describing a collection of cortical areas that form a border (latin: *limbus*) around the brain stem. However, they were not yet associated with emotion but primarily thought to be involved in olfaction. Almost 50 years later the American neuroanatomist James Papez described a neural pathway in the brain as "the anatomical basis of emotion", called the *Papez circuit*. This included the cingulate cortex, anterior thalamic nucleus, hypothalamic mammillary bodies, and hippocampus, with each area being connected to another by a major fiber tract (*Figure 4A*) [271].

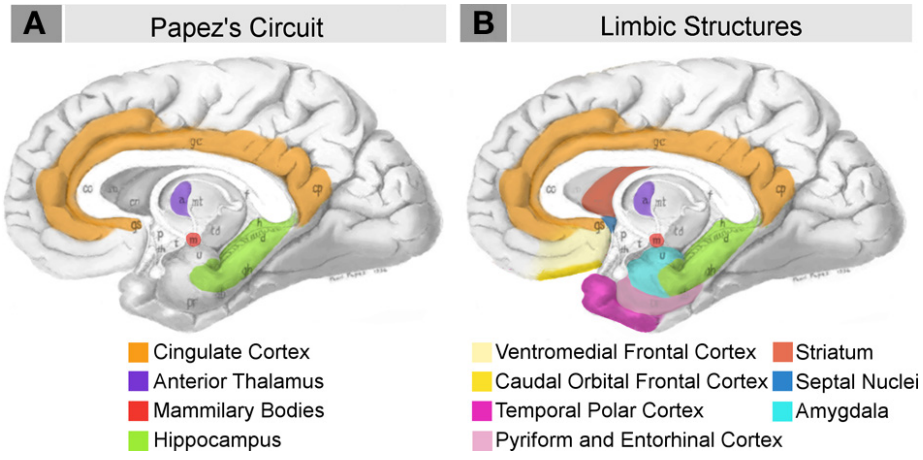

Figure 4. Schematic briefly summarizing neural systems proposed to process emotion, highlighting structures that are visible on the medial surface of the brain. Papez's (1937) original circuit (A) was expanded upon in the concept of the limbic system (B) to include a variety of subcortical and cortical territories. From Barger et al. 2014 [271] *http://journal.frontiersin.org/article/10.3389/fnhum.2014.00277/full.*

Papez believed that the cortex is an important part of the experience of emotions, and that this "emotional system" links the cortex with the

hypothalamus. These structures have been thought to be involved in emotion since then, with the American physiologist Paul MacLean reintroducing the term *limbic system* in the 1950s, expanding the emotion network to include several more areas (*Figure 4B*). However, some of the components central to the limbic system are no longer thought to be central to the expression of emotion (such as the hippocampus), and some structures involved in emotion have also been shown to be involved in other functions [272].

Considering the diversity of emotions that we experience, it seems reasonable, however, that more than one system is involved. Although the term *limbic system* is still frequently used, and its components thought to be involved in emotion, motivation, learning and memory, the current consensus is that it is certain brain structures that are particularly important for the experience and expression of emotions.

The basal ganglia

The basal ganglia are a set of structures including the

- *striatum*: *ventral striatum* (*nucleus accumbens* and the *olfactory tubercle)*
  dorsal striatum (*putamen* and *caudate nucleus*)
- *globus pallidus* in the cerebrum
- *substantia nigra* in the midbrain
- *subthalamic nucleus* in the diencephalon
- *ventral pallidum*

These structures lie deep within each of the cerebral hemispheres in the cerebrum, over and to the sides of the limbic system, with which they interact, and are tightly connected to the cerebral cortex, thalamus, and brainstem, as well as several other brain regions. Their function can be summarized as being "principally involved in the selection and implementation of purposeful actions in response to external and internal cues", as they are an important part of the brain systems that control motor movement (including initiation and

termination of movements), but have also been implicated in other circuits involving emotion, memory, learning, language, decision making, cognitive function and reward [273].

MATERIAL AND METHODS

Ethics in research

All studies in this thesis were approved by the respective ethics committees. The animal studies presented in Paper I and II were approved by the Animal Ethics committee for Animal Experiments in Gothenburg, Sweden, and by the Danish National Committee for Ethics in Animal Experimentation, respectively. In accordance with the "three R's" (refine, reduce, replace), all efforts were made to minimize animal suffering and to reduce the number of animals used to what was deemed necessary for the objectives of each experiment. The human genetic association studies were approved by the Karolinska Institutet Ethical Review Board (Paper III) and by the Stockholm Regional Ethical Review Board (Paper IV). Informed consent was given by all participants.

Animal models in psychiatric research

As it is difficult to study endogenous proteins and their role in e.g. mood disorders in the human brain, animal studies prove to be of importance for exploring these questions. However, since psychiatric diagnoses are based on the verbal description of symptoms reported by the patient and/or relatives, it is problematic to model these conditions in animals. Some symptoms that may be central to some afflicted by mood disorders, such as guilt and worthlessness are difficult to model in animals. Therefore, attempts have been made to develop animal models that focus on other symptoms, modelling more basal fear and stress responses. This can be done either based on the animal itself, e.g. a genetic model, or on the study of a behaviour that can be altered by e.g. pharmacological intervention which shows similar effects as in humans.

The quality of an animal model can be measured by three criteria: face, predictive and construct validity. *Face validity* means that something similar to the studied phenomenon is also present in humans (i.e. symptoms). The second criteria, *predictive validity*, concerns itself with effects of treatments, i.e. a

compound with a known effect in humans has a similar effect in the animal model, while a compound without any effect in humans also shows no response in the model. *Construct validity*, the third criteria, describes how similar the underlying mechanisms between the condition in humans and the animal model are. Since both the etiology and the pathophysiology of mood disorders are far from understood, this is perhaps the most difficult to achieve and can only be speculatively discussed in animal models.

In this thesis we combined two animal models used in depression research; the *Flinders sensitive line* (FSL) rat, a genetic animal model of depression [274], and the *forced swim test*, in which animals can be tested for sickness or depressive-like behaviour and the effect of antidepressant treatment [275,276] The FSL rat, originally bred to develop a resistance to irreversible anticholinesterase agents, instead turned out to be more sensitive to these agents, which has also been noted in depressed patients [277]. FSL animals also share several behavioural characteristics with depressed humans, including reduced appetite, reduced psychomotor function, sleep disturbances, and, interestingly, immune abnormalities such as reduced cytotoxic T lymphocyte activity [278], thus showing a modest degree of face validity. FSL rats have higher immobility scores in the forced swim test than their controls [279], which can be reversed by different antidepressants, including SSRIs [274], indicating good predictive validity. Also, neurochemical abnormalities have been observed in Flinders rats, including imbalances of the serotonergic, cholinergic, and immune systems [274], suggesting construct validity.

The forced swim test is a behavioural test that is widely used to evaluate antidepressant activity in rats and mice. The animals are placed in an inescapable cylinder filled with water, and after escape-oriented movements ("climbing") they either swim or develop an immobile posture ("immobility"). If the test is repeated 24 hours later, they usually resume this posture quickly. This immobility is believed to reflect either giving up escape-directed behaviour (i.e. behavioural despair) [276] or a diminished ability to cope with stressful stimuli [280]. While the forced swim test shows relatively little face or construct validity, its

major advantage is a very high predictive validity, as antidepressant treatment significantly decrease the time spent immobile in the tested animals [274,281].

However, it is important to emphasize that the behaviour of rodents differs greatly from that of humans, and that human depression and depressive-like behaviour in an animal model are two vastly different things. The fact that antidepressant treatment, which suppresses symptoms of depression in humans, also reduces depressive-like behaviour in an animal model, neither means that this behaviour is directly comparable with depression, nor that every other compound with antidepressant effects in humans will inhibit the behaviour in that animal model. Therefore, data from animal models of behaviour should always be interpreted with great caution, although they may nonetheless provide important insights into the underlying mechanisms of e.g. psychiatric disorders.

Human genetic association studies

Genetic association studies analyze the co-occurrence of a trait or phenotype with one or more genotypes, i.e. single-locus allele frequencies or haplotype frequencies are compared between groups (e.g., case-control) or for quantitative traits (e.g., continuous measures). Association analyses have been used in candidate gene studies since the 1990's, and with the introduction of the genome wide SNP-genotyping arrays (see chapter "*Genetic variation*" above), it became possible to perform GWAS. The advantages with GWAS are undoubtedly abundant, enabling the examination of possible associations between a phenotype and thousands of variants across the genome. However, candidate gene studies allow for a more focused, hypothesis driven investigation of endogenous molecules. Considering the intricate and complex relationship between the immune system and brain functions, cause and effect are not always evident, i.e., is dysregulation of the immune system causing a phenotype, or is it a result thereof. The advantage of the candidate gene approach is that possible associations between the genetic code and a phenotype can be established.

Magnetic resonance imaging

MRI is an imaging technique used in radiology to form pictures of the anatomy and the physiological processes of the body in both health and disease. It is a widely used technique for medical diagnosis and research applications, and the method of choice for many conditions of the CNS due to the contrast between white and grey matter in the image. In contrast to computer tomography or positron emission tomography, MRI does not involve the use of radiation; however, it might create discomfort, as the scans usually take longer and are louder, and require the tested subject to enter a narrow, confining tube.

MRI is based on the magnetic properties of atomic nuclei, and uses the nuclear spins of protons in hydrogen nuclei that are contained in a tissue when placed in a magnet field. For that, it uses strong magnetic fields, magnetic field gradients, and radio waves to generate images of the organs in the body. Most MRI examinations target hydrogen in water molecules. As the brain contains approximately 80% water, an image can be constructed with MRI. For that, the nucleus will be excited with electromagnetic energy and taken from a low-energy state to a high-energy state. When the nucleus falls back into the low-energy state, magnetic oscillations from the nucleus induce current in a receiver coil, forming the basis of the MRI signal.

After excitation, each tissue returns to its equilibrium state during a relaxation process. Two types of relaxation can be determined: T1 and T2. To create a T1-weighted image, magnetization is allowed to recover before measuring the MR signal, and this image weighting is useful for obtaining morphological information, e.g., the cerebral cortex or fatty tissue. To create a T2-weighted image, magnetization is allowed to decay before measuring the MR signal, and this image weighting is useful for detecting e.g., edema and inflammation. In our study (Paper IV), the MRI images that were analysed were T1-weighted.

SUPPLEMENTARY MATERIAL

Supplementary Table A. DSM-5 Criteria for Major Depressive Disorder

A.	Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure: 1. Depressed mood most of the day 2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day 3. Significant weight loss when not dieting or weight gain, or decrease or increase in appetite 4. Insomnia or hypersomnia 5. Psychomotor agitation or retardation 6. Fatigue or loss of energy 7. Feelings of worthlessness or excessive or inappropriate guilt 8. Diminished ability to think or concentrate, or indecisiveness 9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide
B.	The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
C.	The episode is not attributable to the physiological effects of a substance or to another medical condition.
D.	The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.
E.	There has never been a manic episode or a hypomanic episode.

DSM-5=*Diagnostic and Statistical Manual of Mental Disorders 5th edition*

Supplementary Table B. DSM-5 Criteria for Bipolar I and II Disorder

For a diagnosis of bipolar I disorder, it is necessary to meet the following criteria for a manic episode. The manic episode may have been preceded by and may be followed by hypomanic or major depressive episodes.

For a diagnosis of bipolar II disorder, it is necessary to meet the following criteria for a current or past hypomanic episode and the following criteria for a current or past major depressive episode.

Manic Episode

A. A distinct period of abnormally and persistently elevated, expansive, or irritable mood and abnormally and persistently increased goal-directed activity or energy, lasting at least 1 week and present most of the day, nearly every day (or any duration if hospitalization is necessary).

B. During the period of mood disturbance and increased energy or activity, three (or more) of the following symptoms (four if the mood is only irritable) are present to a significant degree and represent a noticeable change from usual behavior:
   1. Inflated self-esteem or grandiosity.
   2. Decreased need for sleep (e.g., feels rested after only 3 hours of sleep).
   3. More talkative than usual or pressure to keep talking.
   4. Flight of ideas or subjective experience that thoughts are racing.
   5. Distractibility (i.e., attention too easily drawn to unimportant or irrelevant external
   stimuli), as reported or observed.
   6. Increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation (i.e., purposeless non-goal-directed activity).
   7. Excessive involvement in activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments).

C. The mood disturbance is sufficiently severe to cause marked impairment in social or occupational functioning or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.

D. The episode is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication, other treatment) or to another medical condition.

Note: A full manic episode that emerges during antidepressant treatment (e.g., medication, electroconvulsive therapy) but persists at a fully syndromal level beyond the physiological effect of that treatment is sufficient evidence for a manic episode and, therefore, a bipolar I diagnosis.
Note: Criteria A–D constitute a manic episode. At least one lifetime manic episode is required for the diagnosis of bipolar I disorder.

Hypomanic Episode

A. to D. see Manic Episode

E. The episode is not severe enough to cause marked impairment in social or occupational functioning or to necessitate hospitalization. If there are psychotic features, the episode is, by definition, manic.

F. The episode is not attributable to the physiological effects of a substance (e.g., a drug
of abuse, a medication, other treatment).
Note: A full hypomanic episode that emerges during antidepressant treatment (e.g., medication,
electroconvulsive therapy) but persists at a fully syndromal level beyond the physiological effect of that treatment is sufficient evidence for a hypomanic episode diagnosis. However, caution is indicated so that one or two symptoms (particularly increased irritability, edginess, or agitation following antidepressant use) are not taken as sufficient for diagnosis of a hypomanic episode, nor necessarily indicative of a bi- polar diathesis.

Note: Criteria A–F constitute a hypomanic episode. Hypomanic episodes are common in bipolar I disorder but are not required for the diagnosis of bipolar I disorder.

DSM-5=*Diagnostic and Statistical Manual of Mental Disorders 5th edition*

Supplementary Table C. DSM-IV Criteria for Autistic Disorder

A. A total of six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3).

(1) Qualitative impairment in social interaction, as manifested by at least two of the following:

(a) Marked impairments in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body posture, and gestures to regulate social interaction

(b) Failure to develop peer relationships appropriate to developmental level

(c) A lack of spontaneous seeking to share enjoyment, interests, or achievements with other people

(d) Lack of social or emotional reciprocity

(2) Qualitative impairments in communication as manifested by at least one of the following:

(a) Delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

(b) In individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

(c) Stereotyped and repetitive use of language or idiosyncratic language

(d) Lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(3) Restricted, repetitive and stereotyped patterns of behavior, interests and activities, as manifested by at least two of the following:

(a) Encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

(b) Apparently inflexible adherence to specific, nonfunctional routines or rituals

(c) Stereotyped and repetitive motor mannerisms (e.g hand or finger flapping or twisting, or complex whole-body movements)

(d) Persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years:
(1) social interaction, (2) language as used in social communication, (3) symbolic or imaginative play
C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder

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DSM-IV=*Diagnostic and Statistical Manual of Mental Disorders 4th edition*

Supplementary Table D. DSM-5 Criteria for Autism Spectrum Disorder

A.	Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history: 1. Deficits in social-emotional reciprocity 2. Deficits in nonverbal communicative behaviors used for social interaction 3. Deficits in developing, maintaining, and understanding relationships
B.	Restricted, repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following, currently or by history: 1. Stereotyped or repetitive motor movements, use of objects, or speech 2. Insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal nonverbal behavior 3. Highly restricted, fixated interests that are abnormal in intensity or focus 4. Hyper- or hyporeactivity to sensory input or unusual interests in sensory aspects of the environment
C.	Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities, or may be masked by learned strategies in later life).
D.	Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
E.	These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

DSM-5=*Diagnostic and Statistical Manual of Mental Disorders 5th edition*

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